

# Redox Modulation of Oxidatively Induced DNA Damage by Ascorbate Enhances Melanoma Cancer Cell DNA Damage Formation & Cell Killing

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By

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### Abstract

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Malignant melanoma (MM) is the 5<sup>th</sup> most common cancer in the UK and the most lethal form of skin malignancies. Its incidence has more than quadrupled and mortality more than doubled over the last three decades. With a five-year survival rate for patients with advanced melanoma of  $\leq 20\%$ , there is an obvious need for a better treatment approach.

Heightened genome-instability in cancer-cells suggests a model-scenario for their selective killing via the therapeutic delivery of defined levels of further genomic damage. To interrogate/exploit this model-scenario, it was proposed to investigate intracellular ascorbate's redox-modulation of oxidatively-induced DNA damage in MM cancer-cells, to selectively enhance both DNA damage and cell-killing.

Alkaline comet assay (ACA) data reveals MM-cells to have higher endogenous DNA damage levels than "normal" skin cells. This in turn correlates with MM-cells having higher intracellular ROS and lower catalase activity. ACA data also shows MM-cells to be more sensitive towards the induced-damaging effects of  $H_2O_2$  than "normal" skin cells, and that ascorbate further enhances this effect in MM-cells. This effect was also noted in primary melanoma cancer cells exposed to  $H_2O_2$  and other oxidants (e.g. Elesclomol).

A proposed model for the enhancement of  $H_2O_2$ -induced oxidatively-damaged DNA by ascorbate suggests that an increased local production of hydroxyl radicals (<sup>•</sup>OH) at the DNA may lead to relatively greater increases in complex lesions (i.e. DSBs) relative to single/'isolated' lesions (i.e. SSBs). However, correlated measures of DSBs vs. SSBs reveal only a proportional increase in DSBs relative to SSBs with ascorbate.

Further data shows that ascorbate enhances oxidative-induced cell death in MM-cells and that in HaCaT cells the effect was slightly protective. Together, these results suggest that ascorbate enhances DNA damage/cell-killing through modulation of oxidative stress in MM-cells. This could increase the possibility of using ascorbate plus novel oxidant therapies to treat metastatic melanomas.

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## List of Abbreviations

<b>2-ME</b>	2-methoxy estradiol		
Α	Ascorbate		
AEB	Alkaline electrophoresis buffer		
AJCC	American Joint Committee on Cancer		
ALM	Acral Lentigious melanoma		
ALS	Alkali labile sites		
ATP	Adenosine triphosphate		
BCC	Basal cell carcinoma		
BRAF	Serine/threonine protein kinase B-raf		
BSA	Bovine serum albumin		
BSO	Buthionine sulfoxamine		
Caspase	Cysteine aspartic acid-specific protease		
CAT	Catalase		
DCFHDA	2´,7´-Dichlorofluorescin		
DFO	Desferoxamine		
DHA	Dehydroascorbate		
DMSO	Dimethyl sulfoxide		
DNA	Deoxyribonucleic acid		
DSBs	Double strand breaks		
DTIC	Dacarbazine		
EDTA	Ethylenediaminetetraacetic acid		
ERB	Enzyme reaction buffer		
ЕТС	Electron transport chain		
FCS	Fetal calf serum		
FDA	Food and Drug Administration		
FITC	Fluorescein-5-Isothiocynate		
Fpg	Formamidopyrimidine glycosylase		
Fpg-SS	Formamidopyrimidine glycosylase sensitive site		
FRs	Free radicals		

GPx	Glutathione peroxidase		
GR	Glutathione reductase		
GSH	Glutathione-S-transferase		
GULO	L-gulonolactone oxidase		
HaCaTs	Human cultured Keratinocytes		
HDF	Human dermal fibroblast		
IL-2	Interleukin-2		
LMM	Lentigo maligna melanomas		
LMP-agarose	Low melting point agarose		
MDR	Multi Drug Resistance		
MGMT	Methylguanine-DNA-methyltransferase		
MM	Malignant melanoma		
NAC	N-Acetyl-Lcysteine		
NAD	Nicotinamide		
NADPH	Nicotinamide adenine dinucleotide phosphate		
NM	Nodular melanoma		
	Normal melting point agarose		
NMP-agarose	Normal melting point agarose		
NMP-agarose NMSC	Normal melting point agarose Non-melanoma skin cancer		
NMP-agarose NMSC NOX	Normal melting point agarose Non-melanoma skin cancer NADPH oxidase		
NMP-agarose NMSC NOX ODBLs	Normal melting point agarose Non-melanoma skin cancer NADPH oxidase Oxidatively damaged base lesions		
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<b>Trypsine-EDTA</b>	<b>-EDTA</b> Trypsine-Ethylenediaminetetraacetic acid	
UV	Ultraviolet	
UVA	Ultraviolet A	
UVB	Ultraviolet B	
XO	Xanthine oxidase	
γ-Η2ΑΧ	Gamma H2A Histone family member X	

**CHAPTER I: Introduction** 

#### **1.1** Skin cancers and their health burden

The skin is the body organ within which neoplasms occur most frequently. Cancers that develop in skin are generally classified into non-melanoma skin cancers (NMSC), involving basal and squamous cell carcinoma (BCC and SCC), and malignant melanoma (MM). BCC and SCC are more frequent, but less serious forms of cutaneous malignancies, whereas MM, which accounts for just 5% of skin cancers, is the most aggressive and deadliest form of skin neoplasm (Cancer Research UK, 2014a). Although other rare cutaneous cancers, such as malignant histiocytomas, Paget's disease, cutaneous lymphoma and Merkel cell carcinoma are considered to be NMSCs, the term NMSC is broadly used to encompass SCC and BCC.

The most common of all cancers in the USA are NMSCs, with white populations being the most affected (Skin Cancer Foundation, 2015). The two forms of NMSCs, BCCs and SCCs, share many similarities, but have different incidence rates (Lucas *et al.*, 2006). The first description of BCC was given in 1909 (Janeway, 1909), and it is now considered the most frequent malignant form of all NMSCs (Diepgen and Mahler, 2002). Indeed, statistical reports indicate that 80% of NMSCs are BCCs, the rest being mostly SCCs (Alam and Ratner, 2001). NMSC incidence proportionally increases with age (Cancer Research UK, 2014b) and although NMSC can cause disfigurement, it is seldom lethal, with a cure rate of 99% if treated in its early stages (Young and Rushton, 2012). In comparison to NMSC, much more attention has been paid to MM (Stang *et al.*, 2008) as this form of skin cancer is responsible for the majority of related deaths and develops more frequently in young individuals (Cancer Research UK, 2014c).

The health burden associated with MM is sizeable, with a high mortality rate in proportion to the costs of treatment being reported for MM (Ekwueme *et al.*, 2011). Specifically, a serious public health issue has arisen as a consequence of the substantial economic burden of treating melanoma, as the high expense of managing late stage MM management is significant (Guy Jr *et al.*, 2012). There are also obvious losses to individual productivity and resources for those seeking melanoma care and treatment. Furthermore, the disproportionate mortality rate of MM in young and middle aged groups makes this cancer a common killer of young adults (McMasters *et al.*, 2001).

### 1.2 Malignant melanoma

### 1.2.1 Statistics and incidence rates

Over the past 40 years, an increasing incidence of MM has been recorded among fairskinned populations worldwide (WHO, 2015a). Although the incidence of melanoma varies geographically, the data for most countries reveals an increasing prevalence (MacKie *et al.*, 2009). According to World Health Organisation (WHO) statistics, the incidence of melanoma has increased over past decades, with a global estimation of more than 100,000 melanoma skin cancers reported to occur annually (WHO, 2015b). Cancer Research UK records indicate that MM is the fifth most common cancer in both genders in the United Kingdom (Figure 1.1).



Figure 1.1. Ranking of 20 most common cancers in the UK in 2011 (Cancer Research UK, 2014d).

The annual incidence rate of MM has increased by approximately 7% per year in white populations worldwide (Parkin *et al.*, 2001), although increased public awareness and the early recognition of melanomas may have contributed somewhat to this increased level of recorded incidence. Geographical variation in melanoma incidence has been the focus of several studies (de Vries *et al.*, 2003, Chang *et al.*, 2009). A comparative overview of melanoma epidemiology worldwide indicates that Australia and New Zealand have the highest incidence of cutaneous melanoma affecting both genders, followed by North America, Northern Europe and Western Europe (MacKie *et al.*, 2009) (Figure 1.2).



Figure 1.2. Incidence rate of MM around the world from 1998-2002 (Parkin et al., 2010).

Similarly, in the UK the incidence and mortality rates of MM in both genders has significantly increased over the past three decades (MacKie *et al.*, 2009). Between 1975 and 2011, the incidence rate has increased 4.4 and 7.2-fold in females and males, respectively (Figure 1.3). For instance, in 2010, nearly 13,000 cases of MM were diagnosed in the UK, leading to more than 2,000 deaths (Table 1.1) (Cancer Research UK, 2012a).



Figure 1.3. Incidence rate of MM in both genders in the UK from 1975-2011.

(Cancer Research UK, 2014b).

Table 1.1. (A) Number of MM cases diagnosed per 10<sup>5</sup> of population in the UK in 2010 and (B) number of deaths from total cases

Α	Case	England	Scotland	Wales	Northern Ireland	UK
	Males	5,151	524	410	116	6,201
	Females	5,505	617	330	165	6,617
	Total number	10,656	1,141	740	281	12,818

(Cancer Research UK, 2014b).

B	Patients	England	Scotland	Wales	Northern Ireland	UK
	Males	1,046	114	68	38	1,266
	Females	779	81	52	25	937
	Total number	1,825	195	120	63	2,203

Accordingly, MM has become a global important public health issue, as its incidence, in most developed countries, has increased faster than any other type of cancer since the mid-twentieth century (Hall *et al.*, 1999, Gandini *et al.*, 2005). As well its increased incidence, MM leads to disproportionate mortality in early and middle aged individuals, with each melanoma death representing 18.6 potential life years lost, which is higher than most other cancers (Kang *et al.*, 2005a).

Whilst men tend to have a lower incidence of MM in most countries, they exhibit higher mortality rates than women (MacKie *et al.*, 2009). The differences in the anatomical distribution of melanoma lesions have been extensively studied in literature (MacKie *et al.*, 2002). In males, the trunk is the most common region for melanoma lesions, and their occurrence in this site is associated with a very poor prognosis (Bulliard, 2000). In the UK between 2008 and 2010, the percentage of melanoma lesions arising on the trunk in males was two-fold higher than that in females; by contrast, in women the legs were the predominant site of lesion occurrence (Figure 1.4). Moreover, changes in lifestyle such as clothing, occupation and sun-seeking behaviours all increase the chances of melanoma lesions developing in distal extremities in women, and in the head, neck and trunk in men (Bulliard *et al.*, 1997).



Figure 1.4. Differences in the percentage of anatomical distribution of MM lesions diagnosed between 2008 and 2010 in both genders in the UK (Cancer Research UK, 2014b).

The incidence rates of MM in relation to disease stage are varied. According to Cancer Research UK records, in England the majority of recorded cases (61.4% in male and 71.3% in female) between 2006 and 2010 were stage I; the rest were the stage II and stage III, with the smallest proportion (1.9% in male and 0.7% in female) being metastatic diseases (Figure 1.5).



Figure 1.5. Incidence rate of melanoma in the East of England according to the diagnosed stages of the disease, between 2006 and 2010 (Cancer Research UK, 2014b).

#### **1.2.2** Melanoma staging

Like other types of cancer, cutaneous melanoma has several stages and its classification is important for clinical and research disciplines (Balch *et al.*, 2004). Clinically, melanoma staging provides a consistent nomenclature that informs the prognosis; this also helps categorise melanoma patients into several groups, according to disease risk, and provide them with valuable treatment strategies (Balch *et al.*, 2004). In 1998, the Melanoma Staging Committee of the American Joint Committee on Cancer (AJCC) was formed to revise melanoma staging based on the large clinical databases for melanoma from North America, Europe and Australia (Balch *et al.*, 2000). The current version of the staging system was the result of substantial revision in 2001 by the AJCC Melanoma Staging Database (Balch *et al.*, 2001a), with further improvements being made in 2009 (Balch *et al.*, 2009). The AJCC melanoma staging system focuses on the relevancy of the defined stage, the biology of melanoma and the prognostic outcome of the disease (Balch *et al.*, 2004).

In general, melanoma is classified into four clinical stages (I, II, III and IV). If there is no clinical, radiological and laboratory evidence of regional and/or distant tumour metastasis, patients will be diagnosed as having stage I or II melanoma (Gershenwald *et al.*, 2010). Those with clinical or radiologic evidence of tumour metastasis to the regional lymphatic system are classified as stage III, and stage IV occurs when the tumour has metastasised to distant sites in the body (Gershenwald *et al.*, 2010).

The Tumour-Node-Metastasis classification is the up-to-date system used by the AJCC to describe the extent of the disease. The T category refers to the extent of primary tumour thickness and is assigned a number from 0 to 4, with a small letter, a or b, based on ulceration and mitotic rates. N describes the involvement of the regional lymph nodes, and is assigned a number from 0 to 3, based on whether the lesions have spread to involve the regional lymph nodes or lymphatic channels; it may also be assigned a small letter, a, b, or c (referring to micrometastasis, macrometastasis or in transit metastases). Lastly, category M is used to define the extent of metastasis, and indicate whether melanoma cells have reached far distant organs, which is usually accompanied with an elevation in blood lactate dehydrogenase (LDH) levels (Table 1.2) (Petro *et al.*, 2004, Gershenwald *et al.*, 2010).

Table 1.2. Melanoma Staging system: The 2009 American Joint Committee on Cancer (AJCC) staging system for MM

T Category	Tumour thickness (mm)	Mitosis rate/Ulceration	
Tis	N/A		N/A
T1	≤1.0	a: No ulceration and mitosis < 1/mm <sup>2</sup>	
		b: Wi	th ulceration or mitosis $\geq 1/mm^2$
T2	1.01-2.00		a: No ulceration
			b: With ulceration
Τ3	2.01-4.00		a: No ulceration
			b: With ulceration
T4	> 4.00		a: No ulceration
			b: With ulceration
N Category	Number of metas lymph nodes	tatic	Nodal metastatic burden
N0	0		NA
N1	1		a: Micrometstasis
			b: Macrometastasis
N2	2-3		a: Micrometastasis
			b: Macrometastasis
		c: In transit metastases/satellite	
N3	4+	Ν	Without metastatic nodes
115	41	in transit metastases/satellite with	
		metastatic nodes	
M Category	Site		Serum LDH
<b>M</b> 0	No distant metas	stases	N/A
M1a	Distant skin, subcu	itenous,	Normal
	or lymphatic no metastases	odule	
M1b	Lung metasta	ses	Normal
M1c	All other visce	eral	Normal
	Any distant moto	etacic	Flavated
	Any distant meta	istasis	Elevaled

(Balch et al., 2009, Gershenwald et al., 2010).

#### 1.2.3 Prognosis of melanoma

MM is one of the most common cancers affecting young adults and, once advanced to metastatic disease, it has a high mortality rate (Reed *et al.*, 2012). Approximately 90% of melanoma cases are diagnosed in the early stages, without metastases (as primary tumours), and prognosis is typically favourable (Dickson and Gershenwald, 2011). By contrast, the later stages of this cancer are associated with substantially lower survival rates (Table 1.3).

Several histological prognostic factors for primary melanoma have been discovered by a number of researchers (Spatz *et al.*, 2003, Balch *et al.*, 2009, Thompson *et al.*, 2011). These parameters include vertical tumour thickness (Breslow's depth), the presence of ulceration, number of mitosis per mm<sup>2</sup> (mitotic rates) and the level of tumour invasion (Clark's level). In 1970, Alexander Breslow defined melanoma vertical depth, as being from the top surface of the lesion to the deepest penetration in the skin, and identified a significant relationship between melanoma lesion size, the stage of invasion and prognostic outcomes (Breslow, 1970). These histological features are considered independent prognostic factors for both stage I and stage II melanoma (Marghoob *et al.*, 2000). However, for thin melanomas (T1), the Clark's level is a suitable independent predictive marker (Clark *et al.*, 1969, Balch *et al.*, 2001b). In addition, the presence or absence of ulceration in melanoma also provides a good indicator of future prognosis; the survival rate for those who have ulceration is proportionally lower than those without (Balch *et al.*, 2001a).

Besides stage, there are other factors that also affect survival rates. The age of the patient is one such factor, as elderly individuals have a lower survival rate than young adults (National Cancer Intelligence Network, 2013). Ethnicity also plays a role in prognosis prediction; the incidence of melanoma in black groups is very low (Cancer Research UK, 2014b), but when it occurs, it is more aggressive than it is among white groups (Byrd *et al.*, 2004). It has also been shown that the anatomical site of the melanoma lesion is another important prediction of survival rate. For instance, individuals with melanoma on their extremities have better survival rates than those having melanomas on their head, neck and truck (reviewed by Homsi *et al.*, 2005) (Homsi *et al.*, 2005). Moreover, it has also been shown that immune compromised individuals have a worse prognosis than their otherwise healthier counterparts (Kubica and Brewer, 2012).

#### Table 1.3. Survival rates according to the stages of MM.

The data below show the 5 and 10 years survival rates in different stages of MM. The early stages have highest rates of survival whereas the advanced stages have bad prognosis. Data obtained from American Cancer Society (American Cancer Society, 2015).

Melanoma stage	5-year survival rate	10-year survival rate
IA	97%	95%
IB	92%	86%
ΠΑ	81%	67%
IIB	70%	57%
ПС	53%	40%
IIIA	78%	68%
IIIB	59%	43%
IIIC	40%	24%
IV	15-20%	10-15%

### 1.2.4 Aetiology and risk factors

Skin is the largest organ of the human body, and has many essential physiological functions. For instance, it helps to maintain body temperature, and is the first physical barrier protecting the body from the external environment. The environment contains many sources of potential damage to the body, such as solar radiation, chemicals and pollutants (Thiele *et al.*, 1997). Many of these factors are considered to be causative risks for melanoma development; for instance, studies indicate that solar ultraviolet (UV) radiation has a major etiological role in skin cancer development, with approximately 65% of melanoma directly resulting from sunlight exposure (Whiteman and Green, 1999, Williams and Ouhtit, 2005).

Although the ozone layer of the atmosphere protects humans by absorbing energetic UV light from sunlight, the UV light of longer wavelengths, such as UVA and UVB, passes through the ozone layer and reaches human skin (Narayanan *et al.*, 2010). UV radiation can be absorbed by the double bond of pyrimidine bases in genomic DNA, causing the bond to rupture and so able to react with other adjacent pyrimidine bases in the DNA

(Goodsell, 2001). The resulting products (photoproducts) induced by UVB irradiation (of wavelength 290-320nm) are the main causes of NMSCs (Leiter and Garbe, 2008) whereas photoproducts induced by solar UVA irradiation (320-400nm) are responsible for cutaneous melanoma development, particularly in those with a phenotypic susceptibility (Leiter and Garbe, 2008).

High level of oxidative stress in melanocytes is considered as another risk factor for MM development (Denat et al., 2014). This can be the outcome of several extrinsic and intrinsic sources in melanocytes. In normal cellular metabolism, cellular organelles such as mitochondria, and enzymes including peroxisome's enzymes and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) generates ROS (Denat et al., 2014) In addition to induced photoproducts, for instance, UV light exposure generate huge amounts of cellular inflammatory cytokines and growth factors (the intracellular signalling molecules), which in turn generate free radicals (FRs) and oxidative stress within melanocytes. Furthermore, interactions between UV light and intracellular constituents of melanocytes induce additional oxidative stress, including hydrogen peroxide ( $H_2O_2$ ) and superoxide ( $O_2^{\bullet}$ ) formation. Moreover, the biosynthesis of melanin in melanocytes can also generate reactive oxygen species (ROS) (Thompson et al., 2005). This process is oxygen dependent and is considered a potential source of oxidative stress in pigmented-cells (Smit et al., 2008). During the enzymatic action of tyrosinase enzyme,  $O_2^{\bullet}$  is produced, which eventually converts to  $H_2O_2$  through the action of dismutase (SOD) (Figure 1.6) (Hasegawa, 2010).



#### Figure 1.6. Role of melanin pigment in ROS generation in melanocyte.

During biosynthesis of melanin pigment tyrosinase enzyme converts L-DOPA and dopamine to DOPA semiquinone and dopamine, respectively. Oxidation of L-DOPA and Dopamine by tyrosinase generates  $\mathbf{O_2}^{\bullet}$  which in turn produces  $H_2O_2$  by the action of SOD (Meyskens Jr *et al.*, 2001, Denat *et al.*, 2014).

The above endogenous and exogenous sources of ROS in melanocyte, listed in Figure 1.7 could be possible risk factors for melanoma pathogenesis (Denat *et al.*, 2014, Meyskens Jr *et al.*, 2001).



Figure 1.7. Intrinsic and extrinsic sources of ROS generation in melanocytes.

ROS in melanocytes can be generated endogenously (e.g. melanogensis, mitochondrial activities, NADPH oxidase and peroxisome) and exogenously (e.g. exposure to UV radiation, inflammation).

Although MM occurs within all ethnic and racial groups (Diepgen and Mahler, 2002), the incidence rate of the disease varies from one population to another (MacKie *et al.*, 2007, Gray-Schopfer *et al.*, 2007). The occurrence of melanoma cancer has been increasing among white populations all over the world for several decades (Diepgen and Mahler, 2002). Lack of skin pigmentation in Caucasians makes their skin more susceptible to the deleterious effects of solar radiations (Polefka *et al.*, 2012). In this particular ethnic group, the incidence rate of melanoma is up to 50 per 100,000 annually, while among the dark skinned ethnic population it is  $\leq 1$  per 100,000 per year (Diepgen and Mahler, 2002).

In those with phenotypic susceptibility (e.g.Caucasian individuals), the cause of melanoma is strongly attributed to UV radiation (Tucker and Goldstein, 2003). In the  $20^{th}$  century, epidemiological studies have shown that factors such as heredity and skin colour affect melanoma development (Gellin *et al.*, 1969). The pigment melanin is able to absorb and block the UV radiation from sunlight and so prevent DNA damage. Approximately 80% of white populations with red hair carry genetic variations in the *melanocortin receptor-1 (MC1R)* gene, which leads to the production of pheomelanin instead of melanin; consequently, the fair-skinned population have lower UV protection and thus are more susceptible to melanoma development (Thompson *et al.*, 2005).

In addition to skin colour related predispositions, attention has also been drawn to many other melanoma risk factors; these factors include having red or blonde hair, numerous freckles and a tendency to burn, presence of congenital or acquired nevi, immunosuppression, scars and occupations associated with electronic and chemical industries (occupational exposure) (Ward *et al.*, 1997, Naldi *et al.*, 2000).

As suggested above, in addition to environmental factors, genetic predisposition plays a vital role in the development of melanoma (Jhappan *et al.*, 2003). Individuals with a family history of melanoma are at greater risk of developing of this type of cancer than those with no history of the disease. Some familial mutations have been found to increase susceptibility to melanoma development. The gene serine/threonine protein kinase B-Raf (BRAF) is a family member of the mitogen activated protein kinase pathway RAS RAF MEK ERK MAPK, which mediates cellular response to growth signals. Normally, the BRAF gene-protein stimulates cell growth and proliferation by transmitting a message from stimuli outside the cell to its nucleus (Figure 1.8A). It has been discovered that the BRAF gene is mutated in some cancers (Davies *et al.*, 2002)

and approximately 50-60% of cutaneous melanoma feature mutations of the BRAF gene (Davies *et al.*, 2002, Jang and Atkins, 2013), with most of these cases (~80%) being V600E mutations (Dhomen and Marais, 2009), ~20% V600K mutations (Long *et al.*, 2011) and V600R mutations being the least frequent (5-7%) (Lovly *et al.*, 2012). The activation of BRAF inside the malignant cells occurs without external stimuli and this causes phosphorylation of the MAPK/ERK kinase pathway; this in turn regulates substrates activities, such as transcriptional factors, which control cell proliferation, differentiation and survival and invasion (Wellbrock and Hurlstone, 2010, Kwong *et al.*, 2012). In BRAF mutated melanoma, the mutant gene (BRAF<sup>V600E</sup>) leads to over activation of BRAF kinases (without external stimuli such as growth factors), which promotes the oncogenic activation of cell proliferation and survival pathways of melanoma cancer cells (Thomas, 2006, Ahmed and Davies, 2011, Puxeddu *et al.*, 2008) (Figure 1.8B).


## Figure 1.8. The role of the BRAF gene in induction, differentiation and survival in (A) normal melanocytes and in (B) BRAF mutated MM cancer cells.

A) Under normal conditions, initiation of growth signal cascades occurs via binding of growth factors to receptor tyrosine kinase at the surface of the cell leading to activation of GTPase and RAS. This in turn induces dimer formation of RAF family of kinase which activates kinase and signal transduction for cell survival and differentiation. B) In BRAF mutated melanoma cells, the mutant (V600E amino acid substitution) constitutively activate the BRAF protein kinases which in turn activate growth, differentiation and invasion (Gibney *et al.*, 2013, Ahmed and Davies, 2011, Puxeddu *et al.*, 2008).

#### 1.2.5 Pathogenesis

Melanoma is a melanocyte malignant tumour. Through malignant transformation, melanocytic cells (melanotic or amelanotic melanocytes), which are present in the basal epidermal layer of skin, convert to melanoma. The incidence of pigmented melanoma cancer (melanotic) is higher than the non-pigmented melanoma (Ma *et al.*, 2015). One of the characteristic features of melanoma cancer during progression is the transformation of melanocytes to pigmented nevi, which then progress to radial and consequently to vertical growth phase melanoma (Clark *et al.*, 1984) and later spread to involve other organs in the body (Regad, 2013). At early stages the primary melanoma less than 1 millimetre wide, then undergoes a 'radical growth' (Hershkovitz *et al.*, 2010). Later, the behaviour of the tumour cells changes when they begin to move vertically into the epidermis and papillary dermis (Hershkovitz *et al.*, 2010); then they spread to the regional lymphatic system and distant organs (Figure 1.9).



Figure 1.9. Development of cutaneous melanoma lesion.

Melanoma cells start to grow radically in the epidermis layer then they start to grow vertically to penetrate the dermis and subcutaneous layer (Arizona Advanced Medicine, 2015).

MM is categorised into four different types. In 1969, Clark and colleagues presented the following classifications of MM: Lentigo Maligna Melanomas (LMM), Superficial Spreading Melanoma (SSM), Nodular Melanoma (NM) and Acral Lentiginous Melanoma (ALM) (Clark *et al.*, 1969). Of all diagnosed melanoma cases, LMM, SSM and NM make up to 90%, whereas ALM and some rare melanoma types make up the rest (Cancer Research UK, 2014e). In the UK, the incidence of SSM is 70%, and is considered the commonest form occurring in middle age groups. As it develops, it

begins to grow outwards and then into deeper layers of skin. Likewise, NM more often occurs in middle aged individuals, but only in parts of the body that are occasionally exposed to sunlight, such as the back and chest. NM lesions rarely grow from moles; they are often dark brownish or black in colour and grow very quickly, downwards into deeper skin (Cancer Research UK, 2014e).

LMM accounts for around 10% of all melanoma cases and develops more in old age. This form originates in skin areas that are exposed to sunlight and with pigments called letigo maligna or Hutchinson's melanotic freckles. Physically, LMM are flat and outward growing lesions, which then grow towards the inner layers. ALM is a rare type of melanoma and commonly develops on the palms of the hands and soles of the feet in dark skinned individuals (Cancer Research UK, 2014e). Although most common melanomas are pigmented, approximately 5% are amelaotic, which contain no or very little melanin pigments. This sometimes makes it difficult for clinicians to diagnose and differentiate these from other common forms of skin cancer or conditions (Cancer Research UK, 2014e).

The direct mutagenic effect of UV radiation on melanocyte DNA promotes malignant changes in the skin (Leiter and Garbe, 2008). UV light also stimulates cell membrane receptors, which in turn produce growth factors that activate oncogenes (such as mutated BRAF) and inactivate tumour suppressor genes (for instance, cyclin-dependent-kinase inhibitor 2A (CDKN2A) that prevent cells from uncontrolled growth and rapidly dividing). UV radiation also inhibit the host's immune system and stimulating the production of the pigment melanin in melanocytes, so producing more intracellular ROS which subsequently causes DNA damage, mutation and suppresses apoptosis (Meyskens *et al.*, 2004). In addition, it has been found that exposure of the epidermis to UV leads to a considerable loss of antioxidants in dermal cells (Pence and Naylor, 1990). All these events can initiate melanoma development (Meyskens *et al.*, 2005).

Overall, accumulating evidence indicates that genetic and molecular pathway alteration in melanocytes leads to melanomas with a diverse biology (Palmieri *et al.*, 2012). The ability of cancer cells to invade is controlled by a cluster of intracellular signal transduction molecules (Kato *et al.*, 2002, Kato and Wickner, 2003). Oncogenic changes, such as genetic (gene mutation, deletion, amplification or translocation) and epigenetic alterations (changes in transcriptional activity, DNA methylation alteration and/or changes in chromatin structure or configuration) are strongly linked with the progression of melanoma (Meier *et al.*, 2005).

## **1.2.6** Current treatment

Among skin cancers, cutaneous melanoma remains the most daunting malignancy as the disease is resistant to therapies; its prognosis has not improved over the last three decades (Young *et al.*, 2006). The degree of this resistance to all current chemotherapeutic modalities is a clinical problem with a huge social impact (Soengas and Lowe, 2003).

The current treatment approach for MM depends on the actual stage of the disease (Balch *et al.*, 2001a). An early diagnosis of melanoma lesions is one of the most important aspects of its management, improving the rate of positive prognosis (Ho and Sober, 1990, Diepgen and Mahler, 2002). Early stage primary lesions are curable simply through surgical excision, and this strategy of treatment remains the gold-standard option for most early diagnosed cases (Garbe *et al.*, 2008). More than 95% of successful melanoma treatment is achieved through a complete surgical excision of primary lesions at stage I and II; this method of treatment may even prolong the survival period of patients with stage III melanoma (Balch *et al.*, 2001a). However, the prognosis of patients with advanced stages is still poor, and surgical management in these cases is mostly ineffective (Soengas and Lowe, 2003).

The extent of surgical excision should be carried out based on the depth of the disease, (Breslow's depth). In early stages, when the melanoma lesion thickness is less than 1.0mm, the surgical excision margin must be 1.0 cm; if the lesion is thicker, a wider excision margin should be applied (Marsden *et al.*, 2010). This is because patients with bigger lesions, based on the AJCC melanoma classification, are at high risk of disease recurrence (Balch *et al.*, 2009, Thomas *et al.*, 2004).

If lymph nodes are not involved in stage II, clinical trial enrolment or adjuvant therapies such as Interferon- $\alpha$  (IFN) have beneficial effects. When melanoma develops to stage III, complete lymphadenectomy is required, followed by adjuvant treatment (Garbe *et al.*, 2010, Coit *et al.*, 2009). MM is rarely curable when it has metastasised to deeper organs; even synergistic combinations of different analogues have not improved overall survival rates in clinical trials (Kirkwood *et al.*, 2001, Atkins *et al.*, 1999, Kirkwood *et*  *al.*, 1996, Garbe *et al.*, 2011). The median survival time among patients with stage IV metastatic melanoma does not exceed 10 months, and the 5 year survival rate is approximately 10% (Balch *et al.*, 2009).

In spite of the minimal advantages of therapeutic agents in terms of improving patient survival rates, many drugs approved by the United States Food and Drug Administration (FDA) are still in use to treat patients with unresectable melanomas (Tarhini and Agarwala, 2006). In 1975, Dacarbazine (DTIC), the alkylating agent, was the first anti-tumour agent approved by the FDA to treat metastatic melanoma, although the response rate was very low (Bedikian *et al.*, 2006, Middleton *et al.*, 2000). Later, single chemotherapies such as Cisplatin, Cramustine, Lomustine and Temazolamide were given to patients in an attempt to improve outcome. However, response rates for these agents does not exceed 20% (Garbe *et al.*, 2011).

The combination of chemotherapies for melanoma treatment has been attempted in many clinical trials (Rusthoven *et al.*, 1996, Saxman *et al.*, 1999, McClay *et al.*, 1987, Falkson *et al.*, 1998), with the overall conclusion being that most regimens provide no better effects than single therapies in terms of tumour response and patient survival rates.

There have also been extensive attempts to use biotherapies in the treatment of unresectable melanoma. Studies have used Interlukin-2 (IL-2), either alone or in combination with other chemotherapies or biological compounds (such as interferon, tumour infiltrating lymphocyte and lymphokine activity killer cells), but the response rate for these regimens has unfortunately been low (Rosenberg *et al.*, 1985, Sznol and Parkinson, 1994). Biochemotherapy has also been used for metastatic melanoma, in order to improve patient quality of life and to enhance the treatment response rate. One study has used a complex combination of Cisplatin, Vinblastine and DTIC, with the biologics Interferon Alfa or IL-2, and found a good treatment response rate in some metastatic cases (Legha *et al.*, 1998). However, more recent studies have found no difference between the outcomes of chemotherapy and biochemotherapy regimens (Atkins *et al.*, 2003, Keilholz *et al.*, 2003, Del Vecchio *et al.*, 2003).

Recently, an anti BRAF mutation (BRAF <sup>V600E</sup>) drug has been investigated in clinical trials for treating BRAF-associated MM cases (Flaherty *et al.*, 2012), whereby the downstream MEK/ERK effector is deregulated by BRAF<sup>V600E</sup>. Vemurafenib is the first selective BRAF<sup>V600E</sup> inhibitor to be used to treat MM in phase I and II clinical trials

(Ascierto *et al.*, 2012). This agent blocks the MAPK pathway in melanoma patients who have a BRAF<sup>V600E</sup> mutation, and provides improved survival and progression-free survival rates (Bollag *et al.*, 2010, Chapman *et al.*, 2011, Hauschild *et al.*, 2012). The response rate (81%) in the first clinical trial indicated initially clear success (Flaherty *et al.*, 2010); however, relapses occur quickly, 8-12 months following treatment (Solit and Sawyers, 2010), and 50% of advanced melanoma cases treated with anti-BRAF agents experience disease progression a few months later as a consequence of tumour microenvironment resistance against BRAF inhibitors in some patients (Sosman *et al.*, 2012). This resistance to BRAF inhibitors often occurs via reactivation of receptor tyrosine kinase (Girotti *et al.*, 2015). In addition, BRAF inhibitors have toxic effects that lead to the development of other skin cancers including SCCs and keratocanthoma (Hauschild *et al.*, 2012, Sosman *et al.*, 2012).

## 1.2.7 Drug resistance

Despite a huge number of clinical trials and advances in strategies for melanoma treatment, disease prognosis has remained dismal (Soengas and Lowe, 2003). This is due to the high degree of resistance this cancer possesses against all current drug modalities. The failure of chemical and immunological agents to treat melanoma is mediated by several complex intrinsic and acquired mechanisms (Fukunaga-Kalabis and Herlyn, 2012). These include: alterations in cellular drug-transport mechanisms, detoxification of some drugs by intracellular enzymes, enhanced DNA repair processes and up-regulation of anti-apoptotic pathways (Helmbach *et al.*, 2001).

Resistance to drugs that are chemically unrelated and may have different mechanisms of action is known as multidrug resistance (MDR) (Arias and Jasiulionis, 2013). In tumour cells there are different MDR mechanisms including decreased drug uptake, increased drug efflux, activation of drug detoxification, DNA repair activation and overexpression of anti-apoptotic proteins (Dean *et al.*, 2005, Gillet and Gottesman, 2010).

Classical MDR, which refers to a natural resistance to hydrophobic agents, is mediated by expression of ATP-dependent efflux pumps with a broad drug specificity that lowers the drug concentration inside the cell by increasing the output rate (Dean *et al.*, 2001). These efflux pumps arise from a family of carrier proteins known as ABC transporters (ATP-binding cassette) (Dean *et al.*, 2001). For instance, non-P glycoprotein or multidrug resistance related protein is a member of the ABC transporter superfamily of ATP-binding cassettes. This protein plays a crucial role in the drug resistance process, through an energy-dependent efflux pump mechanism (Helmbach *et al.*, 2001) and has been found to play a role in MM chemoresistance (Ichihashi and Kitajima, 2001). In addition, it has been found that entering of hydrophilic drugs into the cells depends on the carrier proteins that carry nutrients or agents via endocytosis into the cell in exchange with increased cellular outputs. Failure in the cellular output mechanisms could have an inhibitory effect on water soluble drug uptake (Arias and Jasiulionis, 2013).

DNA damage repair following following treatment of MM with alkylating drugs is another form of drug resistance. DNA damage induction as the result of cytostatic agents can be compensated for by the modulation of DNA repair mechanisms (Chaney and Sancar, 1996). Melanoma resistance to alkylating agents such as Fotemustine and Cisplatin is related to its ability to repair the damaged genome (Rünger *et al.*, 2000). The enzyme O<sup>6</sup>-methylguanine-DNA-methyltransferase (MGMT) (the protein encoded by MGMT-gene) is important for genomic stability. MGMT removes the cytotoxic DNA lesions by transferring the methyl group (Alkyl group) from the O<sup>6</sup>-atom of guanine bases to a cysteine residue (the alkyl group acceptor protein) (Ma *et al.*, 2003). Thus, the enzyme recognises and removes the cytotoxic lesions in the DNA molecule caused by alkylating agents such as DTIC (Ma *et al.*, 2003, Lage *et al.*, 1999).

Apoptosis is an essential step in the killing of susceptible cancer cells via chemotherapeutic agents. A defect in the apoptotic pathway has been found in resistant cancers (Lowe *et al.*, 1993). P53 is a tumour suppressor protein encoded by P53 tumour suppressor gene and functions either by causing growth arrest, apoptosis, senesence or autophagy (Vousden and Prives, 2009, Donehower and Lozano, 2009); it is a non-functional mutant in many cancer cells (Lowe *et al.*, 1993). It is suggested that UV light inactivates P53 in melanocyte and that's why melanoma cancer cells prevent UV-induced apoptosis, with non-functional P53 found to be expressed melanoma cancer cells (Perlis and Herlyn, 2004).

Additional to all above mechanisms of drug resistance, anti-apoptotic proteins are also found to be over-expressed in tumour cells, and so enhance resistance to apoptosis (Igney and Krammer, 2002); high levels of these proteins have also been found in melanoma cancer cells following treatment (Krueger *et al.*, 2001, Leiter *et al.*, 2000). Bcl-2 (B-cell lymphoma 2) is one example of anti-apoptotic protein. BcL-2 is a family

of cell death regulator proteins encoded in humans from the gene BCL-2; the Bcl-2 protein is considered to be an oncogene due to its role as an anti-apoptotic (Cleary *et al.*, 1986). Bcl-2 is highly expressed in melanoma cancer cells and may also contribute to resistance against therapies (Leiter *et al.*, 2000, Tron *et al.*, 1995).

Although studies have found a considerable level of shrinkage in melanoma tumour volume when BRAF inhibitors Vemurafenib and Dabrafenib are used (Chapman *et al.*, 2011, Hauschild *et al.*, 2012), most of the responsive cases in these studies eventually became resistant to BRAF inhibitors. This is due to the reactivation of MAPK signalling after prolonged treatment with BRAF inhibitors (Menzies *et al.*, 2014, Shi *et al.*, 2014). Because of the heterogeneity of melanoma tumours, its response to chemotherapies and targeted therapies is poor, and this issue requires further study.

## 1.3 Oxidative stress

#### **1.3.1** Reactive oxygen species

Reactive oxygen species (ROS) is a general term used to describe a group of chemically reactive molecules containing either molecular oxygen ( $O_2$ ) or atomic oxygen (O). Chemically, ROS are classified into two groups: (I) non-radicals, which do not have unpaired electrons in their outer orbital; and (II) radicals that are active species with one or more unpaired electrons in their outer orbital. Because of presence of unpaired electrons, the radical species are FRs. An extra single electron in their orbital means they are able to either donate or to accept an electron from other molecules in order to obtain chemical stability (Trachootham *et al.*, 2009). Donation or acceptance of an electron allows these species to react with other biological molecules, possibly leading to a series structural altering reactions and possibly chain reactions (Polefka *et al.*, 2012, Pelicano *et al.*, 2004).

Common ROS seen in living systems include oxygen (O<sub>2</sub>), superoxide anion (O<sub>2</sub>•<sup>-</sup>), peroxide (O<sub>2</sub>•<sup>2-</sup>), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical ( $^{\circ}$ OH) and hydroxyl anion (OH<sup>-</sup>) (Hemnani and Parihar, 1998). In terms of ROS categories, H<sub>2</sub>O<sub>2</sub> is a non-free radical species, but chemically is an active oxidative compound and can yield  $^{\circ}$ OH, the most potent FR among the family of ROS (Trachootham *et al.*, 2009, Pelicano *et al.*, 2004) (Table 1.4).

<b>ROS member</b>	Chemical symbol
Oxygen	O <sub>2</sub>
Superoxide anion	O2•-
Peroxide	O <sub>2</sub> • <sup>2-</sup>
Hydrogen peroxide	$H_2O_2$
Hydroxal radical	•ОН
Hydroxal anion	OH

Table 1.4. ROS species and their chemical symbols (Hemnani and Parihar, 1998)

In biological systems,  $H_2O_2$  is more stable and can diffuse between extra and intra cellular spaces. By contrast, •OH is more reactive, with a very short half-life (10<sup>-9</sup> s) and reacts with nearby molecules effectively at the site of its formation. In biological systems, •OH has deleterious effects on cellular constituents, or macromolecules, causing cellular injuries such as protein peroxidation and DNA damage (Sonntag, 1987). The intracellular antioxidant system is important in defending cells from the adverse effects of FR. In mammalian cells the reduction-oxidation, or 'Redox', homeostasis is maintained by detoxifying FRs through an enzymatic system, for instance by superoxide dismutases (SOD) and catalase (CAT) (Zelko *et al.*, 2002, Chelikani *et al.*, 2004), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GSH)) (Meister and Anderson, 1983). There are also many known as non-enzymatic exogenous and endogenous metabolites and substances that defend against detrimental oxidants in the human body in addition to the more commonly known cellular antioxidant systems (Figure 1.10).

# Figure 1.10. Antioxidant candidates including enzymatic and non-enzymatic antioxidants in the human body

Enzymatic and non-enzymatic antioxidants protect living systems from FRs (Hingorani, 2003, Hayden and Tyagi, 2002).



In cells, the primary antioxidants are SOD, catalase and glutathione.  ${}^{\bullet}O_2^{-}$  is an oxygen by-product released from mitochondria during aerobic respiration and SOD converts it to H<sub>2</sub>O<sub>2</sub>. The latter is decomposed to H<sub>2</sub>O and O<sub>2</sub>, either by the enzyme catalase, which is localised in cellular cytosol and peroxisomes, or by GPx (Burlakova *et al.*, 2010, Gutteridge and Halliwell, 1999, Wassmann *et al.*, 2004) (Figure 1.11).



Figure 1.11. Production of ROS in mitochondria and their further metabolism by the main antioxidants.

Mitochondria generate  $O_2^{\bullet}$  from the electron transport chain and this is converted to  $H_2O_2$  by the action of SOD. Normally,  $H_2O_2$  is dissociated to water and  $O_2$  by the action of catalase and/or GSH.

#### 1.3.1.1 Sources of endogenous and exogenous ROS

Initially, it was thought that ROS were only generated by biological systems when they are attacking pathogens; it was later found that ROS have a wide range of cellular functions (Hancock *et al.*, 2001, Devasagayam *et al.*, 2004). In normal cells, ROS are generated and eliminated by the functioning of several regulatory cellular biological systems (Dickinson and Chang, 2011). These include cell signal transduction via transcriptional factors for modulation of genes function, cell differentiation and proliferation, induction of cytokines released during inflammation and the attacking of pathogens or foreign bodies by immune cells (Trachootham *et al.*, 2009).

There are many endogenous sources of ROS in mammalian cells. Most FRs are considered to be formed as by-products of mitochondrial activity. Mitochondrial FRs are formed via transportation of electrons chains across the inner layers of mitochondria during adenosine triphosphate (ATP) production (Richter *et al.*, 1995). Hydrogen ion (H<sup>+</sup>) transport across the mitochondrial inner layers occurs during the process of ATP production, which takes place in electron transport chains (ETC). This process releases an electron that escapes and reacts with O<sub>2</sub>, which generates O<sub>2</sub>•, the later then interacts

with SOD to produce  $H_2O_2$ , which can be either utilised by the cell or converted to water by the action of catalase and/or GPx and GR (Trachootham *et al.*, 2009, Collins, 1999). Other endogenous sources of ROS are the endoplasmic reticulum (ER) (Han *et al.*, 2001) or the redox process assisted by metals and oxido-reductase enzymes (Held, 2010), such as the NADPH oxidise complex (NOX) (Trachootham *et al.*, 2009) and xanthine oxidase (XO) (Figure 1.12).



Figure 1.12. Schematic represents the major endogenous sources of ROS in mammalian cells.

Mitochondria, Endoplasmic reticulum, NADPH, NOX and XO all generate  $O_2^{\bullet}$  which then produce  $H_2O_2$  by the action of SOD (Trachootham *et al.*, 2009).

ROS are also introduced into mammalian cells exogenously. For instance, ROSgenerating drugs such as anthracycline, cisplatin and bleomycin (Deavall *et al.*, 2012, Cullen, 2010), Elesclomol (STA-4783) (Kirshner *et al.*, 2008) are all able to produce ROS in cells. Buthionine sulfoxamine (BSO) (the glutathione synthesis inhibitor) (Reliene and Schiestl, 2006) has also been used to enhance endogenous ROS accumulation in tumour cells. A high concentration of intravenous ascorbic acid in patients has also been shown to generate  $H_2O_2$  extracellulary (Chen *et al.*, 2005). Additionally, pollutants, smoking (Loft *et al.*, 1992), radiation (ionisation and nonionisation), and heat exposure (O'Donovan *et al.*, 2005, Devasagayam *et al.*, 2004) are all able to produce intracellular ROS.

#### **1.3.2** Oxidative stress in melanoma and normal skin cells

A disturbance in the equilibrium between ROS generation and the systems that detoxify ROS is widely defined as oxidative stress (Sies, 1994, Mittler, 2002). Normal cells have a low level of ROS in comparison to cancer cells; in normal cells, ROS whilst frequently produced are efficiently removed/dealt with. However, a growing body of evidence suggests that cancer cells generate greater amounts of endogenous ROS. This feature is thought to be the result of abnormal cancer cell metabolism such as higher metabolic rates through an up-regulation of glycolysis (Verrax et al., 2009), accelerated cell division and proliferation and mitochondrial malfunctioning (Pelicano et al., 2004, Cairns et al., 2011). It has been proposed that NADPH oxidase in normal cells is responsible for H<sub>2</sub>O<sub>2</sub> generation, and that the glutathione system mainly serves to regulate this (Nicco *et al.*, 2005). In cancer cells, however, higher levels of  $H_2O_2$  are released from mitochondria and is regulated mainly by catalase activity in these cells (Nicco et al., 2005). As mitochondria are the major site for ROS generation in cells, they normally contain high levels of antioxidants, including GSH, GPx and SOD in their inner and outer membranes (Cadenas and Davies, 2000). In cancer cells, mitochondrial DNA is very susceptible and can easily be mutated and damaged (Copeland *et al.*, 2002). This change in the controlling mitochondrial genome may alter its normal function. For instance, it has been observed that the level of  $O_2^{\bullet}$  (the precursor for H<sub>2</sub>O<sub>2</sub> generation) in mitochondria of liver cancer cells is substantially higher than that found in normal liver cells (Konstantinov et al., 1987).

Several studies have found that  $H_2O_2$  in cancer cells aids their proliferation and survival. However, excessive amounts attack the cell's DNA, inducing damage, mutation and genetic instability (Park *et al.*, 2005, Henle and Linn, 1997). The chemical mechanism of  $H_2O_2$  toxicity in mammalian cells is mediated by the involvement of transition metal (iron ions) via catalytic Fenton reactions, which produce a strong oxidant (°OH) (Henle and Linn, 1997, Das *et al.*, 2015). In the Fenton reaction, Fe<sup>2+</sup> reacts with  $H_2O_2$  and generates °OH and OH<sup>-</sup> (Equation 1.1).

#### **Equation 1.1. Fenton type reaction**



Unlike other body organs, the skin is chronically exposed to endogenous and exogenous oxidative insults, owing to the direct interface of skin cells with endogenous and environmental oxidants (Briganti and Picardo, 2003). The balance between pro-oxidants and antioxidants in skin cells has been shown to be disturbed by UVB and UVA irradiations (Leccia et al., 2001), particularly in melanocytes, which have low but continuous levels of ROS manifest (Meyskens Jr et al., 2001). A reduction in the level of antioxidants has been shown to result from exposure of the epidermis to UVB (Pence and Naylor, 1990). Animal studies found a great reduction in dermal and epidermal catalase following UV irradiation (Shindo et al., 1993). In addition, an increase in intracellular H<sub>2</sub>O<sub>2</sub> is seen when skin cells are exposed to UVB radiation and/or to incubation with H<sub>2</sub>O<sub>2</sub> (Chang et al., 2002). In vitro studies suggest that some human carcinoma cell lines, including MM, have a high amount of endogenous  $H_2O_2$ (Toyokuni et al., 1995, Szatrowski and Nathan, 1991, Meyskens Jr et al., 2001). More specifically, it has been reported that pigmented melanoma cancer cells generate high levels of endogenous  $H_2O_2$  (up to 0.5nmol/10<sup>4</sup> cells) even without any stimulant (Szatrowski and Nathan, 1991). Histopathological examination of melanoma tissue has also revealed a significant increase in oxidative biomarkers, including lipid peroxidation and malondialdehyde (Sander et al., 2003). As mentioned previously, melanin pigment biosynthesis is a source of ROS generation, including H<sub>2</sub>O<sub>2</sub>. Studies have found that dysregulation in melanin synthesis contributes to ROS accumulation in melanoma cancer cells (Meyskens Jr et al., 2001, Fruehauf et al., 1998, Meyskens et al., 2004). Further evidence suggests that the pigment melanin enhances the production of oxidative species and that the interplay between the ROS generation and the scavenging properties of melanin determines the level of ROS in melanocytes (Kipp and Young, 1999, Kvam and Tyrrell, 1999a). Moreover, it is also evident that melanoma cancer cells and melanocytes both respond to oxidative stress, but in different ways. Melanoma cells insufficiently eliminate the stress induced by peroxide (Meyskens *et al.*, 1997). By contrast, other cell types, including melanocytes and keratinocytes, have been shown to effectively neutralise exogenous oxidative stress (Farmer *et al.*, 2003).

