ADAPTATION TO ER STRESS AS A DRIVER OF MALIGNANCY AND RESISTANCE TO THERAPY IN HUMAN MELANOMA

Running Head: ER Stress in Human Melanoma

Peter Hersey* and Xu Dong Zhang¹

Immunology and Oncology Unit, Calvary Mater Newcastle Hospital, New South Wales, Australia.

*Address correspondence to Peter Hersey, email: Peter.Hersey@newcastle.edu.au
Room 443, David Maddison Clinical Sciences Building, Cnr. King & Watt Streets, Newcastle, NSW 2300 Australia
Phone: 61 2 49138828. Fax: 61 2 49138184

¹ Xu Dong Zhang is a Cancer Institute NSW Fellow. Work by the authors referred to in this review was supported by grants from the National Health and Medical Research Council project and program grants, the NSW Cancer Institute and NSW Cancer Council, Australia. We especially thank the Hunter Melanoma Foundation for their continued support.

Word Count: 8,407
SUMMARY

Primary events in the development of melanoma are gradually being pieced together but a more complete picture of evolution of the disease requires additional understanding of secondary events consequent on initiation of the malignancy. Arguably, the most important driver of secondary events are signals resulting from induction of endoplasmic reticulum (ER) stress due for example to hypoglycaemia and anoxia. This may result in a variety of responses such as apoptosis, autophagy and senescence depending on the initiating event and cell type but most importantly it may result in progression of melanoma due to adaptation and selection of melanoma cells to ER stress. The following reviews what is known about the adaptive responses and how this information may provide new initiatives in treatment of the disease.

Key words: ER stress/UPR/melanoma/hypoglycemia/new treatments
INTRODUCTION

Treatment of melanoma once it has metastasized beyond locoregional sites remains unsatisfactory. A range of different treatments based on chemotherapy or biological agents or combination of these agents has had little impact on survival from the disease (Khayat et al., 2004; Tsao et al., 2004). Nevertheless, over the period of these trials substantial advances have been made in understanding the genetic changes associated with onset of this malignancy. As reviewed elsewhere (Bennett, 2008), consensus is emerging about primary events involved in development of melanoma largely from comparative genome hybridization (CGH). These include: (1) upregulation of the RAS/RAF/MEK pathway; (2) downregulation of the retinoblastoma protein (RB) by increased cyclin D1, or CDK4 activity; and (3) inactivation of the CDKN2A p16 suppressor of CDK4, 6 in over 50% of melanoma. Rarely activating mutations in cKit or FGF may occur in melanoma. Inactivation of the CDKN2A gene may also affect production of p14 ARF, which is important in maintenance of p53 protein levels by inhibiting HDM2 ubiquination of p53 that leads to its degradation.

These events are believed to be important in facilitating cell division but also provide anti-apoptotic signals that are essential for development of malignancy. In the case of the MEK pathway this includes phosphorylation of the pro-apoptotic BH3 protein Bim, upregulation of the anti-apoptotic protein Mcl-1 and the chaperone protein GRP78 (Wang et al., 2007; Jiang et al., 2007b). Activating mutations of NRAS also activate the Akt pathway, which has many anti-apoptotic functions. These include activation of NF-
κB and HDM2, inactivation of Bad, Foxo3A, Glycogen synthase kinase 3β (GSK3β), and upregulation of Mcl-1 and Bcl-XL (Manning and Cantley, 2007).

These changes are likely to be important in the initiation of melanoma but the evolution of melanoma also involves development of secondary events. We hypothesize that the main driver of secondary events in melanoma is induction of endoplasmic reticulum (ER) stress due to the demands placed on the cell by the malignant process. In the following we briefly review the biochemical events associated with ER stress and the evidence pointing to its role in progression of melanoma.

**ENDOPLASMIC RETICULUM (ER) STRESS**

The (rough) endoplasmic reticulum is a lacy network of cisternae in the cell that is responsible for folding and glycosylation of proteins before they get exported from the cell or to other regions in the cell (Alberts et al., 1994). This process is highly energy dependent and should the cell be unable to produce enough energy or the protein is abnormal or there are Ca\(^{2+}\) imbalances, the cell undergoes ER stress and responds to protect itself by undergoing the unfolded protein response (UPR). This consists of pathways which inhibit protein synthesis, upregulate chaperone proteins and increase protein degradation, as illustrated in Figure 1. The UPR is initiated when the unfolded proteins exceed the capacity of chaperone proteins, such as GRP78 and GRP 94, in the ER to bind them. The principal chaperone protein is glucose regulated protein 78 (GRP78), which normally binds the N terminal ends of three transmembrane proteins.
referred to as interferon inducible protein kinase regulated by RNA (PKR) like ER kinase (PERK), inositol requiring enzyme 1 alpha (IRE1α) and activating transcription factor 6 (ATF6) (Kim et al., 2006; Szegezdi et al., 2006).

