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Novel Insights into Hyperthermia's Therapeutic Effectiveness in Treating Malignant Melanoma

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ABSTRACT

Malignant melanoma is one of the most aggressive types of human cancers and one of the deadliest malignancies with steadily increasing incidence rates globally. Although early detection and increased surveillance have undoubtedly contributed to the elevation of survival rates, its mortality rates still remain high as the disease does not respond to current therapeutic strategies. Hyperthermia, defined as the application of exogenous heat induction, is one of the most common therapeutic modalities and acts by either directly killing tumor cells and/or sensitizing them against other therapeutic means. Numerous reports have provided proof that it can trigger the activation of cell death pathways in various skin cancer cell lines. Hence, in the present study we have aimed to establish a hyperthermia-induced experimental platform in order to assess its therapeutic efficacy in an *in vitro* human skin cancer model consisting of (1) immortalized keratinocytes (non-malignant), (2) malignant melanoma and (3) epidermoid carcinoma (non-melanoma) cells as well as an *in vivo* mouse model of malignant melanoma (mice injected with mouse malignant melanoma cells). According to our kinetic analyses using our *in vitro* skin cancer model, the optimal hyperthermic experimental conditions range between 43^0C-45^0C, for 2 hours, as there was a significant cell death induction in malignant melanoma cells, whereas, interestingly, non-malignant cells seem to be more resistant. Furthermore, we utilized a real-time PCR microarray-based gene expression profiling system to identify critical gene targets involved at various stages of the apoptotic pathway(s). Moreover, in order to fully characterize the interactions and cross-talks between the different apoptotic cascades (intrinsic, extrinsic, ER- stress mediated), we studied changes in protein expression levels of various genes implicated in those pathways by means of Western immunoblotting. According to our results, there is differential regulation of gene expression of several pro- and anti-apoptotic genes which appears to depend on the hyperthermic exposure conditions. Finally, our previous findings were evaluated by utilizing an *in vivo* mouse malignant melanoma model and according to our data there was a considerable inhibition of tumor growth for both hyperthermic conditions accompanied by the respective changes in several apoptotic genes' expression levels. The results of this study further support hyperthermia's effectiveness in treating malignant melanoma by identifying the underlying molecular mechanisms and various molecules responsible for hyperthermia's beneficial effects on tumor growth inhibition.

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Introduction

1. Introduction

1.1 Skin Cancer

Skin cancer is considered to be one of the most common types of cancer worldwide with steadily increasing incidence rates over the past decades (ACS, 2016). It is categorized into several types according to the kind of skin cells from which they arise. Briefly, there are three main types: (i) basal cell carcinoma (BCC) that originates from basal cells at the basal layer of the epidermis or the follicular epithelium and has the highest prevalence amongst skin cancer rates, (ii) squamous cell carcinoma (SCC) that derives from the cells that make up the top of epidermis and (iii) malignant melanoma that originates from the melanin-producing cells, called melanocytes, and is the most aggressive skin cancer type (Erb et al., 2008). BCC and SCC together are called non-melanoma skin cancers (NMSC) and are the most common histologic subtypes (20%) of all malignancies diagnosed in the UK every year (Brenn, 2014) with almost 132.000 cases (NCRAS, 2014). Approximately, 5.4 million patients are diagnosed with NMSC worldwide per annum, whilst 80% are BCC (Connolly, 2012). Presentation of NMSC has been associated with increased risk of other malignancies (Barton, 2017). BCCs are generally curable through surgical excision and are rarely metastatic or fatal, mainly eroding local anatomical structures, particularly on head and neck. SCCs exhibit a less favorable prognosis with increased metastatic potential and mortality rates. They are characterized by the presence of atypical (dysplastic) keratinocytes although exhibiting a wide range of clinical manifestations. The average treatment costs in the US between 2007 and 2011 did escalate to $4.8 billion for NMSC and $3.3 billion for melanoma (Guy et al., 2015).

1.1.1 Malignant melanoma

Malignant melanoma is the most aggressive form of skin cancer and one of the most lethal amongst all solid tumor types with around 232.000 people diagnosed each year worldwide and incidence rates increasing globally over the past few decades (de Vries et al., 2005, Arnold et al., 2014). This trend is in accordance with recent demographic reports in UK, where 1 in 54 people are developing melanoma during their lifetime, rendering the disease the 5th most common type of cancer, accounting for 4%

of all new cases registered (15.400 in 2014). The disease incidence rates have increased dramatically (by 119%) over the last two decades, while it seems that it is one of the most common cancers among young (between ages 15-35) and fair-skinned people. The incidence rate projections report, for 2035, an estimated additional increase by 7%. Late stage diagnosis is occurring in 1 in 10 cases, whilst approximately 59.000 patients have been reported for 10-year survival post diagnosis (CRUK, 2014).

The disease occurs from accumulation of genetic and metabolic abnormalities at the pigment-producing cells, the melanocytes, that reside in the neural crest and migrate to the skin or other sites during the embryonic development. Melanoma can originate from cutaneous melanocytes or occurs in mucosal surfaces (uveal tract of the eye, oral cavity, leptomeninges, gastrointestinal sites, genital mucosa) (Dennis, 1999, Stang et al., 2003). The pathogenesis of melanoma remains still unclear, however skin phototype, hair color, numerous atypical naevi (benign proliferations of melanocytes), genetic predisposition and solar ultraviolet (UV) radiation have been associated with the disease. Interestingly, extended analysis of the melanoma genome has revealed a close link between frequent mutational events and UV radiation making it the leading risk factor (Russak, 2012 and Fu, 2017).

Clinical diagnosis of malignant melanoma is usually unambiguous and accurate although some non-pigmented malignant melanocytic lesions might be often misinterpreted as NMSC (Ferrara et al., 2013). Clinical examination of the neoplasms can be aided by dermoscopy, computerized imaging, whole-body digital photography and confocal laser microscopy (Marchesini et al., 2002). Excisional biopsy is required for histological classification and staging of the tumor according to the American Joint Commission on Cancer (AJCC) TNM system (Balch et al., 2009, Amin et al., 2017). When diagnosed at an early stage, the disease can be cured by surgical removal, but upon the disease progression to metastatic stages the treatment options become poorer as the current therapeutic strategies do not seem to be quite efficient (Jerant et al., 2000). Several therapeutic regimens based on combined treatment options have been applied in clinical trials. However, none of them have been proven to be advantageous in improving survival rates. Therefore, there is a vital need for the establishment of new strategies that will be able to ameliorate existing therapeutic protocols and/or make them more effective.

Until recently, the major treatment would involve only surgical resection of the tumour. However, 10-15% of melanoma bearing patients develop distant metastases.

Recurrence is more common in subcutaneous tissue and lymph nodes (non-loco-regional metastatic melanoma), lung, brain, gastrointestinal tract, liver and biliary tract, spleen and bone. Prognosis for most of the diagnosed metastatic deposits remains extremely poor, highlighting the need for a more effective eradication of the primary tumor deposits (Garbe et al., 2011, Shain and Bastian, 2016). In addition, increased surveillance as well as improvements in early diagnosis might have contributed to the dsease's increased incidence rates. However, the main reason for the observed trends still remains the prolonged exposure to sunlight (Bataille et al., 2004, Parkin et al., 2011, Rushton et al., 2017). Changes in socio-economic status and in lifestyle habits, such as intense and sporadical exposure to sun - especially during childhood- and use of sun-beds, have both been linked to the steadily rising numbers of new cases, highlighting the importance of sun-protection education programs as a prevention strategy (Perez-Gomez et al., 2008). Technological advances in molecular biology have allowed for the identification of several genes and pathogenic events responsible for melanoma tumorigenesis. UV-induced DNA damage (Cadet et al., 2015) and reactive oxygen species (ROS) production (Joosse et al., 2010) along with secretion of growth factors derived from keratinocytes and suppression of T-cell mediated immune responses are amongst the key drivers of melanoma formation. However, little is known about the primary somatic genetic mutations during melanoma carcinogenesis. The B-Rapidly Accelerated Fibrosarcoma (BRAF) oncogene is well-established as one of the frontline therapeutic targets (Kozar et al., 2017). The mitogen-activated protein kinase (MAPK) pathway (Hartmann et al., 2015), the micropthalmia transcription factor (MITF) (Kawakami et al., 2017), the epidermal growth factor (EGFR) and the N-Ras sarcoma protein (NRAS) proto-oncogene (Posch et al., 2016) are frequently deregulated in melanomas (Griewank, 2016). To this end, more recent molecular drugs targeting melanoma focus on the MAPK pathway which is heavily involved in 90% of melanomas. In fact, 50% of cases present active mutations in BRAF.

Vemurafenib and Dabrafenib are potent and selective BRAF inhibitors whereas Trametinib, a MAPK kinase (MEK) inhibitor, is effective in blocking pathway activation downstream of BRAF (Grimaldi et al., 2017). According to several reports, combinational treatment of BRAF and MEK inhibitors has elicited considerable tumor regression (Ribas et al., 2011, Latimer et al., 2016, Simeone et al., 2017). Another promising approach towards melanoma treatment is based on molecular immunotherapy (Margolin, 2016). Adoptive immunotherapy exploiting tumor infiltrating lymphocytes (TIL) or genetically-engineered T cells expressing chimeric antigen receptors (CAR-T

cells) has been correlated with 50-70% response rates (Sim et al., 2014, Merhavi-Shoham et al., 2017). Administration of interleukin-2 (IL-2) or interferon-alpha (IFN-α) as a single-agent or adjuvant therapy is effective in sustaining the disease under control (Bright et al.,2017, Trinh et al., 2017, Daponte et al., 2013). Treatment with immune check-point regulators has been adopted as a strategy aiming to oppose the induction of immunological tolerance during melanoma formation. Cytotoxic T-lymphocyte associated protein 4 (CTLA4) inhibitors such as Ipilimumab or Tremelimumab are effective as a monotherapy or in combination with the cytotoxic drug Dacarbazine (DTIC) (Lee et al., 2016, Yun et al., 2016). Additionally, Nivolumab and Lambrolizumab are monoclonal antibodies targeting the pro-death receptor-1 ligand 1 (PD-L1) frequently expressed at melanoma cells, giving hope for improvements in the therapeutic management of melanomas (Hassel et al., 2017, Hao et al., 2017). Patients diagnosed with advanced or even intermediate stage of the disease may also undergo adjuvant systemic therapy including administration of intermediate-dose IFN-α or adjuvant radiotherapy post-surgical clearance (Laks et al., 2013, Gonzalez et al., 2016). Moreover, single-agent chemotherapy involving treatment with Dacarbazine, Temozolamide and Fotemustine is frequently used, mainly due to the low toxicity and simplicity of administration although being associated with low response rates (10%) (Quereux et al., 2011). Finally, bio-chemotherapy combines the above mentioned regimens with administration of cytokines and it is considered a promising strategy with potentially better response rates (Diamantopoulos et al., 2016, Chatzistefanou et al., 2016, Biteghe et al., 2017). Last but not least, radiation therapy is widely employed for the treatment of distant metastases (Gampa et al., 2017).

1.1.2 Cancer stem cells (CSCs) in skin cancer progression

It is well known that solid tumor as well as leukaemias do not consist of a single tumor cell type but a quite heterogenous tumor cell population. Over the last few decades, research has provided evidence supporting the stem cell theory of cancer. According to this theory, a small population of cancer cells within a specific tumor type appear to possess stem cell properties (cancer stem cells- CSCs), therefore being able to reproduce themselves, generate the range of cancer cells composing the tumor and retain malignant growth (Visvader, 2011; Visvader and Lindeman, 2008). A critical implication of this theory is that CSCs can promote metastasis of a tumor type to

different sites (migration properties), whereas if they are resistant to the treatment plan followed, they can potentially cause a future relapse (Shiozawa et al., 2013).

One of the most commonly studied tissues with a well structured hierarchical organization is mouse skin, therefore it has been widely used for studying skin carcinogenesis. This process is characterized by multiple steps which evolve from the the development of hyperplasia, dysplasia and benign papilloma to squamus invasive carcinoma and spindle carcinoma (Klein-Szanto et al., 19993). Elevated levels in DNA changes of the target cells have been linked with the progression of this intricate process (Frame and Balmain, 2000), however it remains unclear whether the target CSCs pre-possess the ability to reproduce indefinitely or the accumulation of genetic alterations results in the immortalization of more committed cells with limited life span. Results from studies using chemicals as carcinogenic factors have provided evidence that tumors of different malignancies can arise from different target cells. More specifically, it was argued that malignant tumors are developed from cells residing in the bulge of the hair follicle and have a high self-renewal potential, while papillomas may arise from stem cells of the intefollicular epidermis (Bailleul et al., 1990; Brown et al., 1998). In addition, when mice were treated first with 7,12- dimethylbenz[a]anthracene (DMBA) for the tumor initiation stage and then with the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) after 1 year, it appeared that they developed papilloma in the same pattern as if they were treated soon after the initiation. These findings imply that cells with mutations on RAS gene reside in the epidermis for extended time periods without initiating the formation of lesions, therefore suggesting that those cells belong to a stem cell population (Van Duuren et al., 1975).

Several research reports have suggested the implication of CSCs in the development of malignant melanoma, progression of the disease as well as its resistance to current therapeutic means (Fang et al., 2005; Wang et al., 2006; Monzani et al., 2007). Melanoma cells have been found to form spheres which are groups of non-adherent cells that maintain their self-renewal properties over long time periods, thus supporting the presence of cells with stem cell features within the melanoma population (Fang et al., 2005). Development of melanoma tumors is often characterized by substantial changes in the genome including chromosomal translocations, deletions, amplifications or mutations which suggests the extensive heterogeneity in melanoma populations, however it has been shown that primary, metastatic as well as recurrent tumors arouse from the same initial clone (Chin et al., 2006; Wang et al., 2006).

Furthermore, there is evidence showing that specific factors secreted by melanoma cells are capable of directing the migration of hematopoietic progenitor cells to metastatic sites (Kaplan et al., 2005). Nodal which has been shown to be secreted by melanoma cells is known to act as a morphogen for progenitor cells and may play a role in determining the sites of tumor metastases (Topczewska et al., 2006). Other stem cell markers found to be upregulated in melanomas involve CD133, CD166, nestin, Notch, ALDH1A, etc. (Klein et al., 2007; Massi et al., 2006; Croteau et al., 2013). In contrast, a number of other reports propose that melanoma heterogeneity is based on clonal variation while the formation of tumors arising from different clones may be induced by different molecular mechanisms. Therefore, the potential participation and activation of different mechanisms in the progression of the disease is responsible for the commonly unsuccessful results of certain therapies while revealing the increasing need for developing diverse targeted therapeutic strategies (Brinckerhoff, 2017; Croteau et al., 2013).

1.2 Cell death pathways

The development and interaction of multicellular organisms with their environment is highly dependent on the achievement and maintenance of a relative state of equilibrium between cellular proliferation and death. Tissue homeostasis requires a plethora of tightly linked and regulated processes. However, cell death can also be the result of damage caused by various factors (e.g. UV radiation, viruses, etc.) leading to deregulation of cellular processes. Cellular death involves three major morphologically and biochemically distinct modalities: apoptosis (type I programmed cell death), autophagy (type II) and necrosis (type III) (Galluzzi et al., 2012). These processes are closely related and involve many common regulatory mechanisms.

1.2.1 Apoptosis

This is an evolutionary conserved and precisely tuned mechanism of regulated death occurring in both physiological and pathological cell death instances, thus playing a pivotal role in maintaining cellular homeostasis (Henson et al., 2006) and various developmental processes (Brill et al., 1999) such as organogenesis and tissue architecture establishment (Meier et al., 2000) as well as immunity (Cohen et al., 1992). Apoptosis is a route of programmed cell death (PCD) including a set of morphologically diverse phenomena (as opposed to necrosis-associated processes) and was first reported

as a distinct mode of cell death by Kerr *et al.* (Kerr et al., 1972, Diamantis, 2008). It involves cell detachment and shrinkage, pyknosis (cellular volume reduction), pseudopodia retraction, chromatin condensation, nuclear fragmentation (karyorrhexis) and blebbing of the plasma membrane but minimal or no ultra-structural remodelling of the cytoplasmic organelles (Green, 2005). These processes facilitate cellular fragmentation and creation of membrane-enclosed structures, the apoptotic bodies, which are rapidly phagocytosed by adjacent or recruited cells in absence of triggering inflammatory responses (Khan, 2014). Principal biochemical features involve a set of energy-dependent processes like DNA breakdown, protein cleavage, mitochondrial dysfunction, display of phagocytic markers on the cell surface mediating phagocytic recognition and clearance, an increase in cytoplasmic Ca^{2+} concentration and phosphatidylserine externalisation (Elmore, 2007). The loss of mitochondrial potential and the concomitant lack of adenosine triphosphate (ATP) production elicit the permeabilization of the outer mitochondrial membrane. This event promotes the release of pro-apoptotic factors from the mitochondrial intermembrane space mediating the activation of inducer and effector protease-dependent cascades. These cysteine aspartyl proteases, the caspases, mediate the proteolytic cleavage of diverse cellular substrates (Martin et al., 1995).

Apoptotic deregulation is well-known to be causally linked to various diseases (Hetts, 1998, Mirkes 2001). Excessive apoptosis is one of the key drivers of neuronal loss observed in a wide range of neurodegenerative diseases such as Parkinson's and Alzheimer's, amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (Thompson, 1995). Apoptosis has also been reported to be involved in the progression of infectious diseases such as the acquired immunodeficiency syndrome (AIDS) in addition to heart disease. On the contrary, a deficiency in apoptotis can trigger autoimmunity, allograft rejection and contribute to cancer development. Therapeutic interventions targeting the restoration of either the apoptotic cell death induction or suppression of its excessive triggering hold great promise for the successful treatment of any associated diseases (Elmore, 2007).

1.2.2 Autophagy

This is an intracellular catabolic process involving the engulfment of cytoplasmic material and intracellular organelles though double-membrane vehicles, the

autophagosomes, and the fusion of the latter with lysosomes for the formation of autolysosomes which recycle the removed material by acidic hydrolases (Levine et al., 2008). Nutritional starvation or other environmental and hormonal reasons (trophic factor depletion, hypoxia, etc.) as well as various stress conditions and infections can trigger autophagic vacuolization thus eliciting degradation of intracellular organelles (mitochondria, endoplasmic reticulum, ribosomes) in order to ensure the supply of nucleotides, amino acids and fatty acids (Lin et al., 2015, Fitzwalter et al., 2015). Autophagy is present at low, constitutive levels in most mammalian cell types and therefore it is primarily considered as a rather cytoprotective process playing an important housekeeping role in the recycling of long-lived proteins or organelles. In particular, autophagy is critical for the homeostasis of post-mitotic differentiated cells, such as cardiomyocytes and neurons (Glick et al., 2010). However, excessive autophagy may be harmful as well. Data from genetic knock-out models supports the notion that suppression of autophagy can accelerate cell death (Vicencio et al., 2008). Silencing of key autophagy mediating genes such as BECLIN 1, autophagy protein 5 (ATG5), autophagy protein 10 (ATG10) and autophagy protein 12 (ATG12) evoked starvation-linked cell death in HeLa cells (Boya et al., 2005). On the contrary, pharmacologic inhibition of autophagy has been proven to block cell death in nerve growth deprived sympathetic neurons (Xue et al., 1999) and anti-estrogen treated human mammary carcinoma cells (Bursch et al., 1996). Additionally, silencing of ATG5, ATG7, and BECLIN 1 under apoptotic inhibition conditions has been effective in cell death blockage (Yu et al., 2004). It should be noted that Yoshinori Ohsumi received the 2016 Nobel Prize in Physiology or Medicine for his discoveries on the mechanisms of autophagy. According to his findings from experiments using mutated yeast cells which lacked vacuolar degradation enzymes, there appeared to be accumulation of small vesicles, the autophagic bodies, in the vacuole in response to starvation, thus proving the occurence of autophagy in yeast cells (Takeshige et al., 1992). Moreover, in his following studies he identified genes of critical importance in the autophagic pathway, therefore characterizing and elucidating the complex mechanisms of this cascade (Tsukada and Ohsumi, 1993, Mizushima et al., 1998, Ichimura et al., 2000).

1.2.3 Necrosis

Tis is a rather passive, uncontrolled cellular process provoked by an acute, overwhelming trauma and is characterized by oncosis (cellular volume increase),

swelling of organelles, such as the endoplasmic reticulum (ER) and the mitochondria, rupture of plasma membrane and concomitant release of intracellular contents in the extracellular space. Unlike apoptosis, the nucleus remains intact although distended. These events evoke further damage to the surrounding cells in addition of inducing a robust inflammatory response as well (Leist et al., 2001, Los et al., 2002). Necrosis is typically independent of caspase activation and does not include unique morphological characteristics. The necrotic signalling cascade involves inflammatory responses orchestrated by the release of various factors inluding: (i) the high mobility group protein B1 (HMGB1) and (ii) the hepatoma derived growth factor (HDGF) that triggers the sensitization of the NOD-like receptor family pyrin domain containing 3 (NLRP3) - a core protein of the inflammasome through production of ATP by the mitochondria of the necrotic cells (Iyer et al., 2009).

Necroptosis is a form of programmed necrosis, sharing several key processes with apoptosis. This more physiological and programmed type of necrotic death is present in neurodegeneration and death provoked by ischemia or infection and is also being triggered by the same apoptotic-inducing death signals (Nikoletopoulou et al., 2013). There is evidence that it is involved in the pathogenesis of traumatic brain injury, stroke, neurodegenerative diseases and brain tumour (Favaz et al., 2014). Necroptosis is induced when the pro-apoptotic signalling is blocked and this response is known to be orchestrated by (i) tumour necrosis factor types I (TNFR1) (Weinlich et al., 2016) and II (TNFR2) (Siegmund et al., 2016), (ii) death receptors including Fas (Vercammen et al., 1998) and (iii) TNF related apoptosis inducing ligand receptor types I (TRAILR1) and II (TRAILR2) (Jouan-Lanhouet et al., 2012). The cytoplasmic death domain of TNFR1, following the binding of ligand, binds with TNF-receptor-associated death domain (TRADD), RIPK1 and E3 ligases for the creation of complex I. The removal of ubiquitin from RIP1, results in the formation of complex II which is capable of triggering the apoptotic or necrotic pathway. For apoptotic induction, RIP1 and TRADD recruit FADD and pro-caspase-8, thus leading to its activation. Necroptosis occurs when RIP1 binds to RIP3 and activates it in the absence of FADD and pro-caspase 8 (Green and Llambi, 2015).

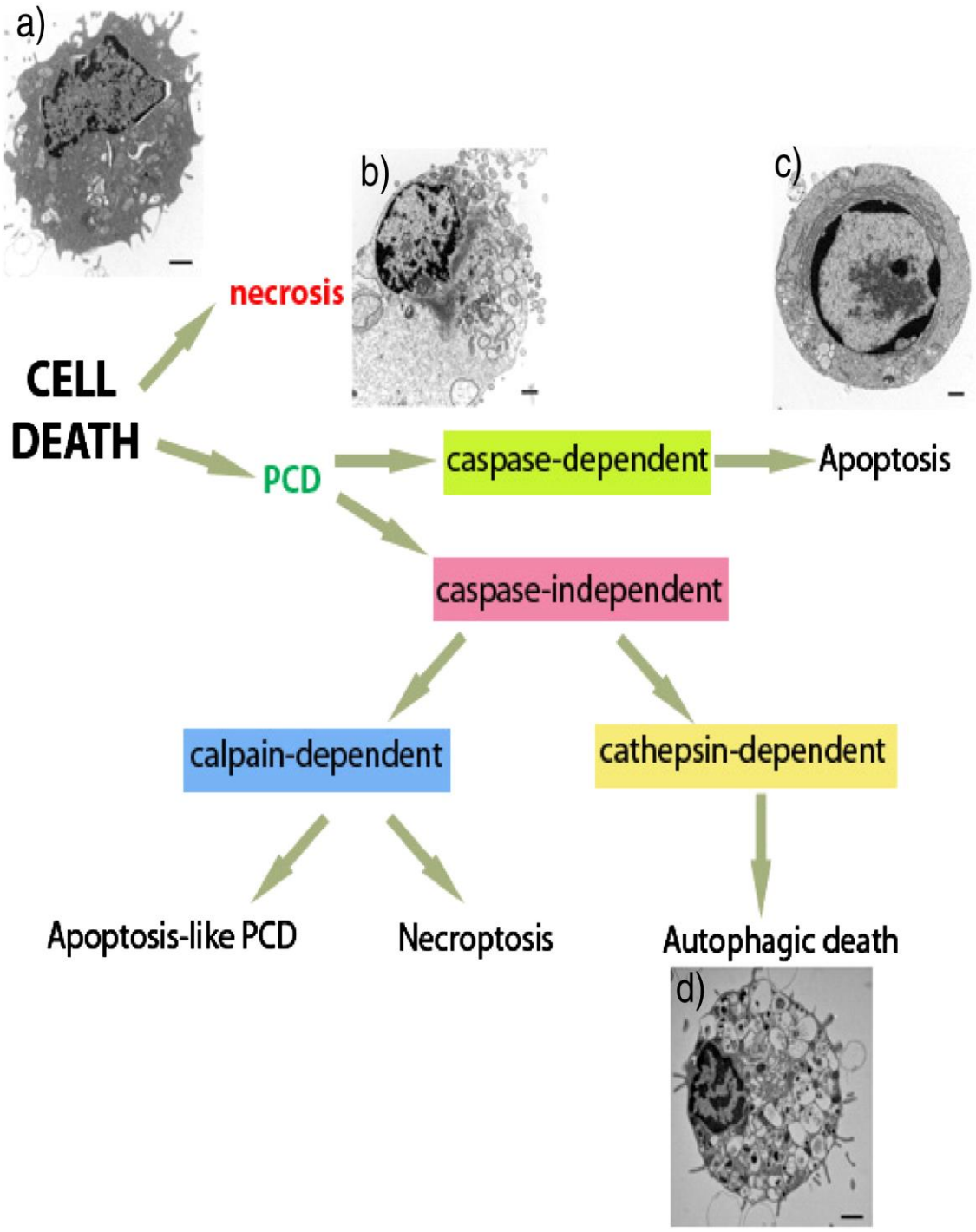


Figure 1.1 The different types of cell death and their morphological characteristics. a) A healthy cell, b) a necrotic cell, c) an apoptotic cell and d) an autophagic cell. PCD: Programmed Cell Death. The activation of different cell death pathways can be triggered by diverse stimuli on healthy cells (a) therefore causing characteristic changes on their morphological features. Necrotic cells (b) are characterized by the disruption of their plasma membrane, swelling of organelles, intact nucleus and cell lysis. On the other hand, characteristics of apoptosis (c) include the permeabilization of

mitochondrial membrane, fragmentation of the nucleus, cell shrinkage, pyknosis and formation of apoptotic bodies. The main feature of autophagy (d) is the formation of autophagosomes which help the cells degrade and recycle their components, however if it is excessive it can lead to the cell death activation. Adapted from (Nikoletopoulou et al., 2013).

1.3 Apoptotis and cancer development

Cancer is a complex disease characterized by the uncontrolled and aberrant accumulation of atypical cells. Additionally, the disease is accompanied by dentrimental defects in apoptotic signalling (Hetts, 1998) as they affect malignant transformation, tumour formation, progression and metastasis (Cotter 2009). The seminal report "Hallmarks of Cancer" by Hanahan and Weinberg has summarised the characteristics that confer survival and growth advantage to cancer cells during tumorigenesis. These include: (i) sustaining proliferative signalling, (ii) evading growth suppressors, (iii) resisting cell death, (iv) enabling replicative immortality, (v) inducing angiogenesis and (vi) activating invasion and metastasis" (Hanahan et al., 2000). Their updated report included (vii) reprogramming of energy metabolism and (viii) evading immune destruction as two additional emerging hallmarks of cancer (Hanahan et al., 2011).

Evasion of apoptosis arises from both the deregulation of the sensory machinery involved in intracellular and extracellular signalling but also from alterations in the pro-apoptotic signalling. Tumour protein p53 (TP53) is a tumour suppressor protein capable of activating the expression of various apoptotic-inducing genes and has a crucial role in the pathogenesis of cancer, tumor aggressiveness and resistance to chemotherapy. Both p53 and its effector murine double minute (MDM2) are capable of regulating the cell cycle arrest in response to DNA damage through upregulation of pro-apoptotic genes (B-cell lymphoma 2; Bcl-2 family members) and genes capable of generating free radical production (Polyak et al, 1997). Furthermore, p53 and MDM2 can activate DNA repair. Overall, p53 is mutated in 55-70% of human cancers (Levine et al., 2009) and it exerts its pro-apoptotic signalling via induction of the breakdown of the outer mitochondrial membrane (Mihara et al.,2003). The activation of oncogenes which mediate apoptotic inhibition such as Bcl-2 and Myc represents another mechanism linking apoptosis with oncogenesis (Strasser et al., 1990). Moreover, the presence of mutations in genes involved in apoptotic suppression confers resistance to anti-

neoplastic agents. Apoptosis-inducing compounds such as cisplatin (Barry et al., 1990), etoposide (Kaufmann et al., 1989) and 5-fluorouracil (Armstrong et al., 1992) have been widely used as the gold standard for the treatment of systemic tumours. A major challenge in enhancing the therapeutic outcome of these compounds is the clinical management of drug-resistant leading to disease recurrence due to positive selection of apoptosis-evading cells (Lowe et al., 2000, Pommier et al., 2004, Fernald et al., 2013).

1.4 Apoptotic signaling

Given the continuously growing interest for a better understanding of the role of the apoptotic deregulation in carcinogenesis, a great number of enzymes and cellular components participating in this complex process have been identified (Elmore, 2007, Plati et al., 2011). Extended research has indicated that there are two main apoptotic pathways: the extrinsic (or death receptor pathway) (Ashkenazi et al., 1998) and the intrinsic (or mitochondrial) pathway (Green et al., 1998). The signalling cascades in both pathways are divided into the following phases: initiation, integration and execution.

The extrinsic pathway is induced by an external ligand binding to its corresponding cell death receptor (DR) located on the cell surface, a process that leads to its activation and the resulting formation of an intracellular signalling complex. This receptor family consists of more than 20 members which belong to the tumor necrosis factor-α (TNF) receptor gene superfamily: (i) TNFR1 (Tartaglia et al.,1993), (ii) Fas (APO-1/CD95) (Dhein et al., 1995), (iii) TNF-related apoptosis inducing ligand (TRAIL/ Apo-2L) and its receptors 1 and 2 (MacFarlane et al.,1997, O'Rourke et al., 1997) and (iv) DR3 (TRAMP) (Kitson et al., 1996), DR4, DR5 (Adams, 2003). Binding of extrinsic ligands (TNF, Lymphotoxin, Fas, Tweak and Trail) to their respective receptors leads to conformational changes that reveal the intracellular death domains (DD) and provokes the binding of adaptor proteins such as Fas-associated death domain (FADD) and TNFR1-associated death domain (TRADD) thus resulting in the formation of the death-inducing signal complex (DISC) (Thorburn, 2004). This complex interacts and eventually activates initiator caspases-8 and -10 which, in turn, trigger the activation of effector caspases. Moreover, the cleavage of caspase-8 can lead to the activation of BH3-interacting domain death antagonist (BID), with the subsequent

formation of truncated BID (tBID) which is capable of facilitating further the cross-talk between the extrinsic and intrinsic pathways (Tait and Green, 2010).

The intrinsic pathway is initiated by intrinsic (non-receptor) death stimuli including (i) damage of an intracellular organelle (nucleus, ER, lysosome, Golgi apparatus, mitochondria), (ii) cellular stress signals (DNA damage, ischemia, increased intracellular Ca^{2+} concentrations, oxidative stress) or (iii) signalling from growth factors and cytokines. These signals activate the mitochondrial signalling pathway (Kroemer, 2007) by affecting the balance between pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family. The functional balance between the pro-apoptotic members of this family [such as Bcl-2-associated X protein (Bax), Bcl-2 associated death promoter (Bad), Bcl-2 homologous antagonist/killer (Bak)] and anti-apoptotic members Bcl-2 and Bcl-xL plays a critical role in inducing the acyivation of this apoptotic pathway. As a consequence, events changing this balance provoke alterations in the mitochondrial membrane integrity (e.g. mitochondrial permeability transition; MPT) (Kim et al., 2003) which in turn results in the formation of the mitochondrial-apoptosis induced channel (MAC) in the outer mitochondrial membrane that facilitates the release of apoptogenic factors (Mayer et al., 2003). These factors include the following: (i) cytochrome c, (ii) the second-mitochondria derived activator of caspases/direct inhibitor of apoptosis (IAP)-associated binding protein with low pI (SMAC/ Diablo) (Du et al., 2000, Adrain et al., 2001), (iii) endonuclease G (Li et al., 2001), (iv) the apoptosis inducing factor (AIF) (Lorenzo et al., 1999) and (v) the high temperature requirement A2 (Omi/ HtrA2) (Suzuki et al., 2001). As soon as cytochrome c is released in the cytoplasm, it binds to the adaptor protein apoptotic protease activating factor-1 (APAF-1) and along with pro-caspase-9 they form a protein complex called the apoptosome which cleaves pro-caspase-9 to its active form (caspase-9) and consequently activates the downstream effector caspase-3 (Zou et al., 1999). AIF and endonuclease G, upon nuclear translocation, elicit chromatin fragmentation and nuclear translocation. Furthermore, SMAC/Diablo and Omi/HtrA2 antagonize cytosolic IAPs namely c-IAP1, c-IAP2, survivin, X-linked IAP and deactivate them thus contributing to the apoptotic induction (Tait and Green, 2010).

Caspases are key players in the activation and execution phases of the apoptotic machinery and can be distinguished into initiators and effectors. Initiator caspases (-2, -8, -9 and -10) upon their response to various stimuli, are stimulated and cleave the inactive pro-forms of the effector caspases (-3, -7 and -6) which can subsequently

cleave other cellular substrates and thus trigger the apoptotic machinery (Green and Llambi, 2015). The targets of such proteolytic cleavage involve diverse cellular substrates including DNA repair enzymes [such as the poly-ADP-ribose-polymerase (PARP) (Lazebnik et al., 1994)], nuclear laminins (Lazebnik et al., 1995) and cytoskeletal proteins (Mashina et al., 1995). Caspase-independent forms of apoptosis have also been reported highlighting the role of other molecules (e.g. cathepsins) as cell death executioners (Leist et al., 2001). The complex interaction of internal and external signals from numerous proteins participating in the apoptotic cascade ultimately leads to the initiation of apoptosis. Nevertheless, the progression of the apoptotic cascade is difficult to orientate due to the cross-talk between all pathways (Hao et al., 2010).

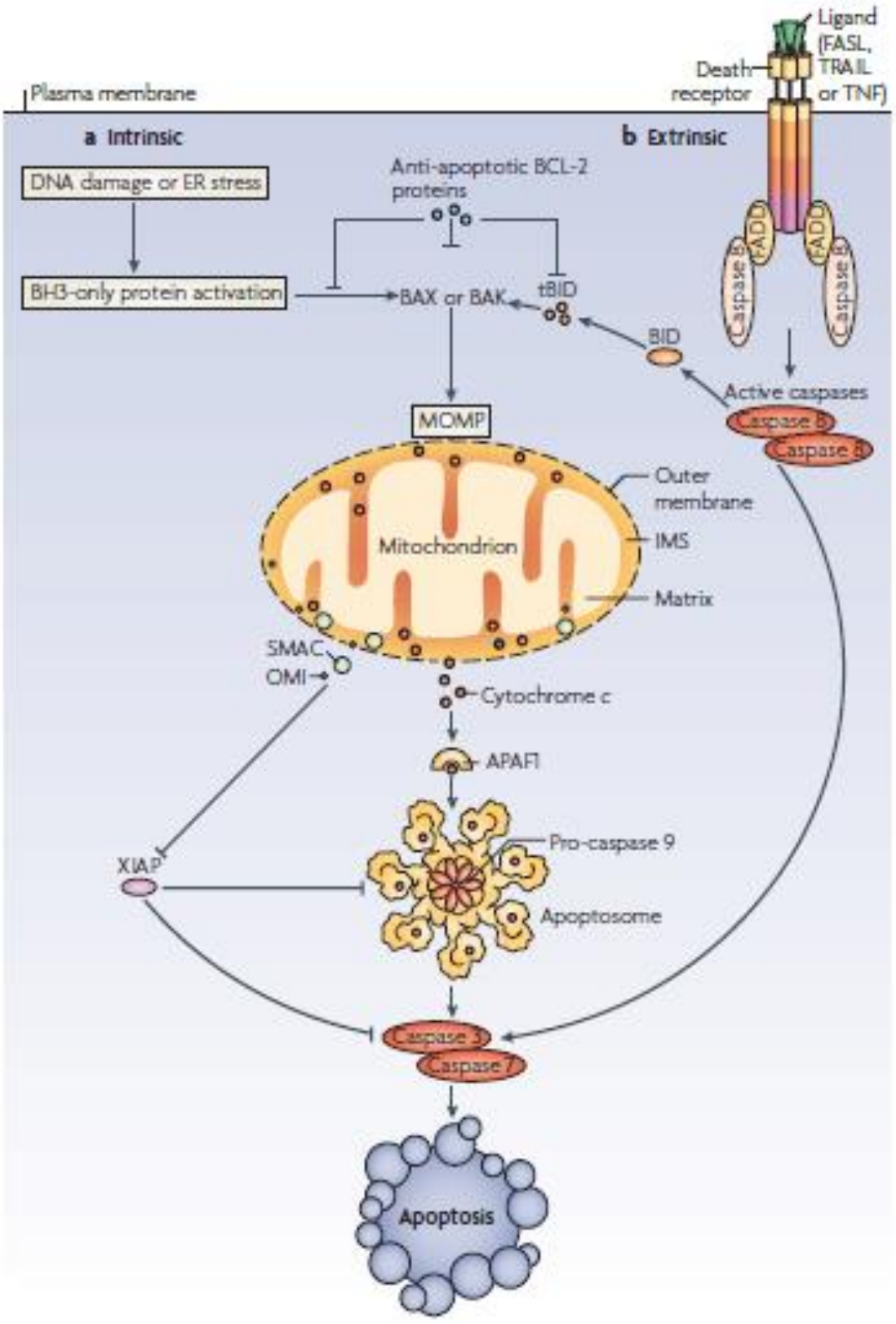


Figure 1.2 The extrinsic and intrinsic pathways of apoptosis. The intrinsic apoptotic pathway (a) is activated upon stimuli of intrinsic origin, such as DNA damage, endoplasmic reticulum stress, etc. and induces the activation of anti-apoptotic proteins of the BCL-2 family as well as pro-apoptotic proteins BAX and BAK, finally leading to

the permeabilization of the outer mitochondrial membrane (MOMP). Members of the anti-apoptotic family BCL-2 bind with active BAX or BAK, thus inhibiting MOMP. However, when the balance between pro- and anti-apoptotic proteins is disturbed, the initiation of the cascade is triggered. MOMP results in the release of cytochrome c along with numerous other proteins which induce apoptotic induction. The apoptosome is formed by the binding of cytochrome c with APAF1 and its following oligomerization and interaction with initiator caspase-9. Active caspase-9 then leads to the activation of executioner caspases-7 and -3. Furthermore, the release of SMAC and OMI from the mitochondrion prevents the action of XIAP which can inhibit apoptosis. The extrinsic pathway (b) is trigerred by the interaction of death receptors with their respective ligands which is followed by the formation of FADD domain and recruitment of initiator caspase-8. Subsequently, caspase-8 gets activated and can in turn cause the activation of caspase-3 and -7. Moreover, activation of caspase-8 can result in the cleavage of BID to tBID which is responsible for the cross-talk between the extrinsic and intrinsic pathways. Adapted from (Tait and Green, 2010).