## 1.3.2.1 Antioxidant levels in normal and cancer cells

The intracellular antioxidant system is important for the balancing and regulation of ROS generation and elimination in cells. Under normal conditions, many antioxidants are required to remove unnecessary oxidative species in living cells. However, this system has been shown to be disturbed in malignant conditions (Laurent et al., 2005). Thus, malignant cells may have lower levels of antioxidants compared with their nonmalignant counterparts. Indeed, gross reduction in antioxidant levels, including SOD and catalase, has been observed in tumour cells (Sykes et al., 1978, Laurent et al., 2005). It has also been demonstrated that in cancer cells, though not in normal cells, there is a low involvement of the glutathione pathway in the control of  $H_2O_2$  production (Nicco et al., 2005). Additionally, by contrast to normal counterpart cells, transformed and malignant cells have low antioxidant activities, including GPx and catalase (Valko et al., 2007). Hepatoma cancer cells have no more than 10% total antioxidants (compared with their normal counterparts); they are deprived of the main cellular antioxidant candidates, including catalase, GPx, SOD and GSH. The western blot analysis reveals a low antioxidant profile with no expression of catalase protein (Verrax et al., 2009). Studies have also reported that melanoma cancer cells exhibit a considerably lower amount of antioxidants involving catalase and glutathione enzymes in comparison to melanocytes (Meyskens et al., 1997, Offner et al., 1992, Picardo et al., 1996). In general, normal cells contain catalase at a concentration of up to 100 times higher than that found in cancer cells (Benade et al., 1969). For instance, catalase gene expression in hepatoma cancer cells is down regulated (Sato et al., 1992). Furthermore, a clinical study has also found that SOD activity in erythrocyte of patients with melanoma cancer is significantly lower than that in healthy subjects (Gadjeva et al., 2008). Further, Pelle et al. (2005) have found that keratinocytes (HaCaTs) cells have higher concentrations of catalase and glutathione peroxidase. In addition, in melanoma cancer cells abnormal lower level of MnSOD has been demonstrated (Valko et al., 2006). The low level of catalase in tumour tissue is also thought to be the result of inadequate blood flow to tumour tissue (Ohno et al., 2009). Consequently, normal tissue receive adequate blood supply which is rich in catalase, so efficiently quenches  $H_2O_2$  and protects the cellular compartments from oxidative stress (Ohno *et al.*, 2009) (Figure 1.13).



Figure 1.13. Inadequate level of catalase in tumour tissue.

Normal tissue is supplied with adequate level of catalase by normal blood flow enriched with catalase, efficiently scavenging  $H_2O_2$ ; however, the tumour tissue has reduced blood supply, thus is low in catalase;  $H_2O_2$  transforms to <sup>•</sup>OH leading to cell damage. Adapted and modified from (Ohno *et al.*, 2009).

#### **1.3.3** Oxidatively assaults and cell injuries

#### **1.3.3.1** Oxidatively mediated DNA damage

Oxidatively damaged DNA is the consequence of ROS interaction with genomic elements, causing a variety of lesions including DNA single and double strand breaks (SSBs and DSBs), sugar lesions, purine and pyrimidine modifications and apurine/apyrimidine (AP) sites (Simic and Jovanovic, 1986, Loft and Poulsen, 1996). The sensitivity of cells to DNA damage is complicated by diversity in the ROS members and their effects (Imlay and Linn, 1988). O<sub>2</sub>•<sup>-</sup> is highly diffusible across membranes, but its reactivity is less than <sup>•</sup>OH reactivity. The latter has the ability to react at near diffusion rates (Hemnani and Parihar, 1998) and consequently no enzyme is involved in its neutralisation from the biological systems (Southorn and Powis, 1988).

Formation of <sup>•</sup>OH from  $H_2O_2$  through the Fenton reaction (Equation 1.1) is responsible for damage to DNA (Dizdaroglu, 1991, Halliwell and Aruoma, 1991, Park *et al.*, 2005, Henle and Linn, 1997). In normal conditions, the level of free intracellular iron is reported to be low, but under stress conditions excess superoxide induces release of free iron ions (Fe<sup>+2</sup>) and these can react with  $H_2O_2$  to from <sup>•</sup>OH (equation 1.1) (de Melo *et al.*, 2013). Intracellular generation of •OH occurs through different mechanisms. Iron released from iron-containing molecules participates in the production of the majority of *in vivo* •OH from H<sub>2</sub>O<sub>2</sub> via the Fenton reaction (Valko *et al.*, 2006). The •OH produced in the nucleus reacts with DNA bases and the deoxyribosyl backbone, thus inducing simple/single damages such as single strand breaks (SSBs) and isolated oxidatively DNA base damage lesions (ODBLs).

Ionising radiation damages DNA, either directly through energy deposition in DNA molecules or indirectly through hydrolysis of water to generate <sup>•</sup>OH at the site of energy deposition. This can induce single damages or local multi-damaged sites (MDS), which are more lethal to the cell (Ward, 1988, Valko *et al.*, 2006).

DNA sugar damage occurs as a result of <sup>•</sup>OH attack, with the latter causing hydrogen atom abstraction from the deoxyribose moieties yielding to SSBs and base loss (Henner et al., 1983). This type of damage occurs due to the susceptibility of deoxyribose to abstraction of its hydrogen atoms by <sup>•</sup>OH (Breen and Murphy, 1995). For ODBLs, it has been shown that oxidative damage to the four nucleobases takes place via complex mechanisms (von Sonntag, 1987). These lesions are generated when <sup>•</sup>OH adds at diffusion-controlled rates to the double bond of purines and pyrimidines, the exact site of this attack depending on the electron density (Evans et al., 2004). Purines are oxidised primarily to yield products such as 7,8-dihydro-8-oxoguanine (8-oxo-dG) and 7,8-dihydro-8-oxoadenine (8-oxo-dA), and two derivatives of ring opened formamidopyrimidines (Aruoma et al., 1989); this mechanism is based on the addition of <sup>•</sup>OH to C8 of DNA purine ring to yield a base radical adduct. Subsequently, oxidation of this adduct generates the 8-oxo-species, while reduction of the adduct results in the formation of the formamidopyrimidine (Fapy) species (Henle and Linn, 1997). Of all the DNA base adducts generated by <sup>•</sup>OH, 8-oxo-dG is considered to be the most common, and an excellent marker for estimating oxidative DNA damage (Weitzman et al., 1994, Wagner et al., 1992, Cooke et al., 2003).

ROS-induced DNA damage is not an unusual event, and occurs even in normal cells when they suffer from oxidative stress. It is claimed that thousands of oxidative-induced DNA damages occur in every human cell each day (Hoeijmakers, 2001), but the damage is not persistent. This is because of the damage repair mechanisms that occurs through the action of DNA repair enzymes notably the base excision repairs (BER) pathways (Wallace, 1988, Hoeijmakers, 2001). Oxidative mediated DNA based damages are efficiently repaired through the multi-step mechanism of BER; briefly this involves the recognition and excision of the damaged DNA bases by DNA glycosylase as the first step of this repair pathway. This process is then followed by cleaving the phosphodiester backbone with endonuclease. Next, a DNA polymerase enzyme replaces the missing nucleotide and the remaining nick is sealed by DNA ligase (Janssen *et al.*, 1993, Friedberg *et al.*, 2005). It is hypothesised that in tumour cells the process of the elimination of ROS-mediated DNA damage lesions may be underwhelmed when the level of damage exceeds the cell's DNA repair capacity (Jackson and Loeb, 2001).

In response to oxidative DNA damage, other enzymes also contribute to the repair mechanism. It is found that oxidatively induced strand breaks activate a nuclear enzyme called poly-ADP-ribose polymerase (PARP) (Schraufstatter *et al.*, 1986). This enzyme helps to repair the DNA strand breaks; however, the enzyme activation reduces the cellular levels of nicotinamide (NAD) and ATP, thus inhibiting cell survival and promoting cell death (Carson *et al.*, 1986). An experimental study has demonstrated that the pigment melanin has an inhibitory effect on the DNA damage repair mechanism. The study found that the pigment melanin reduces the ability of DNA repair enzymes, such as the base excision repair enzymes (Wang *et al.*, 2010).

Metal ion involvement in the  $H_2O_2$ -meditaed DNA damage mechanism has been confirmed by many studies (Barbouti *et al.*, 2001, Duarte and Jones, 2007). Depending on the type of cells, the level of intracellular metal ions also varies. A study by Bedrick and co-workers indicates that melanoma cancer cells have the ability to take-up more metal ions than normal melanocytes (Bedrick *et al.*, 1986). In addition, experimental studies indicate that melanin particles have a tendency to attract more metal ions (Sarzanini *et al.*, 1992). This is because the pigment is colloidal and has metal binding sites (Gidanian and Farmer, 2002, Szpoganicz *et al.*, 2002); Indeed, metal-binding to melanin has been shown to mediate ROS formation (Farmer *et al.*, 2003).

## **1.3.3.2** Oxidative mediated cell death

Programmed cell death or apoptosis is an intrinsic cellular activity that causes an overly damaged cell to self-destruct. Typically, organ or tissue homeostasis relies on the apoptotic pathways and any imbalance in their cellular functioning may lead to the uncontrolled growth of cells. In cancer treatment, the promotion of apoptosis pathways is a promising avenue of tackling the disease and preventing metastasis (WasierskaGadek and Maurer, 2011). Many chemotherapeutics currently used to treat cancers induce apoptosis; however, disturbances in the programmed cell death pathways may make cancer cells resistant to chemotherapy-induced apoptosis (Igney and Krammer, 2002).

It has been demonstrated that stress-induced ROS play a key role in apoptosis. It has been suggested that low levels of H<sub>2</sub>O<sub>2</sub> (3-15 $\mu$ M) stimulates cell division and has a proliferative effect on cells, whereas higher levels may lead to growth arrest, apoptosis and/or necrosis of cells (González *et al.*, 2005). Under stress conditions, a high amount of ROS, including <sup>•</sup>OH, induces cellular damage and death (Kannan and Jain, 2000, Pelicano *et al.*, 2004). The direct link between apoptosis and ROS is based on the fact that apoptosis can be induced by H<sub>2</sub>O<sub>2</sub> and inhibited by catalase (Pierce *et al.*, 1991). The susceptibility of cells to ROS attack varies between cancer cells and normal cells. Additionally, a significant reduction in viability has been demonstrated in melanoma cultured cells when both cell types, melanocyte and melanoma, are exposed to exogenous oxidative stress (Farmer *et al.*, 2003).

Some cytotoxic anticancer modalities target cancer cell DNA, either directly or indirectly, to tackle the disease (Roos and Kaina, 2006). Today, ROS generating drugs are used to kill cancer cells oxidatively; however, the mechanism of oxidative mediated cell death is complex and unclear. Induction of oxidative DNA damage has been found to trigger cell apoptosis and this is likely to occur via the formation of particular DNA lesions, including DNA strand breaks (SSBs and DSBs) and DNA-crosslinks (Roos and Kaina, 2006). In addition, certain gentoxic agents induce cell death by interfering in the DNA repair mechanism (Roos and Kaina, 2006). Another study indicates that induction of  $H_2O_2$ -mediated apoptosis occurs via cellular lysosomes, the cellular organ nicknamed "suicide bags" (Duve, 1969). A correlation between the  $H_2O_2$ -mediated apoptosis and partial lysosomal rupture, which eventually leads to an activation of apoptotic cascades (caspase-3), has been reported (Antunes *et al.*, 2001). In addition to DNA damage, a high amount of ROS oxidises cellular lipids and proteins, and enhances mitochondria to release pro-apoptotic proteins triggering cell death (Ott *et al.*, 2007).

## **1.4 ROS mediated approaches for cancer treatment**

The preferential killing of cancer cells is the major goal in the development of any novel anticancer drug/therapy. To date, studies have targeted tumour tissue, based on the genetic variations between normal and cancer cells. These attempts have resulted in advanced therapies, such as oncogene inhibitors, immunotherapies, and hormonal therapies, delivering some clinical successes in recent years (Comoglio *et al.*, 2008, Sequist *et al.*, 2011, Thakur *et al.*, 2013, Arora and Scholar, 2005). However, these therapies face challenges in the form of drug resistance, tumour heterogeneity and the genetic instability of cancer cells (Trachootham *et al.*, 2009).

ROS performs an essential defence function in the human body. Immune cells, including neutrophils, macrophages, lymphocytes and dendritic cells, release  $H_2O_2$  naturally to attack and eliminate pathogens including bacteria, viruses and cancer cells (Samuni *et al.*, 2001). Research has revealed that monocytes and macrophages produce large amounts of  $H_2O_2$  when they come into contact with malignant cells (Fidler and Schroit, 1988, Mytar *et al.*, 1999).

In cancer cells, ROS can be a double-edged sword. Mounting evidence suggests that in contrast to normal cells, cancer cells from different types of tissue and organs contain higher amounts of  $H_2O_2$  (Szatrowski and Nathan, 1991, Lim *et al.*, 2005, Burdon, 1995, Zieba *et al.*, 2000, Kawanishi *et al.*, 2006). In these cells a certain level of ROS promotes cell differentiation, proliferation of the cancer cells and helps them to grow and survive (Boonstra and Post, 2004, Gibellini *et al.*, 2010). However, if ROS levels are slightly increased in cancer cells, then transient cellular damage can occur; however, overproduction and accumulation of ROS is lethal and may induce toxic chain reactions in cells (Ahmad *et al.*, 2004). This can led to irreversible oxidative damage that disables cellular components including DNA (López-Lázaro, 2007).

Physiologically, normal cells retain a balance between ROS production and their disposal, by maintaining redox homeostasis. It has been reported that normal cells can tolerate a certain amount of exogenous ROS, depending on the capacity of their antioxidant systems. However, in tumour tissues this system may be grossly disturbed because of the reduced blood flow and low level of antioxidant enzymes in the tissues, which makes cancer cells more susceptible to the detrimental effects of ROS (Ohno *et al.*, 2009). In spite of the rationality behind treating ROS-generating tumours with

antioxidant therapies, ironically, the mechanisms of many drugs and ionising radiations for killing cancer cells increase ROS (Fruehauf and Meyskens, 2007). Hence, theoretically, antioxidant therapies may inhibit the effects of some chemotherapies and radiation treatments, by eliminating oxidative damage.

Despite a large body of evidence indicating that cancer cells are under profound oxidative stress, such stress is usually believed to be inadequate to result in cell death (Toyokuni *et al.*, 1995). However, increased intrinsic ROS in cancer cells provides a unique opportunity to selectively kill cancer cells, based on their vulnerability to additional ROS (Pelicano *et al.*, 2004). Thus, theoretically, elevating endogenous ROS in cancer cells could result in a selective ROS-mediated approach as a means to kill these cells. Based on this scenario, it has recently been proposed that providing cancer cells with additional ROS from exogenous sources could increase oxidative stress beyond a toxic threshold, thereby overwhelming their antioxidant defences and inducing higher cell death rates (Trachootham *et al.*, 2009, López-Lázaro, 2007) (Figure 1.14).



Figure 1.14. The proposed model for cancer treatment by ROS-mediated approach.

Normal cells have low endogenous ROS levels, while cancer cells have significant amounts of endogenous ROS. Providing these two groups of cells with therapeutic ROS could raise the level of oxidative stress above the threshold and kill the cancer cells selectively (Lopez-Lazaro, 2007; Trachootham *et al.*, 2009).

The sensitivity of malignant and normal cells to  $H_2O_2$ -induced cell death is obviously different. For instance, cancer cells (Leukemia cells) with high levels of endogenous ROS are more sensitive than normal lymphocytes to 2-methoxyestradiol (2-ME), a novel anticancer agent which generates ROS by inhibiting SOD (Huang *et al.*, 2000,

Mooberry, 2003). In *vitro* studies have also demonstrated that the rate of cell death induced by 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> is higher in Burkitt's lymphoma cancer cells than in normal white blood cells (lymphocytes and monocytes), even when the latter are treated with 250 $\mu$ M H<sub>2</sub>O<sub>2</sub> (Chen *et al.*, 2005). Moreover, an animal study reported that using diluted H<sub>2</sub>O<sub>2</sub> as a substitute for pure water in rats implanted with Walker 256 adenocarcinoma xenograph resulted in 50-60% of them being cured of the disease (Holman, 1957). A linear correlation between renal carcinoma cell death and oxidative markers, such as 8-oxo-dG has been observed after cell exposure to the ROS-inducing agent ferric nitrilotriacetate (Toyokuni *et al.*, 1995).

The direct administration of H<sub>2</sub>O<sub>2</sub> to patients with cancer has not been undertaken because of anticipated toxicity levels (López-Lázaro, 2007). However, as mentioned above, using an  $H_2O_2$ -generating system is a rational way to supply tumour tissues with  $H_2O_2$ . This can be performed by introducing therapeutic  $H_2O_2$  in cancer cells chemically, by certain anticancer drugs such as arsenite (Szymczyk et al., 2006), motexafin gadolinium (Magda and Miller, 2006), doxorubicin, cisplatin, and bleomycin (Deavall et al., 2012), Elesclomol (STA-4783) (Kirshner et al., 2008). Depletion of intracellular glutathione by bothiunine sulfoxamine (BSO) (Sun et al., 2012, Bailey et al., 1994) is another way for elevating endogenous ROS within in cells. These agents induce formation of more intracellular ROS, either directly or indirectly, according to different mechanisms (Schumacker, 2006). BSO acts by irreversibly inhibiting yglutamylcysteine synthetase in cells, thereby inhibiting glutathione levels (Griffith, 1982), leading to an accumulation of oxidative species in cells (Gokce et al., 2009) (Figure 1.15). Elesclomol (STA-4783) is considered a novel oxidative stress inducer. The drug binds with copper ions  $(Cu^{+2})$  in the plasma (extracellular) causing structural changes in the drug, which enable it to enter the cell and eventually the mitochondria. Once inside the mitochondria, the complex (Elesclomol-Cu<sup>+2</sup>) interacts with the electron transport chain (ETC) (the energy producing mechanism within mitochondria). This interaction reduces Cu<sup>2+</sup> to Cu<sup>1+</sup>, triggering sequential redox reactions which ultimately generate oxidative stress (Blackman et al., 2012) (Figure 1.16).



Figure 1.15. Intracellular GSH depletion by BSO enhances endogenous ROS accomulation.

When BSO enters into cells it inhibits the synthesis of GSH, which functions as an antioxidant against  $H_2O_2$ . Depletion of GSH can cause accumulation of intracellular ROS, generated from metabolic process inside the cell. The figure has been adapted and modified from (Higuchi, 2004).



Figure 1.16. ROS production mechanisms by Elesclomol.

Extracellularly, Elescionol binds with  $Cu^{2+}$  and then enters the cells and then the mitochondria. Inside the mitochondria  $Cu^{2+}$  is reduced leading to generation of oxidative stress (Blackman *et al.*, 2012).

The ability of drugs to induce ROS formation varies. 1µM of doxorubicin induces the generation of more than 40 pM of intracellular  $H_2O_2$  in cancer cells; this is the level that can be achieved by treating cells with media containing 500µM  $H_2O_2$  (Wagner *et al.*, 2005). Moreover, a further study, which incubated lymphoma cancer cells (Burkitt's lymphoma cells) with Elesclomol, reported a substantial rise in ROS levels. In the first half an hour there was just a 20% increase in ROS, but 6 hours after incubation the amount had increased by 19-fold (Kirshner *et al.*, 2008). Not only can the drugs generate ROS, but ascorbic acid (AA) autoxidation can also induce the formation of

 $H_2O_2$ . More than  $40\mu$ M of  $H_2O_2$  was detected in media containing mesotheliomia cancer cells when incubated with 1mM AA for 1 hour (Ranzato *et al.*, 2011). The formation of  $H_2O_2$  by AA is a consequence of its interaction with metal ions. Ascorbate participation in metal dependent  $H_2O_2$  generation occurs when it reduces  $Fe^{3+}$  to  $Fe^{2+}$ ; the later reduce  $O_2$  to generate  $O_2^{\bullet^-}$  which is an essential component required to form  $H_2O_2$ (Figure 1.17) (Buettner and Jurkiewicz, 1996, Miller *et al.*, 1990, Duarte and Jones, 2007).



Figure 1.17. Generation of metal dependent H<sub>2</sub>O<sub>2</sub> by ascorbate.

## 1.5 Vitamin C

## 1.5.1 Chemistry of ascorbate

Hexuroic acid was the first term used to describe L-ascorbic acid (AA) (ascorbate) (also known as vitamin C) (Dische, 1947). This vitamin was first discovered and isolated in 1928 (Szent-Györgyi, 1928); since then, its complex biological role in living systems has been the subject of continuous study. AA is a versatile water-soluble vitamin that has a wide range of essential biological functions in the human body. This multifaceted vitamin regulates iron absorption and iron uptake, collagen biosynthesis and hydroxylation of carnitine for connective tissue development, biosynthesis of noradrenaline (the neurotransmitter) from dopamine, tyrosine metabolism and amidation of peptides (Prockop, 1995, Levine *et al.*, 1992, Eipper *et al.*, 1993, Englard and Seifter, 1986) and potentiates the immune system by activating the macrophages (Watson *et al.*, 2010).

Chemically, a ketolactone, 2-oxo-L-threo-hexono-1,4-Lactone-2,3-enediol, refers to vitamin C, which has a molecular weight of 176.13 and two major forms: ascorbate and dehydroasorbate (DHA) (Naidu, 2003, González *et al.*, 2005). A five-membered heterocyclic lactone forms the enediol ring of ascorbate that is responsible for its physical and biochemical properties (Tolbert *et al.*, 1975) (Figure 1.18).



Figure 1.18. Chemical structure of ascorbic acid.

## 1.5.1.1 Antioxidant and prooxidant role of vitamin C

Ascorbate is known as a powerful antioxidant in living organisms (Halliwell, 1996). It is capable of scavenging most biological FRs, and effectively quenches FRs including singlet oxygen, superoxide and hydroxyl radicals. The reaction between those FRs and ascorbate yields the ascorbate radical (a one-electron oxidised product), which can then further be oxidised to from dehydroascorbate (DHA) (Shapiro and Saliou, 2001). This reaction is the result of low, one-electron reduction potentials of both, ascorbate and the ascorbate radical, 282 and -170mV respectively. Furthermore, ascorbate has the ability to be regenerated from the ascorbate radical; the latter is unreactive, which is due to the resonance stabilisation of unpaired electron, and can readily dismutate to AA and DHA (Figure 1.19A) (Carr and Frei, 1999a). In mammalian cells, ascorbate can be rapidly regenerated and trapped intracellularly from its oxidised form (DHA); this happens via the action of GSH/NADPH system (May, 1998) (Figure 1.19B).



Figure 1.19. Ascorbate reduction and oxidation reactions.

A) Ascorbate oxidation to yield ascorbyl radical and dehydroascorbate by two successive one electron steps (Spencer and Bow, 1964, Carr and Frei, 1999a). B) Ascorbate regeneration from DHA (May, 1998).

In addition to its numerous biological functions, ascorbate also acts as a potent reducing agent; this chemical property enables it to be involved in redox reactions (Duarte and Lunec, 2005, May, 1999). The redox properties of AA are based on the fact that ascorbate and ascorbate radical are capable of reducing most biological FRs (Halliwell and Gutteridge, 1985). Although this chemical feature is the reason AA is considered an effective antioxidant within the body, notably, it also acts as a pro-oxidant and a source of ROS generation in living systems (Duarte *et al.*, 2007, Halliwell, 1996). It has been reported that under certain conditions, such as low ascorbate concentration and in the presence of free metal ions, vitamin C behaves as a pro-oxidant (Buettner and Jurkiewicz, 1996). The conditions of and the evidence for ascorbate acting as a pro-oxidant will be discussed further below.

## 1.5.2 Bioavailability, transport, and absorption of AA

With the exception of humans and other primates, most animals are able to synthesise large quantities of ascorbate endogenously through monosaccharaide metabolism. However, humans, higher primates and guinea pigs rely on dietary ascorbate, as they lack the enzyme gulono- $\gamma$ -lactone oxidase (GULO), which converts L-gulono- $\gamma$ -lactone to 2-keto-L-gulonolactone which then spontaneous converts to L-ascorbatic acid (Padayatty *et al.*, 2003). This lack of GULO is the result of multi-inactivating mutations of the gene that encodes GULO (Nishikimi *et al.*, 1988, Nishikimi *et al.*, 1994) (Figure 1.20).



Figure 1.20. The biosynthesis pathway of vitamin C in animals.

The formation of ascorbate acid from monosaccharide (glucose) in some animals occurs by a multistep pathway. Lack of the enzyme gulono- $\gamma$ -lactone in humans and some animals make them deficient in the de novo synthesis of ascorbate (Nishikimi *et al.*, 1988, Nishikimi *et al.*, 1994).

Almost all vegetable foods contain vitamin C, and due to a defect in the *de novo* synthesis of the ascorbate pathway in humans, AA remains one of the essential micronutrients for the body (Griffiths and Lunec, 2001). This vitamin is thus a crucial dietary requirement for ensuring human health. AA also has extensive clinical roles; the first being discovered in the early years of the last century during an investigation of the aetiology, prevention and treatment of scurvy, a classical ascorbate deficiency disease. The disease manifests as a defect in the connective tissue system, and may ultimately lead to death (Li and Schellhorn, 2007). AA has also been used to prevent and treat common colds and infections (Pauling, 1971), wound healing (Hellman and Burns, 1958) and to prevent atherosclerosis (Frei, 1997). Furthermore, AA has long been studied in relation to cancer prevention and treatment (Cameron *et al.*, 1979,

McCormick, 1952, Padayatty et al., 2006, Chen et al., 2005, Chen et al., 2008, Padayatty et al., 2010).

The recommended daily allowance (RDA) for vitamin C is based on several factors: the relationship between the intake doses and plasma concentration steady state, renal excretion threshold, bioavailability and possible side effects. It has been found that approximately 70-90% of AA can be successfully absorbed from a moderate intake of 30-180mg per day (Jacob and Sotoudeh, 2002). Peak absorption, however, falls to less than 50% when the dose exceeds 1gm per day (Jacob and Sotoudeh, 2002). Pharmacokinetics data suggests that a mean peak level of plasma ascorbate reaches 100µM or more through oral supplementation (Padayatty *et al.*, 2004, Woollard *et al.*, 2002, Choi *et al.*, 2004, Levine *et al.*, 2011). A study carried out on 12 volunteers and lasting for 8 weeks found that 500mg/day of vitamin C orally significantly raised the plasma and skin content of ascorbate (McArdle *et al.*, 2002).

Ascorbate and DHA obtained as part of a typical human diet are absorbed via enterocytes of the small intestine. However, this cannot occur by simple diffusion because of the polarity of ascorbate and its relatively high molecular weight. Thus, ascorbate is transported across the cell membrane and trapped intracellularly via two different mechanisms: active transport (sodium-dependent active transport) and facilitated diffusion. The active transport mechanism is mediated by specific classes of membrane proteins known as sodium vitamin C co-trasporters (SVCT), whereas facilitative diffusion is carried out via facilitated glucose (hexose) transporter (GLUT) (Li and Schellhorn, 2007). The reduced form of vitamin C (ascorbate) is transported in and out of the cell via an active SVTC that has two isomers: SVTC1 and SVTC2.

The oxidised form of vitamin C (DHA) can be imported by GLUT1 and GLUT3. When ascorbate is present extracellularly, it is oxidised to DHA, which then diffuses into the cell via the GLUT transporter, where it is then reduced back to ascorbate (Figure 1.21) (Li and Schellhorn, 2007).



Figure 1.21. Mechanisms of ascorbate transportation across cell membrane.

A) Ascorbate is oxidised to DHA extracellularly and is then transported via GLUT into the cell where it reduced back to ascorbate. B) Sodium (Na<sup>+</sup>) is coupled to Asc (AA) and through SVCT directly moves into the cell. The excess intracellular accumulated Na<sup>+</sup> ions are exported in exchange of extracellular potassium (K<sup>+</sup>) ions using Na<sup>+</sup> - K<sup>+</sup> ATPase. These figures are adapted and modified from (Li and Schellhorn, 2007).

Many human cells use the GLUT mechanism to uptake AA. However, melanocytes and melanoma cancer cells utilise both mechanisms of vitamin C transportation. Furthermore, it has been shown that melanoma cells are able to uptake ascorbic acid at a rate 100-fold higher than normal melanocytes do (Spielholz *et al.*, 1997). Additionally, one of the GLUT isomers, GLUT-1, which is responsible for DHA transportation, is highly expressed in tumour tissue (Mochizuki *et al.*, 2001), including melanoma cancer cells (Koch *et al.*, 2013). The high quantities of GLUT in tumour cells is thought to be the result of the greater glucose requirement by these cells; this enhances the uptake of both ascorbate and DHA by these cells (Spielholz *et al.*, 1997).

In the small intestine, vitamin C bioavailability and absorption is regulated by ascorbate concentration and the active SVTC transporters (Jacob and Sotoudeh, 2002). Studies examining ascorbate transporting mechanisms in various cell types have found that high and low affinity transporters exist, with respective  $K_m$  values of 5-150µM and 1-5mM (Rose, 1988, Tsukaguchi *et al.*, 1999). However, uptake ability also depends on the cell type, and concentration and duration of exposure to ascorbate. One study has found that treatment of cultured pancreatic cancer cells (MIA PaCa-2) with 50µM, 1mM and

18mM of AA for 4 hours results in an intracellular accumulation with 6mM, 8mM and 18mM respectively (Ullah *et al.*, 2012). However, when treating skin fibroblasts with 1mM ascorbate for approximately 18 hours, their uptake was just 0.4mM (Vissers *et al.*, 2007).

Ascorbate is the most common form of AA in the human body and in plasma (Welch et al., 1995). The physiological level of AA in human tissue is 50-fold higher than in plasma (Welch et al., 1995). The normal plasma level of ascorbate ranges from 30-100µM, but it is manifest at milimolar concentrations in various tissue types (Shapiro and Saliou, 2001). This is because vitamin C transport takes place against the concentration gradient. Differences in ascorbate levels among tissues and organs, and between tissue layers of the same organ, have been reported. For instance, every 100g of brain (wet-tissue) contains 13-15mg of ascorbate; however, the same amount of skin tissue contains only 0.4 to 1mg of ascorbate (Levine and Hartzell, 1987). Furthermore, in skin layers, ascorbate deposition is higher in the dermis  $(3.8\mu M/g)$  than epidermis  $(0.7\mu M/g)$  (Levine and Hartzell, 1987). Another study has found that in white blood cells the ascorbate concentration is 6-8mM, while plasma ascorbate remains at micromolar levels (Alexandra et al., 1995). Among the various types of tissues and organs in humans, adrenal and pituitary glands have the highest level of ascorbate deposition (Table 1.5) (Hornig, 1975). Ascorbate levels of tissue may vary between healthy and diseased cells; for instance, cancer patients often have abnormally low levels of plasma ascorbate (Head, 1998, Cameron et al., 1979, Mayland et al., 2005, Schleich et al., 2013). Low levels of ascorbate in such cases could indicate over utilisation and a high requirement for vitamin C by tumour tissue (Cameron et al., 1979).

#### Table 1.5. Ascorbate level in different tissues and organs .

Below organs and tissue contain high amounts of ascorbate (Hornig, 1975, Terpstra *et al.*, 2010, Emir *et al.*, 2011).

Tissue	Ascorbate (mmol/Kg wet tissue)
Adrenal gland	1.7-2.3
Pituitary gland	2.3-2.8
Liver	0.8-1
Spleen	0.8-1
Pancreas	0.8-1
Kidneys	0.28-0.85
Skeletal muscles	0.17-0.23
Brain	0.74-0.85
Placenta	0.23-0.72

## 1.5.3 Vitamin C in cancer treatment and prevention

A number of epidemiological and clinical studies have reported beneficial effects for AA, including in the targeting of chronic diseases such as cancer (Weber et al., 1995, Packer, 1997, Gey, 1998, Khaw et al., 2001). Different studies have independently concluded that foods rich in AA, such as fruit and vegetables, afford protection against cancer development (Block et al., 1992, Steinmetz and Potter, 1996, Gey, 1998). Furthermore, some studies have reported an inverse relationship between the plasma level of vitamin C, and cancer risk (Gonzalez and Riboli, 2010, Mayland et al., 2005). From a total of 46 studies, focused on vitamin C status in non-hormone-dependent cancers, 33 have found a significant correlation between ascorbate level and a protective effect (Ullah et al., 2012). Some conditions cause a gross disturbance to occur in the bioavailability of ascorbate in human tissue, and studies show a very low level of vitamin C reserves in cancer patients (Núñez, 1994, Anthony and Schorah, 1982). Furthermore, a correlation has been demonstrated between ascorbate deficiency and decreased survival rates in cancer patients (Mayland et al., 2005). Recent animal studies have reported an inhibitory effect on melanoma cancer cell growth/metastasis in ascorbate supplemented mice (Cha et al., 2011, Cha et al., 2013). A clinical study has also reported a gradual reduction in the level of plasma ascorbate in patients with MM stage, with significant reduction (27.3%) noted in plasma ascorbate levels in patients with stage IV (Schleich et al., 2013).

The application of AA in cancer treatment is not new; indeed, it has been a controversial subject for decades. The first idea to propose using vitamin C in malignant diseases was in 1949 (Klenner, 1949). This was then further supported by the work of Pauling and Cameron in the 1970s, who reported improvements in the outcomes for a variety of advanced cancer cases when administrating high intravenous doses of AA (Cameron and Pauling, 1976, Cameron and Pauling, 1978). This study was later criticised, however, by Moertel et al. (1985), who investigated the effectiveness of ascorbate in progressed cases of cancer using megadoses of ascorbate administered orally, the inconsistency between the two above studies is thought to result from the variation in the plasma levels of ascorbic acid achieved orally vs. intravenously (Li and Schellhorn, 2007). Injecting vitamin C intravenously can result in a plasma level of ascorbate approximately 70-fold greater in healthy individuals than that achieved by oral administration (Padayatty et al., 2004). For this reason, recent pharmacokinetics studies, which have explored the effectiveness of the ascorbate-cancer correlation, has lead to a growing interest in reinvestigating vitamin C feasibility as a cancer treatment (Li and Schellhorn, 2007, Chen et al., 2008, Padayatty et al., 2006). Chen et al. (2005) examined the ascorbate effect in several human and mouse cancer cell lines, including melanoma cancer cells, and found a growth inhibitory effect on cells after incubation for one hour with 0.3-20mM ascorbate (Chen et al., 2005). In addition, a recent metaanalysis study indicated that dietary vitamin C is significantly associated with a risk of breast cancer mortality (Harris et al., 2014).

Data on the mechanism of vitamin C activity against cancer cells is inconsistent. Several mechanisms are proposed to be involved in cancer prevention:

- Initiation and promotion of collagen (connective tissue) synthesis, to act as a strong physical barrier preventing tumour metastasis (McCormick, 1954, Cha *et al.*, 2011);
- 2) Inhibition of carcinogens formation such as nitrosamines (Hecht, 1997);
- Fortification of the immune system against invasion by cancerous cells (Carr and Frei, 1999b, Jacob and Sotoudeh, 2002, Cha *et al.*, 2013);
- 4) Tumour suppressor gene activation (Chiang *et al.*, 1994);
- Neutralisation of free radicals that cause oxidative DNA damage and cell mutations (Li and Schellhorn, 2007, Lutsenko *et al.*, 2002, Sweetman *et al.*, 1997).

The possibility has even been raised that vitamin C kills cancer cells through its effect on the cellular microenvironment. In cancer cells, the transcription hypoxia-inducible factor-1 (HIF-1) drives tumour cells to live and grow in response to metabolic stress. Proposals suggest that in tumour cells, ascorbate deactivates and down regulates the expression of HIF-1, leading to the regression of tumour growth (Kuiper *et al.*, 2010). A particular study, which concerned the influence of ascorbate on MM suggested a positive relationship between ascorbate and transferrin expression (the protein that uptakes iron ions). This was proposed as the mechanism behind ascorbate mediated apoptosis in melanoma (Kang *et al.*, 2005b). Another study suggested that ascorbate stimulates release of inflammatory cytokines to combat cancer cells (Cha *et al.*, 2011).

To elucidate the exact toxic mechanism of ascorbate on cancer cells many hypotheses have been tested in *in vitro* studies in recent years. Although antioxidation is one of vitamin C's main biological roles, it has been found that the pharmacological concentrations of ascorbate, and the formation of H<sub>2</sub>O<sub>2</sub> in extracellular spaces are strongly linked (Chen et al., 2005, Chen et al., 2007). Such concentrations can kill cancer cells in vitro selectively; since, this level of ascorbate mediates high amounts of  $H_2O_2$  formation (mentioned above) in the presence of metal ions, which is large enough to damage cancer cell DNA and other organelles, including mitochondria (Ohno et al., 2009). Based on this mechanism many experimental and clinical studies have been undertaken in previous and recent years. Similar effects have been demonstrated in many in vivo (Podmore et al., 1998, Chen et al., 2007, Verrax and Calderon, 2009) and in vitro studies (Udenfriend et al., 1954, Du et al., 2010, Chen et al., 2005, Chen et al., 2008). The role envisaged for ascorbate in all these studies is thought to be based on the availability of redox-active metal ions (Berger et al., 1997). It is suggested that high intravenous doses of vitamin C induce the generation of H<sub>2</sub>O<sub>2</sub> in extracellular spaces, through the reduction of metal ions (Levine et al., 2011, Chen et al., 2007) (Figure 1.22). More recently it has been shown that  $H_2O_2$ , induced by high concentrations of ascorbate, caused depletion in cancer cell adenosine-triphosphate (ATP) and mediated DNA damage (Ma et al., 2014).

## 1.5.4 Pro-oxidant role of ascorbate

Despite evidence that under physiological conditions ascorbate plays a potent antioxidant role, by scavenging free radicals and inhibiting their cytotoxicity (Carr and Frei, 1999a, Pflaum *et al.*, 1998), paradoxically, evidence indicates that under certain

conditions ascorbate functions as a pro-oxidant. Low micromolar concentrations of ascorbate perform this function when active transitional metal ions are available. Metal ions are reduced by ascorbate and these in turn react with  $H_2O_2$ , which generates damaging <sup>•</sup>OH via the Fenton reaction (Stick *et al.*, 1976, Duarte and Jones, 2007, Satoh and Sakagami, 1996, Gutteridge and Halliwell, 1999, Watson *et al.*, 2010) (Figure 1.22). It is worthwhile mentioning that under normal conditions this is not relevant, because most transition metal ions are inactive and bound to proteins (e.g Transferrin) *in vivo* (Halliwell and Gutteridge, 1986).

#### Figure 1.22. Proposed pro-oxidant mechanism of ascorbate in biological systems.

At the extracellular level, ascorbate generates  $H_2O_2$  and at the intracellular level it interacts with metal ion to generate  ${}^{\bullet}OH$  from  $H_2O_2$ .



Multiple studies have used vitamin C in combination with other therapeutic approaches (ranging from chemo-to-radiotherapy), to examine the efficacy of ascorbate as a synergistic agent in cancer treatment (Kurbacher et al., 1996, Verrax et al., 2004, Taper et al., 1995, Waddell and Gerner, 1980). Hypotheses about ascorbate's co-effect on drug effectiveness in cancer treatments differ. In recent years, unimproved in the sensitisation of tumour cells to therapies in the presence of ascorbate have been reported (Heaney et al., 2008). It was thought that ascorbate enhances drug delivery to cancer cells through activation of membrane transport, thus overcoming a known mechanisms of therapeutic resistance (Chiang et al., 1994). Others have claimed that ascorbate potentiates the drug effectiveness, through the down regulation of apoptotic inhibitors and the up regulation of pro-apoptotic factors (Reddy et al., 2001). Using animal models, a reduction in tumour growth and size was reported with pharmacological doses of vitamin C combined with cupric sulphate. The effect was due to redox-modulation, which generates H<sub>2</sub>O<sub>2</sub> (Reddy et al., 2001). More interestingly, it has been observed that ascorbate modulates the oxidative stress induced by indomethacin (a non-steroid agent that causes oxidative stress by mitochondrial dysfunction), and that this resulted in tumour shrinkage (Waddell and Gerner, 1980). As alluded to above, the majority of ascorbate-cancer related papers cite the pro-oxidant effect of vitamin C against cancer cells. However, most rely on high intravenous concentrations of ascorbate to achieve this result, resulting in considerable debate among clinical researchers. One of the points underlined, is that pharmacological concentrations of ascorbate could have negative effects on some patients. This can lead to haemodialysis in glucose-6-phosphatedehydrogenase deficient patients (Rees et al., 1993). Because oxalate is an ascorbate byproduct, hyperoxaluria was also observed after intravenous administration of vitamin C. This could acidify urine and promote oxalate stone formation (de la Vega et al., 2004, Massey et al., 2005).
### **1.6** Aims and objectives of this project

Of known skin cancers, MM is the most lethal and malignant disease. Its incidence is continually increasing, and it represents a significant health burden to many communities worldwide. Although surgical approaches remain the mainstay of treatment for earlier stage melanomas, later stages are highly resistant to current modalities, with a dismal prognosis; consequently, there is urgent need for superior novel treatments.

A large body of evidence suggests that, unlike normal cells, cancer cells from different tissue sources generate higher amounts of  $H_2O_2$  (Szatrowski and Nathan, 1991, Lim *et al.*, 2005, Burdon, 1995, Zieba *et al.*, 2000, Kawanishi *et al.*, 2006). MM is one of those tumours known to produce abnormally high levels of endogenous ROS (de Melo *et al.*, 2013). A certain level of endogenous ROS promotes cancer differentiation and proliferation, and helps cancer cells to survive (Boonstra and Post, 2004, Gibellini *et al.*, 2010). However, this biological feature is not entirely favourable to cancer cells and when the amount of intracellular ROS exceeds a threshold level, it exhausts the cellular defence system potentially killing the cell (Trachootham *et al.*, 2009).

Therefore, in cancer cells, high levels of endogenous ROS can be exploited to tackle the disease using a radical approach (Trachootham *et al.*, 2009, Dewaele *et al.*, 2010). This strategy has recently become the subject of interest among researchers. For instance, providing cancer cells with additional therapeutic-derived ROS, could raise the cellular oxidative stress from pro-survival levels to a lethal level, resulting from irreversible oxidative damage to the cellular macromolecules, such as the DNA (López-Lázaro, 2007).

Vitamin C is a well-known antioxidant; but interestingly it also functions as a prooxidant in biological systems by modulating oxidative stress through Fenton reactions. Consequently, many clinical/biological studies have been undertaken to elucidate the possible effect of ascorbate on tumour tissues (Levine *et al.*, 2009, Ullah *et al.*, 2012, Du *et al.*, 2010, Chen *et al.*, 2005, Padayatty *et al.*, 2006). Their observations concerning the mechanism of ascorbate activity against cancer have enhanced the interest of clinicians to re-focus on the clinical plausibility of ascorbate in cancer treatments (Ullah *et al.*, 2012, Freilich *et al.*, 2014). However, the majority of these preclinical and in *vitro* studies have relied on high doses of ascorbate to generate  $H_2O_2$  in the extracellular spaces, via the Fenton reaction (Ullah *et al.*, 2012, Chen *et al.*, 2008); and this can have toxic drawbacks including haemodialysis in glucose-6-phosphate-dehydrogenase deficient patients and hyperoxaluria. However, when ascorbate is present inside the cancer cell it could cause exogenously produced  $H_2O_2$  to generate <sup>•</sup>OH by promoting the Fenton reaction, with the produced <sup>•</sup>OH attacking the DNA, causing damage and breakage (Duarte and Jones, 2007).

Therefore it is hypothesised that one of the biological functions of ascorbate is to enable  $H_2O_2$ -induced DNA damage and cell killing, but if the tumour cells are deficient in ascorbate this may compromise the programmed cell death that occurs when cells are subject to oxidants, thereby enabling tumour cells to survive. Therefore, it is proposed that ascorbate accelerates  $H_2O_2$ -induced melanoma cell death via the modulation of oxidative stress, with vitamin C serving as a "DNA damage switch" to promote cell death, notably apoptosis.

In addition, previous studies have reported that ascorbate induces apoptosis and has a negative effect on melanoma cancer cell proliferation (Bram *et al.*, 1980), and it has been reported that melanoma cancers are more susceptible to vitamin C toxicity than other cancer cells (Kang *et al.*, 2005b). Furthermore, a recent study demonstrated an inhibitory effect of ascorbate supplementation on melanoma metastasis and reduced tumour growth in vitamin C deficient mice (Cha *et al.*, 2013).

## **Research objectives:**

- 1. To investigate the hypothesis that ascorbate accelerates  $H_2O_2$ -induced DNA damage in melanoma cancer cells.
- To study the effects of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced melanoma cancer cell apoptosis and cell killing.
- To further investigate the above effects when using drugs to increase cellular oxidative stress instead of H<sub>2</sub>O<sub>2</sub>.
- 4. Investigate the effect of ascorbate on oxidative mediated DNA damage in primary melanoma tumour tissue induced by the H<sub>2</sub>O<sub>2</sub> and ROS-inducing drugs.

The outcomes of this study will establish intracellular oxidative stress mechanisms, resulting from oxidative modulation by ascorbate, in melanoma cancer and normal cell lines, and will contribute to a basic understanding of intracellular oxidative stress in this cancer model.

**CHAPTER II: Materials & Methods** 

# 2.1 Materials

## 2.1.1 Chemicals and cell culture materials

## 2.1.1.1 Cell culture media and additives

Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute-1640 (RPMI-1640), Roswell Park Memorial Institute-8764 (RPMI-8764), Gibco cascade medium 106, foetal bovine serum (FBS), L-glutamine (GlutaMAX-I), non-essential amino acids (NEAA) and sodium pyruvate, Hank's Balanced Salt Solution (HBSS) and media 199 were all purchased from Life Technologies (Paisley, UK).

## 2.1.1.2 Chemicals

Gold antifade/DAPI-SlowFade<sup>®</sup> and ultra-pure dH<sub>2</sub>O were purchased from Life Technologies (Paisley, UK). Elesclomol (STA-4783) was purchased from Selleckchem (Houston, Texas, USA). Desferrioxamine (DFO) was purchased from Santa Cruz Biotechnology Inc (Heidelberg, Germany). Goat milk powder, Trypan blue stain, crystal violet stain, propidium iodide (PI), 2', 7'-Dichlorofluorescin diacetate (DCFH-DA), normal melting point (NMP) agarose and low melting point (LMP) agarose, phosphate buffer saline (PBS), sodium chloride (NaCl), tabs sodium ethylenediaminetetraacetic acid (Na<sub>2</sub>EDTA), sodium hydroxide (NaOH), hydrochloric acid (HCl), acrylamide (30% acrylamide: bis acrylamide), N-Acetyl-L-cysteine (NAC) dimethyl sulfoxide ethylenediaminetetraacetic acid (DMSO), (EDTA), tris hydrochloride (Tris HCl), potassium chloride (KCl), tris-base, triton X-100, normal goat serum, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), L-ascorbate,  $H_2O_2$  (30% v/v), Buthionine sulfoximine (BSO), sodium dodecyl sulphate (SDS), tween-20, glycerol, S, acetic N,N,N,Nethanol, methanol, ponceau acid, glycine, tetramethylethylenediamine (TEMED), ammonium persulphate, 1-butanol. 2mercaptoethanol, bromophenol blue were all obtained from Sigma Aldrich (Gillingham, UK).

## 2.1.1.3 Enzymes and antibodies

Trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA 10x) and catalase from bovine liver were purchased from Sigma Aldrich (Gillingham, UK). Liberase Blendzyme Thermolysin enzyme was purchased from Roche Diagnostics (Sussex, UK). Formamidopyrimidine DNA glycosylase (Fpg) was purchased from New England Biolabs (Hertfordshire, UK), Anti-phospho-histone H2A.X (ser139) antibody, clone JBW301 Mouse Monoclonal Antibody and A21121 Alexa Fluor 488 Goat Anti-mouse IgG were purchased from Life Technologies (Paisley, UK). Goat Anti rabbit IgG (HRP) was obtained from Abacam (Cambridge, UK) and anti-catalase antibody (rabbit polyclonal (IgG) to human CAT / catalase) was purchased from LSBio (Nottingham, UK). Protein ladder (molecular marker) was purchased from Thermo Fisher Scientific (Horsham, UK).

## 2.1.1.4 Buffers and working reagents

All buffers and working reagents were freshly prepared; those that are temperaturesensitive were kept and stored properly prior to utilisation. The following is the list of buffers and working reagents required for the methods and techniques used in this study.

• PBS

PBS buffer (pH 7.4) was prepared by dissolving one tablet of PBS (Sigma Aldrich) in 200ml of deionised water (ddH2O). This yielded 0.01M phosphate buffer, 0.0027M KCl and 0.137M NaCl, pH 7.4, stored at 25°C.

• 1M KOH

The stock of 1M KOH was made by dissolving 5.611g KOH to 100ml  $ddH_2O$ , kept at room temperature.

## • Enzyme reaction buffer (ERB)

10x ERB (stock) was prepared by mixing 400mM HEPES (190.65g), 4.0g BSA, 1M KCl (149.12g) and 5mM Na<sub>2</sub>EDTA (3.7224g) in 1.6 litres of ddH2O; the pH was adjusted to 8.0 with 1M KOH and the final solution made up to 2 litres with ddH<sub>2</sub>O. Aliquots of 50ml of 10 x ERB solutions were stored at  $-20^{\circ}$ C. On the day of experiment, the 10x ERB aliquot was defrosted and 1x ERB was made by adding 50ml of 10x ERB to 450ml of ddH<sub>2</sub>O.

## • Lysis buffer

The stock solution of lysis buffer was made by mixing 100mM Na<sub>2</sub>EDTA (37.2g), 2.5M NaCl (146.1g) and 10mM Tris-HCl (1.2g). The mixture was made up to 1 litre with ddH<sub>2</sub>O and its pH was adjusted to 10.0 with 10M NaOH. The working solution of the lysis buffer was made by adding 1% Triton-X-100 (v/v) to the lysis buffer stock solution and could be stored for 24 hours at 4°C prior to use.