When the capacity of the sensor protein GRP78 to bind the unfolded proteins is exceeded, the individual ER transmembrane proteins undergo homodimerization and autophosphorylation, which trigger the UPR. The signals initiated by the three transmembrane proteins are both separate and overlapping. PERK is a serine threonine kinase that has many targets. Perhaps most important is phosphorylation of the eukaryotic initiation factor α2 (eIFα2) involved in translation of protein. Phosphorylation results in inhibition of protein translation, which relieves the stress on the ER. This aspect of the UPR can be viewed as a physiological mechanism to regulate protein production (Wu and Kaufman, 2006), e.g. by plasma cells (Lai et al., 2007). Another important effect is inhibition of translation of cyclin D resulting in cell cycle arrest. PERK is believed to activate GSK3β and result in GSK3β mediated phosphorylation of p53 on S315, resulting in its relocation to the cytosol where it is ubiquinated and degraded (Qu et al., 2004; Baltzis et al., 2007). Again, this may be viewed as a protective role preventing p53 induced apoptosis of the cells. Phosphorylated eIFα2 may also translate the transcription factor ATF4, which includes a target gene for a phosphatase growth arrest and DNA damage inducible gene 34 (GADD34) (which dephosphorylates eIFα2) and GADD153 [CCAAT/enhancer binding protein-homologous protein (CHOP) (see below)].
IREα1 is an unusual protein in that it acts as both a serine/threonine kinase and an endoribonuclease. The latter activity results in splicing of mRNA for x box binding protein 1 (XBP1), resulting in the strong XBP1 transcription factor which binds both the ER stress response element (ERSE) and the unfolded protein response promoter elements (UPRE). The latter is associated with genes involved in transport of unfolded proteins out of the ER and their degradation by the ubiquitin proteasome system process referred to as ER associated degradation (ERAD) (Kaneko et al., 2007). It also upregulates the HSP40 family protein p53IPK, which inhibits PERK and may act to inhibit the UPR. Among many other target genes are those coding for MHC class II proteins which were shown to be expressed during progression of melanoma (D'Alessandro et al., 1987).

ATF6α shares many of the activities of IREα1. Upon ER stress it transits to the Golgi compartment and is cleaved by the S1P/S2P serine protease to produce a transcription factor which binds to ERSE elements. It appears to be principally involved in induction of the chaperone proteins and to precede IREα1 mediated production of the ERAD pathway (Yamamoto et al., 2007). With persistent ER stress ATF6 and IREα1 activity was found to be attenuated whereas PERK activity was maintained, including GADD153 induction and inhibition of translation. When IREα1 activity was maintained artificially, cell survival was enhanced (Lin et al., 2007). This role of IREα1 is consistent with its role in survival of a number of cell types such as dendritic cells (Iwakoshi et al., 2007). ATF6 also had an important role in adaptation to ER stress in studies on ATF6 knockout mice (Wu et al., 2007).
CONSEQUENCES OF ER STRESS

The consequences of ER stress are illustrated in Figure 2 and described in more detail below.

ER Stress and Apoptosis

In the event that the UPR does not relieve ER stress, normal cells undergo ER stress mediated apoptosis. Several mechanisms have been described that may vary between different cell types and are summarized in Table 1. Activated IREα1 was reported to bind to the adapter molecule TNF receptor-associated factor 2 (TRAF2) and recruit the apoptosis signal regulating kinase (ASK1), which then activates c-Jun N-terminal kinase (JNK). JNK then induces apoptosis by phosphorylating and inactivating Bcl-2 and phosphorylation of Bim, which may increase its apoptotic potential by releasing it from the microtubules (Lei and Davis, 2003; Szegedzi et al., 2006). Phosphorylation of Bcl-2 by JNK appeared to be associated with apoptosis induced by taxols and Vincristine (Mhaidat et al., 2007a,b; Zhu et al., 2008).

