Inhibition of MEK sensitizes paclitaxel-induced apoptosis of human colorectal cancer cells by downregulation of GRP78

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Here we report that paclitaxel induces variable degrees of apoptosis in human colorectal cancer cells. Paclitaxel induces multiple arms of the endoplasmic reticulum stress response, including upregulation of the 78-kDa glucose-regulatory protein (GRP78) and eukaryotic initiation factor α phosphorylation. Inhibition of the MEK/ERK pathway sensitized colorectal cancer cells to paclitaxel-induced apoptosis. A similar result was obtained by the inhibition of GRP78 using small interfering RNA molecules. Knockdown of MEK resulted in a significant downregulation of paclitaxel-induced upregulation of GRP78 indicating that activation of GRP78 is a downstream event of MEK/ERK pathway activation. These results indicate that GRP78 might be a novel mechanism underlying the resistance of colorectal cancer cells to microtubule-targeting drugs. A combination of compounds capable of suppressing GRP78 might be a golden approach for improving the effectiveness of taxanes. *Anti-Cancer Drugs* **20:601–606 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

A number of cellular stress conditions, such as hypoxia, alterations in glycosylation status, and disturbances of calcium flux, lead to the accumulation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen and cause so-called ER stress [1–3]. The ER responds to the stress conditions by activation of a range of stress–response signaling pathways that couple the ER protein-folding load with the ER protein folding capacity and is termed the unfolded protein response (UPR) [1–3].

The UPR of mammalian cells is initiated by three ER transmembrane proteins – activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase-like ER kinase [1–3]. Under unstressed conditions, the luminal domains of these sensors are occupied by the ER chaperon glucose-regulated protein 78 (GRP78) [1–3]. Upon ER stress, sequestration of GRP78 by unfolded proteins activates these sensors by inducing phosphorylation and homo-dimerization of IRE1 and protein kinase-like ER kinase, and relocation and proteolytic cleavage of ATF6 [1–3].

Although the UPR is fundamentally a cytoprotective response, excessive or prolonged UPR results in apoptotic cell death. Various mechanisms have been suggested to play a role in ER stress-induced apoptosis [4–7]. These include activation of the c-Jun NH2-terminal kinase (JNK), the transcription factor C/EBP homologous protein, and the BH3-only proteins – puma, noxa, and bim [4–7]. In addition, processing of caspase-2, 3, 4, 7, 8, 9, and 12 has been observed in ER stress-induced apoptosis [4–7]. As a 'master' regulator of the UPR, GRP78 is believed to play an essential role in counter-acting the apoptosis-inducing potential of ER stress by multiple mechanisms such as binding to the unfolded proteins to alleviate ER stress conditions and binding to calcium to prevent its release from the ER [8].

Previous studies have also shown that ER stress may activate members of the mitogen-activated protein kinases. The JNK was activated by the binding of IRE1 to the scaffold molecule TRAF2 [9,10] and consequently activated ASK1/JNK [10]. In contrast, activation of the prosurvival ERK1/2 pathway after ER stress has been found to protect cells from ER stress-mediated apoptosis [11].

In this study, we report that paclitaxel induces variable degrees of apoptosis of human colorectal cancer cells. Paclitaxel induced ER stress of human colorectal cancer cells by upregulation of GRP78 and phosphorylation of eukaryotic initiation factor α (eIF2α). Inhibition of MEK enhanced ER-induced apoptosis by blockage of ER stress-mediated upregulation of the GRP78. These results provide insights into the mechanisms of colorectal cancer cell resistance to taxane-induced apoptosis.

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Materials and methods

Cell lines

Human colon cancer cell lines COLO205, SW480, SW620, HCT 116, HT29, and WiDr were cultured in Dulbecco's modified Eagle medium containing 10% fetal calf serum (Bio Whittaker, Verviers, Belgium).

Antibodies and other reagents

Paclitaxel was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and stored as a 20 mmol/l solution in dimethyl sulfoxide with a final concentration of 0.1% (v/v), which did not contribute to toxicity, at −80°C and diluted with the Dulbecco's modified Eagle medium before use. The propidium iodide was purchased from Sigma-Aldrich Co. The rabbit polyclonal antibody against ERK1/2 was purchased from Cell Signalling Technology (Beverly, Massachusetts, USA). The ERK1/2 inhibitor (MEK inhibitor, U0126) was purchased from Calbiochem (La Jolla, California, USA). Phosphorylated ERK1/2, the rabbit MAbs against GRP78/Bip, eIF2α, and phosphorylated eIF2α were all purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Apoptosis

Quantitation of apoptotic cells by the measurement of sub-G1 DNA content using the propidium iodide method was carried out as described elsewhere [12].