In addition, the existence of another apoptotic pathway, the ER-mediated one has also been shown to be induced by various apoptotic stimuli. Overall, the endoplasmatic reticulum (ER) is a eukaryotic cellular organelle with a stress-sensing, biosynthetic and signalling role. ER is responsible for synthesizing, modifying and regulating the folding and secretion of proteins along with exporting and transferring them to their final destination (Sasaki et al., 2016). In addition, ER is involved in many cell housekeeping functions, besides folding of proteins, including Ca^{2+} regulation and lipid biosynthesis (Banhegyi et al., 2012, Csala et al., 2012). ER is a pool of molecular chaperone proteins including calnexin, calreticulin, the glucose-related protein 78/ immunoglobulin heavy-chain binding protein (GRP78/BiP or HSP5A), and protein disulfide isomerases (PDI) (Luo et al., 2013). ER-stress can be defined as the response of the organelle to stimuli like disruption of Ca^{2+} homeostasis, misfolding of proteins and abundant accumulation of unfolded ones. ER-stress has also been correlated with chronic pathological conditions, involving inflammation, including: diabetes, obesity, atherosclerosis, neurodegenerative diseases, inflammatory bowel syndrome, etc. (Zhang et al., 2008). Several studies have associated ER-stress with environmental or pathophysiological conditions like hypoglycemia, hypoxia, oxidative stress, ATP depletion, Ca^{2+} depletion, pathogens or inflammatory stimuli (Kaufman et al., 2002, Wang et al., 2014). Under such conditions, ER coordinates a signal transduction pathway, called the unfolded protein response (UPR) in order to maintain ER-

homeostasis and promote cell survival (Schröder et al., 2005, Wang et al., 2014, Tameire et al., 2015, Bhat et al., 2017). UPR activation involves the orchestration of adaptive mechanisms such as attenuation of protein synthesis, increased capacity for protein trafficking, folding and transport through the ER, proteolytic activity including ER-associated degradation (ERAD) and induction of apoptosis or autophagy. When ER-stress is triggered, cells respond by activating chaperone proteins responsible for degrading the excess misfolded proteins and/or assist them to re-fold and thus inhibit formation of protein aggregates. Firstly, mis-folded proteins bind to the chaperone GRP78/BiP (Ng et al., 1992, Kim et al., 1995, Yu et al., 1999, Leach et al., 2004). The reduced levels of unbound BiP exert a positive feedback loop mediating the transcription of BIP and other genes encoding chaperones (Kozutsumi et al., 1988, Dorner et al., 1989). Amongst chaperones, calnexin is a Ca^{2+}-binding protein located in the ER membrane and has the ability to keep newly synthesized glycoproteins inside the ER so that they are folded properly before reaching their final destination (Wada et al., 1991, David et al., 1993, Gelman et al., 1995). Protein disulfide-isomerase (PDI) catalyzes the formation of disulfide bonds (Ellgaard et al., 2005). However, in case of prolonged and severe mis-folded protein-induced stress, the activation of particular pathways results in the induction of the apoptotic machinery. To this end, data from studies using murine cell lines have shown that an important enzyme participating in the ER-mediated apoptotic pathway is caspase-12 (Nakagawa et al., 2000) which ultimately activates caspases-3 and -7 resulting in the triggering of cell death. Other studies have reported that caspase-4 (Bian et al., 2009, Sollberger et al., 2012) is a human counterpart of caspase-12 since it can be activated in a similar way during ER-stress. As part of a third group of caspases, including caspases-1, -5 and -12, caspase-4 has been linked to the inflammatory response according to some studies. Caspase-1, being the best characterized member of that group, is activated and is consequently responsible for the induction of the inflammatory response. Recent studies have demonstrated that caspase-4 plays an essential role on the activation of caspase-1 in keratinocytes, thus supporting its importance in inflammation (Martinon et al., 2004, Sollberger et al., 2012). Moreover, there is evidence suggesting a cross-talk between the mitochondria-initiated and ER-mediated apoptotic pathways, but the underlying mechanisms still remain unclear (Kadowaki et al., 2004, Zhang et al., 2006).

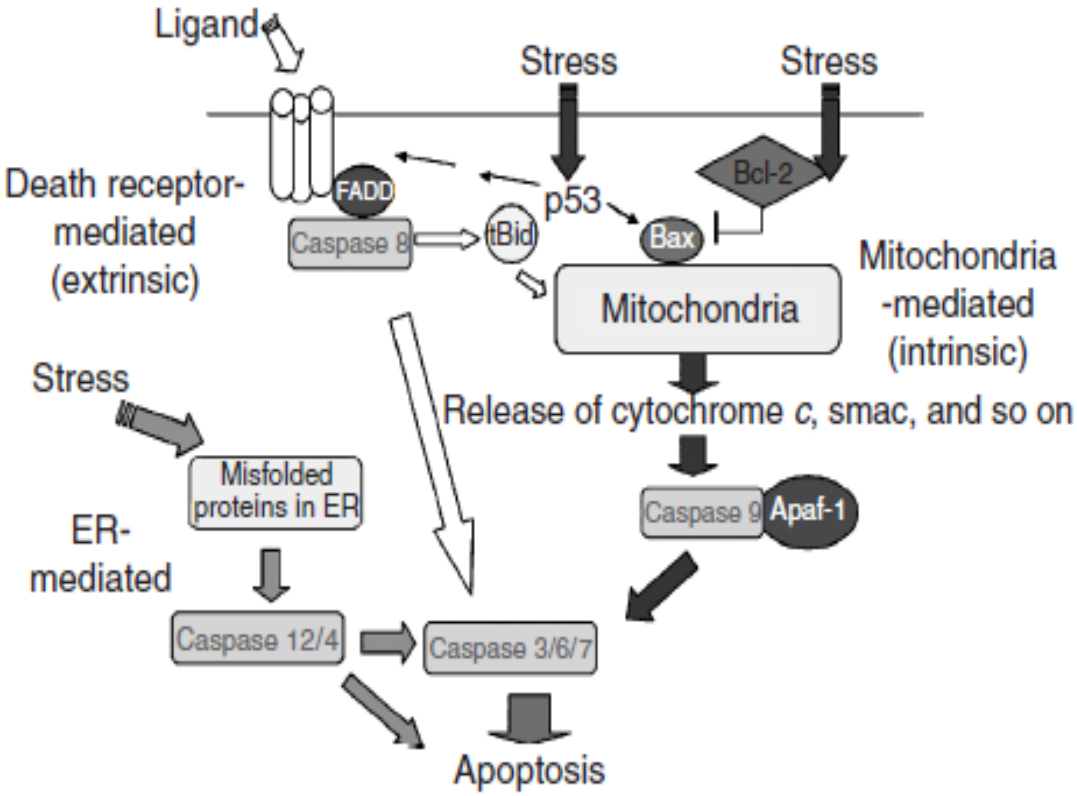


Figure 1.3 The interactions and cross-talk among the extrinsic, intrinsic and ER-induced apoptotic pathways. Activation of the extrinsic apoptotic pathway involves the interaction between death receptors with their respective ligands, formation of FADD domain and caspase-8 activation. tBID is cleaved and activated by caspase-8 while it facilitates the cross-talk between the extrinsic and intrinsic pathways. The induction of the intrinsic pathway includes cytochrome c release from the mitochondrion in response to various stress factors that cause an imbalance between pro- and anti-apoptotic proteins. Caspase-9 is then activated following interaction with APAF1. ER stress causes the accumulation of mis-folded proteins which leads to the activation of caspase-4 and -12, thus triggering the ER-mediated pathway. All three apoptotic pathways finally result in the activation of executioner caspases -3, -6 and -7 leading to apoptosis. Adapted from (Shellman et al., 2008).

1.5 The unfolded protein response (UPR) signalling pathways

UPR involves three signalling cascades: (i) the PRKR-like ER kinase (PERK/ eIF2AK3) eukaryotic translation initiation factor 2α (eIF2α), (ii) the inositol-requiring protein 1α (IRE-1α/ ERN1)-X-box binding protein (XBP1) and (iii) the activating transcription factor 6α (ATF6α). (Fig.1.4).

1.5.1 The PERK–eIF2α pathway

PERK is a type I transmembrane protein residing at mitochondria-associated ER membranes (MAMs) (Verfaillie et al., 2012). In the absence of ER-stress, heat shock protein 90 (HSP90) and BiP bind to PERK in order to prevent its activation (Marcu et al., 2002). Under ER-stress conditions, the BiP-unfolded/misfolded proteins conjugates permits the release of PERK, enabling its homo-trimerization and auto-phosporylation and therefore its activation (Bertolotti et al., 2000, Liu et al., 2000). Then, activated PERK phosphorylates the eukaryotic translation initiation factor 2 α (eIF2α). This, in turn, is a subunit of the heterotrimeric eIF2 complex whose phosphorylation can exert transient abrogation of general protein synthesis (Harding et al., 2000). This event causes polysome disassembly and subsequent availability of ribosomes that can bind newly transcribed mRNAs that encode UPR adaptive functions. Furthermore, PERK is capable of affecting cell survival, function and differentiation through interaction with nuclear factor erythroid 2-related factor 2 (NRF2/ NFE2L2) (Cullinan et al., 2003), forkhead box O (FOXO) (Zhang et al., 2013) and diacylglycerol (Bobrovnikova-Marjon et al., 2012). In addition, membrane fluidity influences PERK oligomerization and activation (Volmer et al., 2013). The PERK- eIF2α activation promotes expression of ATF4/CREB2, ATF5 and amino acid transporters (Yaman et al., 2003). ATF4 promotes cell survival through activation of ER-stress response mediators such as the DNA damage-inducible protein (GADD34/ PPP1R15A). GADD34 in turn dephosphorylates eIF2α and re-establishes global mRNA translation. ATF4 also initiates transcription of C/EBP homologous protein (CHOP/ DDIT3/ GADD153) (Palam et al., 2011) which participates in ER-stress-mediated apoptosis (Marciniak et al., 2004, Song et al., 2008).

Under chronic stress conditions, eIF2α is phosphorylated constitutively, thus resulting in the attenuation of the IRE1α–XBP1 and ATF6α pathways as well as subsequent pro-apoptotic signalling (Scheuner et al., 2006). Depending on the severity of stress, PERK stimulation promotes either adaptive or apoptotic responses varying

amongst cell types and environment, thus setting a particular threshold for ER-stress tolerance. CHOP has been shown to promote cell death through repression of BCL-2 expression (McCullough et al., 2001), up-regulation of BCL-2-interacting mediator of cell death (BIM/ BCL2L11) (Puthalakath et al., 2007) and BAX translocation to mitochondria (Szegezdi et al., 2006). Furthermore, CHOP is able to induce expression of p53 up-regulated modulator of apoptosis (PUMA/ BBC3) (Galehdar et al., 2010), lipocalin 2 (LCN2) (Hsin et al., 2012), tribbles homologue 3 (TRIB3) (Ohoka et al., 2005) and death receptor 5 (DR5/ TNFRSF10B) (Zou et al., 2008). The latter mediates ER stress-induced apoptosis via caspase-8 in cancer cells (Lu et al, 2014). Recent findings support the notion that CHOP coupling with ATF4 and CHOP-mediated protein synthesis serve as stimulus for ER-stress induced apoptosis through aggregation of mis-folded proteins and oxidative stress (Malhotra et al., 2008, Han et al., 2013). However, it cannot be excluded that tumor cells evade the indirect pro-apoptotic signalling of CHOP and exploit diverse pro-survival pathways that are activated downstream of CHOP. Mutations in CHOP have been reported in human tumors, although their impact on protein expression or function and tumorigenesis in general remains to be elucidated (Kan et al., 2010).

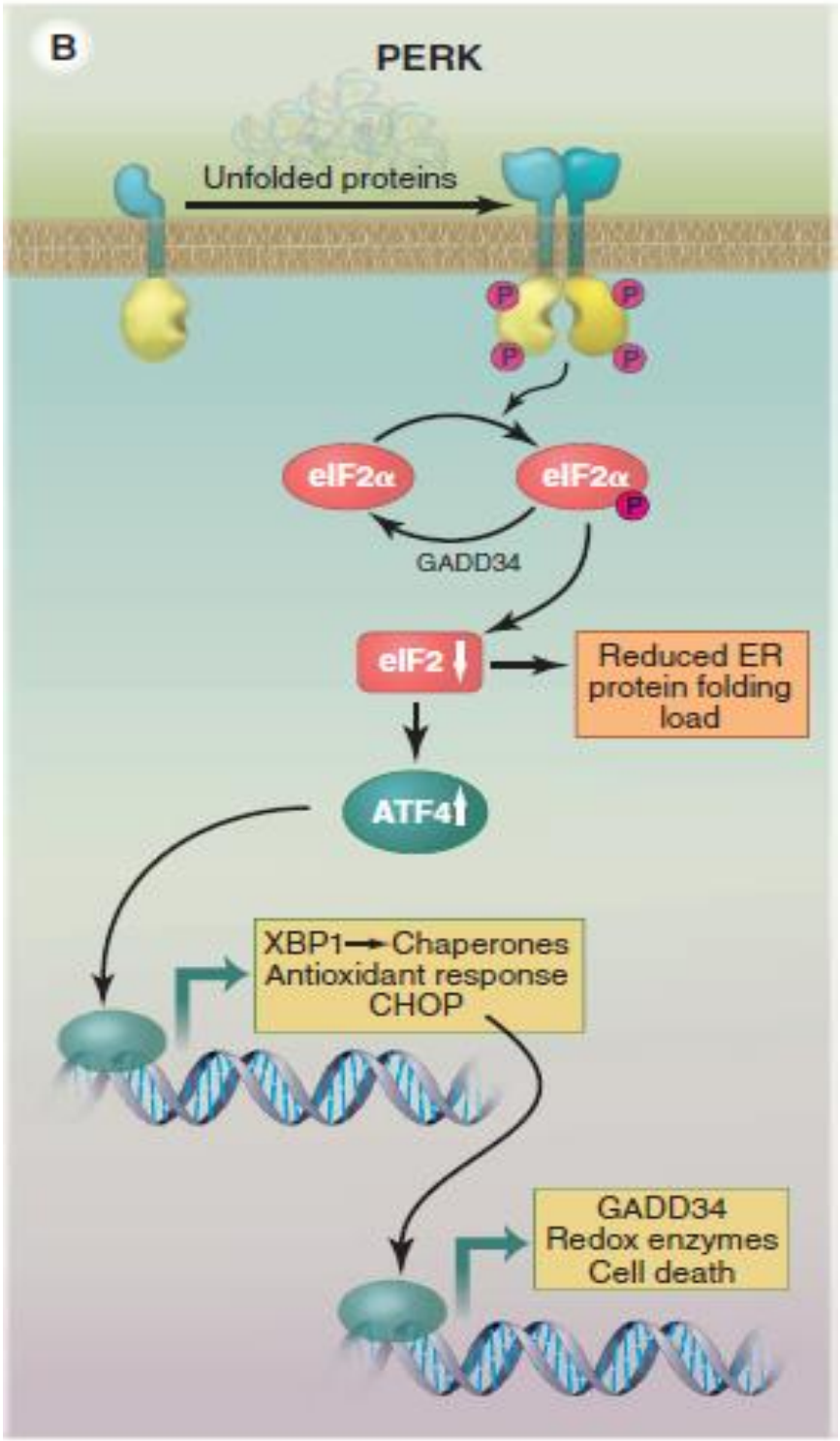


Figure 1.4 The PERK–eIF2α pathway. PERK oligomerizes in response to ER stress and causes the phosphorylation of eIF2α leading to its inactivation and inhibition of mRNA translation. Increased production of ATF4 results in induction of CHOP and GADD34 leading to the activation of cell death. Adapted from (Walter and Ron, 2011).

1.5.2 The IRE-1α- XBP1 pathway

IRE-1α is a type I transmembrane protein ubiquitously and constitutively expressed. In the absence of ER-stress conditions, HSP90 and HSP72 bind to IRE-1α and retain its stability (Gupta et al., 2010). IRE-1α serves also as a target for BiP. Upon ER stress, unfolded and mis-folded proteins bind to BiP. This event results in IRE-1α release and subsequent oligomerization, autophosphorylation and activation of its kinase and endoribonuclease activities. Activated IRE-1α regulates the expression of the transcriptionally active form of XBP1 (XBP-1s) that moves to the nucleus and acts as an effector of the target genes (Lee et al., 2002, Calfon et al., 2002). Localization of the un-spliced Xbp-1u mRNA accelerates the response through interaction with IRE-1α (Yanagitani et al., 2009). Genes that are regulated by this UPR signalling branch resolve protein mis-folding through enhanced protein folding, trafficking and ERAD (Shaffer et al., 2004). XBP-1s expression prevents CHOP expression and thus promotes cell survival by facilitating the adaptation of cells to stress conditions (Guo et al., 2012).

Sustained ER-stress attenuates IRE-1α activation via dephosphorylation, ubiquitylation and degradation (Ishiwata-Kimata et al., 2013, Qui et al., 2013). An ER-resident protein disulphide isomerase family A member 6 (PDIA6), which is required for IRE-1α activation (Groenendyk et al., 2014), restricts IRE-1α signaling under chronic ER-stress conditions (Eletto et al., 2014). Additionally, XBP-1u promotes degradation of XBP1 and ATF6α pathways, thus blocking survival signals (Yoshida et al., 2009). On the other side, prolonged activation of IRE-1α triggers pro-apoptotic signaling and cleaves XBP1 (Tirasophon et al., 2000, Mishiba et al., 2013) through a process called IRE1-dependent decay (RIDD) (Hollien et al., 2006). The activation of IRE-1α kinase is followed by binding of the TNF receptor-associated factor 2 (TRAF2) that recruits apoptosis signal-regulating kinase 1 (ASK1/MAP3K5) and JUN N-terminal kinase (JNK) (Urano et al., 2000). These events mediate BIM activation and suppression of BCL-2. Loss-of-function mutations of IRE-1α and XBP1 have been identified in human cancers and are expected to be linked with reduced kinase/ endoribonuclease activity, suggesting a tumor-suppressive role for IRE-1α and XBP1 (Leung-Hagesteijn et al., 2013). However, research on human triple-negative breast cancers revealed increased XBP1 splicing, suggesting a necessity for XBP1 activation in cancer stem-cell like populations (Chen et al., 2014).

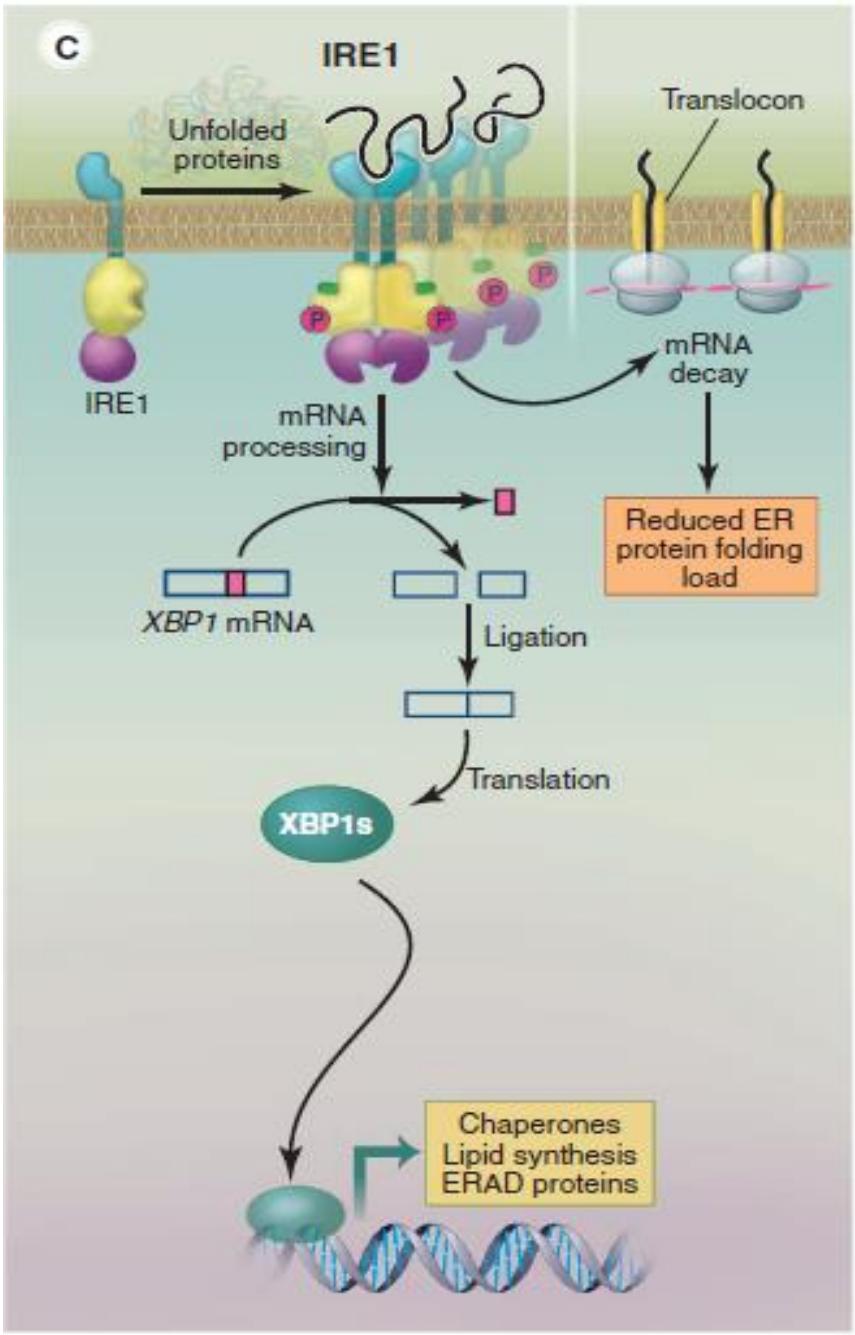


Figure 1.5 The IRE-1α- XBP1 pathway. IRE1 oligomerizes in response to ER stress and when active it cleaves XBP1 mRNA at two specific positions, thus excising an intron. Ligation of the resulting exons leads to the translation of XBP1s which regulates the expression of genes associated with stress conditions. Adapted from (Walter and Ron, 2011).

1.5.3 The ATF6 pathway

ATF6 is a type II transmembrane protein that resides in the ER through interaction with BiP during non-stress conditions. Formation of mis-folded protein aggregates results in the release of ATF6, translocation to the Golgi apparatus and initiation of a process regulating intramembrane proteolysis, generating the cleaved ATF6α, an active transcription factor (Ye et al., 2000). ATF6α promotes the transcription of genes enhancing ER capacity and the expression of XBP1, thus affecting the adaptive response to protein mis-folding (Yoshida et al., 2001, Wu et al., 2007). Its homologue, ATF6β suppresses ATF6α-mediated transcription and activity (Guan et al., 2009). Interestingly, PERK–eIF2α signalling promotes ATF6α synthesis (Teske et al., 2011). The potential role of ATF6α in apoptosis has not been elucidated yet. Missense mutations in ATF6 have been reported for susceptibility to malignant transformation (Wu et al., 2014). Moreover, BiP, a downstream transcriptional target of ATF6α is overexpressed in many human cancers. Although BiP is localized in the ER under normal conditions, it has been detected on the cell surface of malignant cells (Arap et al., 2004). BiP is widely used as malignancy marker for histological grading of neoplasms and has attracted interest as useful prognostic marker and therapeutic target (Lee et al., 2007).

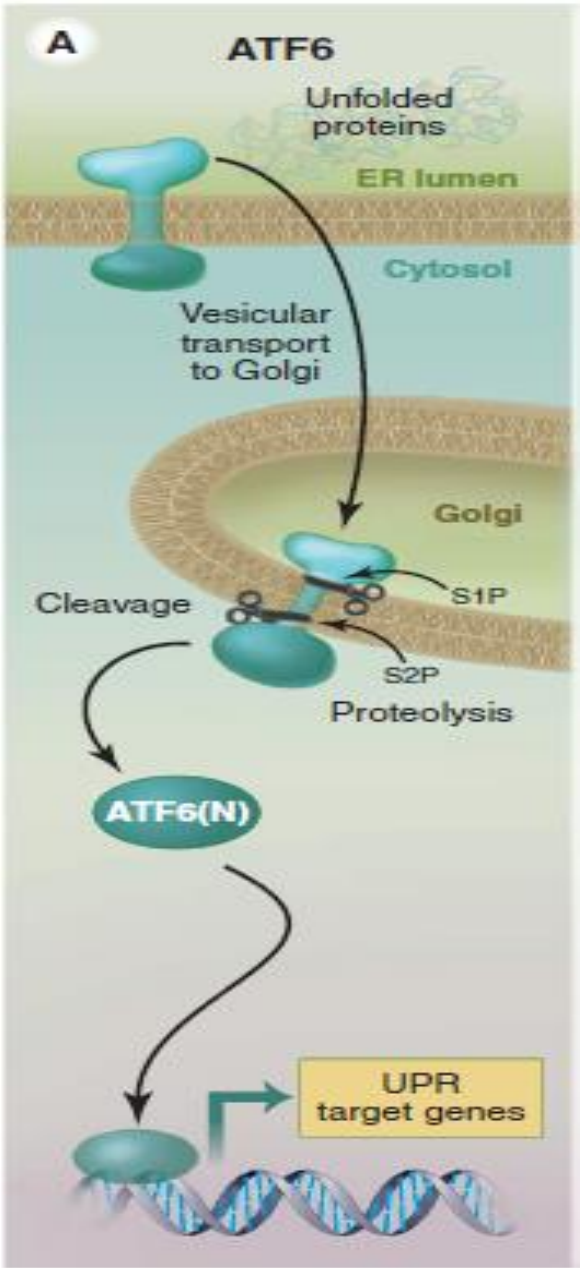


Figure 1.6 The ATF6 pathway. ATF6 resides in the ER as a transmembrane protein and gets transferred to the Golgi apparatus via transport vesicles in response to the accumulation of unfolded proteins due to ER stress. There it gets cleaved by S1P and S2P, therefore generating ATF6α (or ATF6(N)) which then activates the expression of UPR associated genes after moving to the nucleus. Adapted from (Walter and Ron, 2011).

1.6 ER-stress nd UPR activation in cancer developement

Both ER-stress and UPR activation have been documented in oncogenic transformation, cancer development and even chemoresistance associated with various cancer types. Apart from its pro-survival function, unresolved or severe ER-stress leads to cell death, rendering UPR as a target of therapeutic interest.

Various intrinsic factors mediate UPR activation in transformed cells. Hyperactivation of oncogenes or loss-of-function mutations in tumor suppressor genes can result in increased protein synthesis and ER trafficking with the subsequent stimulation of UPR (Ozcan et al., 2008, Kang et al., 2011). Additionally, intrinsic mis-folding provokes UPR activation (Marada et al., 2013). Highly secretory tumor cells are prone to constitutive UPR activation in order to respond to their increased biosynthetic needs (Babour et al., 2010, Mahadevan et al., 2011).

On the other hand, extrinsic factors such as the establishment of a hostile environment for malignant cells can implicate abrogated protein folding resulting in UPR activation. ER stress and UPR activation (i) stimulate angiogenesis, (ii) exert a cytoprotective effect on hypoxic or glucose-/nutrients-deprived cancer cells, (iii) suppress anti-angiogenic factors, such as thrombospondin 1 (THBS1), CXC chemokine ligand 14 (CXCL14) and CXCL10 and (iv) triggers the expression of pro-angiogenic factors like vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2), IL-1β, IL-6 and IL-8. The endothelial cells in the tumor surrounding area undergo ER stress and UPR activation due to VEGFA (Karali et al., 2014) or the accumulation of mis-folded proteins (Hu et al., 2006, Dong et al., 2008).

To summarize, UPR activation can confer cytoprotective as well as deleterious effects to tumor cells and consequently facilitates or suppresses tumorigenesis respectively. Thus, UPR signalling may be targeted as a potentially promising cancer treatment strategy. To date, various ER-stress targeting approaches have been attempted. For instance, there seems to be great potential in the administration of proteasome inhibitors. These molecules aim for the chymotrypsin-like subunits in both the constitutive proteasome and the immuno-proteasome and thus resulting in the accumulation of ubiquitylated proteins leading to ER stress-induced apoptosis. Bortezomib was the first FDA-approved proteasome inhibitor for the clinical management of multiple myeloma and mantle cell lymphoma and it functions as an ER-stress inducer in highly secretory cancer cells. However, in these patients, low levels of

XBP1, XBP-1s, ATF6α and/or loss-of-function mutations in IRE1α or XBP1 are associated with a poor response to the drug (Ling et al., 2012). However, this finding does support the notion that UPR activation can also function as a prognostic indicator of therapeutic outcome. Finally, a plethora of other phase-1/-2 compounds also act by suppressing BiP (BiP inhibitors) or disrupting HSP90 function (Corazzari et al., 2017).

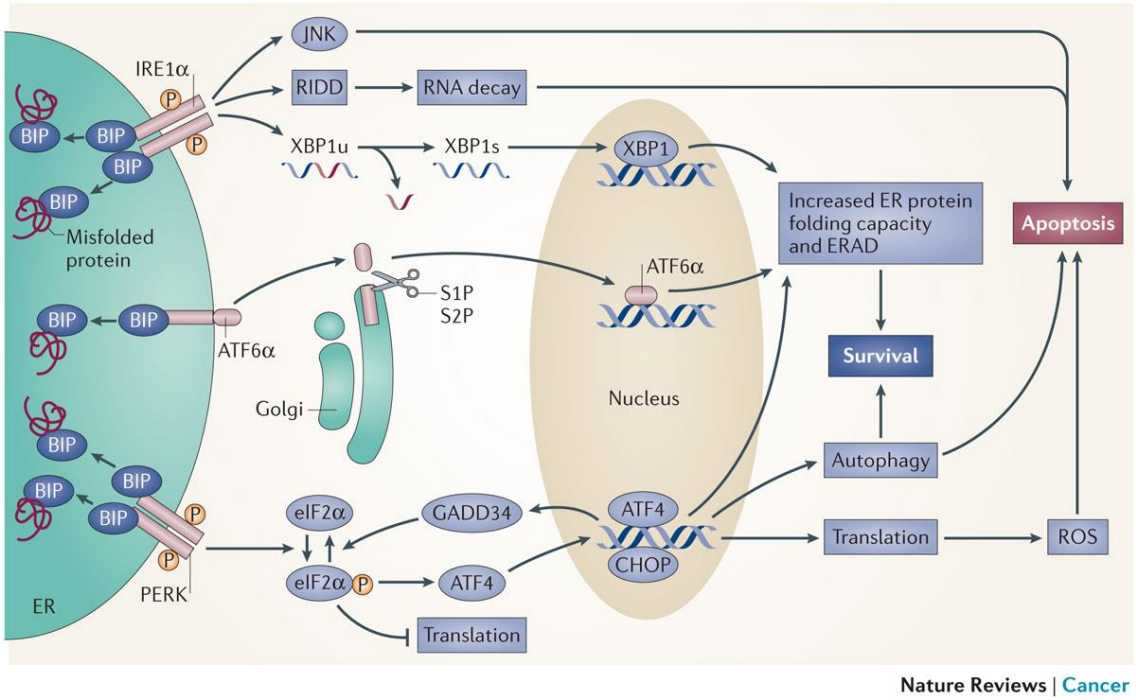


Figure 1.7 The different unfolded protein response (UPR) pathways. The three different signal transducers (IRE1α, ATF6α and PERK) sense the accumulation of unfolded and misfolded proteins in the ER and regulate the induction of various transcription regulators which lead to the translation of UPR related genes after moving to the nucleus. The expression of specific genes results in either cell survival or death. Adapted from (Wang et al,. 2014).

1.7 Hyperthermia

This is defined as the application of exogenous heat induction for treating a wide range of medical conditions including malignancies. Over the past few decades, hyperthermia raised a great interest as a therapeutic modality given that numerous studies aimed to understand the underlined mechanisms of its beneficial therapeutic effects. In principle, the application of exogenous heat results in either directly killing tumor cells, when applied alone (i.e. monotherapy), or sensitizing them against other

therapeutic means such as radiation, chemotherapy, etc. (i.e. adjuvant therapy) (Bettaieb et al., 2013). Mainly, there are two strategies of applying hyperthermia: (i) conventional hyperthermia (at temperatures ranging from 39-45^{o}C) and (ii) ablation therapy (at temperatures over 70^{o}C). The application of heat can be focused on a small area (such as a tumor) in local hyperthermia whereas larger areas of tissue (such as an organ or limb) are heated in regional hyperthermia or even in whole body in cases where metastases have been wide spread. There is a range of methodologies utilized in applying hyperthermia and include water baths and pads, ultrasound, radio frequencies, microwaves, lasers, etc. The great interest in finding new and better means of delivering hyperthermia has resulted in designing more efficient technological platforms and delivery systems with the additional benefit of providing a more accurate control of temperature (Wust et al., 2002).

Early studies have shown that hyperthermia, when applied in combination with radiation therapy (i.e. adjuvant therapy) exerts positive effects in the context of a cancer therapy (Robinson et al., 1974, Gerner et al., 1975). Therefore, during the last decades there has been a rising increase in the number of studies investigating hyperthermia's therapeutic potential on various cancer types (e.g. mammary carcinoma, prostate cancer, osteosarcoma, glioma, melanoma, etc.) by utilizing various *in vitro* as well as *in vivo* models. However, it should be noted that the majority of them use hyperthermia in the context of an adjuvant therapy in combination with a wide range of other regimens (Ito et al., 2003, Garcia et al., 2012, Zhang et al., 2016, Hou et al., 2014). Overall, results from numerous studies have shown that hyperthermia (in the range of 41-47^{o}C) is capable of exerting different effects including: (i) direct cytotoxicity of tumor cells, (ii) modifications in the tumor microenvironment, (iii) induction of heat shock proteins, (iv) alterations in the immune response, (v) stimulation of necrotic and apoptotic cascades, (vi) changes in cell cycle related signaling pathways (vii) changes in blood flow, oxygen and nutrient delivery to the site of tumor and (viii) enhancement of therapeutic outcome when applied in combination with other regimens (Hildebrandt et al., 2002, Dewhirst et al., 2005, Dewhirst et al., 2016).

1.7.1 Alterations in cellular homeostasis

The physiological temperature which allows cells to function and proliferate ranges from 36.5-37.5^{o}C therefore hyperthermia is responsible for triggering several

changes within the cell and results in the loss of homeostasis (Hildebrandt et al., 2002). Synthesis of DNA, RNA and proteins is inhibited at temperatures higher that 42.5°C (Roberts et al., 1985). In addition, elevated heat induction appears to lead to cell cycle arrest, denaturation of proteins, enhanced degradation of aggregated and mis-folded proteins, changes in cellular metabolism (due to reduced levels of ATP) and cytoskeletal structure as well as alterations in the permeability of the cell membrane (Sonna et al., 2002). Early studies have shown that hyperthermia was capable of inducing the generation of DNA breaks in CHO cells (Warters and Henle, 1982). Results from another group demonstrated that there was an increase in mis-rejoined and non-repairable double-stranded DNA breaks together with a reduction in the levels of successfully completed DNA repair after hyperthermia combined with irradiation in human fibroblasts. Therefore, it was suggested that hyperthermia affects the observed elevation in rates of DNA breaks by slowing down DNA repair mechanisms (El-Awady et al., 2001). Moreover, data from several reports demonstrated that there was an increase in γH2AX foci, which indicate the presence of double-stranded DNA breaks, as a response to heat treatments and it appeared to depend on the duration of treatment (Takahashi et al., 2004). In addition, elevated heat has an impact on DNA repair cascades as it appears to hinder the process of DNA replication by inhibiting the activity of DNA polymerases, which eventually results in the detection of increased levels of DNA breaks (Jorritsma et al., 1986, Raaphorst and Feeley, 1994). Denaturation and aggregation of nuclear proteins in response to hyperthermic exposures has an impact on the progression of DNA, RNA and protein synthesis by obstructing access to DNA due to modifications in chromatin (Lepock, 2004). Elevation of heat is capable of affecting the plasma membrane of cells as it causes changes in its fluidity while it also interferes with transmembranal transport proteins and receptors (Bates et al., 1985, Coss and Linnemans, 1996). This, in turn, can lead to changes in membrane functions including those of the sodium-potassium pump and calcium levels (Bates et al., 1985, Vidair and Dewey, 1986). Heat induction has been associated with elevated generation of ROS (Ahmed et al., 2015). According to a number of studies, elevated heat can cause changes in the cellular redox status by affecting the membrane of the mitochondrion thus leading to the induction of DNA damage (Dressler et al., 2006, Wang et al., 2013). In addition, the cytotoxicity of hydrogen peroxide was enhanced when combined with hyperthermia (Lord-Fontaine and Averill, 1999) thus suggesting that an increased production of ROS can result in oxidative damage to lipids, proteins and DNA.

1.7.2 Cytotoxicity

There is an extensive number of studies reporting hyperthermia's effects in inducing cell death by activating the necrotic or apoptotic cascade all of which appear to be dependent on temperature, duration of exposure and cell type (Ahmed et al., 2015). An early study using CHO cells demonstrated that hyperthermia was able to cause a decrease in cell viability which was dependent on the phase of cell cycle and appeared to be attributed to the denaturation of cellular proteins (Westra and Dewey, 1971). At higher hyperthermic conditions (>50^{o}C) cells become destructed due to the immediate denaturation of proteins, irreversible damage in structural proteins and major chemical changes within the cellular environment (Diederich, 2005). On the contrary, milder hyperthermic conditions (<45^{o}C) are capable of delaying and preventing the progression of several cellular processes thus making cells more vulnerable against other regimens such as chemotherapy and radiation (Hildebrandt et al., 2002). In any case, cells are capable of activating specific mechanisms (such as the heat shock response) in order to protect themselves against such damage. Consequently, heat shock proteins (HSPs) are produced in order to facilitate the correct folding (and thus protection) of denatured proteins (Sonna et al., 2002). Still, in cases of prolonged heat stress, cells might not be able to overcome the denaturation and aggregation of these proteins which can then result in the stimulation of apoptotic cell death (Ahmed et al., 2015).

As previously mentioned, hyperthermia-induced cytotoxicity is enhanced against cancer cells. The majority of studies conclude that apoptotis is the main form of cell death triggered under conditions of moderate heat stress. Most of these studies have aimed to characterize the activation of the apoptotic cascade, in response to hyperthermia, and so have provided evidence highlighting the role of the intrinsic apoptotic pathway. In fact, experiments in Jurkat cells demonstrated that initiator caspase-9 plays a crucial role in hyperthermia-induced apoptotic activation while tBid is involved in the process of cytochrome c release (Shelton et al., 2010). Caspase-2 has also been implicated in the initiation of the apoptotic cascade under hyperthermic conditions (Tu et al., 2006) and was also associated with Bid cleavage (Bonzon et al., 2006). Recently, the interaction between pro-apoptotic protein Bim with Bak and Bax has been demonstrated to participate in the activation of the intrinsic pathway as a response to heat induction (Mahajan et al., 2014). Apart from triggering the intrinsic apoptotic pathway, hyperthermia has been suggested to affect the extrinsic pathway as well. In fact, the depletion of c-FLIP due to hyperthermia results in the activation of

death receptors and subsequently caspase-8 (Morle et al., 2015). Furthermore, heat induction has been reported to stimulated the ER-stress mediated apoptotic pathway by increasing calcium levels (Hou et al., 2014) and activating caspases-4 and -12 (Shellman et al., 2008). Finally, the activation of caspase-3 mediates the completion of apoptotic death induction (Shelton et al., 2010, Shellman et al., 2008).

Because of its interference in several cellular processes, hyperthermia has been shown to potentiate the cytotoxic effects of various therapeutic strategies, such as radiation or chemotherapy when applied in combination as an adjuvant therapy (Wust et al., 2002). Several reports have addressed hyperthermia's ability to act as a sensitizer to radiation for treating various types of cancer (Horsman and Overgaard, 2007, Pandita et al., 2009). Moreover, numerous studies have shown an enhancement in the cytotoxic effects of a wide range of chemotherapeutic agents when they are administered in combination with hyperthermia (Hildebrandt et al., 2002, Mallory et al., 2015). However, the exact underlying mechanisms of hyperthermia's potentiation effects still remain largely uknown.

1.7.3 Physiological changes

The sensitivity of cells against increased temperatures varies considerably amongst different tissues. Tumor cells display an increased sensitivity against hypethermia compared to normal tissue whilst specific regions within the same tumor might exert variable cytotoxicity at different temperatures. Delayed cell cycle progression (from phase G0/G1 to S-G2/M) (Yuguchi et al., 2002), glucose deprivation (Lord-Fontaine et al., 2002), differential expression of heat shock-induced proteins (HSPs) and relevant pathways and fluctuations in cellular ATP levels (Oleson et al., 1988) have all been observed when cancer cells were exposed to heat induction. Poor vascularization of the -frequently hypoxic- core of the tumor has been associated with limited capability to dissipate high temperature by circulating blood (Song et al., 1993, Wike-Hooley et al., 1984, Fokas et al., 2012). This altered tumor vascularization allows the use of hyperthermia as a strategy to confer targeted nutrient and oxygen deprivation resulting in interstitial pH decrease and concomitant tumour vasculature breakdown (Song, 1982). These traits have been taken into consideration for the development of combinational treatment approaches (involving hyperthermia and radiation) in order to overcome chemoresistance and promote synergistic anti-proliferative effects (Bogovic

et al., 2001). Mild hyperthermia (39-42^{o}C) is applied on heat-induced radiosensitization strategies, as it results in increasing blood flow around the tumor site in order to render it less hypoxic and thus more susceptible to radiotherapy and/or chemotherapy (Iwata et al., 1996, Song et al., 2001). In addition, hyperthermia has also been adapted as a stimulus aiming to enhance immune surveillance and restrain tumor growth (Wang et al., 2005, Calderwood et al., 2009, Peer et al., 2010). The elevation in temperature leads to increased levels of HSPs but also higher levels of intracellular tumour antigens (Mace et al., 2012, Toraya-Bron et al., 2014) that are taken up by dendritic cells and macrophages thus inducing specific anti-tumor immunity (Frey et al., 2012). The thermal regulation of immune effector cells has been proven to additionally favour: (i) immune cell migration and transfer to target sites, (ii) trigger the release of pro-inflammatory cytokines (TNF, IL-1, IL-6, IL-10, IL-12), (iii) control immune cell growth and (iv) increase the cytotoxic effects of immune cells against malignant ones. Finally, hyperthermia utilization in combination with gene therapy has increased the efficacy of antibodies and drugs targeting different types of tumors (Mallory et al., 2016).

1.8 Hypothesis and Specific Aims

The working hypothesis of our study was that hyperthermia can induce cell death pathways and therefore can exert a therapeutic effect as a mono- and/or adjuvant therapy in malignant melanoma.

To investigate our hypothesis, we focused on characterizing the underlying mechanisms of hyperthermia–induced cell death as the basis of its therapeutic effectiveness in a human *in vitro* and a rodent *in vivo* melanoma skin models.

The specific aims of the project were the following:

(1) To determine the involvement of the apoptotic cell death pathways and reveal the underlined mechanisms in regulating the therapeutic effectiveness of hyperthermia in an *in vitro* model of human malignant melanoma.

(2) To determine key apoptotic genes as targets of regulating the hyperthermia-induced apoptotic cell death response in an *in vitro* model of human malignant melanoma.