The second pathway is by induction of GADD153/CHOP, which is mediated principally by the PERK-eIFα2-ATF4 arm of the UPR. GADD153 downregulates Bcl-2 and thereby increases the relative concentration of BH3 only proteins. The low Bcl-2 levels may also reduce Ca^{2+} and Ca^{2+} fluxes into the ER (Bassik et al., 2004). GADD 153 was reported by Shiraishi et al (Shiraishi et al., 2005) to upregulate TRAIL death
receptors and sensitize prostate cancer cells to TRAIL mediated cell death. We have
reported similar findings in melanoma cells treated with Tunicamycin (Jiang et al.,
2007a) or Thapsigargin (Chen et al., 2007a). GADD153 also induces tribble (TRB),
which may be a negative regulator of GADD153 itself or in prolonged ER stress bind to
and inhibit Akt, thus promoting apoptosis (Kato and Du, 2007). GADD153 was also
reported to upregulate the proapoptotic BH3 only protein Bim. This, together with post
translational activation by phosphatase 2A, was implicated in ER stress mediated
apoptosis of MCF-7 breast carcinoma and other cells (Puthalakath et al., 2007).

The third pathway reported is that of p53 dependent upregulation of the BH3 only
proteins PUMA and Noxa in mouse embryo fibroblasts (Li et al., 2006) exposed to
Tunicamycin or Thapsigargin. p53 independent Noxa mediated apoptosis was reported
in response to Fenretinide or Thapsigargin mediated ER stress by Armstrong et al
(Armstrong et al., 2007). Similarly, p53 independent activation of PUMA was also
observed in human osteosarcoma SAOS-2 cells, and colon cancer HCT116 cells
(Reimertz et al., 2003; Luo et al., 2005).

Additional mechanisms are those related to the anti-apoptotic effects of GRP78.
We reported that inhibition of the MEK pathway in melanoma resulted in downregulation
of GRP78, which binds caspase 4. Under these conditions, apoptosis can be triggered by
Tunicamycin by activation of caspase 4. The same effect can be achieved by knockdown
of GRP78 by siRNA (Jiang et al., 2007b). Others have reported that GRP78 may also
bind caspase 7 (Reddy et al., 2003) and BIK (Fu et al., 2007) and prevent apoptosis.
Another possible apoptotic mechanism is dependent on ER stress induced upregulation of HIF-1α (Werno et al., 2007). This upregulates the Bcl-2 interacting protein 3 (Bnip3), which is a member of the pro-apoptotic Bcl-2 family of proteins that can dimerize and insert into the outer mitochondrial membrane and induce autophagy or atypical cell death with features of apoptosis and necrosis (Bocharov et al., 2007; Graham et al., 2007; Mellor and Harris, 2007).

ER Stress and Senescence

ER stress was reported to induce senescence in human melanocytes transfected with oncogenic forms of HRAS\textsuperscript{G12V}. This resulted in cell cycle arrest associated with vacuolation and expansion of the ER and markers of cell senescence (β-galactosidase activity). The latter was not due to p53 or p16 mediated arrest but was mediated by the UPR (Denoyelle et al., 2006) and involved the Akt pathway. Spitz naevi were reported to express mutated HRAS and to have marked features of ER stress. It was further speculated that the induction of ER stress by HRAS may account for the infrequent detection of HRAS in human melanoma. These results require further study, particularly in relation to adaptive pathways discussed below.

Metabolic Consequences of ER Stress

Cells undergoing ER stress may have profound changes in their metabolism, particularly in relation to glucose metabolism. The latter is due to a switch of glucose
metabolism to glycolysis away from aerobic metabolism through the Krebs cycle. The latter generates 36 ATPs per glucose molecule whereas glycolysis yields only 2 ATPs per glucose molecule (Pan and Mak, 2007; Gillies and Gatenby, 2007). The switch to glycolysis by cancer cells is known as the Warburg effect and was described by Warburg in the 1920s. The reasons for this change are poorly understood but one factor is believed to be induction of hexokinase II (HKII), which is bound to the outer mitochondrial membrane and catalyzes the first step in glycolysis to glucose-6-phosphate (Mathupala et al., 2006).

The Akt pathway may be involved in induction of HKII and hypoxia inducible factor α1 (HIFα1). The latter also induces pyruvate dehydrogenase kinase (PDK), which inactivates pyruvate dehydrogenase and prevents entry of pyruvate into the Krebs cycle (Pan and Mak, 2007; Gillies and Gatenby, 2007). P53 may be involved in that p53 was reported to stimulate the expression of synthesis of cytochrome c oxidase 2 (SCO2), which is critical for oxygen use in the inner mitochondrial membrane. P53 also induces TP53-induced glycolysis and apoptosis regulator (TIGAR), which attenuates glycolysis (Pan and Mak, 2007). Nevertheless, p53 was not shown to play a role in xenograft models that measured glucose uptake by FDG-PET scans (Wang and el-Deiry, 2008).