Western blot and protein expression analysis

Cells were cultured to 80% confluence. The cells were trypsinised and washed twice with the medium followed with cold PBS once. The cells were then lysed with a Triton X-100 based lysis buffer [10% Triton X-100, 10% glycerol, 150 mmol/l NaCl, 20 mmol/l Tris (pH = 7.5), 2 mmol/l ethylene diamine tetraacetic acid, 1 mmol/l phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. Cell lysates were then transferred into microcentrifuge tubes and after placing on ice for 1 h they were centrifuged at 13 000 rpm for 30 min at 4°C. The protein content of cell extracts was determined by the Bradford assay (Bio-Rad, Regents Park, New South Wales, Australia). A total of 20–30 μg of protein was electrophoresed on 10–15% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary Abs at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1 : 3000 dilutions; Bio-Rad). Labeled bands were detected by Immun-Star HRP Chemiluminescent Kit (Bio-Rad), and images were captured. The intensity of the bands was quantitated with the Bio-Rad VersaDoc image system. The relative expression of certain protein was determined by dividing the densitometric value of the test protein by that of the control (GAPDH).

Small RNA interference

The small RNA interference (siRNA) constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon, Lafayette, Colorado, USA), the siGENOME SMARTpool MEK1 (M-003571-01-0010), and the siGENOME SMARTpool GRP78 (M-008198-01). The non-targeting siRNA control, SiConTRolNon-targeting siRNA pool (D-001206-13-20), was also obtained from Dharmacon. Transfection of siRNA pools was carried out as described earlier [12].

Statistical analysis

Data are expressed as mean ± SE. The statistical significance of intergroup differences in normally distributed continuous variables was determined using Student's *t*-test. P values ≤ 0.05 were considered statistically significant. P values ≤ 0.05 and ≤ 0.001 are indicated by * and **, respectively.

Results

Paclitaxel induces apoptosis of human colorectal cancer cells

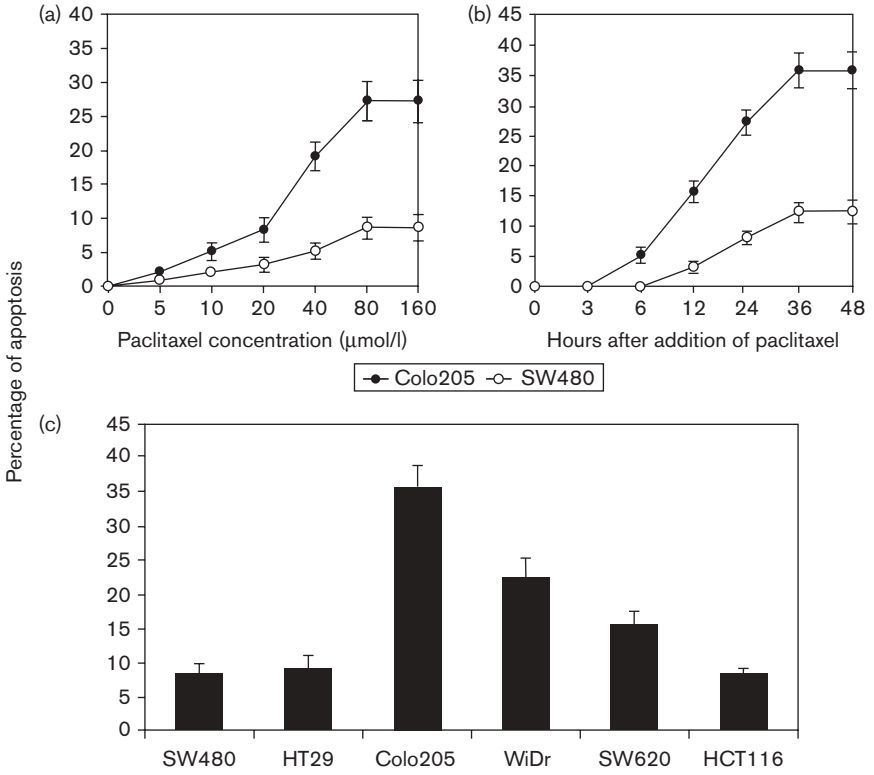
Previous studies have shown a potential for paclitaxel to induce apoptotic cell death in various tumor cell lines [13]. To study this potential in human colorectal cancer cells, Colo205 and SW480 cell lines were treated with a wide range of paclitaxel concentrations: 0, 5, 10, 20, 40, 80, and 160 μmol/l for 24 h. Control cells were treated with the vehicle dimethyl sulfoxide at 0.1% (v/v), and did not contribute to toxicity. As shown in Fig. 1a, paclitaxel induced apoptosis of the human colorectal cancer cells in a dose-dependent manner with the highest percentage of apoptotic cells seen at 80 μmol/l. The kinetics of the induction of apoptosis by paclitaxel is shown in Fig. 1b. Although apoptosis could be detected within 6 h in Colo205 cells, it was not detected in SW480 before 12 h after treatment with paclitaxel at 80 μmol/l. The percentage of apoptotic cells in both cell lines peaked at 36 h after treatment.