## • Alkaline electrophoresis buffer (AEB):

To prepare the AEB, two stock solutions were required. Firstly 10M NaOH was made by dissolving 200g of NaOH in 500ml ddH<sub>2</sub>O, and secondly 200mM Na<sub>2</sub>EDTA was made by dissolving 7.4g of Na<sub>2</sub>EDTA in 100ml ddH<sub>2</sub>O. The AEB working solution was made by mixing 60ml of 10M NaOH with 10ml of 200mM Na<sub>2</sub>EDTA; the final volume of the mixture was made up to 2 litres with ice-cold ddH<sub>2</sub>O; note: the pH of AEB is  $\geq$  13.00.

## • Neutralisation buffer

The neutralisation buffer was made disolving 0.4M Tris (4.85g) a final volume of 100ml ddH<sub>2</sub>O. The pH was then adjusted to 7.5 with concentrated HCL and the solution was kept and stored at room temperature.

# • 1x KCM washing buffer

Prior to preparing the KCM washing buffer, a 10x KCM buffer was made disolving 1.2M KCl (8.946g), 200mM NaCl (1.1688g), 100mM Tris-HCl and 10mM EDTA (0.372g) in 60ml of ddH<sub>2</sub>O and then the final volume was made up to 100ml with fresh ddH<sub>2</sub>O and stored at 4°C. On the day of assay, for each 100ml of 1x KCM washing buffer required, 10ml of 10x KCM buffer, 100 $\mu$ l Triton X-100 and 89.9ml ddH<sub>2</sub>O were mixed thoroughly.

## • KCM blocking buffer

The KCM blocking buffer was made freshly immediately before use by adding 2% of BSA (w/v), 10% (w/v) normal goat serum and 10% (w/v) goat milk powder to the KCM washing buffer (for example, for each 10ml of KCM blocking buffer, 1ml of 10x KCM buffer, 10µl Triton x-100, 0.2g BSA, 1g goat milk powder mixed with 1ml of goat serum; the final volume was made up to 10ml with ddH<sub>2</sub>O).

# • 1x Trypsin-EDTA

1x trypsin-EDTA was made by adding 2ml of 10x trypsin-EDTA to 18ml of sterile PBS and stored at  $4^{\circ}$ C.

# • TBS with tween 20 (1x TBST)

TBST 1x was made by mixing 50mM Tris-base (6.057g), 150mM NaCl (8.76g) and 0.1% tween 20 (v/v) with 800ml of ultra-pure dH<sub>2</sub>O. The pH was adjusted to 7.65 with HCL and the final volume was made up to 11itre with high purity water.

# • TBST-milk

TBST-milk was freshly prepared by adding 5% (w/v) of dried skimmed milk powder to TBST working reagent (for example, 1g of milk powder added to 20ml of 1x TBST).

# • Laemmli buffer

Laemmli buffer was freshly made by mixing 2.5ml of 1M Tris-HCL (pH 6.8), 5ml of 20% SDS and 5ml of 100% Glycerol then 100ml  $ddH_2O$  was added to the mixture.

# • TBST-BSA

TBST-BSA was freshly prepared by adding 5% BSA (w/v) to 1x TBST working reagent.

# • Resolving gel buffer

The gel buffer was made prior to use by dissolving 181.5g of Tris-base in 850ml of  $ddH_2O$  and the pH adjusted to 8.8 by HCl. The solution was kept at room temperature to cool and then more  $ddH_2O$  was added to give the final volume of 1000ml re-adjusting the pH to 8.8.

# • Stacking gel buffer

The gel buffer was freshly prepared by dissolving 60.5g of Tris-base in 850ml  $ddH_2O$  and the pH adjusted to 6.8 with 6M HCl. High purity water was then added to the mixture to give the final volume of 1000ml with pH 6.8.

# Ponceau S Staining Solution

Ponceau S staining solution was prepared by mixing 1g Ponceau S with 50ml Acetic Acid and the mixture was made up to 1000ml with ddH<sub>2</sub>O.

# • Running buffer (10x)

The 10x running buffer was freshly made by mixing 0.25M Tris-HCl (30.3g) with 1.92M (144g) glycine and 35mM (10.08g) SDS and the mixture was made up to 1000ml with ddH<sub>2</sub>O.

# • Transfer buffer (10x)

The 10x transfer buffer was prepared by mixing 60.4g Tris-base and 288g glycine with 1800ml ddH<sub>2</sub>O the volume then made up to 2000ml by adding more ddH<sub>2</sub>O. To make 1x Transfer buffer, 100ml of 10x Transfer buffer was mixed with 100ml of methanol and 800ml of ddH<sub>2</sub>O.

# • 10% SDS polyacrylamide-gel

A 10% SDS gel was made immediately prior to use by mixing 4ml of ddH2O, 3.3ml acrylamide, 2.5ml Tris-HCl (1.5M, pH 8.8), 0.1ml SDS, 0.1ml ammonium persulfate and 0.004 TEMED.

# • 5% Stacking gel:

A 5% stacking gel was made by mixing 3.4ml of ddH2O, 0.83ml acrylamide, 0.63ml Tris-HCl (1.0M, pH6.8), 0.05ml SDS, 0.05ml ammonium persulfate and 0.005 TEMED.

# • 0.6% LMP agarose

The 0.6% LMP agarose gel was prepared by dissolving 0.12g of LMP agarose powder in 20ml PBS using a microwave.

# • 1% NMP agarose

The 1% NMP agarose gel was prepared by dissolving 0.5g of NMP agarose powder in 50ml of  $ddH_2O$  using a microwave.

**N.B**: Calculations of material concentrations in this study and molarities/concentrations of substances and compounds were made using an automatic mathematical formula provided by the GraphPad prism website below:

http://www.graphpad.com/quickcalcs/Molarityform.cfm

# 2.1.1.5 Test compounds

All desired concentrations of the test compounds were freshly made immediately prior to use. The following compounds were dissolved in their specific solvents and diluents.

# • L-Ascorbate (Stock)

The stock of 10mM ascorbate was made by adding 17.6 mg of L-ascorbate to 10ml of sterile PBS (pH 7.4). From stock, the desired concentrations of ascorbate were made using complete culture media.

# • Hydrogen peroxide (30%) v/v

The concentration of the supplied stock is 9.79M. To make a 100mM stock,  $10.2\mu$ L of the main stock was mixed with 989.8 $\mu$ L of PBS (pH 7.4). From the 100mM stock,  $10\mu$ L was taken and mixed with 990 $\mu$ L of PBS to prepare 1mM H<sub>2</sub>O<sub>2</sub> stock. From the final stock, desired concentrations were made using free serum medium (e.g. for 10 $\mu$ M H<sub>2</sub>O<sub>2</sub> concentration, 20 $\mu$ L of 1mM H<sub>2</sub>O<sub>2</sub> was mixed with 2ml of free serum medium).

# • L-Bothionine sulfoxamine (BSO)

A fresh stock (10mM) was prepared by dissolving 22.23mg in 10ml of sterile PBS (pH 7.4). The desired concentrations were then prepared using culture media.

# • Desferrioxamine myselet salt (DFO)

A stock (10mM) was made by adding 65.679mg to 10ml of sterile PBS (pH 7.4). The desired concentrations were then prepared using culture media.

# • Elesclomol (STA-4783)

A stock of 10mM was made by dissolving 2.0025mg in 0.5ml DMSO. From stock, the desired concentration was made by using complete cell culture media. To reduce the level of DMSO, highly concentrated Elesclomol stocks were occasionally made.

# 2.1.1.6 Assay kits

Several ready-to-use working reagents and kits were purchased, as shown in the Table 2.1.

Table 2.1. List of ready-to-use assay kits and reagents

Assay kit	Reagents included	Company name (Town, country)
Annexin V / PI	10x Binding buffer, Annexin V APC, Propodiume iodide stain	Affymetrix (Wooburn Green, London, UK)
ATPlite	ATPlite buffer, mammalian cell lysis solution, lyophilized substrate solution	PerkinElmer (Coventry, UK)
Caspase-Glo® 3/7	Caspase-Glo® 3/7 buffer, Caspase-Glo® 3/7 substrate	Promega (Southampton, UK)
BCA protein assay	Albumin standard, Working reagents A and B	Fisher Scientific (Loughborough, UK)
ECL Western blotting substrates	Luminol enhacer solution, Peroxide solution	Fisher Scientific (Loughborough, UK)
Iron assay kit	Iron assay buffer, iron probe, iron reducer, iron standard	Abacam (Cambridge, UK)
Intracellular ROS Assay Kit (Green Fluorescence)	DCFH-DA reagent	Cambridge Bioscience (Cambridge, UK)

## 2.2 Cell lines:

A panel of human melanoma cancer cell lines, including two non-pigmented (A375M and A375P), a moderately pigmented (SK-MEL-28 (SK28)) and a heavily pigmented (SK-MEL-23(SK23)) cell line and two "normal" human skin cell lines, keratinocytes (HaCaTs) and human dermal fibroblasts (HDF), were all kindly provided by members of the Department of Cancer Studies at the University of Leicester. All cell lines were determined to be free from mycoplasma contamination.

## 2.2.1 Cell line maintenance and conditions

All MM and normal human cell lines were maintained as monolayer cultures via incubation at 37 °C under 5% CO<sub>2</sub>. For the A375M, A375P and SK23 melanoma cancer cells, RPMI-1640 culture medium, supplemented with 10% FCS and 1% glutamax was used; for the SK28 cell line, DMEM culture medium supplemented with 1% NEAA, 10% of FCS and 1% glutamax, plus 1% sodium pyruvate was used. The HaCaT cells were grown in DMEM and supplemented with 10 % FCS, 1% sodium pyruvate and 1% glutamax. HDF cells were grown in Gibco cascade biologics medium 106. Cell splitting was routinely performed after they had achieved *ca*. 80% growth confluence. Before beginning the culturing and sub-culturing, all media, PBS and trypsin were kept at 37°C

in a water bath for 20 minutes to protect cells from temperature shock. For culturing and sub-culturing, the used culture media was first discarded and cells were washed twice with a sterile and pre-warmed PBS to remove dead cells and serum. Cells were then trypsinised by adding 1 x trypsin-EDTA (0.05% trypsin, 0.7mM EDTA) for 3 to 5 minutes, depending on cell types, at 37°C. The trypsin action was then stopped by adding cell specific medium containing 10% FBS, after confirming that cells had detached using an inverted microscope. In order to remove the trypsin, the cell suspension was collected in a sterile 50ml centrifuge tube and centrifuged at 2000xg for 4 minutes; then, the supernatant was removed and the pellet was mixed again with 4-8ml of fresh culture media. The suspension was pipetted up and down to reduce cell clumping. Trypan blue assay was performed to check the cell number and viability of the harvested cells. Once the cells had been counted, they were sub-cultured in small (T25) and medium (T75) flasks at different densities (for instance 1:10 and 1:100) with complete cell specific media. Flasks of cultured cells were then kept incubated at 37°C with 5% CO<sub>2</sub> to maintain cell growth. All cell lines ceased being sub-cultured once they reached a passage number of 50.

### 2.2.2 Cell cryopreserving and thawing

In order to maintain cell lines for longer periods, with earlier passage numbers, they were kept cryopreserved in liquid nitrogen. Cells were cryopreserved when they were nearly confluent (~80%). Briefly, the culture media was removed and cells were washed twice with sterile pre-warmed PBS. Cells were then detached by adding 1x trypsin-EDTA and incubated for 3 to 5 minutes at 37°C with 5% CO<sub>2</sub>. An equal volume of cell specific media containing 10% FBS was added to the trypsinised cells and the suspension was pipetted up and down to reduce cell clumping. Cell suspensions were then transferred to sterile conical tubes and centrifuged for 4 minutes at 2000xg. Any supernatant media was removed and cell pellets were mixed with a freezing media, consisting of the cell specific media plus 10% DMSO. Once the suspension was thoroughly mixed, 1ml aliquots were transferred to sterile vials and kept at -80°C in a Styrofoam rack overnight; they were then transferred to liquid nitrogen.

To thaw and revive the frozen cells, vials were taken out of liquid nitrogen storage and placed on ice immediately to avoid any shock from the sudden change in the temperature. When the cell suspension had completely thawed, cells were thoroughly mixed by pipetting and then transferred to conical tubes. The suspension was washed free of DMSO by centrifuging the tubes at 2000xg for 4 minutes. Then, after removing the supernatant, cells were mixed with 1ml of cell specific complete media and transferred to a sterile T75 flask. The suspension in the flask was mixed with 10-15ml of complete cell specific media and kept incubated at  $37^{\circ}$ C with 5% CO<sub>2</sub> overnight. Once cells had grown and when they nearly confluent, they were split and sub-cultured in different flasks for the new experiments as described above.

### **2.2.3 Primary melanoma tissue (clinical samples)**

Primary tissue samples represent an ex vivo system which better reflect a human cell's *in vivo* response to test agents. In the current study, primary melanoma tumour samples were obtained from patients who underwent surgical excision for MM at Leicester Royal Infirmary. Upon obtaining the samples, they were managed by a histopathologist who trimmed any stromal, fat or necrotic tissue from the tumour to prepare a sample enriched with melanoma tumour cells. Immediately afterwards, samples were kept in media 199 on ice, to keep the tumour cells viable. Once samples had arrived in the lab, the process of cell disaggregation of tumour tissue was carried out. First, samples were minced with a razor blade on a petri dish, into small pieces ( $\leq 1$ mm<sup>2</sup>). The minced tumour sample was collected into a 15 ml tube, to which 5 ml of media 199 containing Liberase Blendzyme TM (60µg/ml) was added. The tube was then placed on the agitating platform where the sample was mixed constantly and incubated at 37°C. Every 15 minutes the sample was taken out and the mixture was pipetted gently to reduce clump formation. This process continued for approximately 60 minutes, until the sample became cloudy. Cell suspension was then centrifuged and the supernatant removed. Then, 5ml of HBSS with 2% FBS was added to the pellet and mixed thoroughly for 3 minutes to neutralise the effect of Liberase Blendzyme TM. A trypan blue assay (see Section 2.4.1) was performed to test cell viability, and to count and split the required number of tumour cells into Eppendorf tubes for further analysis by Comet assay.

### **2.3** Treatment of cells with test compounds

#### 2.3.1 Treatment cells with vitamin C

Fresh L-ascorbic acid stock solution was prepared with PBS (pH 7.4) immediately prior to use. This was further diluted with PBS to obtain a 10mM stock L-ascorbate, from which different concentrations were made using cell culture media. For adherent cells the old media from each well of the 6-well plate was removed and the adhered cells were washed once with sterile pre-warmed PBS (pH 7.4). To treat cells with vitamin C, fresh complete cell culture media containing desirable concentrations of AA was added to cells and incubated at 37°C /5% CO<sub>2</sub> for two hours. Control samples were incubated in media containing no AA. After each treatment, cells were washed once with PBS (pH 7.4). Alternatively, for primary melanoma cancer cells (the clinical samples), the isolated cells were transferred into Eppendorf tubes where they were treated with media containing 100 $\mu$ M ascorbate. After treatment, cells were washed once with PBS and centrifuged to obtain a pellet ready for H<sub>2</sub>O<sub>2</sub> treatment.

### 2.3.2 Cell exposure to H<sub>2</sub>O<sub>2</sub>

 $H_2O_2$  was used as a model oxidant to induce oxidative stress in melanoma cancer cells and normal cells. From the supplied  $H_2O_2$  stock solution (30% w/w; 9.79M), a further diluted stock (1mM) was obtained through serial dilution using fresh PBS (pH 7.4). For each experiment, the desired micromolar concentrations of H<sub>2</sub>O<sub>2</sub> were made by using pre-warmed cell culture media. All preparations were performed immediately prior to treatment, and all diluted samples containing H<sub>2</sub>O<sub>2</sub> were covered and protected from direct light exposure. For the treatment of cells with H<sub>2</sub>O<sub>2</sub>, media was removed and adhered cells were washed twice with pre-warmed PBS (pH 7.4). Cells were then exposed to media containing various concentrations of H<sub>2</sub>O<sub>2</sub>. After treatment, the media was removed and cells were washed once with PBS (pH 7.4) and then harvested using 1x Trypsin-EDTA and collected into pre-labelled Eppendorf tubes, where they were kept on ice ready for comet assay analysis. For gamma H2AX immunoassay, the seeded cells on cover slips were treated as above (but without trypsin harvesting). For primary melanoma cancer cells, the pellets in the Eppendorf were exposed to media containing H<sub>2</sub>O<sub>2</sub> and samples were kept on ice for 30 minutes protected from light. Cells were then centrifuged and the media containing H<sub>2</sub>O<sub>2</sub> was removed from each sample.

#### 2.3.3 Cell exposure to ionising radiation

For the assessment of radiation-induced DNA damage, melanoma cancer cells and the HaCaTs were exposed to X-ray using either a Pantak industrial X-ray machine or an Xstrahl 302 X-ray Unit. For the measurement of radiation-induced SSBs via the comet assay, cells were harvested and mixed with a LMP agarose solution and then dispensed onto the NMP agarose pre-coated slides, and they kept on ice and protected from light for 15 minutes. These slides were then exposed to different doses of X-ray radiation, on ice. However, to assess radiation-induced DSBs (Gamma H2AX immunoassay), cells were seeded and grown on sterile cover slips placed in small individual petri dishes, and were irradiated on ice. All samples during and after radiation were kept on ice and protected from light exposure to avoid any additional DNA damage or DNA repair. Gamma H2AX samples were then directly fixed with ice-cold methanol (100%) and kept at -20°C for overnight until analysis, whereas the comet assay samples were placed directly into ice-cold lysis buffer (kept on ice/protected from light exposure) an then left at 4°C for overnight before running the comet assay.

## 2.3.4 Cell exposure to drug-mediated oxidative stress

Two chemical agents/drugs were used to generate oxidative stress in melanoma cancer cells. One of these agents was BSO, which has been widely used in many *in vitro* and *in vivo* studies and has also been used in combination with other chemotherapeutics in a phase I clinical trial (Bailey *et al.*, 1994) and in a phase II clinical study, (reviewd by Trachootham *et al.*, 2009). The pro-oxidant role of BSO is mediated by the inhibition of glutathione synthesis (Trachootham *et al.*, 2009). A fresh stock of L-BSO (10mM) was prepared by dissolving 22.23mg in 10ml of sterile PBS (pH 7.4). The desired concentrations were then prepared using culture media.

The second drug used in this study was Elesclomol (STA-4773), a chemical agent that is considered to be a novel oxidative stress inducer (O'Day *et al.*, 2009). The Elesclomol stock solutions (10mM) were made using DMSO, and then further diluted to obtain the desired concentrations.

Before cells were exposed to drugs, media from each well of a 6-well plate was removed and adhered cells were washed gently with pre-warmed sterile PBS (pH 7.4), and the media containing drugs (BSO/Elesclomol) was added to cells and kept incubated at  $37^{\circ}C/5\%$  CO<sub>2</sub> for the time periods indicated. In order to treat primary

melanoma cells with Elesclomol, suspended cells were washed once with PBS after being treated with ascorbate and then incubated with complete cell culture media containing Elesclomol for the indicated time at 37°C/5% CO<sub>2</sub>.

## 2.4 Methods

### 2.4.1 Cell counting and viability assay

In this study, cell viability and counting was performed using the trypan blue exclusion test as described by Phillips (Phillips, 1973). This assay is based on the observation that the intact cell membrane of live cells prevents Trypan blue entering into the cell, allowing cells to remain unstained (white/clear); however, the stain traverses the cell membrane of any dead cells, which become a distinctive blue colour.

To perform this assay, an equal volume  $(20\mu)$  of cell suspension and trypan blue stain were thoroughly mixed in an Eppendorf tube, by slowly pipetting. Then, approximately 10µl of the mixture was pipetted slowly into a haemocytometer chamber (Neubauer chamber) under a cover slip. Cells were then counted as either viable cells (white) or non-viable cells (blue) in four 1mm<sup>2</sup> areas of the chamber; this was performed within two minutes to avoid recording false negative results. The number of viable cells per 1ml of cell suspension was obtained by multiplying the average number of white cells per 1mm<sup>2</sup> area by the dilution factor and by 10<sup>4</sup> (the conversion of 0.1mm<sup>3</sup> to 1ml). The viable percentage was calculated by dividing the number of viable cells by the total number of cells (viable plus non-viable cells) and multiplied by 100. The average cells in one square (1mm<sup>2</sup>) of the chamber must be between 20-50 cells. If the number of cells exceeds this range, a further dilution is required to obtain an accurate cell count, but if the number of cells per square is less than 15, a less diluted sample is recommended. The calculation of cell numbers and cell viability was based on the following equations:

#### Equation 2.1. Cell counting and cell viability formula

Cell density (cells/ml) = Number of cells counted x 
$$\frac{\text{Dilution factor}}{\text{Number of squares}} \times 10^4$$
Viability (%) = 
$$\frac{\text{Number of viable cells}}{\text{Total number of cells counted}} \times 100$$

### 2.5 Measurement of DNA damage

#### 2.5.1 Standard and modified alkaline comet assay (ACA)

One of the most useful techniques for quantifying DNA damage is the comet assay, or single gel electrophoresis (Tice *et al.*, 2000). This technique is a rapid, flexible, versatile and relatively simple method for measuring and analysing DNA damage in individual eukaryotic cells. Although the data obtained by this assay is sometimes not consistent and its standardisation is difficult, the simplicity and sensitivity of this technique are its main advantages (Collins, 2002). This assay is widely used in a variety of research areas for the assessment of genomic damage resulting from various genotoxins, including chemicals and radiations. This is because of its high sensitivity, allowing detection of even low levels of DNA lesions; the assay can also be performed relatively rapidly (Speit *et al.*, 2004).

Briefly, DNA damage assessment using the comet assay follows several steps, including: slide preparation, the embedding of an appropriate number of cells in thin agarose gels on microscope slides; release of DNA by lysis buffer; slide exposure to alkaline, of pH=13; electrophoresis under alkaline conditions; neutralisation, to negate the alkali; staining and DNA comet visualisation (Tice *et al.*, 2000). In the modified version of ACA, lysed embedded cells are exposed to an endonuclease enzyme such as formamidopyrimidine glycosylase (Fpg) before the electrophoresis to detect ODBLs.

The comet assay was first developed by Östling and Johansson in 1984, and the basic principles are based on the fact that DNA strand breaks reduce the large size and effective molecular weight of genomic DNA (Ahnström, 1988, Olive, 1992). Additionally, single and double strand breaks DNA have a huge impact on the DNA structure relaxing the tight supercoiling of the DNA. The sensitivity of the neutral comet assay, introduced by Östling and Johansson, is limited with the assay having a low sensitivity. The reason for the low sensitivity of the neutral assay is the likely ineffectiveness of the lysis buffers used to remove all proteins, which in turn hold the DNA supercoiling and so cause the broken fragments to migrate less during electrophoresis (Fairbairn *et al.*, 1995).

Singh *et al.* (1988) modified the comet assay protocol by introducing an alkaline version. This allowed for an increase in the sensitivity of the assay, enabling the measurement of low numbers of single strand breaks (Singh *et al.*, 1988). In the alkaline

comet assay, the unwinding and denaturing of the DNA double strands is an important step, allowing for the sensitive detection of single strand breaks. An alkaline buffer with a pH greater than 12.5 is used to promote the DNA denaturation, unwinding and expression of single strand lesions and alkali labile sites (ALS) following damage (Fairbairn *et al.*, 1995).

In this study, to assess DNA damage, both standard and the modified ACA were used to measure various levels of SSBs and ODBLs, respectively, in treated melanoma and normal cells. The assays were run according to general protocol described by Singh *et al.* (1988) with modifications as detailed by Zainol and colleagues (Zainol *et al.*, 2009) as described below:

## • Harvesting the treated cells

First, treated cells were harvested by adding 0.5 ml of 1x trypsin-EDTA to each well of the 6-well plate, and incubated for 3 minutes  $(37^{\circ}C/5\% CO_2)$  to deadhere cells from the well's base. 1.5ml of cell specific medium was then added into each well and mixed thoroughly. After counting, *ca.* 30,000 cells were taken from each well and transferred into labelled Eppendorf tubes and the gels were made (as previously mentioned). For each sample two gels were made.

## • Slide preparation:

After treatment, cells were kept on ice and protected from the light to negate DNA repair and to avoid any further DNA damage. Treated cells were then centrifuged in a refrigerated microfuge for 4 minutes (2000 rpm/4C°). This was followed by removing the supernatant, leaving a cell pellet. For each sample, the cell pellets were mixed with 170µl of 0.6% LMP agarose dissolved in PBS at  $37^{\circ}$ C.80µl of this mixture was gently dispensed on to a slide previously precoated with 1% NMP agarose and then covered with 22 x 22 mm cover slips. Slides were kept on ice for 10 minutes and protected from light so the agarose could solidify forming a gel. The number of cells that can be embedded in LMP agarose should range between 10,000-30,000; higher densities of cells can cause overlapping comets, particularly when the damage is high (Liao *et al.*, 2009).

## • Cell lysis

Once the gels had solidified, slides were placed in a lysis buffer (100mM Na<sub>2</sub>EDTA, 2.5M NaCl and 10mM Tris-HCl plus 1% Triton-X-100) and left overnight at 4°C, protected from light exposure. This removes the cell's membrane and DNA-bound histone proteins. The remaining entity is called the nucleoid/nucleoid body and contains supercoiled DNA attached to remaining scaffolding proteins (the nuclear matrix).

## • Enzyme treatment

This step was performed in the modified ACA to detect oxidised base lesions in DNA post-treatment; the Fpg enzyme was exploited for this purpose. Each gel (on slides) was exposed to either 50 $\mu$ l of ERB or 50 $\mu$ l of Fpg diluted in ERB for 30 minutes at 37°C in a dark and moist condition before electrophoresis.

# • Alkali unwinding

Slides were incubated in cold AEB pH  $\geq$ 13 at 4°C for 20 minutes prior to electrophoresis. This step, which was introduced by Singh *et al.* (1988), enables DNA unwinding and expresses ALS as SSBs.

# • Electrophoresis

After incubation of gels in AEB, the samples in the electrophoresis tank were subject to electrophoresis using 30 volts and 300 mA for 20 minutes. The negatively charged broken DNA migrates toward the anode electrode generating a 'tail' and the unbroken DNA remains in place forming head; the head and the migrated tail resembling the shape of a comet (Figure 2.1).



Figure 2.1. DNA breaks migration in an electric field

### • Neutralisation

Neutralisation (0.4M Tris-base in 100ml ddH<sub>2</sub>O with pH 7.5) facilitates the renaturing of the supercoiled DNA strands in the head, which are separated by the action of alkaline treatment, whereas the strands in the tail remain single stranded (Liao *et al.*, 2009). After electrophoresis, slides were removed from the electrophoresis tank, washed for 20 minutes with neutralisation buffer, and then for 10 minutes with ddH<sub>2</sub>O. After this, the slides were placed in an oven  $(37^{\circ}C)$  to dry overnight before staining.

## • Staining

A DNA binding dye, such as PI can be used to label the DNA. When this fluorescent dye binds to DNA, it intercalates between the bases (Liao *et al.*, 2009). Prior to staining, slides were rehydrated with ddH<sub>2</sub>O for 30 minutes and then 1 ml of freshly made PI ( $2.5\mu g/ml$ ) was dispensed on each slide and left for 20 minutes at room temperature. After this, the slides were washed with water and left with more fresh water for a further 30 minutes in the dark; the water was then removed and slides were placed in trays and kept overnight in an oven to dry before scoring.

### Visualisation and scoring the comets

To assess the level of DNA damage, 50 comets per gel were randomly selected for scoring, and with there being 2 gels per slide this equates to 100 comets scored. This number of scored comets provides representative results for the population of treated and untreated cells (Price *et al.*, 2000). This was performed after adding one drop of water on to each gel and it being covered with a coverslip for visualisation by fluorescence microscope. To capture the comet images and to analyse the images, Komet software (Version 5.5, Andor Technology, Belfast, UK) was used.

The microscope system consists of a computer connected to an epi-fluorescence microscope (Olympus BH2) fitted with an excitation filter of 515 - 535 nm, a barrier filter of 590 nm and a 100 W mercury lamp. This was operated at a magnification of X 200. Percentage Tail DNA (%TD) was selected as the parameter that best reflected DNA damage (Collins, 2002). The %TD marker correlates with the dose of genotoxic agents (Kumaravel and Jha, 2006). The percentage of tail DNA is calculated using the following equation (Equation 2.2).

Tail DNA (%) = 
$$\frac{\text{Tail DNA intensity}}{\text{Total intensity of whole DNA}} \times 100\%$$

Equation 2.2. The mathematical formula for calculation of Tail DNA damage

All of the above steps of the comet assay are presented in a figure below (Figure 2.2).



scoring

Figure 2.2. Schematic figures show the steps of the ACA and the modified ACA.

#### 2.5.1.1 Modified ACA

In order to measure DNA base lesions in treated cells, a modified version of the comet assay was carried out. In this assay, Fpg enzyme was used to digest the nucleoid body DNA post-lysis, before running the alkaline electrophoresis. After the slides were removed from the lysis buffer, they were washed three times with ERB for 5 minutes each time. 50µl of the appropriately diluted Fpg (the Fpg enzyme being diluted using ERB) was then dispensed onto each gel and then covered with a cover slip to spread it evenly over the entire gel. These slides were then incubated in moistened dark boxes for 30 minutes at 37°C. Slides were then taken out from the boxes and placed onto the electrophoresis tank containing ice-cold AEB for further 20 minutes before running the electrophoresis (see above).

## 2.5.2 γ-H2AX-immunoassay

H2A is a member of histone protein family and is a constituent of the DNA packaging nucleosome. It has been shown that H2A family member X (H2AX) constitutes a major species of H2A in mammalian nucleosomes (Fernandez-Capetillo *et al.*, 2004). Serine-139 residues of the histone variant H2AX undergo a rapid phosphorylation in response to DSB formation, forming  $\gamma$ -H2AX (Mah *et al.*, 2010). H2AX in the chromatin surrounding DSBs is hugely phosphorylated, serving as a signal to recruit the repair apparatus. This phosphorlyation can be observed, by using  $\gamma$ -H2AX phosphor-specific antibody, as a nuclear foci (Löbrich *et al.*, 2005). Foci detection has thus become a diagnostic tool to assess the exact amount of DSB resulting from the effects of genotoxic agents, such as chemicals and radiation.

In this study, a  $\gamma$ -H2AX assay was performed to measure the level of DSBs in the treated cells. For the  $\gamma$ -H2AX assay, cells were seeded and grown on sterile cover slips attached to the bottom of wells of 6-well plates at approximately 2 x 10<sup>4</sup> cells per well, and left overnight incubated at 37°C/5% CO<sub>2</sub>. The next day, the cells were pre-incubated for 2 hours with ascorbate. Then, the cell specific medium was removed from each well and cells were washed with ice cold PBS before being exposed to various micromolar concentrations of H<sub>2</sub>O<sub>2</sub> (prepared in serum free medium) for 30 minutes on ice and protected from the light. Following treatment, cells were washed again with PBS and fixed in 100% methanol for 24 hours at -20°C. After this, the methanol in each well was

removed and cells were washed twice with ice cold PBS for 10 minutes each. This was followed by 15 minutes incubation with fresh KCM blocking buffer.

After removing the blocking buffer, the primary anti-phosphohistone H2AX (ser<sup>139</sup>) antibody (Clone JBW301, Mouse Monoclonal Antibody; Upstate Millipore Corp) was diluted in blocking buffer at 1:200, added to the cells and incubated at room temperature for 2 hours on a shaker. Afterwards, cells were washed for four times with KCM washing buffer, and then the secondary antibody (A21121 Alexa Fluor 488 Goat Anti-mouse IgG), which was diluted in blocking buffer at the same concentration as the primary antibody dilution, was added to the cells and incubated for a further one hour, on a shaker, at room temperature. Finally, the cells were washed four more times with KCM washing buffer before being mounted upside down on the labelled slides. A drop of SlowFade<sup>®</sup> Gold antifade reagent with DAPI (10µl) was dispensed on each labelled slide, and the relevant (identified) cover slip removed and mounted on the slide (treated cells being between the cover slip and the slide surface). After the slides had been dried at room temperature, they were stored at 4°C for 24 hours ready for image analysis.

To measure the foci number per cell, images of the treated cells were taken at 40x magnification using a fluorescent Zeiss Axioskop 2 plus microscope (Carl Zeiss Ltd, Welwyn Garden City), which was fitted with a 100W mercury bulb and an Axio-CamHRc camera (Zeiss), and accessed using Axio-Vision software. The analysis of the captured images was carried out using ImageJ (WCIF Image J version 1.42, available from Research Services branch of NIH). From each sample, 10 fields of view (*ca.* 400 cells) were randomly chosen for analysis. Images of clear  $\gamma$ -H2AX foci were captured using a 485µM filter, whereas the number of DAPI stained nuclei images were captured using a DAPI filter. As a part of the ImageJ software's functioning,  $\gamma$ -H2AX foci and nuclei numbers were counted automatically. After exclusion of cells with more than one nucleus, the actual numbers of  $\gamma$ -H2AX foci by the total number of cells per field. The procedure for foci counting is represented in (Figure 2.3).



#### Figure 2.3. Analysis steps for foci counting per field of treated sample using imageJ software.

Immunocytochemistry image shows H2AX phosphorylation (H2AX Foci) (ser 139, green) which represents DSBs and the nuclear dye DAPI for measuring cell number per examined field.

### 2.6 Measurement of intracellular ROS

The fluorescent dye 2',7'-Dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) probe was used to measure intracellular ROS in melanoma cancer cells and normal cells. The principle of this assay is based on the oxidation of non-fluorescent DCFH to highly fluorescent DCF by ROS once the dye enters into the cell (Figure 2.4). This was performed using two different methods using either a plate reader or flow cytometry.



Figure 2.4. Principle of DCFH-DA assay for detection of intracellular ROS.

### 2.6.1 ROS measurement by plate reader

For the plate reader method, cells were seeded in 96-microwell black plates and incubated for 24 hours at 37°C/5% CO<sub>2</sub>. On the day of assay, media was removed from all of the microwells and cells were washed once, gently, with 200 $\mu$ l PBS (pH 7.4); then, under dark conditions, 1 $\mu$ l of freshly made H<sub>2</sub>DCFDA solution with DMSO (25mM) was added to each well (blank, controls and samples) and the plate was covered and incubated for 30 minutes. This incubation allows intracellular ROS to oxidise the flourogenic dye and change it to highly fluorescent dihydrofluorescein. Following this incubation time, 200 $\mu$ l of PBS was added to each well and the relative fluorescent intensity of the plate was read immediately using a fluorometric plate reader

(BMG FLUOstar OPTIMA Microplate Reader) at an excitation of 480nm and emission of 530nm.

### 2.6.2 ROS measurement by flow cytometry

Using flow cytometry, 5 x  $10^5$  cells were seeded in each well of the 6-well plate and kept incubated for 24 hours to allow attachment. The dye H<sub>2</sub>DCFDA was diluted with DMSO (10mg/ml), with a minimal exposure to air, and was then added directly to cells (0.5µl of diluted H<sub>2</sub>DCFDA per 1ml of media) and incubated for 30 minutes at  $37^{\circ}$ C. After incubation, media was removed from each well and cells were washed once with PBS (pH 7.4). Cells were then collected by trypsin (0.5ml/well), centrifuged and then re-suspended in 0.5ml PBS (pH 7.4). Then, cells were transferred to FACS tubes and protected from light and kept at 4°C until analysis by flow cytometry. The principle of intracellular ROS detection by flow cytometry is that when the compound H<sub>2</sub>DCFDA is cleaved by the intracellular esterases, it produces a non-fluoresent product, H<sub>2</sub>DCF. This nonfluoresent molecule accomulated within the cells, and consequently becomes fluoresence when oxidised by the intracellular oxygen species. This fluoresence at 530nm can be detected and measured by flowcytometry (Eruslanov and Kusmartsev, 2010).

## 2.7 Measurement of intracellular iron ions

Intracellular iron ions were measured in melanoma cancer cells using a colorimetric iron assay kit. The assay is based on the principle that the ferric carrier protein and the ferric ions are dissociated in the presence of acidic buffer. During the reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , iron ions react with Ferene S, a provided reagent, to produce a stable coloured complex. The assay kit is sensitive and measures iron levels ranging between 0.4-20nmol/50µl of sample.

Before running the assay, different standards were freshly prepared from the iron standard stock provided by the assay kit. Briefly, cells were seeded in a large flask (T175) and incubated at  $37^{\circ}$ C / 5% CO<sub>2</sub> until they become confluent (*ca.* 80%). Cells were then washed with PBS (pH 7.4), trypsinised and collected in a 15ml centrifuge tube. Cells were then washed twice with PBS (pH 7.4) and centrifuged again. To lyse the cells, a pellet of 2 x  $10^{6}$  cells was mixed with 250µl of assay buffer. To remove insoluble materials, the mixture was centrifuged at 16,000xg for 10 minutes. The supernatant was then pipetted into a clean sterile Eppendorff tube.

To measure the total intracellular iron level, different volumes of the diluted iron standard (1mM) were added to empty wells (0, 2, 4, 6, 8, and 10µl). Similarly, different volumes of the cell lysate were added to microwells. Then, the volume of the standards and samples in each microwell was brought to 100µl with assay buffer. Afterward, to each microwell (standards and samples) 5µl of iron reducer was added and the plate was incubated at room temperature for 30 minutes. After incubation, 100µl of iron probe (Ferene S) was added to each microwell and again the plate was incubated at room temperature for 60 minutes, protected from light. The optical density (O.D) at 593nm, of the plate was then measured.

## 2.8 Cell killing and viability assessments

### 2.8.1 Clonogenic cell survival assay

A clonogenic assay was used to assess the ability of cells to grow and form colonies consisting of at least 50 individual cells after being treated. This assay is the method of choice for assessing treatment effect and is commonly used to determine the survival ability of cancer cells after treatment with anti-cancer agents. Based on a standard protocol, 5 x 10<sup>4</sup> cells were seeded in each 6-well plate and incubated for 24 hours at  $37^{\circ}C / 5\%$  CO<sub>2</sub>. After cells had been exposed to treatment they were washed with sterile PBS (pH 7.4), harvested, counted and then seeded in 9cm labelled sterile petri dishes. In this study, the number of seeded cells varied depending on the plate efficiency (PE) of each cell line, and ranged from 50 to 200 cells. Fresh cell specific complete media was added to each petri dish and incubated at  $37^{\circ}C / 5\%$  CO<sub>2</sub> until small colonies were observed; old media being replaced with fresh complete media every 7 days.

When visible colonies appeared, the media was removed from each plate and colonies were fixed with 2ml of 100% ethanol for 1 minute. Following this, the ethanol was removed and the plates were gently rinsed with water and left overnight at room temperature to dry. The next day, colonies were stained by adding 2ml of 0.5% crystal violet into each plate, left for 1 minute. The stain was then removed and plates were rewashed with water and left for 3 hours at room temperature to dry. Finally, colonies were counted and recorded for each plate. Based on the number of cells seeded, the respective PE was calculated according to the equation 2.3. Surviving fraction (as a %) was then calculated as the relative percentage of PE for the treated cells with respect to the PE of untreated cells (which was taken to represent 100% survival) (equation 2.4).

#### **Equation 2.3. Plate efficiency measurement**

Plate efficiency (PE %) = 
$$\frac{\text{Number of colonies counted}}{\text{Number of cells plated}} \times 100$$

#### **Equation 2.4. Survival fraction measurement**

Surviving fraction (SF %) = **2.8.2 Catalase activity assay** PE of treated cells PE of control PE of control

Catalase activity in HaCaT cells and melanoma cancer cells was measured using a simple qualitative method, as described by Iwase and colleagues (Iwase *et al.*, 2013). Cells were seeded in T175 flasks and incubated for 3-4 days in order to grow and yield an adequate number of cells: between  $1 \times 10^6$  and  $1 \times 10^7$  cells per sample were suitable for this assay. Media from each flask was removed and cells were washed twice with sterile pre-warmed PBS (pH 7.4). Cells were harvested with trypsin and 15ml of media containing FCS was added to stop the effect of trypsin. Afterwards, cells were counted using trypan blue, and the desired cell numbers were collected in 15ml tubes and then centrifuged for 4 minutes at 4000g. Media was removed and cells were washed twice with sterile pre-warmed PBS (pH 7.4).

Cells from each tube were suspended with  $100\mu$ l PBS, mixed thoroughly and placed into labelled Pyrex tubes. For each cell suspension,  $100\mu$ l of 1% Triton-X 100 and  $100\mu$ l of concentrated H<sub>2</sub>O<sub>2</sub> (30%) were added; the mixture was mixed thoroughly and left for three minutes at room temperature. Cellular catalase reacts with H<sub>2</sub>O<sub>2</sub> forming an O<sub>2</sub>-foam in the Pyrex tubes, and the height of O<sub>2</sub>-foam, which represents catalase activity, was measured with a ruler. The steps of the above method for measuring the intracellular catalase are represented in figure 2.5.



Figure 2.5. Schematic figures show steps for the measurement of catalase in cells by a visual technique

#### 2.8.3 Cell viability assay

ATPlite<sup>TM</sup> kit is a monitoring system for measuring the intracellular ATP in metabolically active cells based on the action of luciferase. This assay is luminescence-based and is considered highly sensitive for the detection of the anti-proliferative effect of a wide range of drugs. The principle of this assay is the production of light, which is the result of a reaction between luciferase and D-luciferin, supplied in the kit solutions and the cellular ATP of viable cells (equation 2.5) (Crouch *et al.*, 1993)

ATPlite<sup>TM</sup> kit were used in this study to measure the number of metabolically active cells (viable cells), based on the total amount of ATP in adhered cells. This viability assay was performed following treatment of melanoma cancer cells and HaCaT cells with the studied compounds. Briefly, equal numbers of cells were seeded into each well of a 96-black microwell plate (to avoid well-to-well interference) and incubated for 24 hours at 37 °C / 5% CO<sub>2</sub>; the cells were then treated. After treatment, the media from each microwell was removed and cells were washed once with PBS (pH 7.4). Meanwhile, an apropriate volume of reagent buffer was added to the lypholised substrate supplied by the assay kit, and mixed gently until clear and transparent. Following this, 50µl of provided cell lysis buffer was added to each microwell. The plate was then shaken thoroughly for 5 minutes, using an orbital plate shaker. This was followed by the addition of 50µl of provided substrate to each microwell and the plate was shaken for further 5 minutes. After this, the plate was incubated at room temperature in dark conditions for 10 minutes before measuring the luminescence signal on the plate reader (BMG FLUOstar OPTIMA Microplate Reader) at a wavelength of 562nm.

#### Equation 2.5. The principle of cell viability measurement by ATPlite assay

 $ATP + D-luciferin + O_2 \xrightarrow{Luciferase} Oxyluciferin + AMP + PPi + CO_2 + light$ 

#### 2.8.4 Annexin V / PI for apoptosis

Annexin V / PI was used to measure early and late apoptosis in treated cells. This assay is based on changes that occur in the layers of the phospholipids during apoptosis and/or necrosis. In normal cells, the distribution of phospholipids is maintained asymmetrically, whereas this symmetry is lost in apoptotic cells. In viable cells, the principal location of phosphatidylserine (PS) is on the inner surface of the cell membrane; however, in apoptotic cells this phospholipid rotates to outside the plasma membrane, causing the membrane to lose its asymmetrical feature. Annexin V is calcium ( $Ca^{2+}$ )-dependent phospholipid binding protein with high affinity to PS, Thus, in apoptotic cells, the Annexin V, in the presence of  $Ca^{2+}$ , binds with PS on the outer membrane surface. Based on the fact that fluorescein-5-isothiocyanate (FITC) conjugates to Annexin V that binds with PS, flow cytometry is capable of distinguishing between apoptotic and non-apoptotic cells. In order to distinguish between early apoptosis and late apoptosis or necrotic cells, a DNA permeable stain (PI) is added to cell suspensions following Annexin V. In late apoptotic or necrotic cells, Annexin V binds to PS in the cell surface and the disruption in the cell membrane integrity allows PI to enter cells and bind with DNA so both Annexin V positive and PI positive cells recorded by flow cytometry. Whereas in early apoptotic cells only Annexin V binds and so only Annexin V positive cells recorded by flow cytometry) (Figure 2.6).

Apoptosis in treated and untreated cells was measured using Annexin V/ PI. Briefly, after cell treatment, media from each sample containing any floating cells was pipetted and collected in labelled 15ml tubes. The attached cells were rinsed with PBS (pH 7.4), and this was also collected and added to the same tube. Attached cells were then harvested with trypsin and collected in 15ml tubes. All the fractions of each sample (media with floating cells / harvested adhered cells) were combined in the same, labelled tube and centrifuged at 350 x g, 20°C for 5 minutes. Supernatant media was removed and then 5ml of cell culture media with 10% FCS was added to the pellet, mixed and incubated at 37°C for 30 minutes, to permit cell recovery from trypsin. Cells were then centrifuged again and washed once with 0.5ml PBS (pH 7.4). Meanwhile, the appropriate volume of 1x binding buffer was prepared. Cells were then washed once with 0.5 ml of 1x binding buffer and pelleted. Afterwards, 195µl of 1x binding buffer was added to each sample and the pellet was mixed thoroughly by gently pipetting and transferring to FACs tubes. 5µl of Annexin V was added to each sample, then mixed

and protected from the light and left at room temperature for 10 minutes. Finally,  $10\mu$ l of PI ( $1.5\mu$ g/ml) was added to each sample, then covered and kept on ice whilst analysis by FACS was conducted.



Figure 2.6. The difference in the cell membrane anatomy in live cells, apoptotic cells and late apoptotic cells detected by the annexin V/PI stains.

In a live cell the phospholipids in the bilayer of the cell membrane is asymmetry. The PS is located in the inner layer of the intact normal cell membrane; so neither Annexin V nor PI can bind to cell's membrane and nucleus/DNA. In early apoptotic cell, the membrane become symmetric and the PS are translocated from the inner to the outer leaflet of cell membrane allowing Annexin V to binds the cell membrane. In late apoptosis and necrosis, Annexin V binds the translocated PS in the outer membrane and the loss of cell membrane integrity allows the PI to enter the cell and bind the DNA. The figure was adapted and modified from <u>www.flow-cytometry.us</u>

## 2.8.5 Caspase-3/7 assay

Activation of cysteine aspartic acid-specific protease (Caspase) 3 and 7 family play an essential role in mammalian cell apoptosis (Garcia-Calvo *et al.*, 1999). The Caspase-Glo® 3/7 assay kit was used to measure Caspase 3/7 activity post-treatment. The principle of this assay is based on luminescence. When a mixture of the supplied Caspase-Glo® 3/7 substrate is added to cell lysate, the Caspase cleavage of the substrate leads to generation of a luminescence signal caused by luciferase provided in the reagent (Figure 2.7). The intensity of the luminescence generated is proportional to the level of caspase 3/7 activity in the cell. Following the standard protocol provided, equal numbers of cells ( $15 \times 10^3$ ) (based on assay kit instructions) for each microwell were seeded in a 96-well plate and incubated overnight. Adhered cells were then treated with

and without ascorbate for 2 hours at  $37^{\circ}C/CO_2$  5%. Cells were then washed with PBS and incubated with the test compound (concentrations of exposing agents will be mentioned in the result chapters) in a total volume of 100µL of media per microwell. After treatment, 100µL of Caspase-Glo® 3/7 substrate was added to each microwell, and the plates were gently mixed using a plate shaker for 30 seconds. Plates were then incubated at room temperature for 30 minutes before reading the luminescence intensity (which represent the level of caspase-3/7 activity) using a plate reader (BMG FLUOstar OPTIMA Microplate Reader).



Figure 2.7. Principle of the Caspase-Glo® 3/7 assay for detection of caspase 3/7 expression.

Cleavage of the proluminogenic substrates such as DEVD by caspase 3/7 to release of luciferase, which in turn results in the production of light. Measurement of luminescence intensity represents the amount of caspase-3/7 activity in treated cells.

### 2.9 Western blot

Western blot was used to measure catalase protein levels in melanoma cancer cells and HaCaT cells.

### 2.9.1 Protein isolation

Cells were grown in T75 flasks; when they reached 60-70% confluence they were washed twice with PBS (pH 7.4). Cells were then dislodged using a scraper and lysed by adding 0.7ml of a pre-warmed laemmli buffer (see Section 2.1.1.4, Chapter 2) to each flask; each sample was then collected in 1.5ml Eppendorf tubes. To further lyse and degrade the cells, the Eppendorf were heated at boiling point for 3-4 minutes using a thermo-shaker (Grant-Bio) and then sonicated for one minute. The supernatant was then transferred to another clean Eppendorf tube. After the samples were centrifuged for 5 minutes at 1500 rpm, total protein concentration was quantified for each sample using a Bicinchoninic acid (BCA) protein assay kit. After the protein concentration of the samples had been estimated, a concentration of  $1000\mu g/ml$  was prepared for western blot. Bromophenol blue was added to a final concentration of 0.005% (w/v) and 2-mercaptoethanol to a final concentration of 1% (v/v), and this sample made up to  $200\mu l$  with laemmli buffer and stored at  $-20^{\circ}$ C until analysis.

### 2.9.2 Preparation of gel and sample loading

As the catalase protein is 60kD, a fresh 10% SDS polyacrylamide-gel was prepared. Gels were then made by using gel casting apparatus (Bio-Rad, mini gel apparatus); a stacking gel (5%) was poured on top of the SDS polyacrylamide-gel then the combs were the placed in the top of the gel and left for few minutes to form the wells. Samples were boiled at 98°C for 3 to 5 minutes before being loaded into the gel. 4µl of molecular marker was carefully pipetted into the first well, and  $20\mu l$  ( $20\mu g$ ) of each sample was pipetted into the other wells, in duplicate. The electrophoresis was run by applying a current of 170 V for 1 hour.

### **2.9.3** Transfer of proteins from gel to membrane

With the electrophoresis run complete, a transfer cassette was assembled containing filter layers, nitrocellulose membrane and the gel, with care being taken to ensure that no air bubbles were trapped between layers. The cassette was then transferred into the transfer tank. A piece ice was placed beside the cassette. The tank was then filled with transfer buffer and the current was applied (100 V for 1 hour).