(3) To determine hyperthermia's efficacy in potentiating the therapeutic effectiveness of non-targeted (Dacarbazine and a novel palladium-based chemotherapeutic agent) versus targeted (Dabrafenib and Vemurafenib) pharmacological approaches when utilized as an adjuvant therapy in an *in vitro* model of human malignant melanoma.

(4) To evaluate our *in vitro* findings of hyperthermia's therapeutic effectiveness, in the context of monotherapy, by utilizing an *in vivo* rodent model of malignant melanoma.

Chapter Two

Materials & Methods

2. Materials & Methods

2.1 Cell Culture Methodologies

2.1.1 Cell lines

Our experimental system consists of three different human skin cell lines: **(1)** a normal, immortilized keratinocyte (HaCaT) cell line, **(2)** an epidermoid carcinoma (A431) cell line and **(3)** a malignant melanoma (A375) cell line.

Table 2.1: List of cell lines used for the *in vitro* human malignant melanoma model.

Cell line	Company
HaCaT	A kind gift from Dr Sharon Broby (Dermal Toxicology and Effects Group; Centre for Radiation, Chemical and Environmental Hazards; Public Health England, UK).
A431	Purchased from Sigma-Aldrich (St. Louis, MO, USA)
A375	Purchased from Sigma-Aldrich (St. Louis, MO, USA)

Table 2.2: Characteristics of the different cell lines used for the *in vitro* human malignant melanoma model.

Characteristics	HaCaT	A431	A375
Organism	*Homo sapiens* (Human)	*Homo sapiens* (Human)	*Homo sapiens* (Human)
Ethnicity	Caucasian	Caucasian	Caucasian
Age	62 years old	85 years old	54 years old
Gender	Male	Female	Female

Tissue	Skin	Skin/epidermis	Skin
Properties	Monolayer, adherent	Epithelial, adherent	Epithelial, adherent
Description	*In vitro* spontaneously transformed keratinocytes from histologically normal skin	Established from solid tumors in the skin/epidermis of an 85-year-old patient.	Derived from the skin of a 54-year-old patient.
Disease	N/A	Epidermoid carcinoma	Malignant melanoma
Tumorigenic	No	Yes	Yes

2.1.2 Materials for cell culture

Table 2.3: List of materials used for cell culture [unless otherwise stated, chemicals were from Sigma-Aldrich (St. Louis, MO, USA), Applichem (Darmstadt, Germany), Invitrogen (Carlsbad, CA, USA) and were of analytical grade].

Reagent	Components
Complete Growth Media	DMEM (Dulbecco's Modified Eagle Medium) (Labtech International Ltd, East Sussex, UK) high glucose was supplemented with 10% fetal bovine serum heat-inactivated (Labtech), 2mM L-glutamine (Labtech) and 1% (v/v) penicillin/streptomycin mix.
Phosphate Buffered Saline (PBS)	Concentration: 1X 140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.7mM KH_2PO_4, pH 7.4

	(Labtech)
Trypsin-EDTA Solution	Concentration: 1X Trypsin-EDTA 0.25% in PBS w/o Mg, w/o Ca. (Labtech)
Cell Culture Plates & Flasks	Corning Inc., NY, USA
Stripettes	Corning Inc., NY, USA

2.1.3 Recovery of cells

All cell lines were stored in the vapor phase of liquid nitrogen, for long term storage, in fetal bovine serum (FBS) containing 10% (v/v) DMSO. Prior to use, cells were rapidly thawed and resuspended in 5ml complete DMEM. Cells were then centrifuged at 2,000rpm for 2min at room temperature. Normal and freezing media were removed by aspiration. Cells were resuspended in complete DMEM and placed in 25cm^2 cell culture flasks. All cell lines were cultured for up to 25 passages before new aliquots were utilized.

2.1.4 Propagation of cells

All cell lines were cultured in flasks containing DMEM complete media and maintained in 5% atmospheric CO_2, at 37^oC, in a humidified incubator. Cells were subcultured via trypsinization 2-3 times per week prior to reaching 100% confluence. More specifically, cells were washed with 6-7ml PBS after aspirating growth media and were then washed with 1ml of trypsin-EDTA (1X). Fresh trypsin-EDTA was added in the flask (1ml for A375 cells or 2ml for HaCaT and A431 cells) and then cells were placed in the incubator until they detached from the flask's surface (5-10min). The cells were then observed under the microscope to ensure that they had detached and 4-5ml of complete growth media was added into the flask in order to deactivate trypsin-EDTA. Finally, cells were resuspended and the wanted number of them was returned in the flask while more media was added (10-15ml).

2.1.5 Plating of cells

A previously described protocol was used (section 2.1.4) along with some modifications including additional steps. After trypsinization and suspension, the cells in complete media were transferred into a 15ml falcon tube and then centrifuged at 2,000rpm for 2min at room temperature. Media was then aspirated, and cells were thoroughly suspended in fresh media (5-12ml according to the size of cell pellet) while a 50μl sample was transferred into a microcentrifuge tube. An equal amount of trypan blue solution 0.4% (v/v) (HyClone Inc, South Logan, USA) was added, into the tube, and mixed with the sample of cell suspension. For the determination of cell number, a small amount (10μl) of the previously mentioned mix was loaded into a Neubauer counting chamber (hemocytometer) and the number of viable cells were counted, under the microscope. The concentration of cells (number of cells/ml) was calculated by using the following formula:

$$Concentration\ of\ cells = N/4 \times dilution\ factor \times 10^4$$

where N= the number of cells counted in the 4 squares of the counting chamber, dilution factor = 2 (1:1 dilution with trypan blue solution) and 10^4 = conversion factor to convert 10^{-4}ml to 1ml (calculated according to the dimensions of the small square).

After estimating the concentration of cells in the suspension, the desired number of cells were seeded into 100mm^3 cell culture dishes, 96-well plates, etc. (according to the requirements of the various experimental protocols).

2.1.6 Hyperthermia (HT) exposures (monotherapy)

Cells were exposed to various temperatures ranging from 37-50^{o}C for different time courses in standard 5% CO_2 incubators. Briefly, following trypsinization and estimation of cell concentration, the appropriate number of cells was plated and incubated at 37^{o}C overnight. On the next day, cells were observed under the microscope to ensure that they reached confluency while media was changed prior to hyperthermic exposure. Plates were then transferred into a 5% CO_2 incubator set at the desired hyperthermic temperature (37-50^{o}C) and exposed for various periods of time. Finally, plates were allowed to cool down, for a few minutes, at room temperature and then were returned to the 37^{o}C incubator for additional incubation periods.

2.1.7 Hyperthermia exposures combined with non-targeted and targeted therapeutic agents (adjuvant therapy)

Cells were trypsinized, plated and incubated, at 37°C, overnight. On the following day, plates were transferred into an incubator set at 43°C (hyperthermia) or 37°C (normothermia) for 2h. After the end of the exposure period, all media was aspirated, and various concentrations of non-targeted and targeted therapeutic agents were added (after being dilluted in complete growth medium or in the appropriate solvent as recommended by the manufacturer).

Table 2.4: List of non-targeted (chemotherapeutic) and targeted (small molecule) therapeutic agents.

Drug	Type of therapy	Company
Dacarbazine (DTIC)	Chemotherapeutic (non-targeted therapy)	Abcam
Palladium (II) saccharinate complex of terpyridine	Chemotherapeutic (non-targeted therapy)	A kind gift from Prof. Engin Ulukaya (Uludag, University, Turkey)
Vemurafenib (PLX4032, RG7204)	Small molecule (targeted therapy)	Selleckchem
Dabrafenib (GSK2118436)	Small molecule (targeted therapy)	Selleckchem

2.1.8 Cell viability assay

Cell viability was determined by utilizing the Celltiter-Blue Assay (Promega, UK) which is based on the Alamar Blue Assay. The assay uses the indicator dye (resazurin) which is converted to a highly fluorescent product (resorufin) by metabolically active cells. Non-viable cells lose their metabolic capacity; thus, they are not able to reduce resazurin into the fluorescent product and consequently cannot generate a fluorescent signal. Briefly, a desired concentration of cells was plated into 96-well plates and incubated overnight at 37°C. On the following day, the media was aspirated and fresh

one was added into each well (100μl). Cells were exposed to various hyperthermic conditions, for various time periods, and then were returned to 37ºC. At a range of time points (0, 24, 48 and 72h after exposure), 20μl of Celltiter-Blue reagent was added into each well of α 96-well plate and mixed by gentle shaking. The plates were incubated at 37ºC for 2h and then the samples were transferred into the wells of a black opaque plate. Fuorescence was monitored with an emission of 560nm and an excitation of 590nm by using a SpectraMax M5 multimode plate reader (Molecular Devices, LLC, Sunnyvale, USA). The levels of viable cells were estimated according to the amount of fluorescent product that was produced. Cell viability was expressed as percentage of control cells (incubated at 37ºC. Five replicates (N=5) of each condition were used in each experiment.

2.1.9 CytoTox-Fluor cytotoxicity assay

The CytoTox-Fluor cytotoxicity assay (Promega, UK) was utilized in a manner capable of determining the relative number of dead cells in any cell population. The assay involves a fluorigenic peptide substrate (bis-alanyl-alanyl-phenylalanyn-rhodamine 110; bis-AAF-R110) which can measure the activity levels of a specific protease released from dead cells which have lost membrane integrity. This particular peptide substrate cannot produce a signal in viable cells as it cannot cross their cell membrane. However, it should be mentioned that this method has some limitations; the activity of the protease has a limited half-life, so the assay is not recommended to be used at incubation periods longer than 24h. A375 and HaCaT cells (10,000 cells/well) were plated into 96-well plates and cultured overnight at 37ºC. On the following day, the media was aspirated and fresh one was added into each well (100μl). Cells were exposed to 43ºC or 45ºC, for 2h, and then were transferred back to 37ºC for additional incubation periods. For preparing the 2X working reagent an appropriate amount of bis-AAF-R110 substrate was added to a specific volume of assay buffer (1:1000 dilution). The reagent was mixed by vortexing and then an equal volume (100μl) of the assay reagent was added into each well, samples were mixed briefly by orbital shaking and were incubated for 2h at 37ºC. Finally, samples were transferred into the wells of a black opaque plate and fluorescence was monitored with emission at 485nm and excitation at 520nm by using a SpectraMax M5 multimode plate reader (Molecular Devices, LLC, Sunnyvale, USA). The generation of fluorescent product is proportional to the protease activity of the marker associated with cytotoxicity so that higher

fluorescence values represent increased levels of dead cells. Five replicates (N=5) of each condition were used in each experiment.

2.1.10 Trypan blue staining

The trypan blue staining protocol was utilized in order to determine the proliferating potential of cells. More specifically, A375 cells were plated in 100mm dishes (10^6cells/plate) and then were incubated at 37°C, overnight. The following day, they were exposed to 43°C or 45°C for 2h and then were then transferred back to 37°C for additional incubation periods. Control cells were kept at 37°C (normothermia). At specific time points (immediately after and 24h after exposures), cells were trypsinized and collected. A sample of each cell suspension was mixed with the trypan blue stain and cells were counted under the microscope (see section 2.1.5). According to their staining, cells were categorized into two groups: viable (unstained) and dead (blue colour-stained) while their total number, in the cell suspension, was calculated. Three replicates (N=3) of each condition were used in each experiment.

2.1.11 Caspase activity assay

The Caspase Family Fluorometric Substrate kit II Plus (Abcam, Cambridge, UK) was utilized in order to detect changes in the activity levels of various caspases after exposing cells to hyperthermic conditions. The assay is based on the ability of an active enzyme to cleave the chromophore from a peptide substrate specific for a respective caspase. The peptide substrates are specific for different caspases (Table 2.5) and they are linked with the fluorophore -AFC. During the assay, active caspases will cleave their substrate between the peptide and the fluorophore AFC, therefore generating highly fluorescent AFC which can be detected by using a fluorescence reader. Substartes will be cleaved only in the lysates of apoptotic cells so the amount of AFC produced is proportional to the number of apoptotic cells in the sample. Briely, an appropriate number of cells (based on specific experimental requirements) was seeded into 100mm^3 plates and cultured overnight at 37°C. On the next day, their media was replaced, and cells were exposed to specific hyperthermic conditions. Then, cells were transferred back to 37°C for additional incubation periods while control cells were kept at 37°C at all times. At specific time points, cells were collected via trypsinization,

washed twice with PBS and stored in microcentrifuge tubes at -20°C until being further processed. For performing the assay, cell pellets were mixed with the appropriate amount of lysis buffer (depending on pellet size) (50mM HEPES, pH 7.4, 0.1% Triton X-100, 100mM NaCl, 0.1mM EDTA) and were kept on ice for 10-20min while being periodically vortexed. Then, they were centrifuged at 14,000 x g for 15min at 4°C. Supernatants were transferred into fresh pre-chilled microcentrifuge tubes and kept on ice until protein concentration was determined. Protein concentration was determined by utilizing the Bicinchoninic Acid Assay (BCA Assay) (see section 2.3.1). An appropriate sample volume (50μg of protein content) was diluted with lysis buffer up to 50μl and was mixed with equal amount of reaction buffer 2X (50mM HEPES, pH 7.4, 0.1% CHAPS, 10% glucose, 100mM NaCl, 1mM EDTA, 10mM DTT). Next, 5μl from 1mM AFC conjugated substrate (final concentation = 50μM) was mixed with each sample individually and the samples were incubated at 37°C for 1h in the dark. The assay was performed in a 96-well black opaque plate. Fluorescence was monitored at 400 Exc / 505 Emm (nm) by using a SpectraMax M5 multimode plate reader (Molecular Devices LLC, Sunnyvale, USA). Results were expressed as fold increase in caspase activity by comparing the readings of control samples with those of the treated ones. Three replicates (N=3) of each experimental condition were used for each experiment.

Table 2.5: List of various caspases and their respective peptide substrates for determining their activity levels after exposure to hyperthermic conditions.

Caspase	Substrate
Caspase-3/7	Ac-DEVD-AFC
Caspase-4	Ac-LEVD-AFC
Caspase-6	Ac-VEID-AFC
Caspase-8	Ac-IETD-AFC
Caspase-9	Ac-LEHD-AFC

2.2 Molecular Biology Methodologies

2.2.1 RNA extraction

Total RNA was extracted using the TRIzol Reagent according to the manufacturer's instructions. Briefly, cells (1-5 x 10^6) were collected via trypsinization, washed twice with PBS, suspended in 1ml TRIzol Reagent and stored in microcentrifuge tubes at -20°C until being further processed. Samples were allowed to thaw at room temperature for a few minutes before adding 0.2ml of chloroform per 1ml of TRIzol and tubes were then shaken vigorously by hand for 15sec and incubated for 5min at room temperature. Afterwards, they were centrifuged at 12,000 x g for 10min at 4°C. After this step, there were 3 layers formed: a lower red phenol-chloroform phase, an interphase and an upper aqueous phase. DNA and proteins were extracted from the interphase/organic phase while RNA remained exclusively in the aqueous phase. This phase was transferred, from each sample, into a fresh tube while avoiding the interphase containing the DNA. For precipitating the RNA, 0.5ml of 100% isopropanol (per 1ml of TRIzol used) was added to the aqueous phase and samples were incubated for 10min at room temperature. Then, they were centrifuged at 12,000 x g for 10min at 4°C and supernatant was removed from the tubes, while leaving the RNA pellet at the bottom. Pellets were washed with 1ml 75% ethanol (per 1ml of TRIzol used) vortexed briefly and centrifuged at 7,500 x g for 5min at 4°C. All ethanol was discarded and pellets were allowed to air dry on the bench for 5-10min before they were suspended in RNAase free water (20-50µl according to pellet size) and then stored at -20°C. RNA quantity was determined by using the NanoDrop (ThermoScientific, Waltham, MA, USA). RNA quality was assessed by running 2µg of each RNA sample on a 1% agarose gel as well as checking the A260/280 and A260/230 ratios.

2.2.2 cDNA synthesis

Complimentary DNA was synthesized by using the SuperScript VILO cDNA synthesis kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 2.5µg RNA was diluted in RNAase free water to a total volume of 14µl. Four µl of 5X VILO Reaction Mix (random primers, $MgCl_2$, dNTPs) and 2µl 10X SuperScript Enzyme Mix (SuperScript III RT, RNaseOUT recombinant ribonuclease inhibitor and a proprietary helper protein) were then added to the diluted RNA (total reaction volume = 20µl). The mixture was gently mixed and incubated at 25°C for 10min. Next, the

mixture was heated at 42°C for 60min to generate cDNA and then at 85°C for 5min to inactivate the enzyme before chilling to 4°C. The resulting product was stored at -20°C until further use.

2.2.3 Quantitative real-time polymerase chain reaction (qPCR)

The quantitative polymerase chain reaction was carried out by utilizing the TaqMan Array Human Apoptosis 96-well plates (Applied Biosystems, Carlsbad, CA, USA). Taqman qPCR was used as the primers and probes, specific for the gene of interest, provide accurate quantification of the cDNA present. The predesigned 96-well plates contained a collection of dried-down TaqMan gene expression assays, which were composed of gene-specific primers and probe sets, for screening candidate genes involved at various stages of the apoptotic pathway. TaqMan Universal master mix (2X) was thawed and 550µl were mixed with the equal amount of diluted in RNAase free water cDNA (5-50ng per well) up to a final volume of 1,100µl (increased 10% for running one 96-well plate). Then, the reaction mixture was gently vortexed and spinned down before adding 10µl into each well of the 96-well plate. The plate was covered with a clear adhesive film and was briefly centrifuged to ensure that the reaction mixture was collected at the bottom of the wells. The plates were run at the StepOne Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA).

Table 2.6: Thermal cycling conditions for running the TaqMan Array 96-well plates.

Fast thermal cycling conditions for TaqMan array fast plates			
Hold	Hold	PCR (40 cycles)	
		Melt	Anneal/Extend
50°C	95°C	95°C	60°C
2min	20sec	3sec	30sec

The TaqMan Array Human Apoptosis 96-well plates contain 90 assays for genes implicated at various stages of the apoptotic cascade along with 6 assays for candidate

endogenous control genes. Gene expression data were analyzed by the ΔΔCt method and differences observed were expressed as fold change in gene expression by using the DataAssist v3.01 software.

Table 2.7: List of apoptotic gene sub-families in the human apoptosis qPCR array.

Apoptosis Induction: *BAD, BAK1, BAX, BID, DFFA, DFFB, DIABLO (SMAC),* DAPK3, *FASLG (TNFSF6), PARP1*
Anti-Apoptosis: *ATM, BCL2, BCL2L1 (BCL-X), BCL2L11, CFLAR (CASPER), EGFR, ENDOG, IGF1, IGF1R, NFKB1*
Apoptosis Regulation: (i) Negative Regulation: *BCL2, BCL2L1 (BCL-X), BCL2L11, BIRC2 (c-IAP2), IGF1, IGF1R, TP53;* (ii) Positive Regulation: *AKT1, BAD, BAK1, BAX, BBC3 (PUMA), BCL2L11, BID, CDKN2A, CFLAR, CHEK2, CHUK, CYCS, DIABLO (SMAC), FADD, FASLG (TNFSF6), HTRA2, PMAIP1, TNFRSF10A, TNFSF10 (TRAIL), TP53, TRADD, TRAF2, VDAC1, VDAC2, VDAC3*
Death Domain Proteins: *FADD, FAS (TNFRSF6), DAXX, RIPK1, TNF, TNFRSF10A, TNFRSF1A, TNFSF10, TNFSF12, TRADD, TRAF2*
Caspases & Regulators: *CASP3, CASP7, CASP8, CASP9, APAF1*
Endogenous controls: *18S, AIFM1, AKT1, GAPDH, GUSB, HPRT1*

2.3 Protein Methodologies

2.3.1 Preparation of cell lysates and protein determination by the bicinchoninic acid assay (BCA)

Cells were exposed to the required experimental conditions and then collected at various time points by trysinization, washed twice with PBS and stored at -20°C until further use. Cell pellets were suspended in the appropriate amount of lysis buffer containing: 10mM HEPES, pH 7.9, 10mM KCl, 0,1mM EDTA, 1,5mM $MgCl_2$, 0,2% NP40, supplemented with protease inhibitors cocktail tablets (ThermoFisher), as well as 10mM

NaF, 20mM β-glycerophosphate and 0,2mM Na_3VO_4 (phosphatase inhibitors) and were left on ice while periodically being vortexed for 15min. Then, they were sonicated (by using a probe sonicator) at 30% amplitude for 3 cycles of 15sec (with 30sec intervals) on ice. Cell lysates were centrifuged at 14,000 x g for 15min at 4°C and supernatants were transferred into fresh microcentrifuge tubes. Protein content was determined by utilizing the Bicinchoninc Acid assay (BCA) kit from ThermoScientific (Waltham, MA, USA) according to the manufacturer's recommendations. To carry out the protein concentration quantification, a standard curve using Bovine Serum Albumin (BSA) was used. Briefly, a series of various BSA concentrations (0, 0.2, 0.5, 1, 2μg/μl) were added into the wells of a 96-well plate by mixing the appropriate volumes of dH_2O and BSA stock to a final volume of 20μl. Samples were also added at a 1:10 dilution. All standards and samples were run in duplicates. The appropriate volume of working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1), 200μl was added to each well and the plate was shaken gently by hand so that samples were thoroughly mixed. The plate was covered and incubated at 37°C for 30min. Next, the plate was centrifuged at 2,000rpm for 2min to avoid presence of bubbles in the wells (responsible for false readings) before the absorbance was measured at 562nm by using a SpectraMax M5 multimode plate reader (Molecular Devices LLC, Sunnyvale, USA). The unknown protein concentrations of the samples were calculated by preparing a standard BSA curve. The required volume of samples was transferred to new microcentrifuge tubes and loading buffer (5X) was added as 4 parts of sample in 1 part of loading buffer. Then, the samples were either immediately used or they were stored at -20°C until future usage.

2.3.2 Western immunoblotting

Samples were heated on a heat-block at 95°C for 5min (to denature proteins and reduce protein-protein interactions), were allowed to cool down for a few minutes and then vortexed and spinned down briefly. Proteins were separated by using SDS-polyacrylamide gels of different acrylamide concentration (8, 10, 12, 10-20%) according to the molecular weight of the protein of interest. Fifty micrograms (50μg) of protein extracts were loaded onto a polyacrylamide gel and electrophoresis was carried out in SDS running buffer 1X at 160V for 2h. A protein ladder (PageRuler Plus Prestained Protein Ladder, 10-250kDa, ThermoScientific), containing multiple proteins of known molecular weights labeled with dye for visualization, was used to determine

approximately the molecular weight of the proteins of interest. Separated proteins were then transferred electrophoretically onto PVDF membranes (ThermoScientific), 0.2 or 0.45μm depending on protein molecular weight, by wet transfer in transfer buffer 1X at predetermined running conditions. The blots were blocked in 5% (w/v) non-fat milk powder in TBST buffer for 1h at room temperature under gentle agitation. Then, the blots were incubated with the desired primary antibody (see table) overnight at 4°C under gentle agitation. On the following day, the membranes were washed in TBST buffer for 10min for three times and then were incubated with the appropriate secondary antibody (Table 2.9) for 1h at room temperature under agitation. Moreover, the blots were incubated with SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) according to the manufacturer's protocol. Finally, the reagent contains an enhanced chemiluminescent HRP (horseradish peroxidase) substrate which maintains and enhances the light emitted from the oxidation of luminol in the presence of HRP and H_2O_2. The blots were imaged by using a ChemiDoc XRS+ system from Bio-Rad (Perth, UK).

Table 2.8: List of materials used for Western immunoblotting.

Reagents	Components
SDS running buffer 10X	250mM Tris-base, 1.92M Glycine, 1% SDS, pH 8.3-8.5
SDS running buffer 1X	100ml SDS running buffer 10X diluted with 900ml dH_2O 25mM Tris-base, 192mM Glycine, 0.1% SDS, pH 8.3-8.5
Transfer buffer 10X	250mM Tris-base, 1.92M Glycine, pH 8.3-8.5
Transfer buffer 1X	100ml Transfer buffer 10X, 5ml SDS 10% (w/v), 695ml dH_2O, 200ml methanol 25mM Tris-base, 192mM Glycine, 0.5% SDS, 20% (v/v) methanol, pH 8.3-8.5
Tris Buffered Saline-Tween 20 (TBS-T) buffer 1X	20mM Tris, 150mM NaCl, 0.1% Tween 20 in dH_2O, pH 7.6

Stripping buffer	25mM Glycine, 0.1% SDS, 1% Tween 20 diluted in dH_2O pH 2.2

Table 2.9: List of antibodies used for Western immunoblotting.

Antibody	Dilution	Blocking buffer	Isotype	Company	Catalogue Number
Anti-caspase-3 (8G10)	1:1000	5% dry milk in TBS-T	Rabbit	Cell Signaling	#9665
Anti-caspase-6	1:1000	5% dry milk in TBS-T	Rabbit	Cell Signaling	#9762
Anti-caspase-7 (D2Q3L)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#12827
Anti-caspase-8 (1C12)	1:1000	5% BSA in TBS-T	Mouse	Cell Signaling	#9746
Anti-caspase-9 (C9)	1:1000	5% dry milk in TBS-T	Mouse	Cell Signaling	#9508
Anti-lamin A/C (4C11)	1:1000	5% dry milk in TBS-T	Mouse	Cell Signaling	#4777
Anti-PARP	1:1000	5% dry milk in TBS-T	Rabbit	Cell Signaling	#9542
Anti-Bad (D24A9)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#9239
Anti-Bax (D2E11)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#5023
Anti-Bim (C34C5)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#2933
Anti-BID	1:1000	5% dry milk in TBS-T	Rabbit	Cell Signaling	#2002
Anti-Bak (D4E4)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#12105
Anti-TNF-R1 (C25C1)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#3736
Anti-FADD	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#2782

Anti-TRADD (7G8)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#3684
Anti-RIP (D94C12)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#3493
Anti-BiP (C50B12)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#3177
Anti-IRE1a (14C10)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#3294
Anti-PERK (D11A8)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#5683
Anti-CHOP (L63F7)	1:1000	5% BSA in TBS-T	Mouse	Cell Signaling	#2895
Anti-HSP60 (D6F1)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#12165
Anti-HSP70	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#4872
Anti-HSF1	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#4356
Anti-HSP40 (C64B4)	1:1000	5% BSA in TBS-T	Rabbit	Cell Signaling	#4871
Anti-HSP90 (C45G5)	1:1000	5% dry milk in TBS-T	Rabbit	Cell Signaling	#4877
Anti-β-tubulin (SAP.4G5)	1:20000	5% dry milk in TBS-T	Mouse	Sigma-Aldrich	#T7816-.2ML
Anti-β-actin (AC-74)	1:2000	5% dry milk in TBS-T	Mouse	Sigma-Aldrich	#2228
Rabbit Anti-mouse HRP conjugated secondary antibody	1:5000	5% dry milk in TBS-T	Mouse	Sigma-Aldrich	#A9044-2ML
Horse Anti-mouse HRP conjugated secondary antibody	1:2000	5% dry milk in TBS-T	Mouse	Cell Signaling	#7076
Goat Anti-rabbit HRP conjugated secondary antibody	1:2000	5% dry milk in TBS-T	Rabbit	Cell Signaling	#7074

2.3.3 Stripping and re-probing of blots

Membranes were stripped (using mild stripping buffer; see table) to remove all primary and secondary antibodies in order to allow for re-probing for various other proteins (depending on their molecular weight). Briefly, the membranes were incubated twice with stripping buffer for 10-15min at room temperature on a shaker and then were washed twice in PBS for 10min. Another two washes with TBS-T buffer for 5min followed before proceeding to blocking in 5% (w/v) non-fat dry milk powder in TBS-T for 1h at room temperature. The blot was then incubated with different primary antibodies as previously described.

2.4 *In Vivo* Xenograft Rodent Models of Melanoma and Colon Carcinoma

2.4.1 Cell lines

For the *in vivo* xenograft model of malignant melanoma, the B16-F10 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) while cells were cultured in DMEM, high glucose, supplemented with 10% fetal bovine serum heat-inactivated, 2mM L-glutamine and 1% (v/v) penicillin/streptomycin mix. On the other hand, the colon carcinoma (CT26) cell line was a kind gift from Dr. Chlichlia (Departnment of Molecular Biology & Genetics, Democritus University of Thrace, Alexandroupolis, Greece) and was used for the *in vivo* model of colon carcinoma. CT26 cells were grown in DMEM, high glucose, supplemented with 10% fetal bovine serum heat-inactivated, 4mM L-glutamine and 1% (v/v) penicillin/streptomycin mix. Both cell lines were kept at 37°C, 5% CO_2 in a humidified incubator. Materials and protocols used for cell culturing were described previously (see paragraphs 2.1, 2.1.1, 2.1.2, 2.1.3).

Table 2.10: Characteristics of the cell lines used for the *in vivo* xenograft melanoma and colon carcinoma rodent models.

	Cell lines	
Characteristics	B16-F10	CT26
Organism	*Mus Musculus* (Mouse)	*Mus Musculus* (Mouse)

Strain	C57BL/6	BALB/c
Tissue	Skin	Colon
Properties	Adherent Mixture of spindle-shaped and epithelial-like cells	Adherent Morphology: fibroblast
Description	B16-F10 were derived from the parental B16 cell line as they display significantly greater tissue-invasive abilities	CT26 cells were derived from BALB/c mice treated with *N*-nitroso-*N*-methylurethane.
Disease	Malignant melanoma	Colon carcinoma
Tumorigenic	Yes	Yes

2.4.2 Animals

C57BL/6J mice were purchased from the Hellenic Pasteur Institute (Athens, Greece) and were raised in the Animal House Unit (Laboratory of Experimental Surgery and Surgical Research at Democritus University of Thrace, Alexandroupolis, Greece Nr. EL71 BIO2). BALB/c mice were kindly provided by Dr. Chlichlia (Departnment of Molecular Biology & Genetics, Democritus University of Thrace, Alexandroupolis, Greece) and were maintained at the Animal House Unit. A total of 30 female mice (8-12 weeks old, weight 20-25gr) were used under each experimental group. They were housed in sterile polycarbonate cages (maximum 5 mice per cage) at room temperature, on a 12h light-12h dark cycle. Finally, all mice were provided with tap water and commercial pelleted diet (Mucedola).

2.4.3 Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of the Veterinary Department of Evros Prefecture (License No. EL1BIOexp1) and complied with the requirements set by Directive 86/609/EEC and PD160/91 (the legislation in force at the time of experimentation). All experiments were conducted in

light of the 3R's (Replacement, Refinement, Reduction) policy while all mice were not subjected to any painful or discomforting conditions.

2.4.4 Hyperthermia exposures- tumor induction protocols

All experiments were performed at the Department of Molecular Biology and Genetics and Laboratory of Experimental Surgery and Surgical Research, Democritus University of Thrace, Alexandroupolis, Greece. Thirty female C57BL/6J mice (8-12 weeks old) were separated into 3 groups (10 mice per group): Group 1: control at 37°C (untreated), Group 2: hyperthermia-treated (at 43°C) and Group 3: hyperthermia-treated (at 45°C). B16-F10 melanoma cells were plated in $100mm^3$ dishes and on the following day were exposed to either hyperthermic (43°C and 45°C) or normothermic (37°C) conditions for 1h. Then, the cells were returned to 37°C for an additional 24h incubation period. All cells were trypsinized and collected while their total number was determined as described previously. Overall, 0.5×10^6 cells were suspended in 150μl PBS and then injected subcutaneously into the tissue of the back of the neck of each mouse. Tumors were allowed to grow for a period of 12-14 days until they became visible. Then the animals were sacrificed by cervical dislocation and the tumors were excised. Tumor volume and incidence were determined. Tumor dimensions were measured by an electronic caliper and tumor volume was calculated by using the modified ellipsoid formula ($width^2 \times length$) / 2. The weight of each mouse was monitored during the course of the experiment, as well as signs of disease and/or discomfort. All efforts were made to ameliorate suffering whereas two independent experiments were performed in order to confirm the results.

Figure 2.1: Images descriptive of handling C57BL/6J mice during the tumor induction protocol.

On the other hand, thirty female BALB/c mice (8-12 weeks old) were separated into 3 groups (10 mice per group): Group 1: control at 37°C (untreated), Group 2: hyperthermia-treated (at 43°C) and Group 3: hyperthermia-treated (at 45°C). CT26 colon carcinoma cells were plated, exposed to hyperthermia and further processed as already described above for the C57BL/6J mice during the tumor induction protocol.

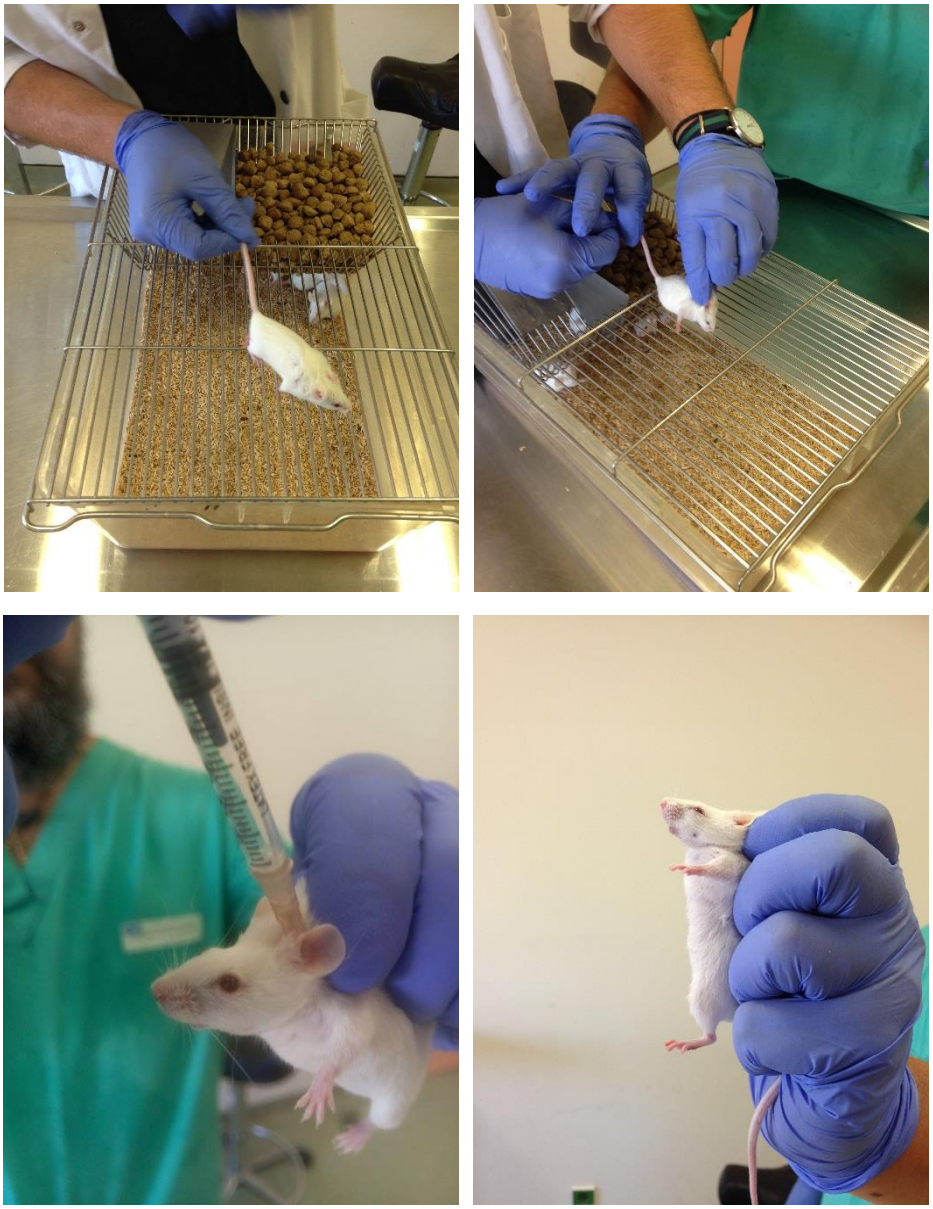


Figure 2.2: Images descriptive of handling BALB/c mice during the tumor induction protocol.

2.4.5 Immunohistochemical analysis

Tumors were fixed in 10% formalin and then dehydrated in graded concentrations of ethanol and xylole and then embedded in paraffin. Serial sections (3µm thick) from the formalin-fixed paraffin-embedded tissue blocks were prepared and floated onto charged glass slides. A hematoxylin and eosin stained section was obtained from each tissue block. Immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections by the streptavidin-biotin peroxidase labeled (LSAB) method. Sections were

deparaffinized after being washed 3 times (for 5min each) in xylene. Afterwards, they were hydrated by using graded concentrations of ethanol to dH_2O, 2 washes in 100% ethanol (for 10min each), 2 washes in 95% ethanol (for 10min each), 2 washes in 70% ethanol for (5min each), 2 washes in 50% ethanol (for 5min each) and finally 2 washes in dH_2O (for 5min each). Tissue sections were subjected to antigen retrieval (by heating them in 1X citrate unmasking solution using a microwave until boiling was initiated) and then were incubated at a sub-boiling temperature (95-98^oC) for 10min. After being allowed to cool down (on the bench top for 30 minutes), they were washed 3 times in dH_2O (for 5min each). For blocking endogenous peroxidase activity, the slides were incubated with 3% (v/v) hydrogen peroxide (for 10min), washed twice with dH_2O (for 5min each) and once in TBST buffer (for 5min). During the next step, the sections were incubated with blocking buffer (for 1h at room temperature) followed by overnight incubation with primary antibody at 4^oC. The sections were washed with TBST buffer 3 times (for 5min each) after removing the primary antibody, covered with a few drops of SignalStain Boost Detection Reagent (HRP, rabbit) and then were further incubated in a humidified chamber (for 30min at room temperature). Moreover, they were washed 3 times with TBST buffer (for 5min each) before adding DAB chromogen solution onto each slide, incubated for further 1-10min (while staining was monitored under a microscope) washed 3 times with TBST buffer (for 10min each) and finally washed in dH2O. Staining was followed by counterstaining with hematoxylin and eventually mounted and covered with coverslips. An image analysis system composed of the Olympus BX43 upright microscope, digital camera Olympus Cam-SC30 and soft analysis (analySISH) was used for all tumor sections (stained with antibodies and counterstained with hematoxylin). Immunostaining was assessed by a developed continuous score system where the number of immunopositive cells was divided by the total number of the counted cells while the expression was defined as the percentage of positive cells of the total number of counted cells.

Table 2.11: List of materials used for immunohistological staining.

Reagents

Formalin (Sigma-Aldrich)	Paraffin (Sigma-Aldrich)	Hematoxylin (Sigma Aldrich)
Eosin (Sigma-Aldrich)	Xylene (Sigma-Aldrich)	Ethanol (VWR)

1X Citrate buffer, pH 6.0 (Cell Signaling)	Hydrogen Peroxide solution (Sigma-Aldrich)	Bovine Serum Albumin (BSA) (Amresco, VWR)
1X TBST buffer	SignalStain Antibody Diluent #8112 (Cell Signaling)	SignalStain Boost Detection Reagent (HRP, rabbit) #8114 (Cell Signaling)
SignalStain DAB Substrate kit #8059 (Cell Signaling)	SignalStain Mounting Medium #14177 (Cell Signaling)	

Table 2.12: List of antibodies used for immunohistological staining.

Antibody	Dilution	Antibody diluent	Incubation time	Isotype	Company	Cat #
Anti-COX2 (D5H5)	1:600	SignalStain Antibody Diluent #8112	1h at RT	Rabbit	Cell Signaling	#12282
Anti-cleaved-caspase 3 (Asp175) (5A1E)	1:1000	SignalStain Antibody Diluent #8112	O/N at 4°C	Rabbit	Cell Signaling	#9664
Anti-phospho-H2A.X(Ser139) (20E3)	1:250	SignalStain Antibody Diluent #8112	O/N at 4°C	Rabbit	Cell Signaling	#9718

2.5 Statistical analysis

Experimental conditions for all set of experiments were identical and comparisons were made between control and treatment groups. Calculations were performed by using the Microsoft Office Excel 2007 software. Means were compared by one-way ANOVA with Tukey's test for multiple comparisons. SPSS or PRISM software were used for statistical tests. A value of $p<0.05$ was considered statistically significant. At least two independent experiments were performed for each experimental design while results were repeatable with similar SEM values for independent experiments.

Chapter Three

Results I - Effects of hyperthermia in an *in vitro* model of human malignant melanoma

3. Results

3.1 Optimization of a hyperthermia-induced experimental platform by using an in vitro model of human malignant melanoma

3.1.1 Kinetic analyses based on cell viability

The first step in setting up our experimental exposure platform was to determine the optimal experimental conditions capable of inducing cell death in human malignant melanoma (A375) and epidermoid carcinoma (A431) cell lines. For this purpose, we performed kinetic analyses including temperature-response and time-course experiments where cells were exposed to various temperatures (37-50^{o}C) (Fig.3.1.1). Cell viability levels were determined (by utilizing the Alamar-Blue assay) immediately after the 2h exposure to hyperthermia as well as after a further 24h incubation, at 37^{o}C, which provided the time needed for the potential activation of cell death pathways.

Overall, exposure to temperatures below 43^{o}C did not seem to have a significant effect on cell viability levels in both cell lines. On the other hand, when cells were exposed to higher temperatures (43^{o}C onwards), there was a decline in the number of viable cells which was to a greater extent in the case of A375 cells. In addition, at temperatures above 45^{o}C, there was excessive reduction in cell viability suggesting complete cellular destruction. Collectively, our data suggest that the optimum hyperthermic temperatures for our *in vitro* model of human malignant melanoma were those of 43^{o}C and 45^{o}C as there was a significant reduction in cell viability levels in both cell lines.