A summary of studies on a panel of colorectal cancer cell lines treated with paclitaxel at 80 μmol/l for 36 h is shown in Fig. 1c. Results showed different degrees of colorectal cancer sensitivity to paclitaxel-induced apoptosis.

Inhibition of MEK sensitizes colorectal cancer cells to paclitaxel-induced apoptosis

Activation of the MEK/ERK pathway is a common cause for resistance of cells to apoptosis mediated by the death receptor and mitochondrial apoptotic pathways [14–19]. To better understand the varying sensitivity of colorectal cancer cells to paclitaxel, we were promoted to study whether inhibition of MEK/ERK sensitizes colorectal cells to paclitaxel-induced apoptosis. The kinetics of activation of ERK1/2 kinase in Colo205 and SW480 cells treated with paclitaxel at different time intervals is shown in Fig. 2a. Results indicated that ERK1/2 pathway

Fig. 1



Paclitaxel-induced apoptosis of colorectal cancer cells. (a) Titration of paclitaxel on induction of apoptosis. Colo205 and SW480 cells were treated with paclitaxel at the indicated doses for 24 h before measurement of apoptosis by the propidium iodide (PI) method using flow cytometry. The data shown are the mean ± SE of three individual experiments. (b) Kinetics of induction of apoptosis by paclitaxel. Colo205 and SW480 cells were treated with paclitaxel at 80 μmol/l for the indicated time periods before measurement of apoptosis by the PI method using flow cytometry. The data shown are the mean ± SE of three individual experiments. (c) Paclitaxel-induced apoptosis in a panel of colorectal cancer cell lines. Cells were treated with paclitaxel at 80 μmol/l for 36 h and apoptosis was measured by the PI method using flow cytometry. Columns, mean of three individual experiments; bars, SE.

is constitutively activated in both cell lines. Although paclitaxel induced a marked activation of ERK1/2 in SW480 cells, the phosphorylated form of ERK1/2 was weakly induced in Colo205 cells at an early time of treatment followed by continuous downregulation.

To explore the role of MEK/ERK signal pathways in pacliatxel-induced apoptosis of colorectal cancer, Colo205 and SW480 cells were treated with inhibitor of ERK1/2 (U0126) 1 h before adding paclitaxel for another 36 h. As shown in Fig. 2b, inhibition of the ERK1/2 pathway enhanced killing of cells by paclitaxel. The latter was significantly evident in the SW480 cell line ($P \leq 0.05$).

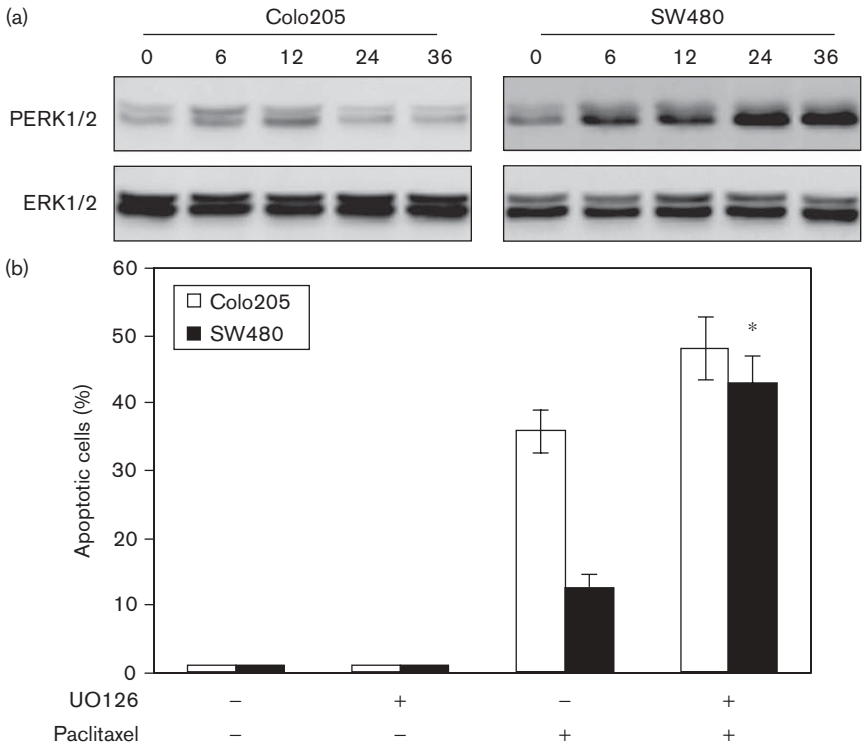
Paclitaxel induces unfolded protein response in colorectal cancer cells