### 2.9.4 Primary and secondary antibodies

Once blots were transferred, the nitrocellulose membrane was placed in TBST-milk blocking buffer and left for 1 hour at room temperature. After this, the nitrocellulose membrane was washed twice with TBST and then the primary antibody for the catalase protein, diluted in TBST-BSA, was added to the nitrocellulose membrane and left to incubate overnight in a cold room at 4°C. The primary antibody was then poured off and the nitrocellulose membrane was washed to the nitrocellulose membrane off and the nitrocellulose membrane was washed four times with TBST. The secondary antibody was then added to the nitrocellulose membrane and left to incubate for a further 1 hour at room temperature, on a shaker (20 rpm).

### 2.9.5 Blotting development

After incubation with primary and secondary antibodies, the membrane was washed with TBST five times, drained and kept in a plastic bag without air bubbles. To view the blot of the catalase protein, the membrane was placed into the X-ray film cassette (DBio Aluminium cassette), and then the cassette was taken to the dark room (film developing room). In the dark room a X-ray film (Thermo Scientific CL-XPosure Film) was placed on the membrane (in the X-ray film cassette) and the cassette was closed for various times (30 seconds, 1 minute and 2 minutes). Immediately, the cassette was then opened and the X-ray film was taken out from the cassette and placed in the automatic film processor (AGFA CP1000 automatic film processer). To analyse and quantify the data (the blots), X-ray films were scanned to the imageJ software.

#### 2.10 Statistical analysis

GraphPad Prism software version 6 (San Diego, CA, USA) was used in this study to determine statistical differences among sample groups. The One-Way Analysis of Variance (ANOVA) followed by Tukey *post hoc* test was used to determine statistical differences between means for multiple comparisons. A student *T*-test (unpaired) was used to compare the difference between two comparisons. The probability value (*p*) was considered as significant if < 0.05. \* if  $P \le 0.05$ , \*\* if  $p \le 0.01$ , \*\*\* if  $p \le 0.001$ , NS, non-significant.

CHAPTER III: Investigation of factors that may render melanoma cancer cells susceptible to H<sub>2</sub>O<sub>2</sub>-induced DNA damage

## 3.1 Introduction

Literature to date, has established that cancer cells exhibit more endogenous ROS than normal cells (Lim *et al.*, 2005). Some types of tumour generate more endogenous ROS than others, and of these MM is considered to be an oxidatively mediated cancer (Szatrowski and Nathan, 1991). This biological feature can cause cancer cells to differ from normal cells, in terms of their oxidative balance.

Human genomes are continuously exposed to attacks of an oxidative nature from both exogenous and endogenous oxidative sources (Kryston *et al.*, 2011). DNA damage resulting from endogenous oxidative assaults is extensive. In a normally functioning cell, oxidant hits DNA probably causing more than 10,000 oxidative damages every day (Ames *et al.*, 1993, Dipple, 1995). But this, in a short term, does not have an unduly detrimental effect to the cell (Evans *et al.*, 2004). Normal cells, with full functioning repair pathways, can efficiently recover from such damage. By contrast, in cancer cells the damage often overwhelms the repair capacity, leading to the accumulation of multiple lesions, causing genomic instability (Jackson and Loeb, 2001).

ROS are highly reactive species. They induce damage and can generate several types of lesions, including DNA strand breaks and oxidative base modifications of DNA molecule (e.g. 8-oxo-dG) (Randerath *et al.*, 1996, Lloyd *et al.*, 1997). Evidence of oxidative DNA damage in the form of lesions, such as 8-oxo-dG, has been used as an indicator of oxidative stress and genomic instability in cancerous tissue (Altieri *et al.*, 2008).

Theoretically, higher levels of endogenous ROS in cancer cells is the basis for a ROSmediated strategy to kill these cells preferentially (Trachootham *et al.*, 2009). It is, therefore, of value to assess endogenous oxidative DNA damage and its consequences on melanoma cancer cells. Such findings might underlie the relative susceptibility of melanoma cancer cells to oxidative-induced damage, and may advance redoxmechanisms, and their modulation as an approach to kill melanoma cancer cells.

This chapter aims to:

• Study the endogenous level of DNA damage, in terms of SSBs and ODBLs (Fpg-sensitive sites (Fpg-SS), in a panel of melanoma cancer cells and HaCaT cells.
- Investigate the level of endogenous ROS in melanoma cancer cells, and HaCaT cells.
- Examine the status of intracellular antioxidants status in melanoma cancer cells and HaCaT cells.
- Identify any relationship between endogenous DNA damage and endogenous ROS plus antioxidant defence.

### 3.2 Results

# 3.2.1 Assessment of endogenous oxidative DNA damage levels in melanoma cancer cells and HaCaT cells

A standard and modified-ACA (see Section 2.4.1.1 in Chapter 2) were conducted to investigate the background levels to oxidative DNA damage, in terms of SSBs and ODBLs (Fpg-SS), in melanoma cancer cells and HaCaT cells.

The modified-ACA detected a significant level of endogenous oxidative DNA damage in all melanoma cancer cells and in HaCaTs relative to the damage enhanced by ERB. By both techniques, a greater amount of endogenous oxidative DNA damage lesions, including SSBs and Fpg-SS, were observed in melanoma cancer cells than in the HaCaTs. Among the melanoma cancer cells, the heavily pigmented melanoma cells (SK23) were the most endogenously damaged, followed by the moderately pigmented (SK28) and two non-pigmented (A375P & A375M) melanoma cancer cells; the HaCaT cells were the least damaged (Figure 3.1).



Figure 3.1. Endogenous DNA damage including SSBs and ODBLs in heavily pigmented (SK23), moderately pigmented (SK28), and non-pigmented (A375M and A375P) melanoma cancer cells, and HaCaT cells.

The cells were seeded in 6-well plates for 24 hours at  $37C^{\circ}/5\%$  CO<sub>2</sub> to allow attachment. Cells were then washed and collected into Eppendorf tubes and the standard and modified-ACA assays were then performed, using indicated levels of Fpg enzyme. Each bar represents the mean % of Tail DNA of 300 comets  $\pm$  SEM determined from three independent experiments. One-way ANOVA test was used to compare the mean of endogenous ODBLs determined by various levels of Fpg enzyme versus the damage enhanced by ERB. Ns, non-significant, \*\*\*p<0.001, \*\*\*\*p<0.0001.

A further analysis of the above data was undertaken using the two melanoma cancer cell lines (A375P & SK23) plus HaCaT cells. For each cell line the level of endogenous ODBLs, as detected by 0.8U/gel Fpg was measured after subtracting the SSBs +ALS. The results were then plotted to illustrate the difference. The findings were that the endogenous SSBs in A375P and SK23 melanoma cancer cells were, respectively, 1.4-fold and 3-fold higher than the endogenous SSBs in HaCaT cells. Likewise, the endogenous ODBLs (Fpg-SS) for A375P and SK23 were, respectively, 1.4-fold and 1.9-fold higher in the melanoma cancer cells than in HaCaT cells (Figure 3.2).



## Figure 3.2. Difference in the level of endogenous oxidative DNA damages lesions in heavily pigmented (SK23) and non-pigmented (A375P) melanoma cells and the HaCaTs.

The endogenous ODBLs (Fpg-SS) detected by 0.8 U/gel Fpg in the three cell lines with subtracted SSBs + ALS. SSBs + ALS of the three cell lines were also plotted to show the difference among three cell lines. One-way ANOVA test was used to compare the mean of endogenous ODBLs and SSBs between melanoma cancer cells and the HaCaT cells. Ns=non-significant, \*\*\*\*p=<0.0001. (The figure adapted from Figure 3.1).

# 3.2.2 Measurement of endogenous ROS level in melanoma cancer cells and in the HaCaTs

The above experiments found clear evidence of endogenous oxidatively damaged DNA in melanoma cancer cells and HaCaT cells, with the higher ODBLs in melanoma cancer cells, particularly the pigmented cells. Therefore, it was proposed to examine the level of endogenous ROS in these cells, to uncover possible causative reasons for such an effect. Thus, a non-pigmented (A375P) and a heavily pigmented (SK23) melanoma cancer cell lines plus the HaCaTs cell line were selected to study their level of endogenous ROS, as these three cell lines had the highest, intermediate, and lowest levels of endogenous DNA damage, respectively, as measured using ACA.

To assess endogenous ROS in these cells, H2DCFDA fluorescent probe was implemented using two different techniques, plate reader and flow cytometry. In using a plate reader, the number of cells needs to be optimised. For this purpose, HaCaT cells were used, because they stick firmly to the microwell plate bases, and are hardly removed during washing. The cells were stained with H2DCFDA fluorescent probe, and the relative fluorescent intensity measured. Among the series of cell numbers investigated, 5 x  $10^4$  was the optimum cells density required for each microwell, to show the level of intracellular ROS, as detected by 1µl /microwell of 25µM fluorescent probe (Figure 3.3).



Figure 3.3. Endogenous ROS level measured in different cells numbers of HaCaT cells.

Cells were seeded in a 96-microwell at different indicated densities for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. Cells were washed with PBS and then stained with H2DCFDA fluorescent probe for 30 minutes at 37°C and protected from light. Fluorescent intensity was then measured using the plate reader. Each bar represents mean  $\pm$  SD determined from three independent experiments run in duplicate. One-way ANOVA test was used to compare the mean of fluorescence intensity (endogenous ROS) determined in different cell numbers *versus* the mean of fluorescence intensity of the background (the blank). Ns, non-significant, \*p=<0.005, \*\*p=<0.001, \*\*\*\*p=<0.0001.

After the cell number had been optimised, for the detection of intracellular ROS by H2DCFDA fluorescent probe using the plate reader, ROS was measured in heavily pigmented (SK23), non-pigmented (A375P) melanoma cancer cells and the HaCaT cells.

Data obtained from this experiment revealed that melanoma cancer cells exhibit a substantially higher level of endogenous ROS than HaCaT cells (Fluorescence intensity (endogenous ROS):  $76.17 \pm 5.69$ ,  $56.33 \pm 8.25$  in SK23 and A375P melanoma cancer cells, respectively, *versus* 29.71  $\pm$  3.24 in HaCaT cells) (Figure 3.4). This was denoted by the fact that the fluorescence intensity of A375P and SK23 melanoma cancer cells stained was higher than that in HaCaT cells, by 1.9-fold and 2.6-fold, respectively.



Figure 3.4. Endogenous ROS levels measured in heavily pigmented (SK23), non-pigmented (A375P) melanoma cancer cells and HaCaT cells detected by H2DCFDA fluorescence probe and measured by plate reader.

A) Fluorescence images show the amount of endogenous ROS in melanoma cancer cells and HaCaTs. Images were captured from microplates using a fluorescence Zeiss Axioskop 2 plus microscope. B) Cells were seeded in a 96-microwell at different indicated densities for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Cells were washed with PBS and then stained with H2DCFDA fluorescent probe for 30 minutes at  $37^{\circ}C$  and protected from light. Fluorescence intensity was then measured using the plate reader. Each bar represents mean  $\pm$  SD of relative fluorescence intensity determined from three independent experiments run in triplicate. One-way ANOVA test was used to compare the mean of fluorescence intensity (endogenous ROS) in melanoma cancer cells *versus* the mean of fluorescence intensity in HaCaT cells.

To further substantiate the above observations, flow cytometry was also used for detection and measuring endogenous ROS in the above different cell lines using the H2CDFDA fluorogenic probe. This method is widely used for assessment of reactive species in cells (Negre-Salvayre *et al.*, 2002). Data obtained by this method agrees with the results obtained using the plate-reader. The level of endogenous ROS observed in SK23 and A375P melanoma cancer cells was significantly higher than that in HaCaT cells (Fluorescence intensity (endogenous ROS):  $14334 \pm 969.6$  *versus*  $2632 \pm 582.9$ , p<0.0001, in comparing SK23 melanoma cancer cells and HaCaT cells, respectively, and  $6998 \pm 643.2$  *versus*  $2632 \pm 582.9$ , p=0.0008, in comparing A375P melanoma

cancer cells and HaCaT cells, respectively). Further analysis of data revealed that the intracellular level of ROS observed in SK23, and A375P melanoma cancer cells was greater by 5.4-fold and 2.8-fold, respectively, compared with that in HaCaT cells (Figure 3.5).



Figure 3.5. Endogenous ROS level measured in heavily pigmented (SK23), non-pigmented (A375P) melanoma cancer cells and the HaCaTs detected by H2DCFDA fluorescence probe and measured by flow cytometry.

A) The flow cytometer hsitograms show the difference in the fluoresence intensity (endogenous ROS) detected in melanoma cancer cells and HaCaT cells. B) Cells were seeded in 6-well plate for 24 hours at  $37C^{\circ}/5\%$  CO<sub>2</sub> to allow attachment. Cells were then washed with PBS and then stained with H<sub>2</sub>DCFDA fluorescence probe for 30 minutes  $37C^{\circ}/5\%$  CO<sub>2</sub>. Cells were then washed once with PBS and collected into FACS tubes for measuring endogenous ROS by flow cytometry. Each bar represents mean  $\pm$  SD of fluorescence intensity determined from three independent experiments each run in triplicate. One-way ANOVA test was used to compare the mean of fluorescence intensity (endogenous ROS) between the two melanoma cell lines *versus* that in HaCaT cells.

## **3.2.3** Assessment of intracellular catalase enzyme activity in melanoma cancer cells and in HaCaT cells

Previous studies have indicated that cancer cells exist under an altered redox status with a disturbed oxidant and antioxidant balance (Nicco *et al.*, 2005, Valko *et al.*, 2007). Melanoma cancer cells are suggested to have low levels of primary cellular antioxidants, including catalase enzyme (Meyskens *et al.*, 1997, Offner *et al.*, 1992). High levels of endogenous ROS in melanoma cancer cells, as detected in the above experiments, may therefore relate to a lower cellular antioxidant status of these cells. Therefore, measures of the intracellular level of catalase enzyme activity in heavily pigmented (SK23), and non-pigmented A375P melanoma cancer cells, and in the HaCaT cells. A visual approach to measuring recent intracellular catalase activity, developed by Iwase *et al.* (2013), was exploited in this study (Iwase *et al.*, 2013). Briefly, this assay is based on the reaction between the intracellular catalase enzyme, released from the cells after cell lysis, with the added H<sub>2</sub>O<sub>2</sub> leading to formation of O<sub>2</sub>foam in the test tube. The higher level of O<sub>2</sub>-foam formation, the more catalase activity in the cell lysate.

Before assessing the intracellular catalase enzyme activity of these cells, the sensitivity of the method was established using bovine liver catalase enzyme as a standard. Different amounts (enzyme units) of bovine liver catalase enzyme were freshly prepared, and each was added to labelled Pyrex tubes. The added catalase enzyme reacted with the  $H_2O_2$  caused  $O_2$ -foam formation, which was then measured using a ruler. Analysis revealed a positive linear relationship (R2=0.9520) between the amount of catalase activity, and the level of the test indicator ( $O_2$ -foam height) (Figure 3.6).



#### Figure 3.6. Standard curve of catalase activity.

A) The photo shows the  $O_2$ -foam formation in Pyrex tubes containing different levels of bovine liver catalase activity (units). B) A constant volume (100µl) of catalase/PBS mixture was maintained by adding each specific amount of catalase to PBS in a Pyrex tube with total volume of 100µl then to each tube 100µl of 1% Triton X-100 and 100µl of concentrated H<sub>2</sub>O<sub>2</sub> (30%) were added. The mixtures were then mixed thoroughly and left at room temperature for 3 minutes to develop an O<sub>2</sub>-foam. The level of O<sub>2</sub>-foam formation from each tube was measured and recorded using a ruler. A linear relationship was found between the level of catalase activity and the height of the foam, over a range of 40-200 units of the catalase enzyme. Each points represents mean ± SEM of O<sub>2</sub>-foam height (mm) as determined in three independent experiments.

To measure the intracellular catalase enzyme activity in heavily pigmented (SK23), non-pigmented (A375P) melanoma cancer cells and HaCaT cells, a high quantity of cells (3 x  $10^6$  cells) were seeded in T175 flasks and incubated for 48 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>, to allow attachment. Cells were then washed and collected into 15ml tubes for assessment of intracellular catalase activity. According to the principle of the assay which is developed by Iwase *et al.* (2013), equal numbers of cells were transferred into Pyrex tubes in which cells were exposed to lysis buffer (Triton X-100) to release their contents into the cell lysate and then H<sub>2</sub>O<sub>2</sub> was added. The reaction between cellular catalase enzyme and H<sub>2</sub>O<sub>2</sub> caused O<sub>2</sub>-foam formation.

A clear reaction between the intracellular catalase and  $H_2O_2$  was observed ( $O_2$ -foam formation), which was linear with the cell numbers used for each cell line. A considerably higher level of catalase enzyme activity was observed in HaCaT cells followed by the A375P melanoma cancer cells, with the lowest level being observed in the SK23 cells, (Figure 3.7). The level of catalase activity measured ( $O_2$ -foam height (mm)), in a lysate of 10 x  $10^6$  cells in HaCaTs was significantly higher than that in A375P and SK23 melanoma cancer cells ( $27.32 \pm 1.53$  versus 16.67  $\pm 2.51$ , p=0.0037, in HaCaTs and A375P melanoma cancer cells, respectively, and  $27.32 \pm 1.53$  versus 10.33  $\pm 3.21$ , p=0.0003 in HaCaTs and SK23 melanoma cancer cells, respectively, and 27.32  $\pm 1.53$  versus 10.33  $\pm 3.21$ , p=0.0003 in HaCaTs and SK23 melanoma cancer cells, respectively, and 27.32  $\pm 1.53$  versus 10.33  $\pm 3.21$ , p=0.0003 in HaCaTs and SK23 melanoma cancer cells, respectively, and 27.32  $\pm 1.53$  versus 10.33  $\pm 3.21$ , p=0.0003 in HaCaTs and SK23 melanoma cancer cells, respectively, and 27.32  $\pm 1.53$  versus 10.33  $\pm 3.21$ , p=0.0003 in HaCaTs and SK23 melanoma cancer cells, respectively) (Figure 3.7).



Figure 3.7. Intracellular level of catalase activity in heavily pigmented (SK23), non-pigmented melanoma cancer cells and the HaCaT cells.

A) Photo shows the  $O_2$ -foam height formed from the reaction between intracellular catalase and  $H_2O_2$  in melanoma cancer cells and HaCaT cells. B) The cells were seeded in T175 flasks for 48 hours at 37°C/5% CO<sub>2</sub> to allow attachment and grow. They were then washed with PBS and harvested.Three different cell numbers (1 x 10<sup>6</sup>, 5 x 10<sup>6</sup> and 10 x 10<sup>6</sup>) were collected into each labelled 15ml tube. The cells were then washed with PBS. The pellets were then transferred into Pyrex tubes and mixed with 100µl of 1% Triton X-100 and 100µl of concentrated H<sub>2</sub>O<sub>2</sub> (30%). The contents of the tubes were then mixed thoroughly and incubated for 3 minutes at room temperature to develop an O<sub>2</sub>-foam. The height of O<sub>2</sub>-foam height (mm) as determined from in three independent experiments. One-way ANOVA test was used to compare the mean of O<sub>2</sub>-foam level (catalase activity) generated by 10 x 10<sup>6</sup> cells between SK23 and A375P melanoma cell cells *versus* that in HaCaT cells.

### **3.2.4** Estimation of catalase protein expression in heavily pigmented (SK23), nonpigmented (A375P) melanoma cancer cells and in HaCaT cells

For further confirmation of variations in the intracellular catalase activity in heavily pigmented (SK23) and non-pigmented (A375P) melanoma cancer cells and HaCaT cells, a western blot assay was performed to assess protein levels. Based on a standard protocol (see Section 2.9 in Chapter 2), the catalase protein was measured in the cell lysates of the above three cell lines. In this assay, a very low level of catalase protein (measured as % of catalase protein band density over the band density of  $\beta$ -actin protein of each cell line) was observed in the two melanoma cancer cell lines; whereas, the level of catalase protein in HaCaT cells was significantly higher (196.0 ± 55.57% versus 44.72 ± 9.22%, *p*=0.0326, in HaCaT cells and A375P melanoma cancer cells, respectively, and 196.0 ± 55.57% versus 50.37 ± 31.04, *p*=0.0388, in HaCaTs and SK23 melanoma cancer cells, respectively (Figure 3.8).



Figure 3.8. Catalase levels in heavily pigmented (SK23), non-pigmented (A375P) melanoma cancer cells and HaCaT cells.

Cells were seeded in T75 flasks for 48 hours at 37°C/5% CO<sub>2</sub> to grow and become confluent. Cells were washed with PBS and collected by scraping and adding 200µl of laemmli buffer and the suspension was added to Eppendorf tubes. From each sample the total protein was estimated before performing western bloting. The samples were then analysed for catalase protein using western blot. Each bar represents mean  $\pm$  SD of relative catalase protein expression versus expression of endogenous  $\beta$ -actin, as determined from two independent experiments. One-way ANOVA test was used to compare the mean of intracellular catalase protein levels between SK23 and A375P melanoma cancer cells *versus* that in HaCaT cells.

# 3.2.5 Correlation of endogenous DNA damages with endogenous ROS level and intracellular catalase enzyme activity in heavily pigmented (Sk23), non-pigmented (A375P) melanoma cancer cells and in HaCaT cells

In melanoma cancer cells, an increase in the level of endogenous oxidative DNA damages was observed, when compared to the HaCaT cells (Figure 3.1). Likewise, the level of intracellular ROS was higher in these melanoma cancer cells when compared with the HaCaTs (Figure 3.4 & 3.5). In contrast, the catalase enzyme activity was significantly lower in melanoma cancer cells (Figure 3.7 & 3.8).

To further analyse this data, and to uncover the relationship between these endogenous parameters, scatter graphs were plotted (Figure 3.9). Interestingly, a positive relationship was clearly apparent between the level of intracellular ROS and the level of endogenous oxidative DNA damage (SSBs & Fpg-ss) in melanoma cancer cells and HaCaT cells. Notably, the melanoma cancer cells with the highest intracellular ROS levels presented the highest level of endogenous DNA damage. In contrast, HaCaT cells, with a low level of endogenous ROS had a lowest level of endogenous DNA damage (Figure 3.9).

Interstingly, the cells with the highest amounts of catalase enzyme have lower levels of endogenous DNA damage including SSBs and endogenous oxidative base lesions (Fpg-SS) (Figure 3.10). Moreover, an inverse relationship between endogenous background ROS, and the intracellular level of catalase enzyme activity was observed in melanoma cancer cells and HaCaT cells. Cells with the lowest amount of endogenous ROS were found to have the highest level of intracellular catalase enzyme activity and vice versa (Figure 3.11).



Figure 3.9. Relationships between endogenous oxidative DNA damage and the intracellular background level of ROS in heavily pigmented (SK23) and non-pigmented (A375P) melanoma cancer cells and HaCaT cells.

A) The graph shows the background DNA SSBs in the three cell lines plotted versus endogenous ROS level measured by flow cytometry in each cell line. B) The graph shows the endogenous oxidative base DNA damage (Fpg-SS) as detected by Fpg-ACA (0.8U/gel) (with background SSBs subtracted) plotted against the endogenous measures of ROS for each cell line. (Figure adapted from Figures 3.2 & 3.5).



Figure 3.10. Relationship between intracellular catalase enzyme activity and the intracellular background level of ROS in heavily pigmented (SK23) and non-pigmented (A375P) melanoma cancer cells and HaCaT cells.

A) The graph shows background DNA SSBs plotted versus the intracellular catalase enzyme activity level detected from  $10 \times 10^6$  cells of each cell line. B) The graph shows the endogenous oxidative base DNA damage (Fpg-SS) as detected by Fpg-ACA (0.8U/gel) (with background SSBs subtracted) plotted against the intracellular catalase activity level detected from  $10 \times 10^6$  cells of each cell line. (Figure adapted from Figures 3.2 & 3.8).



Figure 3.11. Relationships between intracellular catalase enzyme activity and the endogenous ROS in heavily pigmented (SK23) and non-pigmented (A375P) melanoma cancer cells and HaCaTs cells.

The intracellular catalase activity level detected from  $10 \ge 10^6$  cells of each cell line was plotted against the intracellular measures of ROS for each cell line. (Figure adapted from Figures 3.5 & 3.7).

### 3.3 Discussion

ROS, produced either endogenously or exogenously, attack cellular components including DNA, leading to the generation of oxidatively DNA damage including ODBLs. The antioxidant system in normal cells withstands the detrimental (the damaging effect) effects of intracellular ROS (Kryston *et al.*, 2011). It protects the cell from oxidants and reduces DNA damage (Gutteridge and Halliwell, 1999). However, tumour tissues are deficient in antioxidants and the protective systems that target oxidants fail to protect cellular compartments from oxidative damage (Olinski *et al.*, 1995, Glorieux *et al.*, 2011).

Many studies have found elevated measures of oxidatively damaged base lesions (e.g 8oxo-dG) to be present in tumour cells. As reviewed extensively by Kryston *et al.*, (2011) numerous studies have found a higher level of 8-oxo-dG in different tumour tissues (Kryston *et al.*, 2011). In various cancer types, an elevation in urinary 8-oxo-dG, which indicate higher oxidative stress, has been noted (Wu *et al.*, 2004). In addition, formation of DNA 8-oxo-dG has been shown to be increased in breast cancer tissue when compared to normal tissue (Matsui *et al.*, 2000, Musarrat *et al.*, 1996).

Murtas *et a*l. (2010) found an increase in the levels of nuclear 8-oxodG in primary cutaneous melanoma tissue (Murtas *et al.*, 2010). This might indicate that cancer cells have more endogenous oxidatively damaged DNA lesions than normal tissue. Data from the current study (Figure 3.1) confirmed a difference between melanoma cancer cells and the HaCaTs, since the damage observed in melanoma cancer cells, including pigmented and non-pigmented cell lines, was dramatically higher than the DNA damage in HaCaT cells (Figure 3.1). An additional interesting point observed from the current data, was that, of all the melanoma cancer cells, the highly pigmented melanoma cancer cells (SK23) exhibited the highest level of endogenous DNA damage.

Endogenous oxidatively damaged DNA is strongly linked to the level of oxidative stress in tumour cells. In cancer cells, endogenous ROS production is abnormally high, and this could be the reason for the insufficiency in the antioxidant's capacities; thus the antioxidant system failure may be responsible for the higher endogenous DNA damage (Jackson and Loeb, 2001). In contrast, in normally functioning cells, the rate of intracellular  $H_2O_2$  generation is maintained in a steady state and rangies between submicromolar levels (Gardner *et al.*, 1997, Takeda *et al.*, 1999). Other studies suggest that melanoma cancer cells generate a high amount of endogenous H<sub>2</sub>O<sub>2</sub> (Toyokuni et al., 1995, Meyskens Jr et al., 2001), with a greater amount of endogenous H<sub>2</sub>O<sub>2</sub> observed in pigmented melanoma cancer cells (Szatrowski and Nathan, 1991, Meyskens et al., 2004). In melanoma cancer cells, because of the malformed melanosomes and the disruption of melanin synthesis during the melanogenesis process, melanin acts as another source of ROS generation (Sarangarajan and Apte, 2006). In support of this, the data obtained in the present study indicates that melanoma cancer cells have a significantly higher amount of ROS background than HaCaT cells, and that pigmented melanoma (SK23) cells have even greater endogenous ROS levels than non-pigmented melanoma cancer cells (figure 3.4 & 3.5). The difference in the level of endogenous ROS between pigmented (SK23) and nonpigmented (A375P) may be the result of melanin pigment content. A recent experimental study has found a significant level of enhancement in the level of intracellular H<sub>2</sub>O<sub>2</sub> in SK23 melanoma cancer cells after exposure them to UV radiation whereas the level of ROS generated in non-pigmented cells (A375) was considerably lower (Swalwell et al., 2012).

Studies have provided evidence indicating that expression of intracellular antioxidants, including catalase, is tissue, organ and cell dependent (Jeon et al., 2007, Oberley et al., 1996). It has been reported that melanoma cancer cells are deficient in primary antioxidant systems, including catalase and glutathione (Offner et al., 1992, Picardo et al., 1996, Meyskens et al., 1997). Catalase enzyme is the major antioxidant that tackles and disassociates  $H_2O_2$  in the cellular environment, generating  $H_2O$  and  $O_2$ (Schraufstätter et al., 1988). Strong evidence indicates that UV light is one of the main etiological factors for melanoma development (Williams and Ouhtit, 2005, Chang et al., 2009) and a recent animal study has found that exposure of skin to UV-B light can cause a significant inhibition in cutaneous catalase enzyme activity (Sullivan et al., 2012). The data reported in this Chapter indicates that catalase enzyme activity is significantly lower in melanoma cancer cells than in HaCaT cells (Figure 3.7). This effect possibly increases the sensitivity of melanoma cancer cells to H<sub>2</sub>O<sub>2</sub> damage. To support this further, a western blot assay was conducted to measure the catalase protein levels in HaCaT cells and melanoma cancer cells. Data obtained from the experiment, again demonstrated a very high level of expression of catalase protein in HaCaT cells, while it was substantially lower in both the A375P and SK23 melanoma cancer cells

(Figure 3.8). As mentioned in the literature, catalase is the main intracellular antioxidant that converts  $H_2O_2$  to water and  $O_2$ . A recent experimental study has found that suppression of catalase protein expression in tumour cells (HeLa, SKOV3 and A549 tumour cell lines) lead to accumulation of endogenous  $H_2O_2$  (Yang *et al.*, 2011). Consistent to their observations, data from the current study may indicate that high endogenous ROS is due to the low level of intracellular catalase expression in melanoma cancer cells (Figure 3.11).

To further support this, evidence suggests an increase in the level of oxidative DNA damage in acute lymphoblastic leukaemia accompanied with a reduced antioxidant capacity, including catalase (Honda *et al.*, 2000). Data regarding the relationship of endogenous DNA damage to the level of intracellular ROS has not been available for melanoma cancer cells. Data in the current Chapter of this study indicates a positive linear relationship between these two parameters (Figure 3.9). It revealed that, heavily pigmented melanoma cancer cells (SK23) present the highest endogenous DNA damage and a greater level of intracellular ROS. In contrast further analysis determined an inverse relationship between intracellular ROS and cellular catalase enzyme activity in melanoma cancer cells and HaCaT cells (Figure 3.11). Likewise, the amount of endogenous DNA damage in melanoma and HaCaT cells is inversely related to the level of their intracellular catalase enzyme activity (Figure 3.10).

Lack of intracellular catalase enzyme activity in melanoma cancer cells could be one factor explaining the accumulation of high intracellular ROS. Consequently, this together with higher metabolism may enhance the levels of oxidative DNA damage in melanoma cancer cells if they exposed to exogenous therapeutic ROS.

CHAPTER IV: Optimisation and characterisation of H<sub>2</sub>O<sub>2</sub>-induced DNA damage dose response in melanoma cancer cells and in HaCaT and HDF cells

#### 4.1 Introduction

 $H_2O_2$  is an oxidant, which has long been used as a model in studies related to oxidative stress (Coyle and Kader, 2007, Collins, 2004, Meneghini, 1997).  $H_2O_2$  is a small ROS molecule. It is relatively unreactive, mostly stable (compared to other ROSs), highly diffusible and crosses the cell membrane, travelling long distances in intracellular and extracellular spaces and to reach its molecular targets (Mishina *et al.*, 2011, Panieri *et al.*, 2013).

DNA is particularly sensitive to  $H_2O_2$ , an oxidant that induces oxidative stress within cells and causes DNA damage (Barbouti *et al.*, 2002, Ott *et al.*, 2007). Many studies have used  $H_2O_2$  to induce circumstances of oxidative stress and oxidative DNA damage in different types of cells (Driessens *et al.*, 2009, Duarte *et al.*, 2007, Panieri *et al.*, 2013). Duarte and Jones, (2007) investigated the mechanism by which  $H_2O_2$  causes oxidative DNA damage (Duarte and Jones, 2007). Their data suggested that when  $H_2O_2$ enters the cell, it reacts with iron ions through a Fenton reaction, generating <sup>•</sup>OH, which can attack the DNA molecule, producing strand breaks and other lesions.

In the previous Chapter, a clear difference in the level of endogenous oxidatively damaged DNA between melanoma cancer cells and normal HaCaT cells was observed. The comet assay detected significant levels of endogenous oxidatively damaged DNA in melanoma cancer cells, compared with levels of the same damage in HaCaT cells. Such a difference was also positively correlated with the amounts of endogenous ROS, but negatively correlated with catalase enzyme activity. Based on this data, it was proposed to study the effect of induced DNA damage by the model oxidant  $(H_2O_2)$  in melanoma cancer cells and normal skin cells. This could illustrate the relative degree of sensitivity of melanoma cancer cells and normal skin cells to induced DNA damage.

Consequently,  $H_2O_2$  was used as a model oxidant to induce oxidative DNA damage in a panel of melanoma cancer cell lines, including non-pigmented (A375M & A375P), moderately (SK28) and heavily (SK23) pigmented melanoma cancer cells, and two "normal" skin cell lines (HaCaTs & HDF cells). Using the standard protocol described in the Materials and Methods section of the current study (see Section 2.5.1, in Chapter 2), the ACA and its modified version were used in this study to measure DNA damage after cell treatment with  $H_2O_2$ .

This chapter aims to:

- Optimise the concentrations and time of H<sub>2</sub>O<sub>2</sub> exposure necessary for it to be able to induce measurable levels of DNA damage as detected by the ACA.
- Study the degree of sensitivity of melanoma cancer cells and HaCaT and HDF cells to oxidative mediated DNA damage induced by H<sub>2</sub>O<sub>2</sub>.
- Study the role of iron ions in the H<sub>2</sub>O<sub>2</sub>-induced DNA damage mechanism.

### 4.2 Results

### 4.2.1 H<sub>2</sub>O<sub>2</sub>-induced DNA damage in A375M melanoma cancer cells.

The A375M melanoma cancer cell line was initially used to optimise the exposure time and doses of  $H_2O_2$  for the induction of DNA damage measured by the standard ACA. A clear  $H_2O_2$ -induced DNA damage response was observed that was both time- and dosedependent. Low micromolar concentrations of  $H_2O_2$  were generally able to generate DNA damage in A375M melanoma cancer cells proportional to the concentration of  $H_2O_2$  and time of exposure (Figure 4.1).



Figure 4.1. H<sub>2</sub>O<sub>2</sub>-induced DNA damage dose responses in A375M melanoma cancer cells.

Cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> prepared in a cell-specific medium for different time points, on ice, protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 300 comets  $\pm$  SEM determined from three independent experiments.

### 4.2.2 H<sub>2</sub>O<sub>2</sub>-induced DNA damage in non-pigmented (A375M and A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells and in HaCaT cells treated in cell-specific media

A panel of four melanoma cancer cell lines, A375M, A375P, SK28 and SK23 melanoma cancer cells and HaCaT cells, were treated in their cell-specific serum-free culture media, with varying low levels of H<sub>2</sub>O<sub>2</sub> concentrations (ranging from 0 to 30 $\mu$ M) for 30 minutes on ice, protected from the light. ACA was then immediately carried out to measure the level of oxidative DNA damage in the cells. Unexpectedly, the three melanoma cell lines (A375M, A375P & SK23) clearly formed H<sub>2</sub>O<sub>2</sub>-mediated DNA damage, while one of the melanoma cell lines (SK28) and the HaCaT cells did not. Of the cell lines that responded to H<sub>2</sub>O<sub>2</sub>-induced DNA damage, the SK23 cells (the heavily pigmented melanoma cancer cells) were the most sensitive, followed by the A375P and A375M cells (Figure 4.2).



# Figure 4.2. $H_2O_2$ -induced DNA damage in non-pigmented (A375M and A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells HaCaTs treated using low doses of $H_2O_2$ in cell-specific media.

Cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> before exposure to H<sub>2</sub>O<sub>2</sub>. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> prepared in a cell-specific medium for 30 minutes, on ice, protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 200 comets ± SEM determined from two independent experiments.

The insensitivity of the cell lines SK28 and HaCaTs to  $H_2O_2$  was unexpected, and it was thought that the levels of  $H_2O_2$  used may not have been high enough to induce DNA damage in these cells, and that the other cell lines (A375M, A375P & SK23) were more sensitive to  $H_2O_2$ . So, in subsequent further studies, the experiment was repeated using higher doses of  $H_2O_2$  (up to 100µM). Surprisingly, the reaction of A375M, A375P and SK23 melanoma cells to  $H_2O_2$  was almost the same as in the previous experiment, although clearly higher. However, SK28 and HaCaT cells did not respond to  $H_2O_2$ . Even with the highest concentration of  $H_2O_2$  (100µM), the  $H_2O_2$ -induced DNA damage observed in SK28 and HaCaT cells did not exceed 10% (Figure 4.3).



Figure 4.3.  $H_2O_2$ -induced DNA damage in non-pigmented (A375M and A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells and HaCaT cells treated using high doses of  $H_2O_2$  in cell-specific media.

Cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> prepared in a cell-specific medium for 30 minutes, on ice, protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 200 comets ± SEM determined from two independent experiments.

The lack of effect of  $H_2O_2$  for the SK28 and normal HaCaT cells drew attention to the composition of the culture media in which the cells were treated. It was therefore proposed to investigate the possible influence of media composition on  $H_2O_2$ -induced DNA damage. Two cell lines, one sensitive to  $H_2O_2$  (A375M) and one insensitive to  $H_2O_2$  (SK28), were used. Three different media, including A375M-media, SK28-media and PBS, were used for the treatment of SK28 and A375M cells with  $H_2O_2$ . However, before conducting this experiment, a trypan blue assay (see Section 2.4.1, Chapter 2) was performed in order to assess the effect of the different media on the cell viability of both cell lines (A375M & SK28) for the duration of the treatment. No gross difference in the cell viability was observed for the two cell lines (A375M & SK28) treated with the three different media (PBS, A375M-media and SK28-media), with all cell lines showing similar high levels of viability (Table 4.1).

Table 4.1. Viability of melanoma cancer cells (A375M and SK28) in three different media

Media	A375M melanoma cells	SK28 melanoma cells
	Viability (%)	Viability (%)
PBS	85.5	73.0
A375M-media	90.5	71.0
SK28-media	81.0	74.0

A375M and SK28 melanoma cancer cells were seeded in each well of the 6-well plate for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>, using their own specific media to allow attachment. The cells where then washed once with PBS, and new different media (PBS, A375M-media and SK28-media) were then added to each well separately and incubated on ice for 30 minutes. The media were removed from each well and the cells were washed with PBS and collected by trypsin. A trypane blue exclusion assay was then performed (as described in section 2.4.1, Chapter 2).

After confirming the effect of these three media on cell viability, a standard ACA experiment was conducted using one sensitive cell line (A375M) and one insensitive cell line (SK28) and each experiment for each cell line was carried out separately. Cells were exposed to  $H_2O_2$  using cell-specific media, an alternative non-cell specific medium or PBS. The results showed that neither cell line (A375M or SK28) responded to  $H_2O_2$  when treated in SK28-media, revealing that SK28-media has an inhibitory effect on  $H_2O_2$ -induced DNA damage. In contrast, the two cell lines were found to be sensitive to  $H_2O_2$  when cells were treated in A375M-specific media and PBS (Figure 4.4 & 4.5).



Figure 4.4. H<sub>2</sub>O<sub>2</sub>-induced DNA damage in A375M (A) melanoma cancer cells treated either in A375M-media (A), SK28-media (B) or PBS (C).

Cells were seeded in 6-well plates for 24 hours at 37°C/5% CO<sub>2</sub> before exposure to H<sub>2</sub>O<sub>2</sub>. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> prepared in PBS, A375M-media and SK28-media for 30 minutes, on ice, protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 300 comets  $\pm$  SEM determined from three independent experiments.



Figure 4.5. H<sub>2</sub>O<sub>2</sub>-induced DNA damage in SK28 melanoma cancer cells treated either in A375Mmedia (A), SK28-media (B) or PBS (C).

Cells were seeded in 6-well plates for 24 hours at 37°C/5% CO<sub>2</sub> before exposure to  $H_2O_2$ . The cells were then exposed to indicated concentrations of  $H_2O_2$  prepared in PBS, A375M-media and SK28-media for 30 minutes, on ice, protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 300 comets  $\pm$  SEM determined from three independent experiments.

In order to further examine this media effect, it was proposed that one of the media constituents could be the reason for the observed inhibitory effect. For a better cell growth, the media for A375M, A375P and SK23 were usually supplemented with 10% FCS, 1% glutamine, 1% non-essential amino acids whereas, additional to FCS and glutamine, 1% of sodium pyruvate was usually added as a supplement to SK28 and HaCaTs-specific media to promote cell growth. Indeed, a previous study suggested that pyruvate in culture media scavenges  $H_2O_2$  generation (Long and Halliwell, 2009). In order to test this, another ACA experiment was conducted using A375M melanoma cancer cells to examine the level of DNA damage that can be induced by  $H_2O_2$  in different media (A375M-media and PBS), supplemented with and without 1% sodium pyruvate, substantially protected cellular DNA from oxidative damage mediated by  $H_2O_2$ , whereas the same media without sodium pyruvate allowed  $H_2O_2$  to induce DNA damage (Figure 4.6); since the damage induced by the highest dose of  $H_2O_2$  prepared in media containing pyruvate did not exceed 10%.



### Figure 4.6. Effect of sodium pyruvate on the level of $H_2O_2$ -induced DNA damage in A375M melanoma cancer cells.

Cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> before exposure to H<sub>2</sub>O<sub>2</sub>. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> prepared in two different media (A375M-media and PBS), with and without 1% sodium pyruvate for 30 minutes, on ice, protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 300 comets ± SEM determined from three independent experiments.

### 4.2.3 H<sub>2</sub>O<sub>2</sub>-induced DNA damage in non-pigmented (A375M and A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells and HaCaT cells treated in A375M-media

From the previous experiments, it was decided to use A375M-media as the H<sub>2</sub>O<sub>2</sub>treatment medium for all future experiments. Next, an ACA experiment was conducted involving A375M, A375P, SK28 and SK23 melanoma cancer cells and HaCaT cells. Cells were seeded and treated with different levels of H<sub>2</sub>O<sub>2</sub> prepared in serum free A375M-media, and the ACA experiment performed. From the scored comets, a clear H<sub>2</sub>O<sub>2</sub>-induced DNA damage dose response was observed in all melanoma cancer cells and in the HaCaT cells. As with the previous experiments, SK23 melanoma cancer cells were the most sensitive, followed by the SK28, A375P and A375M melanoma cancer cells, with the HaCaT cells being least sensitive to H<sub>2</sub>O<sub>2</sub> (Figure 4.7).



# Figure 4.7. $H_2O_2$ -induced DNA damage in non-pigmented (A375M and A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells and in HaCaT cells treated in A375M-media.

Cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> prepared in A375M-media for 30 minutes, on ice and protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 300 comets ± SEM determined from three independent experiments.

## 4.2.4 H<sub>2</sub>O<sub>2</sub>-induced DNA damage in non-pigmented (A375M) melanoma cancer cells, HaCaTs and HDF cells

A further study was carried out to examine the sensitivity of normal skin cells, including HaCaTs and HDF cells and melanoma cancer cells, to H<sub>2</sub>O<sub>2</sub>-induced DNA damage. For this puropose, HDF and HaCaT cells plus A375M melanoma cancer cells were all exposed to H<sub>2</sub>O<sub>2</sub> and then analysed by ACA in order to measure the level of DNA damage. The data observed in this study confirmed that the normal cells (HaCaTs & HDF cells) are less sensitive than melanoma cancer cells to DNA damage induced by H<sub>2</sub>O<sub>2</sub>, and of the two normal skin cell lines, HDF cells were less sensitive than HaCaT cells (Figure 4.8). Analysis of data has shown that in melanoma cancer cells, the damage induced by 20µM H<sub>2</sub>O<sub>2</sub> was significantly higher than that in HaCaT and HDF cells (% tail DNA:  $36.82 \pm 1.06$  *versus*  $23.94 \pm 1.44\%$ , *p*<0.0001, in comparing A375M melanoma cancer cells and HDF cells, respectively, and  $36.82 \pm 1.06$  *versus*  $21.08 \pm 1.17\%$ , *p*<0.0001, in comparing A375M melanoma cancer cells and HDF cells, respectively).



### Figure 4.8. H<sub>2</sub>O<sub>2</sub>-induced DNA damage in A375M melanoma cancer cells and in HaCaTs and HDF cells.

Cells were seeded in 6-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. The cells were then exposed to indicated concentrations of  $H_2O_2$  prepared in A375M-media for 30 minutes, on ice, protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 300 comets  $\pm$  SEM determined from three independent experiments. A one-way ANOVA was used for statistical analysis between each of the three groups.

### 4.2.5 Investigation of the role of iron ions in H<sub>2</sub>O<sub>2</sub>-induced DNA damage

Previous studies have shown that iron ions play a crucial role in the H<sub>2</sub>O<sub>2</sub>-induced DNA damage mechanism in cells exposed to H<sub>2</sub>O<sub>2</sub> (Duarte *et al.*, 2007b, Barbouti *et al.*, 2001). In the Fenton reaction (see Section 1.3.2, Chapter 1), reduced redox-active translational metal ions reduces H<sub>2</sub>O<sub>2</sub> to generate <sup>•</sup>OH, which in turn attacks DNA and induces oxidative damage. In this reaction, H<sub>2</sub>O<sub>2</sub> oxidises ferrous ions (Fe<sup>2+</sup>) to ferric ions (Fe<sup>3+</sup>), producing <sup>•</sup>OH. In order to investigate this and confirm the involvement of metal ions, two melanoma cancer cell lines (A375P & SK23) were selected and seeded 24 hours before treatment with and without an iron chelating agent (DFO) for different times. The cells were then exposed to H<sub>2</sub>O<sub>2</sub> in order to induce DNA damage, which was measured by the standard ACA. The data obtained from this experiment indicated a significant reduction in the level of DNA damage in cells pre-incubated with DFO for  $\geq$  5 hours, whereas DNA damage was evident in those not treated or treated with DFO immediately (Figure 4.9).



## Figure 4.9. H<sub>2</sub>O<sub>2</sub>-induced DNA damage in non-pigmented (A375P) (A) and heavily pigmented (SK23) (B) melanoma cancer cells, in the presence and absence of DFO.

Cells were seeded for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment, before exposure to DFO (300µM) for indicated time points. The cells were then washed with PBS and exposed to H<sub>2</sub>O<sub>2</sub> for 30 minutes, on ice, protected from the light. After the cells were harvested, DNA damage was measured by the standard ACA. Each bar represents the mean percentage of Tail DNA of 200 comets ± SEM determined from two independent experiments.

## 4.2.6 Measurement of total intracellular iron level in non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer cells

Studies have suggested that melanoma cancer cells trap and contain more iron ions than do normal cells (Bedrick *et al.*, 1986), and that the pigment melanin has an affinity for metal ions (Sarzanini *et al.*, 1992). As was observed in the above experiments, iron ions play a crucial role in the H<sub>2</sub>O<sub>2</sub>-induced DNA damage mechanism. An important thing to note in all the ACA experiments conducted so far was the higher sensitivity of the pigmented melanoma cancer cells (SK28 & SK23) to H<sub>2</sub>O<sub>2</sub>-induced DNA damage compared with the non-pigmented melanoma cancer cells (A375P & A375M). It was proposed that the iron ion content in pigmented and non-pigmented melanoma cancer cells could play a role in the level of cellular sensitivity to H<sub>2</sub>O<sub>2</sub>-induced DNA damage.

In order to assess the intracellular concentration of total iron ions in pigmented and nonpigmented melanoma cancer cells, SK23 and A375P melanoma cell lines were selected. An equal number of the two cell lines was seeded in T75 flasks and allowed to attach, and from this the total intracellular iron concentration was measured in  $2 \times 10^6$  cells of each cell line using the iron assay kit. The test was conducted according to the manufacturer's instructions (see Section 2.7, Chapter 2). Different iron standards were prepared from the (stock) iron standard with iron assay buffer (provided by the kit) (Table 4.2). The estimation of total iron was run parallel to the determined standard curve, which showed a clear linear response (R2=0.9553) (Figure 4.10). When the cell extracts were examined, the pigmented melanoma cancer cells were found to have a significantly greater level of total intracellular iron ions than the non-pigmented melanoma cancer cells (Figure 4.11). The levels of total intracellular iron measured in 20µl cell extract lyaste were 2.33  $\pm$  0.03 nmol/L versus 2.16  $\pm$  0.04 nmol/L, p=0.0002, in SK23 and A375P melanoma cancer cells, respectively. Similarly, the amount of total intracellular iron measured in 30µl cell extract lyaste were  $2.53 \pm 0.01$ nmol/L versus  $2.34 \pm 0.03$  nmol/L, p=0.0005, in SK23 and A375P melanoma cancer cells, respectively.



Table 4.2. Iron standards preparation with iron assay buffer



Figure 4.10. A standard curve of iron concentrations as detected by the colourometric method.

Various concentrations of iron standards were prepared and added to each microwell, of a 96-microwell plate, in triplicate, in order to obtain a final concentration of 0, 2, 4, 6, 8 and 10nmol/well. Optical density (O.D.) was measured at 593nm. A standard curve was then constructed using linear regression analysis. The assay kit measures iron ranging from 0.4nmol to 20nmol per  $50\mu$ l of sample. Each point represents mean  $\pm$  SD of absorbance, measured from different standards containing different levels of total iron (prepared from stock standard) from two independent experiments.



## Figure 4.11. Intracellular total iron ions in non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer cells.

 $2x10^6$  cells were transferred into 15ml tubes and washed with cold PBS.  $250\mu$ l of iron assay buffer was added to each pellet and mixed thoroughly. To remove the insoluble substances, the mixture was centrifuged for 10 minutes at 16,000g. From supernatants, different sample volumes (5, 10, 20, and 30µl) were transferred to 96-microwell plates and each was adjusted to 100µl with the iron assay buffer. 5µl of iron-reduced reagent was added to each and incubated at room temperature for 30 minutes. 100µl of iron probe was then added to each microwell, mixed and incubated for further 60 minutes at room temperature in the dark. The O.D. was then measured at 593nm. Each point represents mean ± SD of the total intracellular iron level (nmol) per cell lysate volume (µl) measured depending on the standard curve. The data was determined from three independent experiments, each run in triplicate. A *T*-test (unpaired) was used for statistical analysis between each of the two groups.