One of the consequences of glycolysis is the production of lactate within the cell which is pumped out by monocarboxylate transporters (MCT) (Brahimi-Horn and Pouyssegur, 2007) and proton pumps. This results in an acidic microenvironment which may provide selective growth advantages for the melanoma cells over that of normal
cells. Lactic acid from tumors was also reported to inhibit T cell function (Fischer et al., 2007). Hypoxia is an additional cause of ER stress and the UPR (Bi et al., 2005). ER stress in normoxia increases HIF-1α mRNA and protein levels (Werno et al., 2007) so that it is likely that even in relatively low levels of hypoxia, ER stress may be associated with stabilization and transcriptional activation of HIF-1α. The target genes for HIF-1α are numerous and include those for angiogenesis, glycolysis, vasodilation, erythropoiesis and apoptosis (Brahimi-Horn and Pouyssegur, 2007).

**ER Stress in Melanoma**

There is ample evidence that most melanoma have some degree of ER stress even at early stages of development. GRP78 is a marker of ER stress and can be readily detected in most melanoma cell lines and, as shown in Figure 3, tissue sections of melanoma. Our studies on tissue sections also show that expression of GRP78 correlates with progression of the disease and other markers of prognosis such as tumor thickness and mitotic rate (Zhuang et al., submitted). Several of the metabolic consequences of ER stress, such as increased glycolysis and high lactic acid dehydrogenase (LDH), are readily apparent in patients with melanoma. Indeed, LDH levels in sera are the single most powerful predictor of prognosis in metastatic disease (Keilholz et al., 2007). Increased glycolysis also underlies the effectiveness of FDG PET scanning for diagnostic imaging of melanoma. A number of factors may induce ER stress in melanoma but the principal cause may be the relative hypoglycemia resulting from the primary malignant events discussed above and the relatively inefficient utilization of glucose for energy production.
As for other cancers, hypoxia in some areas of the tumor would also be expected to play a role. It is an open question as to whether there are other properties of melanoma which predispose to ER stress or whether defects in pigmentation in melanocytes may lead to ER stress and thereby predispose to development of melanoma.

**ADAPTATION OF MELANOMA TO ER STRESS**

As discussed by others, adaptation to ER stress may be a key driver of malignancy and resistance to therapy (Rutkowski and Kaufman, 2007). Under laboratory conditions of extreme ER stress induced by Tunicamycin or Thapsigargin, melanoma cell lines are relatively resistant to apoptosis even though varying degrees of apoptosis occur in melanocytes and fibroblasts (Jiang et al., 2007a). This implies that protective mechanisms against apoptosis are active in melanoma. This is also implied by evidence of constitutive activation of the UPR in cultured melanoma even in the absence of additional ER stress induction. This includes expression of GADD 153/CHOP and to a lesser extent GRP78 (Jiang et al., 2007b). As indicated above, studies in murine embryonic fibroblasts (MEF) and human embryonic kidney (HEK) 293 cells suggested that the IREα1 and ATF6 pathways were self-limiting during prolonged ER stress but the PERK pathway was maintained (Lin et al., 2007). Mice with deletion of genes for ATF6 were reported not to adapt to ER stress - most probably due to failure to increase the chaperone proteins (Wu et al., 2007). Our studies on cultured melanoma cells have shown constitutive levels of GADD153/CHOP (Jiang et al., 2007b) which is believed to be mainly driven by the PERK/ATF4 arm of the UPR. However, pPERK and pElfα2
were barely detectable in cultured melanoma (Shiraishi et al., 2005). Low levels of XBP1 were also seen consistent with some ongoing activation of the IREα1 arm of the UPR response. The reasons for constitutive levels of GADD153 in melanoma and the role of XBP1 in the adaptation of melanoma to ER stress remain open questions.

Some of the known adaptive mechanisms that allow cells to survive ER stress are summarized in Table 2 and are described in more detail below.