Previous studies have shown interaction between ER stress and activation of members of the mitogen-activated protein kinases including MEK/ERK pathway [9–11]. To determine whether paclitaxel would impinge on stress or the UPR, we first examine the potential of paclitaxel to induce ER stress of colorectal cells. Cells sensitive (Colo205) and resistant (SW480) to paclitaxel-induced apoptosis were treated with paclitaxel for the indicated time periods and the levels of protein expression were assessed by western blots. As shown in Fig. 3a, exposure of the sensitive Colo205 line to paclitaxel was associated with initial increase (at 6 h) followed by a continuous decrease in GRP78 and there was no increase in the phosphorylated form of eIF2. In contrast to Colo205, paclitaxel induced protein levels of GRP78 and the phosphorylation of eIF2α significantly in SW480 cells.

Sensitization of paclitaxel-induced apoptosis by inhibition of MEK/ERK pathway is associated with downregulation of induction of GRP78

In view of the above results showing consistent kinetics of ERK1/2 phosphorylation and induction of GRP78, we studied whether sensitization of colorectal cancer cells to paclitaxel-induced apoptosis by inhibition of MEK/ERK is related to regulation of GRP78. As shown in Fig. 3b, inhibition of MEK/ERK in SW480 cells with U0126 or by

Fig. 2



Involvement of MEK/ERK signaling pathway in paclitaxel-induced apoptosis. (a) Colo205 and SW480 cells were treated with paclitaxel at 80 μmol/l for the indicated time periods. Whole-cell lysates were subjected to western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. (b) Colo205 and SW480 cells were treated with inhibitor of ERK1/2 (U0126) 1 h before adding paclitaxel for another 36 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE. PERK, protein kinase-like ER kinase. $*P < 0.05$.

siRNA knockdown of MEK1 resulted in reduced levels of GRP78 and eIF2α expressions. Inhibition of GRP78 by siRNA significantly ($P \leq 0.05$) sensitized SW480 cells to paclitaxel-induced toxicity to a similar degree of MEK/ERK pathway inhibition (Fig. 3c).