#### 4.3 Discussion

ACA is a technique that has been broadly used in the field of genotoxicity. This assay is a simple and reliable method of quantifying DNA strand breaks, alkali-labile sites (ALS) and crosslinks, induced either physically or chemically. Migration of the damaged DNA in an electronic field is proportional to the extension of the DNA strand breaks, and is considered to be an estimate of the degree of DNA damage (Duez *et al.*, 2003).

 $H_2O_2$  was used as a model oxidant to induce oxidative stress-mediated DNA damage, measured by ACA (Duarte and Jones, 2007). It is thought that  $H_2O_2$  rapidly enters cells via water channels (aquaporin) to reach the nucleus, where it generates <sup>•</sup>OH, which in turn attacks the sugar residue of the DNA backbone, resulting in strand breaks. It can also modify the DNA bases to generate oxidatively damaged purine and pyrimidine base lesions (Jaruga and Dizdaroglu, 1996, Henzler and Steudle, 2000).

Optimisation of the exposure time and doses of  $H_2O_2$  was an important step for this study. A recent study concluded that the degree of  $H_2O_2$ -induced DNA damage is doseand incubation time-dependent (Benhusein *et al.*, 2010). Exposure of cells to  $H_2O_2$  for long periods could result in a reduction in the level of DNA damage by repair mechanisms or by the decomposition of  $H_2O_2$  (Benhusein *et al.*, 2010).

In the present study, time and dose-dependent  $H_2O_2$ -induced DNA damage was observed in A375M melanoma cancer cells. The damage caused by  $H_2O_2$  was very low in the first 15 minutes, but increased substantially after further incubation. DNA damage was highest when exposing cells to 10 and  $30\mu M H_2O_2$  for an hour, but with evidence of a 'limit' being reached. This may be the result of  $H_2O_2$  decomposition in media with time. Therefore, 30 minutes' incubation time was chosen as an ideal period for the induction of a clear  $H_2O_2$ -induced DNA damage dose response for all future work (Figure 4.1).

The study aim in this Chapter was to investigate  $H_2O_2$ -induced DNA damage dose response in different melanoma cancer cell lines (according to the degree of pigmentation) and normal skin cells. The first two  $H_2O_2$ -induced DNA damage experiments, which involved all the melanoma cancer cell lines and the HaCaT cells, surprisingly revealed an apparent high level of resistance of some cell lines to  $H_2O_2$ - (Figures 4.2 & 4.3). The highest level of  $H_2O_2$ -induced DNA damage was observed for the SK23 melanoma cells, and the lowest level of damage was observed in the SK28 melanoma cells and the HaCaT cells.

Further analysis has shown that a media constituent affects  $H_2O_2$ -induced DNA damage (Figure 4.4 & 4.5). Media such as DMEM contain pyruvic acid, which has been found to have an inhibitory effect on  $H_2O_2$ -induced DNA damage. This effect was confirmed when cells were exposed to  $H_2O_2$  in media supplemented with and without 1% of sodium pyruvate (Figure 4.6).

Thus, the media effect must be taken into account during investigation of oxidative mediated DNA damage by  $H_2O_2$  or other oxidants. From this experiment, it was decided to use A375M-media as the source for preparing  $H_2O_2$  concentrations for all cell lines in all future work. When the melanoma cancer cells and the HaCaT cells were treated with  $H_2O_2$  in A375M-media, a clear  $H_2O_2$ -induced DNA damage dose response was observed for all the cells. Interestingly, the highest level of  $H_2O_2$ -induced DNA damage was demonstrated in the heavily pigmented (SK23) and moderately pigmented (SK28) melanoma cancer cells, followed by the two non-pigmented melanoma cancer cell lines (A375M & A375P), and the HaCaT cells were the least sensitive to this effect (Figure 4.7).

The observed high level of  $H_2O_2$ -induced damage in the pigmented melanoma cancer cells raises many questions. On one hand, it has been suggested that melanin can act as an antioxidant, scavenging FRs (Riley, 1997, Godic *et al.*, 2014), and this could protect pigmented cells from damaging ROS. Heavily pigmented skin also protects cells from light-induced DNA damage because of melanin, which absorbs the damaging photons (Agar and Young, 2005, Kobayashi *et al.*, 1993). On the other hand, other studies have found an enhancement effect of melanin on induced DNA damage. UV irradiation directly and indirectly induces intracellular oxidative stress (Stepien, 2010), it has been found that pigmented melanocytes are more sensitive to UV irradiation-induced oxidative DNA damage in terms of SSBs (Wenczl *et al.*, 1998). Kvam and Tryrrell, (1999), also reported high sensitivity of pigmented human melanoma cells to UV-induced ODBLs (Kvam and Tyrrell, 1999). Furthermore, it has been reported that both keratinocyte and skin fibroblast are less vulnerable than melanocytes to oxidative stress-induced DNA damage (Valverde *et al.*, 1996, Furukawa *et al.*, 1988). Moreover, it has been shown that melanin pigment in melanocytes enhances DNA strand breaks, and
damage is inhibited by blocking melanogensis with tyrosinase inhibitors (Hoogduijn *et al.*, 2004). Researchers carrying out an *in vitro* study also observed a higher sensitivity of DNA to UV-induced oxidative DNA damage in the presence of melanin (Kvam and Tyrrell, 1999).

Data from the present study suggests that pigmented melanoma cancer cells are more susceptible to  $H_2O_2$ -induced DNA damage than non-pigmented cells (Figure 4.7). Consistent with these findings, Kvam and Tyrrell (1999) (Kvam and Tyrrell, 1999) found no protective effect of melanin pigment in human melanoma cells against oxidative-induced DNA damage caused by UV irradiation. Their data shows a 2-fold greater accumulation of oxidatively damaged DNA lesions in melanoma cancer cells with high content of melanin pigment compared with cells with low melanin pigment.

More importantly, data from the present study suggests that melanoma cancer cells are more susceptible to  $H_2O_2$ -induced DNA damage than HaCaTs and HDF cells (Figure 4.8). This could indicate that melanoma cancer cells are more sensitive to oxidative-mediated DNA damage than normal cells.

Metal ions have a crucial role in the H<sub>2</sub>O<sub>2</sub>-induced DNA damage mechanism (Barbouti et al., 2001). Although some studies have reported that copper ions are more efficient than iron ions in initiating H<sub>2</sub>O<sub>2</sub>-induced DNA damage in non-cellular systems (Stohs and Bagchi, 1995, Chevion, 1988, Lloyd and Phillips, 1999, Oikawa and Kawanishi, 1998), Barbouti et al. (Barbouti et al., 2001) suggested that, unlike iron ions, free intracellular copper ions are undetectable; thus they suggested that intracellular iron ions play a critical role in  $H_2O_2$ -induced DNA damage. This is further supported by more recent studies, which utilised the iron ion chelating agent (DFO) before the induction of H<sub>2</sub>O<sub>2</sub>-induced DNA damage (Duarte et al., 2007). In the current study, the role of intracellular iron ions was reinvestigated. The damage induced by H<sub>2</sub>O<sub>2</sub> was substantially prevented when cells were incubated with DFO for five hours or longer (Figure 4.9). However, the damage induced by  $H_2O_2$  without the effect of DFO was significant, particularly in SK23 melanoma cells. This suggests that iron ion is the key element for the generation of  $^{\circ}OH$  from  $H_2O_2$ . When the two melanoma cancer cell lines, pigmented (SK23) and non-pigmented (A375P), were examined for their total iron content, it was found that pigmented cell lines contain a significantly higher concentration of total iron than that non-pigmented melanoma cancer cells (Figure 4.11). This could be one of the reasons why pigmented melanoma cancer cells are more sensitive to  $H_2O_2$  than non-pigmented melanoma cancer cells. It may also be conceivable that the pigment melanin in melanoma cells could sensitise its DNA molecule to  $H_2O_2$ -induced DNA damage because the pigmented cells have a larger amount of intracellular iron ions.

Matching the data of the previous chapter (Chapter 3) with the findings of the current one, it can be concluded that melanoma cancer cells with higher amount of endogenous ROS and with more oxidatively damaged DNA are more susceptible to  $H_2O_2$ -induced DNA damage. The heavily pigmented (SK23) and non-pigmented (A375P) melanoma cells contain much more endogenous ROS and are substantially lower in catalase enzyme activity than the HaCaTs, and are highly sensitive to  $H_2O_2$ -induced DNA damage. This means that adding exogenous ROS ( $H_2O_2$ ) to melanoma cancer cells, which have more endogenous ROS and low catalase enzyme activity, can induce more oxidative DNA damage.

Overall, the data shown indicates that melanoma cancer cells are more sensitive to  $H_2O_2$ -induced DNA damage than normal skin cells, including HaCaTs and HDF cells, with the highest sensitivity observed in pigmented melanoma cancer cells. Media composition plays a key role a role in  $H_2O_2$ -induced DNA damage and this has to be taken in to account in any in *vitro* studies trying to investigate the oxidative-mediated DNA damage.

Additionally, it has been found that iron ion is an essential component in  $H_2O_2$ -induced DNA damage, and that the pigmented melanoma cancer cells have greater concentrations of total intracellular iron ions than the non-pigmented melanoma cancer cells. Such differences play a major role in the degree of melanoma cell susceptibility to  $H_2O_2$ -induced DNA damage.

CHAPTER V: Effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage in melanoma cancer cells and in HaCaT and HDF cells

#### 5.1 Introduction

The pro-oxidant activity of ascorbate has been a subject of interest in many studies (Podmore *et al.*, 1998, Halliwell, 1996, Carr and Frei, 1999a, Duarte and Jones, 2007, Halliwell, 2013). As was alluded to in the literature review section (see Section 1.5.4, Chapter 1), the pro-oxidant role of vitamin C is thought to involve two different mechanisms. One of the pro-oxidant roles of ascorbate is through autoxidation, which can yield  $H_2O_2$  in the presence of transition metal ions (Chen *et al.*, 2005). Injection of high doses of ascorbate systematically can result in the production of extracellular  $H_2O_2$  (Ullah *et al.*, 2012). Ascorbate can also function as a pro-oxidant agent inside the cell. When it enters into the cell, in the presence of redox-active metal ions, it mediates the further reduction of  $H_2O_2$  via the Fenton reaction generating damaging **°**OH (Satoh and Sakagami, 1996, Watson *et al.*, 2010). Duarte and Jones (2007) investigated the intracellular pro-oxidant effect of ascorbate on metal ion-dependent  $H_2O_2$ -induced DNA damage has been observed in cells pre-treated with ascorbate (Duarte and Jones, 2007).

In the previous Chapters of the current study, it was clearly demonstrated that melanoma cancer cells have a higher degree of sensitivity to  $H_2O_2$ -induced DNA damage than HaCaTs and HDF cells. Indeed, melanoma cancer cells have a greater amount of endogenous ROS than HaCaT cells, and when they were exposed to further exogenous  $H_2O_2$ , a higher level of DNA damage was observed in the melanoma cancer cells than in "normal" skin cells. The degree of cell sensitivity to  $H_2O_2$  among melanoma cancer cells also vary; with the more pigmented cells being more sensitive to  $H_2O_2$ -induced DNA damage. Therefore, it is proposed to investigate the effect of ascorbate on  $H_2O_2$ -induced DNA damage in melanoma cancer cells and "normal" skin cells.

This chapter aims to:

- Study the effects of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage in terms of SSB formation in melanoma cancer cells and in HaCaTs and HDF cells.
- Study the effects of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage in terms of ODBLs formation in melanoma cancer cells.
- Study the effects of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage in terms of DSBs formation in melanoma cancer cells and in HaCaTs.

• Study the effects of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage complexity, as assessed by comparing measures of simple single damage (SSBs) to that of measures of multiple/complex damage (DSBs).

#### 5.2 Results

# 5.2.1 Effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage in A375M melanoma cancer cells

The initial comet assay experiments conducted aimed to investigate the effect of very low concentrations of ascorbate (10µM) on levels of H<sub>2</sub>O<sub>2</sub>-induced DNA damage. A375M melanoma cells were used to examine the effects. Cells were seeded in 6-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment; then the cells were incubated with and without 10µM ascorbate for 2 hours, prior to exposure to H<sub>2</sub>O<sub>2</sub>. ACA was carried out, and DNA damage was measured. In this experiment, significant enhancements in levels of H<sub>2</sub>O<sub>2</sub>-induced DNA damage were observed in the cells pretreated with ascorbate (P<0.0001) (Figure 5.1).



Figure 5.1. H<sub>2</sub>O<sub>2</sub>-induced DNA damage dose responses in A375M melanoma cancer cells in the presence and absence of low concentration of ascorbate.

Cells were seeded in 6-well plates for 24 hours at 37 C/5% CO<sub>2</sub> to allow attachment. Cells were then incubated, for a further 2 hour, with and without 10 $\mu$ M of ascorbate. Cells were then exposed to the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 30 minutes, on ice, protected from light. The cells were then harvested and DNA damage was measured using standard ACA. Each bar represents the mean % of Tail DNA of 400 comets ± SEM determined from two independent experiments. The *T*-test (unpaired) was used for statistical analysis and comparison between each of the two groups. \*\*\*\*P<0.0001 versus ascorbate untreated cells.

To further confirm the above effect of vitamin C on H<sub>2</sub>O<sub>2</sub>-induced DNA damage, some melanoma cancer cells were pre-treated with 1 $\mu$ M and 100 $\mu$ M ascorbate, before being incubated with low concentrations of H<sub>2</sub>O<sub>2</sub>. The ACA experiment was conducted, and DNA damage measured. As in the previous experiment, a significant enhancement in levels of H<sub>2</sub>O<sub>2</sub>-induced DNA damage was clearly observed in the cells treated with 100 $\mu$ M ascorbate, when compared to the very low concentration (1 $\mu$ M) (% tail DNA: 19.76 ± 0.98% *versus* 11.37 ± 0.81%, *p*<0.0001, caused by 20 $\mu$ M H<sub>2</sub>O<sub>2</sub> in cells pre-treated with 100 $\mu$ M and 1 $\mu$ M ascorbate, respectively, and 36.32% *versus* 25.41 ± 1.19%, *p*<0.0001, caused by 30 $\mu$ M H<sub>2</sub>O<sub>2</sub> in cells pre-treated with 100 $\mu$ M and 1 $\mu$ M ascorbate, respectively, and 100 $\mu$ M and 1 $\mu$ M



Figure 5.2. H<sub>2</sub>O<sub>2</sub>-induced DNA damage dose responses in A375M melanoma cancer cells in the presence of low and high concentrations of ascorbate.

Cells were seeded in 6-well plates for 24 hours at 37 °C /5% CO<sub>2</sub> to allow attachment. Cells were then incubated, for a further 2 hours either with 1 $\mu$ M or 100 $\mu$ M of ascorbate. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 30 minutes, on ice, protected from light. After the cells were harvested, DNA damage was measured using the standard ACA. Each bar represents the mean % of Tail DNA of 400 comets  $\pm$  SEM determined from two independent experiments. The *T*-test (unpaired) was used for statistical analysis and comparison between each of the two groups. \*\*\*\*P<0.0001 versus cells treated with1 $\mu$ M ascorbate.

## 5.2.2 Effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage in A375M melanoma cancer cells and HaCaTs and HDF cells

Further investigation of ascorbate's effect on  $H_2O_2$ -induced DNA damage was conducted with one of the MM cancer cell lines (A375M) and HaCaTs and HDF cells. A375M melanoma cancer cells, HaCaTs, and HDF cells were seeded in 6-well plates for 24 hours at 37°C /5% CO<sub>2</sub> to allow attachment; the cells were then incubated with 0µM, 100µM, and 300µM ascorbate prior to exposure to  $H_2O_2$ . After cell treatment, ACA was conducted, and DNA damage was measured in all samples.

As observed in Chapter four of this study, HDF, and HaCaT cells were found to be less sensitive to H<sub>2</sub>O<sub>2</sub>-induced DNA damage, while A375M melanoma cancer cells were more sensitive to its effects. DNA damage induced by  $H_2O_2$  in melanoma cancer cells was higher than the damage induced in the HaCaTs and HDF cells (Figure 5.3A). Analysis of data showing that the damage induced by 20µM H<sub>2</sub>O<sub>2</sub> in melanoma cancer cells is significantly higher than the damage occurred in HDF and HaCaT cells by the same dose of  $H_2O_2$  (% tail DNA: 29.2 ± 1.01% versus 17.08 ± 0.86% and 17.1 ± 0.89%: p < 0.0001, respectively). Interestingly, an enhancing effect of ascorbate, on the level of H<sub>2</sub>O<sub>2</sub>-induced DNA damage, was observed in melanoma cancer cells and in HaCaTs and HDF cells (Figure 5.3B & C). More interestingly, a further analysis of the data revealed that, in melanoma cancer cells, the damage induced by  $20\mu M H_2O_2$  in the presence of 100µM ascorbate was also significantly higher than that in HDF and HaCaT cells (% tail DNA:  $48.13 \pm 1.04\%$  versus  $31.99 \pm 1.23\%$  and  $23.35 \pm 1.06\%$ : p<0.0001, respectively). Similarly, in melanoma cancer cells, the damage induced by 20µM H<sub>2</sub>O<sub>2</sub> in the presence of  $300\mu$ M ascorbate was also significantly higher than that in HDF and HaCaT cells (% tail DNA:  $60.27 \pm 1.45\%$  versus  $46.92 \pm 1.53\%$  and  $36.37 \pm 2.04\%$ : p < 0.0001, respectively). This indicates that melanoma cancer cells are more sensitive than HDF and HaCaT cells, to DNA damage induced by H<sub>2</sub>O<sub>2</sub> alone and H<sub>2</sub>O<sub>2</sub> plus ascorbate (5.3A-C). Further analysis of the data revealed that, in melanoma cancer cells the enhancing effect of 100µM ascorbate on DNA damage, induced by 20µM H<sub>2</sub>O<sub>2</sub> was 1.3-fold and more than 3-fold higher than that in HDF and HaCaT cells, respectively. Similarly, the damage enhancement by 300µM ascorbate on the level of DNA damage induced by the same dose of H<sub>2</sub>O<sub>2</sub> was 1.3-fold and more than 2-fold higher than that in HDF and HaCaT cells, respectively (5.4A & B).



Figure 5.3. H<sub>2</sub>O<sub>2</sub>-induced DNA damage dose responses in A375M melanoma cancer cells and in HaCaT and HDF cells, in the presence and absence of high concentrations of ascorbate.

The cells were seeded in 6-well plates for 24 hours at 37  $^{\circ}$ C/5% CO<sub>2</sub> to allow attachment. The cells were then incubated, for a further 2 hour without ascorbate (A) and with either 100µM (B) or 300µM (C) ascorbate. The cells were then exposed to selected concentrations of H<sub>2</sub>O<sub>2</sub> for 30 minutes on ice, protected from light. After the cells had been harvested, DNA damage was measured using the standard ACA. Each bar represents the mean % of Tail DNA of 300 comets ± SEM determined in three independent experiments. A one-way ANOVA was used for statistical analysis between each of the three groups. \*\*\*\*p<0.0001.



Figure 5.4. Level of  $H_2O_2$ -induced DNA damage enhanced by  $100\mu M$  (A) and  $300\mu M$  (B) ascorbate in A375M melanoma cancer cells and in HaCaTs and HDF cells.

By conducting the comet assay experiments detailed above, it was found that ascorbate plays an effective pro-oxidant role, to modulate H<sub>2</sub>O<sub>2</sub>-induced DNA damage, particularly in melanoma cancer cells. The effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage was clear, and it was dependent on ascorbate concentrations. However, it was hypothesised that using high doses of vitamin C could induce greater DNA damage through the mechanism of ascorbate autooxidation (generation of H<sub>2</sub>O<sub>2</sub> in the cell culture media). As observed in the above experiment (Figure 5.3B & C), in melanoma cancer cells, the level of DNA damage enhanced by ascorbate alone was slightly increased in cells pre-treated with 300µM ascorbate compared to the damage enhanced by 100µM ascorbate (% tail DNA:  $9.09 \pm 0.42\%$  *versus* 7.11 ± 0.51%, respectively). Therefore, it was proposed to determine the concentration of ascorbate that can maximally enhance H<sub>2</sub>O<sub>2</sub>-induced DNA damage and still be achievable by oral supplementation.

Thus, A375M melanoma cancer cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment; the cells were then incubated with 0µM, 1µM, 3µM, 10µM, 30µM, 100µM and 300µM of ascorbate for 2 hour prior to exposure to H<sub>2</sub>O<sub>2</sub>. After the cells had been treated, ACA was conducted, and DNA damage was measured.

Different levels of enhancing effects from ascorbate on  $H_2O_2$ -induced DNA damage were observed in cells treated with ascorbate. The highest concentration of ascorbate caused the highest level of enhancement in the level of  $H_2O_2$ -induced DNA damage.

The level of induced damaged caused by  $20\mu M H_2O_2$  was subtracted from the damage induced by the same concentration of  $H_2O_2$  ( $20\mu M$ ) but in the presence of ascorbate. Figures adapted from figure 5.3.

However, increasing levels of DNA damage were observed when treating with ascorbate alone. Almost certainly,  $300\mu$ M of ascorbate causes substantial levels DNA damage (% tail DNA:  $14.20 \pm 1.16\%$  versus control) through autoxidation processes (the formation of H<sub>2</sub>O<sub>2</sub> in media by ascorbate) (Figure 5.5A); and this effect was more than 2-fold higher than with lower concentrations of ascorbate. Therefore  $100\mu$ M of AA was chosen as the standard concentration for the future work; this level of ascorbate effectively enhances H<sub>2</sub>O<sub>2</sub>-induced DNA damage (Figure 5.5B) and is achievable by administration of oral supplementations, and does not mediate extensive extracellular autooxidation as noted at the highest concentration ( $300\mu$ M).



### Figure 5.5. $H_2O_2$ -induced DNA damage in A375M in the presence and absence of different concentrations of ascorbate.

A) Cells were seeded in 6-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. They were then incubated for a further 2 hours with and without indicated levels of ascorbate. Cells were then exposed to  $30\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 minutes on ice, protected from light. After the cells had been harvested, DNA damage was measured, using the standard ACA. Each bar represents the mean % of Tail DNA of 300 comets ± SEM determined from three independent experiments. One-way ANOVA was used for statistical analysis to compare between groups. Ns, non-significant, \*\*p< 0.01, \*\*\*\*P<0.0001 *versus* the damage induced by H<sub>2</sub>O<sub>2</sub> alone. B) Line graph shows the level of H<sub>2</sub>O<sub>2</sub>-induced DNA damage in melanoma cancer cells treated with different concentrations of ascorbate; the background of DNA damage enhanced by ascorbate alone were subtracted from the H<sub>2</sub>O<sub>2</sub>-induced DNA damage in cells pre-treated with ascorbate. The figure adapted from figure 5.5A.

# 5.2.3 Effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced SSBs damage in non-pigmented (A375M and A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells in HaCaT cells

Using ACA, an examination of the effect of ascorbate on  $H_2O_2$ -induced DNA damage in a panel of melanoma cancer cell lines, and HaCaT cells was performed. HaCaT cells were used as the standard control "normal" cell line for all future work, because the HDF cells were too slowly growing for frequent/routine use.

Herein, the different melanoma cancer cells, including non-pigmented (A375M & A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells plus the HaCaTs were seeded in 6-well plates and ACA conducted after the cells were treated with  $H_2O_2$  in the presence and absence of ascorbate. DNA damage was measured in all melanoma cancer cells, and in the HaCaT cells. Data obtained in these experiments showed a significant enhancement in the levels of DNA damage, as mediated by  $15\mu$ M and  $30\mu$ M  $H_2O_2$  in all the melanoma cancer cells in the presence of ascorbate was less effective in the HaCaT cells, since the  $H_2O_2$ -induced DNA damage enhancement by ascorbate was less significant in these cells (Figure 5.6E).

In A375M, the % tail DNA damage induced by  $15\mu$ M H<sub>2</sub>O<sub>2</sub> in cells pre-treated with ascorbate was significantly increased (% tail DNA:  $21.12 \pm 0.605$  versus  $13.70 \pm 0.57$ , p<0.0001). Likewise, a highly significant in the damage was observed when ascorbate-pre-treated cells when exposed to  $30\mu$ M H<sub>2</sub>O<sub>2</sub> (% tail DNA:  $45.80 \pm 0.82$  versus 24.09  $\pm 0.743$ , p<0.0001). In A375P melanoma cancer cells the % tail DNA damage induced by  $15\mu$ M H<sub>2</sub>O<sub>2</sub> in the presence of ascorbate in was significantly increased (% tail DNA:  $32.33 \pm 1.527$  versus  $14.15 \pm 0.785$ , p<0.0001). Similarly, a significant level of DNA damage was observed in cells exposed to  $30\mu$ M H<sub>2</sub>O<sub>2</sub> in the presence of ascorbate (% tail DNA:  $48.55 \pm 1.182$  versus  $35.80 \pm 1.384$ , p<0.0001). In SK28 melanoma cancer cells, the level of tail DNA damage induced by  $15\mu$ M H<sub>2</sub>O<sub>2</sub> in cells pre-treated with ascorbate was also increased significantly (% tail DNA:  $48.06 \pm 1.26$  versus  $23.73 \pm 0.842$ , p<0.0001) and the effect of ascorbate was same when SK28 cells exposed to  $30\mu$ M H<sub>2</sub>O<sub>2</sub> (% tail DNA:  $72.33 \pm 1.046$  versus  $41.96 \pm 1.24$ , p<0.0001). In SK23 melanoma cancer cells, the level of % tail DNA:  $72.33 \pm 1.046$  versus  $41.96 \pm 1.24$ , p<0.0001). In SK23 melanoma cancer cells, the level of % tail DNA:  $72.33 \pm 1.046$  versus  $41.96 \pm 1.24$ , p<0.0001). In SK23 melanoma cancer cells, the level of % tail DNA damage induced by  $15\mu$ M H<sub>2</sub>O<sub>2</sub> in the presence of ascorbate was also highly significant (% tail DNA:  $60.51 \pm 1.342$  versus

46.84  $\pm$  1.253, *p*<0.0001) and the effect of ascorbate was same when these cells exposed to 30µM H<sub>2</sub>O<sub>2</sub> (% tail DNA: 74.51  $\pm$  0.687 versus 54.01  $\pm$  1.08, *p*<0.0001).

Whereas in HaCaT cells, the enhancement effect of ascorbate was less significant than that in melanoma cancer cells. The ascorbate enhancement effect on the % tail DNA damage induced by  $15\mu$ M H<sub>2</sub>O<sub>2</sub> in these cells was less significant than the effect of ascorbate on the damage induced by the same dose of H<sub>2</sub>O<sub>2</sub> in all melanoma cancer cells (% tail DNA:  $17.25 \pm 1.02$  versus  $12.24 \pm 0.809$ , p=0.001) and the damage observed when ascorbate-pre-treated HaCaT cells exposed to  $30\mu$ M H<sub>2</sub>O<sub>2</sub> was also even less significant (% tail DNA:  $27.97 \pm 1.161$  versus  $24.53 \pm 1.23$ , p=0.0431).

Among all the cell lines studied, the highest levels of damage induced by the higher dose of  $H_2O_2$  (30µM) in both the presence and absence of ascorbate was observed in heavily pigmented (SK23), then in moderately pigmented (SK28), followed by that in non-pigmented (A375P & A375M) melanoma cancer cells, with HaCaT cells being the least sensitive (Figure 5.6F).

In line with Chapter 3 (notably section 3.2.1), further analysis of the data was carried out to investigate the effects of ascorbate on  $H_2O_2$ -mediated DNA damage specifically in heavily pigmented (SK23) and non-pigmented (A375P) melanoma cancer cells, and HaCaT cells. Herein, the effect of a single dose of  $H_2O_2$  (30µM) was measured for the three different cell lines, both in the presence and absence of ascorbate. The background DNA damage was subtracted from the damage induced by  $H_2O_2$ , and the enhancement effect of ascorbate calculated. Indeed, the enhancement effect was greater in melanoma cancer cells, than on HaCaT cells. The enhancement effects of ascorbate were higher in SK23 than in A375P, by 1.5-fold and 1.4-fold, respectively, with the smallest enhancement effect observed in HaCaT cells (Figure 5.7).



### Figure 5.6. H<sub>2</sub>O<sub>2</sub>-induced DNA in non-pigmented including A375M (A) and A375P (B), moderately pigmented (SK28) (C) and highly pigmented (SK23) (D) melanoma cells and in HaCaT (E) cells.

The cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. The cells were then incubated, for a further 2 hours with and without ascorbate. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 30 minutes, on ice, protected from light. After the cells were harvested, DNA damage was measured using the standard ACA. Each bar represents the mean % of Tail DNA of 300 comets  $\pm$  SEM determined in three independent experiments. A *T*-test (unpaired) was used for statistical analysis and comparison between each of the two groups. F) The figure adapted from figure 5.6A-E to show the difference between all the cell lines after treatment with 20µM H<sub>2</sub>O<sub>2</sub> for induction of SSBs in the presence and absence of ascorbate.



Figure 5.7. H<sub>2</sub>O<sub>2</sub>-induced DNA damage enhanced by ascorbate in heavily pigmented (SK23), and non-pigmented (A375P) melanoma cancer cells and HaCaT cells.

### 5.2.4 Measurement of $H_2O_2$ -induced oxidised DNA base lesions (ODBLs) in melanoma cancer cells pre-treated with and without ascorbate

The evidence above clearly demonstrates the enhancing effect of vitamin C on the level of H<sub>2</sub>O<sub>2</sub>-induced DNA strand break damage in the cells studied. To extend this further, the modified version of ACA was used to examine whether the ascorbate-dependentenhancement affects the extent to which oxidatively damaged base lesions were formed. To achieve this, the base excision endonuclease enzyme (Fpg) was used to treat the nucleoid bodies generated post-lysis. This endonuclease enzyme can be used to characterise particular classes of DNA damage, such as ODBLs (Speit *et al.*, 2004). To detect oxidative DNA damage, it is recommended to use the Fpg enzyme. This enzyme detects 8-oxo-dG lesions and other oxidatively damaged DNA purines (Tice *et al.*, 2000). The enzyme Fpg removes ODBLs and cuts the DNA at the resulting abasic sites (AP sites); this results in an enhanced measures of strand breaks as detected by ACA and this additional strand break damage reflects the level of ODBLs induced.

To run the Fpg-ACA experiment, it is important to optimise the level of Fpg enzyme to maximally/optimally detect and reveal the level of ODBLs (revealed as Fpg-sensitive sites (Fpg-SS) in the damaged DNA. The SK23 melanoma cancer cell line was selected

DNA damage background levels were subtracted from the damage induced by  $30\mu M H_2O_2$  in the above three cell lines, both in the absence and presence of ascorbate. The enhancement effect of ascorbate was then measured by calculating the fold increase. Figure adapted from figure 5.6F.

for this purpose, because these cells are more sensitive to  $H_2O_2$ -induced DNA damage than the other melanoma cancer cells.

As in the previous comet assay work, cells were seeded in 6-well plates for 24 hours at  $37^{\circ}$ C /5% CO<sub>2</sub> to allow attachment; the cells were then incubated with and without  $20\mu$ M H<sub>2</sub>O<sub>2</sub>. The cells were then harvested and embedded into LMP agarose gels on pre-coated slides. The gel-embedded cells were then pre-treated with different levels of Fpg enzyme units diluted in ERB, post-lysis and subsequently ACA was conducted.

The data obtained indicates that  $H_2O_2$ -induced DNA damage contained Fpg-SS and a gradual increase in the level of  $H_2O_2$ -induced Fpg-SS was observed with increasing concentration of Fpg. The levels of background endogenous Fpg-SS were almost constant, although the damage level seemingly increased when cells were treated with the highest amount of Fpg (8.0U/gel) suggesting adventitious/non-specific cleavage of the DNA by Fpg at this high concentration of the enzyme (Figure 5.8A).

Further analysis of this data, suggests that 0.8U/gel can optimally reveal the level of  $H_2O_2$ -induced ODBLs (Fpg-SS) in the treated cells (the highest level of Fpg-SS) measured as a ratio of the damage observation for between  $H_2O_2$  treated and untreated cells (Figure 5.8B).



Figure 5.8. Optimisation of the level of Fpg enzyme for detection of  $H_2O_2$ -induced ODBLs in melanoma cancer cells.

A) SK23 melanoma cells were seeded in 6-well plates for 24 hours at 37°C /5% CO<sub>2</sub> to allow attachment before exposure to  $H_2O_2$ . The cells were then exposed either to media alone or media containing 20µM  $H_2O_2$ , on ice, and protected from the light. After harvesting and collecting the cells, they were mixed with LMP agarose on slides and incubated with lysis buffer for overnight at 4°C. The gel-embedded cells were then pre-incubated for a further 30 minutes, at 37°C in black and moist boxes, to either ERB or to indicated levels of Fpg enzyme. After incubation with Fpg, ACA was conducted, and the DNA damage measured. Each bar represents the mean % of Tail DNA of 300 comets ± SEM determined from three independent experiments. B) The optimal effect of Fpg for revealing  $H_2O_2$ -induced ODBLs measured as a ratio of DNA damage in  $H_2O_2$  treated and untreated cells (The figure adapted from the figure 5.8A).

To investigate the pro-oxidant effect of ascorbate on  $H_2O_2$ -induced DNA damage, Fpg-ACA was performed using non-pigmented (A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells. The cells were pre-treated with and without ascorbate for 2 hour at 37°C/5% CO<sub>2</sub> and then exposed to 30µM H<sub>2</sub>O<sub>2</sub> for 30 minutes on ice and protected from the light.

After harvesting and collection, the treated cells were mixed with LMP agarose, dispensed onto slides and incubated in lysis buffer overnight at 4°C. Gel-embedded melanoma cells, including controls and treated cells, were then pre-incubated with either ERB or Fpg enzyme for a further 30 minutes at 37°C in moist and dark boxes. After this the standard ACA was conducted. The outcomes of this experiment revealed that ascorbate is able to enhance the formation of H<sub>2</sub>O<sub>2</sub>-induced ODBLs (Fpg-SS), in addition to induced SSBs (Figure 5.9A-C). As can be seen from the figure 5.9D, the extent of H<sub>2</sub>O<sub>2</sub>-induced DNA damage was further increased by Fpg in melanoma cancer cells pre-treated with ascorbate.

Further analysis by subtraction of the effect of  $H_2O_2 + ERB$ ,  $H_2O_2 + ERB + AA$ ,  $H_2O_2 + Fpg$  from the combined effect of  $H_2O_2 + Fpg$  +AA on melanoma cancer cells was performed. The extent of  $H_2O_2$ -induced DNA damage in cells pre-treated with ascorbate, detected by the Fpg enzyme (Fpg-SS), in melanoma cancer cells is noticeably higher than the level of  $H_2O_2$ -induced DNA damage detected as Fpg-SS in the absence of ascorbate. In A375P melanoma cancer cells, ascorbate caused the additional formation ODBLs (Fpg-SS) as measured by a 14.17% increase in %TD (Fpg-SS); whilst in SK28 and heavily pigmented SK23 melanoma cancer cells, pre-treated with ascorbate yielded further increases of 15.76% and 18.46%, respectively (Figure 5.9A-C).



Fpg

 $A375P + 30\mu M H_2O_2/AA + ERB$ 



A375P +  $30\mu$ M H<sub>2</sub>O<sub>2</sub> + Fpg



A375P +  $30\mu M H_2O_2/AA + Fpg$ 



Figure 5.9. H<sub>2</sub>O<sub>2</sub>-induced ODBLs (Fpg-SS) in non-pigmented A375P (A), moderately pigmented SK28 (B) and heavily pigmented SK23 (C) melanoma cancer cells treated with and without ascorbate.

The cells were seeded in 6-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment; the cells were then incubated, for a further 2 hours with and without ascorbate. The cells were then exposed to 30µM H<sub>2</sub>O<sub>2</sub>, kept on ice, protected from the light. They were then pre-incubated for a further 30 minute.s, at 37°C in black and moist boxes, to ERB or Fpg enzyme (0.8U/gel). After incubation with Fpg, ACA was conducted and DNA damage measured. Each bar represents the mean % of Tail DNA of 300 comets  $\pm$ SEM determined in three independent experiments. D) Shows the extent of the DNA damage migration induced by  $H_2O_2$  and enhanced by ascorbate in A375P melanoma cancer cells treated with Fpg enzyme.

# 5.2.5 Effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DSBs in non-pigmented (A375M and A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells in HaCaT cells

Cell exposure to radiation or certain genotoxic agents induces DSBs, which are considered one of the most lethal DNA lesions (Svetlova *et al.*, 2010). This type of genomic damage effectively induces cell death (Lliakis, 1991). As observed in the above experiments, ascorbate enhances  $H_2O_2$ -induced DNA damages in terms of SSBs (Figure 5.6A-D) and ODBLs (e.g. 8-oxo-dG) (Figure 5.9A-C) the latter being indicative/tell-tale of oxidative effects.

H<sub>2</sub>O<sub>2</sub>, as a model oxidant, has the ability to induce DSBs in cells (Driessens *et al.*, 2009). Therefore, it was proposed to examine the effect of ascorbate on the level of H<sub>2</sub>O<sub>2</sub>-induced DSBs in melanoma cancer cells and in HaCaT cells. The formation of DSBs in DNA leads to extensive phosphorylation of the histone H2AX, resulting in the formation of  $\gamma$ -H2AX foci (Banáth *et al.*, 2010).

The  $\gamma$ -H2AX immunoassay was used for the detection of  $\gamma$ -H2AX foci in cells after treatment. A375P melanoma cancer cells were first tested for H<sub>2</sub>O<sub>2</sub>-induced  $\gamma$ -H2AX foci formation (DSBs), both in the presence and absence of ascorbate. As expected, a significant enhancement in the number of H<sub>2</sub>O<sub>2</sub>-induced foci (representing DSBs) was demonstrated in cells incubated with ascorbate. As can be seen in Figure 5.10A, more H<sub>2</sub>O<sub>2</sub>-induced foci were present in the ascorbate pre-treated A375M melanoma cancer cells than in those only treated with H<sub>2</sub>O<sub>2</sub>. The ability of ascorbate to enhance the number of H<sub>2</sub>O<sub>2</sub>-induced DSB formation in cells is significant (number of foci/cell: 27.63 ± 1.71 *versus* 21.91 ± 1.76, *P*=0.0231, in cells pre-treated with 10µM H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate, respectively, and 33.14 ± 2.79 *versus* 24.2 ± 1.39, *P*=0.0058, in cells pre-treated with 20µM H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate, respectively) (Figure 5.10B).



Figure 5.10.  $H_2O_2$ -induced DSBs in terms of  $\gamma$ -H2AX foci formation in A375M melanoma cancer cells in the presence absence of ascorbate.

A) Fluorescence images (40x magnified) show  $\gamma$ -H2AX foci (green) per DAPI stain nuclei (blue) of cells exposed to H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate. B) Cells were seeded on coverslips in the 6-well plate for 24 hours at 37°C/5% CO<sub>2</sub>, to allow attachment. The cells were then incubated, for a further 2 hours with and without ascorbate. They were then washed with PBS and exposed to 30µM H<sub>2</sub>O<sub>2</sub> for 30 minutes on ice, protected from light. The cells were then fixed with methanol and incubated at -20°C overnight, prior to the  $\gamma$ -H2AX immunoassay. Each bar represents mean  $\pm$  SEM of foci number per cell, as determined from 10 fields per sample in two independent experiments. A *T*-test (unpaired) was used for statistical analysis between each of the two groups. T-test (unpaired) was used to test the difference between the mean of each to groups. \**p*= 0.0231, \*\**p*<0.0058 versus ascorbate untreated cells.

The effect of ascorbate on  $H_2O_2$ -induced DSBs was next investigated in the panel of MM cancer cell lines including non-pigmented (A375M & A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells and HaCaT cells.

As in the previous  $\gamma$ -H2AX experiments, the cells were pre-incubated, for 2 hours, with ascorbate (100 $\mu$ M), and then exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> (0 $\mu$ M, 15 $\mu$ M and 30 $\mu$ M). The purpose of using low levels of H<sub>2</sub>O<sub>2</sub> treatment was to avoid oversaturation of the cells with foci as was observed when melanoma cancer cells exposed to 50 $\mu$ M H<sub>2</sub>O<sub>2</sub> in the presence of ascorbate. If a nucleus is saturated with H<sub>2</sub>O<sub>2</sub>-induced DSBs (Foci), the image analysis software (e.g. ImageJ) cannot determine/measure the number of foci (Figure 5.11).



Figure 5.11. Saturation of nuclei with γ-H2AX foci H<sub>2</sub>O<sub>2</sub>-induced foci in the presence of ascorbate.

SK23 melanoma cancer cells were seeded on coverslips in the 6-well plate for 24 hours at 37°C/5% CO<sub>2</sub>, to allow attachment. The cells were then incubated, for a further 2 hours with ascorbate. They were then washed with PBS and exposed to  $50\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 minutes on ice, protected from light. The cells were then fixed with methanol and incubated at -20°C overnight before conducting the  $\gamma$ -H2AX immunoassay. Fluoresence images (40 x magnifications) show  $\gamma$ -H2AX foci per nuclei from cells exposed to H<sub>2</sub>O<sub>2</sub> in the presence of ascorbate. A) Shows foci phosphorylation (green). B) Shows foci saturation per nuclei after transforming the image to black and white.

As expected, an enhancement effect by ascorbate on  $H_2O_2$ -induced DSBs (Foci) was observed in all melanoma cancer cell lines and in HaCaT cells when they were exposed to low concentrations of  $H_2O_2$ . The role of ascorbate in melanoma cancer cells was obvious. However, in HaCaT cells, the enhancement effect of ascorbate on the level of  $H_2O_2$ -induced DNA damage was less effective. In the majority of the melanoma cancer cell lines the level of DSBs mediated by  $H_2O_2$ in the presence of ascorbate was statistically higher than the damage induced by  $H_2O_2$ alone (Figure 5.12A-D). In A375M, the number of DSBs induced by  $15\mu$ M  $H_2O_2$  in cells pre-treated with ascorbate was significantly increased (number of foci/cell:  $32.95 \pm$ 2.51 *versus* 24.18  $\pm$  1.61, *p*=0.0051). Likewise, a significant increase in the number of  $H_2O_2$ -induced DSBs was observed when ascorbate-pre-treated cells were exposed to  $30\mu$ M  $H_2O_2$  (number of foci/cell:  $32.17 \pm 1.87$  *versus*  $22.25 \pm 1.81$ , *p*=0.0003) (Figure 5.12A). In A375P melanoma cancer cells, the number of DSBs induced by  $15\mu$ M  $H_2O_2$ in cells pre-treated with ascorbate was also significantly increased (number of foci/cell:  $32.67 \pm 1.06$  *versus*  $26.04 \pm 1.55$ , *p*=0.0014). Similarly, a significant number of  $H_2O_2$ induced DSBs was demonstrated when ascorbate-pre-treated cells were exposed to  $30\mu$ M  $H_2O_2$  (number of foci/cell:  $34.53 \pm 2.13$  *versus*  $28.44 \pm 1.58$ , *p*=0.0321) (Figure 5.12B).

In SK28 melanoma cancer cells, the number of DSBs induced by  $15\mu$ M H<sub>2</sub>O<sub>2</sub> in cells pre-treated with ascorbate was also increased (number of foci/cell:  $34.00 \pm 3.12$  versus  $28.24 \pm 1.92$ , p=0.1209) and when the H<sub>2</sub>O<sub>2</sub> concentration increased to  $30\mu$ M the number of induced DSBs in ascorbate-pre-treated cells increased significantly (number of foci/cell:  $38.50 \pm 3.35$  versus  $29.32 \pm 2.17$ , p=0.00242) (Figure 5.12C).

In SK23 melanoma cancer cells, there was an increase in the number of DSBs induced by 15µM H<sub>2</sub>O<sub>2</sub> in cells pre-treated with ascorbate (number of foci/cell: 33.30 ± 3.39 *versus* 26.36 ± 2.16, *p*=0.0925). A significant number of H<sub>2</sub>O<sub>2</sub>-induced DSBs was demonstrated when ascorbate-pre-treated cells were exposed to 30µM H<sub>2</sub>O<sub>2</sub> (number of foci/cell: 42.85 ± 2.25 *versus* 27.38 ± 3.78, *p*=0.0011) (Figure 5.12D).

However, in HaCaT cells, enhancement of H<sub>2</sub>O<sub>2</sub>-induced DSBs by ascorbate was less effective. The increase in the number of DSBs induced by 15µM H<sub>2</sub>O<sub>2</sub> in cells pretreated with ascorbate was not significantly increased (number of foci/cell:  $20.45 \pm 3.24$ *versus* 15.50 ± 1.86, *p*=0.2029); and a similar effect was observed when ascorbate-pretreated cells exposed to 30µM H<sub>2</sub>O<sub>2</sub> (number of foci/cell:  $23.10 \pm 2.23$  *versus* 19.24 ± 2.03, *p*=0.2197) (Figure 5.12E).

Further analysis of this data reveals melanoma cancer cells, exposed to  $30\mu$ M H<sub>2</sub>O<sub>2</sub>, particularly the pigmented cells (SK28 & SK23), are the most sensitive cells with

respect to  $H_2O_2$  induced DSBs in the presence and absence of ascorbate, and that HaCaT cells are the least sensitive (Figure 5.12F).





Cells were seeded on coverslips in the 6-well plate for 24 hours at 37°C/5% CO<sub>2</sub>, to allow attachment. The cells were then incubated, for a further 2 hours with and without ascorbate. They were then washed with PBS and exposed to indicated concentrations of  $H_2O_2$  for 30 minutes on ice, protected from light. The cells were then fixed with methanol and incubated at -20°C overnight, before conducting the  $\gamma$ -H2AX immunoassay. Each bar represents mean  $\pm$  SEM of  $\gamma$ -H2AX foci number per cell determined from 10 fields per each sample. The data was obtained from three independent experiments. The *T*-test (unpaired) was used for statistical analysis between each of the two groups. Ns, non-significant, \*p<0.05, \*\*P<0.01, \*\*\*P<0.001 versus ascorbate untreated cells. F) Figure adapted from figures 5.12A-E to show the difference between all the cell lines after treatment with 30µM H<sub>2</sub>O<sub>2</sub> for induction of DSBs in the presence and absence of ascorbate.

With respect to the data observed in Chapter 3 (see Section 3.2.5) and this Chapter 5 (see Section 5.2.3), additional analysis was performed on the above data, to study the effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DSBs in heavily pigmented (SK23), nonpigmented (A375P) and in HaCaT cells. Simply, the effect of one concentration of H<sub>2</sub>O<sub>2</sub> ( $30\mu$ M) has been examined in the above three different cell lines, both in the presence and absence of ascorbate. The enhancing effect of ascorbate on DNA damage was assessed as the fold increase in damage, after the subtraction of background DNA DSB damage from the DSB damage induced by H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate. The sensitivity of melanoma cancer cells to the enhancement of AA towards oxidatively-mediated DNA DSBs was obvious. The enhanced damage caused by ascorbate in the pigmented (SK23) and non-pigmented (A375P) melanoma cancer cells was *ca*. >2-fold and more than 1.5-fold, respectively, higher than the damage in the HaCaT cells (Figure 5.13).



Figure 5.13. Fold change in  $\gamma$ -H2AX foci members induced by 30 $\mu$ M H<sub>2</sub>O<sub>2</sub>, in the presence and absence of ascorbate, in heavily pigmented (SK23) and non-pigmented (A375P) melanoma cancer cells and in HaCaT cells.

DNA damage background levels were subtracted from the damage induced by  $30\mu M H_2O_2$  in the above three cell lines, both in the absence and presence of ascorbate. The enhancement effect of ascorbate on  $H_2O_2$ -induced DSBs was then calculated using the fold change increase for each cell line. Figure adapted from figure 5.12F.

## 5.2.6 Assessment of DNA damage complexity (SSBs vs DSBs) in melanoma cancer cells induced by $H_2O_2$ in the presence and absence of ascorbate

 $H_2O_2$ , as mentioned in the literature, has the ability to diffuse freely within the cell to reach the nucleus, where it attacks the DNA inducing damage (Termini, 2000, Dizdaroglu *et al.*, 1991). This process occurs in the presence of transition metal ions, including iron ions, which convert  $H_2O_2$  to a potentially damaging <sup>•</sup>OH (Loeb *et al.*, 1988, Barbouti *et al.*, 2001).

In the present study, it was anticipated that upon entering the cell  $H_2O_2$  reaches the nucleus where, upon encountering DNA-bound redox active metal ions, notably ferrous iron ions (Fe<sup>2+</sup>), it produces mostly single <sup>•</sup>OH ions, which in turn induce mostly DNA SSBs and only a few DNA DSBs. However, it was proposed that if the cell is preloaded with ascorbate, ascorbate reduces ferric ions to ferrous iron (so effectively 'recycling' the metal ions), enabling further reactions with  $H_2O_2$ . The outcome of this is the generation of sequential <sup>•</sup>OH. This may in turn locally attack and damage the DNA in close vicinity, resulting in more DSBs, relative to SSBs (Figure 5.14).

A similar mechanistic scenario is anticipated to also occur when cells are exposed to ionising radiation. Ionising radiation, directly and indirectly induces a spectrum of DNA damage lesions (Gulston *et al.*, 2002). Since radiation produces instant clusters of <sup>•</sup>OH, ionising radiation will produce 'clustered lesions' locally within DNA (known as multiply damaged sites (MDS)) (Ward, 1994, Nikitaki *et al.*, 2015). These complex lesions occur when there is a combination of two or more DNA lesions in close proximity, on both DNA strands (Harrison *et al.*, 1999, Eot-Houllier *et al.*, 2005).

DSBs are harder to repair and are considered lethal and toxic to the cell (Gulston *et al.*, 2002). In the current study, it was anticipated that ionising radiation can yield more MDS (high DSB:SSB ratio), than that induced oxidatively by  $H_2O_2$  alone (Figure 5.15). To test this hypothesis, A375M and HaCaT cells were treated with either ionising radiation or  $H_2O_2$ . Following treatment, the ACA and the  $\gamma$ -H2AX immunoassay were conducted to measure DNA damage in terms of SSBs and  $\gamma$ -H2AX foci (DSBs). As expected, a comparison of the data obtained indicated the formation of complex damage (MDS; more DSBs but less SSBs) resulting from ionising radiation exposure; whereas, the type of DNA damage induced by  $H_2O_2$  was indicative of simpler/single damage (more SSBs and less DSBs) (Figure 5.16A-D & 5.17A-D). Scatter plots comparing the ACA versus  $\gamma$ -H2AX foci data clearly show the difference in the quality of the DNA

damage induced by ionising radiation and by  $H_2O_2$  with ionising radiation inducing more complex damage (Figure 5.18A & B).