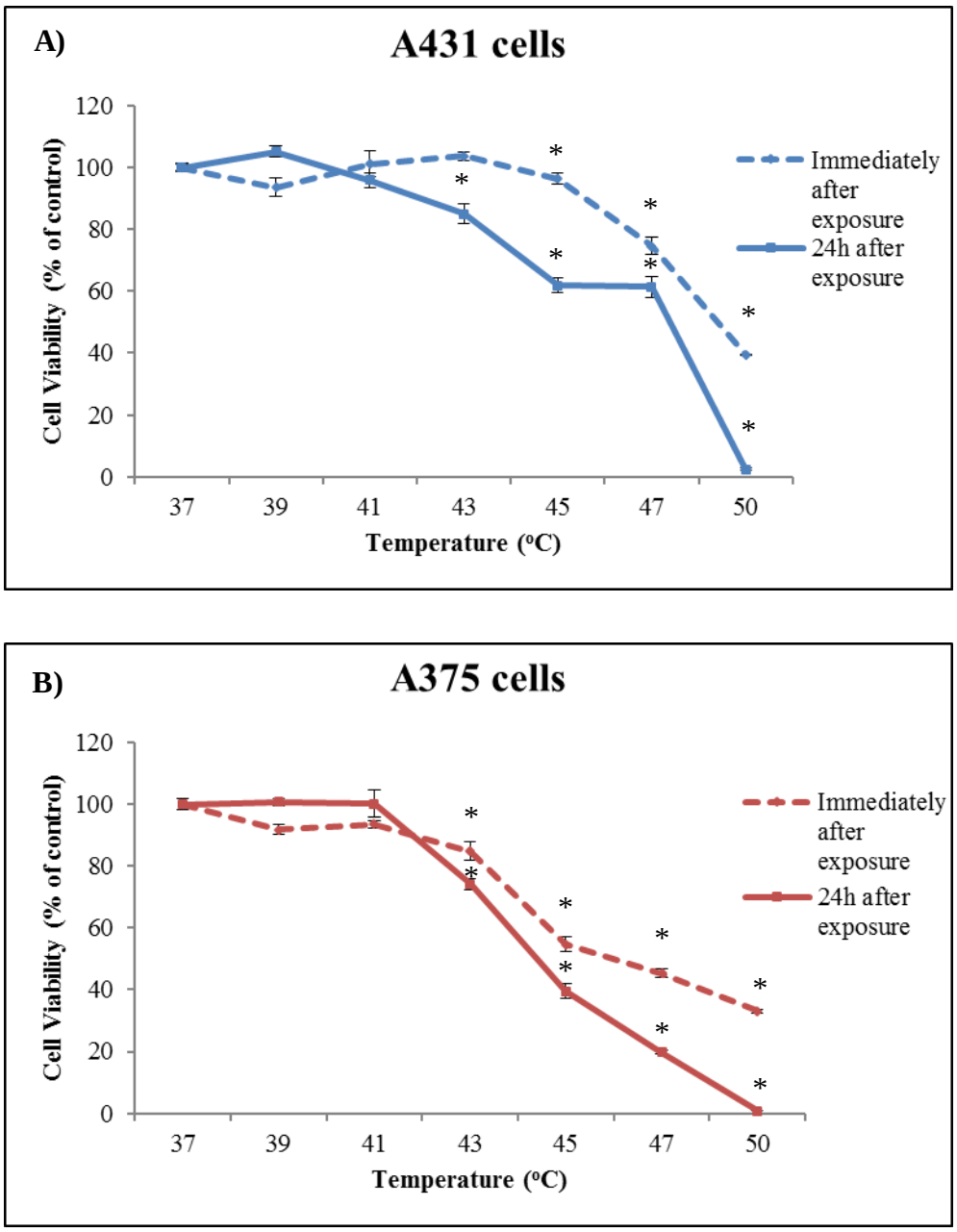


Figure 3.1.1: The effect of hyperthermia at various temperatures (37-50°C) on cell viability levels in A) a human epidermoid carcinoma (A431) and B) human malignant melanoma (A375) cell line. Briefly, cells were exposed to different temperatures, ranging from 37°C to 50°C, for 2h, and cell viability was determined by utilizing the Alamar-Blue assay either immediately after the exposures or after an additional 24h incubation period at 37°C. Cell viability was expressed as percentage of control (37°C) cells. Data shown are mean values (n=5) ± SEM and represent one of three independent experiments. Asterisk (*) indicates statistical significance at $p<0.05$.

Furthermore, we aimed to determine the optimum time course response at 43°C and 45°C of hyperthermic exposures (Fig.3.1.2). In this set of experiments, we exposed A431 and A375 cell lines to these temperatures over various time courses (30, 60 and 120 min). Cell viability levels were determined immediately after the hyperthermic exposure or following an additional 24h incubation period at 37°C by using the Alamar-Blue assay. According to our results, we did not observe any significant reduction in A431 cells when exposed to 43°C at shorter incubation periods (i.e. 30 and 60min) (Fig.3.1.2.A), whereas A375 cells appeared to be more sensitive, although the decline in cell viability observed was around 10-15% (Fig.3.1.2.B). Overall, it was shown that short exposures of A375 and A431 cells, at 43°C, had a minor effect on cell viability, while a 15-25% reduction was observed (at 24h after exposure) in each cell line respectively when treated for 2h (Fig.3.1.2.A & B). On the contrary, exposure of both cell lines at 45°C had a much more profound effect even after shorter time courses of exposure when compared to 43°C. Overall, there was a 40-60% decrease in cell viability levels for each of the cell lines when incubated for a further 24h after the original 2h exposure (Figs.3.1.2.C & D). Collectively, our data suggest that the optimum experimental hyperthermic conditions were those at 43°C and 45°C, for 2h, either immediately after or 24h after the initial (2h) exposure period.

In another set of experiments, we introduced an additional non-tumorigenic, immortalized human keratinocyte (HaCaT) cell line in an attempt to investigate if hyperthermia would exert the same effects in non-tumorigenic cells as it did with the A375 and A431 cancer cell lines. This cell line was utilized in the context of healthy, non-tumorigenic cells that surround the primary tumor. Once again, cells were exposed to hyperthermia either at 43°C or 45°C, for 2h, and cell viability was determined right after and/or 24, 48 or 72h post hyperthermic exposure at 37°C. The reason for the extended incubation periods was to determine if these were associated with a more prolonged decline in cell viability levels suggestive of increased rates of cytotoxicity. According to our data, A375 cells were more sensitive to 43°C as there was a significant decline in the levels of viable cells (around 20-30%) at 24 and 48h respectively while A431 cells were more resistant (Fig.3.1.3.A). Moreover, exposure at 45°C induced a more profound decrease in cell viability (around 30-40%) for each of the A375 and A431 cell lines respectively at 24h post hyperthermic exposure while even lower viability rates were observed at 48 and 72h post hyperthermic exposure respectively (Fig.3.1.3.B). Finally, the HaCaT cell line was shown to be more resistant to hyperthermia irrespectively of the experimental condition (Fig.3.1.3.A & B). More

specifically, it was evident that exposure of HaCaT cells to 43°C did not cause a significant decline in cell viability levels while this was also the case even at 45°C exposure (Fig.3.1.3.A & B).

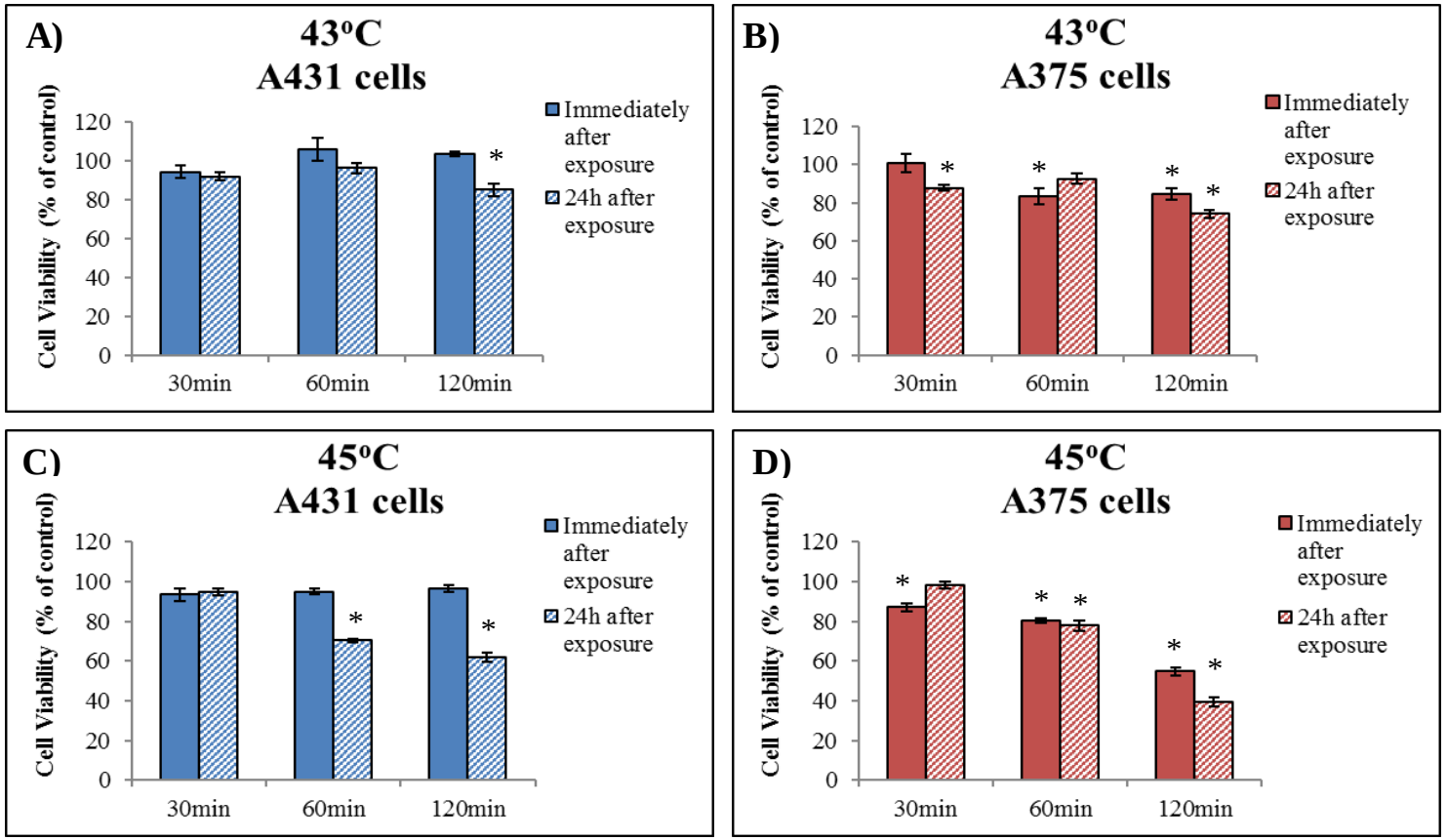


Figure 3.1.2: The effect of hyperthermia (43°C and 45°C) at different time courses in a human epidermoid carcinoma (A431; A & C) and melanoma (A375; B & D) cell line. Cells were exposed to 43°C and 45°C over 30, 60 and 120min. Cell viability was determined by utilizing the Alamar-Blue assay immediately after each exposure (filled bars) as well as 24h after the initial (2h) exposure (hatched bars), at 37°C. Cell viability was expressed as percentage of control cells incubated at 37°C. Data shown are mean values (n=5) ± SEM and represent one of three independent experiments. Asterisk (*) indicates statistical significance at $p<0.05$.

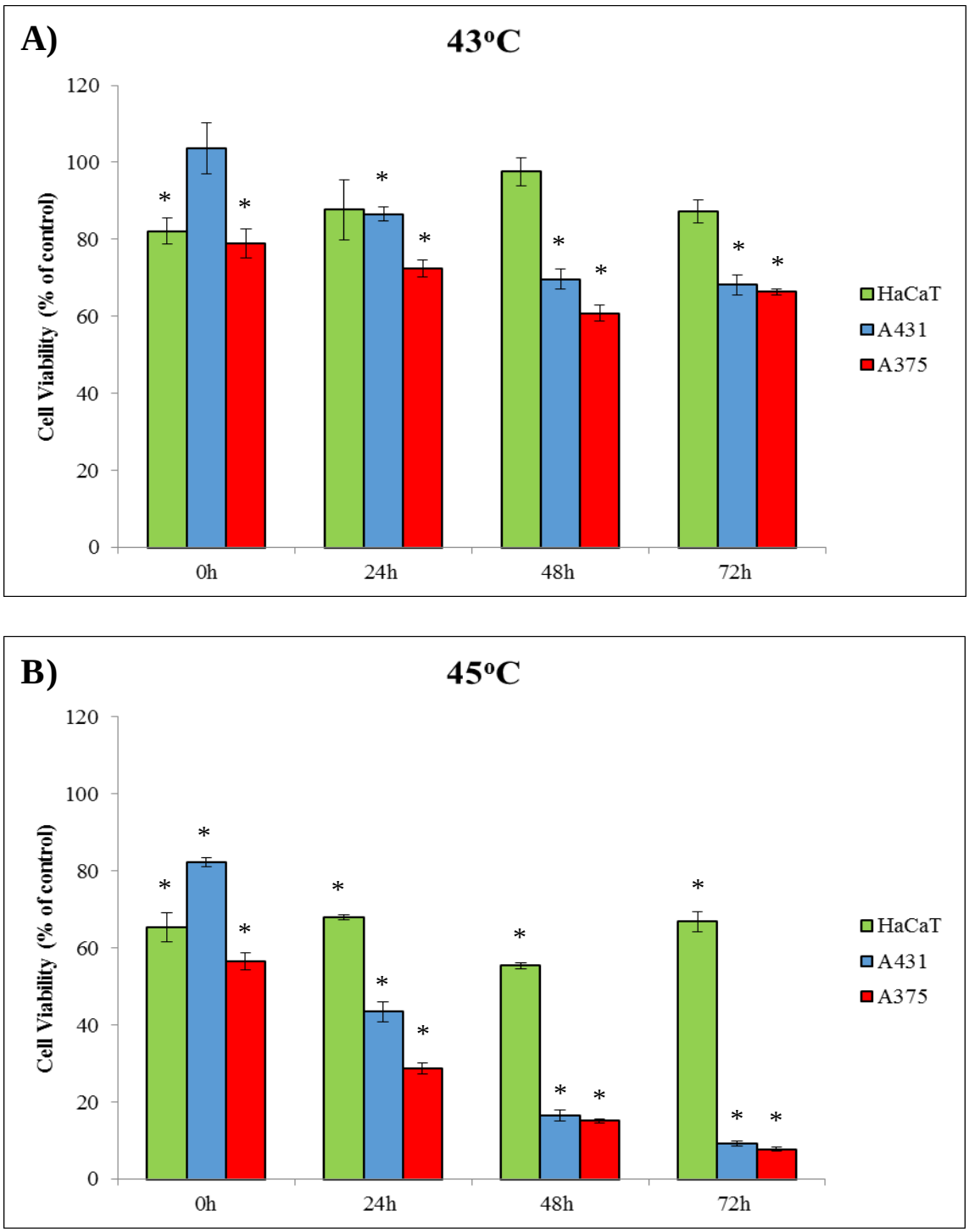


Figure 3.1.3: The effect of hyperthermia at 43°C (A) and 45°C (B) in three different human skin cell (HaCaT, A431, A375) lines. Cells were seeded into 96-well plates and on the next day, cells were exposed to 43°C or 45°C for 2h. Cell viability was determined by using the Alamar-Blue assay immediately after the exposures and 24, 48 or 72h post exposure at 37°C. Cell viability was expressed as percentage of control cells incubated at 37°C. Data shown are mean values (n=5) ± SEM and represent one of three independent experiments. Asterisk (*) indicates statistical significance at $p<0.05$.

3.1.2 Hyperthermia-induced cytotoxicitty

In addition to cell viability, we also utilized cytotoxicity as another endpoint of the hyperthermia exposure platform optimization. For this reason, we used the CytoTox Fluor assay (Promega, UK) which is capable of determining relative levels of dead cells. The assay utilizes a specific fluorigenic peptide substrate (bis-AAF-R110) in order to measure the activity levels of a protease which is released from cells that have lost membrane integrity. Due to the fact that the activity of this protease marker has a short half-life, it is not recommended that the assay is used for extensive incubation times (longer than 24h). Given that, we utilized the assay for time courses equal or shorter than 24h post exposure (6, 12 and 24h). Our data indicate that there was a much higher increase of fluorescence levels in A375 compared to HaCaT cells, at all time points, when exposed to 43^{o}C (Fig.3.1.4.A) or 45^{o}C (Fig.3.1.4.B) suggestive of increased levels of dead cells at both hyperthermic temperatures. In addition, we have used exposure to 50^{o}C, for 2h, as a positive control because according to our previous experiments there was excessive cell death observed at temperatures above 45^{o}C (Fig.3.1.4.C).

In another set of experiments, we utilized trypan-blue staining as an additional means of determining the levels of viable and dead cells after exposing A375 cells to hyperthermia. Similarly to our previous findings, A375 cells were exposed to 43^{o}C or 45^{o}C, for 2h, and then were collected immediately after the exposures and 24h post exposure at 37^{o}C. Our data indicated that there was a slight increase in the levels of dead cells immediately after the exposure to 45^{o}C, however, a dramatic change was observed 24h incubation post exposure (Fig.3.1.5.B). Furthermore, data showed that when A375 cells were exposed to 43^{o}C they appeared to have a reduced proliferating potential compared to control cells whereas the levels of dead cells were the same as those indicated at control conditions (Fig.3.1.5.A). It should be noted that although trypan-blue staining is not a preferred method for determining levels of cell proliferation, it nevertheless provides an indication that perturbations in the levels of viable cells, at 43^{o}C, might be related to cell cycle which could potentially induce growth arrest.

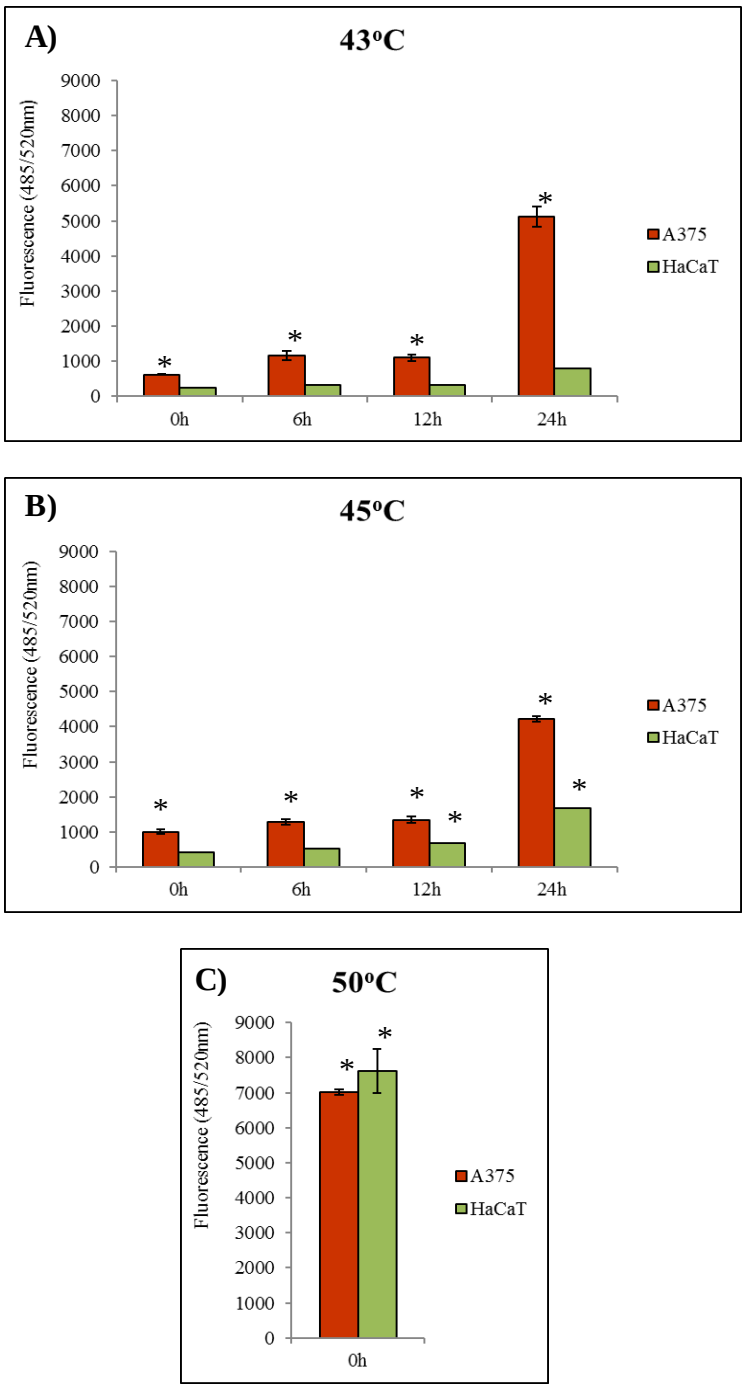


Figure 3.1.4: The effect of hyperthermia at 43°C (A), 45°C (B) and 50°C (C), expressed as relative levels of dead cells, in melanoma (A375) and non-tumorigenic, immortalized keratinocyte (HaCaT) cell lines. Briefly, cells were plated into 96-well plates and on the following day they were exposed to 43°C or 45°C, for 2h. Cytotoxicity was determined by the CytoTox-Fluor Cytotoxicity assay (Promega, UK) immediately after (2h) and 6, 12 and 24h post exposure to hyperthermia. In addition, both cell lines were exposed to 50°C, for 2h, to demonstrate the significance of a positive control. Cytotoxicity is expressed as fluorescence values ($485nm_{Exc}/520nm_{Em}$). Data shown are mean values (n=5) ± SEM and represent one of two independent experiments. Asterisk (*) indicates statistical significance at $p<0.05$.

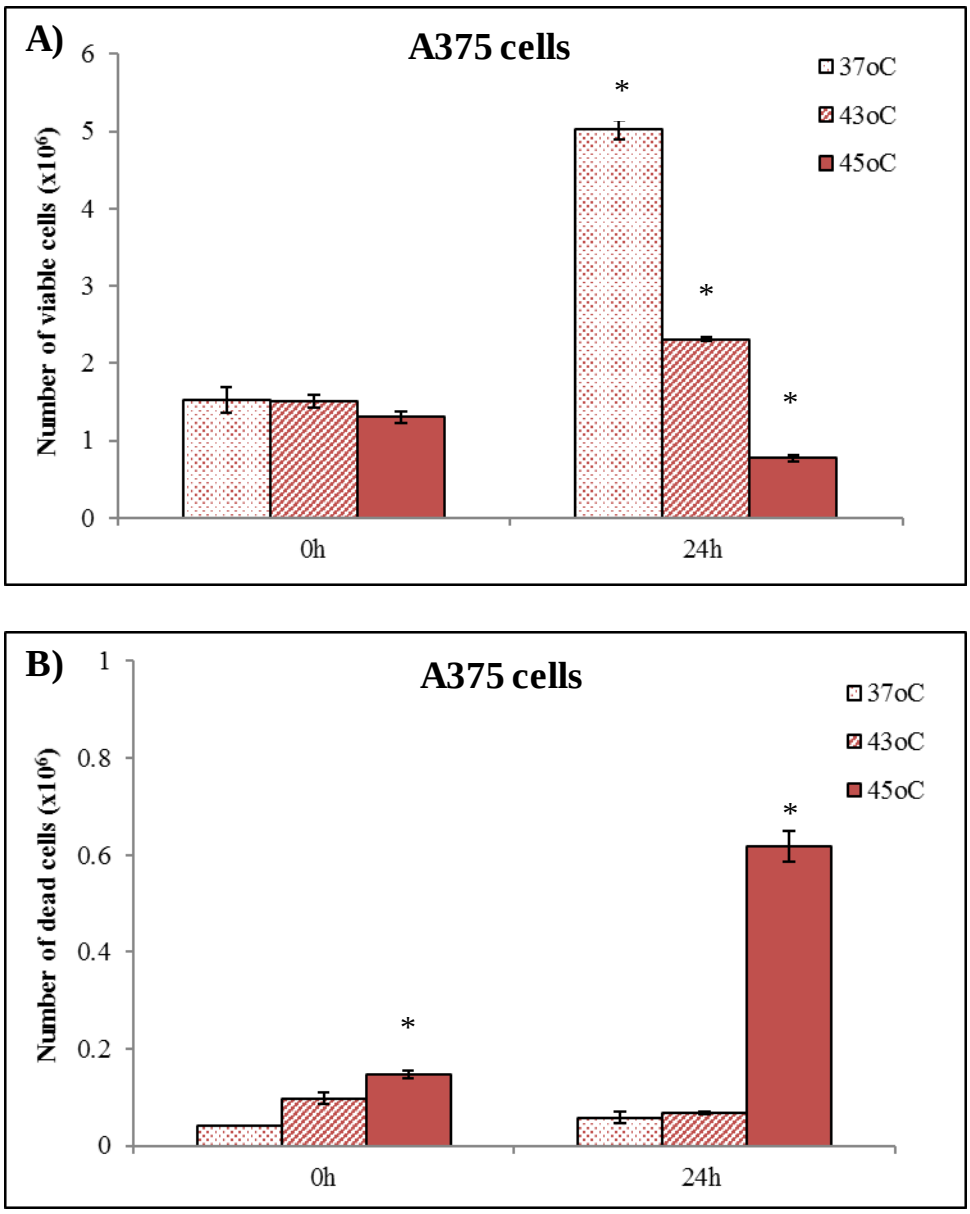


Figure 3.1.5: The effect of hyperthermia (43°C and 45°C) on cell viability (A) and dead cell (B) levels determined by trypan blue staining in human malignant melanoma (A375) cells. Briefly, cells were plated in 100mm dishes and incubated overnight at 37°C. The following day, they were exposed to 43°C and 45°C, for 2h, and then were returned to 37°C for an additional 24h incubation period (post exposure). Control cells were kept at 37°C. Cells were collected and stained with trypan-blue. Data shown are mean values (n=3) ± SEM and represent one of three independent experiments. Asterisk (*) indicates statistical significance at $p<0.05$.

3.2 Hyperthermia induces the apoptotic pathway response in human malignant melanoma (A375) cells

In this set of experiments, we aimed to investigate the involvement of the apoptotic machinery in an attempt to better characterize the apoptotic response to hyperthermia in A375 cells. To this end, different caspases were chosen (caspases-3, -4, -6, -8 and -9) given that they are involved at different stages and pathways of the apoptotic cascade. In these experiments, we determined caspase activity levels at various time points [immediately after 2h exposure (0h) as well as 2, 4, 8, 24, 48 and 72h post exposure]. Justification for the inclusion of each caspase comes from the rationale that each one of them represents a different apoptotic cascade by participating at a different stage within such cascade. For instance, caspase-8 is known to participate in the activation of the extrinsic apoptotic pathway by involving the activation of death receptors. Data showed that there was a slight induction of caspase-8 activity levels, in A375 cells, at 45°C reaching a peak value at 24h (>1.5 fold) and then returning to control levels at the longer post exposure time points (48 and 72h) (Fig.3.1.6). On the other hand, caspase-9 is known to participate in the intrinsic apoptotic pathway (also known as mitochondria-dependent apoptotic pathway) by taking part in the formation of the apoptosome. This caspase was shown to be activated immediately after exposure (0h) and up to 8h post exposure, at both 43°C and 45°C, while its activity was elevated only at 45°C, 24h post exposure (Fig.3.1.7). Finally, the involvement of the ER stress-mediated apoptotic pathway was determined by examining the activation levels of caspase-4. According to our data, its activity levels were shown to be elevated by about a 3-fold induction after exposure to 45°C, at 24h, whereas there was no change observed at 43°C (Fig.3.1.8).

Moreover, we monitored the activation levels of two more executioner caspases namely caspases-3 and -6. Both of these enzymes although do not represent the activation of a specific apoptotic pathway they are, however, involved in the execution signal of the various apoptotic stimuli. To this end, data showed a substantial increase (4-5-fold) in the activity levels of caspase-6 at both 43°C and 45°C immediately after exposure and up to 8h post exposure. However, at longer time points (24 and 48h) there was only activation at 45°C (Fig.3.1.9). On the other hand, the activity of caspase-3 was elevated 8h post exposure (3.5 fold), at 45°C, onwards with a peak (16-fold) at 24-48h post exposure. On the contrary, a modest increase (2-fold) was observed at 43°C at 24h post exposure with a more substantial increase at 48-72h (Fig.3.1.10).

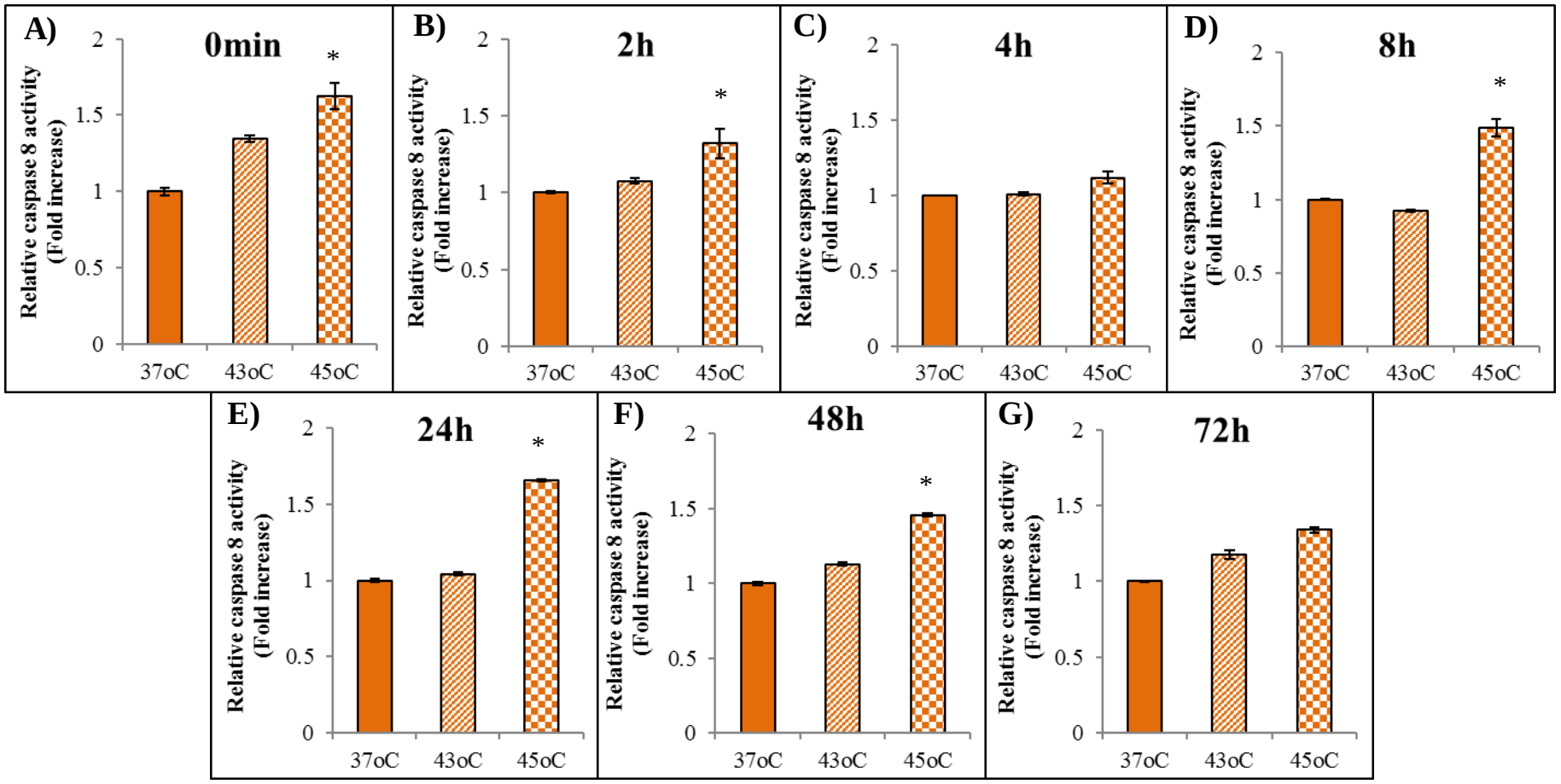


Figure 3.1.6: The effect of hyperthermia (43°C and 45°C) on the activity levels of caspase 8, at various time points, in A375 cells. Briefly, cells were exposed to 43°C and 45°C for 2h (0h) and then were returned back to 37°C for an additional 2-72h while control cells were kept at 37°C. Samples were collected at each time point and following lysis, cell extracts were incubated with the appropriate caspase substrate with fluorescence being monitored. Results shown are expressed as fold increase of caspase activity compared to untreated control. Data shown are mean values (n=3) ± SEM and represent one of three independent experiments. Asterisk (*) indicates statistical significance at $p<0.05$.

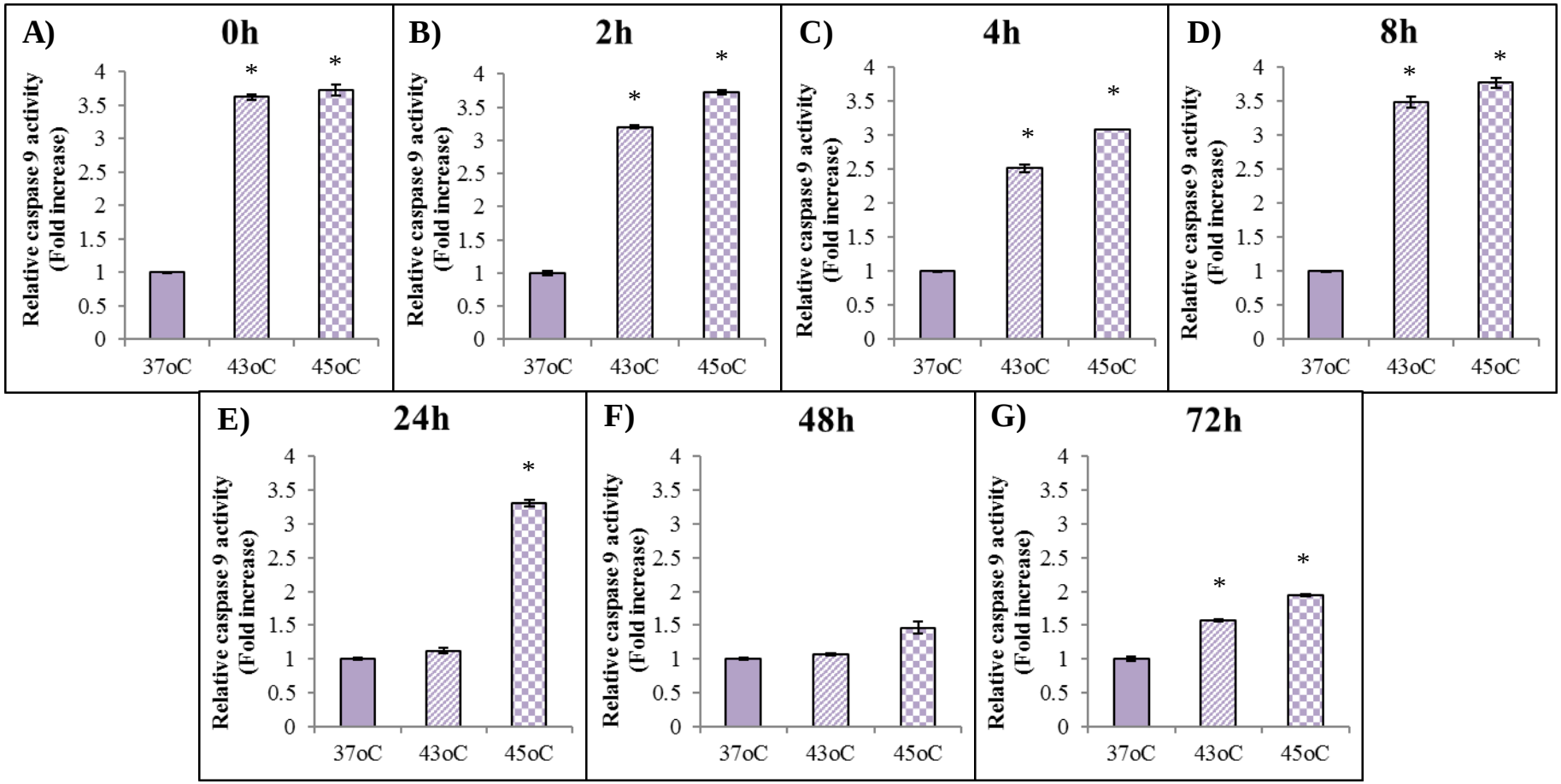


Figure 3.1.7: The effect of hyperthermia (43°C and 45°C) on the activity levels of caspase 9, at various time points, in A375 cells. Briefly, cells were exposed to 43°C and 45°C for 2h (0h) and then were returned back to 37°C for an additional 2-72h while control cells were kept at 37°C. Samples were collected at each time point and following lysis, cell extracts were incubated with the appropriate caspase substrate with fluorescence being monitored. Results shown are expressed as fold increase of caspase activity compared to untreated control. Data shown are mean values (n=3) ± SEM and represent one of three independent experiments. Asterisk (*) indicates statistical significance at $p<0.05$.

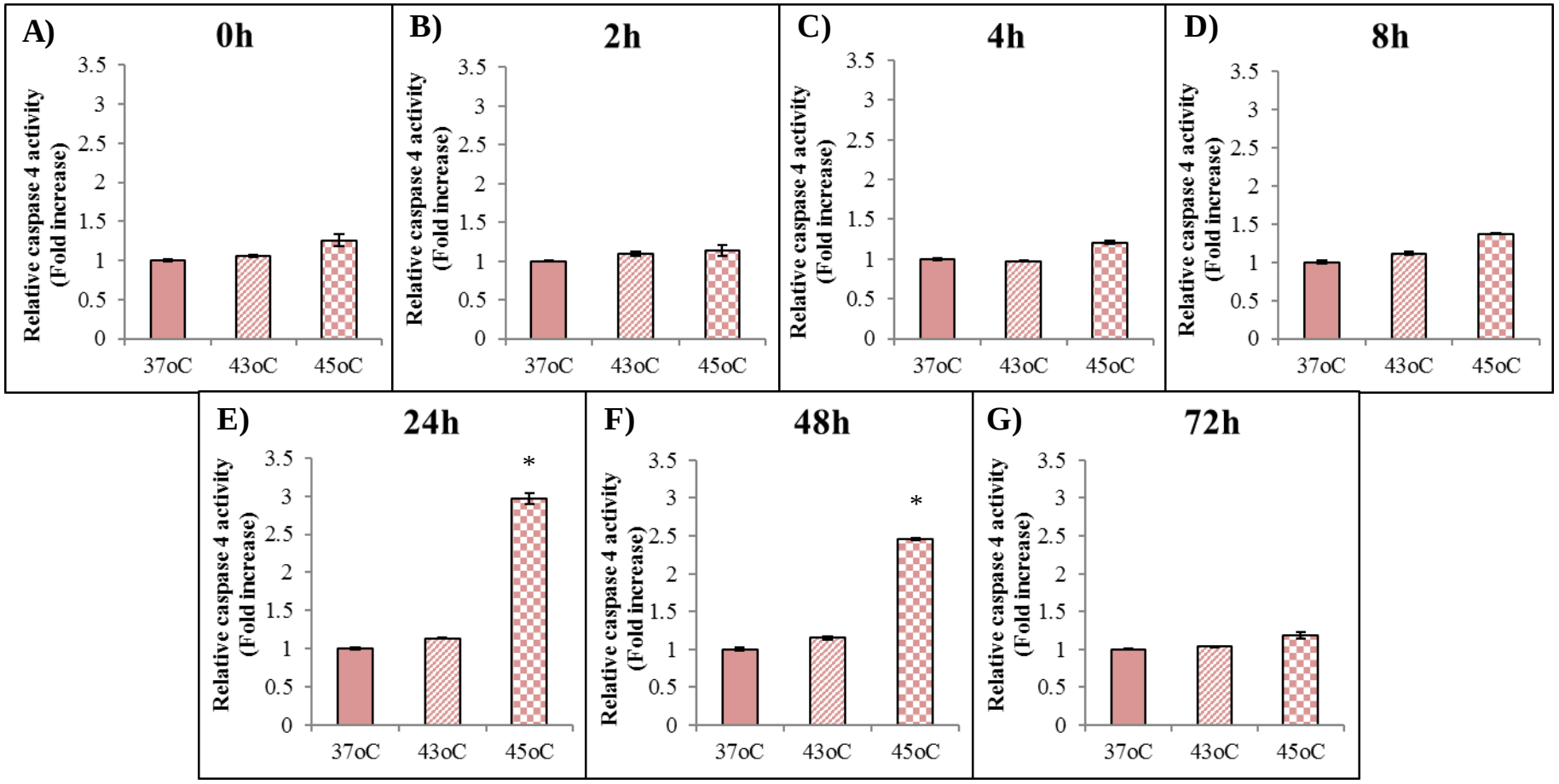


Figure 3.1.8: The effect of hyperthermia (43°C and 45°C) on the activity levels of caspase 4, at various time points, in A375 cells. Briefly, cells were exposed to 43°C and 45°C for 2h (0h) and then were returned back to 37°C for an additional 2-72h while control cells were kept at 37°C. Samples were collected at each time point and following lysis, cell extracts were incubated with the appropriate caspase substrate with fluorescence being monitored. Results shown are expressed as fold increase of caspase activity compared to untreated control. Data shown are mean values (n=3) ± SEM and represent one of three independent experiments. Asterisk (*) indicates statistical significance at $p<0.05$.