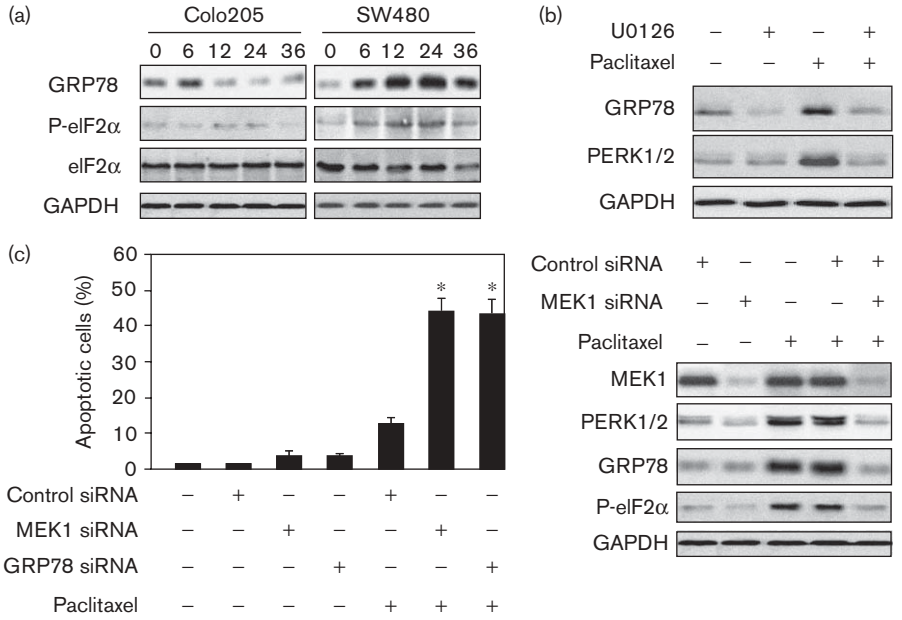
Discussion

Resistance of colorectal cancer cells to chemotherapeutics is a major obstacle to successful treatment of colorectal cancer. In this study, we showed that activation of the MEK/ERK pathway contributes to resistance of colorectal cancer cells against paclitaxel-induced apoptosis, and this is, at least in part, because of MEK/ERK-mediated upregulation of the ER chaperone GRP78. Paclitaxel induced different degrees of apoptosis in colorectal cancer cells. Inhibition of MEK/ERK pathway sensitized paclitaxel-induced apoptosis of colorectal cancer cells, particularly in the resistant SW480 cells. Paclitaxel induces multiple arms of the ER stress response, including upregulation of GRP78 and eIF2α phosphorylation. Sensitivity of colorectal cancer cells to paclitaxel-induced apoptosis was associated with down-regulation of GRP78 by inhibition of the MEK/ERK pathway.

Constitutive activation of Ras/Raf/MEK/Erk signaling is a hallmark of many human cancers such as breast, lung, colorectal cancers, and melanoma [15,16,20]. ERK1/2 lies downstream of a group of kinases including protein kinase C, Raf-1, and MEK1. On stimulation by extracellular signals, they are successively activated by phosphorylation [19]. Previous studies have shown that MEK/ERK signaling pathway is associated with suppression of apoptosis [14,15,18]. Furthermore, activation of the ERK1/2 pathway has been found during ER stress, and governs cell survival during ER stress-induced apoptosis [20,21]. Recently, it has been shown that the ER chaperone GRP78 expression contributes to antiapoptotic effects and chemotherapy resistance in many cancers [8,22]. The levels of GRP78 were correlated well with ERK1/2 pathway activation in renal epithelial cells [11]. These findings promoted us to investigate the role of MEK/ERK inhibition in paclitaxel-induced cell death of colorectal cancer cells.

Investigation of the mechanism involved in the MEK/ERK-mediated inhibition of ER stress-induced apoptosis led to focus on GRP78, which is known to inhibit apoptosis by multiple mechanisms [8]. The findings that

Fig. 3



Paclitaxel induces ER protein chaperone GRP78 and the eukaryotic initiation factor α (eIF2α). (a) Colo205 and SW480 cells were treated with paclitaxel at 80 μmol/l for the indicated time periods. Whole cell lysates were subjected to western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. (b) Inhibition of MEK downregulates GRP78 expression and blocks its upregulation by paclitaxel. Upper panel: SW480 cells were treated with ERK1/2 inhibitor (U0126) for 1 h before adding paclitaxel at 80 μmol/l for another 12 h. Whole-cell lysates were subjected to western blot analysis. Western blot analysis of GAPDH levels was included to show that equivalent amounts of protein were loaded in each lane. Data are representative of two individual experiments. Lower panel: SW480 cells were transfected with the control or MEK small RNA interference (siRNA). Twenty-four hours later, whole-cell lysates were subjected to western blot analysis of GRP78 expression. The data shown are representative of three individual experiments. (c) Downregulation of MEK1 or GRP78 expression in SW480 cells using siRNA. SW480 cells were transfected with either a non-targeting siRNA (control siRNA) or with a MEK1 or with GRP78-specific siRNA sequences (Dharmacon) at 100 nmol/l for 24 h. Transfected cells were exposed to paclitaxel for 36 h and assessed for apoptosis using the propidium iodide method. Columns, mean of three individual experiments; bars, SE. PERK, protein kinase-like ER kinase.*$P<0.05$.

enhancement of paclitaxel-induced apoptosis by inhibition of MEK was closely associated with blockage of GRP78 upregulation strongly suggest that GRP78 plays a role in MEK/ERK-mediated inhibition of apoptotic signaling in cells subjected to ER stress. Inhibition of MEK/ERK signaling with U0126 or by siRNA knockdown of MEK1 resulted in a decrease in the levels of expression of GRP78, indicating that MEK/ERK pathway might be an upstream regulator of GRP78.