The RAS/RAF/MEK/ERK Pathway

Under laboratory conditions of extreme ER stress, inhibition of the MEK pathway by siRNA or MEK inhibitors induced marked levels of apoptosis. This appeared to be, at least in part, due to downregulation of GRP78 and release of caspase 4 by the MEK inhibitor. Similar results were obtained by siRNA knockdown of GRP78 (Jiang et al., 2007b) and is the first indication that the MEK pathway regulates GRP78. It was noticeable that ER stress did not increase activation of MEK in melanoma, most likely due to near maximum activation of this pathway by events unrelated to ER stress. Nevertheless, ER stress was a strong activator of the MEK pathway in normal cells such as melanocytes (unpublished data). These results are consistent with the idea that ER stress provides further selection pressure for outgrowth of melanoma cells with strong constitutive activation of the RAS/RAF/MEK/ERK pathway because of its ability to inhibit apoptosis induced by ER stress. Although upregulation of GRP78 may be the main mechanism involved in inhibition of apoptosis, other mechanisms include
phosphorylation of Bim (Ser69), phosphorylation of Bad and upregulation of Mcl-1 (Wang et al., 2007).

**GRP78 in Adaptation to ER Stress**

Expression of GRP78 in several cancers such as breast, lung, gastric, prostate and hepatocellular cancers was correlated with an adverse prognosis and in breast carcinoma was related to resistance to chemotherapy (Li et al., 2006). Immunohistological studies on melanoma have shown that GRP78 expression is related to progression of the disease but is not an independent predictor of prognosis (Zhuang et al., submitted). GRP78 is regulated principally by transcription factors binding to multiple copies of the ER stress response element (ERSE) in its promoter. The main transcription factor involved is ATF6 after it has been cleaved to the nuclear form in the Golgi apparatus. However, XBP1 and ATF4 may also upregulate GRP78. Other known transcription factors are NF-Y, YY1, TFII-I and SP1 transcription factors (Li et al., 2006). Constitutive levels of the processed nuclear form of ATF6 were barely detectable in the melanoma lines prior to induction of ER stress (Jiang et al., 2007b), suggesting that other transcription factors may be involved to account for the high GRP78 levels. GRP78 appears to inhibit apoptosis by its ability to bind caspases such as caspase 4 (Jiang et al., 2007b) and caspase 7 (Reddy et al., 2003) or the pro-apoptotic ER related BH3 protein BIK (Fu et al., 2007).
Antiapoptotic Mcl-1 Proteins in Adaptive Responses to ER Stress

An important component of many survival pathways is upregulation of antiapoptotic pathways (Hetz, 2007), particularly Mcl-1. In melanoma Bcl-2 was found to be downregulated during progression of the disease and correlated with Stat-3 expression (Zhuang et al., 2007). Moreover, sensitivity of melanoma cells to MEK inhibitors was related to whether Mcl-1 levels were reduced during exposure to the MEK inhibitors (Wang et al., 2007). Similar findings were reported in studies on the multikinase inhibitor, Sorafenib, in lung cancer (Yu et al., 2005) and human leukemia cells (Rahmani et al., 2005). The latter effect was attributed to inhibition of translation of Mcl-1 due to dephosphorylation of eIF4E translation initiator factor.

In vitro studies on human melanoma lines have shown that exposure to the ER stress inducer Tunicamycin resulted in upregulation of both Bcl-2 and Mcl-1 but it was only knockdown of Mcl-1 by shRNA that resulted in induction of apoptosis by Tunicamycin. The increase in Mcl-1 was due to increased transcription and this was inhibited by siRNA knockdown of IRE-α1 and ATF6. The transcription factors involved in upregulation of Mcl-1 are unlikely to be the UPR proteins as the Mcl-1 promoter does not contain ERSE or UPRE elements (Jiang et al., submitted 2008).
Adaptation to ER Stress by the PI3K/Akt Pathway

The serine/threonine kinase Akt, also referred to as protein kinase B (PKB), is a central signaling pathway for growth factors, cytokines and other stimuli (Manning and Cantley, 2007). It ranks of equal importance to the mitogen activated protein kinase in proliferation and survival of cells and dysregulation of the pathway is implicated in the development of a number of cancers (Vivanco and Sawyers, 2002) such as colon carcinoma (Parsons et al., 2005). It has been implicated in development of melanoma and the predominant Akt3 isotype increased with progression of melanoma (Robertson, 2005; Karst et al., 2006). Factors implicated in upregulation of Akt include activation of cell surface receptors, increased copy number of Akt3 genes and loss of PTEN lipid phosphatase activity due to alterations in the gene in 10-30% of melanoma (Robertson, 2005; Goel et al., 2006).