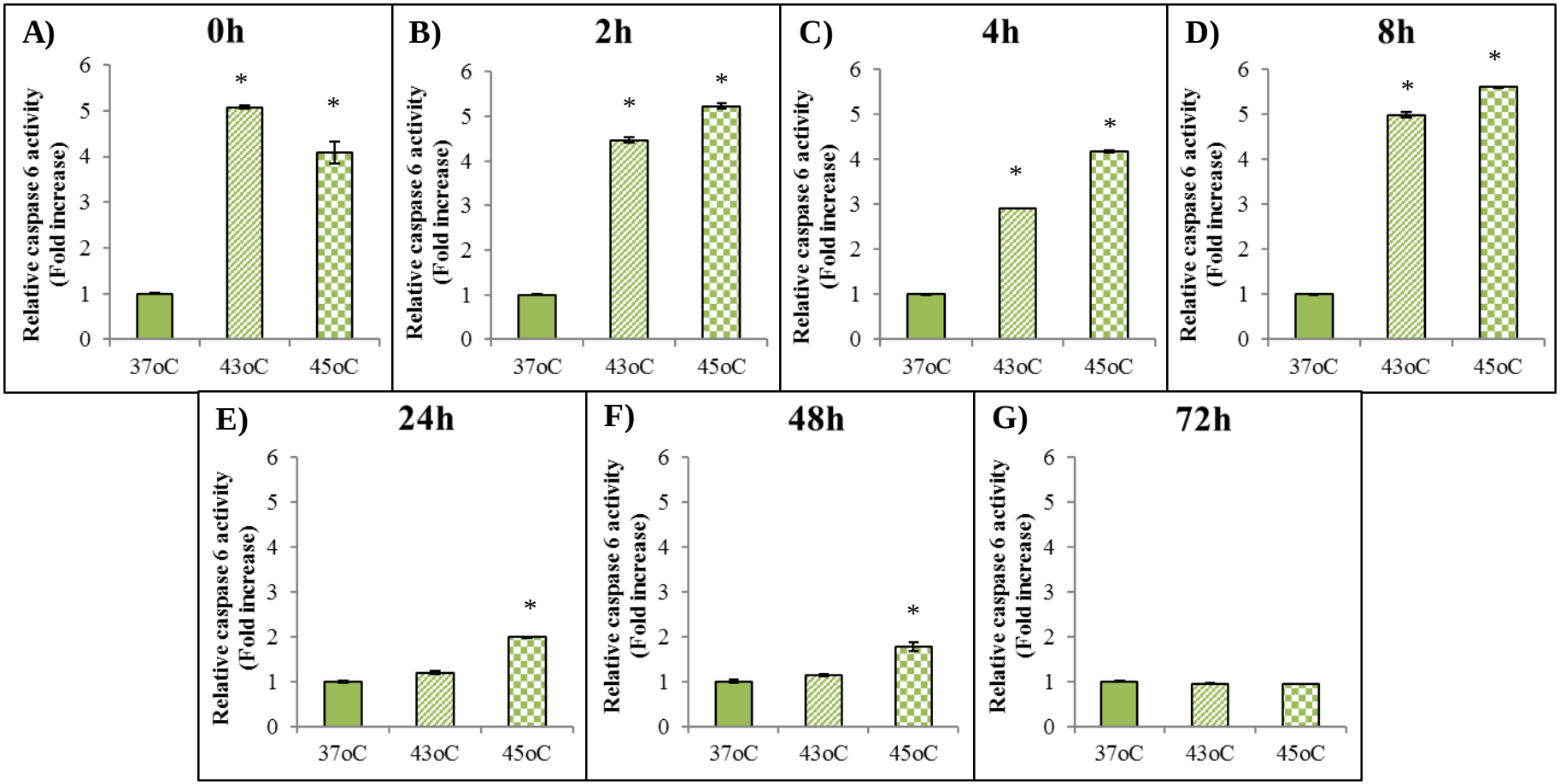


Figure 3.1.9: The effect of hyperthermia (43°C and 45°C) on the activity levels of caspase 6, at various time points, in A375 cells. Briefly, cells were exposed to 43°C and 45°C for 2h (0h) and then were returned back to 37°C for an additional 2-72h while control cells were kept at 37°C. Samples were collected at each time point and following lysis, cell extracts were incubated with the appropriate caspase substrate with fluorescence being monitored. Results shown are expressed as fold increase of caspase activity compared to untreated control. Data shown are mean values (n=3) ± SEM and represent one of three independent experiments ($p<0.05$). Asterisk (*) indicates statistical significance at $p<0.05$.

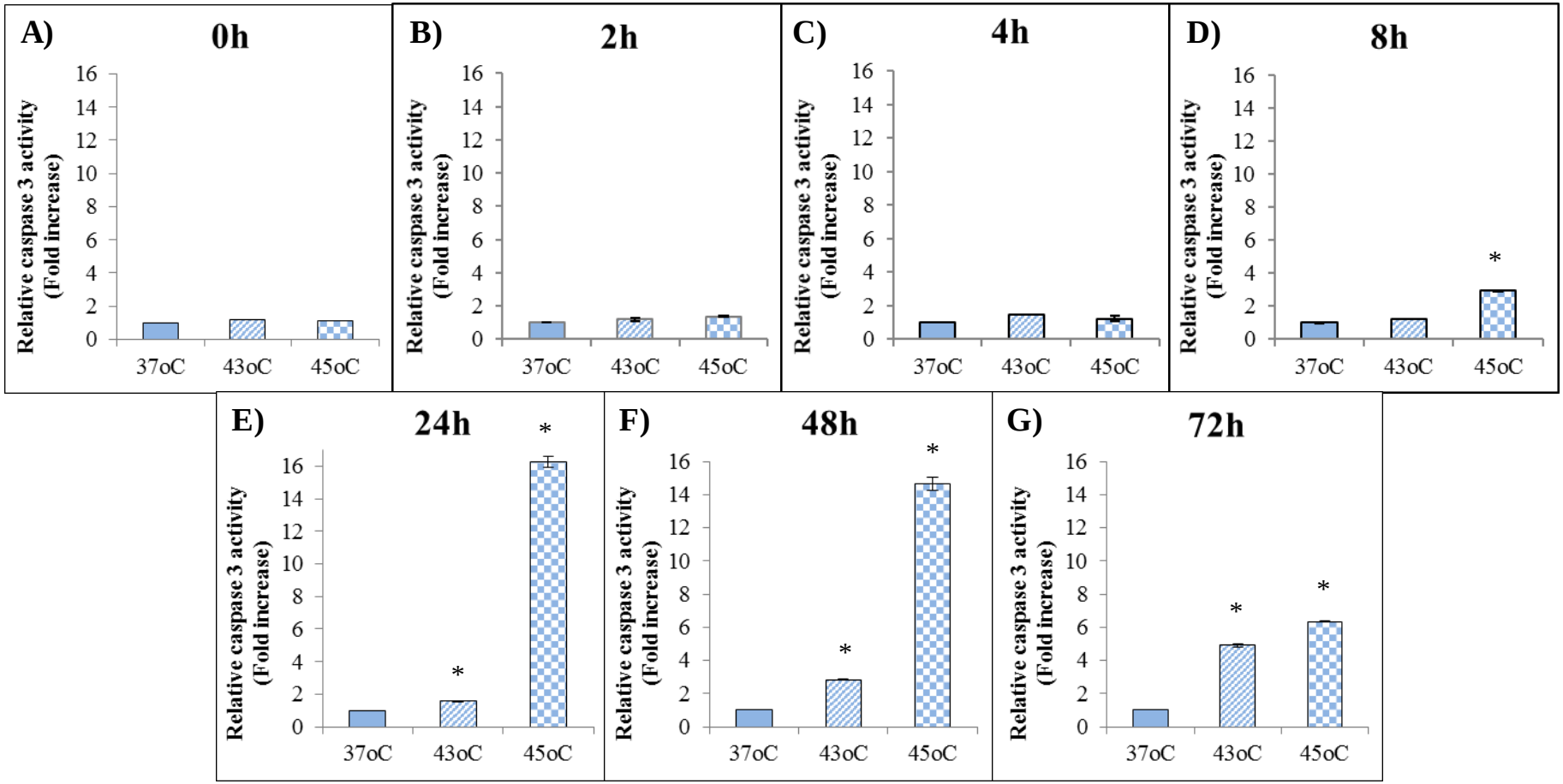


Figure 3.1.10: The effect of hyperthermia (43°C and 45°C) on the activity levels of caspase 3, at various time points, in A375 cells. Briefly, cells were exposed to 43°C and 45°C for 2h (0h) and then were returned back to 37°C for an additional 2-72h while control cells were kept at 37°C. Samples were collected at each time point and following lysis, cell extracts were incubated with the appropriate caspase substrate with fluorescence being monitored. Results shown are expressed as fold increase of caspase activity compared to untreated control. Data shown are mean values (n=3) ± SEM and represent one of three independent experiments ($p<0.05$). Asterisk (*) indicates statistical significance at $p<0.05$.

3.3 Hyperthermia-induces alterations in the expression of key apoptotic genes in human malignant melanoma (A375) cells

We have utilized a genomic approach for screening critical gene targets descriptive of the various stages of the apoptotic response by means of a real-time PCR microarray-based gene expression profiling system. A total of 90 candidate genes were screened in order to profile their differential gene expression pattern in response to 43°C and 45°C at 24h post exposure time point and compare it to 37°C. Our data showed that there were several fold differences in the induction of various apoptotic genes after 24h post exposure to 43°C and 45°C. For the purpose of simplicity, we categorized such altered gene expression into three apoptotic pathways namely (i) the intrinsic, (ii) extrinsic and (iii) ER stress-mediated ones (Table 3.1.1). According to our data, a number of genes, in the intrinsic apoptotic pathway, were found to be up-regulated (after exposure to 43°C and 45°C) including the following: *APAF1, BAK1, BBC3, BCL2L11, BIRC2, CASP7, KIT, PMAIP1* and *VDAC2*. It is noteworthy that although all of these genes were up-regulated at 45°C, only *BAK1, BBC3* and *BIRC2* demonstrated higher expression levels at 43°C (Table 3.1.1). In the case of the extrinsic pathway, *DAPK3, FAS* and *FASLG* were shown to be up-regulated at both 43°C and 45°C while *TNF, TNFRSF10A, TNFSF10, TNFSF12* and *TRAF2* were up-regulated only at 45°C. Finally, *BBC3* (implicated in the intrinsic as well as ER-mediated apoptotic pathways) showed increased expression levels at both temperatures (Table 3.1.1). Moreover, we further categorized genes into three additional groups involved in other cellular processes namely (i) immune response and inflammation, (ii) cell growth and proliferation and (iii) the p-53 dependent apoptotic pathway. Our data showed that a number of genes involved in the immune response and inflammation appeared to be up-regulated in response to either one of the temperatures including: *IL6, NFKBIA, NFKBIE* and *PIK3CD* whereas *CHUK, NFKB2, NFKBIB* and *RELB* demonstrated an increase in expression levels only at 45°C (Table 3.1.2). Furthermore, *IGF-1* was shown to be up-regulated at both hyperthermic temperatures while *KDR* exhibited elevated expression levels at 43°C and *KIT* at 45°C respectively. These three genes are known to play a role in cell growth and proliferation processes (Table 3.1.2). Another group of genes with increased expression levels were those participating in the p53-dependent apoptotic pathway and more specifically *MDM2* and *P53AIP1*. These were up-regulated at both temperatures whereas *PMAIP1* demonstrated higher expression levels only at 45°C (Table 3.1.2).

Table 3.1.1: List of apoptotic genes being differentially regulated as a response to hyperthermic exposures at 43°C and 45°C.

PATHWAY	GENE	DESCRIPTION	Fold induction 43°C	Fold induction 45°C
Intrinsic	***BAK1***	BCL2-Antagonist/Killer 1	↑ 2.1	↑ 31.7
	BBC3	BCL2-Binding Component 3	↑ 4.3	↑ 16.0
	KIT	Mast/stem cell growth factor receptor Kit	-	↑ 2.4
	APAF1	Apoptotic Protease Activating Factor 1	-	↑ 2.9
	BCL2L11	Bcl-2-like protein 11	-	↑ 2.2
	CASP7	Caspase 7	-	↑ 2.2
	PMAIP1	Phorbol-12-Myristate-13-Acetate-Induced Protein 1	-	↑ 4.0
	VDAC2	Voltage-Dependent Anion-selective Channel protein 2	-	↑ 2.3
	BIRC2	Baculoviral IAP Repeat Containing 2	↑ 2.5	↑ 2.2
Extinsic	***CASP7***	Caspase 7	-	↑2.0
	DAPK3	Death-associated protein kinase 3	↑ 2.1	↑ 2.0
	FAS	Fas Cell Surface Death Receptor	↑ 3.0	↑ 11.6
	FASLG	Fas Ligand (TNF Superfamily, Member 6)	↑ 5.0	↑ 176.0
	TNF	Tumor Necrosis Factor	-	↑186.0
	TNFRSF10A	Tumor Necrosis Factor Receptor Superfamily, member 10a	-	↑ 2.0
	TNFSF10	Tumor Necrosis Factor ligand Superfamily member 10	-	↑ 4.1

	TNFSF12	Tumor Necrosis Factor Ligand Superfamily member 12	-	↑ 2.0
	TRAF2	TNF Receptor-Associated Factor 2	-	↑ 2.0
ER-mediated	***BBC3***	BCL2-Binding Component 3	↑ 4.3	↑ 16.0

Table 3.1.2: List of genes associated with other cellular processes being differentially regulated as a response to hyperthermic exposures at 43°C and 45°C.

CELLULAR PROCESS	**GENE**	**DESCRIPTION**	**Fold induction 43°C**	**Fold induction 45°C**
Immune response, Inflammation	***CHUK***	Conserved Helix-Loop-Helix Ubiquious Kinase	-	↑ 2.0
	IL6	Interleucin 6	↑ 23.8	↑ 23.0
	NFKB2	Nuclear Factor Kappa B Subunit 2	-	↑ 2.2
	NFKBIA	NFKB Inhibitor Alpha	↑ 2.1	↑ 6.3
	NFKBIB	NFKB Inhibitor Beta	-	↑ 2.0
	NFKBIE	NFKB Inhibitor Epsilon	↑ 2.2	↑ 2.0
	PIK3CD	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta	↑ 2.5	↑ 2.2
	RELB	RELB Proto-Oncogene, NF-KB Subunit	-	↑ 6.3
Cell growth, Cell proliferation	***IGF-1***	Interleucin 6 Insulin Like Growth Factor 1	↑ 4.4	↑ 2.0
	KDR	Kinase Insert Domain Receptor	↑ 3.0	-

	KIT	KIT Proto-Oncogene Receptor Tyrosine Kinasewe	-	↑ 2.0
p-53 dependent pathway	***MDM2***	MDM2 Proto-Oncogene	↑ 6.1	↑ 16.2
	P53AIP1	P53 Regulated Apoptosis Inducing Protein 1	↑ 2.2	↑ 5.7
	PMAIP1	Phorbol-12-Myristate-13-Acetate-Induced Protein 1	-	↑ 4.0

Chapter Three

Results II - Mechanisms of cell death induction in response to hyperthermia in an *in vitro* model of human malignant melanoma

3. Results - Part II

3.4 Underlined apoptotic mechanisms characteristic of the hyperthermic response in human malignant melanoma (A375) cells

3.4.1 Hyperthermia induces the activation of initiator caspases-8 and -9 in A375 cells

In this part of the study, we aimed to characterize in more depth the molecular mechanisms underlying the activation of cell death in response to 43°C and 45°C. To this end, we focused on determining protein expression levels of several molecules involved at different stages and pathways of the apoptotic machinery. A375 cells were treated with 43°C and 45°C, for 2h, and protein expression levels of initiator caspases-8 (extrinsic pathway) and -9 (intrinsic pathway) were monitored by means of Western blotting at several time points including immediately after hyperthermic exposure (0min) and (2, 4, 8, 24, 48 and 72h) post exposure.

According to our data, there was a drop in protein levels of the uncleaved form of caspase-8 at both temperatures but it was even more evident at 45°C. Caspase-8 appeared to be activated immediately after hyperthermic exposure (0min) and up to 8h post exposure at 43°C in comparison to 45°C where the enzyme remained active even at 24h post exposure. At 48h and 72h post exposure, caspase-8 was retained at control levels at both temperatures (Fig.3.2.1). Furthermore, the pattern of caspase-9 activation was remarkably similar to that of caspase-8. More specifically, there was a reduction of its protein levels at both 43°C and 45°C temperatures. In the case of 43°C, the enzyme was activated up to 8h post exposure as opposed to 45°C at which temperature the enzyme remained active even at 24h post exposure (Fig.3.2.1).

Collectively, our data suggest that at shorter post exposure periods of time (2-8h), the initiator caspases-8 and -9 were activated as a response to hyperthermia (at both temperatures) and that the activation was more intense at 45°C than 43°C. Such protein expression pattern was not observed at longer post exposure periods of time (24h) during which only caspase-9 was activated at 45°C alone (Fig.3.2.1).

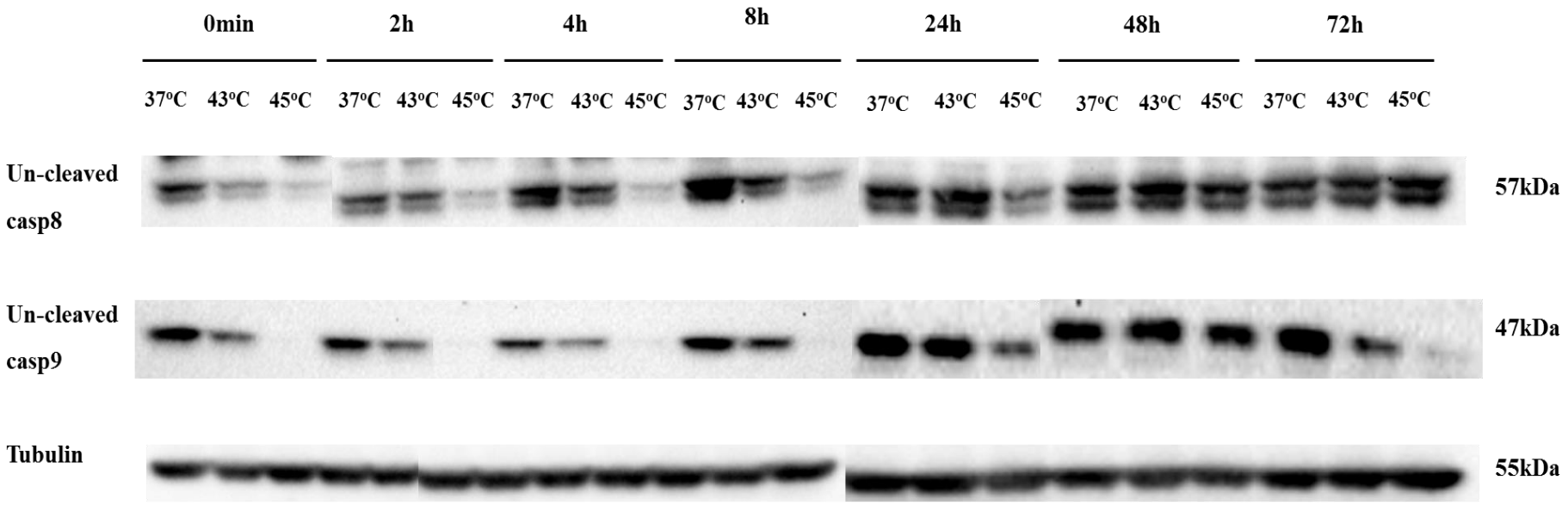


Figure 3.2.1: The activation of caspases-8 and -9 in response to hyperthermic exposures at 43°C and 45°C. Image shows changes in protein expression levels of initiator caspases-8 and -9 immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting conditions. Control cells were kept at 37°C. β-tubulin was used as loading control.

3.4.2 Hyperthermia induces the activation of executioner caspases-6, -7 and -3 in A375 cells

In this set of expetiments, we aimed to study the protein expression profile of caspase-6 which is one of the executioner caspases along with caspases-7 and -3. According to our data, there was a significant reduction in the protein levels of its un-cleaved form after exposing A375 cells to 43^{o}C and 45^{o}C for 2h. This observation indicated the activation of the enzyme which appeared to have a more profound effect at 45^{o}C as it was completely active up to 24h post exposure (Fig.3.2.2). On the contrary, after exposure to 43^{o}C, the protein levels of the pro-caspase form increased from 2-8h post exposure until reaching control levels after 8h and onwards (Fig.3.2.2). Next, we intended to determine the protein expression levels of Lamin A/C, as it represents the target molecule for cleavage by activated caspase-6. As shown in Figure 3.2.2 there is a reduction in the protein levels of the uncleaved Lamin A/C form in response to 43^{o}C and 45^{o}C. In the case of 43^{o}C, the molecule appeared to be cleaved immediately after exposure (0min) and up to 8h post exposure. On the other hand, the molecule was shown to get cleaved all the way through from 2h to 72h post exposure after exposure to 45 ^{o}C. This data is supportive of the increased activation levels of caspase-6 at these time points as well.

On the other hand, caspase-7 and -3 are the two major executioner caspases responsible for cleaving downstream target molecules and account for the well-described characteristics of apoptosis. Our data revealed that caspase-7 was activated from 2-24h post exposure but only after cells were exposed at 45^{o}C (and not at 43^{o}C) (Fig 3.2.3). In the case of caspase-3, we found that the protein remained inactive at 43^{o}C throughout the entire post exposure time course whereas its activity was evidently induced 24-48h post exposure at 45^{o}C. The latter results were in agreement even when determining changes in protein expression levels of the cleaved form of the enzyme. More specifically, we showed that there was a substantial increase in the protein levels of cleaved caspase-3 at 24-48h post exposure at 45^{o}C (Fig 3.2.3). Finally, we also chose to monitor alterations in protein levels of Poly ADP Ribose Polymerase (PARP) as it is one of the main cleavage targets of caspase-3. According to our data, PARP remained uncleaved at each time point post exposure to 43^{o}C while its cleaved form was evident between 24-48h post exposure to 45^{o}C (Fig 3.2.4). In turn, these data is concominant with the protein expression profile of caspase-3.

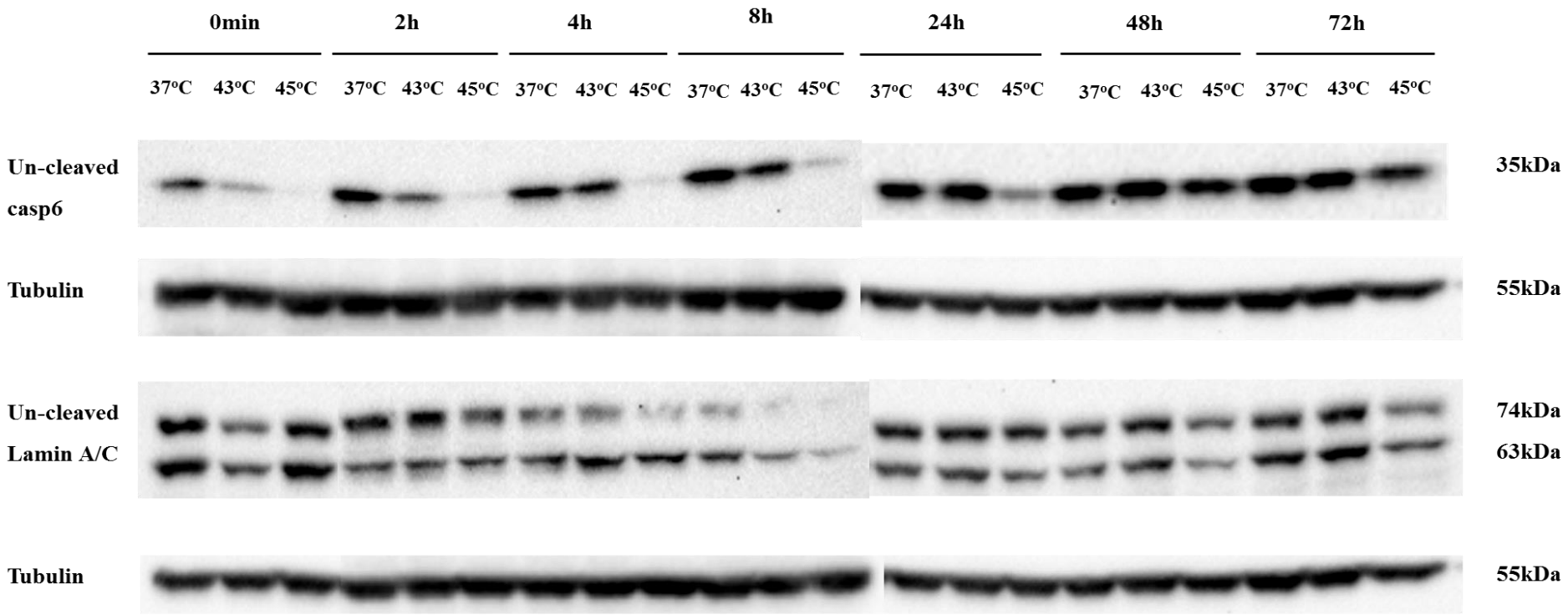


Figure 3.2.2: The activation of caspase-6 in response to hyperthermic exposures at 43°C and 45°C. Image shows changes in protein expression levels of executioner caspase-6 and downstream target Lamin A/C immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting conditions. Control cells were kept at 37°C. β-tubulin was use as a loading control.

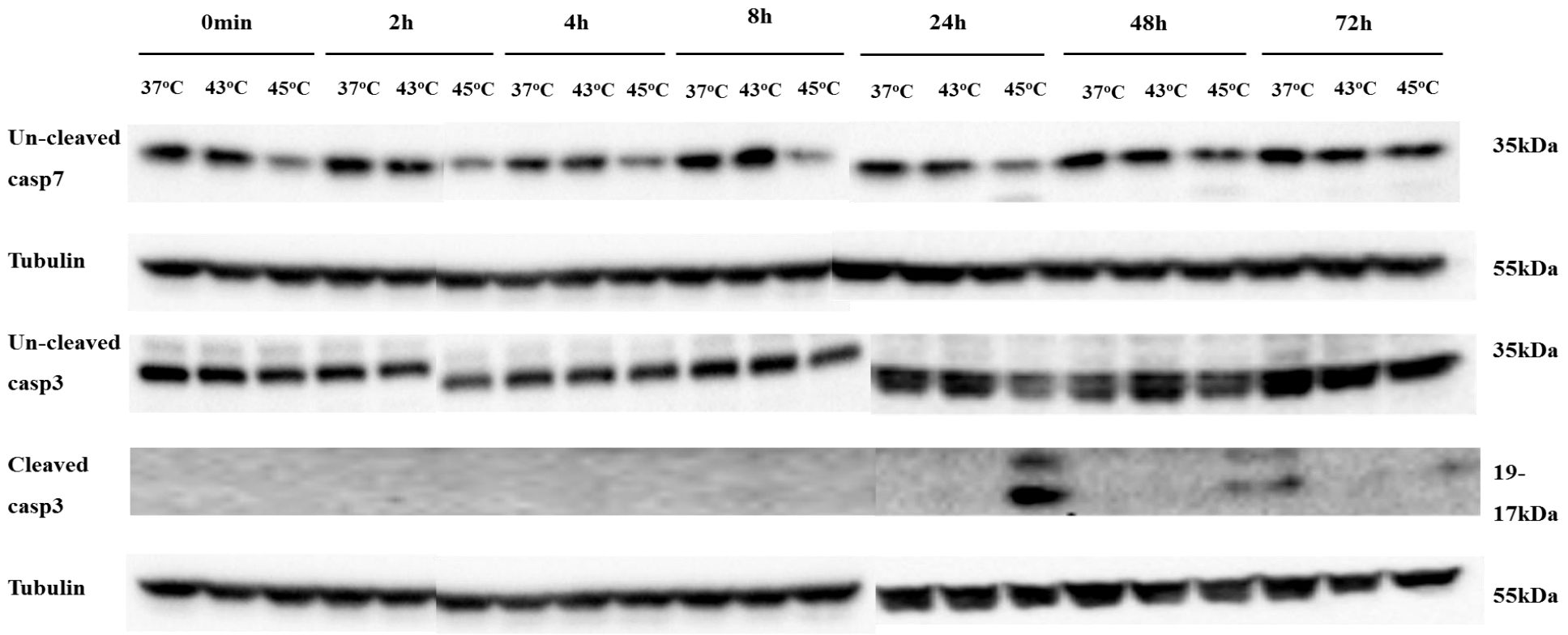


Figure 3.2.3: The activation of executioner caspases-7 and -3 in response to hyperthermic exposures at 43°C and 45°C. Image shows changes in protein expression levels of executioner caspases-7 and -3 immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting conditions. Control cells were kept at 37°C. β-tubulin was used as loading control.

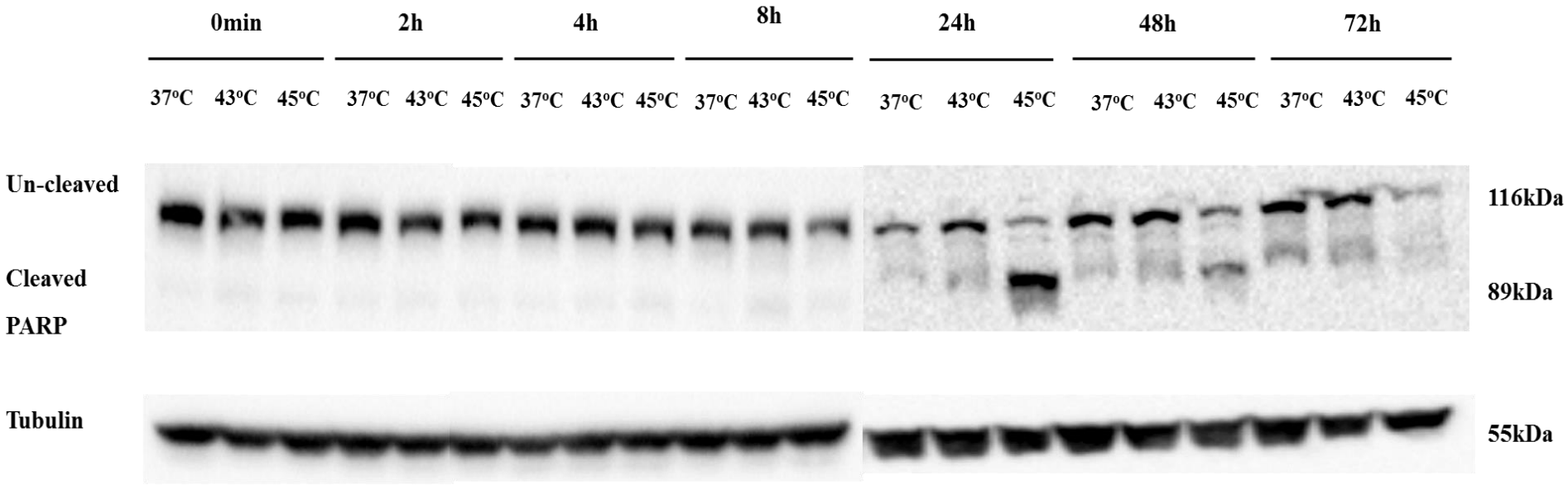


Figure 3.2.4: The cleavage of Poly ADP Ribose Polymerase (PARP) in response to hyperthermic exposures at 43°C and 45°C. Image shows changes in protein expression levels of the un-cleaved and cleaved forms of Poly ADP Ribose Polymerase (PARP) immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting conditions. Control cells were kept at 37°C. β-tubulin was used as loading control.

3.4.3 Hyperthermia induces the activation of death receptor molecules in A375 cells

In this part of the study, we aimed to characterize in more detail the involvement of the extrinsic (death receptor) apoptotic pathway, at 43°C and 45°C, as indicated by our previous findings regarding caspase-8 activation. Firstly, we determined changes in protein levels of TNF-R1 (one of the two receptors for TNFa which, in turn is implicated in initiating a caspase-dependent apoptosis). Our data showed that when cells were exposed to 43°C, TNF-R1 levels were reduced at 2-8h post exposure, suggestive of its activation as opposed to 45°C when its levels were further reduced up to 48h post exposure (Fig.3.2.5).

Another protein playing an important role in the formation of the death domain is TRADD. This protein interacts and binds with TNF-R1 for the creation of the death domain. Alterations in protein expression levels of TRADD presented a similar pattern to those of TNF-R1. More specifically, there was a decline in protein levels at 43°C between 2-8h post exposure while at 45°C the decline was sustained up to 48h post exposure (Fig.3.2.5).

Last but not least, we aimed to look into the involvement of the death domain receptor Fas by monitoring the action of RIP, which contains a death domain known to interact with that of Fas and TRADD. Results demonstrated a reduction in the protein levels of RIP at 45°C at each time point up to 48h post exposure. In contrast, at 43°C there was no activation of RIP at any time point (Fig.3.2.5). In any case, the data thus far seem to support the notion of an interaction between TNF- and Fas-induced extrinsic pathways leading to the subsequent activation of a caspase-8 induced triggering of apoptosis evident at 45°C.

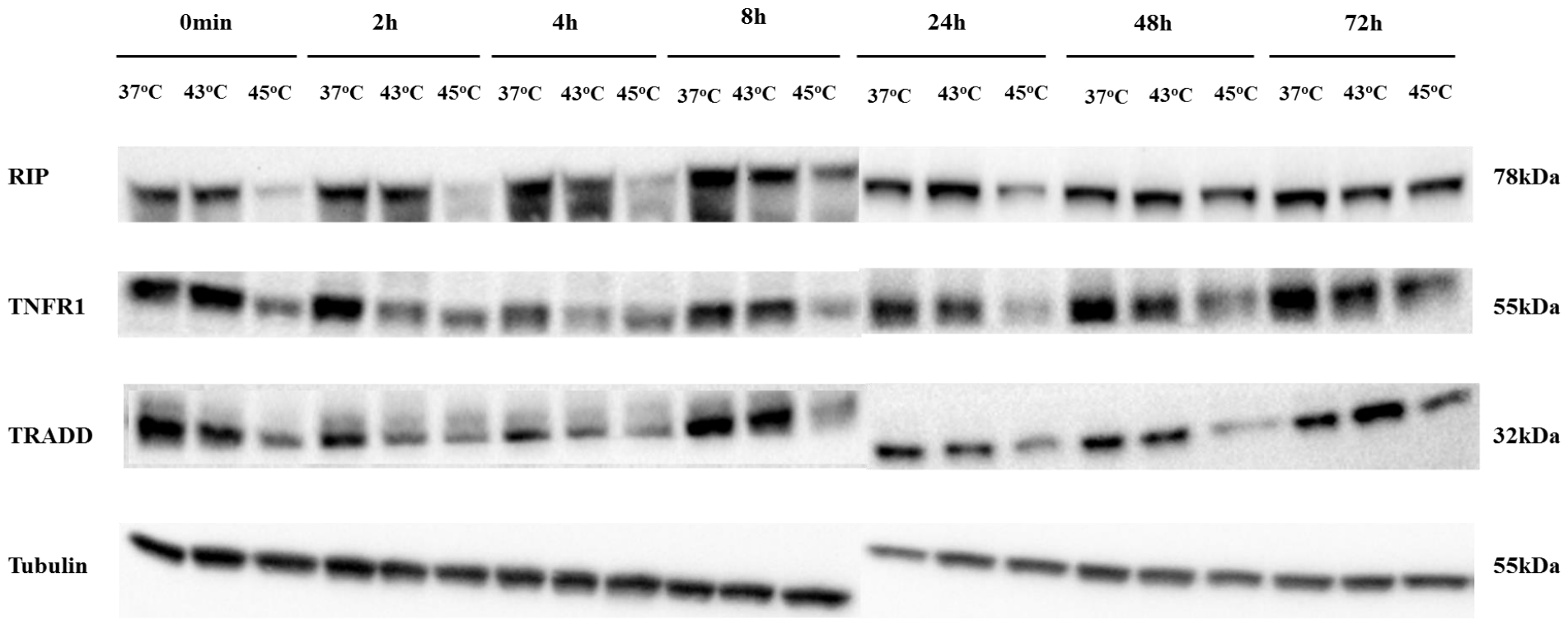


Figure 3.2.5: The activation of death receptor (extrinsic) pathway activation in response to hyperthermic exposures at 43°C and 45°C. Image shows changes in protein expression levels of RIP, TNF-R1 and TRADD immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting conditions. Control cells were kept a t 37°C. β-tubulin was used as loading control.

3.4.4 Hyperthermia induces the upregulation of various proapoptotic proteins in A375 cells

The next set of experiments, was focused on investigating the role of several pro-apoptotic proteins in hyperthermia-induced cell death. It is well known that an imbalance between pro-apoptotic and anti-apoptotic factors can lead to the activation of programmed cell death. For this reason, several pro-apoptotic proteins were selected and their expression levels were determined by Western blotting.

Bak is located on the outer mitochondrial membrane and plays a role in the formation of channels in the membrane in order to facilitate the cytochrome c release when apoptosis is triggered. According to our results, there was an up-regulation of Bak when A375 cells were exposed immediately after 45°C and up to 8h post exposure. Similarly, at 43°C, there was also an up-regulation of the protein at 4-8h post exposure (Fig.3.2.6).

On the other hand, Bax is responsible for increasing the mitochondrial membrane permeability by interacting with other proteins. It was also found to be slightly elevated immediately after exposure and up to 48h post exposure, in the case of 43°C. In contrast, exposure at 45°C was also associated with increased protein expression levels but only up to 8h post exposure (Fig.3.2.6).

Finally, we aimed to determine Bad protein levels as it is a molecule known to act by displacing Bax from binding to antiapoptotic proteins such as Bcl-2 and Bcl-xL. Our data demonstrated that there was an elevation in the protein levels of Bad immediately after 45°C followed by a slight decrease for both hyperthermic conditions following longer incubation periods (from 2 to 24-48h) (Fig.3.2.6). Overall, all such observations support further the complexity of the interactions amongst apoptotic pathways in promoting cell death as a response to hyperthermia.

Furthermore, we decided to examine the involvement of BID in regulating apoptosis as it represents a critical molecule participating in the apoptotic machinery. In doing so, it interacts with caspase-8 which, in turn, cleaves BID thus causing the translocation of its cleaved fragment (t-BID) to the outer mitochondrial membrane where it interacts with caspase-9 and aids in cytochrome c release. In fact, this molecule is known to act as a cross talk point between the extrinsic (caspase 8 induced) and intrinsic (caspase 9 induced) apoptotic pathways. According to our findings, both hyperthermic temperatures were shown to cause an elevation in the protein expression

levels of BID immediately after exposures (0min), an effect which was maintained up to 8h post exposure at 43°C. In contrast, there was a significant decrease in the protein's content from 2-48h post exposure at 45°C (Fig.3.2.7).

In addition, we determined changes in protein expression levels of three different isoforms of Bim, namely Bim_{EL}, Bim_{L}, Bim_{S} as all of them have been implicated in promoting apoptosis. The protein expression levels of Bim_{EL} were shown to be slightly increased up to 24h post exposure at 45°C while the opposite effect (a reduced protein content) was observed at the same time points of post exposure at 43°C. Moreover, there were no major changes in the protein expression levels of Bim_{L} at both hyperthermic temperatures. Finally, Bim_{S}, was found to be elevated immediately after exposure to both hyperthermic temperatures while such increase was even more apparent up until 8h post exposure at 43°C and 24-48h post exposure at 45°C (Fig.3.2.7).