In summary, the results shown in this study seem to provide several new insights into potential interaction between the UPR and the MEK/ERK signaling pathway in colorectal cancer cells. They show that sensitivity of colorectal cancer cells to paclitaxel is determined by levels of the ER chaperone protein GRP78. Sensitization of colorectal cancer cells to paclitaxel-induced apoptosis by inhibition of the MEK/ERK pathway was closely associated with downregulation of GRP78. Thus, combination of compounds capable of suppressing GRP78 might be a novel approach for improving the effectiveness of paclitaxel in the treatment of colorectal cancer.

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References

1 Harding HP, Calfon M, Urano F, Novoa I, Ron D. Transcriptional and translational control in the mammalian unfolded protein response. *Annu Rev Cell Dev Biol* 2002; **18**:575–599.
2 Zhang K, Kaufman RJ. Signaling the unfolded protein response from the endoplasmic reticulum. *J Biol Chem* 2004; **279**:25935–25938.
3 Schroder M, Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem* 2005; **74**:739–789.
4 Yamaguchi H, Wang HG. CHOP is involved in endoplasmic reticulum stress-induced apoptosis by enhancing DR5 expression in human carcinoma cells. *J Biol Chem* 2004; **279**:45495–45502.
5 Boyce M, Yuan J. Cellular response to endoplasmic reticulum stress: a matter of life or death. *Cell Death Differ* 2006; **13**:363–373.
6 McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by downregulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol* 2001; **21**:1249–1259.
7 Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. *Nat Cell Biol* 2001; **3**:255–263.
8 Lee AS. GRP78 induction in cancer: therapeutic and prognostic implications. *Cancer Res* 2007; **67**:3496–3499.

9 Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 2000; **287**:664–666.

10 Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, *et al*. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 2002; **16**:1345–1355.

11 Hung C, Ichimura T, Stevens J, Bonventre J. Protection of renal epithelial cells against oxidative injury by endoplasmic reticulum stress preconditioning is mediated by ERK1/2 activation. *J Biol Chem* 2003; **278**:29317–29326.

12 Mhaidat NM, Wang Y, Kiejda KA, Zhang XD, Hersey P. Docetaxel-induced apoptosis in melanoma cells is dependent on activation of caspase-2. *Mol Cancer Ther* 2007; **6**:752–761.

13 Rowinsky EK, Donehower RC. The clinical pharmacology and use of antimicrotubule agents in cancer chemotherapeutics. *Pharmacol Ther* 1991; **52**:35–84.

14 Erhardt P, Schremser EJ, Cooper GM. B-Raf inhibits programmed cell death downstream of cytochrome c release from mitochondria by activating the MEK/ERK pathway. *Mol Cell Biol* 1999; **19**:5308–5315.

15 Adeyinka A, Nui Y, Cherlet T, Snell L, Watson PH, Murphy LC. Activated mitogen-activated protein kinase expression during human breast tumorigenesis and breast cancer progression. *Clin Cancer Res* 2002; **8**:1747–1753.

16 Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, *et al*. BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res* 2002; **62**:6997–7000.

17 Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM. Serine/threonine protein kinases and apoptosis. *Exp Cell Res* 2000; **256**:34–41.

18 Hersey P, Zhuang L, Zhang XD. Current strategies in overcoming resistance of cancer cells to apoptosis in melanoma as a model. *Int Rev Cytol* 2006; **251**:131–158.

19 Mhaidat NM, Zhang XD, Jiang CC, Hersey P. Docetaxel-induced apoptosis of human melanoma is mediated by activation of c-Jun NH2-terminal kinase and inhibited by the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway. *Clin Cancer Res* 2007; **13**:1308–1314.

20 Hu P, Han Z, Couvillon AD, Exton JH. Critical role of endogenous Akt/IAPs and MEK1/ERK pathways in counteracting endoplasmic reticulum stress-induced cell death. *J Biol Chem* 2004; **279**:49420–49429.

21 Guichard C, Pedruzzi E, Fay M, Marie JC, Braut-Boucher F, Daniel F, *et al*. Dihydroxyphenylethanol induces apoptosis by activating serine/threonine protein phosphatase PP2A and promotes the endoplasmic reticulum stress response in human colon carcinoma cells. *Carcinogenesis* 2006; **27**:1812–1827.

22 Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? *Nat Rev Cancer* 2004; **4**:966–977.