There is increasing evidence that ER stress is an important activator of the Akt pathway. It was speculated that this may be due to release of Ca^{2+} from the ER resulting in activation of PI3K upstream of Akt3 (Hu et al., 2004). Tunicamycin induced phosphorylation of Akt in short term but not long term (>12hrs) treatment of cultured glial cells (Hosoi et al., 2007). Studies on melanoma lines also showed that exposure to Tunicamycin activated Akt with peak levels of pAkt at 1 hour and a gradual decrease after 6 hours (unpublished data). Inhibition of Akt in melanoma lines by an inhibitor of PI3K (LY294002) resulted in induction of apoptosis by Tunicamycin, indicating that ER
stress may exert a selection pressure to generate melanoma cells with high levels of activated Akt (unpublished data).

The inhibitory effects of Akt on apoptosis are reviewed elsewhere (Manning and Cantley, 2007). They include upregulation of NF-κB (Dhawan et al., 2002) (and thereby inhibitor of apoptosis proteins [IAPs]), activation of HDM2 (and thereby reduction in p53 dependent apoptosis via PUMA and NOXA), phosphorylation and inhibition of BAD, Caspase 9, GSK3β, FOXO transcriptional factors (and thereby downregulation of Bim). GSK3β is phosphorylated on S9,S21 and inactivated. GSK3β down regulates Mcl-1 so that inhibition of GSK3β can be expected to inhibit apoptosis via increased Mcl-1 levels. The effects of Akt in promoting glucose uptake and glycolysis have also been implicated in inhibition of Bax activation (Rathmell et al., 2003).

Downregulation of p53

Although p53 is believed to be upregulated by a variety of stresses, there is some evidence that activation of the UPR may inhibit the p53 response. The main evidence for this came from studies on mouse embryonic fibroblasts (MEFs) and human fibrosarcoma cells HT1080, which showed that downregulation of p53 after exposure to Thapsigargin was dependent on activation of PERK. This was associated with export of p53 to the cytosol and proteasomal degradation by HDM2 (Pluquet et al., 2005). Nuclear export of p53 was however dependent on activation of GSK3β by PERK, which entered the nucleus and phosphorylated p53 on Ser^{315} and Ser^{376}. The mechanism of activation of
GSK3β was not clear (Baltzis et al., 2007). These results are consistent with reports that inhibitors of GSK3β (DW1/2) or of HDM2 (Nutlin 3a) could upregulate p53 in melanoma cells and induce apoptosis (Smalley et al., 2007). Similarly, sustained upregulation of p53 was reported in colorectal cancer cells treated with inhibitors of GSK3β.

In contrast, clear evidence of p53 stabilization was seen in HCT116 colon carcinoma cells after culture in glucose free media or after exposure to Tunicamycin (Zhang et al., 2006; Li et al., 2007). This was attributed to inhibition of HDM2 activity by binding to ribosomal proteins that were in excess due to eIFα2 inhibition of protein synthesis. p53 dependent production of Noxa and PUMA and apoptosis was also reported in MEFs (Li et al., 2006).

Studies on melanoma lines have shown no or at best transient induction of p53 by ER stress inducers (work in preparation) so that muted or absent p53 responses may be more typical of melanoma in response to ER stress perhaps for the reasons described by Baltzis et al (Baltzis et al., 2007). Interpretation of p53 responses in melanoma are also complicated by the presence of inhibitory isotypes in some cell lines (Avery-Kiejda et al., 2008).
Targeting the adaptive mechanisms resulting from ER stress could be expected to overcome some of the resistance of melanoma to treatment, e.g. ER stress adds to the other known causes of increased activation of the RAS/RAF/MEK and Akt pathways and provides further rationale for treatment with inhibitors of these pathways. Those available are reviewed elsewhere (Haluska et al., 2007). Inhibitors of GSK3β are at preclinical stages of development and which might be better considered as agents which restore p53 function (Smalley et al., 2007).

Additional agents which may find a role in treatment of ER stressed cells are summarized in Table 3. Restoration of p53 function has been the subject of much research and includes agents such as CP-31398 which restore function to mutant p53 (Wang and el-Deiry, 2008). The most appropriate agents to combat the effects of ER stress would appear to be those that target the HDM2 degradation pathway. The best known of these is Nutlin 3a, which has progressed to testing in xenograft models (Vassilev, 2006). Studies in cell lines revealed restoration of cell cycle arrest function of p53 but the effects on induction of apoptosis were variable, suggesting that downstream targets of p53 involved in apoptosis may be regulated by other factors (Tovar et al., 2006).