Figure 5.14. Proposed models showing the differing levels of DSBs versus SSBs proposed be induced by  $H_2O_2$  in the presence and absence of ascorbate.



Figure 5.15. Proposed models showing the level of DSBs versus SSBs proposed to be induced by ionising radiation.



Figure 5.16.  $H_2O_2$  and radiation-induced DNA damage in terms of SSBs and DSBs in A375M melanoma cancer cells.

A) Cells were seeded in six-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. The cells were then exposed to indicated doses of H<sub>2</sub>O<sub>2</sub> for 30 minutes, on ice and protected from the light. After harvesting, DNA damage was measured using the standard ACA. Each bar represents the mean % of Tail DNA of 200 comets ± SEM, as determined from two independent experiments. B) Cells were seeded in six-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. Cells were then collected and embedded in LMP agarose on slides than exposed to indicated doses of ionising radiation on ice, protected from light. DNA damage was measured by the standard ACA. Each bar represents mean  $\pm$  SEM of % Tail DNA determined from 200 individual comets from two independent experiments. C) Cells were seeded on coverslips in the 6-well plate for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. The cells were then exposed to indicated doses of  $H_2O_2$  on ice protected from light. The cells were then fixed with 100% ice cold methanol prior conducting  $\gamma$ -H2AX immunoassay. Each bar represents mean  $\pm$  SEM of foci number per cell, as determined from 10 fields per sample from two independent experiments. D) Cells were seeded on coverslips in the 6-well plate for 24 hours at 37°C/5% CO2 to allow attachment. Cells were then exposed to indicated doses of ionising radiation on ice and then kept on ice before being fixed with 100% ice cold methanol prior to conducting the  $\gamma$ -H2AX immunoassay. Each bar represents mean  $\pm$  SEM of foci number per cell determined from 10 fields per sample from two independent experiments.



Figure 5.17. H<sub>2</sub>O<sub>2</sub> and radiation-induced DNA damage in terms of SSBs and DSBs in the HaCaT cells.

A) Cells were seeded in six-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. The cells were then exposed to indicated doses of  $H_2O_2$  for 30 minutes, on ice and protected from the light. After harvesting, DNA damage was measured using the standard ACA. Each bar represents the mean % of Tail DNA of 200 comets ± SEM, as determined from two independent experiments. B) Cells were seeded in six-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. Cells were then collected and embedded in LMP agarose on slides than exposed to indicated doses of ionising radiation on ice, protected from light. DNA damage was measured by the standard ACA. Each bar represents mean ± SEM of % Tail DNA determined from 200 individual comets from two independent experiments. C) Cells were seeded on coverslips in the 6-well plate for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. The cells were then exposed to indicated doses of  $H_2O_2$  on ice protected from light. The cells were then fixed with 100% ice cold methanol prior conducting  $\gamma$ -H2AX immunoassay. Each bar represents mean  $\pm$  SEM of foci number per cell, as determined from 10 fields per sample from two independent experiments. D) Cells were seeded on coverslips in the 6-well plate for 24 hours at 37°C/5% CO2 to allow attachment. Cells were then exposed to indicated doses of ionising radiation on ice and then kept on ice before being fixed with 100% ice cold methanol prior to conducting the  $\gamma$ -H2AX immunoassay. Each bar represents mean  $\pm$  SEM of foci number per cell determined from 10 fields per sample from two independent experiments.



Figure 5.18. Scatter plot showing the correlation between the DNA damage complexity (SSBs vs DSBs) induced by radiation versus the DNA damage complexity induced chemically by  $H_2O_2$  in A375M melanoma cells (A) and HaCaT cells (B).

The data for the above two figures is derived from figures 5.16 and 5.17.

Next, an attempt was made to observe the above effects when cells were pre-treated with AA prior to  $H_2O_2$  treatment. The hypothesis was that in the presence of ascorbate, iron ions in the nucleus can be recycled, thus generating sequential <sup>•</sup>OH from exogenous  $H_2O_2$  within the cells, which in turn attack the DNA and lead to the formation of complex damage lesions (more DSBs relatively to SSBs).

Based on the sensitivity of melanoma cancer cells to  $H_2O_2$ , three cell lines (nonpigmented (A375P), moderately pigmented (SK28) and highly pigmented (SK23)), were selected to examine this effect. Data of the ACAs which were run parallel with the previous  $\gamma$ -H2AX immunoassay (Figure 5.12A, C and D) were used to compare SSBs (tail DNA damage %) versus DSBs ( $\gamma$ -H2AX foci).

The findings of these experiments showed an enhancing effect by ascorbate on the level of  $H_2O_2$ -induced DNA SSBs and DSBs (Figure 5.19A & B - 5.21A & B). However, analysis of the data demonstrates that ascorbate enhanced  $H_2O_2$ -induced DSB formation, but this was entirely proportional to the level of AA enhancement of  $H_2O_2$ -induced SSBs (Figure 5.19C-5.21C); there is no substantial difference in the noted gradients between the two sets of data (+/- AA).



Figure 5.19. Relationships between the DNA damage complexity (SSBs vs DSBs) in A375M melanoma cancer cells induced by  $H_2O_2$  in the presence and absence of ascorbate.



Figure 5.20. Relationships between the DNA damage complexity (SSBs vs DSBs) in SK28 melanoma cancer cells induced by  $H_2O_2$  in the presence and absence of ascorbate.



Figure 5.21. Relationships between the DNA damage complexity (SSBs vs DSBs) in SK23 melanoma cancer cells induced by  $H_2O_2$  in the presence and absence of ascorbate.



Figure 5.22. Proposed models for generating types of DNA damage lesions induced by  $H_2O_2$  in the presence of ascorbate.

A) If the metal ions are present at local sites on DNA, the Fenton reactions would occur locally and in the presence of ascorbate this generates numbers of  ${}^{\bullet}OH$  which in turn attack the DNA inducing clustered DNA damage lesions. B) Fe<sup>2+</sup> or Fe<sup>3+</sup> binds to the DNA, the other partner being unbound and so free to migrate. This will lead to the formation of dispersed  ${}^{\bullet}OH$  causing simple damage rather than complex damage.

#### 5.2.7 Assessment of H<sub>2</sub>O<sub>2</sub>-induced γ-H2AX foci formation (DSBs) in the presence and absence of ascorbate following incubation post-treatment

It is suggested that  $\gamma$ -H2AX foci can be visualised 3 minutes after exposure to ionising radiation (Rogakou et al., 1999). More recently, different kinetics for the formation of  $\gamma$ -H2AX foci have been observed in cells at different times post treatment with ionising radiation (Staszewski et al., 2008). Furthermore, the formation of more DSBs can arise from repair processing of SSBs and AP sites or from aborted BER (Löbrich et al., 2010, Ma et al., 2011). Therefore, it was proposed to study the effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DSBs formation post-treatment in melanoma cancer cells at different times. SK23 melanoma cancer cells were pre-treated with and without ascorbate for 2 hours at 37°C/5% CO<sub>2</sub>, and then exposed to 30µM H<sub>2</sub>O<sub>2</sub> on ice, protected from light. The  $\gamma$ -H2AX immunoassay was then performed after the cells were incubated for different times at 37°C/5% CO<sub>2</sub> following treatment. The data presented in these experiments showed a clear further formation of  $\gamma$ -H2AX foci per cell at different time points, between 0-2 hours with a noticeable enhancement with ascorbate pre-treatment. The number of foci was found to be sharply increased between 30 minutes and 2 hours incubation post-treatment, in cells exposed to ascorbate. The foci number after 2 hours incubation following treatment had substantially decreased (Figure 5.22).



### Figure 5.23. Kinetic formation of $\gamma$ -H2AX foci post-treatment of SK23 melanoma cancer cells with H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate.

Cells were incubated, for a further 2 hours with and without ascorbate at  $37^{\circ}C/5\%$  CO<sub>2</sub>, to allow attachment. The cells were then washed with PBS and exposed to  $30\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 minutes on ice, while protected from light. They were then washed with PBS and new fresh media was added to them and they were then incubated for indicated time points. After each incubation period, the cells were fixed with methanol, and incubated at -20°C overnight, before conducting the  $\gamma$ -H2AX immunoassay. Each bar represents mean  $\pm$  SEM of foci number per cell, determined by testing 10 fields per sample. Data was obtained from three independent experiments.

#### 5.3 Discussion

Investigation of the pro-oxidant role of ascorbate has been the subject of interest in many recent studies (Chen *et al.*, 2008, Mandl *et al.*, 2009, Du *et al.*, 2012, Duarte and Jones, 2007). Previous work has shown that ascorbate has the ability to modulate oxidative stress inside mammalian cells. Studies have found that HDF cells preloaded with ascorbate become more sensitive to  $H_2O_2$ -induced DNA damage, compared to cells untreated with ascorbate (Duarte and Jones, 2007). Theoretically, it was proposed that when ascorbate enters the cell it modulates  $H_2O_2$ -induced oxidative stress via Fenton reactions (see section 1.4.5, Chapter 1), which eventually results in the formation of sequential <sup>•</sup>OH that attack the DNA causing damage.

Data from Chapter 4 of the current study clearly showed that micromolar concentrations of H<sub>2</sub>O<sub>2</sub> induce DNA damage in melanoma cancer cells and "normal" skin cells, with greater damage sensitivity being observed in melanoma cancer cells (Figure 4.7 & 4.8). In this Chapter, it was proposed to use physiological concentrations of ascorbic acid to test its effect on H<sub>2</sub>O<sub>2</sub>-induced DNA damage in melanoma cancer cells and "normal" skin cells. In the first experiments, an enhancing effect of ascorbate on the level of H<sub>2</sub>O<sub>2</sub>-induced DNA damage dose response was demonstrated in A375M cancer cells, even though the cells were treated with 10µM ascorbate (Figure 5.1). This was, then further confirmed when the cells were pre-incubated with 1µM and 100µM of ascorbate, prior to exposing them to  $H_2O_2$ ; the difference in the levels of  $H_2O_2$ -induced DNA damage enhancement with the two different ascorbate concentrations was significant (Figure 5.2). These results are in agreement with the data presented in previous work, which showed the pro-oxidant function of ascorbate on the modulation of H<sub>2</sub>O<sub>2</sub>-induced DNA damage (Duarte and Jones, 2007) despite using ascorbate concentrations equal or less than physiologically relevant levels. A large number of previous studies have also reported an increase in the sensitivity of tumour cells (U937 cells) to ROS in the presence of intracellular ascorbate (Guidarelli et al., 2004, Guidarelli et al., 2001).

Reports about ascorbate's effect on cultured cells are also controversial. Some authors suggest ascorbate has cytotoxic effect on cultured cells, while others have reported protective effects from ascorbate. Clément *et al* (2001) claimed that ascorbate interacts with some culture mediums to produce  $H_2O_2$  at different rates, and this may explain the

conflicting results obtained regarding vitamin C's impact on cells in different culture media (Clément *et al.*, 2001). Their studies indicate that a high concentration of ascorbate (1mM) generates different levels of  $H_2O_2$  depending on the types of culture media used. In the present study, to reduce any media dependent variable, all investigations of the effect of ascorbate were consistently compared with negative controls (cells being treated without  $H_2O_2$  in the presence and absence of ascorbate).

Suggestions concerning the effect of ascorbate on  $H_2O_2$ -induced DNA damage also vary. Some refer to ascorbate's free-radical scavenging efficiency. A study carried out by Arranz *et al* (2007) using the comet assay reported a protective effect from ascorbate against  $H_2O_2$ -mediated DNA damage in human hepatoma cells (Arranz *et al.*, 2007). This study used DMEM media, when treating cells with ascorbate and  $H_2O_2$ . Recently, Azqueta *et al.* (2013) also have reported that ascorbate, even at a level of 200µM, does not have significant effect on the level of  $H_2O_2$ -induced DNA damage in HeLa tumour cells (Azqueta *et al.*, 2013). In their study, Hela cells were treated with ascorbate for 30 minutes, and they were then exposed to  $H_2O_2$  prepared in DMEM for 5 minutes on ice. However, DMEM media contains pyruvate, a potent  $H_2O_2$  scavenger. In the current study, a clear inhibitory effect on  $H_2O_2$ -induced DNA damage was noted for media (A375M-media) has been used for exposing cells to  $H_2O_2$  to avoid any variable and to obtain a consistent effect on all studied cell lines.

In the previous work, it has been illustrated that melanoma cancer cells are more sensitive to  $H_2O_2$  induced DNA damage than HaCaTs & HDF cells (see Chapter 4, Figure 4.7 & 4.8). Moreover, among melanoma cancer cells, pigmented melanoma cancer cells are the most responsive to  $H_2O_2$  induced DNA damage, more so than the non-pigmented cells (see Chapter 4, Figure 4.8). Studying the effect of ascorbate on  $H_2O_2$ -induced damage in melanoma cancer cells and "normal" skin cells was the key focus in this study. A375M melanoma cancer cells, HDF and HaCaT cells were initially used to examine this effect. Cells were incubated with and without ascorbate ( $0\mu$ M,  $100\mu$ M and  $300\mu$ M), and then exposed to  $H_2O_2$ . Data obtained in these experiments showed an obvious enhancement effect of ascorbate on the level of  $H_2O_2$ -induced DNA damage in A375M melanoma cancer cells and in the two "normal" skin cells were the most sensitive to  $H_2O_2$ -induced DNA damage in the presence and absence of ascorbate;

more so than "normal" HaCaTs and HDF cells (Figure 5.3A-C). This has been further confirmed by calculating fold changes in the enhancement effect of the two different concentrations of ascorbate. The data revealed that, in melanoma cancer cells the enhancing effect of 100µM ascorbate on DNA damage, induced by 20µM H<sub>2</sub>O<sub>2</sub> was 1.3-fold and more than 3-fold higher than that in HDF and HaCaT cells, respectively. Similarly, the damage enhancement by 300µM ascorbate on the level of DNA damage induced by the same dose of H<sub>2</sub>O<sub>2</sub> was 1.3-fold and more than 2-fold higher than that in HDF and HaCaT cells, respectively (Figure 5.4A & B). The latter two "normal" skin cell lines may have a potent defence against the oxidants, with the HaCaTs being the most resistant. A study has found that in cultured normal human lymphocytes, ascorbate when used in combination with H<sub>2</sub>O<sub>2</sub> has an inhibitory effect against H<sub>2</sub>O<sub>2</sub>-induced DNA damage (Yen et al., 2002). To further support this, an in vitro study has also reported that ascorbate "partly" prevents DNA damage induced by H<sub>2</sub>O<sub>2</sub> in human keratinocytes (Petersen et al., 2000). Other studies have found neither a protective effect nor a damaging effect from ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage (Szeto and Benzie, 2002). More recently, Siddique *et al* (2009) have also used high levels of  $H_2O_2$ (100-200µM) to induce DNA damage in lymphocytic cells in the presence of ascorbate. They found a protective effect for ascorbate, countering damage induced oxidatively by  $H_2O_2$ . However, 100-200µM  $H_2O_2$  is high enough to cause massive DNA damage in melanoma cancer cells. This could indicate that in normal human cells with normal level of antioxidants, ascorbate may function as an antioxidant rather than as a prooxidant.

High concentrations of vitamin C are supposed to yield  $H_2O_2$  formation in media (Chen *et al.*, 2005). Even when it is at low levels,  $H_2O_2$  enters into the cells and can cause DNA damage. Therefore, optimisation of ascorbate concentrations ranging between lower and upper physiological levels was tested to determine the actual amount of vitamin C that enhances  $H_2O_2$ -induced DNA damage. To do so, A375M melanoma cancer cells were pre-treated, either with or without ascorbate, and then exposed to media either without  $H_2O_2$  or with  $30\mu M H_2O_2$ . Comet assay data from this experiment indicated that, ascorbate at a level equal or higher than  $10\mu M$  significantly enhances  $H_2O_2$ -induced DNA damage. More than  $100\mu M$  yielded a greater enhancement effect on  $H_2O_2$ -induced DNA damage. However, when the ascorbate-mediated DNA damage enhancement of ascorbate pre-treated cells was subtracted from  $H_2O_2$ -induced DNA
damage in cells pre-treated with ascorbate, it was found that above  $100\mu$ M ascorbate gave the highest level of damage. However, the high level of damage is thought to be due to the formation of additional H<sub>2</sub>O<sub>2</sub> in the media from ascorbate autooxidation (Figure 5.5A & B). Therefore,  $100\mu$ M ascorbate was proposed for use in all future work also; this level is achievable orally (Cameron and Campbell, 1974, Levine *et al.*, 2011, Padayatty *et al.*, 2004).

Further investigation into the effect of ascorbate on  $H_2O_2$ -induced DNA damage was performed in a panel of MM cancer cell lines, including A375M, A375P, SK28 and SK23, and HaCaT cells. Data analysis of these experiments illustrates a significant enhancement in the level of  $H_2O_2$ -induced DNA damage in all melanoma cancer cells and HaCaT cells. However, the HaCaT cells were less sensitive to  $H_2O_2$ -induced DNA damage in the presence and absence of ascorbate (Figure 5.6A-E). In a very recent study, it has been found that ascorbate protects normal human keratinocytes from the damage induced oxidatively by ozone exposure (Valacchi *et al.*, 2015).

Among melanoma cells, the level of  $H_2O_2$ -induced DNA damage observed in the absence and presence of ascorbate was noticeably higher in pigmented cells than in non-pigmented cells (Figure 5.6A-D). Further data analysis showed a higher level of enhancement of ascorbate's effect on  $H_2O_2$ -induced DNA damage in heavily pigmented (SK23) than non-pigmented (A375P) melanoma cancer cells, with only a small effect noted in the HaCaTs (Figure 5.7).

Among all the melanoma cancer cell lines, pigmented melanoma cancer cells were the most sensitive to  $H_2O_2$ -induced DNA damage and to its modulation by ascorbate (Figure 5.7F). The cells' sensitivity could be due to the noted higher intracellular status of iron ions in the pigmented melanoma cells, as compared to that in non-pigmented melanoma cancer cells (see Chapter 4, Figure 4.11). Therefore, it is proposed that the greater the metal ion content of melanoma cells is responsible for their higher susceptibility to  $H_2O_2$ -induced DNA damage, and as a consequence, the modulation of damage by ascorbate. Additional to this, pigmented melanoma cancer cells have enormous amounts of endogenous ROS, in comparison to non-pigmented melanoma cancer cells (see Chapter 3, Figure 3.4 & 3.5). Thus, providing these cells with further exogenous ROS and modulating it by ascorbate could exacerbate the DNA damage in these cells. Furthermore, lack or deficiency of the intracellular catalase enzyme in melanoma cancer cells (see Chapter 3, Figure 3.7 & 3.8) might be a further reason of

why these cells are significantly more affected by  $H_2O_2$ -induced DNA damage in the presence and absence of ascorbate than "normal" skin cells.

A previous study has found that low micromolar cencentrations of  $H_2O_2$  efficiently induce oxidative damage in DNA (Nakamura *et al.*, 2003). However, in the present study, the effect of ascorbate leading to enhancement in the level of  $H_2O_2$ -induced DNA damage raised the question of whether the effect is truly oxidative. To investigate this effect a modified version of the ACA was conducted using the Fpg enzyme. The enzyme is capable of detecting and cleaving the oxidatively damaged DNA at the sites of oxidised purines (8-oxo-dG), and ring opened purine lesions (formamidopyrimidines) (Collins *et al.*, 1993) with the additional strand breaks indicating/representing the presence oxidised purine lesions, which are specific tell-tale markers of oxidative effects.

In the current study, an enhancement effect is reported for the level of  $H_2O_2$ -induced ODBLs (Fpg-SS) in cells pre-treated with ascorbate (Figure 5.9A-C). This substantiates the role of ascorbate in the enhancing of oxidatively damaged DNA in melanoma cancer cells.

DNA damage caused by <sup>•</sup>OH attack not only involves SSBs and ODBLs, but also participates in the generation of DSBs (Driessens *et al.*, 2009). DSB damage results in one of the most lethal lesions, affecting the DNA molecules and causing cell apoptosis rather than cell necrosis (Lips and Kaina, 2001); these lesions have considerable cytotoxic effects (Ghardi *et al.*, 2012). DSBs are induced by ionising radiation and can be induced by certain genotoxic agents (Svetlova *et al.*, 2010, Jekimovs *et al.*, 2014). The repair mechanism of DSBs is complex and difficult (Riballo *et al.*, 2004) and it is suggested that the presence of even single unrepaired DSBs could induce cell death (Zhou, 2011, Scott and Pandita, 2006).

In the current study, a  $\gamma$ -H2AX immunoassay experiment was performed to measure the effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DSBs in melanoma cancer cells. In the first  $\gamma$ -H2AX immunoassay experiment, using A375M melanoma cancer cells, more H<sub>2</sub>O<sub>2</sub>-induced  $\gamma$ -H2AX foci per cell were observed in the presence of ascorbate, compared with cells incubated without ascorbate.  $\gamma$ -H2AX foci counts per cell revealed a significant enhancement in the level of H<sub>2</sub>O<sub>2</sub>-induced DSBs in melanoma cancer cells treated with ascorbate, compared with untreated cells. Also, some  $\gamma$ -H2AX foci formations were found in untreated melanoma (control) cells, even in the presence or

absence of ascorbate (Figure 5.10). Far from being experimental artefacts, it has been found that some tumour cell lines have the ability to express different numbers of endogenous  $\gamma$ -H2AX foci than others (Rogakou *et al.*, 1999). H2AX foci are also formed in cells in the mitotic phase (during replication phase) (Takahashi and Ohnishi, 2005). Additionally, the high level of background foci in A375M melanoma cells might have arisen from the endogenous ROS attack within melanoma cancer cells, with a slight enhancement in cells loaded with ascorbate (Figure 5.10).

When the  $\gamma$ -H2AX immunoassay was performed using all melanoma cell lines and the HaCaT cells, a clear H<sub>2</sub>O<sub>2</sub>-induced DSBs dose response was seen in all cells, with the highest number of DSBs being observed in melanoma cancer cells, particularly in the pigmented cells (SK23 and SK28) (Figure 5.12A-D) . It is noteworthy to say that HaCaT cells were the least sensitive to H<sub>2</sub>O<sub>2</sub>-induced DSBs. And even when the HaCaT cells were treated with ascorbate, no significant enhancement in the number of  $\gamma$ -H2AX foci was visualised, compared to those exposed to H<sub>2</sub>O<sub>2</sub> alone (Figure 5.12A-E).These findings are analogous with the comet assay findings (see Figure 5.6A-E).

Analysis of the  $\gamma$ -H2AX assay data demonstrated that the average results for H<sub>2</sub>O<sub>2</sub>induced DSBs treated with  $30\mu M H_2O_2$  in the presence and absence of ascorbate were noticeably higher in melanoma cancer cells than in HaCaT cells (Figure 5.12F). A further interpretation of this data shows that the enhancement of  $H_2O_2$ -induced DNA DSBs by ascorbate in heavily pigmented (SK23) and non-pigmented (A375P) melanoma cells was 2.1-fold, and 1.4-fold increased; whereas this effect in HaCaT cells was less than a 1-fold increased (Figure 5.13). This may indicate that the endogenous ROS and the antioxidants status could influence the sensitivity of melanoma cancer cells to H<sub>2</sub>O<sub>2</sub>-induced DSBs and the synergistic effect of ascorbate in these cells.Similar to the previous observations put forward in this discussion, low levels of intracellular catalase enzyme, plus higher levels of endogenous ROS in melanoma cancer cells could render melanoma cells more susceptible than HaCaT cells to H<sub>2</sub>O<sub>2</sub>-induced DSBs and their modulation by ascorbate. Taken together, it should be pointed out that a physiologically relevant ascorbate level is sufficient to produce more H<sub>2</sub>O<sub>2</sub>-induced DSBs in melanoma cancer cells. Such an enhancement in the level of lethal DNA damage lesions could increase the likelihood of melanoma cell death.

Ionising radiation is an efficient way to induce bistranded clustered DNA lesions, which are refractory to repair (Sutherland *et al.*, 2002, Hada and Sutherland, 2006). Clustered

genomic lesions induced via ionising radiation, are cytotoxic because they are difficult/problematic to repair (Eccles *et al.*, 2011). This is because the location of the damages is close on the opposing strands, within a few helical turns of the DNA (Sutherland *et al.*, 2002). Thus, clustered DNA damage, also referred to as MDS, are considered more biologically relevant for the induction of cell death (Ward, 1994). In a recent work, a considerably higher number of DSBs than SSBs have been observed in rat thyroid cells being treated ionising radiation. However, the damage induced by  $H_2O_2$  in these cells generated more SSBs than DSBs (Driessens *et al.*, 2009).

In the current study, A375M melanoma and the HaCaT cells were used to study the complexity of DNA damage induced by ionising radiation and  $H_2O_2$ . The data obtained, here was consistent with the anticipated results. Unlike the effect of  $H_2O_2$ , ionising radiation induced a much higher ratio of DSB:SSB. Consistent with the literature, these data suggest that radiation provoked more complex, MDS in cells treated with ionising radiation, when contrasted with those treated chemically with  $H_2O_2$  (Figure 5.18A & B).

MDS was hypothesised to be observed in ascorbate pre-treated melanoma cancer exposed to  $H_2O_2$ . The prediction was based on the supposition that acceleration in the Fenton reaction by ascorbate might generate sequential <sup>•</sup>OH from  $H_2O_2$ , which in turn attacks different sites of DNA molecules, causing more of DSBs relative to SSBs. However, when the investigation was conducted with three different melanoma cancer cell lines (A375P, SK28 & SK23), it was found that in cells treated with ascorbate, enhancement in the number of  $H_2O_2$ -induced DSBs was proportional to the enhancement in the number of  $H_2O_2$ -induced SSBs (Figure 5.19C-5.21C); so there was no increase in damage complexity.

The high level of damage complexity observed in irradiated melanoma cancer cells, indeed, is the result of direct and indirect effects. The mechanism of DNA damage by ionising radiation is metal ion-independent (Carante *et al.*, 2015). The direct DNA damage occurs via the interaction between the emitted electrons, released from ionising radiation, and the DNA. The indirect DNA attack is through <sup>•</sup>OH generation from radiolysis of H<sub>2</sub>O that then locally attack the DNA (Ravanat *et al.*, 2014). However, induction of DNA damage chemically by H<sub>2</sub>O<sub>2</sub> depends on the availability of redox metals (necessary for metal-dependent process) on the DNA molecule. Damaged sites induced by <sup>•</sup>OH occur at the site of <sup>•</sup>OH production (Lenton *et al.*, 1999). This is because the <sup>•</sup>OH formed by the Fenton reactions are highly unstable and react rapidly

and do not migrate far from their site of their generation (Lenton *et al.*, 1999). Therefore, in view to the above data (Figure 5.19C-5.21C), a reasonable explanation for this, is that either  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  bind to the DNA, the other partner being unbound and so free to migrate. This will lead to the formation of dispersed <sup>•</sup>OH causing simple damage (Figure 5.22).

DSBs are not only formed immediately post treatment. It has been well known that many SSBs and much unrepaired ROS-induced damage is converted to DSBs via replication and DNA repair mechanisms (formation of secondary DSBs post the damage recovery) (Maxwell and Roskelley, 2014, Ward and Chen, 2001). This form of DNA damage lesion, formed during attempted repair following treatment, has a potential effect on cell fate (Ward, 1994).

A DNA damage repair experiment was performed using  $\gamma$ -H2AX immunoassay, to examine any change in the number of H<sub>2</sub>O<sub>2</sub>-induced DSBs in the presence and absence of ascorbate, immediately after and at different time points post-treatment. Indeed, a sharp enhancement in the number of DSBs ( $\gamma$ -H2AX foci) was observed during the first 2 hours of a cell's recovery from induced DNA damage at 37°C. The number of DSBs induced in the presence of ascorbate was substantially higher than that in the absence of ascorbate (Figure 5.23). Generation of higher numbers of the DSBs in the cells incubated following treatment with H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate may be the result secondary to the repair process. The repair processing of the SSBs and AP sites and the aborted repair of base lesions all can lead to formation of secondary DSBs (Ma *et al.*, 2011). Or this could be due to development of more  $\gamma$ -H2AX foci during the recovery post-treatment.

CHAPTER VI: Assessment of ascorbate's effect on the H<sub>2</sub>O<sub>2</sub>induced viability, apoptosis and cell killing of melanoma cancer cells

#### 6.1 Introduction

The DNA molecule is a vital intracellular element and damage to it could have an effect on a cell's lifecycle. When ROS enters into cells, it causes damage to cellular components, and when ROS attacks the DNA molecule it results in oxidatively damaged lesions (Roos and Kaina, 2006). It is well-known that extensive DNA damage can induce cell death (Panieri *et al.*, 2013), and that  $H_2O_2$  can mediate cell apoptosis which has been demonstrated by the fact that apoptosis can be prevented by treating cells with catalase (Pierce *et al.*, 1991, Simon *et al.*, 2000). A moderate concentration of  $H_2O_2$  can cause cell apoptosis, although the effect of this is largely dependent on the cell type (Ott *et al.*, 2007, Nicotera and Melino, 2004). It has been reported that up to  $15\mu$ M of  $H_2O_2$  has a pro-proliferative effect on tumour cells, whereas a higher amount of  $H_2O_2$ could have a deleterious impact on cancer cells, causing growth arrest and cell killing (González *et al.*, 2005).

In agreement with previous studies (Szatrowski and Nathan, 1991, Toyokuni *et al.*, 1995), the findings of Chapter 3 of the current study indicate that MM cells contain a greater amount of endogenous ROS (Figure 3.4 & 3.5). Although cancer cells are oxidatively stressed, the stress they contain does not usually induce cell death (Toyokuni *et al.*, 1995). However, a high level of endogenous ROS in tumour tissue may permit a ROS-mediated approach for cancer treatment; providing tumour cells with further exogenous (therapeutic) ROS could increase the amount of intracellular ROS and so exceed at toxic threshold level (i.e. the level at which ROS become toxic) and so may selectively kill tumour cells (Trachootham *et al.*, 2009, Thompson, 1995).

In the previous Chapter, it was clearly observed that melanoma cancer cells are more sensitive to  $H_2O_2$ -induced DNA damage; also, this effect was exacerbated and triggered in the presence of ascorbate (Figure 5.6 & 5.12). It was therefore proposed to study the effect of ascorbate on  $H_2O_2$ -induced cell death and cell killing.

This chapter aims to:

- Investigate the effect of H<sub>2</sub>O<sub>2</sub> on cell viability in melanoma cancer cells and HaCaT cells, in the presence and absence of ascorbate
- Study the effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis in melanoma cancer cells and HaCaT cells

• Examine the effect of H<sub>2</sub>O<sub>2</sub> on melanoma cancer cell and HaCaT cell survival in the presence and absence of ascorbate

### 6.2 Results

### 6.2.1 Investigation of cell viability in melanoma cancer cells and in HaCaT cells exposed to H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate

### 6.2.1.1 Optimisation of ATPlite assay

Measurement of cell viability is a method employed to assess the impact of drugs or toxic agents on cells. In the present study, the ATPlite assay kit was used to measure the effect of  $H_2O_2$  in combination with ascorbate on cell viability. The ATPlite assay is an extremely sensitive tool for assessing cytotoxicity, because it can detect ATP levels even for as few as 10 cells (Germain *et al.*, 2003). ATP amount may change within a cell, but low levels has been found to be directly linked to cell apoptosis or cell necrosis (Germain *et al.*, 2003).

To measure the level of ATP, optimisation of the assay is required to ensure the linearity between the ATP levels and the luminescence signals using the ATPlite standards provided. A stock of 10mM ATPlite standards was prepared according to the instructions provided by the assay kit. From the ATPlite stock, serial dilutions were made using ddH<sub>2</sub>O (0.0001 $\mu$ M to 1 $\mu$ M), and the assay was performed based on instructions provided by the assay kit. A clear linear relationship was found between ATP concentrations and luminescence signals (Figure 6.1).

ATP is a good marker for the detection and measurement of cell viability following treatment. It detects metabolically active cells; thus a lowering of the level of ATP in a sample of cells, would indicate a reduction in metabolically active cells (Cree and Andreotti, 1997). To confirm this, further assay optimisation was carried out, using different numbers of A375P melanoma cancer cells.

Ranges of cell numbers (500-16000 cells/microwell) were seeded in a 96-microwell plate for 24 hours. Adhered cells were then washed twice with PBS, and the ATPlite assay was then conducted. The relationship found between the ATP luminescence and cell numbers was positive and linear ( $R^2$ =0.9883). Additionally, it was found that 16,000 cells is an ideal cell number for the ATPlite assay for assessing a likely reduction in cell number (Figure 6.2).



Figure 6.1. The standard curve for the ATPlite assay.

Different ATPlite standards  $(0.0001\mu$ M to  $1\mu$ M) were prepared from the provided lyophilised material using ddH<sub>2</sub>O. 100µl of cell culture media was added to each identified microwell of the 96-well blak plate, and then 50µl of provided cell lysis buffer was added to each well. The plate was then shaken thoroughly for 5 minutes using an orbital plate shaker. 10µl of each standard was added to each microwell, and again the plate was shaken by the plate shaker for a further 5 minutes. This process was followed by the addition of 50µl of provided substrate to each microwell, and the plate was then shaken for a further 5 minutes. After this, the plate was incubated at room temperature in dark conditions for 10 minutes, before measuring the luminescence signals using the plate reader (BMG FLUOstar OPTIMA Microplate Reader) at a wavelength of 562nm. Each point on the graph represents mean ± SD of relative luminescence intensity determined from three independent experiments.



Figure 6.2. Optimisation of the cell number for the ATPlite assay.

Different cell numbers were seeded in a 96-microwell plate (in a total volume of  $100\mu$ /microwell) and incubated for 24 hours 37°C/5% CO<sub>2</sub> to allow attachment.  $50\mu$ l of provided cell lysis buffer was added to each microwell. The plate was then shaken thoroughly for 5 minutes using an orbital plate shaker. This process was followed by the addition of  $50\mu$ l of provided substrate to each microwell and the plate was shaken for a further 5 minutes. After this, the plate was incubated at room temperature in dark conditions for 10 minutes, before measuring the luminescence signals using the plate reader (BMG FLUOstar OPTIMA Microplate Reader) at a wavelength of 562nm. Each point on the graph represents mean  $\pm$  SD of relative luminescence intensity determined from two independent experiments.

# 6.2.1.2 Effect of H<sub>2</sub>O<sub>2</sub> on cell viability for non-pigmented (A375P), moderatly pigmented (SK28) and and heavily pigmented (SK23) melanoma cancer cells and in HaCaT cells, in the presence and absence of ascorbate

An ATPlite assay kit then was used to measure cell viability in the HaCaTs and in melanoma cancer cells including non-pigmented (A375P), moderately pigmented (SK28) and heavily pigmented (SK23), following treatment with  $H_2O_2$  in the presence and absence of ascorbate.  $16 \times 10^3$  melanoma cancer cells and HaCaT cells were seeded in each microwell of the 96-microwell plates for 24 hours. The cells were then gently washed with PBS and pre-treated with ascorbate for 2 hours, prior to exposure to  $H_2O_2$ for 30 minutes on ice, protected from light. After treatment, cells were washed with PBS and incubated with cell specific media for 18 hours at 37°C/5% CO<sub>2</sub>. The ATPlite assay was then performed and the luminescence intensity measured. Data indicates that in most of the cell lines studied (SK28, SK23 & HaCaTs), up to 30µM H<sub>2</sub>O<sub>2</sub> has a proproliferative effect in the absence of ascorbate (Figure 6.3A-D). However,  $H_2O_2$  in combination with ascorbate becomes more toxic to cells, particularly for the melanoma cancer cells, since a clear reduction in cell viability in all cell lines was observed when the cells were pre-treated with ascorbate before exposing them to  $H_2O_2$ ; most  $H_2O_2$ doses caused a significant reduction in the viability rates of ascorbate pre-treated melanoma cancer cells. Melanoma cancer cells were the most sensitive to this effect, more so than the HaCaT cells. In addition, the pigmented melanoma (SK28 and SK23) cells were also clearly more affected than the non-pigmented cells (Figure 6.3A-D).



Figure 6.3. Effect of  $H_2O_2$  on cell viability rates for non-pigmented (A375P) (A), moderately pigmented (SK28) (B) and heavily pigmented (SK23) (C) melanoma cancer cells and the HaCaT cells (D).

(16 x  $10^3$  cells per microwell of a 96-microwell plate were seeded for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. The cells were either incubated without ascorbate or with ascorbate for 2 hours. The cells were then washed once with PBS and then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 30 mins on ice, while protected from light. The media was then removed and the cells washed with PBS. 100µl of fresh cell culture media was then added to each microwell and incubated for 18 hours at 37°C/5% CO<sub>2</sub>. 50µl of provided cell lysis buffer was then added to each microwell, after which the plate was shaken thoroughly for 5 minutes using an orbital plate shaker. This was followed by adding 50µl of provided substrate to each microwell, and the plate was again shaken for 5 minutes. After this, the plate was incubated at room temperature in dark conditions for 10 minutes before measuring luminescence signals with the plate reader (BMG FLUOstar OPTIMA Microplate Reader) at a wavelength of 562nm. Each bar represents the mean ± SD of the relative luminesce intensity (presented as % control), as determined in three independent experiments each run in triplicate. *t*-test (unpaired) was used to assess the statistical difference between each two groups.

## 6.2.1.3 Assessment of cell viability for non-pigmented (A375P), heavily pigmented (SK23) melanoma cancer cells and HaCaT cells incubated with H<sub>2</sub>O<sub>2</sub> for prolonged duration in the presence and absence of ascorbate

It was thought that the incubation of cells for a longer time with  $H_2O_2$  at 37°C/5% CO<sub>2</sub> would have more effect on cell viability. To assess this, ATPlite assay was repeated in non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer cells and in the HaCaT cells. Cells were seeded in 96-microwell plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment, and were then exposed to  $H_2O_2$  for 15 hours at 37°C/5% CO<sub>2</sub> in the absence and presence of ascorbate. An ATPlite assay was then performed, and the luminescence signals analysed. Data from these experiments showed a clear reduction in the cell viability rates in melanoma cancer cells with a further effect noted in the presence of ascorbate. Interestingly, HaCaT cells were almost entirely unaffected (Figure 6.4A-C).



## Figure 6.4. Cell viability for non-pigmented (A375P) (A) and heavily pigmented (SK23) (B) melanoma cancer cells, and HaCaTs (C) cells pre-treated with and without ascorbate and exposed to $H_2O_2$ for long periods.

16 x  $10^3$  cells were seeded per microwell of the 96-microwell plate for 24 hours at  $37^\circ$ C/5% CO<sub>2</sub> to allow attachment. The cells were either incubated with or without ascorbate for 2 hours. The cells were then washed once with PBS and exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> (with a total volume 100µl media) for 15 hours at  $37^\circ$ C/5% CO<sub>2</sub>. After this, 50µl of provided cell lysis buffer was added to each microwell. The plate was then shaken thoroughly for 5 minutes, using an orbital plate shaker. This was followed by the addition of 50µl of provided substrate to each microwell and the plate was shaken for further 5 minutes. After this, the plate was incubated at room temperature in dark conditions for 10 minutes before measuring the luminescence signal on the plate reader (BMG FLUOstar OPTIMA Microplate Reader) at a wavelength of 562nm. Each bar represent mean ± SD of luminesce relative intensity (presented as % control), determined from three independent experiments each run in triplicate.

### 6.2.2 Effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced apoptosis in non-pigmented (A375M & A375P) melanoma cancer cells

To investigate the effect of ascorbate on  $H_2O_2$ -induced cell apoptosis in melanoma cancer cells and HaCaT cells, the annexin V/PI assay was performed. A375M and A375P melanoma cancer cells were initially used to test positive and negative controls, and to deduce the extent of apoptosis induced by  $H_2O_2$  in the presence and absence of ascorbate.

Cells were first incubated with and without ascorbate, and then exposed to different concentrations of  $H_2O_2$  for 30 minutes on ice /while protected from light. After treatment, the cells were washed with PBS, new fresh complete culture media was then added to the cells, and they were then incubated for 72 hours before conducting the annexin V/PI assay. Data obtained from this experiment suggests that  $H_2O_2$  induces apoptosis in A375M and A375P melanoma cancer cells. The level of apoptosis caused by  $H_2O_2$  was obvious when compared to the controls. Interestingly, ascorbate was capable of enhancing  $H_2O_2$ -induced cell apoptosis in A375M and A375P cells (Figure 6.5A & B), although the effect was small.

Further analysis of this data indicates that the average of the total apoptosis induced by  $15\mu$ M and  $30\mu$ M H<sub>2</sub>O<sub>2</sub> in the presence of ascorbate (background subtracted) was enhanced by more than 2-fold in A375M and A375P cells (Figure 6.5C & D). In A375M cells, the recorded level of endogenous apoptosis was high, whilst in the A375P cells the level of induced apoptosis was higher compared to A375M cells (Figure 6.5A & B).



### Figure 6.5. Effect of ascorbate pre-treatment on H<sub>2</sub>O<sub>2</sub>-induced apoptosis in A375M (A) and A375P (B) melanoma cancer cells.

Equal numbers (5 x  $10^5$ ) of cells were seeded in T25 flasks for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. The cells were then washed with PBS and pre-treated with and without ascorbate for a further 2 hours. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 30 minutes on ice, while protected from light. They were then washed once with PBS and new cell specific complete media added and incubated at  $37^{\circ}C/5\%$  CO<sub>2</sub> for 72 hours. The cells were then collected in FACS tubes and the annexin V/PI assay was carried out. Each bar represents mean  $\pm$  SD of apoptosis determined from three independent experiments. Melanoma cells were treated with Etoposide for 24 hours as +ve controls. Figures C and D represent total H<sub>2</sub>O<sub>2</sub>-induced apoptosis (%) in the absence and presence of ascorbate, adapted from figures 6.5A & B.

### 6.2.3 Assessment of caspase-3/7 expression in A375P melanoma cancer cells following treatment with H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate

Apoptosis is an event that can be initiated either by extrinsic factors, which stimulate cell surface pro-apoptotic receptors, called death receptors (e.g. Tumour Necrosis Factor-Alpha (TNFR), or intrinsically through cellular events including DNA damage (Cotter, 2009). Traditionally, caspase proteins have been divided into two different groups. The first members including caspase (2, 8, 9 and 10) are considered initiators, whereas the second group are called effectors, and are caspase (3, 6 and 7). Caspase-3 is a reliable marker for apoptosis detection, because the protein's activation occurs during the irreversible commitment of a cell to undergo apoptosis. It has been found that oxidative DNA damage induced by  $H_2O_2$  or other oxidants lead to caspase-3/7 expression cells (Jin *et al.*, 2001, Mendivil-Perez *et al.*, 2015).

To confirm the enhancement effect of ascorbate on  $H_2O_2$ -induced apoptosis in melanoma cancer cells, caspase-3/7 cleavage was measured in A375P melanoma cancer cells, following exposure to  $H_2O_2$  in the presence and absence of ascorbate. Interestingly, an increase in the level of caspase-3/7 expression was demonstrated in cells exposed to 15µM and 30µM  $H_2O_2$  in the presence of ascorbate, by 1.2-fold and 1.4-fold respectively (Figure 6.6).



Figure 6.6. Caspase 3/7 expressions in A375P melanoma cancer cells induced by  $H_2O_2$  in the presence and absence of ascorbate.

Based on the manufacturer's instructions of the assay kit,  $15 \times 10^3$  A375P melanoma cells were seeded in each microwell of the 96-micrwell plate for 24 hours to allow attachment. The cells were then treated with and without ascorbate for 2 hours. The cells were then exposed to indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 30 minutes on ice, while protected from light. The cells were then washed once with PBS, and 100µL of new fresh cell culture media was added. The plates were incubated at  $37^{\circ}C/5\%$  CO<sub>2</sub> for a further 15 hours before measurement of the caspase-3/7 activity using Caspase-Glo® 3/7 assay kit. Each bar represents the mean ± SD (presented as % control) of the relative fluorescence intensity, from three independent experiments, each run in triplicate. A *T*-test (unpaired) was used for statistical analysis between each of the two groups *versus* ascorbate untreated cells.

## 6.2.4 Effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced apoptosis in non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer cells and HaCaT cells exposed to H<sub>2</sub>O<sub>2</sub> for prolonged duration

For the above annexin V/PI experiments, the level of apoptosis was clearly observed with an enhancement noted in melanoma cancer cells pre-loaded with ascorbate. In the literature, cells were usually exposed to high concentrations of  $H_2O_2$  and for longer times for induction of apoptosis (Woo *et al.*, 2012). Therefore, it was thought that incubation of cells with  $H_2O_2$  at 37°C/5% CO<sub>2</sub> for a longer time would increase the level of apoptosis induced and that ascorbate may further enhance the effect. To assess this it was proposed to incubate cells with different concentrations of a  $H_2O_2$  at 37°C/5% CO<sub>2</sub> for 24 hours before assessing the apoptosis level. The annexin V/PI assay was conducted in A375M melanoma cancer cells to measure the degree of  $H_2O_2$ -induced cell apoptosis dose response. The cells were seeded in T25 flasks for 24 hours prior to treatment. The cells were then washed once with PBS and incubated with freshly made  $H_2O_2$  for a further 24 hours at 37°C/5% CO<sub>2</sub>. Next, the annexin V/PI was then performed. Data obtained from this experiment showed a clear  $H_2O_2$ -induced cell apoptosis dose response. The average for the total apoptotic cells increased with  $H_2O_2$  concentrations. A dose of 200µM and 400µM  $H_2O_2$  induced a significant increase in the level of apoptosis (% total apoptosis:  $15.55 \pm 0.88$  versus control, p=0.0149, and 17.08  $\pm$  3.04 versus control, p=0.0037, respectively) (Figure 6.7). The two highest concentrations of  $H_2O_2$  were selected to study oxidative mediated apoptosis further in future work. This is because the lower concentrations (30µM-60µM  $H_2O_2$ ) did not cause measurable significant apoptosis when the cells were treated with  $H_2O_2$  for long periods.



Figure 6.7.  $H_2O_2$ -induced cell apopotosis dose response in A375M melanoma cancer cells exposed to  $H_2O_2$  for a longer period.

Equal numbers (5 x  $10^5$ ) of cells were seeded in T25 flasks for 24 hours prior to treatment to allow attachment. The cells were then washed with PBS and then incubated with H<sub>2</sub>O<sub>2</sub> for 24 hours at 37°C/5% CO<sub>2</sub>. The cells were washed with PBS, and then collected in the FACS tubes, and the annexin V/PI assay was then carried out. Each bar represents the mean ± SD, determined of three independent experiments. One-way ANOVA was used for statistical analysis between the groups *versus* control.

The same treatment protocol as that described above was used to test  $H_2O_2$ -induced cell apoptosis in the HaCaT cells, non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer cells, in the presence and absence of ascorbate. In this instance, the cells were seeded for 24 hours in T25 flasks and then pre-treated with and without ascorbate for 2 hours. The cells were then washed once with PBS and incubated with indicated concentrations of  $H_2O_2$  for 24 hours at 37°C/5% CO<sub>2</sub>. The annexin V/PI assay was then conducted to measure the level of cell apoptosis.

Overall, the data obtained indicated a clear  $H_2O_2$ -induced cell apoptosis dose response in all cell lines, with an enhancing effect noted for ascorbate, particularly in the melanoma cancer cells; a typical flow cytometry result showing the level of  $H_2O_2$ induced apoptosis in A375P melanoma cancer cells in the presence and absence of ascorbate are shown in Figure 6.8. Data obtained indicate an enhancing effect of ascorbate on  $H_2O_2$ -induced cell apoptosis in the melanoma cancer cells (Figure 6.9A-C). Statistical analysis for the late apoptosis data of three cell lines was carried out. The outcome reveals a significant enhancement effect of ascorbate on the level of  $H_2O_2$ induced apoptosis mediated by  $200\mu$ M and  $400\mu$ M  $H_2O_2$  in A375P melanoma cancer cells (% of late apoptosis:  $36.38 \pm 2.55$  versus  $29.38 \pm 0.96$ , p=0.0031,  $49.54 \pm 4.67$  *versus*  $35.54 \pm 1.1$ , p=0.0195, respectively). Likewise, the enhancement effect of ascorbate was observed in SK23 melanoma cancer cells exposed to the same above doses of H<sub>2</sub>O<sub>2</sub> (% of late apoptosis:  $38.37 \pm 3.91$  *versus*  $18.8 \pm 4.02$ , p=0.00073,  $41.42 \pm 5.78$  *versus*  $30.86 \pm 6.68$ , p=0.2665, respectively). The enhancement effect of ascorbate on the apoptosis mediated by 200µM and 400µM H<sub>2</sub>O<sub>2</sub> in HaCaT cells was very small (% of late apoptosis:  $27.25 \pm 2.5$  *versus*  $25.73 \pm 1.91$ , p=0.6411,  $32.50 \pm 0.6$  *versus*  $33.7 \pm 2.4$ , p=0.6756, respectively) (Figure 6.9A-C).

Further analysis of the data obtained indicates that, unlike the HaCaT cells, melanoma cancer cells are more susceptible to  $H_2O_2$ -mediated apoptosis in the presence of ascorbate. The average for the total  $H_2O_2$ -induced cell apoptosis, mediated by two doses of  $H_2O_2$  (200µM & 400µM) in A375P and SK23 is enhanced by ascorbate by more than 1.4-fold and 1.9-fold, respectively; that is, higher than that in the HaCaT cells (Figure 6.9D-F).



Figure 6.8. Typical flow cytometry results showing the level of H<sub>2</sub>O<sub>2</sub>-induced apoptosis in A375P melanoma cancer cells in the presence and absence of ascorbate.



## Figure 6.9. Effect of ascorbate pre-treatment on $H_2O_2$ -induced apoptosis, in non-pigmented (A375P) (A), and heavily pigmented (SK23) (B) melanoma cancer cells and in the HaCaT cells (C) exposed to $H_2O_2$ for longer periods.