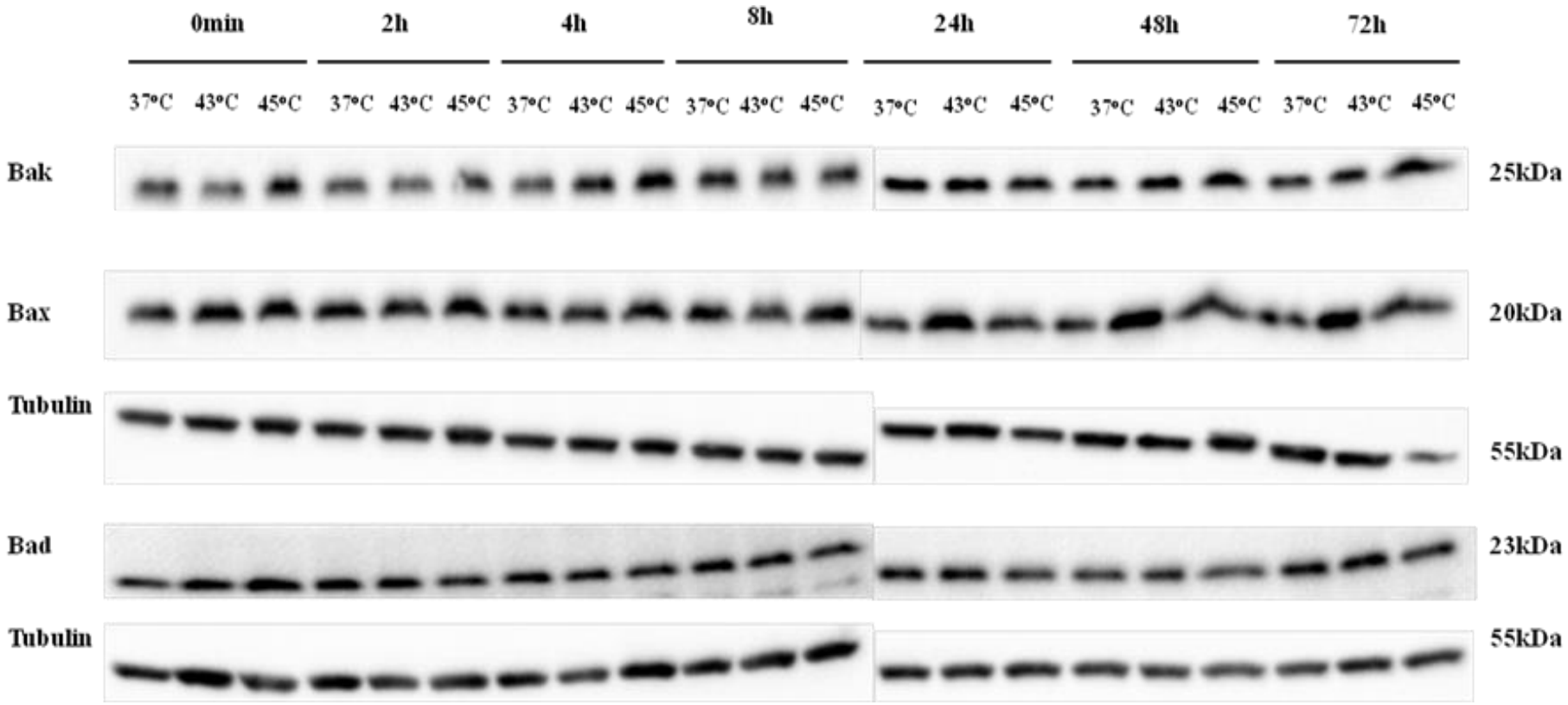


Figure 3.2.6: The involvement of proapoptotic proteins Bak, Bax and Bad in response to hyperthermic exposures at 43°C and 45°C. Image shows alterations in protein expression levels of Bak, Bax and Bad immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting. Control cells were kept at 37°C. β-tubulin was used as loading control.

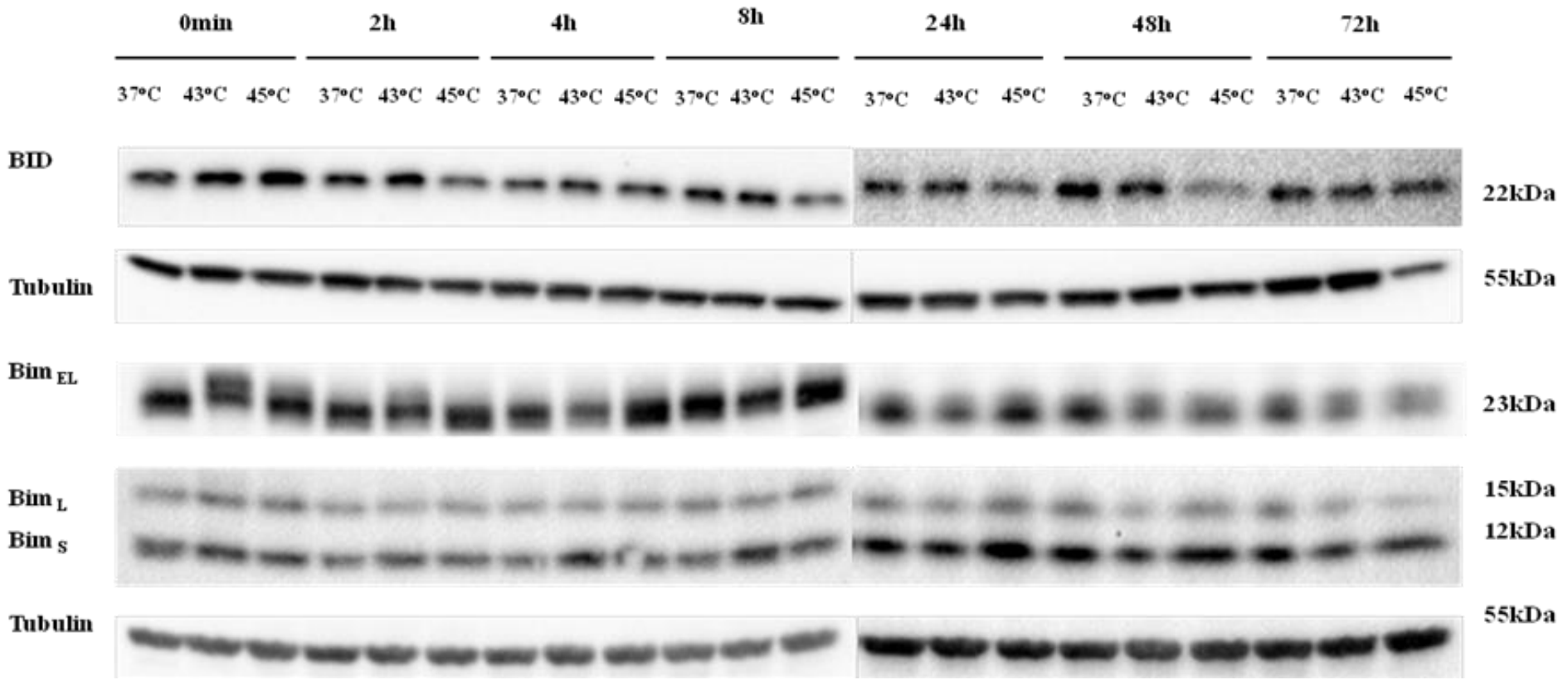


Figure 3.2.7: The involvement of proapoptotic proteins BID, Bim_{EL}, Bim_L and Bim_S in response to hyperthermic exposures at 43°C and 45°C. Image shows alterations in protein expression levels of BID, Bim_{EL}, Bim_L and Bim_S immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting. Control cells were kept at 37°C. β-tubulin was used as loading control.

3.4.5 Hyperthermia induces ER stress response in A375 cells

In this part of the study, we focused on investigating the role of hyperthermia-induced ER stress response in regulating apoptosis. For this purpose, alterations in protein expression levels of several regulators taking part in ER stress induction (UPR response) and also playing a role in activating the apoptotic cascade were studied. First, we examined changes in protein content of Grp78/BiP, a chaperone protein which is induced due to irregular protein folding taking place in ER. This molecule is known to bind to PERK, IRE-1a and ATF-6, under normal conditions, however when ER stress is induced it dissociates from them and thus activates their respective UPR pathways. According to our results, there was a significant increase in Grp78 protein expression levels up until 8h post exposure at 43ºC and 24-48h post exposure at 45ºC (Fig.3.2.8). Second, we looked at PERK as it is responsible for increasing the phosphorylation of eIF2a which in turn promotes a decrease in protein translation. Data showed a reduction in PERK protein levels up to 24h post exposure at 45ºC. In contrast, there were no alterations in its protein content at any time point post exposure at 43ºC (Fig.3.2.8). Third, we monitored the protein expression levels of IRE-1a, a molecule capable of splicing X-box binding protein (XBP-1) mRNA leading to the up-regulation of additional genes implicated in UPR. According to our findings, there was a profound decrease in IRE-1a protein levels up to 8h post exposure at both hyperthermic temperatures. Finally, the observed decline was even more profound at 24-72h post exposure at 45ºC only (Fig.3.2.8).

ATF-6 upon cleavage and subsequent translocation to the nucleus, activates the transcription of various ER stress-related genes. Upon examining alterations in the protein content of the uncleaved ATF-6 form, our findings revealed a decrease in its protein expression levels, at 43ºC, immediately after (0min) and up to 8h post exposure while this decline was maintained immediately after as well as at all time points post exposure at 45ºC (Fig.3.2.9). As mentioned previously, one of the downstream targets of UPR is XBP-1. According to our data, XBP-1s was found to be induced immediately after exposure (0min) and 2h post exposure at 43ºC and 4-8h post exposure at 45ºC. Finally, its protein levels were completely undetected at any time point after 8h post exposure at both temperatures (Fig.3.2.9). Last but not least, CHOP is known to be a major contributor in regulating the ER stress response and consequently ER-induced apoptosis. Our data revealed a significant alteration in its protein expression levels, at

43°C, immediately after (0min) and 2-8h post exposure whereas at 45°C its induction became evident only after 24h post exposure (Fig.3.2.9).

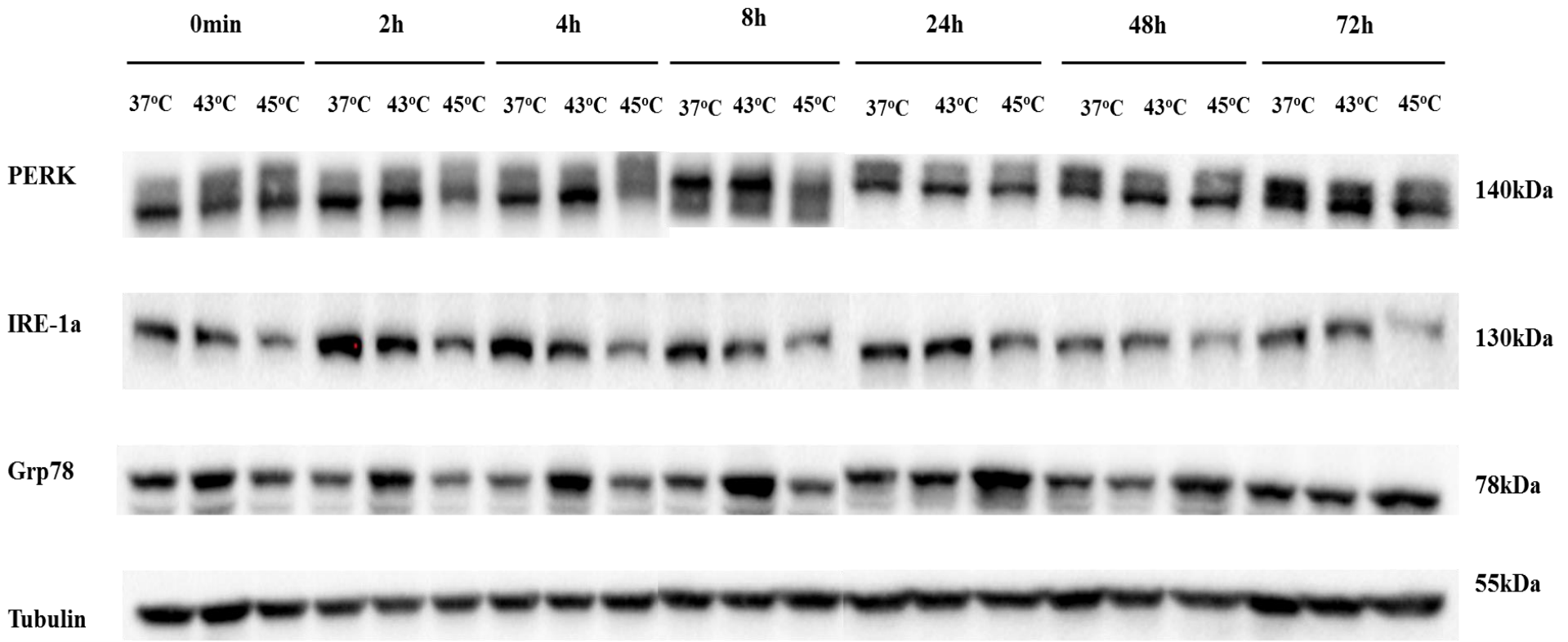


Figure 3.2.8: The participation of PERK, IRE-1a and Grp78 in promoting ER stress response to hyperthermic exposures at 43°C and 45°C. Image shows alterations in protein expression levels of PERK, IRE-1a and Grp78 immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting. Control cells were kept at 37°C. β-tubulin was used as loading control.

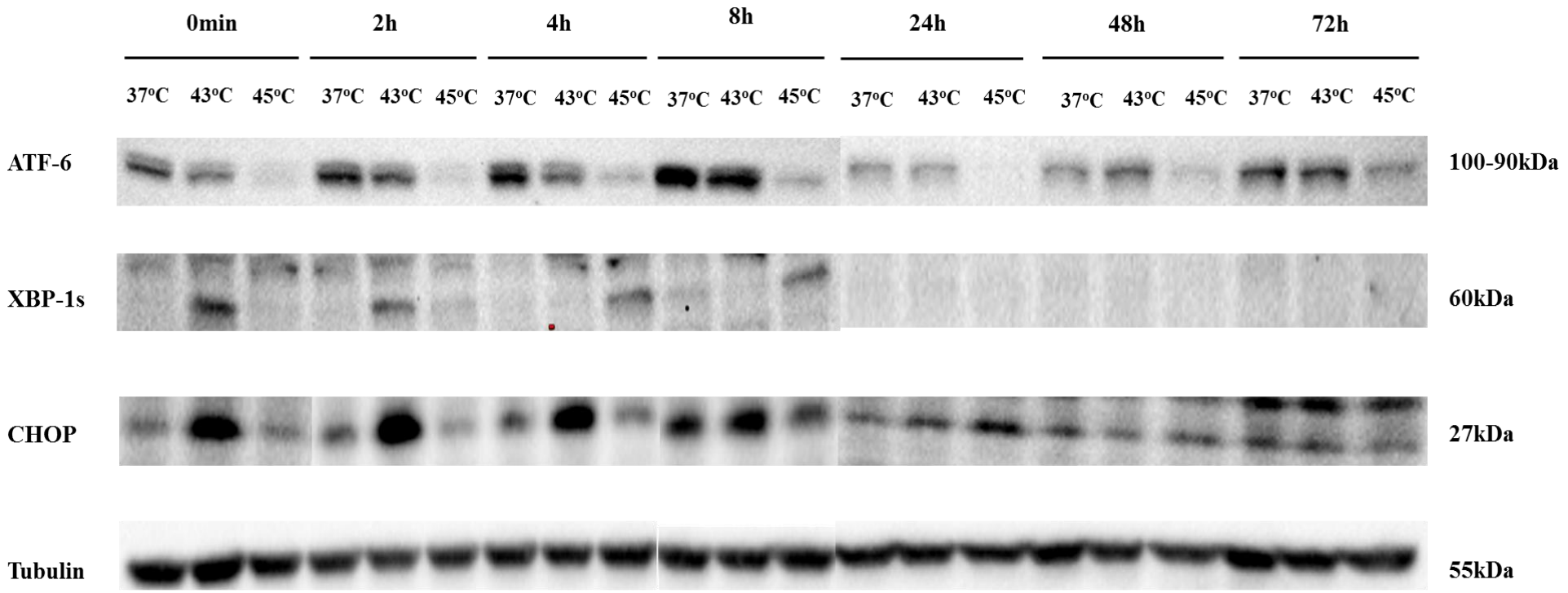


Figure 3.2.9: The participation of ATF-6, XBP-1s and CHOP in promoting ER stress response to hyperthermic exposures at 43°C and 45°C. Image shows alterations in protein expression levels of ATF-6, XBP-1s and CHOP immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting. Control cells were kept at 37°C. β-tubulin was used as loading control.

3.4.6 Hyperthermia induced regulation of heat shock proteins in A375 cells

In this part of the study, we aimed to document the effect of hyperthermia on various heat shock protein regulators. Two major proteins induced by heat stress are HSP90 and HSP70 known to act as molecular chaperones capable of binding and interacting with unfolded proteins thus preventing their aggregation.

Based on our data, protein expression levels of HSP90 were increased following 4-48h post exposure hyperthermic exposures at 45°C whereas remained at control levels, at 43°C, at any time point right after or post exposure (Fig.3.2.10).

In addition, our data showed a remarkable elevation in protein expression levels of HSP70 at both 43°C and 45°C although evidently more profound at 43°C. This trend was shown to persist right after exposure (0min) and all the way up to 48h post exposure (Fig.3.2.10).

Next, we studied alterations in protein expression levels of HSP40. Results demonstrated that its levels were significantly higher right after exposure (0min) and all the way to 8h post exposure at 43°C when compared to 45°C. It was only until 24h post exposure at 45°C when its protein expression levels were significantly higher than 43°C (Fig.3.2.10).

On the other hand, HSP60 is considered to act as a mitochondrial chaperonin responsible for transporting and refolding proteins from the cytoplasm to the mitochondrial matrix. An increase in the protein expression levels of HSP40 was detected in a manner where it was more apparent from 2-24h post exposure at both 43°C and 45°C and 24-72h post exposure only at 45°C (Fig.3.2.11).

Finally, the highly conserved (in multicellular organisms) HSF1 is known to act not only by ensuring the proper folding of damaged proteins but as an important transcription factor for heat shock proteins. Results showed a decrease in HSF1 in the protein expression levels in a manner where it was more evident right after (0min) and up to 4h post exposure at 43°C while this trend continued at each time point post exposure thereafter (2-72h) at 45°C (Fig.3.2.11).

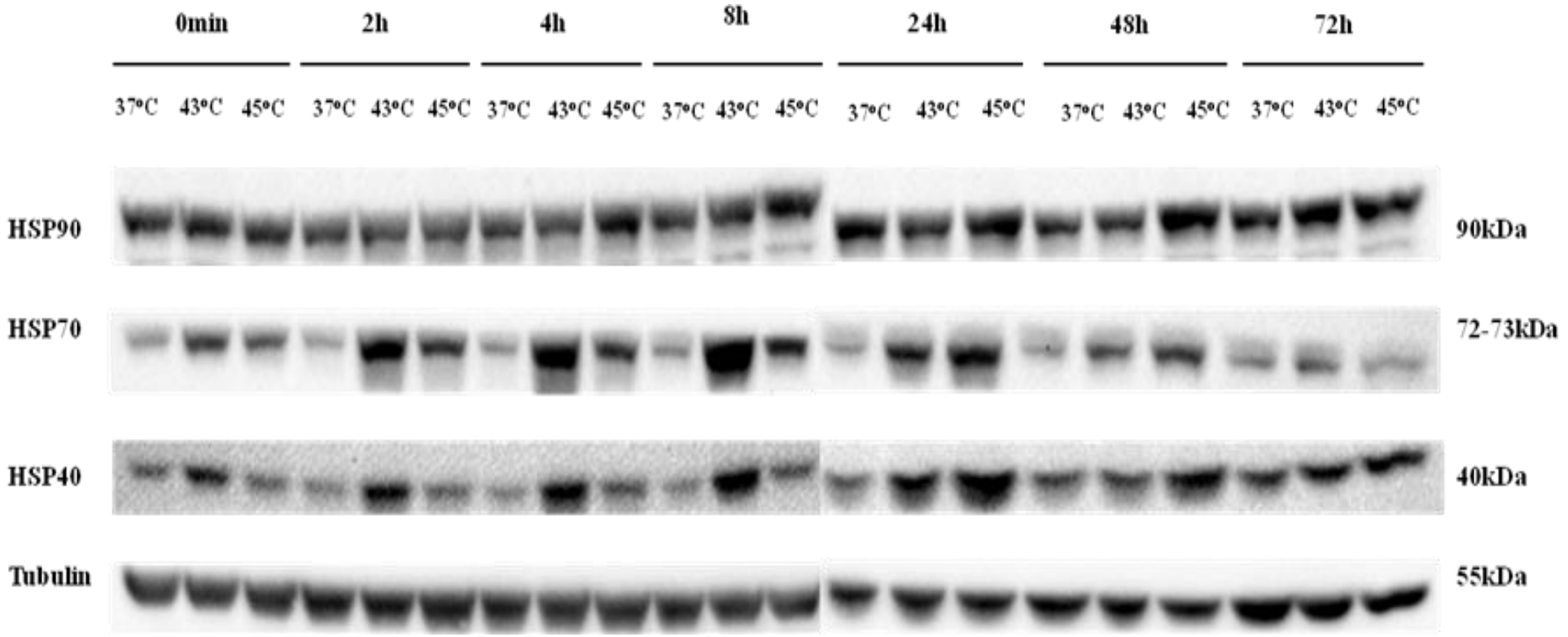


Figure 3.2.10: The response of HSPs 90, 70 and 40 following hyperthermic exposures at 43°C and 45°C. Image shows alterations in protein expression levels of HSP90, HSP70 and HSP40 immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting. Control cells were kept at 37°C. β-tubulin was used as loading control.

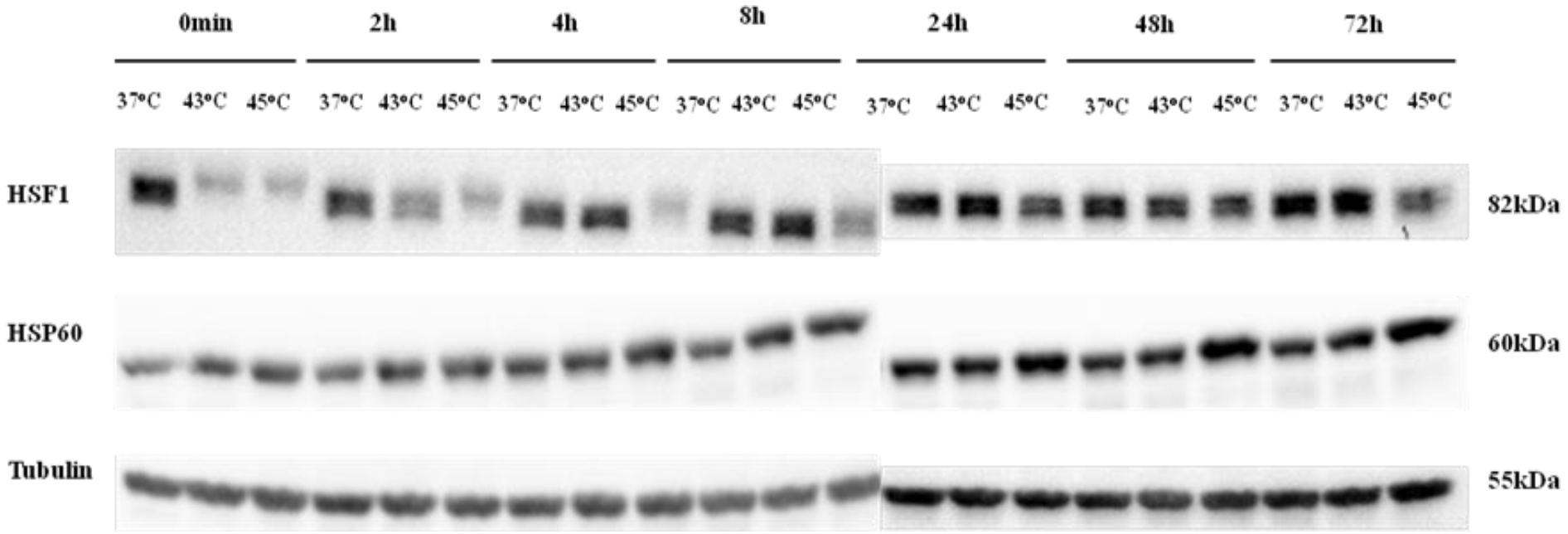


Figure 3.2.11: The response of HSP60 and HSF1 following hyperthermic exposures at 43°C and 45°C. Image shows alterations in protein expression levels of HSF1 and HSP60 immediately after exposure to 43°C and 45°C, for 2h, (0min) as well as at 2-72h post exposure in A375 cells. Cells were grown overnight at 37°C followed by exposure to hyperthermia and then transferred back to 37°C for the indicated post exposure incubation times. Cell lysates were prepared (for all indicated time points) before being subjected to western blotting. Control cells were kept at 37°C. β-tubulin was used as loading control.

Chapter Three

Results III - Hyperthermia as an adjuvant therapy in an *in vitro* model of human malignant melanoma treatment

3. Results - Part III

3.5 The effect of Dacarbazine (DTIC) in combination with hyperthermia in A375 cells

In this part of the study, we aimed to investigate the role of hyperthermia in potentiating the therapeutic effectiveness of other treatments (targeted and non-targeted) and more specifically the efficacy of various chemotherapeutic drugs (a non-targeted treatment) when applied as a combinational protocol. For this reason, we have utilized DTIC which is used widely as an FDA-approved therapy in patients with malignant melanoma. Firstly, we determined changes in cell viability levels of A375 cells after exposure to various concentrations of DTIC at different time periods, 24-72h, (data not shown). We chose 7.81μM and 31.25μM concentrations of DTIC for the reason of the first one being associated without any significant cytotoxicity while the second one showed considerable cytotoxicity when administered individually. For the combined treatment protocol, we exposed A375 cells at 41, 43 and 45°C, for 2h, and immediately after each hyperthermic exposure we administered each DTIC concentration for different time courses (24-72h). Finally, the levels of viable cells were monitored by using the Alamar Blue assay (described in "Materials & Methods"). According to our results, exposure at 41°C showed no statistically significant differences when comparing the effect of 41°C on each concentration of DTIC at 24h post exposure. However, considerable reduction in cell viability levels was recorded at 48h and 72h post exposure in the case of 31.25μM combined with 41°C (from 75% to 55.5% and from 43.0% to 31.0% respectively) (Fig.3.3.1). Next, we used a combinational exposure protocol involving 43°C with both DTIC concentrations. Similarly, there was no potentiation effect observed at 24h post exposure with neither DTIC concentration whereas significant reduction in cell viability levels was recorded at 48h post exposure with 31.25μM DTIC (from 53% to 36.0%). Moreover, considerable reduction was also observed with 7.81μM and 31.25μM DTIC at 72h post exposure (from 59% to 34.0% and from 37.0% to 15.0% respectively) (Fig.3.3.2). Finally, at 45°C, our data showed that the reduction in cell viability was of the same magnitude for both DTIC concentrations at all time points (24-72h). The rates of viable cells were recorded to be around 40%, 20% and 10% at 24h, 48h and 72h post exposure respectively (Fig.3.3.3). This observation indicated that no potentiation effect could be observed under this experimental condition due to the great degree of cytotoxicity observed at 45°C on its own (monotherapy).

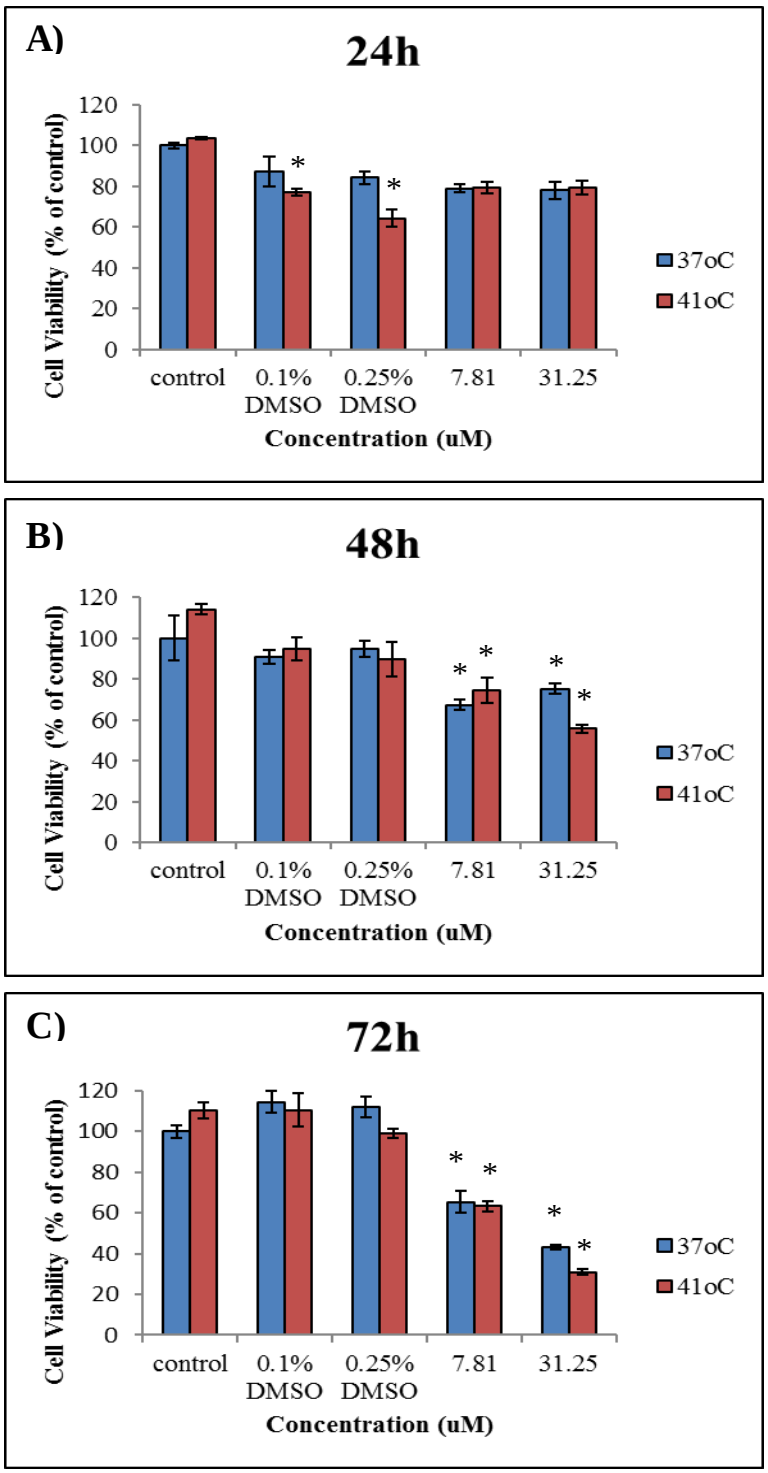


Figure 3.3.1: The effect of an adjuvant therapeutic protocol combining 41°C and DTIC at 24h (A), 48h (B) and 72h (C) post exposure in A375 cells. Briefly, cells were seeded in 96 well-plates and kept at 37°C overnight. Next day, cells were subjected at 41°C, for 2h, and 7.81μM as well as 31.25μM DTIC concentrations were added. Cells were then returned at 37°C for additional post exposure incubation periods (24-72h). Cell viability levels were recorded by utilizing the Alamar Blue assay. Control cells were kept at 37°C. Data shown are mean values ± SEM (n=5). Asterisk (*) indicates statistical significance at $p<0.05$.

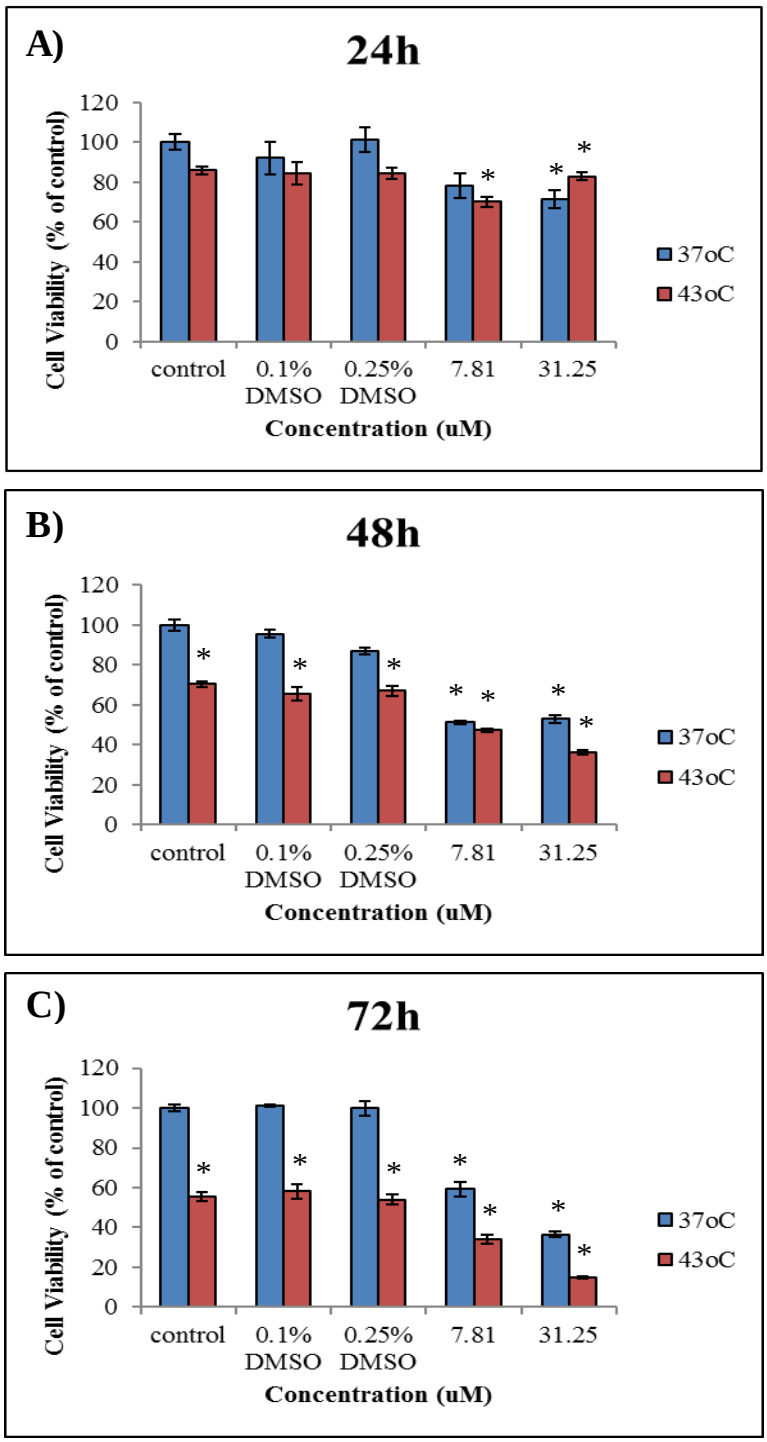


Figure 3.3.2: The effect of an adjuvant therapeutic protocol combining 43°C and DTIC at 24h (A), 48h (B) and 72h (C) post exposure in A375 cells. Briefly, cells were seeded in 96 well-plates and kept at 37°C overnight. Next day, cells were subjected at 43°C, for 2h, and 7.81μM as well as 31.25μM DTIC concentrations were added. Cells were then returned at 37°C for additional post exposure incubation periods (24-72h). Cell viability levels were recorded by utilizing the Alamar Blue assay. Control cells were kept at 37°C. Data shown are mean values ± SEM (n=5). Asterisk (*) indicates statistical significance at $p<0.05$.

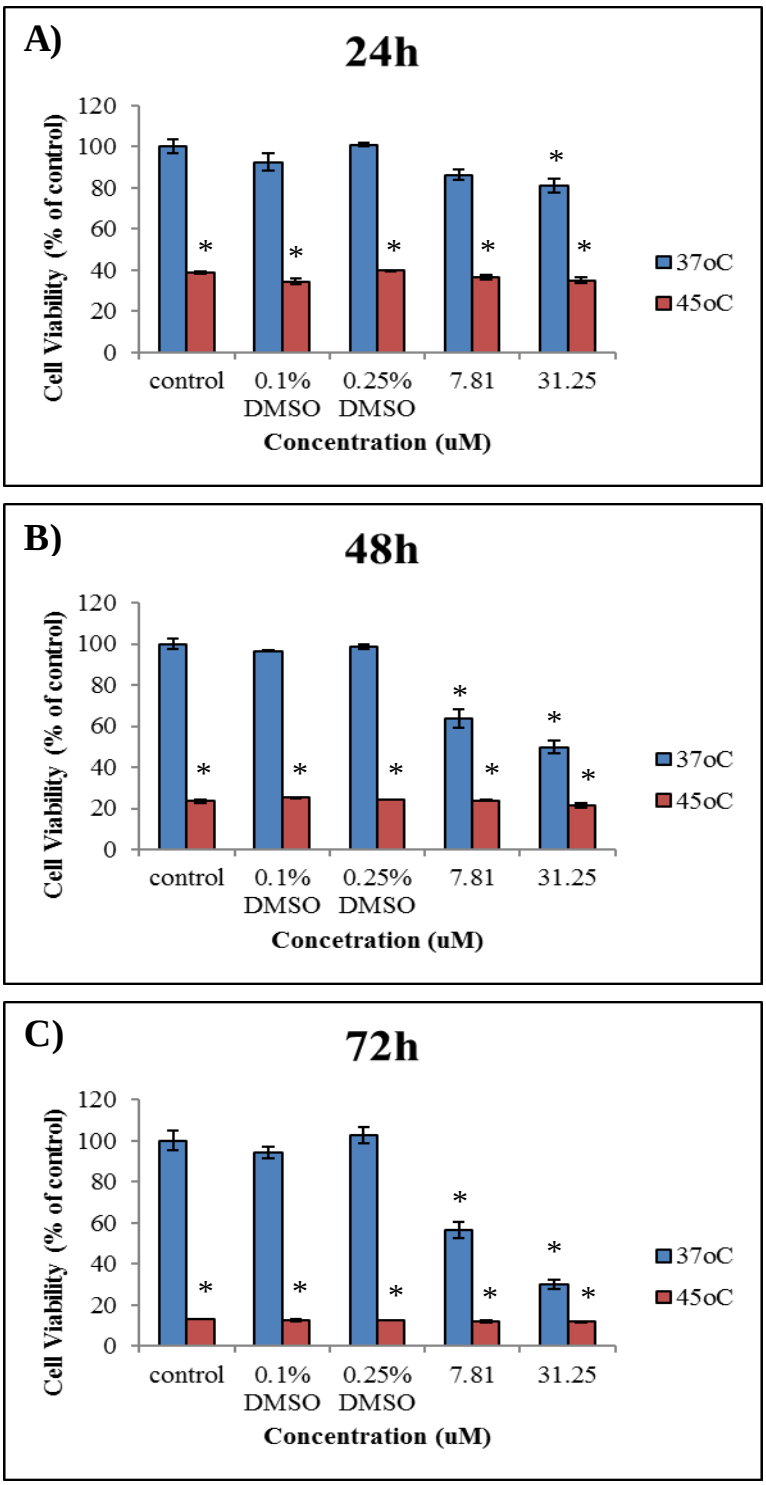


Figure 3.3.3: The effect of an adjuvant therapeutic protocol combining 41°C and DTIC at 24h (A), 48h (B) and 72h (C) post exposure in A375 cells. Briefly, cells were seeded in 96 well-plates and kept at 37°C overnight. Next day, cells were subjected at 41°C, for 2h, and 7.81μM as well as 31.25μM DTIC concentrations were added. Cells were then returned at 37°C for additional post exposure incubation periods (24-72h). Cell viability levels were recorded by utilizing the Alamar Blue assay. Control cells were kept at 37°C. Data shown are mean values ± SEM (n=5). Asterisk (*) indicates statistical significance at $p<0.05$.

3.6 The effect of a novel palladium-based chemotherapeutic agent as well as two targeted drug compounds (Vemurafenib and Dabrafenib) in combination with hyperthermia in A375 cells

In this set of experiments, we utilized two targeted drugs compounds (Vemurafenib and Dabrafenib) which have been very recently FDA approved and are also currently used in clinical trials. Their mechanism of action is based upon acting and inhibiting a mutated (V600E) BRAF form present in almost 40-50% of all melanoma patients. Firstly, we determined the effect of various concentrations of each targeted drug alone (monotherapy) in A375 cells at different time courses (24-72h) (data not shown). Next, we chose a range of concentrations of each drug for combining with hyperthermia. Given, from our previous observations, that an optimal hyperthermic temperature was exposure at 43^{o}C, we tested this temperature in its ability to potentiate the therapeutic efficacy of each of these two targeted drug compounds. In addition, we included an experimental palladium-based novel compound, which has never been tested in melanoma before, in order to determine its ability to induce cytotoxicity and if so, whether it is also potentiated in the presence of hyperthermia (Fig.3.3.4 & 3.3.6). Briefly, A375 cells were exposed at 43^{o}C, for 2h, then a wide range of predetermined concentrations for each drug compound was utilized over different time courses (24-72h) and finally cell viability levels were determined. We selected the 48h post exposure time point as it was shown to be the optimum experimental condition, for each drug compound, in detecting significant changes in their cell viability levels. Data showed a decline in A375 viability levels when combined with hyperthermia at 43^{o}C indicating a possible potentiation effect. Interestingly, this decline appeared to be more profound in the case of the palladium-based drug compound and to a lower extent with the two targeted ones (Figs.3.3.6.A). More specifically, when looking at the EC50 (effective concentration which kills 50% of cells) value for each drug compound (50nM for Vemurafenib, 5nM for Dabrafenib and 0.2μM for the palladium-based one), it was apparent that exposure at 43^{o}C induced a lower than 10% reduction in both targeted drug compounds and slightly higher in the palladium-based one (Fig.3.3.4 & 3.3.6.A). Next, we included the non-tumorigenic, immortalized human keratinocyte (HaCaT) cell line in our exposure protocol in order to examine if the cytotoxicity observed at the above-mentioned EC50 values was also the case in HaCaT cells. The rationale for the inclusion of this cell line (as a control one) is based on the fact that, under normal conditions, melanocytes are surrounded by keratinocytes and so when a non-targeted

drug is administered it is expected to induce cytotoxicity in the surrounding "healthy" area as part of its non-targeted action. According to our results, when HaCaT cells were exposed to the same experimental conditions, their viability rates remained either at control levels or at much higher levels compared to the corresponding ones for each drug's concentration tested in A375 cells (Fig.3.3.5).