Agents which target the chaperone GRP78 could be expected to have a major role in sensitizing cells to therapy. Our studies suggest that inhibitors of the RAS/RAF/MEK
pathway have a major role in downregulation of GRP78 either directly or indirectly. In addition, agents that specifically target GRP78 are under study, such as versipelostatin, which inhibits upregulation of GRP78 induced by hypoglycemia (Park et al., 2007; Li and Lee, 2006). Epigallocatechin from green tea was also reported to inhibit GRP 78 by competing for ATP binding (Ermakova et al., 2006). Aspirin may have a similar role (Li and Lee, 2006). Inhibition of GRP78 led to increased sensitivity of glioma cells to Temozolomide (Pyrko et al., 2007). Similarly there is interest in targeting UPR signal pathways such as IRE1α. One such drug, Irestatin, was reported to strongly inhibit xenografts of HT1080 fibrosarcoma cells (Feldman and Koong, 2007). Specific inhibitors of the downstream XBP1 transcription factor were also reported to be under development (Koong et al., 2006).

Adding to ER stress may be effective in overcoming cancer cells that have relatively low levels of adaptation. Several agents which upregulate GADD153/CHOP may have such a role. These include cyclooxygenase 2 inhibitors such as celecoxib and an analogue 2,5dimethyl-celecoxib which increased free Ca^{2+} levels in the cytosol and triggered ER responses (Pyrko et al., 2007). Curcumin (Scott and Loo, 2007) and Dipyramidole (Thompson et al., 2003) were reported to upregulate GADD 153 and increase ER stress response. Agents which reduce degradation of proteins such as the proteasome inhibitor Velcade/Bortezomib may also induce ER stress and contribute to their antitumor effects. More recent work has suggested that Bortezomid may increase proapoptotic Noxa via reduced degradation of c-Myc (Nikiforov et al., 2007). Sorafenib,
the multikinase inhibitor was also shown to induce apoptosis of leukemia cells by induction of ER stress (Rahmani et al., 2007).

Targeting metabolic changes induced by ER stress has also attracted much attention. This includes blockade of proton pumps to trap $\text{H}^+$ in the cells with agents such as Omeprazole. Induction of apoptosis by Emoprazole has been reported in pre B cell cancer cells (De Milito et al, 2007) and reduction of resistance to cationic drugs such as Cisplatin (Luciani et al., 2004). The use of monocarboxylate transport inhibitors (MCT1 and 4) are also under evaluation. More direct targeting of glycolysis includes use of halogenated pyruvate derivatives (3-Bromopyruvate) to deplete ATP. This was reported to be effective against hepatocellular carcinomas in animal models (Mathupala et al., 2006). Dichloroacetate (DCA) is another agent that has attracted attention because of its ability to inhibit PDK and reduce lactate production (Pan and Mak, 2007). HIF-$\alpha_1$, 2, transcription factors play a key role in induction of metabolic changes needed to survive hypoxia (Bi et al., 2005; Kim et al., 2007) and hypoglycemia but so far inhibitors have been largely an in vitro tool.

As discussed above, agents which target the antiapoptotic Bcl-2 proteins, particularly Mcl-1, should be valuable in overcoming some of the adaptive mechanisms set up by ER stress. Similar findings were made in relation to treatment of melanoma with Bortezomid (Qin et al., 2006). The Abbott ABT 737 agent has affinity for and inhibits Bcl-2, Bcl-XL and Bcl-W but not Mcl-1. Preclinical studies have shown that many tumors were resistant to this agent due to Mcl-1 proteins in the tumor.
Downregulation of Mcl-1 resulted in sensitivity to ABT 737 (van Delft et al., 2006; Chen et al., 2007b). GSK3β may also downregulate Mcl-1 (Maurer et al., 2006). There are however a number of small mol. wt. inhibitors of the anti-apoptotic proteins which have selectivity for all the anti-apoptotic proteins, including Mcl-1, e.g. Obatoclax is now in preliminary trials in patients with hematological malignancies (Trudel et al., 2007) and was shown to overcome resistance to apoptosis mediated by Mcl-1 (Nguyen et al., 2007). TW37 and related compounds are also undergoing preclinical evaluation (Mohammad et al., 2007) and were shown to be effective with MEK inhibitors (Verhaegen et al., 2006). At this stage we would expect that these broad spectrum Bcl-2 inhibitors would be valuable in overcoming ER stress induced resistance pathways.