Equal numbers (5 x  $10^5$ ) of cells were seeded in T25 flasks for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. The cells were then washed with PBS and pre-treated with and without ascorbate for a further 2 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. The cells were then exposed to H<sub>2</sub>O<sub>2</sub> for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. They were then washed with PBS and collected in FACS tubes and the annexin V/PI assay carried out. Each bar represents mean  $\pm$  SD of apoptosis determined of three independent experiment. Melanoma cells were pre-treated with Etoposide for 24 hours as + ve controls. Figures D, E and F represent the total apoptosis (%) in the presence and absence of ascorbate, adapted from figure 6.9A-C. *T*-test (unpaired) was used to test the difference between the late apoptosis between each two groups.

# 6.2.5 Effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced cell killing in non-pigmented (A375M & A375P), moderately pigmented (SK23) and heavily pigmented (SK23) melanoma cancer cells and HaCaT cells

Clonogenic potential has been used to measure the ability of cells to survive post-treatment and is considered by many to be the 'gold-standard' for the assessment of cell killing (Pauwels *et al.*, 2003). In the current study a clonogenic assay was used to estimate the survival of melanoma cancer cells and HaCaT cells post-treatment with  $H_2O_2$  in combination with and without ascorbate.

First, the plating efficiency (PE) was assessed for all melanoma cells including nonpigmented (A375M & A375P), moderately pigmented (SK28) and heavily pigmented (SK23), and HaCaT cells. To achieve this, a number of cells from different lines were seeded and colonies developed without subjecting them to any treatment. The data obtained demonstrates that the ability of cells to develop colonies varied among the studied cell lines (Table 6.1). The average of plate efficiency (PE) was highest in A375P melanoma cancer cells (59.91%) and lowest in HaCaT cells (7.29 %).

#### Table 6.1. Plate efficiency (PE) in melanoma cancer cells and HaCaT cells.

A different number of cells (50 cells, 100 cells and 200 cells) were added to each Petri dish. These were seeded and incubated at  $37^{\circ}C/5\%$  CO<sub>2</sub> for 7 days until small colonies appeared. Old media was then removed and new fresh cell specific media added to each petri dish and incubated at  $37^{\circ}C/5\%$  CO<sub>2</sub>. Colonies were counted after staining with crystal violet dye. The data presented are the average of three independent experiments.

Cell line	Cell number seeded per plate	Average number of Colonies	Plate efficien cy (PE)	Average of Plate efficiency
			(/0)	
	50	8.35	16.7	
A375M	100	16.7	16.7	
	200	27.6	13.3	15.56%
	50	24.0	48.0	
A375P	100	71.0	71.0	
	200	121.5	60.75	59.91%
	50	13.33	26.66	
SK28	100	26.67	26.67	
	200	54.33	27.16	26.83%
	50	17.0	34.0	
SK23	100	28.0	28.0	
	200	49.75	24.87	28.59%
	50	2.25	4.5	
HaCaTs	100	8.5	8.5	
	200	17.75	8.87	7.29%

Based on the determined PE of each cell line, the number of cells for future clonogenic experiment was selected. The number of cells seeded as controls for A375M, A375P, SK28, SK23 and the HaCaT cells were 200, 50, 100, 100 and 200 cells, respectively.

Data obtained from the clonogenic experiments reveals that  $H_2O_2$  in the presence of ascorbate reduces the ability of the cells to from colonies, so kills more melanoma cancer cells (Figure 6.10 & 6.11). Data analysis indicates that ascorbate enhances  $H_2O_2$ -induced cell killing in melanoma cancer cells, whereas in HaCaT cells, ascorbate slightly protected cells against  $H_2O_2$  (Figure 6.12A-E).

Further statistical analysis of the data obtained, using the determined 'area under the curve' (AUC), clearly demonstrates that ascorbate enhances  $H_2O_2$ -induced cell killing in all melanoma cancer cells. A decrease in the area under the curve (AUC) was observed in melanoma cancer cells treated with  $H_2O_2$  and ascorbate, compared to the AUC of the cells treated with  $H_2O_2$  alone. Notably, this effect was reversed in the HaCaT cells. The ratios obtained between the AUC for  $H_2O_2$ -exposed cells in the presence and absences of ascorbate were 0.79, 0.63, 0.63, and 0.68 in A375M, A375P, SK28, SK23, respectively, whereas it was 1.3 for the HaCaT cells (Table 6.2).



Figure 6.10. Clonogenic assay showing the number of colonies formed by non-pigmented (A375M & A375P) melanoma cancer cells treated with H<sub>2</sub>O<sub>2</sub> in the presence and absence of ascorbate.

An equal number of cells were pre-incubated with and without ascorbate for 2 hours. The cells were then exposed to indicated concentrations of  $H_2O_2$  for 30 minutes on ice and protected from light. The cells were then collected, counted and seeded, at the indicated cell numbers, in petri dishes and then incubated to develop colonies. Old media was replaced with fresh every 6 days, until visible colonies were appeared. These colonies were then stained with crystal violet to become visible. Photos of each plate were taken using iPhone 4 cameras.



Figure 6.11. Clonogenic assay showing the number of colonies formed by moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells treated with  $H_2O_2$  in the presence and absence of ascorbate.

An equal number of cells were pre-incubated with and without ascorbate for 2 hours. The cells were then exposed to indicated concentrations of  $H_2O_2$  for 30 minutes on ice and protected from light. The cells were then collected, counted and seeded, at the indicated cell numbers, in petri dishes and then incubated to develop colonies. Old media was replaced with fresh every 6 days, until visible colonies were appeared. These colonies were then stained with crystal violet to become visible. Photos of each plate were taken using iPhone 4 cameras.



Figure 6.12. Clonogenic assay showing the surviving fraction (% control) for non-pigmented, (A375M) (A) and A375P) (B), moderately pigmented (SK28) (C) and heavily pigmented (SK23) (D) melanoma cancer cells and in HaCaT cells (E) treated with  $H_2O_2$  in the presence and absence of ascorbate.

An equal number of cells were pre-treated with and without ascorbate for 2 hour and were then exposed to different micromolar concentrations of  $H_2O_2$  for 30 minutes on ice while protected from light. Cells were then collected, counted and seeded in petri dishes and incubated at  $37^{\circ}C/5\%$  CO<sub>2</sub> to develop colonies. Old media was replaced with fresh media every 6 days until visible colonies appeared. The colonies were then stained with crystal violet and counted. Each point represents mean  $\pm$  SD of survival fraction (% control) determined from three independent experiments run in triplicate. A *T*-test (unpaired) was used to compare the mean of survival fraction (% control) between each group *versus* untreated cells with ascorbate.

Cell lines	Area Under the Curve (AUC)		Fold changes
	H <sub>2</sub> O <sub>2</sub>	$H_2O_2 + AA$	in AUC
A375M	3553	2819	0.79
A375P	3499	2237	0.63
SK28	3714	2322	0.63
SK23	4288	2920	0.68
HaCaTs	2585	3382	1.3

Table 6.2. Survival curve analysis by using area under the curve

In order to find a correlation between enhancement effect of  $H_2O_2$ -induced DNA damage (SSBs & DSBs) presented in the Chapter 5 of this study and the above clonogenic assay results, further analysis of the above data was performed using HaCaT cells, non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer cells. The enhancing effect of ascorbate on oxidative mediated cell killing induced by  $30\mu$ M  $H_2O_2$  was evaluated in each of these three cell lines. Indeed, it has been found that the level of enhancement increased by 1.9-fold and 1.6-fold in SK23 and A375P melanoma cancer cells, respectively; that is more than 2-fold higher than that in the HaCaT cells (Figure 6.13).



Figure 6.13. H<sub>2</sub>O<sub>2</sub>-induced cell killing enhanced by ascorbate in HaCaT cells and heavily pigmented (SK23) and non-pigmented (A375P) melanoma cancer cells.

The enhancing effect of ascorbate on cell killing induced by  $30\mu M H_2O_2$  was measured by fold changes. The figure adapted from figures 6.12B, D & E.

## 6.2.6 Correlation between the effect of ascorbate on $H_2O_2$ -induced DNA damage and the effect of ascorbate on $H_2O_2$ -induced cell killing in melanoma cancer cells

The main hypothesis of this study was that if ascorbate enhances DNA damage via the modulation of oxidative stress, this may also enhance melanoma cell killing. To investigate this, an analysis was performed to find a correlation between the enhancement effects of ascorbate on  $H_2O_2$ -induced DNA damage (as assessed by ACA and  $\gamma$ -H2AX immunoassay) and the enhancement effects of ascorbate on  $H_2O_2$ -induced cell killing (as assessed by clonogenic assay) in the HaCaT cells, non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer cells. A positive correlation was observed between ascorbate enhancement of  $H_2O_2$ -induced DNA damage (SSBs (ACA)) & DSBs ( $\gamma$ -H2AX immunoassay)) and  $H_2O_2$ -induced cell killing enhancement by ascorbate in cells treated with 30 $\mu$ M  $H_2O_2$  (Figure 6.14).





The scatter plot shows correlation of the enhancement effect of ascorbate on  $H_2O_2$ -induced SSBs (A) and DSBs (B) with enhanced  $H_2O_2$ -induced cell killing induced by  $30\mu M H_2O_2$ . The figures were adapted from Figures 5.7 and 5.13 (Chapter 5), and figure 6.13.

#### 6.3 Discussion

Collective evidence indicates that tumour tissue generates higher amounts of  $H_2O_2$  compared to normal tissue (Szatrowski and Nathan, 1991, Lim *et al.*, 2005, Burdon, 1995, Zieba *et al.*, 2000, Kawanishi *et al.*, 2006). The high amount of oxidative stress within tumour cells is usually insufficient to causes cell death (Toyokuni *et al.*, 1995); however, providing these cells with exogenous ROS could exhaust cellular resistance, and trigger cancer cell death preferentially (López-Lázaro, 2007, Trachootham *et al.*, 2006).

Cultured cancer cells die if they are exposed to either high acute doses of  $H_2O_2$  (<1mM) or low chronic doses of  $H_2O_2$  (2µM) (Antunes and Cadenas, 2001, Barbouti *et al.*, 2002).  $H_2O_2$ -induced DNA damage is one of the main mechanisms of ROS mediated cytotoxicity (Schraufstatter *et al.*, 1986, Yin *et al.*, 2000)

Previous studies have shown that cancer cells survive despite having high levels of  $H_2O_2$ , and it has been observed that normal cells are less susceptible than cancer cells to  $H_2O_2$ -induced cell death (López-Lázaro, 2007). This effect was further supported by a recent study which found high sensitivity for  $H_2O_2$ -induced cell death in leukemic cells, but not in normal hematopoietic cells (Nogueira-Pedro *et al.*, 2013).

In this Chapter, cell viability was the first method used to assess the effect of ascorbate on  $H_2O_2$ -induced cytotoxicity in melanoma cancer cells and HaCaTs. The ATPlite luminescence assay was used to measure the viability of treated and untreated cells after the assay had been optimised (Figure 6.1 & 6.2). In the optimisation of this assay, the intensity of luminescence signal (representing ATP level) was proportional to the number of cells used (Figure 6.1 & 6.2). Indeed, in cancer treatment the intracellular level of ATP determines cell death and the effect of cytotoxic compounds (Verrax *et al.*, 2011b). The suggested mechanism for  $H_2O_2$ -induced depletion in ATP after cell exposure to oxidative stress is through ROS-mediated DNA damage (Lee and Shacter, 1999). Indeed, tumour cells are generally highly active, metabolically, and therefore they require high level of ATP for uncontrolled proliferation and growth (Hlavatá *et al.*, 2003); thus depletion in ATP level by ROS may kill these cells.

ATPlite experiments were performed following cell treatment with  $H_2O_2$  in the presence and absence of ascorbate. Data obtained in these experiments show a general reduction in cell viability in melanoma cancer cells treated with  $H_2O_2$  and ascorbate (Figure 6.3A- C). Compared to controls, the viability measured was increased in treated HaCaT cells (Figure 6.3D). In agreement with the present study's findings, Farmer *et al* (2003) demonstrated that melanoma cancer cells are sensitive to exogenous ROS, and that the viability rate in these cells fell after the cells were exposed to exogenous oxidative stress (Farmer *et al.*, 2003). This suggestion is also compatible with the data reported in a previous study, which found that cancer cells are substantially more sensitive to  $H_2O_2$  than normal cells (Chen *et al.*, 2005). As shown in the figure 6.3, in most of the cell lines studied a slight proliferative effect was seen for  $H_2O_2$  does up to  $30\mu M H_2O_2$ ; however, with higher concentrations, the cell viability rates reduced. This effect, to some extent, agrees with data reported by previous studies. It has been suggested that low levels of  $H_2O_2$  (3-15 $\mu$ M) stimulates cell division and has a proliferative effect on cells, whereas higher levels may lead to growth arrest, apoptosis and/or necrosis of cells (González *et al.*, 2005).

The most important observation from these experiments is that ascorbate exacerbated the effect of  $H_2O_2$  on cell viability; this effect was more obvious in melanoma cancer cells than in HaCaT cells. This can indicate that melanoma cancer cells are likely to be more sensitive than to  $H_2O_2$  alone and plus ascorbate, than HaCaTs cells. In melanoma cancer cells, the reduction in the cell viability, caused by some  $H_2O_2$  doses, in the presence of ascorbate, is statistically significant; this was more apparent in pigmented melanoma cancer cells than in other non-pigmented melanoma cells (Figure 6.3A-D). This indicates that the pro-proliferative concentrations of  $H_2O_2$  become toxic to melanoma cancer cells pre-treated with ascorbate. Furthermore, when melanoma and HaCaT cells incubated with  $H_2O_2$  for 15 hours at 37°C/5% CO<sub>2</sub> in the absence and presence of ascorbate, the oxidative stress effect and its modulation by ascorbate in melanoma cancer cells became more apparent. Interestingly, HaCaT cells were almost entirely unaffected (compared to its controls) (Figure 6.4A-C).

Despite reports suggesting that the ATPlite assay kit is highly sensitive for the detection of cell proliferation and viability (based on the level of detected ATP in cells) (Grimsey *et al.*, 2012), it was thought that low amount of ATP detected in a group of treated cells may not guarantee that all cells could die. To validate the data and to see the effect of ascorbate on the level of H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity, apoptosis assays were conducted by performing annexin V/PI assays and by measurement of caspase-3/7 expression in treated cells.

The effect of  $H_2O_2$  mediating apoptosis in cancer cells has been observed in previous studies (Kanno *et al.*, 2003, Whittemore *et al.*, 1995). Induced oxidative cellular injuries, such as DNA damage, has been shown as a general trigger for cellular apoptosis mediated by  $H_2O_2$  (Whittemore *et al.*, 1994, Panieri *et al.*, 2013). The first annexin V/PI experiment was conducted using A375M and A375P melanoma cancer cells to examine apoptosis level 72 hours after treatment with  $H_2O_2$  for 30 minutes on ice, in the presence and absence of ascorbate. Indeed, there was an obvious enhancement of  $H_2O_2$ -induced apoptosis, resulting from the pre-treatment of the cells with ascorbate (Figure 6.5A & B).

Further analysis of this data indicates that the average apoptosis induced by the two doses of  $H_2O_2$  (15µM & 30µM), in the presence of ascorbate was enhanced by more than 2-fold in A375M, and A375P melanoma cancer cells (Figure 6.5C & D). The low level of induced apoptosis in both above melanoma cell lines was not expected; perhaps the oxidative damage happened to some cells could be repaired after treatment at  $37^{\circ}C/5\%$  CO<sub>2</sub>. It was also noted that a considerable level of background apoptosis (endogenous apoptosis) was present in A375M melanoma cancer cells. This may be due to overgrowth of melanoma cancer cells (control groups), as cells may die when they become confluent.

 $H_2O_2$ -induced apoptosis and the enhancing effect of ascorbate, was further confirmed by measuring caspase-3/7 expression following treatment. The expression of caspase-3/7 has noticeably increased in the ascorbate pre-treated melanoma cancer cells (Figure 6.6). The level of caspase-3/7 demonstrated in melanoma cancer cells exposed to 15µM and 30µM H<sub>2</sub>O<sub>2</sub> in the presence of ascorbate was increased by 1.2-fold and 1.4-fold, respectively (Figure 6.6). This confirms apoptotic cell death induced by H<sub>2</sub>O<sub>2</sub> was enhanced by ascorbate.

The effect of  $H_2O_2$  on melanoma cancer cells at physiological temperature (37°C) may represent a more realistic situation in which oxidative agents would exert their function. Li *et al.*, (2000) found more than 60% apoptosis in human hepatoma cells exposed to 200µM  $H_2O_2$  for 48 hours (Li *et al.*, 2000). Consequently, more annexin V/PI experiments were performed in which cells were treated with  $H_2O_2$  for longer incubation periods (24 hours) at 37°C/5% CO<sub>2</sub>. It has been suggested that up to 400µM  $H_2O_2$  causes apoptosis, but that a concentration between 400-800µM leads to cell necrosis (Demelash *et al.*, 2004). When A375P melanoma cells are exposed to  $H_2O_2$  (0µM, 30µM, 60µM, 200µM and 400µM) the average total apoptosis was increased with escalating H<sub>2</sub>O<sub>2</sub> concentrations. The averages apoptosis observed in melanoma cells exposed to  $200\mu$ M and  $400\mu$ M H<sub>2</sub>O<sub>2</sub> was significantly higher than the background apoptosis (untreated cells) (p=0.0149 and p=0.0037, respectively) (Figure 6.7). This revealed an actual effect from H<sub>2</sub>O<sub>2</sub> on melanoma cancer cells at physiological temperature. Using the above H<sub>2</sub>O<sub>2</sub> doses (0µM, 200µM and 400µM), but in the presence and absence of ascorbate, annexin V/PI was repeated to further investigate the effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced apoptosis in non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer and HaCaT cells under physiological temperature, for 24 hours. Interestingly, a considerable enhancement in the level of H<sub>2</sub>O<sub>2</sub>-induced apoptosis was observed in the ascorbate-treated melanoma cancer cells. Statistical analysis reveals a significant enhancement effect of ascorbate on the level of H<sub>2</sub>O<sub>2</sub>induced apoptosis (late apoptosis) mediated by 200µM and 400µM H<sub>2</sub>O<sub>2</sub> in A375P melanoma cancer cells (p=0.0031 and p=0.0195, respectively). Similar effect of ascorbate was observed in SK23 melanoma cancer cells when exposed to the same above doses of  $H_2O_2$  (p=0.00073 and p=0.2665, respectively). However, in HaCaT cells, only a small effect of ascorbate on the the level of apoptosis mediated by 200µM and 400 $\mu$ M H<sub>2</sub>O<sub>2</sub> was observed (*p*=0.6411 and *p*=0.6756, respectively). An enhancing effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-mediated apoptosis was also found for ascorbate pre-loaded human T-cell leukaemia (Jurket) cells exposed to H<sub>2</sub>O<sub>2</sub> (Sane et al., 2004). In the current study, it was noted that melanoma cancer cells are more sensitive to H2O2induced apoptosis in the presence of ascorbate than the HaCaT cells. Further data analysis demonstrate that the enhancing effect of ascorbate on  $H_2O_2$ -induced apoptosis in non-pigmented (A375P) and heavily pigmented (SK23) melanoma cancer is more than 1.4-fold, and 1.9-fold, respectively; that is, higher than that in the HaCaT cells (which were almost unaffected) (Figure 6.9).

Based on the ACA and  $\gamma$ -H2AX data of this study (see Chapter 5, Figure 5.6 & 5.12), the pigmented melanoma cancer cells were the most sensitive to H<sub>2</sub>O<sub>2</sub> and its modulation by ascorbate. Therefore, it was, indeed, expected to observe more H<sub>2</sub>O<sub>2</sub>-induced apoptosis in SK23 melanoma cells in the presence and absence of ascorbate. Indeed, annexin V/PI data clearly demonstrates the sensitivity of SK23 melanoma cancer cells to H<sub>2</sub>O<sub>2</sub>-induced apoptosis in the presence of ascorbate.

Further assessment of  $H_2O_2$ -mediated cell killing in the presence and absence of ascorbate was undertaken using the clonogenic assay. The experiment was performed based on the plate efficiency of melanoma cancer cells and HaCaT cells. Data obtained in these assays indicated that ascorbate enhances  $H_2O_2$ -induced cell killing in melanoma cancer cells, but the effect was slightly protective in HaCaT cells (Figure 6.12A-E). The enhancement of the level of  $H_2O_2$ -induced melanoma cancer cell killing was clearly observed in all melanoma cancer cells. The enhancing effect of ascorbate on  $H_2O_2$ -mediated cell killing induced by  $30\mu M H_2O_2$  was assessed in A375P and SK23 melanoma cancer cells and in HaCaTs. Indeed, it has been found that the level of enhancement increased by 1.9-fold and 1.6-fold in SK23 and A375P melanoma cancer cells, respectively; that is more than 2-fold higher than that in the HaCaT cells (Figure 6.13).

Further analysis of the data demonstrates that AUC for  $H_2O_2$ -induced cell killing in the presence of ascorbate is lower than the AUC for  $H_2O_2$ -induced cell killing in the absence of ascorbate; this effect completely opposed that noted in the HaCaT cells (Table 6.2).

Taken together, the data analysis indicates a positive relationship between DNA damage and cell killing. It suggests that those cells that are more sensitive to  $H_2O_2$ -induced DNA damage and its enhancement by ascorbate more easily die under the oxidative conditions promoted by ascorbate. A positive linearity was observed between ascorbate enhancement of  $H_2O_2$ -induced DNA damage and ascorbate enhancement of  $H_2O_2$ induced cell killing (Figure 6.14). CHAPTER VII: The assessment of ascorbate's effect on induced DNA damage by oxidative drugs and cell death in melanoma cancer cells

#### 7.1 Introduction

Many known drugs are able to mediate oxidative stress in tumour tissue to kill cancer cells (Szymczyk *et al.*, 2006, Magda and Miller, 2006, Deavall *et al.*, 2012, Kirshner *et al.*, 2008). The ROS-mediated approach to killing cancer cells is based on the hypothesis that cancer cells, unlike normal cells, have a greater level of endogenous ROS; thus, providing tumour cells with additional therapeutic ROS, could generate a lethal level of intracellular oxidative species so selectively killing cells (López-Lázaro, 2007, Trachootham *et al.*, 2009).

Studies have used ascorbate in combination with other therapeutic modalities, to examine its efficacy as a synergistic model in cancer treatment (Kurbacher et al., 1996, Verrax et al., 2004, Taper et al., 1995, Waddell and Gerner, 1980, Verrax et al., 2011b). Most studies carried out to date, have used pharmacological concentrations of ascorbate in combination with other agents. The principle of using high doses of ascorbate, is to generate  $H_2O_2$  extracellularly (via ascorbate autooxidation) which in turn can kill tumour cells (Espey et al., 2011). A recent in vitro study has shown data indicating high pharmacological concentration of ascorbate kills melanoma cancer cells through generation of  $H_2O_2$  and, indeed, the effect was successfully inhibited by treating cells with catalase (Serrano et al., 2015). Using animal models, a reduction in tumour growth and size was also noted with pharmacological doses of vitamin C, combined with cupric sulphate. This effect was due to redox-modulation reactions which generates  $H_2O_2$ (Reddy et al., 2001). Other researchers have discovered a synergistic cytotoxic prooxidant effect of ascorbate, in combination with manganese porphyrin in cancer cells. It is suggested that manganese porphyrin increases ascorbate oxidation, therby generating H<sub>2</sub>O<sub>2</sub> fluxes and cytotoxicity (Ye et al., 2009, Rawal et al., 2013).

All of the above studies have tried to deliver  $H_2O_2$  by using high doses of ascorbate alone or plus other redox-active elements. Ascorbate, at micromolar concentrations also becomes an excellent pro-oxidant (Buettner and Jurkiewicz, 1996). Interestingly, a previous study has discovered evidence indicating ascorbate modulates the oxidative stress induced by indomethacin (A drug that causes oxidative stress), resulting in tumour shrinkage (Waddell and Gerner, 1980). Therefore, it was of interest to investigate the effect of ascorbate on oxidative drugs in melanoma cancer cells. Data in Chapters 3 and 4 of the present study indicate that melanoma cancer cells are more susceptible to  $H_2O_2$ -induced DNA damage than "normal" skin cells, and that melanoma cancer cells exhibit more endogenous ROS background than "normal" skin cells. Data in Chapter 5 concluded that, when melanoma cancer cells were preincubated with vitamin C, a noticeable synergistic effect could be observed in the levels of  $H_2O_2$ -induced DNA damage, particularly in pigmented melanoma cancer cells. Moreover, the results in Chapter 6 revealed enhancement in the level of  $H_2O_2$ -induced cell apoptosis, cell killing and proliferative inhibition occurred in cells pre-treated with ascorbate, and that melanoma cancer cells were more sensitive to these effects than HaCaT cells. Therefore, it was proposed to use oxidative-generating drugs in place of the model oxidant  $H_2O_2$ , to induce DNA damage and cell killing in melanoma cancer cells, both in the presence and absence of ascorbate.

Two chemical agents were used to generate oxidative stress in melanoma cancer cells. BSO, as mentioned in the Introduction (see Section 1.4, Chapter 1), was used to modulate cellular redox status, leading to the accumulation of ROS in melanoma cancer cells. When BSO enters into the cell it inhibits GSH (Schnelldorfer *et al.*, 2000), thus, enabling cells to accumulate more endogenous ROS (Kramer *et al.*, 2004). Elesclomol is a novel therapy that generates intracellular  $H_2O_2$  via its interaction within mitochnodria (Kirshner *et al.*, 2008). Elesclomol binds with copper ions (Cu<sup>+2</sup>) outside the cell and this enables it to enter the cell and eventually the mitochondria. Once inside the mitochondria, the complex (Elesclomol-Cu<sup>+2</sup>) interacts with the electron transport chain (ETC) (see Section 1.4, Chapter 1). This interaction reduces Cu<sup>2+</sup> to Cu<sup>1+</sup>, triggering sequential redox reactions which ultimately generate oxidative stress (Blackman *et al.*, 2012). The drug has, recently, been used to kill melanoma cancer cells oxidatively (Cierlitza *et al.*, 2015).

This chapter aims to:

- Investigate the level of intracellular ROS in melanoma cancer cells treated with oxidative mediated agents (BSO and Elesclomol).
- Investigate the effect of above agents on the induction of DNA damage.
- Investigate the effect of ascorbate in combination with above agents on melanoma cells in terms of DNA damage and cell death.

### 7.2 Results

### 7.2.1 Assessment of endogenous ROS in A375P melanoma cancer cells treated with BSO

BSO is capable of initiating endogenous ROS accumulation in cells, via suppression of the important step of GSH synthesis pathway (Tian *et al.*, 1997, Maeda *et al.*, 2004). BSO acts by irreversibly inhibiting  $\gamma$ -glutamylcysteine synthetase in cells, thereby inhibiting glutathione levels (Griffith, 1982), leading to an accumulation of oxidative species in the cells (Gokce *et al.*, 2009). BSO has also been used in combination with oxidative mediated agents. Reportedly, incubation of cells with glutamate plus BSO for a period of 8 hours, induces generation of endogenous ROS at a level 50 times higher than that in untreated cells (Maher and Hanneken, 2005).

Based on this fact, BSO was used to enhance an oxidative environment in melanoma cancer cells. The A375P melanoma cancer cells were initially used to test this effect. The H2DCFDA-flourogenic probe was used to measure the ROS level in cells following treatment with BSO. Equal numbers of melanoma cells were seeded in each microwell of the 96-microwell plate, for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. Cells were then washed with PBS and then, for 20 hours, they were exposed to different concentrations of BSO prepared in the culture media. After treatment, cells were washed once with PBS and then incubated with H2DCFDA for 30 minutes at 37°C/protected from light. After incubation, 100µL of PBS was added to each microwell and mixed thoroughly. DCF fluorescence intensity was then measured using a plate reader (BMG FLUOstar OPTIMA Microplate Reader) at an excitation of 480nm and emission of 530nm. The levels of ROS detected in treated cells with 1000, 2000 and 3000µM were significantly higher than that measured in untreated cells (DCF fluorescence intensity:  $164.4 \pm 16.2$  versus  $68.85 \pm 4.72$ , p = 0.0026,  $230.6 \pm 19.4$  versus  $68.85 \pm 4.72$ , p<0.0001, and 274.9  $\pm 29.5$  versus  $68.85 \pm 4.72$ , p<0.0001, respectively). These data suggest that BSO enhances intracellular ROS in melanoma cancer cells, and that the level of ROS generation in these cells is dose-dependent. This experiment also found 20 hours incubation was sufficient for ROS generation in cells by BSO (Figure 7.1).


Figure 7.1. Effect of BSO on endogenous ROS generation in A375P melanoma cancer cells.

50 x  $10^3$  cells were seeded in each microwell of the 96-microwell plate, and incubated for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Cells were then pre-incubated with different concentrations of BSO, and incubated for 20 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. Treated and untreated cells were washed once with PBS. Cells were then incubated with H2DCFDA for 30 minutes at  $37^{\circ}C$ /protected from light. After incubation,  $100\mu$ L of PBS was added in to each microwell and mixed thoroughly. DCF fluorescence intensity was then measured using a plate reader. Each bar represents mean ± SEM of DCF fluorescence intensity, determined from three independent experiments run in triplicate. Results were blank/background subtracted. One-way ANOVA was used to test the difference between DCF fluorescence intensity in treated cells and untreated cells.

# 7.2.1.1 Investigation of endogenous DNA damage caused by BSO in melanoma cancer cells

It has been proposed that accumulated endogenous ROS within cells, resulting from the pro-oxidative effect of BSO, could attack DNA molecules, causing genomic damage in melanoma cancer cells. To study the effect of BSO on endogenous DNA damage, cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment; then they were incubated with and without BSO ( $3000\mu$ M) for different time periods (0-20 hours), and the ACA then conducted.

Data from these experiments (Figure 7.2) showed that DNA damage was caused by BSO, and confirmed that the effect was time-dependent. Incubation of cells with BSO for 15 hours or more caused a significant level of DNA damage in A375P melanoma cancer cells *versus* that BSO untreated cells (% tail DNA: 8.41  $\pm$  0.5045 and 9.69  $\pm$  0.6132 *versus* 4.59  $\pm$  0.2808, *p*<0.0001, respectively). In this experiment, a 20-hour time period was found to be appropriate for mediating of BSO-induced DNA damage in melanoma cancer cells.



Figure 7.2. Endogenous DNA damage in A375P melanoma cancer cells mediated by BSO.

Cells were seeded for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. Cells were then washed once with PBS, treated with 3000 $\mu$ M BSO, and incubated at 37°C/5% CO<sub>2</sub> for a range of different time periods (0-20hrs). After the cells were harvested, DNA damage was measured, using standard ACA. Each bar represents mean  $\pm$  SEM of 200 comets determined from two independent experiments. One-way ANOVA was used to test the difference between the damage caused by BSO at different times *versus* that in untreated cells.

# 7.2.1.2 Endogenous DNA damage in BSO-treated melanoma cancer cells in the presence and absence of ascorbate

To investigate the effect of ascorbate on the level of endogenous DNA damage in BSO treated cells, three melanoma cancer cell lines were selected, including non-pigmented (A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells. The cells were seeded in 6-well plates for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. Adherent cells were then washed with PBS and incubated with and without ascorbate for 2 hours. Cells were then washed once PBS and treated with BSO for 20 hours at 37°C/5% CO<sub>2</sub>. The standard ACA was then performed to measure DNA damage. Interestingly, ascorbate significantly enhanced the endogenous level of DNA damage caused by BSO in all melanoma cancer cells (SK28 & SK23) (Figure 7.3).



Figure 7.3. Endogenous DNA damage in non-pigmented (A375P) (A), moderately pigmented (SK28) (B) and heavily pigmented (SK23) melanoma cancer cells treated with BSO in the presence and absence of ascorbate.

Cells were seeded for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Cells were then incubated with ascorbate for 2 hours. Cells were then washed once with PBS, and treated with different doses of BSO (0µM, 100µM, 1000µM and 3000µM) for 20 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. After the cells were harvested, DNA damage was measured, using standard ACA. Each bar represents mean ± SEM of 300 comets, determined from three independent experiments. *T*-test was used to test the difference between the mean of each group *versus* ascorbate untreated cells.

#### 7.2.2 Induction of oxidative stress in melanoma cancer cells by Elesclomol

Elesciomol is a novel therapy for mediating oxidative stress in tumour tissue (Blackman *et al.*, 2012) and its cytotoxicity is related to the oxidative stress it induces (Hasinoff *et al.*, 2014, Cierlitza *et al.*, 2015). Recently, this agent has been used in combination with Paclitaxel in phase II and III clinical trials, in an attempt to tackle MM cancer cells by an oxidative-mediated approach and delivered an improvement in progression free survival (O'Day *et al.*, 2009, O'Day *et al.*, 2013b).

Induction of oxidative stress by Elesclomol was evaluated by treating SK23 melanoma cancer cells both with and without Elesclomol, in the presence and absence of the antioxidant, N-acetyl cysteine (NAC). To perform this test, equal numbers of melanoma cells were seeded in each microwell of the 96-microwell plate, for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Cells were then washed with PBS. One group of cells was incubated with a medium containing DMSO, whilst the other two groups of cells were treated with Elesclomol (500nm) for 5 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. Before examining the ROS, a group of Elesclomol-treated cells was exposed to NAC (500µM) for 30 minutes at  $37^{\circ}C/5\%$  CO<sub>2</sub>. After this, media was discarded from the microwells and cells were washed with PBS. This was followed by addition of H2DCFDA fluorescent probe onto the cells and incubated for 30 minutes at  $37^{\circ}C$ , protected from light. Fluorescence images were then captured by fluorescence microscope. Indeed, images showed that Elesclomol induces ROS generation in melanoma cancer cells and the effect was inhibited when cells were pre-treated with NAC (Figure 7.4).

After confirming that Elesclomol induces endogenous ROS, the level of ROS was measured in Elesclomol-treated cells. A375P melanoma cancer cells were used, as these cells firmly adhere to the plate bases. Cells were seeded in each microwell of the 96-microwell plate, for 24 hours at 37°C/5% CO<sub>2</sub> to allow attachment. Cells were then washed with PBS. Cells were incubated with either a medium containing DMSO or Elesclomol (500nm) for different time points, at 37°C/5% CO<sub>2</sub>. Following treatment, A375P cells were washed once with PBS and then incubated with H2DCFDA for 30 minutes at 37°C/protected from light. After incubation, 100µL of PBS was added in to each microwell and mixed thoroughly. ROS was then measured by using plate reader (BMG FLUOstar OPTIMA Microplate Reader) at an excitation of 480nm and emission of 530nm).

Data obtained from this experiment indicated the ability of Elesclomol to generate ROS in melanoma cancer cells, and confirmed the effect was time-dependent. The level of endogenous ROS generated by Elesclomol after 1 hours of incubation was significant (p<0.05) and became highly significant after 3 hours incubation (p<0.001), when compared to the endogenous ROS in cells treated with DMSO alone (Figure 7.5).



Figure 7.4. DCF-Fluorescence images showing generation of ROS by Elesclomol in SK23 melanoma cells.

Cells were seeded in 96-well plate for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Cells were then washed with PBS; one group of them was incubated with a medium containing DMSO, whilst the other two groups of cells were treated with Elesclomol (500nm) for 5 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. Before adding the H2DCFDA, a group of Elesclomol-treated cells was exposed to NAC (500µM) for 30 minutes at  $37^{\circ}C/5\%$  CO<sub>2</sub>. Media was then removed and cells were washed with PBS and then stained with the H2DCFDA fluorescent probe for 30 minutes at  $37^{\circ}C$ , protected from light. DCF fluorescence images, which show the amount of endogenous ROS in treated cells, were captured from microplates using a fluorescence Zeiss Axioskop 2 plus microscope. (20x magnification).



Figure 7.5. Endogenous ROS generation in A375P melanoma cancer cells treated with Elesclomol.

A) DCF fluorescence images show the amount of ROS generated in treated cells following incubation cells with either DMSO or 500nM Elescionol, in different times. Images were captured from microplates using a fluorescence Zeiss Axioskop 2 plus microscope (20x magnifications). B) 50 x  $10^3$  were seeded in a 96-microwell plate for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Cells were then washed and preincubated with 500nM of Elescionol for indicated time periods at  $37^{\circ}C/5\%$  CO<sub>2</sub>. Treated and untreated cells were washed once with PBS. Cells were then incubated with H2DCFDA for 30 minutes at  $37^{\circ}C$ , protected from light. After incubation,  $100\mu$ L of PBS was added in to each microwell and mixed thoroughly. DCF fluoresence intensity was then measured using a plate reader. Each bar represents mean  $\pm$  SEM, determined from three independent experiments run in triplicate. Results were the blank / background subtracted and calculated as % over DMSO control. One-way ANOVA was used to test the difference between the mean of DCF fluorescence intensity between treated cells (in different times) and DMSO treated cells.

### 7.2.3 Induction of DNA damage in melanoma cancer cells and HaCaT cells by Elesclomol

All melanoma cancer cell lines, including non-pigmented (A375M & A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells and HaCaT cells, were investigated whether Elesclomol induces DNA damage in them. To study this, cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment; before treatment. Adherent cells were then washed with PBS and incubated with Elesclomol for 7 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. Afterwards, a standard ACA was then performed to measure the level of induced DNA damage.

The duration of treatment with Elesclomol, was based on the ability of drug to generate ROS. The previous experiment (Figure 7.5) showed an increase in ROS with time of exposure and this was further confirmed from evidence in the literature (Kirshner *et al.*, 2008). Thus it was expected to observe damage in the DNA if cells exposed to Elesclomol for 5 hours or more.

The data obtained indicated that 50 and 100nM of Elesclomol induced slight DNA damage in three of cell lines including A375M and SK28 melanoma cancer cells and HaCaT cells (Figure A, C & E). However, the heavily pigmented melanoma cancer cells (SK23) were the most sensitive to Elesclomol-induced DNA damage; although the damage induced did not exceed 25% tail DNA (Figure 7.6D).

Statistical analysis of data shows that the damages induced by 50nM and 100nM Elesciomol in A375M are not significant (P=0.5198 and P=0.5531, repspectively) whereas in A375P the damages are significant (P=0.0001 and P<0.0001, respectively). Similarly, the same above doses of Elesciomol induced non-significant damages in SK28 melanoma cancer cells (P=0.8785 and P=0.1389, respectively) while in SK23 cells the effect caused by the two concentrations of Elesciomol was highly significant (P<0.0001). In HaCaT cells the drug doses (50nM and 100nM) also caused damage in DNA but not significant (P=0.0574 and P=0.249, respectively) (Figure 7.6A-E).



# Figure 7.6. Elesclomol-induced DNA damage dose responses in non-pigmented including A375M (A) and A375P (B), moderately pigmented (SK28) (C) and heavily pigmented (SK23) (D) melanoma cancer cells and HaCaT cells (E).

Cells were seeded and incubated for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Cells were then preincubated with indicated concentrations of Elesclomol for 7 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. After the cells were harvested, DNA damage was measured, by the standard ACA. Each bar represents the mean % of Tail DNA of 200 comets ± SEM, determined from two independent experiments. One-way ANOVA was used to test the difference between the means of induced DNA damage *versus* the background DNA damage.

# 7.2.4 Elesclomol-induced DNA damage in melanoma cancer cells in the presence and absence of ascorbate

To study the effect of ascorbate on the level of DNA damage induced by Elesclomol, melanoma cancer cells, including non-pigmented (A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells were selected. The reason for this, was to observe the level of induced DNA damage by Elesclomol in the presence of ascorbate and to study this effect among pigmented and non-pigmented cells.

To carry out the experiment, cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Adherent cells were then washed with PBS and incubated with and without ascorbate for 2 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. After preloading cells with ascorbate, they were washed with PBS and exposed to different concentrations of Elesclomol for 7 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. After treatment, standard ACA was carried out to measure the level of DNA damage induced by Elesclomol.

Data obtained from these experiments revealed an enhancement to the level of induced DNA damage in cells treated with ascorbate. It is also important to notice that the damage, which occurred within the three cell lines, was highest in pigmented melanoma cancer cells (SK23), than the two other cell lines (SK28 & A375P) (Figure 7.7). In A375P melanoma cancer cells, the damage induced measured as % tail DNA by 50nM and 100nM Elesclomol, in the presence and absence of ascorbate was  $7.52 \pm 0.5382$  *versus*  $4.484 \pm 0.3432$ , *p*<0.0001, and  $7.088 \pm 0.1469$  *versus*  $6.07 \pm 0.4221$ , *p*<0.1469, respectively. In SK28 melanoma cancer cells, the damage induced by the above two doses of Elesclomol, in the presence and absence of ascorbate was  $9.433 \pm 0.7627$  *versus*  $8.83 \pm 0.6152$ , *p*<0.481, and  $14.52 \pm 0.6554$  *versus*  $11.03 \pm 0.7393$ , *p*<0.0005, respectively. The damage occurred in SK23 melanoma cancer cells by 50nM and 100nM Elesclomol, in the presence and absence was  $19.84 \pm 1.007$  *versus*  $16.20 \pm 0.0091$ , *p*<0.0091, and  $27.58 \pm 1.277$  *versus*  $15.4 \pm 0.7627$ , *p*<0.0001, respectively. This clearly indicates that SK23 melanoma cells are more sensitive to the effect of Elesclomol and its enhancement by ascorbate (Figure 7.7).



# Figure 7.7. Elesclomol-induced DNA damage dose responses in non-pigmented (A375P) (A), moderately pigmented (SK28) (B) and heavily pigmented (SK23) (C) melanoma cancer cells, in the presence and absence of ascorbate.

The cells were seeded and incubated for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Cells were then preincubated with and without ascorbate for a further 2 hours, and then exposed to indicated concentrations of Elesclomol for 7 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. After the cells were harvested, DNA damage was measured, using standard ACA. Each bar represents the mean % of Tail DNA of 300 comets ± SEM, determined from three independent experiments. *T*-test was used to test the difference between the means of induced DNA damage in ascorbate treated cells *versus* ascorbate untreated cells.

# 7.2.4.1 Elesclomol-induced ODBLs in melanoma cancer cells in the presence and absence of ascorbate

To investigate the effect of ascorbate on Elesclomol-induced DNA damage, and to determine whether it was truly oxidative, a modified version of ACA was conducted, using the heavily pigmented (SK23) melanoma cancer cells, as they are the melanoma cancer cells that were most sensitive to Elesclomol.

To examine this, melanoma cells were seeded in 6-well plates for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub> to allow attachment. Adherent cells were then washed with PBS and incubated with and without ascorbate for 2 hours. Afterwards, they were washed with PBS and exposed to Elesclomol (100nM) for 7 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>; subsequently the modified-ACA was conducted, using Fpg enzyme.

As expected, data obtained from this work revealed that ascorbate had an enhancing effect on ODBLs in cells treated with Elesclomol, accounting for an additional 10.6% tail DNA damage (Figure 7.8).



### Figure 7.8. Elesclomol-induced oxidatively damaged DNA in SK23 melanoma cancer cells in the presence and absence of ascorbate.

Cells were seeded and incubated for 24 hours at 37°C/5% CO<sub>2</sub> to allow attchment. Cells were then preincubated with and without ascorbate for a further 2 hours and were then exposed to 100nM Elesclomol for 7 hours at 37°C/5% CO<sub>2</sub>. They were then pre-incubated for a further 30 minutes, at 37°C in black and moist boxes, with ERB or ERB+Fpg enzyme (0.8U/gel). After incubation, ACA was conducted and DNA damage measured. Each bar represents the mean % of Tail DNA of 200 comets  $\pm$  SEM determined from two independent experiments.

# 7.2.5 Elesclomol-induced apoptosis in melanoma cancer cells in the presence and absence of ascorbate

Data from recent studies suggest that Elesclomol causes apoptosis in tumour cells via an oxidative stress mechanism (Kirshner *et al.*, 2008, Nagai *et al.*, 2012). In the current study, the enhancing effect of ascorbate on Elesclomol-induced DNA damage was observed. To determine whether this might have an impact on the survival of melanoma cancer cells, the extent of apoptosis was assessed using the heavily pigmented (SK23) melanoma cancer cells, as these cells are more sensitive to Elesclomol-induced DNA damage.

In this instance, the cells were seeded for 24 hours in T25 flasks and then pre-treated with and without ascorbate for 2 hours. The cells were then washed once with PBS and incubated with indicated concentrations of Elesclomol (prepared in complete cell culture medium with 10% FCS) for 15 hours at 37°C/5% CO<sub>2</sub>. The treated and untreated cells were then then collected in FACS tubes and the annexin V/PI assay for apoptosis was conducted. 15 hours incubation with Elesclomol was expected to causes apoptosis. An in *vitro* study found that 18 hours causes up to 11-fold increase in human melanoma (Hs294T) cell apoptosis exposed to 200nM Elesclomol (Kirshner *et al.*, 2008).

As expected, an obvious level of Eleschomol-induced cell apoptosis (although small) was observed in treated melanoma cancer cells, with a clear enhancing effect due to ascorbate also being observed (Figure 7.9). Analysis of late apoptosis revealed a considerable increase in extent of apoptosis caused by 50nM and 100nM Eleschomol, in the presence of ascorbate than that occurred in the absence of ascorbate (% late apoptosis:  $6.1 \pm 1.0$  versus  $5.133 \pm 0.6506$ , p=0.2332, and  $6.83 \pm 0.6807$  versus  $5.2 \pm 0.2082$ , p=0.0213, respectively) (Figure 7.9A).

Further analysis showed that the total apoptosis in cells treated with 50nM and 100nM Elesciomol, in the presence of ascorbate increased to approximately 3-fold; to a level much higher than that induced by treating cells with Elesciomol alone (Figure 7.9B).



### Figure 7.9. Effect of Elesclomol in inducing apoptosis in SK23 melanoma cancer cells in the presence and absence of ascorbate.

A) Cells were seeded in 25T flasks for 24 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>, to allow attachment and grow. Cells were then incubated with and without ascorbate for a further 2 hours. Cells were then washed with PBS, and incubated with media containing either DMSO, or indicated concentrations of Elesclomol prepared in complete media for 15 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. They were then collected in FACS tubes and the annexin V/PI assay was carried out. Each bar represents mean  $\pm$  SD of apoptosis determined from three independent experiments. Figure B, represents total apoptosis (%) in the presence and absence of ascorbate adapted from figure 7.9A.

# 7.2.6 Assessment of caspase-3/7 expression in heavily pigmented (SK23) melanoma cancer cells following treatment with Elesclomol in the presence and absence of ascorbate

The effect of ascorbate on Elesclomol-induced cell death in SK23 melanoma cancer cells was further studied by measuring caspase-3/7 expression in Elesclomol-treated cells. It has been found that at an early time (3hrs to less than 18 hours) of Elesclomol exposure, caspase 3 is elevated (Kirshner *et al.*, 2008); thus, 3 hours of incubation with Elesclomol was chosen to investigate the level of caspase-3/7 in the presence and absence of ascorbate. To test this, SK23 melanoma celles were pre-treated with and without ascorbate for 2 hours then exposed to Elesclomol (100nM and 200nM) for 3 hours at  $37^{\circ}C/5\%$  CO<sub>2</sub>. After treatment,  $100\mu$ L of Caspase-Glo® 3/7 substrate (provided by the assay kit) was added to each microwell, and the plates were gently mixed using a plate shaker for 30 seconds. Plates were then incubated at room temperature for 30 minutes before reading the luminescence intensity (which represent the level of caspase-3/7 activity) using a plate reader (BMG FLUOstar OPTIMA Microplate Reader).

Data obtained in these experiments supported the enhancment effect of ascorbate on Elesclomol-induced apoptosis, since a slightly higher (more than 1-fold) level of caspase 3/7 activity was observed in cells treated with Elesclomol in the presence of ascorbate (Figure 7.10B & C). When ascorbate pre-treated cells were exposed to 100nM and 200nM Elesclomol, the level of caspase-3/7 was clearly increased more than the level induced by the same concentrations of Elesclomol in the absence of ascorbate (Luminescence intensity (% control):  $123 \pm 17.34$  versus  $108.4 \pm 13.74$ , p=0.198, and  $137.6 \pm 8.7$  versus  $118 \pm 13.8$ , p=0.2736, respectively). When the treated cells were examined under the microscope to observe any possible physical changes, those treated with Elesclomol plus ascorbate looked like separated cells and slightly round and whitish, whereas those treated with Elesclomol alone were still normally agregated (Figure 7.10A).



### Figure 7.10. Effect of Elesclomol on caspase 3/7 expression in SK23 melanoma cancer cells in the presence and absence of ascorbate.

A) Images show microscopical examination of the physical appearance of SK23 melanoma cancer cells post treatment with Elesclomol with and without ascorbate. B & C) Equal numbers of cells  $(15 \times 10^3)$  were seeded in a 96-microwell plate for 24 hours, and then preincubated with and without AA for a further 2 hours. Media was removed, and cells were washed once with PBS. Cells were then treated for 3 hours with 100nM Elesclomol and 200nM Elesclomol. Following treatment, 100µL of Caspase-Glo® 3/7 substrate was added to each microwell and the plates were gently mixed using a plate shaker for 30 seconds. Plates were then incubated at room temperature for 30 minutes before reading the luminescence intensity (which represent the level of caspase-3/7 activity) using a plate reader (BMG FLUOstar OPTIMA Microplate Reader).

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#### 7.3 Discussion

Oxidative stress mediated by agents is one of the proposed therapeutic strategies for killing cancer cells selectively (Trachootham *et al.*, 2009, Pelicano *et al.*, 2004, Trachootham *et al.*, 2006, Nagai *et al.*, 2012). This therapeutic approach is believed to be successful in killing malignant cells which are in a chronic condition of oxidative stress. In therapeutic terms, mediated oxidative stress could kill these cells by raising the level of endogenous ROS beyond a perceived lethal level (Trachootham *et al.*, 2009).