In another set of experiments, we selected two concentrations of each drug (one at an EC50 level and another one of a more cytotoxic profile) and we determined their potential to induce an apoptotic response either on its own (37^{o}C) or in combination with hyperthermia (43^{o}C) by using western immunoblotting. According to our findings, the combination of 43^{o}C and Vemurafenib treatment caused the activation of initiator caspase-9 whereas caspase-8 did not get activated at these experimental conditions (Fig.3.3.7). Moreover, there were no profound changes in protein expression levels of pro-caspase-6 (and its downstream target Lamin A/C) as well as that of pro-caspase-7 and -3 (Fig.3.3.8). On the other hand, the combinational treatment of Dabrafenib and hyperthermia at 43^{o}C was shown to induce a reduction in protein expression levels of pro-caspase 8 thus indicating its activation whereas no changes in the protein content of un-cleaved caspase 9 were detected (Fig.3.3.7). A minor decline in protein levels of pro-caspase 6 was also observed, while it was even more obvious for Lamin A/C, therefore showing the activation of the corresponding proteins (Fig.3.3.8). Moreover, there were no significant changes detected in protein expression levels of procaspase-3 at these experimental conditions (Fig.3.3.9). Finally, exposure to 43^{o}C followed by treatment with the palladium-based drug compound resulted in the activation of initiator caspase-8 but not of initiator caspase-9 whose protein content remained unchanged (Fig.3.3.7). In addition, effector caspases-6 and -7 appeared to be activated under these experimental conditions as a modest decline in the protein content of their un-cleaved enzyme forms was observed (Fig.3.3.8 & 3.3.9). The activation of caspase-6 was also indicated by a reduction in the protein levels of Lamin A/C (Fig.3.3.8). In contrast, effector caspase-3 did not appear to be induced since there were no changes in the protein content of the un-cleaved enzyme form (Fig.3.3.9). Collectively, it was evident that each of the drug compounds was capable of triggering the apoptotic process by activating different caspases thus implying the involvement of different apoptotic pathways (e.g. extrinsic, intrinsic) in inducing cell death.

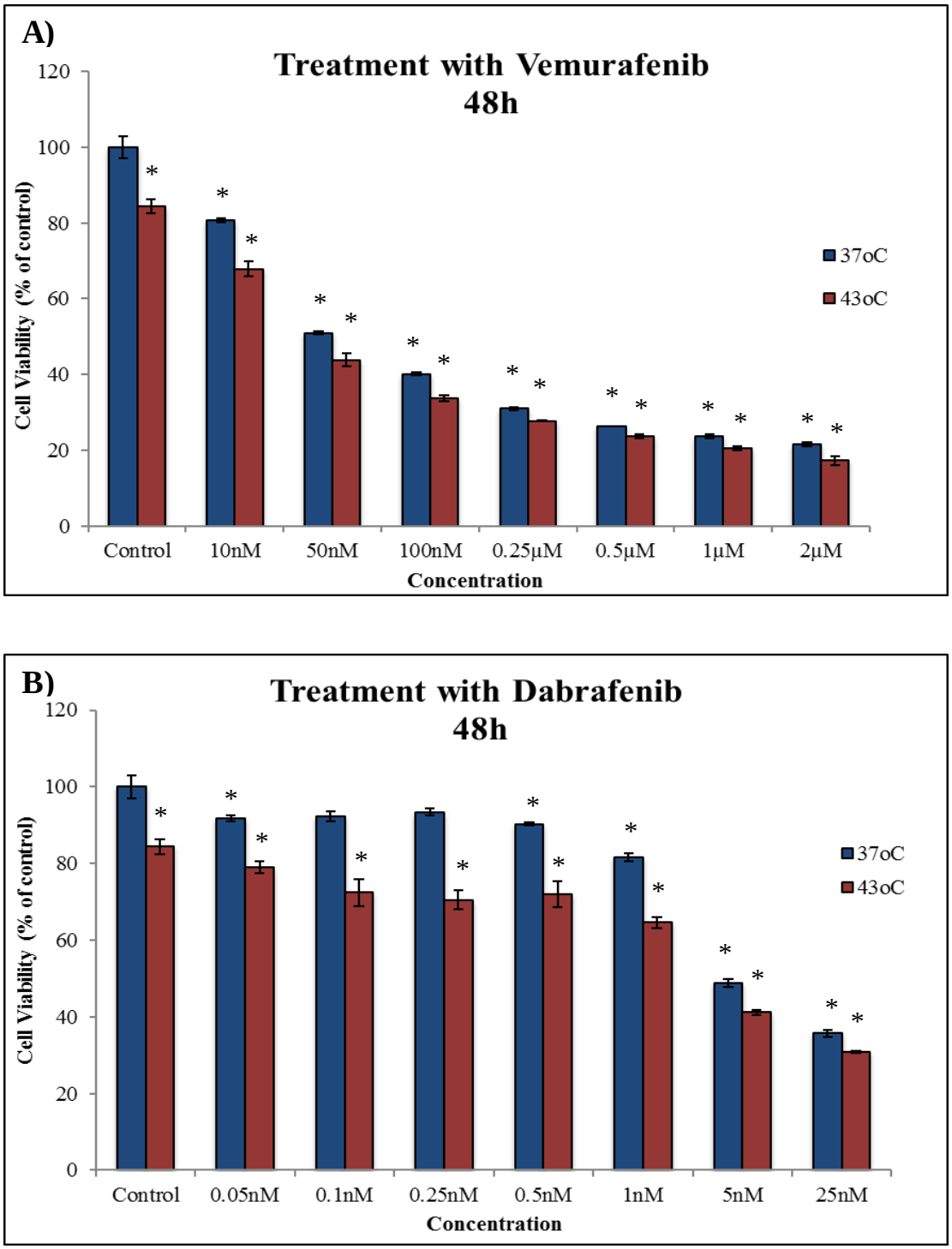


Figure 3.3.4: The effect of a combined therapeutic protocol at 43°C and treatment with Vemurafenib (A) and Dabrafenib (B) 48h post exposure in A375 cells. Briefly, cells were seeded in 96 well-plates and kept at 37°C overnight. Next day, cells were subjected at 43°C, for 2h, and different concentrations of each drug were added. Cells were then returned to 37°C for an additional 48h post exposure incubation period. Cell viability levels were recorded by utilizing the Alamar Blue assay. Control cells were kept at 37°C. Data shown are mean values ± SEM (n=5). Asterisk (*) indicates statistical significance at $p<0.05$.

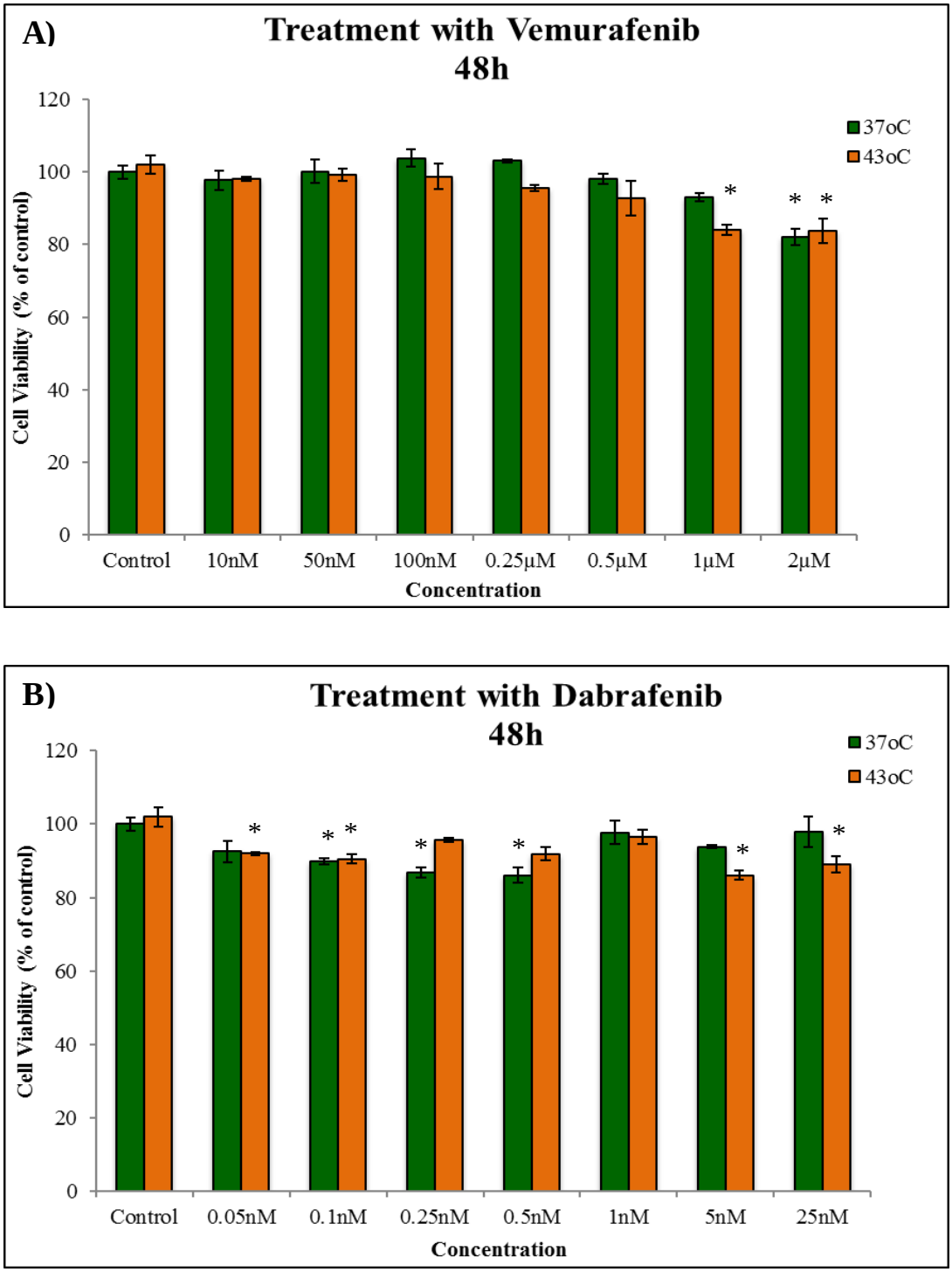


Figure 3.3.5: The effect of a combined therapeutic protocol at 43°C and treatment with Vemurafenib (A) and Dabrafenib (B) 48h post exposure in HaCaT cells. Briefly, cells were seeded in 96 well-plates and kept at 37°C overnight. Next day, cells were subjected at 43°C, for 2h, and different concentrations of each drug were added. Cells were then returned to 37°C for an additional 48h post exposure incubation period. Cell viability levels were recorded by utilizing the Alamar Blue assay. Control cells were kept at 37°C. Data shown are mean values ± SEM (n=5). Asterisk (*) indicates statistical significance at $p<0.05$.

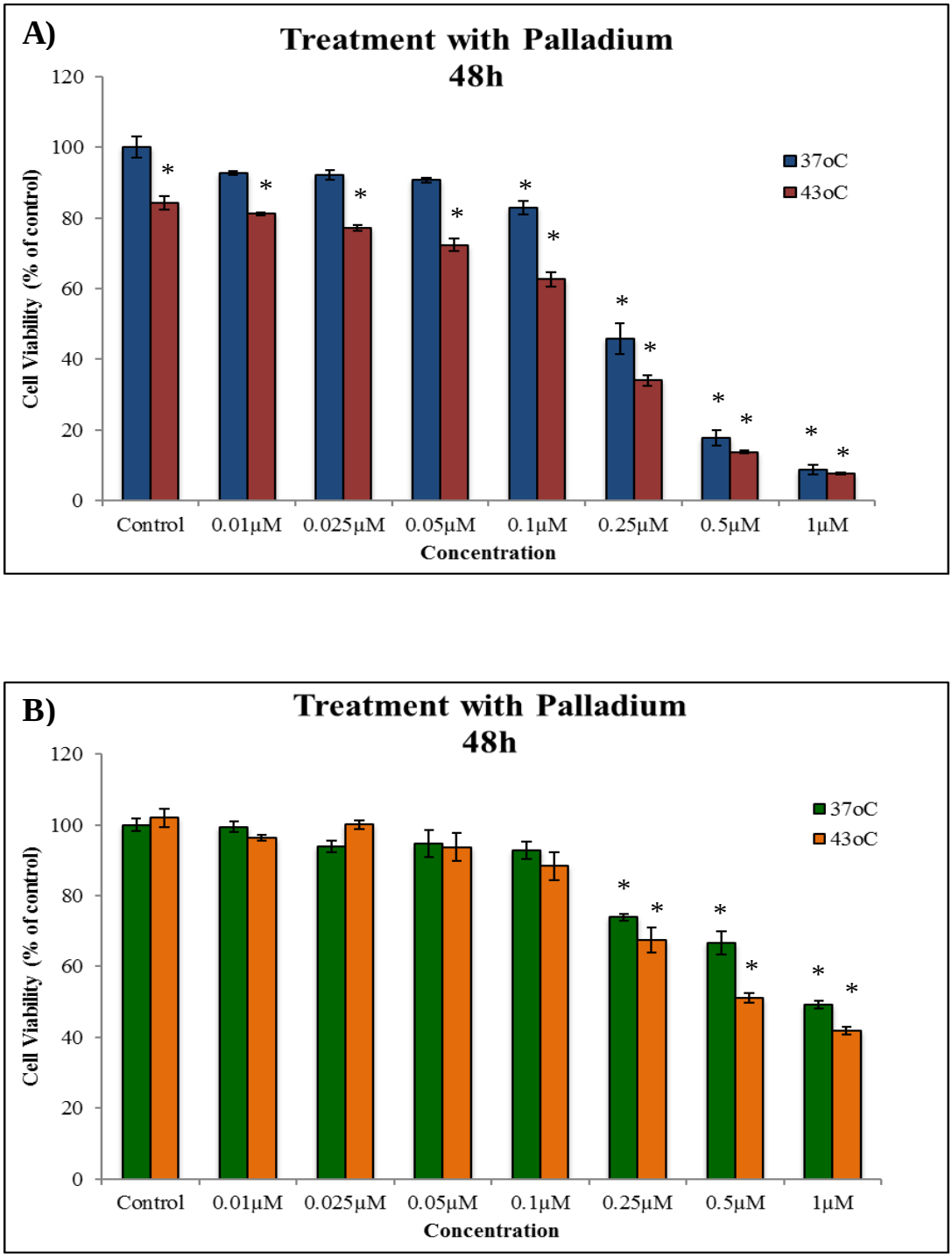


Figure 3.3.6: The effect of a combined therapeutic protocol at 43°C and treatment with a palladium-based drug compound in A375 (A) and HaCaT (B) cells 48h post exposure. Briefly, cells were seeded in 96 well-plates and kept at 37°C overnight. Next day, cells were subjected at 43°C, for 2h, and different concentrations of the drug were added. Cells were then returned to 37°C for an additional 48h post exposure incubation period. Cell viability levels were recorded by utilizing the Alamar Blue assay. Control cells were kept at 37°C. Data shown are mean values ± SEM (n=5). Asterisk (*) indicates statistical significance at $p<0.05$.

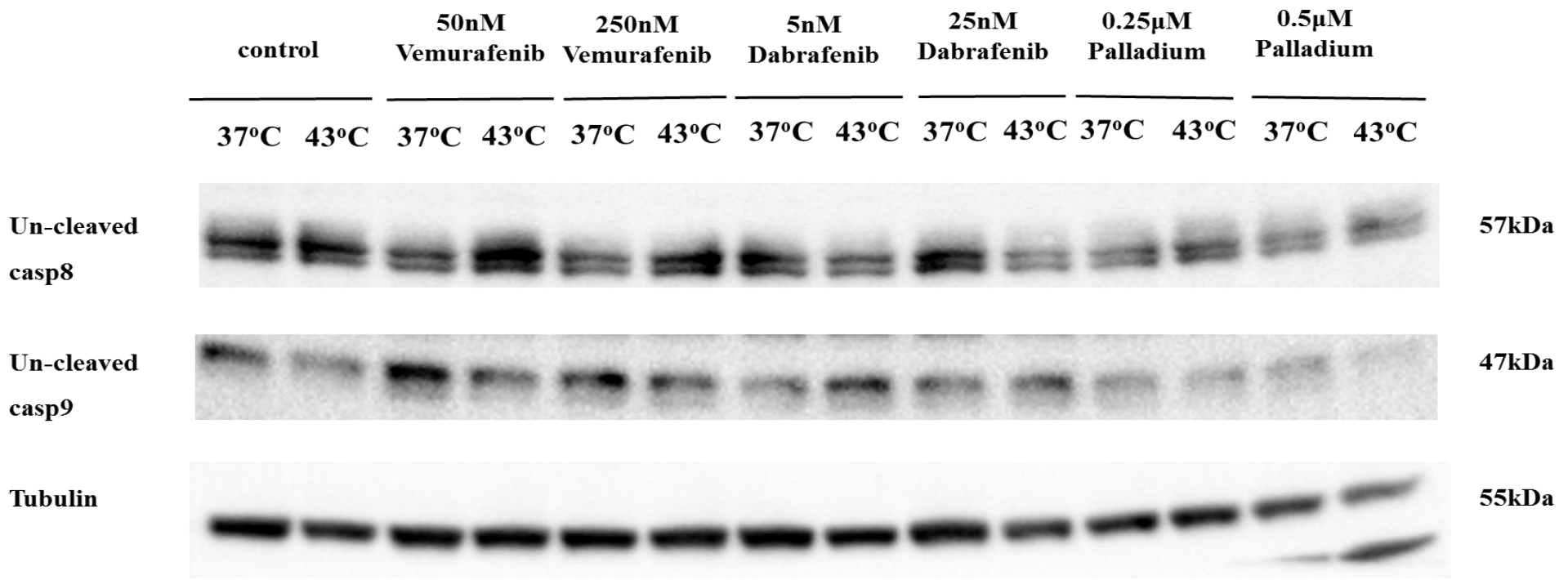


Figure 3.3.7: The effect of Vemurafenib, Dabrafenib and an experimental palladium-based novel drug compounds on the levels of un-cleaved caspases-8 and -9 in the absence (37°C) and presence (43°C) of hyperthermia in A375 cells. Image shows protein expression levels of initiator caspases -8 and -9 at 37°C and 43°C (for 2h) after an initial incubation period with each drug compound, at 37°C, for 48h in A375 cells. Cells were grown overnight before any exposures. Cell lysates were prepared before being subjected to western blotting. β-tubulin was used as loading control.

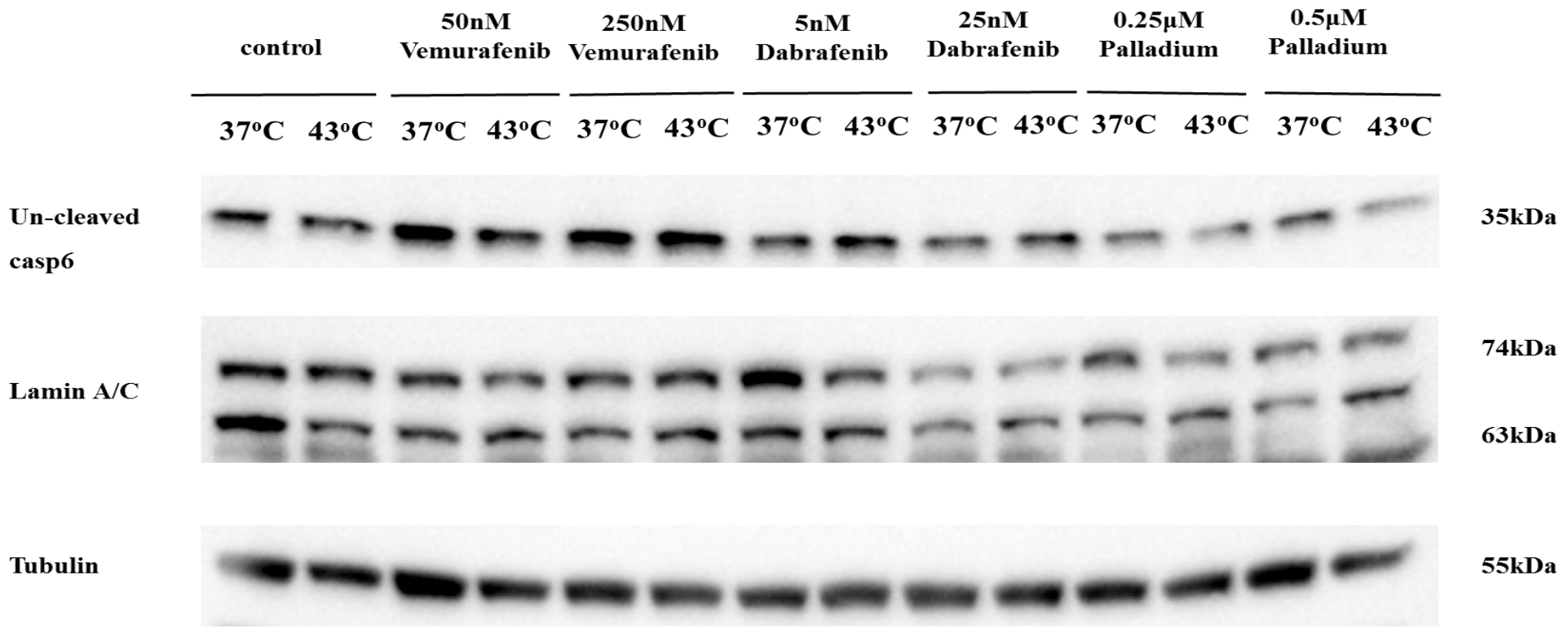


Figure 3.3.8: The effect of Vemurafenib, Dabrafenib and an experimental palladium-based novel drug compounds on the levels of un-cleaved caspases-6 and Lamin A/C in the absence (37°C) and presence (43°C) of hyperthermia in A375 cells. Image shows protein expression levels of initiator caspases -8 and -9 at 37°C and 43°C (for 2h) after an initial incubation period with each drug compound, at 37°C, for 48h in A375 cells. Cells were grown overnight before any exposures. Cell lysates were prepared before being subjected to western blotting. β-tubulin was used as loading control.

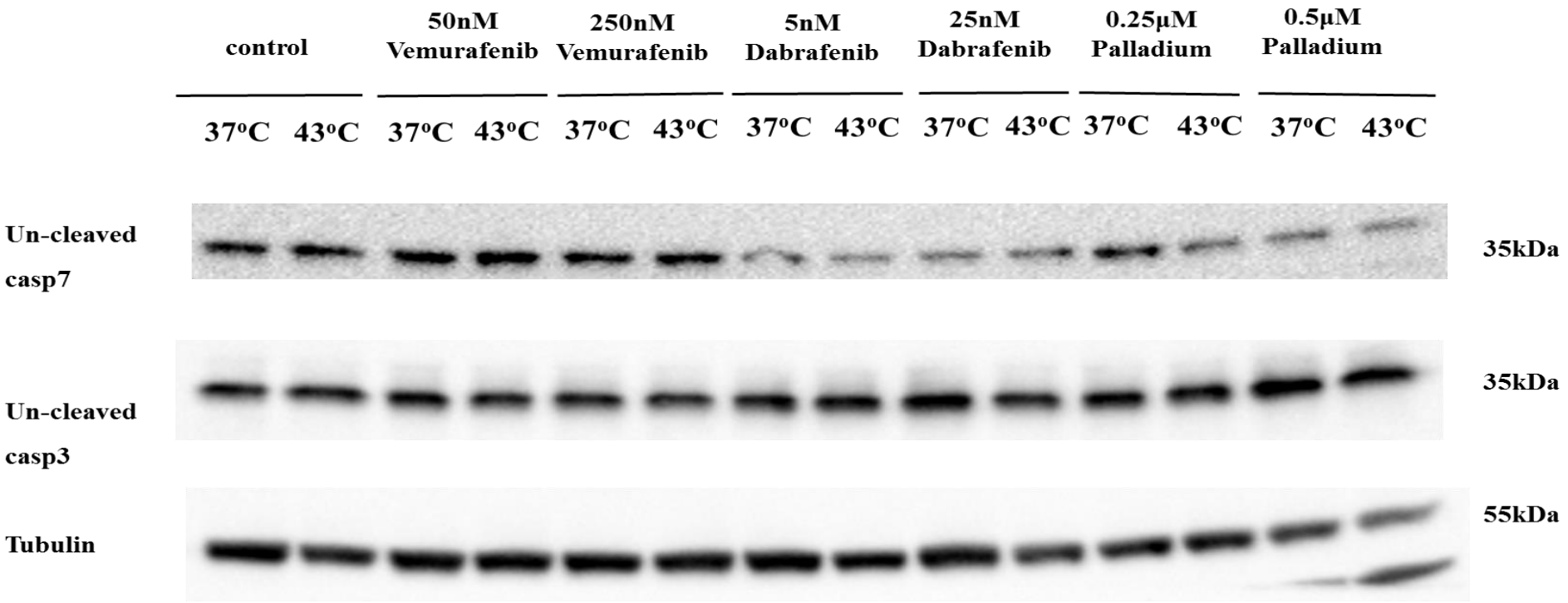


Figure 3.3.9: The effect of Vemurafenib, Dabrafenib and an experimental palladium-based novel drug compounds on the levels of un-cleaved caspases-7 and -3 in the absence (37°C) and presence (43°C) of hyperthermia in A375 cells. Image shows protein expression levels of initiator caspases -8 and -9 at 37°C and 43°C (for 2h) after an initial incubation period with each drug compound, at 37°C, for 48h in A375 cells. Cells were grown overnight before any exposures. Cell lysates were prepared before being subjected to western blotting. β-tubulin was used as loading control.

Chapter Three

Results IV – Therapeutic efficacy of hyperthermia in an *in vivo* model of rodent malignant melanoma

3. Results - Part IV

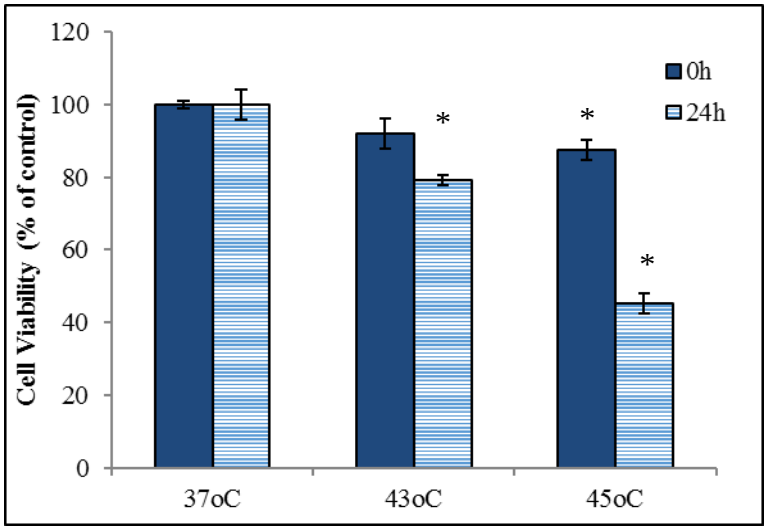
3.7 The effect of hyperthermia at 43°C and 45°C in an *in vivo* xenograft rodent malignant melanoma model

In the final part of this study, an *in vivo* xenograft malignant melanoma murine model was utilized in order to evaluate and validate our previous findings. This model was chosen to study hyperthermia's therapeutic effectiveness as it resembles better the disease's pathophysiology. To this end, the mouse malignant melanoma B16-F10 cell line was utilised for the induction of tumours in C57BL/6 female mice. Our first experiments included establishing optimal *in vitro* hyperthermic conditions for this cell line. More specifically, B16-F10 cells were exposed to 43°C and 45°C for various time periods and cell viability levels were recorded by using the Alamar blue assay immediately after the exposure as well as at 24h post exposure incubation at 37°C. According to our data, 1h of hyperthermia at 43°C and 45°C led to significant drop in the levels of viable cells as recorded 24h post exposure. More specifically, the percentage of living cells declined to around 80% and 45% after being exposed to 43°C and 45°C respectively (Fig.3.4.1.A). Shorter or longer hyperthermia exposure protocols caused either no significant or very extensive induction of cell death, so the 1h hyperthermic exposure protocol was chosen as the optimal one for this model. For the induction of tumours, B16-F10 cells were exposed at 43°C, 45°C (hyperthermia) and 37°C (physiological conditions) and 24h post exposure they were injected subcutaneously in female C57BL/6 mice and were allowed to grow for 12-14 days. Then, all animals were sacrificed, tumours were removed and their size was determined. According to our findings, there was a significant effect of hyperthermia on the inhibition of tumour growth at 43°C with an even more profound one at 45°C (Fig.3.4.1.B & 3.4.1.C). More specifically, the average tumour size dropped from around 3,000mm^3 (at 37°C) to approximately 2,000 (at 43°C) and lower than 1,000mm^3 (at 45°C) (Fig.3.4.1.B & 3.4.1.C).

Apart from determining the size of the excised tumours, we sought to determine any changes in protein expression levels of different markers descriptive of therapeutic effectiveness by means of immunohistochemistry. First, we determined changes in the protein levels of activated caspase-3 as an endpoint of apoptotic induction in response to hyperthermia. According to our data, there was a clear elevation in the protein levels of

cleaved caspase-3 in the samples from both hyperthermia-exposed groups (at 43°C and 45°C) compared to those from the control (37°C) one (Fig.3.4.2). Second, COX-2 was tested as an additional marker whose inhibition has been associated with the activation of cell death pathways as well as the prevention of proliferation. Our findings showed that there was a substantial reduction in its levels in the tumours from the hyperthermia-treated groups in contrast to those obtained from the control group (Fig.3.4.2). Last, we stained for phosphoH2A.X (Ser139), a common DNA damage marker. Results demonstrated a clear increase in the protein levels of phosphoH2A.X (Ser139) in the samples exposed to hyperthermia at 43°C and 45°C (Fig.3.4.2). Overall, the data from these experiments confirmed the therapeutic efficacy of hyperthermia in inducing cell death and inhibiting the growth of cancer cells in an *in vivo* xenograft rodent model of malignant melanoma.

A)



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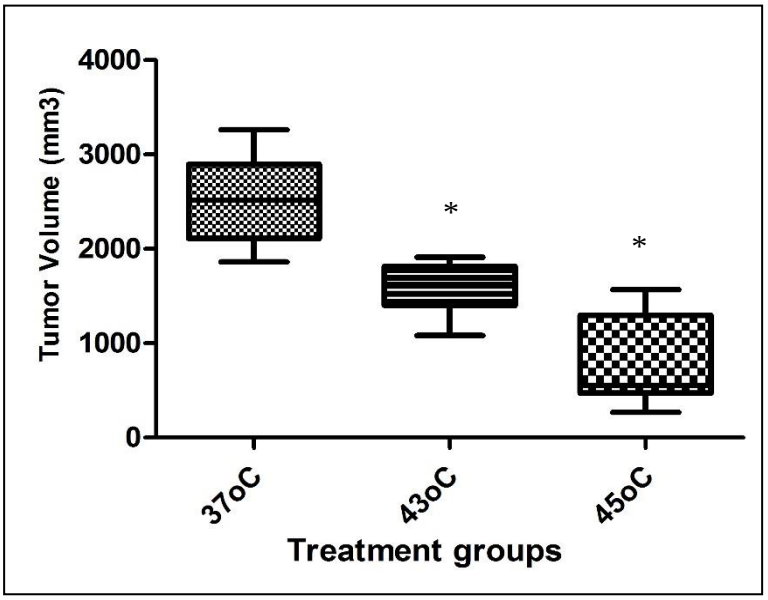

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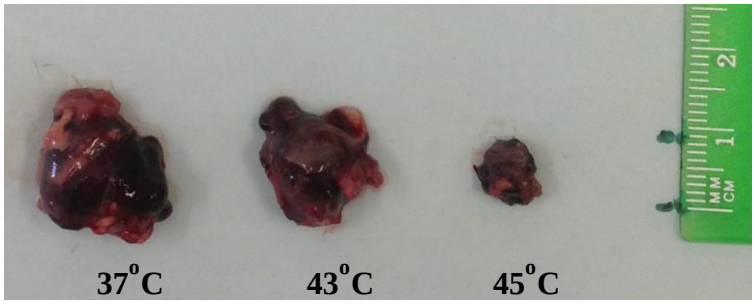
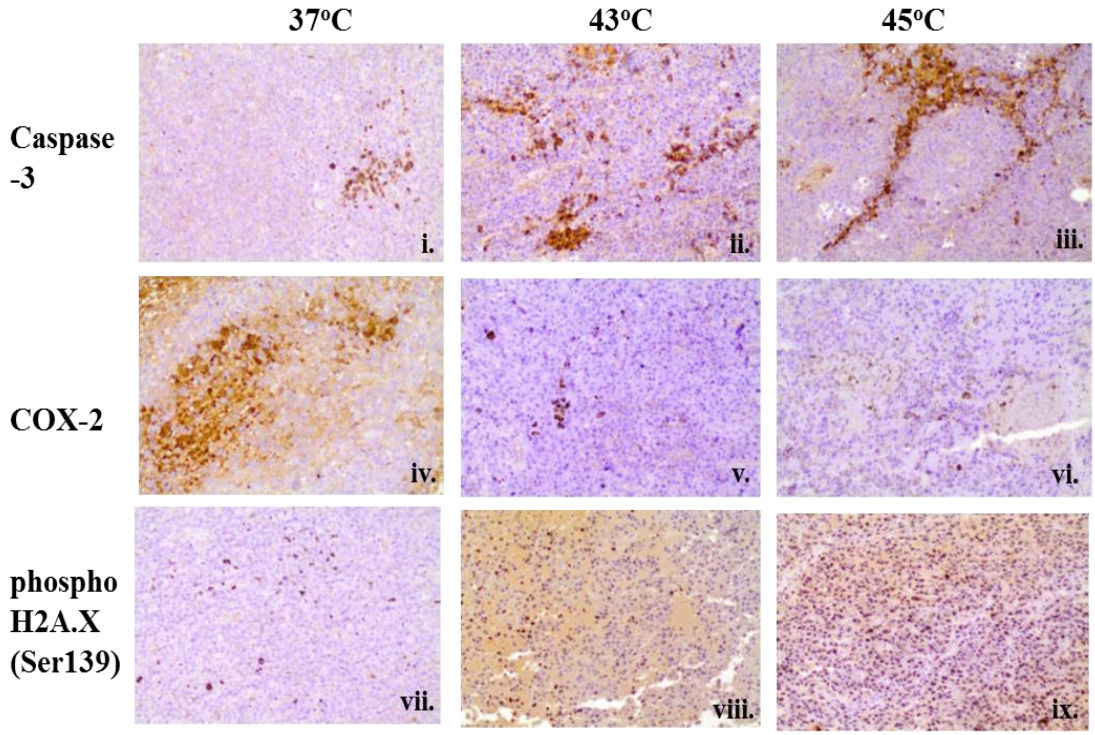


Figure 3.4.1: The effect of hyperthermia in an *in vivo* xenograft rodent malignant melanoma model. A) Cell viability levels of B16-F10 cells immediately after and 24h post exposure at 37°C, 43°C and 45°C for 1h (n=5). B) Boxplot showing average tumor volume growth of B16-F10 cells at 37°C, 43°C and 45°C (n=10). Asterisk (*) indicates statistical significance at $p<0.05$. C) Image showing representative tumors at 37°C, 43°C and 45°C.

A)



B)

	Treatment groups		
Genes	**37°C**	**43°C**	**45°C**
Caspase-3	20.00	33.75	32.5
COX-2	47.50	12.50	12.50
phosphoH2A.X (Ser139)	28.75	41.25	45.00

Figure 3.4.2: The effect of hyperthermia on the expression levels of various proteins in an *in vivo* xenograft model of rodent malignant melanoma. (A) Immunohistological staining for caspase-3 (upper panel), COX-2 (middle panel) and H2A.X (Ser139) (lower panel) for the following treatment groups: i, iv and vii (control group at 37°C), ii, v and viii (hyperthermia-treated group at 43°C) and iii, vi and ix (hyperthermia-treated group at 45°C). (B) Table showing quantification of protein expression levels for the above-mentioned proteins in all three experimental groups (37°C, 43°C and 45°C).

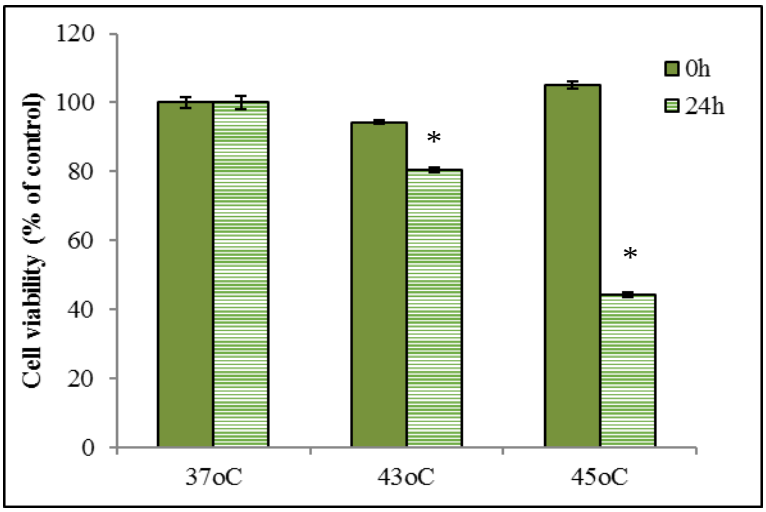
3.8 The effect of hyperthermia at 43°C and 45°C in an *in vivo* xenograft rodent colon carcinoma model

In this set of experiments, we utilized an additional *in vivo* model in order to examine if hyperthermia would exert the same therapeutic efficacy in a different type of cancer. In this case, an *in vivo* xenograft rodent colon carcinoma model was used by utilizing the CT-26 cell line. Similarly to our previous *in vivo* xenograft rodent malignant melanoma model, a series of *in vitro* experiments determined the optimum experimental conditions for the hyperthermic exposures of CT-26 cells. The cells were exposed at 43°C and 45°C over different time courses and the level of viable cells was determined by the Alamar blue assay immediately after exposure and 24h post exposure at 37°C. Our results showed that 1h at 43°C and 45°C respectively had a similar effect on the rates of viable cells as the one observed in the case of B16-F10 cells. In particular, cell viability levels declined to 80% and 40% following exposure at 43°C and 45°C respectively (Fig.3.4.3.A). Based on these observations, we used an 1h exposure protocol at 43°C and 45°C in the next set of experiments. CT-26 cells were injected subcutaneously in female BALB/c mice and allowed to grow for 12-14 days. The animals were then sacrificed and their tumours were removed. The size of these tumours was determined by using an electronic calliper and according to the results there was a considerable reduction in tumour size in both hyperthermia-treated groups (43°C and 45°C) compared to the control (37°C) group. More specifically, average tumour size for the control was about 2,000mm^3 whereas it was reduced to about 1,500mm^3 and lower than 1,000mm^3 at the 43°C and 45°C hyperthermia-treated groups respectively (Fig.3.4.3.B & 3.4.3.C). Overall, hyperthermia was shown to inhibit tumour growth in two different *in vivo* mouse carcinoma models thus supporting hyperthermia's beneficial effects in experimental cancer treatment.

Next, various tumour sections, from BALB/c mice, were subjected to immunohistological staining with the same markers used in the *in vivo* xenograft malignant melanoma rodent model. Staining with the cleaved caspase-3 antibody demonstrated a considerable increase in its protein expression levels in the tumours from the 43°C hyperthermia-treated group compared to the control (37°C) group. Moreover, it should be noted that the tumour sections from the 45°C hyperthermia-treated group exhibited a much stronger staining of the protein (Fig.3.4.4). Protein expression levels of COX-2 were also affected by exposure to hyperthermic conditions

as we detected a remarkable decrease in its protein expression levels in the tumours from both hyperthermia-treated groups (Fig.3.4.4). Finally, according to our results with phosphoH2A.X (Ser139) staining, the levels of its protein content were elevated in the tumour sections from the two hyperthermia-treated groups (at 43^{o}C and 45^{o}C) compared to those from the control (37^{o}C) group (Fig.3.4.4). Overall, our data document that hyperthermia could potentially exert its beneficial effects in other distinctively different types of cancers including colon carcinoma, etc.