In summary, secondary events in the evolution of melanoma set in train by ER stress arguably underlie much of the malignant behaviour of melanoma and its resistance to current therapies. Insights into the adaptive mechanisms involved in response to ER stress provide new initiatives in treatment of the disease.

ACKNOWLEDGEMENTS

Work of the authors referred to in the review was supported by the National Health and Medical Research Council, NSW Cancer Institute, NSW State Cancer Council and the Hunter Melanoma Foundation.
REFERENCES


Keilholz, U., Suciu, S., Bedikian, A.Y., Punt, C.J., Gore, M., Kruit, W., Pavlick, A.C., Spatz, A., Gilles, E., and Eggermont, A.M. (2007). LDH is a prognostic factor in stage IV melanoma patients (pts) but is a predictive factor only for bcl2 antisense treatment efficacy: Re-analysis of GM301 and EORTC18951 randomized trials. American Society of Clinical Oncology Meeting, Abstract 8552, 485s.


Table 1. Mechanisms of Apoptosis Induced by ER Stress

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IREα1 / TRAF2 / ASK1 / JNK</td>
<td>(Szegezdi et al., 2006)</td>
</tr>
<tr>
<td>Gadd153 / CHOP</td>
<td>(Bassik et al., 2004; Shiraishi et al., 2005; Jiang et al., 2007; Chen et al., 2007)</td>
</tr>
<tr>
<td>p53 or non-p53</td>
<td>(Li et al., 2006; Armstrong et al., 2007)</td>
</tr>
<tr>
<td>Downregulation of GRP78 via Caspase 4, 7, BIK, Ca^2+</td>
<td>(Jiang et al., 2007; Reddy et al., 2003; Fu et al., 2007)</td>
</tr>
<tr>
<td>HIF-1α via Bnip3</td>
<td>(Mellor et al., 2007)</td>
</tr>
<tr>
<td>Bim ↑ due to ↑ CHOP transcription or dephosphorylation by PP2A</td>
<td>(Puthalakath et al., 2007)</td>
</tr>
</tbody>
</table>
Table 2. Adaptation of Melanoma to ER Stress

- RAS / RAF / MEK / ERK activation
- Increased GRP78 chaperone proteins
- Upregulation of Mcl-1 antiapoptotic pathways
- Akt3 activation
- Suppression of p53
- Glycolysis and acidification of the tumor microenvironment
Table 3. Additional Agents to Target ER Stress Induced Resistance to Apoptosis

- Agents that target HDM2 & increase p53, eg Nutlin 3a (Vassilev, 2006)
- Inhibitors of GSK3β that target S 315,376 on p53, eg DW 1/2 (Smalley et al., 2007)
- Inhibitors of GRP78 - IRE1α (epigallocatechin) (Ermakova et al., 2006)
  Versipelostatin (Park et al., 2007)
  Irestatin (Feldman and Koong, 2007)
- Inhibitors of Mcl-1 - Obatoclax (Nguyen et al., 2007; Trudel et al., 2007)
  TW-37 (Verhaegen et al., 2006, Mohammed et al., 2007)
  Gossypol (Wang et al., 2006)
- Proton pump inhibitors - Omeprazole (De Milito et al., 2007)
- Monocarboxylate transporters (MCT) inhibitors (Brahimi-Horn and Pouyssegur, 2007)
- Depletion of ATP - 3-Bromopyruvate (Mathupala et al., 2006)
  lactate production (DCA) (Pan and Mak, 2007)
- Agents in clinical use that increase ER stress
  - Velcade/Bortezomib (Linder and Shoshan, 2005)
  Sorafenib (Rahmani et al., 2007)
  Cisplatin (Mandic et al., 2003)
  Celecoxib (Pyrko et al., 2007)
  Curcumin (Scott and Loo, 2007)
  Dipyramidole (Thompson et al., 2003)
Figure 1. Schematic outline of key aspects of the unfolded protein response (UPR)
Primary Events Initiating Melanoma

ER Stress

UPR

Apoptosis
Autophagy

Selection &
Adaptation to Death Signals

Senescence

Progression of Melanoma

Figure 2. Suggested Outcomes from ER Stress in Melanoma
Figure 3. GRP in: A) compound naevus; B) thin <1mm melanoma; C) thick melanoma; D&E) metastases in skin and lymph nodes; F) staining of plasma cells (400x magnification).