A number of studies have used chemical agents to target tumour cells oxidatively (Szymczyk *et al.*, 2006, Magda and Miller, 2006, Deavall *et al.*, 2012, Kirshner *et al.*, 2008, Nagai *et al.*, 2012). For instance, previous *in vitro* studies have shown that GSHdepletion is associated with BSO toxicity in melanoma cancer cells (Kable *et al.*, 1989, Thrall and Meadows, 1991). This effect could be caused by high levels of endogenous ROS in melanoma cancer cells treated with BSO (Voshavar *et al.*, 2015). Melanoma cancer cells are sensitive to BSO toxicity; possibly as a result the of inability of BSO treated-cells to scavenge endogenously-generated free radicals (Chance *et al.*, 1979, Kable *et al.*, 2012, Nagai *et al.*, 2012), which has been used in phase III clinical trials to tackle advanced MM tumours, via the induction of oxidative stress (Kirshner *et al.*, 2008); with an improvement in progression free survival been reported in advanced melanoma recently (O'Day *et al.*, 2009, O'Day *et al.*, 2013b).

In the present Chapter, BSO and Elesclomol were used instead of  $H_2O_{2,}$  in an attempt to mediate intracellular oxidative stress in melanoma cancer cells. It was proposed to study oxidative-induced DNA damage, and cell death mediated by these ROS-generating agents in melanoma cancer cells, both in the presence and absence of ascorbate.

BSO had been used in *vitro* (Tagde *et al.*, 2014), and in *vivo* (phase I clinical trials), with only slight side effects (O'Dwyer *et al.*, 1992, Bailey *et al.*, 1994). BSO selectivly inhibits GSH, and it has therefore been exploited to reduce tumour cell resistance against alkylating agents (Anderson *et al.*, 1999). It was suggested that oxidative stress could be induced when glutathione level is depleted by BSO (Gokce *et al.*, 2009).

In this study, a significant level (p<0.0001) of endogenous ROS was observed in melanoma cancer cells treated with BSO, compared to that observed in untreated cells;

the effect was dose-dependent. BSO caused melanoma cancer cells to accumulate more endogenous ROS (Figure 7.1), and this result agreed with the data from a previous study, in which tumour cells were exposed to BSO (Armstrong *et al.*, 2002).

More DNA damage was expected in melanoma cells exposed to BSO. Assessment of DNA damage was conducted by the standard ACA, after A375P melanoma cancer cells were incubated with BSO for different time periods. The data obtained clearly demonstrated an induction of DNA damage dose response in BSO treated-melanoma cancer cells, with the highest level of damage being observed after 15 hours of incubation (P<0.0001) (Figure 7.2). The level of DNA damage did not exceed 10%, possibly due to DNA damage repair during incubation time at 37°C/5% CO<sub>2</sub>.

The above effect was further studied, using three melanoma cancer cell lines, including non-pigmented (A375P), moderately pigmented (SK28) and heavily pigmented (SK23). Cells were pre-treated with ascorbate for 2 hours, and then incubated with different levels of BSO. ACA was then performed and obtained data from these experiments demonstrated clear BSO-induced DNA damage dose response in all cell lines, with the highest level of damage being observed in pigmented cells (SK28 & SK23). Interestingly, the level of DNA damage in BSO-treated cells was increased significantly by the presence of ascorbate (Figure 7.3). This suggests that pre-loading cells with BSO causes the accumulation of a greater amount of endogenous ROS within melanoma cells and in the presence of ascorbate the Fenton-mediated proccess is accelerated, generating more <sup>•</sup>OH, which in turn causes DNA damage. However, in the absence of ascorbate only smaller level of damage occurs.

As mentioned in above, Elesclomol has been used in recent clinical trials to treat advanced MM cases (Gonzalez *et al.*, 2008, Hauschild *et al.*, 2009, O'Day *et al.*, 2009, O'Day *et al.*, 2013). Using Elesclomol, a significant free-progression survival from metastatic melanoma has been recorded in a randomised, double-blinded, phase II clinical trial, which involved 81 patients (Korn *et al.*, 2008). This chemical agent is a novel oxidative stress-inducer, and has been found to induce cell apoptosis and kill cancer cells selectively by this means (Nagai *et al.*, 2012). Elesclomol kills tumour cells by generating H<sub>2</sub>O<sub>2</sub> within cells and this effect was inhibited when cells were exposed to catalase, following Elesclomol treatment (Kirshner *et al.*, 2008).

In the current sudy, Elesclomol was also used to mediate oxidative stress in melanoma cancer cells. Induction of oxidative stress in melanoma cancer cells was confirmed by

examining the endogenous ROS in melanoma cancers incubated with Elesclomol, with and without antioxidant (NAC). The effect was abolished by treating Elesclomol-treated SK23 melanoma cells with NAC (Figure 7.4). When ROS level assessed, indeed, a significant level of endogenous ROS was observed in melanoma cancer cells, after cells were incubated with Elesclomol for different time points with a greatest level been observed in cells treated for 5 hours (P=0.00012) (Figure 7.5). These data are compatible with the key results reported recently by Kirshner *et al.* (2008).

After confirmation of enhanced oxidative stress in melanoma cancer cells treated with Elesclomol, the standard ACA was carried out in order to examine whether Eleclomol induced DNA damage in in these cells. Non-pigmented (A375M & A375P), moderately pigmented (SK28) and heavily pigmented (SK23) melanoma cancer cells plus HaCaT cells were pre-incubated with different doses of Elesclomol for 7 hours at 37°C/5%CO<sub>2</sub>. Elesclomol was found to have an oxidative DNA damage effect on melanoma cancer cells. Indeed, among all cell lines, SK23 melanoma cells were found to be more sensitive to Elesclomol genotoxicity. Although DNA damage in A375P cells was small, comparing to the background damage it was significant (Figure 7.6B). However, Elesclomol was close to the level of their background DNA damage (Figure 7.6A, C & D). This is probably due to low level of metal ion in these cells.

Interestingly, when melanoma cancer cells were exposed to ascorbate prior Elesclomol, ascorbate was shown to have a promotive effect on Elesclomol-induced DNA damage in these cells, since the level of Elesclomol-induced DNA damage was significantly enhanced by ascorbate. The effect was more prominent in pigmented (SK23) melanoma cancer cells (Figure 7.7A-C). In line to previous Chapters of this study, this can be an important point illustrating the higher sensitivity of SK23 melanoma cells to oxidative stress.

To confirm the pro-oxidant effect of ascorbate on Elesclomol-induced DNA damage, a modified-ACA was performed, using Fpg enzyme. In the presence of ascorbate, a noticeable level of Fpg-SS was found in Elesclomol-treated cells. This suggests that ascorbate is able to modulate and further promote the oxidative mediated DNA damage induced by Elesclomol (Figure 7.8).

A previous study has clearly identified oxidative stress as the mechanism of Elesclomol for cell apoptosis (Kirshner *et al.*, 2008). The effect of Elesclomol on cell death was

also studied, both in the presence and absence of ascorbate. SK23 melanoma cancer cells were incubated with and without ascorbate for 2 hours, and then exposed to Elesclomol. As in Kirshner's study, the annexin V/PI assay data showed an increase in the level of cell apoptosis caused by Elesclomol. Importantly, the level of Elesclomol-induced apoptosis was further enhanced by ascorbate (approximatly 3-fold increased) (Figure 7.9A & B). This indicates that oxidative DNA damage induced by Elesclomol could be a possible cause of cell apoptosis, and that ascorbic acid promotes this effect.

To further confirm apoptosis induced by Elesclomol, and the enhancment effect of ascorbate, caspase 3/7 expression was measured in treated cells. In two independent experiments, a higher level of caspase 3/7 was observed in cells treated with Elesclomol in combination with ascorbate, than in cells exposed to Elesclomol alone (Figure 7.10A & B).

CHAPTER VIII: Ascorbate modulation of H<sub>2</sub>O<sub>2</sub> and drug-induced DNA damage in primary human melanoma tissue

### 8.1 Introduction

Cell lines provide us with valuable information about cellular behaviour, such as signal transduction, gene expression, proliferation, senescence and cell death in biological experiments; and since they are immortalised, they are convenient systems to study and they can be repeatedly used and expanded in the laboratories for decades. However, cultured cells may have different cellular behaviour compared to in *vivo* cells (primary tissue) (Halliwell, 2003). But despite of this limitation they are more relevant and easier to compare data with other researches. Unlike cell line models, primary cells are difficult to grow, and are slow to proliferate. However, inspite of the added complexity of primary cells they are often preferred (Pan *et al.*, 2009), as they are not manipulated biologically and may represent an ex-*vivo* system.

In the current study, primary melanoma tissue was investigated, in order to estimate the effect of ascorbate on oxidative-induced genomic damage. Investigation of ascorbate effects on  $H_2O_2$ -induced DNA damage in primary melanoma cancer cells was an important part of this study. These cells may reflect a better biological system of melanoma cancer cells, and their response to a redox-modulatory approach.

Standard ACA was used to measure the level of oxidative-induced DNA damage in the presence and absence of ascorbate, in disaggregated primary melanoma cells obtained from patients who had undergone surgical treatment of cutaneous melanoma.

This Chapter aims to:

- Measure the level of  $H_2O_2$ -induced DNA damage in primary human melanoma cells, in the presence and absence of ascorbate.
- Assess the effect of Elesclomol on the induction of DNA damage in primary human melanoma cells, in the presence and absence of ascorbate.

#### 8.2 Results

# 8.2.1 Number of melanoma cases and characteristics of primary melanoma tissue samples

Primary melanoma tissue samples were obtained from six patients, following informed conscent. Each melanoma tissue sample was obtained separately, and provided with a specific NHS-identification code (Table 8.1). Three samples were obtained from females, and three from males. The samples were examined by a histopathologist to identify viable tumour lesion. Upon the samples being delivered, they were disaggregated, in order to generate cell suspensions of primary melanoma cancer cells ready for treatment with test compounds (see section 2.2.3, in Chapter 2).

#### Table 8.1. Primary melanoma tissue samples

Participant ID	Gender	Age	Sample location	Lesion description
B33/15CRB33	Female	76	Regional (axilla) lymph node metastasis	Pigmented
B45/15CRB42	Female	53	Regional (axilla) lymph node metastasis	Non-pigmented
B46/15CRB43	Female	41	Regional skin metastasis	Pigmented
B57/15CRB46	Male	75	Regional (groin) lymph node metastasis	Pigmented
B63/15CRB47	Male	75	Regional (axilla) lymph node metastasis	Non-pigmented
B64/15CRB48	Male	73	Regional (axilla) Lymph node metastasis	Pigmented

# 8.2.2 Assessment of H<sub>2</sub>O<sub>2</sub>-induced DNA damage in primary melanoma cancer cells in the presence and absence of ascorbate

Disaggregated primary melanoma cells were checked for viability by trypan blue assay, and then counted. From each cell suspension the required numbers of cells (equal numbers) were transferred into labelled Eppendorf tubes, in which they were exposed to  $H_2O_2$  in the presence and absence of ascorbate. Cells were then centrifuged, and the treated and untreated pellets of cells were used for the standard ACA (see section 2.3, Chapter 2).

Data acquired from comet analysis were in agreement with the ACA findings obtained from the melanoma model cell lines (Chapter 5 & Chapter 6). Melanoma cancer cells from three patients (B33/15, B45/15 and B46/15), were used to test the effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced DNA damage. Primary melanoma cells from the three patients responded significantly to the  $H_2O_2$  treatment, although the sensitivity to  $H_2O_2$ differed between the three samples. The findings of these experiments suggest that primary melanoma cells are sensitive to H<sub>2</sub>O<sub>2</sub>-induced DNA damage, and that this effect is significantly enhanced by ascorbate (Figure 8.1). Among the three studied cases, the two pigmented primary melanoma samples (B33/15 and B46/15) were more sensitive to H<sub>2</sub>O<sub>2</sub>-induced DNA damage in the presence of ascorbate than the nonpigmented primary melanoma sample (B45/15) (Figure 8.2A-B). The damage induced by  $50\mu$ M H<sub>2</sub>O<sub>2</sub> in ascorbate pre-treated B33/15 melanoma cancer cells was significantly greater than that occurred in the absence of ascorbate (% tail DNA:  $52.43 \pm 2.605$ versus  $19.63 \pm 1.633$ , p<0.0001) (Figure 8.2A). Similarly, the damage occurred by H<sub>2</sub>O<sub>2</sub> in ascorbate pre-treated B46/15 melanoma cancer cells was increased significantly compared to that induced by the same dose of H<sub>2</sub>O<sub>2</sub> in the absence of ascorbate (% tail DNA:  $53.5 \pm 1.629$  versus  $31.08 \pm 1.505$ , p<0.0001) (Figure 8.2B). However, the damage induced in B45/15 melanoma cancer cells in the presence of ascorbate was significantly less than that in these cells in the absence of ascorbate (% tail DNA: 26.81 ± 1.219 versus 21.85 ± 1.213, p<0.0042) (Figure 8.2C).



### Figure 8.1. Fluorescent images of comets induced by $H_2O_2$ in the presence and absence of ascorbate in primary melanoma cancer cells.

Primary MM tissue sample (B33/15) was obtained from the patient, and processed for cell disaggregation. Cells were then transferred into Eppendorf tubes, in which they were treated for 2 hours with and without ascorbate, at  $37C^{\circ}/5\%$  CO<sub>2</sub>. The cells were then exposed on ice to  $50\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 minutes, protected from light. After the cells had been washed and centrifuged, the standard ACA was performed. Fluoresent images of DNA damage were captured by a fluorescent microscope (20x magnification) connected to the Komet (v5.5) software (AndorImaging, Belfast UK).



### Figure 8.2. H<sub>2</sub>O<sub>2</sub>-induced DNA damage in primary melanoma cancer cells in the presence and absence of ascorbate.

Primary MM tissue samples were obtained from patients B33/15 (A), B46/15 (B) and B45/15 (C), and processed for cell disaggregation. Cells were then transferred into Eppendorf tubes, in which they were treated for 2 hours with and without ascorbate. Cells were then exposed on ice to indicated concentrations of  $H_2O_2$  for 30 minutes, and protected from light. After the cells had been washed and centrifuged, DNA damage was measured, by the standard ACA. Each bar represents the mean % of Tail DNA of 300 comets  $\pm$  SEM, determined from one single experiment run in triplicate. The *T*-test (unpaired) was used for statistical analysis between each group *versus* ascorbate untreated cells.

# **8.2.3** Induction of DNA damage by Elesclomol in primary melanoma cancer cells in the presence and absence of ascorbate

Primary human melanoma tissue was also exposed to Elesclomol, in order to examine induced DNA damage in the presence and absence of ascorbate. Three primary melanoma tissue samples, identified as B57/15, B63/15 and B64/15, were obtained from patients who had undergone surgical removal of the melanoma lesions. B57/15 and B64/15 melanoma samples were pigmented, and B63/15 was non-pigmented melanoma tissue.

B57/15 melanoma cancer cells were exposed to a high concentration of Elesclomol (1mM), both with and without ascorbate. Despite the high endogenous background of DNA damage, the comet assay was able to detect more induced DNA damage in cells treated with Elesclomol, with a significant enhancing effect being observed in cells preloaded with ascorbate (% tail DNA:  $75.35 \pm 1.204$  versus  $63.29 \pm 1.492$ , *p*<0.0001) (Figure 8.3).





Disaggregated primary melanoma cells obtained from patients, and identified as B57/15, were incubated with and without ascorbate for 2 hours at  $37C^{\circ}/5\%$  CO<sub>2</sub>. After washing cells with PBS, they were then exposed at  $37C^{\circ}/5\%$  CO<sub>2</sub> to indicated concentrations of Elesclomol for 15 hours. After the cells had been washed and centrifuged, DNA damage was measured, by the standard ACA. Each bar represents the mean % of Tail DNA of 300 comets ± SEM, determined from a single experiment. The *T*-test (unpaired) was used for statistical analysis between each group *versus* ascorbate untreated cells.

The other two primary melanoma tissue samples, obtained from patients B64/15 and B63/15, were exposed to a lower micromolar concentration of Elesclomol (50µM), in the presence and absence of ascorbate. There was an obvious response by the melanoma cells to the test compound, particularly in the pigmented primary melanoma cells. Elesclomol caused DNA damage in B64/15 melanoma cells, with a significant enhancement effect being noted in the presence of ascorbate (% tail DNA:  $37.03 \pm 1.47$  *versus* 28.06 ± 1.192, *p*<0.0001) (Figure 8.4A). However, the damage that that occurred in (B63/15) primary melanoma cancer cells by Elesclomol in the presence of ascorbate was not so prominent (enhancing effect of ascorbate was small compared to the induced DNA damage in cells treated with Elesclomol alone) (% tail DNA:  $18.94 \pm 1.023$  *versus* 16.65 ± 0.939, *p*<0.1006) ( (Figure 8.4B).



Figure 8.4. Elesclomol-induced DNA damage in primary melanoma cancer cells in the presence and absence of ascorbate.

Disaggregated primary melanoma cells obtained from patients, and identified as B64/15 (A) and B63/15 (B), were incubated with and without ascorbate for 2 hours at  $37C^{\circ}/5\%$  CO<sub>2</sub>. After washing cells with PBS, they were then exposed exposed at  $37C^{\circ}/5\%$  CO<sub>2</sub> to indicated concentrations of Elesclomol for 15 hours. After the cells had been washed and centrifuged, DNA damage was measured by the standard ACA. Each bar represents the mean % of Tail DNA of 300 comets ± SEM, determined from a single experiment. The *T*-test (unpaired) was used for statistical analysis between each group *versus* ascorbate untreated cells.

The effect of oxidative-induced DNA damage in the presence of ascorbate in primary melanoma tissue was studied using the model oxidant  $H_2O_2$  and the drug Elesclomol. Primary melanoma tissues obtained from patients were (visibly) pigmented and non-pigmented.

Three primary melanoma tissue samples, including B33/15 (pigmented), B45/15 (nonpigmented) and B46/15 (pigmented), were exposed to  $H_2O_2$  in the presence and absence of ascorbate. ACA was conducted for each sample independently. DNA damage induced by  $H_2O_2$  in all primary melanoma cancer cells from all tissue samples was clearly observed, with a significantly enhanced effect noted when ascorbate was present (Figure 8.2A-C).

The two pigmented primary melanoma tissue samples (B33/15 & B46/15) responded the most to  $H_2O_2$  modulation by ascorbate. These data are consistent with the effect of  $H_2O_2$  plus ascorbate on cultured human melanoma cells, which were observed in Chapters 5 and 6 of this study. The pigmented primary melanoma cells were the most sensitive to the modulatory effect of  $H_2O_2$ -induced DNA damage (Figure 8.2A & B). However, surprisingly, the damage induced by  $H_2O_2$  alone in most of these cells (B33/15 and B45/15) was almost the same (Figure 8.2A & C). But in the presence of ascorbate the effect was enhanced for the pigmented cells (p<0.0001). This might be the result of higher metal content in pigmented cells as observed in pigmented melanoma cancer cells in Chapter 4 (Figure 4.10). More metal ions in pigmented melanoma cells, is expected to accelerate the Fenton reaction in the presence of ascorbate, thus generating more <sup>•</sup>OH and inducing more DNA damage.

Induced DNA damage was also observed in primary melanoma cells treated with Elesciomol, with a considerable enhancing effect noted in the presence of ascorbate. Similar to the effect of  $H_2O_2$  on the above primary melanoma tissue samples, Elesciomol caused DNA damage in primary melanoma cancer cells. The first tissue sample was heavily pigmented melanoma cells (B57/15) and was treated with a very high dose of Elesciomol (1mM) in the presence and absence of ascorbate. Indeed, the background DNA damage in cells pre-treated with and without ascorbate was very high ( $\geq$ 50%) but inspite of this, an enhancment effect of ascorbate was observed in Elesciomol treated cells (Figure 8.3). The high background DNA damage may be due to

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the effect of high level of endogenous ROS in these cells (as they were heavily pigmented and thought to have more ROS).

A further two primary melanoma samples were exposed to lower concentration of Elesciomol (50µM) in the presence and absence of ascorbate. Interestingly, even at lower concentrations of Elesciomol, DNA damage still occurred in primary melanoma cells obtained from these two samples. The damage induced by Elesciomol was more effective in the pigmented primary melanoma cells (B64/15) (p<0.0001) (Figure 8.4A), than in the non-pigmented melanoma cells (B63/15) (p=0.1009) (Figure 8.4B). Again, this is probably due to the effect of more metal content in pigmented cells.

Taken together, this suggests that ascorbate is able to modulate oxidative stressmediated DNA damage in primary human melanoma tissue, and that the melanin pigment levels in melanoma cancer cells sensitise these cells toward more oxidativeinduced DNA damage. **CHAPTER IX:** Concluding discussion

#### 9.1 Concluding discusion

MM is one of the ten commonest cancers in the UK (Cancer Research UK, 2014d), and is increasingly, affecting both males and females, worldwide (WHO, 2015a). In the latter stages, melanoma becomes very aggressive and resistant to all current therapeutic modalities (Fukunaga-Kalabis and Herlyn, 2012). In recent years the BRAF inhibitors were developed to tackle advanced melanoma. However, these targeted agents are only suitable to those who have a BRAF<sup>V600</sup> mutation, and unfortunately the majority of patients eventually develop resistance to anti-BRAF (Girotti *et al.*, 2014). Furthermore, the BRAF inhibitors have potential toxicities (Sosman *et al.*, 2012). Therefore, a better treatment for advanced MM is urgently required.

Cancer cells, generally, produce higher levels of endogenous ROS than normal cells, with decreased expression of antioxidant enzymes including catalase (Oberley and Oberley, 1997, Glorieux *et al.*, 2015); a biological feature that stimulates cell proliferation, and makes the tumour cell genetically unstable. Persistent oxidative stress promotes cancer cell adaptation that helps them to survive under oxidative environments (Ivanova *et al.*, 2012). However, this represents a specific vulnerability that treatments can potentially be targeted (Verrax *et al.*, 2011b). Among cancer cells, melanoma is unique; the biosynthesis of the pigment melanin leads to an elevated level of the endogenous ROS in these cells (Meyskens Jr *et al.*, 2001, Wittgen and van Kempen, 2007).

Heightened genome-instability in cancer cells suggests a model-scenario for their selective killing via the therapeutic delivery of defined levels of further genomic damage. Therefore, it has recently been proposed that adding ROS from exogenous sources to cancer cells could increase oxidative stress within them, resulting in a toxicity that overwhelms their likely limited endogenous antioxidant defences, inducing higher cell death rates (Trachootham *et al.*, 2009, López-Lázaro, 2007, Glorieux *et al.*, 2015). It has recently been shown that melanoma cancer cells are potentially sensitive to agents that mediate oxidative stress (Burgeiro *et al.*, 2013). To test this further, the present study proposed the modulation of induced DNA damage by modulating intracellular oxidative stress via ascorbate's redox activity in melanoma cancer cells, as an attempt to kill these cells preferentially.

Vitamin C is a well-known antioxidant that also functions as a pro-oxidant in biological systems, modulating oxidative stress through the Fenton reaction. This has prompted numerous clinical and biological studies to discover possible theraputic effects of ascorbate on tumour tissues (Levine *et al.*, 2009, Ullah *et al.*, 2012, Du *et al.*, 2010, Chen *et al.*, 2005, Padayatty *et al.*, 2006, Cieslak and Cullen, 2015). To date, these observations concerning the mechanism of ascorbate activity against cancer have encouraged clinicians to re-focus on the clinical plausibility of utilising ascorbate in cancer treatments (Ullah *et al.*, 2012, Freilich *et al.*, 2014, Mastrangelo *et al.*, 2015). However, the majority of pre-clinical and in *vitro* studies have relied on high doses of ascorbate to generate  $H_2O_2$  in the extracellular spaces, via a metal dependent proccess (Ullah *et al.*, 2012, Chen *et al.*, 2008, Cieslak and Cullen, 2015).

Recycling and accumulation of ascorbate within the cells against a concentration gradient suggests important intracellular function of vitamin C (Duarte and Lunec, 2005).  $H_2O_2$  is highly diffusible across cell membranes, but less reactive than other ROS candidates. However, when ascorbate is present inside a cell it induces redoxactive elements to generate <sup>•</sup>OH from  $H_2O_2$  via the Fenton reaction, which then attacks the DNA causing oxidative damage (Duarte and Jones, 2007).

Studies have found ascorbate induces apoptosis, which has a negative effect on melanoma cancer cell proliferation (Bram *et al.*, 1980). Reportedly, melanoma cancers are more susceptible to vitamin C toxicity than other cancer cells (Kang *et al.*, 2005b), with a recent study demonstrating an inhibitory effect from ascorbate supplementation on melanoma metastasis, and reduced tumour growth in vitamin C deficient mice (Cha *et al.*, 2013). Thus, it is proposed, ascorbate accelerates H<sub>2</sub>O<sub>2</sub>-induced melanoma cell death via the modulation of oxidative stress, with vitamin C acting as a "DNA damage switch" to promote cell death, notably apoptosis.

Chapter 3 describes the first section of lab work in which the melanoma cancer cells were examined for endogenously damaged DNA; it was found that melanoma cells, particularly pigmented ones, have greater DNA damage than the "normal" HaCaT cells studied. Using a modified-ACA it was found that damage is oxidatively induced by endogenous sources of ROS. Further investigation found melanoma cancer cells, particularly pigmented ones, also have significantly higher amounts of endogenous ROS. These findings concur with those of previous studies, suggesting melanoma tumour cells generate greater level of endogenous  $H_2O_2$  (Toyokuni *et al.*, 1995, Szatrowski and Nathan, 1991, Meyskens Jr *et al.*, 2001). The pigment melanin has both beneficial and toxic effects on cells. On one hand, it protects the cells from the damaging effect of UV-light (Kvam and Tyrrell, 1999b). On the other hand, it has been reported that melanin acts as a photosensitizer by producing more ROS after UV-radiation (Korytowski *et al.*, 1987). Furthermore, it has been suggested that melanin in normal melanocytes scavenge FRs generated by cellular metabolism. But in melanoma cancer cells, this process is reversed because of malfunctioning melanosomes (the cellular organelle that synthesize and store melanin) which in turn produce high amounts of  $H_2O_2$ ; this eventually causes further endogenous DNA damage in melanoma cancer cells (Fruehauf and Trapp, 2008).

In the current study a positive linear relationship was demonstrated between the amount of endogenous ROS and endogenously damaged DNA with higher levels more obvious in melanoma cancer cells, particularly in the pigmented (SK23) melanoma cells, than that in the HaCaT cells. This observation concurs with other studies, which suggest that the pigment melanin generates more ROS (Wang *et al.*, 2010).

When the cells were investigated for intracellular catalase enzyme activity (the primary intracellular antioxidant enzyme, that neutralisies H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O plus O<sub>2</sub>), it was found melanoma cancer cells have lower levels of catalase enzyme activity than HaCaTs. This was confirmed using two different techniques. This agrees with studies suggesting tumour cells including melanomas, but not normal cells, have very low catalase enzyme activity (Offner et al., 1992, Picardo et al., 1996, Meyskens et al., 1997). Furthermore, the amount of endogenous ROS observed was inversely related to the intracellular level of catalase. This relationship has also been reported in a recent study (Song and Gao, 2014). To further support this, it has been found that inhibition of intracellular catalase leads to H<sub>2</sub>O<sub>2</sub> accumulation (Nicco et al., 2005), and recently, an inverse relationship has been observed between histological grades and the antioxidant level in tumour tissue (Verrax et al., 2011a). Consequently, a low level of catalase plus the pro-oxidant effect of melanin might explain the accumulation of increased amounts of endogenous ROS in melanoma cancer cells. Thus, in line with the current study's hypothesis, the above findings indicate a possible vulnerability of melanoma cancer cells to treatment by a ROS-mediated approach.

In the second section of this work in Chapter 4, a panel of melanoma cancer cell lines including non-pigmented (A375M & A375P), moderately pigmented (SK28) and

heavily pigmented (SK23) and a panel of "normal" skin cells (HDF & HaCaTs) were examined for their sensitivity to  $H_2O_2$ -induced DNA damage. During the investigation it became apparent that culture media has a key effect on  $H_2O_2$ -induced DNA damage, in that media with pyruvate (DMEM) quenches the effect of  $H_2O_2$  by scavenging and preventing it from causing cellular genomic damage. This finding emphasises that the media effect must be taken into account when investigating oxidative mediated DNA damage by  $H_2O_2$  (or other  $H_2O_2$ -inducing oxidants).

To study the effect of  $H_2O_2$  in all the test cell lines, a single non pyruvate-containing media was chosen when exposing the cells to  $H_2O_2$ , and clear dose responses were observed in melanoma cancer cells and the HaCaT cells. Interestingly, the sensitivity of melanoma cancer cells to  $H_2O_2$ -induced DNA damage was greater than that of the HaCaTs. In agreement with this data, it has been reported that melanoma cancer cells are more susceptible to oxidative stress induced by  $O_2$  exposure than normal melanocytes (Farmer *et al.*, 2003). More interestingly, in the present study, higher levels of  $H_2O_2$ -induced DNA damage was found in pigmented melanoma cancer cells than in non-pigmented melanoma cancer cells.

The level of intracellular iron ions and their role in  $H_2O_2$ -induced DNA damage in melanoma cancer cells were also examined. Pre-incubation of melanoma cancer cells with DFO (a strong iron chelator) significantly decreased  $H_2O_2$ -induced DNA damage, revealing  $H_2O_2$ -induced DNA damage as an iron dependent mechanism; this was further supported by previous studies (Duarte *et al.*, 2007, Riviere *et al.*, 2006). In the literature, it is reported that, unlike normal cells, cancer cells have higher levels of metal ions including iron (Kontek *et al.*, 2013). In addition, the level of total intracellular iron ions (examined in this present study) was higher in pigmented (SK23) melanoma cancer cells than in the non-pigmented cells (Chapter 4). A high level of intracellular iron ions in pigmented cells are most likely causative of the generation of higher endogenous ROS in these cells as reported in Chapter 3. This may also further explain why pigmented melanoma cells are more sensitive to  $H_2O_2$  than non-pigmented cells (Chapter 4).

A key part of this project was to investigate the effect of ascorbate on  $H_2O_2$ -induced DNA damage/cell killing in melanoma cancer cells and "normal" skin cells. The work of Chapter 5, was to examine the effect of ascorbate on  $H_2O_2$ -induced damage in the studied cells. As predicted, there was a clear pro-oxidant impact of ascorbate on  $H_2O_2$ -

induced DNA damage. This effect was confirmed when the damage to DNA bases was explored using a modified-ACA; ODBLs, validated markers of oxidative stress (Cooke *et al.*, 2003), were clearly found enhanced in melanoma cancer cells exposed to  $H_2O_2$  plus ascorbate.

In this study, the sensitivity of melanoma cancer cells to  $H_2O_2$ -induced DNA damage was significantly increased by ascorbate, when used at a concentration equivalent to plasma levels (Padayatty *et al.*, 2004, Woollard *et al.*, 2002, Choi *et al.*, 2004, Levine *et al.*, 2011) achievable after supplementation orally (Levine *et al.*, 2011). The highest effect was observed in pigmented melanoma cancer cells, with the HaCaTs and HDF cells being the least sensitive to  $H_2O_2$  and its modulation by ascorbate.

This suggests intracellular ascorbate in the ascorbate-pre-treated cells accelerated the Fenton reaction by recycling and regenerating  $Fe^{2+}$  ions, so generating more <sup>•</sup>OH from  $H_2O_2$ , which in turn attack the DNA inducing more damage, such as strand breakage and ODBLs. The role of intracellular ascorbate in Fenton reactions that stimulate formation of <sup>•</sup>OH has previously been reported when rat brain tissue was exposed to a mixture of ascorbate and iron ions (Chakraborty *et al.*, 2001) and is consistent with reports from other studies which found that ascorbate enhances the genomic damage caused by  $H_2O_2$  (Kontek *et al.*, 2013, Duarte and Jones, 2007).

Other published reports regarding the pro-oxidant effect of ascorbate on oxidative mediated DNA damage assessed by standard ACA show contrary data. Arranz *et al.* (2007) reported that ascorbate, via scavenging, has a protective effect against H<sub>2</sub>O<sub>2</sub>-induced DNA damage in hepatoma cancer cells (Arranz *et al.*, 2007). However, in their study hepatoma cancer cells were treated with H<sub>2</sub>O<sub>2</sub> prepared in DMEM media for only for 5-10 minutes at 37°C, 5% CO<sub>2</sub> and then exposed them to 1-10 $\mu$ M ascorbate for 30 minute; so the pro-oxidant effect of ascorbate would not have been detected. Azqueta *et al.*, (2013) also reported no gross effect from ascorbate in HeLa tumour cells, even at levels of 200 $\mu$ M, nor induced any H<sub>2</sub>O<sub>2</sub>-induced DNA damage (Azqueta *et al.*, 2013). In their study HeLa cells were treated with ascorbate for 30 minutes and then exposed them, for only 5 minutes, to H<sub>2</sub>O<sub>2</sub> but in DMEM media.The treatment protocol of these studies was different to the protocol used in the current study (see section 2.3, in Chapter 2). Herein, cells were exposed to a higher concentration of ascorbate (100 $\mu$ M) and for 2 hours at 37°C then they were exposed, for 30 minutes on ice, protected from the light, to H<sub>2</sub>O<sub>2</sub> prepared in a serum and pyruvate-free media. Also a single cell

culture media (A375M-media) has been used for all cell lines for cell exposure to  $H_2O_2$  to minimise any media dependent effect, and all cells were pre-incubated with ascorbate prior to exposure to  $H_2O_2$ .

Moreover, according to the literature, the effect of ascorbate on  $H_2O_2$ -induced DNA damage varies between different cell types. One study found that in cultured normal human lymphocytes, ascorbate has an inhibitory effect against H<sub>2</sub>O<sub>2</sub>-induced DNA damage (Yen et al., 2002) and others reported a protective effect of ascorbate on H<sub>2</sub>O<sub>2</sub>induced DNA damage in normal human cells. For instance, Siddique et al (2009) used high levels of  $H_2O_2$  (100-200µM) to induce DNA damage in lymphocytic cells in the presence of ascorbate, reporting a protective effect of ascorbate against damage induced oxidatively by H<sub>2</sub>O<sub>2</sub>. The data of the current study demonstrates an obvious enhancing effect of ascorbate on the level of H<sub>2</sub>O<sub>2</sub>-induced DNA damage in melanoma cells whereas the HaCaTs and HDF cells were the least affected. This is probably due to effective an antioxidant system in normal human cells that keep the cellular oxidative stress status balanced. The role of intracellular antioxidants in normal cells against H<sub>2</sub>O<sub>2</sub> was also previously reported; in 1994, Ward reported that  $H_2O_2$  targets cellular compartments in normal cells, but antioxidants, such as catalase, limit its effects preventing it from inducing more DNA damage (Ward, 1994). To further support this, more recently, it has been found that knocking-down catalase in human tumour cells can sensitize these cells to  $H_2O_2$  (Klingelhoeffer *et al.*, 2012). This data agrees with very recent findings which have found an inhibitory effect of ascorbate on cytotoxicity induced by ozone in normal human keratinocytes (Valacchi et al., 2015).

DNA damage caused by <sup>•</sup>OH attack not only involves SSBs and ODBLs, but can also generate DSBs (Driessens *et al.*, 2009). When examining melanoma cancer cells and HaCaT cells for the effect of ascorbate on  $H_2O_2$ -induced DSBs, an enhancing effect from ascorbate was observed on  $H_2O_2$ -induced DSBs, as measured by  $\gamma$ -H2AX immunoassay in all melanoma cancer cells. Similar to the effect of ascorbate on  $H_2O_2$ -induced SSBs in melanoma cancer cells, the number of DSBs induced by  $H_2O_2$  and enhanced by ascorbate were greatest in melanoma cells, particularly in pigmented cells.

Of all of the lesions induced by oxidation, those constituting bistrand clustered DNA lesions are the most refractory to repair (Sutherland *et al.*, 2002, Hada and Sutherland, 2006). This class of lesion is a challenge for the DNA repair mechanism, because the damage is located close on the opposing strands, within a few helical turns of the DNA
(Sutherland *et al.*, 2002). Thus, clustered DNA damage, also referred to a multiply damaged sites (MDS), are more relevant biologically for the induction of cell death (Ward, 1994, Ward, 1988, Valko *et al.*, 2006). The current study also investigated the efficacy of radiation and  $H_2O_2$  to induce MDS in melanoma and HaCaT cells. Indeed, ionising radiation induced a much higher ratio of DSB:SSB than  $H_2O_2$ . The relative number of  $\gamma$ -H2AX foci measured in the cells exposed to radiation was higher than that in the same cells when treated with  $H_2O_2$ . Moreover, the relative number of SSBs induced by radiation was lower than the SSBs, in cells treated with  $H_2O_2$ ; suggesting radiation provokes more complex MDS. This finding is in agreement with a recent study in which relatively higher numbers of DSBs compared to SSBs were reported in rat thyroid cells treated with ionising radiation compared to that induced by  $H_2O_2$  in these cells (Driessens *et al.*, 2009).

The DNA damage complexity was predicted to occur in melanoma cancer cells following exposure to  $H_2O_2$  in the presence of ascorbate. Acceleration of the Fenton reaction by ascorbate was proposed to generate greater number of local  $^{\circ}OH$  from H<sub>2</sub>O<sub>2</sub>, which in turn would attack local sites of the DNA molecule, causing a greater number of DSBs relatively to SSBs. However, when the investigation was conducted in melanoma cancer cells, the enhancing effect of ascorbate on the number of H<sub>2</sub>O<sub>2</sub>induced DSBs was found to be in proportion to the enhancing effect of ascorbate on the level of H<sub>2</sub>O<sub>2</sub>-induced SSBs. DNA damage induced by H<sub>2</sub>O<sub>2</sub> depends on the presence of redox active metals ions on the DNA molecule. Genomic damage induced by a Fentonmediated mechanism occurs at the site of <sup>•</sup>OH production (Lenton *et al.*, 1999). This is because the <sup>•</sup>OH formed by the Fenton reactions are highly reactive and do not migrate far from the site of their formation (Lenton et al., 1999). However, to account for the noted proportional increase in both SSBs and DSBs mediated by ascorbate, one possible explanation is that whilst either  $Fe^{2+}$  or  $Fe^{3+}$  binds to the DNA, the other partner is unbound and so free to migrate. This will lead to the formation of dispersed 'OH causing simple damage rather than complex damage (Figure 5.22).

The other goal of this study was to evaluate the effect of ascorbate on  $H_2O_2$ -induced cell death. Evidence suggests DNA is one of the main targets of  $H_2O_2$ -induced cell killing (Samuni *et al.*, 2001) and strong evidence suggests that  $H_2O_2$  induces cell killing via induction of metal-dependent DNA damage; inhibition of  $H_2O_2$ -induced cell killing can

be successfully initiated by metal chelating agents such as DFO (Samuni *et al.*, 2001, Davies, 1999).

In the present study, cell viability, annexin V/PI staining, clonogenic assay and caspase-3/7 expression were all used to assess  $H_2O_2$ -induced cell death in the presence and absence of ascorbate. Interestingly, in all assays ascorbate enhanced the effect of H<sub>2</sub>O<sub>2</sub>induced cell death in melanoma cancer cells, whereas in HaCaT cells ascorbate only weakly enhanced H<sub>2</sub>O<sub>2</sub>-induced cell killing/apoptosis and, indeed, for clonogenic assay the effect was noted to be protective rather than enhancing (The enhanced effect of ascorbate on H<sub>2</sub>O<sub>2</sub>-induced cell killing in melanoma cancer cells was more than 2-fold higher than that in the HaCaTs) (see Chapter 6). This may indicate that in normal cells, ascorbate functions as an antioxidant rather than as a pro-oxidant. To further support this, it has also been shown that ascorbate at "pharmacological doses" induce toxicity in cancer cells (by delivering H<sub>2</sub>O<sub>2</sub> through ascorbate autooxidation to tumour cells) but not in normal cells (Chen et al., 2008), with a high level of intracellular catalase being thought to be one of the potent factors that protect these normal cells from the exogenous oxidative stress and its modulation by ascorbate. A very recent study involving B16 murine melanoma cancer cells has shown data indicating that treatment of cells with catalase almost completely abolish the killing effect of the exogenous H<sub>2</sub>O<sub>2</sub> (Serrano et al., 2015). Also a recent study using a mouse model to test the effect of antioxidant supplementations on MM cancer cells found evidence indicating that NAC increases metastasis of melanoma cells (Le Gal et al., 2015). This suggests that the effective antioxidants such as NAC inhibit the endogenous ROS to a certain level so favouring for cell survival and metastasis. Indeed, the abolishing effects of NAC on the intracellular ROS in melanoma cancer cells has been clearly observed in the current study (see Chapter 7). However, pre-treating melanoma cancer cells with ascorbate enhanced the oxidative damage in these cells, and it has been reported that ascorbate supplementation has inhibitory effects on melanoma metastasis and tumour growth in vitamin C deficient mice (Cha et al., 2013).

The data reported in Chapter 5 and Chapter 6 suggests that the effect of ascorbate on  $H_2O_2$ -induced cell killing is positively related with the effect of ascorbate on  $H_2O_2$ -induced DNA damage in the studied cells; with melanoma cell killing, as measured by clonogenic assay, correlating with oxidatively induced DNA damage enhanced by ascorbate. From such observations emerges the answer to the study's hypothesis

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claiming that ascorbate within melanoma cancer cells renders these cells more susceptible to  $H_2O_2$ -mediated cell damage inducing greater death in these cells. This observation is highly relavent, as the project's findings clearly illustrate the intracellular pro-oxidant function of ascorbate on the induction of melanoma DNA damage and in promoting cell killing.

Chemical agents including BSO and Elesclomol were tested to induce intracellular oxidative stress in melanoma cancer cells, instead of the model oxidant H<sub>2</sub>O<sub>2</sub>. This aimed to test the pro-oxidant effect of ascorbate on the modulation of oxidative stress within melanoma cancer cells mediated by clinically relavent agents rather than the model oxidant. The attempt to induce more intracellular ROS via the two agents in melanoma cancer cells was successful. More DNA damage was also observed by the standard ACA in melanoma cancer cells treated with BSO and Elesclomol; with a significant enhancing effect from ascorbate on the level of DNA damage induced, particularly in the pigmented melanoma cancer cells (SK23). Other melanoma cancer cells were resistant to Elesclomol induced DNA damage, possibly due to the noted lower level of metal ions. The modified-ACA detected additional ODBLs in melanoma cancer cells treated with Elesclomol plus ascorbate, indicating the effect was authentically oxidative. Melanoma cells (SK23) were also examined for apoptotic cell death using annexin V/PI and by measuring caspase-3/7 expression, after cell exposure to Elesclomol in the presence and absence of ascorbate; apoptosis was enhanced in melanoma cancer cells exposed to Elescloml combined with ascorbate. This suggests that oxidative mediated therapies plus ascorbate could synergistically kill melanoma cancer cells via modulation of oxidative stress within the treated cells (see Chapter 7).

In the final part of the current work, an assessment of the effect of intracellular oxidative stress and its modulation by ascorbate was undertaken using clinical samples (primary melanoma tumours tissues). A standard-ACA was used to test genomic damage after exposing the primary melanoma cells to either  $H_2O_2$  or Elesclomol, both in the presence and absence of ascorbate. The enhancement effect of ascorbate on  $H_2O_2$ -induced DNA damage observed in primary melanoma cancer cells was similar to that noted in previously using the established melanoma cell lines. A key finding was that pigmented primary melanoma tissues were more sensitive than non-pigmented primary melanoma tissues to the effects of oxidants ( $H_2O_2$  and Elesclomol) plus ascorbate. The above observations are of possible importance clinically; the primary melanoma tissues,

representing a primary *ex-vivo* system, respond positively to oxidatively-mediated DNA damage and its modulation via ascorbate. The noted data also confirms that the pigmented primary melanoma tumour cells are again more sensitive to the ROS-mediated therapeutic approach (see Chapter 8).

To sum up and conclude, all the above evidence provided by this study indicates that  $H_2O_2$ -induced oxidative DNA damage and cell killing in melanoma cancer cells is enhanced in the presence of ascorbate, and that the pigmented melanoma cells are the most affected. The oxidant and antioxidant imbalance in melanoma cancer cells allows the selective for treatment of melanoma cancer cells to ROS-mediated therapy and its modulation by ascorbate; making this cancer type vulnerable and appropriate for a potentially ROS-mediated therapeutic approach.

This project provides further new information regarding the intracellular oxidative stress mechanism, and its modulation by ascorbate in melanoma cancer, contributing to a better basic understanding of oxidative stress in this tumour model. This study also shows the sensitivity of primary melanoma tissue to the combinatory effect of ascorbate with the oxidative stress mediated by a model oxidant ( $H_2O_2$ ) or novel therapeutic oxidants (e.g. Elesclomol). The benefits of the study primarily relate to cancer treatment and the possibility of using ascorbate as a combination therapy to treat melanoma.

#### 9.2 Future studies

This project involved human melanoma cancer cells including the melanoma model cell lines plus immortalised normal skin cells and also human primary melanoma tissues. The intracellular pro-oxidant action of ascorbate on the level of oxidative-mediated DNA damage and cell killing in melanoma cancer cells has clearly been observed. This suggested that ascorbate treatment in melanoma cancer cells could increase tumour cell sensitivity toward H<sub>2</sub>O<sub>2</sub>-mediated drugs.

For induction of intracellular oxidative stress, BSO and Elesclomol were used. It may be better to use different types of oxidative mediated drugs or means of  $H_2O_2$ -delivering systems to study the effect of ascorbate.

A very recent study, showed evidence indicating an enhancement effect in radiosensitivity of pancreatic cancer cells by using pharmacological doses of ascorbate (Du *et al.*, 2015). It has been suggested that ionising radiation stimulate cancer cells to generate more intracellular ROS (Leach *et al.*, 2001, Claro *et al.*, 2014). Thus, it is also may be recommended to investigate the use of physiological concentrations of ascorbate in combination with ionising radiation to kill melanoma cancer cells.

To further validate the pro-oxidant effect of ascorbate on oxidative damage, cells can be either pre-treated with ascorbate or other antioxidants such as NAC prior to exposing them to  $H_2O_2$ . Consequently, the ACA can be conducted to assess the induced DNA damage in treated cells. This might differentiate the role of different antioxidants in tumour cells.

This project can also be further developed to investigate the pro-oxidant effect of ascorbate on MM tumour and its metastasis using transgenic mouse models with melanoma (Milagre *et al.*, 2010). Here, the animals can be supplemented with vitamin C, and the animals then will be treated with oxidants.

# Thesis related work:

- Conference Abstracts
- Hishyar Najeeb, Matthew W Stuttard, Karen J Bowman, Rachel Haywood and George DD Jones. "The role of Ascorbate in Oxidative Stress in Malignant Melanoma Cancer Cells" (Poster). 34th EORTC-PAMM – BACR Winter Meeting, 23rd–26th January 2013. National Museum Cardiff, Wales, U.K.
- 2- Hishyar Najeeb, Karen J Bowman, Jesvin J Samuel, Rachel Haywood, Salvador Macip, George DD Jones. "Modulation of intracellular oxidative stress via ascorbate: A possible mechanism to kill melanoma cancer cells!" (Poster). The third special conference on Advances in Cancer Drug Discovery. BACR. 30<sup>th</sup> March-1<sup>st</sup> April 2014. Homerton College, Cambridge, UK.
- 3- Hishyar Najeeb, Karen J Bowman, Jesvin J Samuel, Rachel Haywood, Salvador Macip, George DD Jones. "Modulation of intracellular oxidative stress via ascorbate: A possible mechanism to kill melanoma cancer cells!" (Poster). ELTU Research Festival held on Wednesday 19th February in Fraser Noble Hall. University of Leicester, Leicester, UK. "Awarded for a best presentation among the international students".
- 4- Hishyar Najeeb, Karen Bowman, George Jones. "Redox modulation of oxidatively-induced DNA damage by ascorbate selectively enhances DNA damage formation in melanoma cancer-cells and enhances cell-killing" (Oral presentation). 41<sup>st</sup> Annual Meeting of the Radiation Research Society (ERR2014). 14<sup>th</sup> – 19<sup>th</sup> September 2014. Rhodes, Greece.
- 5- Hishyar Najeeb, Karen Bowman, George Jones. "Redox modulation of oxidatively-induced DNA damage by ascorbate selectively enhances DNA damage formation in melanoma cancer-cells and enhances cell-killing" (Poster). 2014 NCRI Cancer Conference. 2 - 5 November 2014. The BT Convention Centre, Liverpool, UK.
- 6- Hishyar Najeeb, Karen Bowman, George Jones. "Redox modulation of oxidatively-induced DNA damage by ascorbate selectively enhances DNA damage formation in melanoma cancer-cells and enhances cell-killing"(Oral presentation).15<sup>th</sup> International Congress of Radiation Research (ICRR 2015), Kyoto, Japan.
- 7- Hishyar A Najeeb, Victor Alfred, Charlotte Lee, Soteroula Panayiotou, Michael Weinfeld, Karen J Bowman & George DD Jones. "Redox modulation of oxidative and radiation-induced DNA strand break damage" (Oral presentation). 61<sup>st</sup> Annual Meeting of the Radiation Research Society. September 19th-22nd, 2015, Weston, FL, USA.

## **Collaborative work**

### **A- Conference abstracts**

1- Jesvin J Samuel, BTech, MRes, Alice H Wignall, BSc, Hishyar Najeeb, BVMS, MSc, Aneela Majid, PhD, Sandrine Jayne, PhD, George D Jones, BSc, MSc, D.Phil., Salvador Macip, MD, PhD and Martin JS Dyer, MA, DPhil, FRCP, FRCPath. "Proliferating CLL Cells Express Abundant But Transcriptionally Compromised TP53 Protein" (Oral presentation). 55<sup>th</sup> American Society of Haematology (ASH) Annual Meeting and Exposition. 7<sup>th</sup>-10<sup>th</sup> December 2013. New Orleans, USA.

# **B-** Publications

- 1- Jesvin Samuel, Sandrine Jayne, Aneela Majid5, Alice Wignall1, **Hishyar Najeeb**, Yixiang Chen, Jin-Li Luo, George. Don Jones, Salvador Macip, and Martin J. S. Dyer. *ROS-dependent up-regulation of p53 in proliferating chronic lymphocytic leukemia cells*. Blood. (Submitted).
- 2- Mohammad Althubiti, Miran Rada, Kamaldeep Chana, Jesvin Samuel, Josep M. Escorsa, **Hishyar Najeeb**, George D. D. Jones, Nickolai Barlev, Eran Tauber, and Salvador Macip1,2\*.*BTK phosphorylates p53 to regulate senescence and ageing* (Submitted).

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