A)



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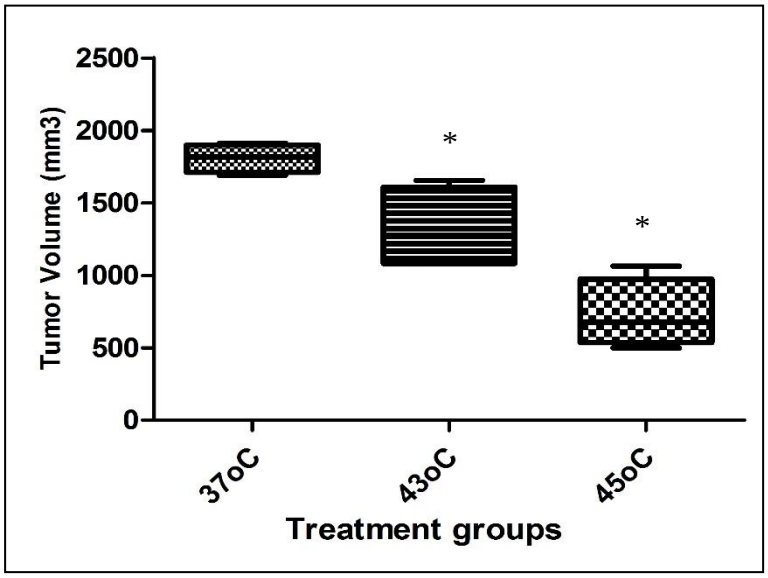


C)

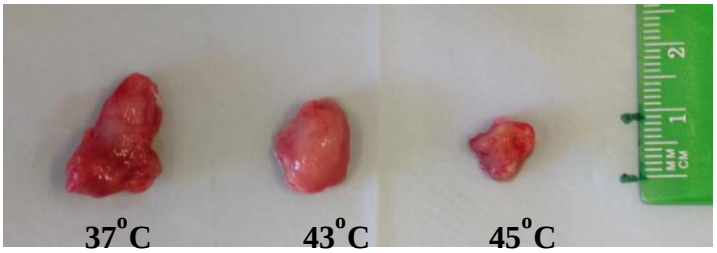
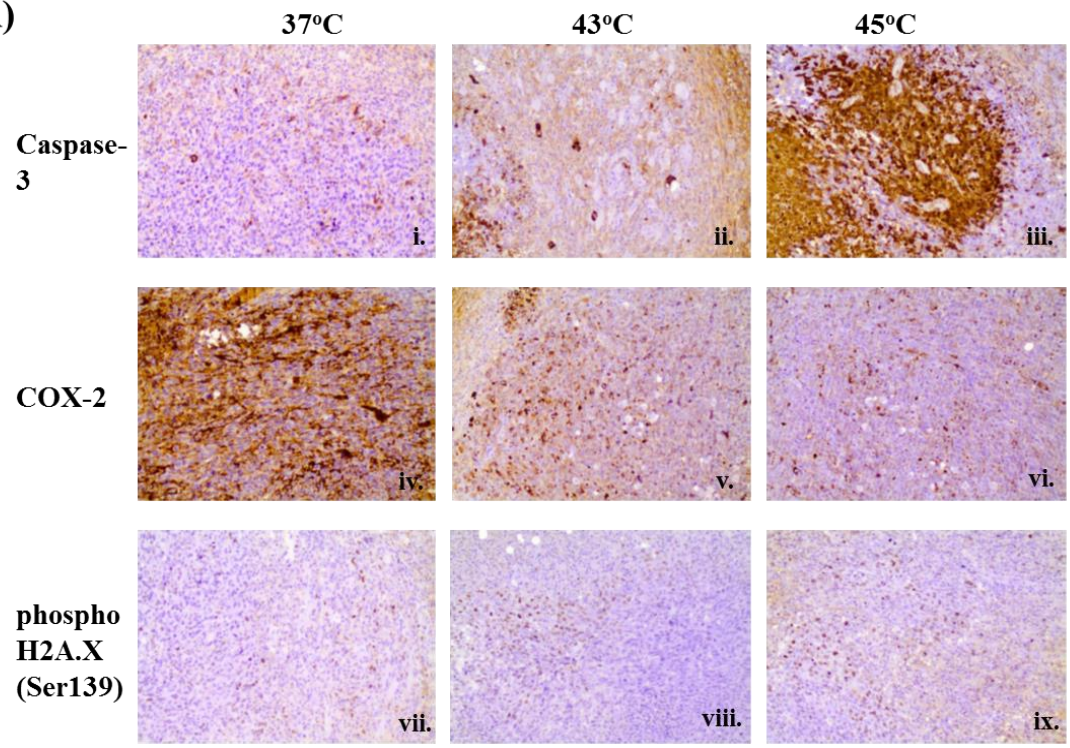


Figure 3.4.3: The effect of hyperthermia in an *in vivo* xenograft rodent colon carcinoma model. A) Cell viability levels of B16-F10 cells immediately after and 24h post exposure at 37°C, 43°C and 45°C for 1h (n=5). B) Boxplot showing average tumor volume growth of B16-F10 cells at 37°C, 43°C and 45°C (n=10). Asterisk (*) indicates statistical significance at $p<0.05$. C) Image showing representative tumors at 37°C, 43°C and 45°C.

A)



B)

	Treatment groups		
Genes	**37°C**	**43°C**	**45°C**
Caspase-3	20.00	47.50	45.00
COX-2	82.50	60.00	55.00
phosphoH2A.X (Ser139)	17.50	42.50	35.00

Figure 3.4.4: The effect of hyperthermia on the expression levels of various proteins in an *in vivo* xenograft rodent model of colon carcinoma. (A) Immunohistological staining for caspase-3 (upper panel), COX-2 (middle panel) and H2A.X (Ser139) (lower panel) for the following treatment groups: i, iv and vii (control group at 37°C), ii, v and viii (hyperthermia-treated group at 43°C) and iii, vi and ix (hyperthermia-treated group at 45°C). (B) Table showing quantification of protein expression levels for the above-mentioned proteins in all three experimental groups (37°C, 43°C and 45°C).

Chapter Four

Discussion

4. Discussion

Malignant melanoma is considered one of the deadliest types of cancer and the 5th most common type, in UK (2014), while its incidence rates have been steadily increasing over the last decades, according to Cancer Research UK, 2014. Its survival rates are increased when it is diagnosed at an early stage, however if it progresses to a metastatic stage then current treatment options become less efficient (Cancer Research UK, 2014). Therefore, there is an increasing number of studies focusing on the establishment of new strategies which will improve the existing therapeutic protocols and make them more effective (Harries et al., 2016). Since the 1970s there has been a rising interest in investigating hyperthermia's therapeutic potential as a cancer treatment modality as the need for more effective therapeutic strategies has increased dramatically. Early studies showed that when hyperthermia is applied, in combination with radiation therapy, it can exert beneficial effects in treating tumor cells (Gerner et al., 1975). Various studies utilizing different cancer cell lines and models have looked into the role of apoptosis as a consequence of heat application and according to their findings, the induction of cell death pathways (apoptosis and/or necrosis) is dependent on the cancer cell type, temperature and duration of heat exposure (Hildebrandt et al., 2002, Wrzal et al., 2008). The aim of this study was to investigate hyperthermia's effectiveness in triggering cell death pathways as a therapeutic basis for treating malignant melanoma.

To this end, we have utilized an *in vitro* human malignant melanoma model consisting of three different cell lines: a human, non-tumorigenic immortalized keratinocyte (HaCaT) cell line, an epidermoid human carcinoma (A431) cell line and a human malignant melanoma (A375) cell line. During the initial experiments of this study, we optimized the experimental hyperthermic conditions for inducing cell death in A431 and A375 cell lines. According to our results, exposing cells to mild temperatures (39-41^{o}C) had no significant effect on cell viability levels whereas when they were exposed to higher temperatures (>43^{o}C) the activation of cell death was more prominent. Temperatures higher than 45^{o}C were shown to result in an excessive cell death induction (Fig.3.1.1). In addition, time course experiments determined the optimum duration for hyperhermic exposures (Fig.3.1.2). Overall, the optimal exposure conditions for our hyperthermia-inducing platform were 2h at 43^{o}C and 45^{o}C as these conditions caused a significant drop in the rates of viable cells thus indicating the

activation of cell death pathways. Our results (with the Alamar blue assay) are consistent with other studies using different viability assays (on A375 cells) as well as utilizing other malignant melanoma cell lines (Shellman et al., 2008). Furthermore, it was evident that the epidermoid carcinoma cells (A431) appeared to be more resistant to heat treatments when compared to melanoma ones. Moreover, we monitored changes in cell viability levels after more extended post hyperthermic exposure incubation times (at 37^{o}C) including 24, 48 and 72h in order to determine the presence of a more prolonged decline in cell viability rates or whether there would be a recovery by the cells representing their adaptation to hyperthermia (Fig.3.1.3). An important advantage of our study is that we have further validated the cytotoxic effects of hyperthermia in non-tumorigenic keratinocytes in an attempt to obtain a "perspective" of a non tumor (i.e. control) cell line representing the majority of cells, in epidermis, that are normally surrounding the malignant melanocytes (Choi and Lee, 2015). Our results indicated that HaCaT cells showed significantly higher rates of cell viability after being exposed at 43 and 45^{o}C compared to A431 and/or A375 cells. Most specifically, they appeared to maintain high levels of cell viability (around 60%) even when utilizing hyperthermic exposure at 45^{o}C (Fig.3.1.3). These findings were further validated when utilizing an additional cytotoxicity assay capable of determining levels of dead cells. In doing so, we determined a remarkable induction of cell death in A375 cells while HaCaT cells were more resistant (Fig.3.1.4). Finally, we selected trypan-blue staining as an additional means of determining the levels of viable as well as dead cells, in the same sample, in order to confirm all our previous findings with A375 cells. As expected, there was a considerable increase in the rates of dead cells while the rates of viable cells declined at 45^{o}C, however cells exposed at 43^{o}C did not exert significant induction of cell death although reduced proliferation potential was observed (Fig.3.1.5). Interestingly, the observed reduction in cell viability levels, at 43^{o}C, could be attributed to hyperthermia's interaction with other cellular processes (e.g. cell cycle observation) rather than induction of cell death. In fact, several research reports have associated hyperthermia's anti-proliferative effects with alterations in cell cycle regulation as well as DNA repair mechanisms in various cell lines (Furusawa et al., 2012, Harnicek et al., 2016, Oei et al., 2015). Collectively, our data support the therapeutic effectiveness of hyperthermia against malignant melanoma (A375) and non-melanoma (A431) cell lines, at 43^{o}C and 45^{o}C, presumably by inducing relevant cell death pathways and/or altering cell cycle, DNA repair mechanisms, etc., while at the same time had no (or very limited) cytotoxic effect in non-tumorigenic keratinocyte (HaCaT) cells.

There is a vast number of studies addressing the involvement of the apoptotic pathway in hyperthermia-induced cell death by using a wide range of *in vitro* and *in vivo* models (Shellman et al., 2008, Ahmed et al., 2015, Hildebrandt et al., 2002). To this end, the next part of our study was focused on elucidating the underlying mechanisms of apoptotic cell death in our *in vitro* human malignant melanoma model. According to our results, all three apoptotic pathways (intrinsic, extrinsic and ER-mediated) were activated as a response to hyperthermic exposures at 43°C and 45°C although it should be noted that the activation of various caspases appeared to be temperature-dependent. For instance, initiator caspases-8 and -9 were both found to be activated after hyperthermic exposures at 43°C and 45°C, however it became apparent that both remained active over a short period of time in the case of 43°C while they appeared to be further activated at longer periods of time (e.g. 24h post exposure) at 45°C (Fig.3.2.1). The activation of caspases 8 and 9 provides evidence for the triggering of the extrinsic and intrinsic apoptotic pathways respectively. This observation was further supported by real-time PCR data as several genes were shown to be implicated and up-regulated in both apoptotic pathways. More specifically, regarding the extrinsic pathway *DAPK3, FAS* and *FASLG* were shown to be up-regulated at 43°C and 45°C while *TNF, TNFRSF10A, TNFSF10, TNFSF12* and *TRAF2* were up-regulated only at 45°C. As for the intrinsic pathway, *BAK1, BBC3* and *BIRC2* demonstrated higher expression levels at 43°C while *APAF1, BCL2L11, CASP7, KIT, PMAIP1* and *VDAC2* were additionally up-regulated at 45°C (Table.3.1.1). The importance of caspase-9 activation (intrinsic pathway) in hyperthermia-induced apoptotic cell death has been previously reported in Jurkat cells (Shelton et al., 2010) while the implication of the death receptor (extrinsic) pathway has also been shown to be activated (Bettaieb and Averill-Bates, 2008). However, conflicting data from another study has shown no activation of both initiator caspases in melanoma cells (Shellman et al., 2008). This observation can be attributed to the utilization of different experimental conditions and/or protocols including the hyperthermia-induced platforms, exposure kinetics, etc. Regarding the involvement of the effector caspases, we found that only caspase-6 becomes activated after a hyperthermic exposure at 43°C, whereas all of them (i.e. caspase-3, -6 and -7) are induced at 45°C (Fig.3.2.2 & 3.2.3). Taking into account these findings, it can be hypothesized that hyperthermic exposure at 43°C induces the activation of both intrinsic and extrinsic pathways driven by the effector caspase-6 in a manner that is time dependent and up to 8h post treatment. On the other hand, exposure at 45°C induces the activation of all caspases (e.g. initiator as well as effector)

determined by our experimental methodologies. Our results are consistent with previous reports demonstrating the induction of caspases-3 and -7 in response to hyperthermic stress (Jeon et al., 2016, Shellman et al., 2008). Finally, in an attempt to further validate our previous findings confirming the role of the extrinsic pathway in hyperthermia-induced apoptosis, we investigated the activation of several molecules involved in this pathway. Our data support the activation of TNF-R1 and TRADD, in response to both hyperthermic conditions at 43°C and 45°C, as there was a significant reduction in their protein levels indicating their interaction in forming a death domain which can then recruit caspase-8 (Fig.3.2.5). Other studies have also demonstrated the induction of death receptors as a response to thermal stress (Morle et al., 2015) while additional reports have shown that combined administration of TNFa with hyperthermia appears to have promising results in clinical trials (Bonvalot et al., 2009, Bonvalot et al., 2012). Moreover, our data demonstrated the activation of RIP1, only in the case of exposure at 45°C, an observation which could be indicative either of the protein's interaction with FADD and TRADD (in stimulating the extrinsic pathway) (Festjens et al., 2007) or alternatively, its interaction with RIP3 for the formation of the necrosome (which is required for necroptotic cell death) (Cho et al., 2009, Li et al., 2012). Taken together, our results propose the induction of both necroptotic and apoptotic pathways as a response to higher hyperthermic temperatures (45°C) in contrast to milder ones (43°C), however further experiments are needed in formulating such hypothesis. Another important factor in contributing towards activation of the apoptotic cascade is changes in the balance between pro- and anti-apoptotic proteins. To obtain further insight into possible alterations causing this imbalance, as a response to hyperthermic exposures, we selected various pro-apoptotic proteins and monitored changes in their expression levels. Our data illustrated that Bak, Bax, Bad and Bim were all up-regulated, at both hyperthermic exposures, although the increase in their protein levels was detected at different time periods post exposures (Fig.3.2.6 & 3.2.7). Several anti-apoptotic proteins such as Bcl-2 and Bcl-xL have been shown to be down-regulated whereas several pro-apoptotic ones like Bak, Bax, Puma and Noxa are up-regulated under heat stress conditions (Glory et al., 2014, Hou et al., 2014). Bim, on the other hand, has been reported to be of critical importance in promoting ER-stress mediated apoptotic cell death as its up-regulation is modulated by CHOP (Puthalakath et al., 2007). On the contrary, down-regulation of Bim prevents melanoma cells from undergoing apoptotis induced by ER stress conditions (Tay et al., 2012). Altogether, our data support triggering of the apoptotic mechanisms by enhancing the expression levels of numerous

pro-apoptotic proteins with Bim being the most important factor shown to be up-regulated under hyperthermia-induced ER stress. Finally, Bid (another pro-apoptotic protein) was shown to be considerably reduced after exposure at 45°C (Fig.3.2.7) which, in turn, can indicate its cleavage to t-Bid thus promoting the cross-talk between the extrinsic and intrinsic apoptotic pathways (Billen et al., 2008). Our previous findings documenting the participation of both apoptotic pathways (extrinsic and intrinsic) in activating cell death, at 45°C, might also be supported by the possible formation of t-Bid, however its presence does not seem to be necessary for the modulation of the apoptotic process at 43°C. Nevertheless, in order to highlight the importance of t-Bid further experiments are needed in utilizing this form of the molecule instead of its total protein content.

Next, we aimed to investigate the participation of the ER-mediated pathway in triggering hyperthermia-induced cell death. According to our findings, there was a clear increase in the protein content of Grp78 following hyperthermic exposure at 43°C and 45°C (Fig.3.2.8). This observation is consistent with the existing literature and can be explained in the context of cellular adaptation by means of producing higher quantities of chaperone proteins that will bind to denatured proteins (caused by hyperthermia) and help them regain their normal form and function (Shellman et al., 2008). Interestingly, a slight decrease in protein levels of PERK was only detected at 45°C exposure (Fig.3.2.8), which can be explained by its increased homodimerisation for phosphorylating the eIF2 factor in order to inhibit protein synthesis in stressed cells (Teske et al., 2011). The determination of the expression protein levels of the phosphorylated forms of both proteins (PERK and eIF2) could further validate the activation of this pathway. In contrast, exposure to both hyperthermic temperatures were shown to trigger the induction of IRE-1a and ATF-6 as well. This was indicated by a marked decline in their protein expression levels suggesting that IRE-1a gets homodimerised and binds to downstream proteins while ATF-6 is cleaved to its active form under ER-stress conditions (Fig.3.2.8 & 3.2.9). Consistent with these results, the downstream target of IRE-1a, XBP-1s, was shown to be up-regulated and along with active ATF-6 are responsible for directing the activation of UPR pathways (Yoshida et al., 2001, Zhang and Kaufman, 2004). Finally, induction of CHOP was shown to be dependent on ATF-6 and XBP-1s activation as well (Nishitoh, 2012). Furthermore, the presence of active caspase-4 and CHOP, at 45°C, can be associated with the stimulation of apoptosis (Fig.3.1.8 & 3.2.9) (Bettaieb and Averill-Bates, 2008, Shellman et al., 2008). Collectively, our data suggest that PERK, IRE-1a and ATF-6 pathways take part

in the UPR-induced response at 45°C whereas only the IRE-1a and ATF-6 pathways are activated at 43°C. Interestingly, the induction of IRE-1a and ATF-6 pathways has been suggested to play an anti-apoptotic role under ER stress conditions, in contrast to PERK pathway which was demonstrated to have pro-apoptotic effects instead (Lin et al., 2007, Lin et al., 2009, Rutkowski et al., 2006). Moreover, the continuous induction of IRE-1a and ATF-6 pathways has been shown to provide a survival advantage to melanoma cells undergoing prolonged ER stress according to a recent report (Tay et al., 2014). Taking into consideration all our previously described findings, it can be proposed that activation of IRE-1a and ATF-6 pathways appears to exert anti-apoptotic effects when cells are exposed at 43°C hyperthermia as there is no induction of active caspase-3 following CHOP up-regulation. On the other hand, stimulation of PERK signaling is presented to be of great importance in leading towards apoptotic induction in the case of 45°C hyperthermic exposure. On another note, it is well known that heat shock proteins (HSPs) are produced in higher rates in cells in response to several stress conditions as a means of protecting them from proteins' misfolding and aggregation that could, in turn, provoke the induction of cell death. Since hyperthermia is the main reason for the up-regulation of this group of proteins, we examined alterations in the expression levels of several of them as a response to our hyperthermia exposure protocols. In particular, our data showed that the expression of HSP70, HSP40 and HSP60 was up-regulated, whereas that of HSF1 was down-regulated at both hyperthermic temperatures while HSP90 was elevated but only at 45°C (Fig.3.2.10 & 3.2.11). The up-regulation of HSP70 and HSP90 has been found to have anti-apoptotic effects by preventing the formation of the apoptosome (Beere et al., 2000, Pandey et al., 2000). Furthermore, inhibition of HSP70 appears to have anti-cancer effects by preventing tumor growth and enhancing the cytotoxic effects of cisplatin in an *in vivo* melanoma model (Schmitt et al., 2006). According to results from a recent paper, the absence of JB12 (an ER-associated HSP40 protein) is linked with the stimulation of ER-stress-mediated apoptosis (Sopha et al., 2017). Similarly, HSP60 is known to exert its anti-apoptotic effects by acting as a mitochondrial chaperone while its inhibition is responsible for promoting apoptosis and preventing tumor growth in an *in vivo* glioblastoma model (Ghosh et al., 2008, Ghosh et al., 2010). Interestingly, the suppression of HSF1 appeared to exert anti-proliferative effects in melanoma cells under hyperthermic conditions (Nakamura et al., 2010). Collectively, our findings suggest that the up-regulation of HSP70, HSP40 and HSP60 might prevent apoptotic induction at 43°C, an observation which is additionally supported by the absence of cleaved caspase-3. On the

contrary, down-regulation of HSF-1 (which has opposing effects) was determined only for a short post exposure period of time. On the other hand, the prolonged decrease in HSF-1 protein levels is capable of enhancing apoptotic stimulation at 45°C, due to the delayed up-regulation of all other HSFs (HSP90, HSP70, HSP40, HSP60) as they display maximum expression levels at 24h post exposure to hyperthermia (Fig.3.2.10 & 3.2.11). To the best of our knowledge, our study provides novel insights into characterizing and consequently understanding the interplay between different apoptotic cascades and the complex apoptotic signaling amongst them, as a response to hyperthermia, in an *in vitro* model of human malignant melanoma.

In the next part of our study, we focused on investigating the effect of hyperthermia in potentiating the induction of cell death when applied in combination with other chemotherapeutic agents (combinational treatment). The rationale for such combinational protocol is based on hyperthermia's ability to activate cell death pathways and thus sensitize human malignant melanoma cells to the action of various chemotherapeutic agents. If this is so, then the cytotoxic effects of various chemotherapeutic agents could be enhanced (in the presence of hyperthermia) in a way where their lower concentrations are comparable to the ones observed at higher concentrations thus eliminating the risk for any potential side effects (Bettaieb et al., 2013). The first chemotherapeutic drug we tested was Dacarbazine (DTIC) and our data showed that hyperthermic exposure of A375 cells at mild temperatures (41°C and 43°C) potentiated the drug's cytotoxicity potential (Fig.3.3.1 & 3.3.2). It should be noted that exposure at 43°C was the most effective one since it potentiated the cytotoxic effect of both DTIC concentrations whereas exposure at 41°C was capable of increasing the cytotoxicity of the highest DTIC concentration only. The latter was also supported by other studies where lower hyperthermic temperatures (<41°C) combined with the use of chemotherapy were accompanied by a limited effect on cell viability levels. This appeared to due to the thermo-tolerance or thermo-sensitivity of different cell lines used (Kalamida et al., 2015, Hildebrandt et al., 2002). The reason behind the observed hyperthermia-induced potentiation of DTIC could be attributed to the impairment of DNA repair pathways which would otherwise act by repairing any DTIC-induced damage (Oei et al., 2015, Mantso et al., 2016). Moreover, we tested the effect of a higher temperature (45°C) in this combined protocol, although we had previously shown that exposing cells at this hyperthermic temperature resulted in a dramatic increase in the rate of dead cells (Fig.3.3.3). Our results are in agreement with the existing literature suggesting that the combination of mild hyperthermia (40-43°C) and

chemotherapy can present increased cytotoxicity against cancer cells (Hildebrandt et al., 2002, Issels, 2008) whereas higher temperatures (>45°C) are associated with the induction of necroptotic death (Harmon et al., 1990, Shellman et al., 2008), thus not being suitable for combinational treatment studies. It is worth mentioning that an important limitation in these experiments is the fact that DTIC needs to be bioactivated in the liver (Marchesi et al., 2007) so the use of the drug directly on its target cancer cell type is not an ideal experimental model for studying its therapeutic effectiveness. On the other hand, almost half of melanoma patients carry a mutation (V600E) in the *BRAF* oncogene. Consequently, there has been growing interest in developing new drugs capable of targeting this mutation and inhibiting the continuous activation of MAPK/ERK signaling pathways which can lead to accelerated tumor growth (Taube et al., 2009, Ascierto et al., 2012). Two *BRAF*-targeted drugs, currently administered in melanoma patients, are Vemurafenib and Dabrafenib both of which have been approved by FDA (in 2011 and 2013 respectively) (NIH, 2011 & 2013). We decided to include these two targeted drugs in our study model in order to examine whether their efficacy could be enhanced in combination with hyperthermia. Our results demonstrated that the application of 43°C induced a potentiation effect on the cytotoxicity of both targeted drugs, in A375 cells, but it was quite limited as it was recorded to be 10% difference in cell viability rates at 48h post exposure (Fig.3.3.4). Because these two drugs target the same mutated form of *BRAF*, it was assumed that they would not be capable of exerting any cytotoxic effects if administered to cells carrying the wild type gene. Indeed, when HaCaT cells were treated with the same concentrations of drugs there was no significant cytotoxicity observed as this cell line does not carry a mutated *BRAF* gene (Fig.3.3.5). However, it should be mentioned that during a recent study there was development of non-melanoma skin cancer after Vemurafenib administration and according to these data, treatment of HaCaT cells with low doses of Vemurafenib (2μM) resulted in an increased activation of CRAF kinase accompanied by elevated growth rates and invasive capacity of cells (Roh et al., 2015). In addition, Dabrafenib appeared to have the same effects as reported by a different group of investigators (King et al., 2013). Finally, we sought to test the cytotoxic action of another experimental but novel chemotherapeutic agent based on palladium (II) saccharinate complex of terpyridine. Once again, our data demonstrated that hyperthermia at 43°C was able to increase the drug's cytotoxicity in A375 cells (Fig.3.3.6). In fact, hyperthermia was shown to enhance the drug's effectiveness to a slightly greater extent compared to both targeted drugs in these melanoma cells, however it did also present higher cytotoxicity levels

when used in the control, immortalized keratinocyte (HaCaT) cells (Fig.3.3.6). These findings revealed the therapeutic potential of this experimental drug in the development of new strategies in melanoma treatment as this is the first study utilizing its potential use against this type of cancer. When comparing the cytotoxicity profile of the drug obtained in A375 cells with that of lung and breast carcinomas, it becomes apparent that it was more potent in melanoma cells (Ulukaya et al., 2011b, Ari et al., 2014). More specifically, its EC50 levels in the other cancer cell types were much higher compared to that of melanoma. For instance, the EC50 values in lung and breast cancers were recorded as 1.8-2.1μM and 2.71-46.5μM respectively whereas those in melanoma as approximately 0.2μM. However, data from another study demonstrated that the EC50 value of the complex dropped to 0.1-1.0μM when treating fibrosarcoma cell lines (Coskun et al., 2013), which suggests that the therapeutic effectiveness of the complex is cell type dependent and can potentially exert anti-cancer effects against other types of tumors as well. Finally, a considerable advantage of our *in vitro* model is that keratinocytes were also exposed at the same experimental conditions in order to determine the safety levels (i.e. free of any associated cytotoxicity) of the treatment protocol. It is noteworthy that the previously mentioned reports lack the addition of such non-tumorigenic, control cell line in their *in vitro* models. Currently, there are no scientific reports investigating the underlying mechanisms leading to the induction of cell death after the application of hyperthermia in combination with any of the previously mentioned therapeutic agents (Vemurafenib, Dabrafenib and novel palladium-complex) in malignant melanoma. All data, thus far, are based on viability assays and so it remained largely unclear if the observed potentiation of cytotoxicity was due to the triggering of programmed cell death (apoptotic) pathways. To this end, we determined protein expression levels of several caspases involved at various stages of the apoptotic cascade and soon became obvious that each of the different therapeutic agents showed a different pattern of caspase activation. As previously described, in the case of Vemurafenib, hyperthermia's potentiation effect was observed by the activation of initiator caspase-9 but not -8 suggesting primarily the activation of the intrinsic apoptotic pathway (Fig.3.3.7). Futhermore, effector caspases-6 and -7 were unaffected whereas a minor activation of caspase-3 was detected (Fig.3.3.8 & 3.3.9). Despite the observed activation of intrinsic apoptosis, clearly further experiments are required in order to better characterize the involvement of proteins downstrean in this pathway. The reduction in pro-caspase-3 protein expression content confirms the participation of this apoptotic pathway in response to the combined hyperthermia and Vemurafenib

treatment as there was no activation of the extrinsic (caspase-8-dependent) pathway. Evidence from a recent report revealed the importance of caspase-3 activation in triggering cell death as the use of PAC-1 (a pro-caspase-3 activator) along with Vemurafenib increased the drug's anti-cancer activity in melanoma cells (Peh et al., 2016). Our findings agree with the results from another report showing that the administration of Vemurafenib in melanoma cells was responsible for triggering the intrinsic apoptotic pathway while also initiating the ER stress response in melanoma cell lines (Beck et al., 2013). Thus, it may be that the combination of hyperthermia and Vemurafenib can be a more effective therapeutic strategy against melanoma due to the combined elevation of the ER stress-based and intrinsic apoptotic activation. In the case of Dabrafenib, caspase-8 (rather than caspase-9) appeared to be activated, thus indicating the stimulation of the extrinsic apoptotic pathway (Fig.3.3.7). In addition, a reduction in the protein levels of Lamin A/C suggests the activation of effector caspase-6 (although this was not evident perhaps due to technical reasons) (Fig.3.3.8). Intriguingly, there was no significant activation of caspase-3 either (Fig.3.3.9), an observation that has previously been reported to be the case in Dabrafenib-induced cell death (King et al., 2013). However, although the conflicting data between our study and the work of others, there is data suggesting that Dabrafenib is capable of preventing necroptosis and partially apoptosis in melanoma cells. This is due to the inhibition of RIP3, which acts by regulating the induction of necroptosis when completion of apoptosis is prevented, by Dabrafenib (Geserick et al., 2015). On the other hand, if this is the reason for the absence of caspase-3 activation, caspase-8 should have remained inactive since it does not take part in the necroptotic pathway (Vandenabeele et al., 2010). An interplay between the stimulation of apoptotic and necroptotic pathways could explain these findings while additional experiments focusing on the relation of the two pathways are necessary. Last but not least, the combination of hyperthermia with the palladium-complex appeared to provoke the induction of the extrinsic apoptotic pathway which was indicated by reduced protein levels of pro-caspase 8. In addition, effector caspases-6 and -7 were found to be active whereas caspase-9 and -3 appeared to be unaffected. Even though there is no data pertaining to the cytotoxicity induced by this complex in melanoma cells the involvement of death receptor pathway was documented when breast cancer cells were treated with this drug agent. More specifically, various death receptor genes were found to be up-regulated while caspase-3 was also shown to be active (Ulukaya et al., 2011a, Ari et al., 2013). The results from our study agree, in part, with these findings as we provided evidence for the extrinsic

pathway's participation in mediating cell death via active caspase-8 although in the absence of caspase-3 activation. This could be due to experimental discrepancies or different experimental conditions required for the detection of the active form of the enzyme (i.e. different concentration or incubation period, etc.). Another reason could be the fact that effector caspases-6 and -7 rather than caspase-3 drive the final stages of the apoptotic process in melanoma cells. Collectively, our data show that each drug triggers the activation of different caspases and consequently different apoptotic pathways. In order to better understand how the effectiveness of these drugs is potentiated, in our experimental human malignant melanoma model, more elaborate experiments have to be performed while focusing on the molecular mechanisms underlining the interplay amongst these apoptotic pathways.

Finally, in the last part of our study, we utilized two different *in vivo* rodent models in order to evaluate the significance of our previous *in vitro* findings regarding hyperthermia's therapeutic effectiveness. Firstly, we demonstrated that hyperthermia at 43^{o}C and 45^{o}C had a comparable effect on viability levels of mouse melanoma (B16-F10) cells to that observed in human melanoma (A375) cells (Fig.3.4.1.A). However, it should be noted that the duration of the exposure was reduced to 1h due to the excessive cell death observed at the 2h exposure period suggesting that the mouse melanoma cells were more sensitive to our previously-described hyperthermia exposure protocol. Studies, by other groups, have also utilized this mouse cell line in documenting hyperthermia's effect on proliferation and therapeutic potential in these cells (Garcia et al., 2012, Jin et al., 2013). After cells were exposed to hyperthermia, they were injected in female C57BL/6 mice for the induction of tumors. Then, the size of the excised tumors, from the different hyperthermia-treated groups together with the control group, was determibed. Results showed a remarkably prominent difference in tumor volume which was temperature-dependent (Fig.3.4.1.B & 3.4.1.C). As expected, tumors from the 45^{o}C-hyperthermia treated group appeared to be considerably smaller in size compared to those from the group treated at 43^{o}C. These observations further support the significance of hyperthermia's therapeutic potential in an *in vivo* xenograft murine model of malignant melanoma. Several scientific reports have also provided evidence regarding hyperthermia's beneficial effects on tumor growth inhibition utilizing the same animal model, however it should be noted that the majority of such studies were mainly focused on determining potential enhancement in the efficacy of various therapeutic protocols when combined with hyperthermia (Portela et al., 2013, Kim et al., 2015, Haghniaz et al., 2016, Misir Krpan et al., 2012, Werthmoller et al., 2016).

According to our findings, the observed inhibition in tumor growth was due to the stimulation of the apoptotic pathway, as there was a clear activation of caspase-3 detected in the samples from both hyperthermia-treated groups (Fig.3.4.2). Active caspase-3 was found to be expressed to a greater extent in tumors from the 45°C hyperthermia-treated group which is in accordance with our previous *in vitro* findings. Also, the excised tumors in this experimental group were of considerably smaller size when compared to those from the group treated at 43°C. Our data are consistent with various studies showing the involvement of caspase-3 in triggering the apoptotic response in a number of hyperthermia-based therapeutic protocols (Portela et al., 2013, Haghniaz et al., 2016). In contrast to caspase-3, COX-2 was shown to be expressed in significantly lower levels in the tumors of both hyperthermia-treated groups when compared to those of the control group (Fig.3.4.2). There is evidence showing that COX-2 is highly expressed in metastatic melanoma lesions as well as various melanoma cell lines while being undetected in lesions at primary stages (Goulet et al., 2003) and thus its use as a prognostic marker for melanoma development has been suggested (Becker et al., 2009). Furthermore, results from a recent study monitoring COX-2 expression in a broad number of clinical samples have associated its increased detection with poor prognosis and high metastatic risk (Kuzbicki et al., 2016). For this reason the observed decline in COX-2 protein levels after hyperthermia treatment further supports its potential as a promising melanoma therapy. Interestingly, there appeared to be a remarkable increase in the protein content of phospho-H2A.X (Ser139) in tumors excised from both hyperthermia-treated groups and particularly from the group treated at 45°C (Fig.3.4.2). This increase indicates an elevation in double-stranded DNA breaks as a response to hyperthermic exposures which also appears to be temperature-dependent. A large number of previous studies have demonstrated that exposing cells to hyperthermic conditions (>40°C) can lead to the generation of double-stranded DNA breaks which, in turn, can induce cell death as shown in several *in vitro* models (Takahashi et al., 2004, Takahashi et al., 2008, El-Awady et al., 2001). Other scientific reports have argued that the observed increase in double-stranded DNA breaks is not a direct effect of hyperthermia but the consequence of its effects on other cellular processes such as DNA repair pathways (Laszlo and Fleischer, 2009, Hunt et al., 2007, Furusawa et al., 2012). Nonetheless, our findings illustrate the significance of implementing hyperthermia as a therapeutic strategy perhaps by targeting the DNA of tumor cells. Finally, in an attempt to investigate if hyperthermia would exert a beneficial therapeutic effect against a cancer type other than melanoma, we also utilized

an *in vivo* murine model of colon carcinoma. The same hyperthermia-based exposure protocol was utilized for the induction of tumors in female BALB/c mice. According to our data, murine colon carcinoma cells (CT-26) presented a similar pattern of cytotoxicity as the one observed with the mouse melanoma cells (Fig.3.4.3.A). Overall, there was significant inhibition of tumor growth in both hyperthermia-treated groups and the reduction in tumor size was more profound in the hyperthermia-treated group at 45^{o}C (Fig.3.4.3.B & 3.4.3.C). Furthermore, numerous studies have also utilized the same *in vivo* murine colon carcinoma model for investigating hyperthermia's role in improving the therapeutic outcome of other standard treatment protocols when applied in combination (Costa Lima et al., 2017, Lim et al., 2014, Nie et al., 2014, Tsang et al., 2015, Li et al., 2011). These studies have also provided evidence about the induction of apoptosis and particularly the induction of caspase-3 in triggering the apoptotic cell death response. Moreover, the expression of COX-2 was decreased while this of phospho-H2A.X (Ser139) was increased (Fig.3.4.4) thus displaying a similar pattern of expression as with the *in vivo* mouse melanoma model (between control and hyperthermia-treated groups). Similarly, there is clear evidence for the role of elevated COX-2 expression levels concominant with an increased metastatic potential and poor prognosis in colorectal carcinomas (Rahman et al., 2012, Wang and Dubois, 2010). However, the remarkable decrease in COX-2 in the hyperthermia-treated groups provides evidence for hyperthermia's role as a more efficient therapeutic strategy. On another note, according to the results from a recent report, elevated phospho-H2A.X levels in clinical samples from colon carcinoma patients at various stages is associated with poor prognosis and is more evident in metastatic stages as well (Lee et al., 2015). However, because an increased expression of the protein was detected in the hyperthermia-treated groups (while remaining at low levels in the control group) it can be argued that hyperthermia could lead in the generation of double-stranded DNA breaks or even perhaps interfere with DNA repair mechanisms (Schaaf et al., 2016, Devun et al., 2014). Collectively, our data from both *in vivo* xenograft murine models provide strong support for the significance of hyperthermia's anti-tumor effects as a very promising therapeutic modality against melanoma and colon carcinomas.

Chapter Five

Conclusions

5. Conclusions

Utilization of hyperthermia, either alone or in combination with chemotherapy or radiation, has been the subject of extensive studies during the last few decades due to its promising effects in cancer therapy. A number of studies have provided evidence about hyperthermia's capacity to enhance the cytotoxicity of other therapeutic regimens when applied in combination with them. Despite its advantages in terms of negligible side effects and non-evasiveness, it is not used as a routine therapeutic strategy in cancer treatment currently. The main reasons for that are the insufficient heating of tumor areas as well as the lack of knowledge regarding the molecular mechanisms underlying hyperthermia-induced cell death. A large number of studies have demonstrated that the application of hyperthermia in killing cancer cells results in the induction of cell death via the activation of the apoptotic pathway. However, the underlying molecular mechanisms still remain unclear. The significance of our study is that it enhances our current understanding of how hyperthermia exerts its therapeutic effectiveness in malignant melanoma by characterizing and elucidating the underlying molecular mechanisms in regulating the apoptotic pathway(s) involvement by utilizing an *in vitro* as well as an *in vivo* model of human and rodent malignant melanomas respectively.

Our study provides evidence about hyperthermia's therapeutic potential against malignant melanoma (A375) as well as non-melanoma (A431) cell lines by exerting its cytotoxic effects. However, non-tumorigenic keratinocytes (HaCaT) have been shown to be less sensitive against heat induction. This is of great importance as it suggests that the application of hyperthermia in treating melanoma does not result in damaging healthy, normal cells that surround melanocytes like in the case of keratinocytes. This, in turn, constitutes hyperthermia a safe treatment option. Obviously, additional experiments can be performed in focusing on examining hyperthermia-induced cytotoxicity in cell lines obtained from melanoma metastatic sites (e.g. brain, lung, lymph nodes, etc.) and/or other primary tumors. In fact, such studies would indicate if this treatment modality can be equally effective against a potentially more aggressive cell population (metastatic cells) originating from the same primary tumor site.

Activation of the complex apoptotic cascade was demonstrated to be the major signaling pathway responsible for hyperthermia's cytotoxicity. In particular, both

intrinsic and extrinsic apoptotic pathways were induced in response to hyperthermic conditions at 43°C and 45°C in melanoma cells. Moreover, different caspases were shown to be activated upon different exposure temperatures. According to our findings, exposure to higher temperatures led to the activation of all major caspases, death receptor related proteins and pro-apoptotic factors, thus indicating the complete activation of the cascade. On the other hand, exposure at 43°C presented a more "orchestrated" effect in terms of apoptotic induction as only specific caspases were activated in a time-dependent manner. Furthermore, we observed the induction of the ER-mediated apoptotic pathway as a response to hyperthermic exposure conditions. Similarly to our previous findings, there was a differential pattern of protein activation according to the degree of hyperthermic temperatures. Finally, although the majority of heat shock proteins were activated, at both hyperthermic conditions, they were shown to be expressed in higher levels at 43°C. Overall, our data support the presence of an intricate interplay amongst various proteins participating in different signaling cascades. It is obvious that the most important factor for the activation of each one of these proteins is temperature dependance. To this end, at 45°C of hyperthermia it was shown that cells could not manage to adapt effectively to such altered conditions whereas at 43°C of exposure the cells appeared to be able to adapt more efficiently to hyperthermia by being able to regulate the apoptotic response in a more efficient manner. A promising direction, for future experiments, could be studying the underlying mechanisms of caspase-6 induction which was shown to be the only effector caspase activated at 43°C. As for the ER-mediated apoptotic pathway, further work could be focused on investigating the role of CHOP induction in the stimulation of cell death processes as its up-regulation is associated with subsequent transcription and translation of several genes implicated in death pathways.

Furthermore, our findings from the combinational treatment protocols involving hyperthermic exposures followed by the administration of various therapeutic agents, in melanoma cells, supported further hyperthermia's potentially promising application in clinical practice. In most cases, the use of hyperthermia combined with other therapeutic modalities (e.g. targeted and non-targeted) in melanoma was accompanied by a variable degree of potentiation in the drug's cytotoxicity profile. Consequelty, this is of great importance as lower drug concentrations can achieve very effective therapeutic profiles without the presence of unwanted side effects in melanoma patients. In addition, we have shown that the observed potentiation effect of hyperthermia was associated with the activation of different caspases at various apoptotic pathways according to the

specific drug agent that was utilized in our experimental conditions. Most importantly, the application of combinational treatments was shown to be significantly safer for normal, healthy cells as the cytotoxicity observed with various treatment protocols was significantly lower than when compared to malignant melanoma cells. Obviously, future studies can utilize an *in vivo* rodent model to investigate further into the complexity of the interplay between different cell death pathways involved in enhancing the therapeutic effectiveness of combinational treatment protocols in melanoma therapy.

Finally, our *in vitro* findings were validated by utilizing an *in vivo* model of rodent malignant melanoma as hyperthermic exposures were demonstrated to affect tumor growth by activating the apoptotic cascade in a caspase-dependent manner. Interestingly, a marker (COX-2) associated with poor prognosis of the disease was found to be downregulated in response to hyperthermic exposures thus providing promising evidence about hyperthermia's therapeutic potential in melanoma treatment. Moreover, the detection of DNA damage markers due to heat treatments could be the basis for the design of more targeted and efficient compounds which, if applied in combination with hyperthermia, will result in a targeted way of causing damage to tumor cells specifically. Last but not least, we utilized an additional murine *in vivo* model of colon carcinoma which was also highlighted the significance of hyperthermia's therapeutic effectiveness. This additional observation, although not directly relevant to malignant melanoma, it does nevertheless warranty the clinical significance of our results in documenting the great potential of hyperthermia as a therapeutic modality in other types of human cancers as well.

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