Noxa/Mcl-1 balance influences the effect of the proteasome inhibitor MG-132 in combination with anticancer agents in pancreatic cancer cell lines

Katrin Naumann[a], Kathrin Schmich[a], Christoph Jaeger[a], Felix Kratz[b] and Irmgard Merfort[a]

Pancreatic cancer progresses aggressively and owing to chemoresistance responds poorly to chemotherapy. Thus, there is an urgent need to understand the mechanisms of cancer cell resistance to generate effective strategies to circumvent intrinsic chemoresistance in this tumour indication. In this study, three pancreatic cancer cell lines, MIA PaCa-2, MDAPanc-3 and AsPC-1, were treated with the proteasome inhibitor MG-132 together with camptothecin, doxorubicin or paclitaxel, and cytotoxicity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The combination of MG-132 and camptothecin at a ratio of 5 : 1 gave the most promising results and enhanced cytotoxicity compared with the single compounds in MIA PaCa-2 cells. The increase was shown to be due to enhanced caspase-3 activity resulting in apoptosis. Moreover, this combination upregulated the levels of the proapoptotic protein Noxa and reduced the levels of the antiapoptotic protein Mcl-1, as demonstrated by western blotting. In contrast, the combination of MG-132 with doxorubicin also induced increased cytotoxicity, but apoptosis was decreased. The lack of an enhanced apoptosis induction could be correlated with high levels of Mcl-1 in response to the combined treatment with MG-132 and doxorubicin. Thus, the results indicate that regulation of the antiapoptotic and proapoptotic Bcl-2 family members Noxa and Mcl-1 is predicative of the effectiveness of the combination of MG-132 with different anticancer agents on apoptosis induction in pancreatic cancer cells. *Anti-Cancer Drugs* 23:614–626 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2012, **23**:614–626

Keywords: camptothecin, doxorubicin, Mcl-1, MG-132, MIA PaCa-2, Noxa, paclitaxel, pancreatic cancer cell lines

[a]Department of Pharmaceutical Biology and Biotechnology, University of Freiburg and [b]Division of Macromolecular Prodrugs, Tumour Biology Center, Freiburg, Germany

Correspondence to Irmgard Merfort, Department of Pharmaceutical Biology and Biotechnology, Albert-Ludwigs Universität Freiburg, Stefan-Meier-Str. 19, Freiburg 79104, Germany
Tel: +49 761 203 8373; fax: +49 761 203 8383;
e-mail: irmgard.merfort@pharmazie.uni-freiburg.de

Received 1 September 2011 **Revised form accepted** 12 December 2011

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the US, with high aggressiveness and poor survival prognosis [1]. This tumour shows rapid growth and metastatic distribution, as well as frequent resistance to anticancer drug treatment, and if surgery is not possible this type of cancer can be treated only palliatively. Thus, effective treatment of metastatic pancreatic cancer remains a challenging task. Gemcitabine and 5-fluorouracil are at present the only drugs that have been approved, leading to a response rate of approximately 10% and an overall survival benefit of about 2 months [2].

In conventional chemotherapy, anticancer drugs camptothecin, paclitaxel and doxorubicin are commonly used to treat solid tumours [3–6]. The antitumour activity of camptothecin is attributable to its specific interference with topoisomerase-1 (TOP-1), which induces single-strand breaks. Camptothecin inhibits the rejoining step of the cleavage/relegation reaction of TOP-1 by forming a reversible covalent reaction intermediate. The cell cycle machinery is blocked and consequently apoptosis is induced. It has been shown that camptothecin can also induce TOP-1 downregulation, which can contribute to a decreased sensitivity towards it [7–10]. In the case of doxorubicin, several mechanisms have been demonstrated for its cytotoxic activity, such as intercalation with DNA and inhibition of DNA synthesis, DNA damage by inhibition of TOP-2, or free radical formation [11]. Paclitaxel is a polymerizing antimicrotubule agent that induces apoptosis by preventing dissociation of tubulin moieties during cell division [12].

It is well known that anticancer agents can activate the transcription factor nuclear factor-κB (NF-κB) contributing to resistance towards these drugs [13–16]. NF-κB is a transcription factor involved in immune responses, inflammation and tumourigenesis. In its inactive form it is located in the cytoplasm, where it is sequestered by the inhibitory protein IκBα. On phosphorylation of IκBα, the inhibitory subunit is ubiquitinated and degraded by the proteasome [17], thus releasing NF-κB in the cytoplasm and enabling the transcription factor to translocate to the nucleus. In the nucleus, NF-κB regulates transcription of several target genes, including antiapoptotic and apoptotic

All supplementary data are available directly from the authors.

0959-4973 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e3283504e53

ones. Hence, NF-κB can inhibit apoptosis by induction of antiapoptotic genes and/or suppression of proapoptotic genes [18]. Moreover, some tumour cells constitutively express high levels of active NF-κB, thus protecting themselves from the effects of anticancer agents. It has already been reported that most pancreatic adenocarcinomas show constitutive NF-κB expression [19]. Therefore, inhibition of NF-κB activity would be a promising target for circumventing chemoresistance towards anticancer drugs [15,20].

One possible way to target NF-κB is to prevent its activation by inhibiting the proteasome. Bortezomib (Velcade; USA Millennium Pharmaceuticals, Boston, Massachusetts, USA) was the first proteasome inhibitor approved in 2008 by the US Food and Drug Administration for treatment of multiple myeloma. This small molecule inhibits degradation of IκBα and retains NF-κB in the cytoplasm, thereby preventing antiapoptotic effects mediated by this transcription factor. Besides IκBα, other intracellular proteins that have undergone ubiquitination, such as the cell cycle regulators p21, p27, cyclins and p53, are also degraded by the proteasome, indicating that the proteasome plays an important role in cell growth and apoptosis. Thus, proteasome inhibition is an interesting therapeutic target for inhibiting tumour growth and when combined with an anticancer drug can enhance cell kill [21]. The effect of the combination of a proteasome inhibitor with chemotherapeutic agents has already been shown in several in-vitro studies: for example, with the proteasome inhibitor PS-341 and the active metabolite of camptothecin (SN-38) in human colorectal cancer cells [22], or with PS-341 and gemcitabine in pancreatic MIA PaCa-2 cells [23]. A further potent, reversible and cell-permeable proteasome inhibitor is MG-132, a tripeptide aldehyde Z-Leu-Leu-Leu-CHO (Z = benzyloxycarbonyl). It was reported that this proteasome inhibitor prevents degradation of TOP-1 by camptothecin, thereby enhancing its apoptotic effect [7], and that MG-132 enhances the apoptotic effect of doxorubicin in the pancreatic cell lines Capan-1 and A818-4 [15].

In general, there are two different signalling pathways that induce apoptosis, both of which converge in cleavage of different procaspases to generate their active forms [24]. The extrinsic pathway (death receptor pathway) is activated upon binding of death-inducing ligands, such as FasL or TNF-related apoptosis-inducing ligand, to cell surface receptors. The intrinsic pathway triggers apoptosis in response to DNA damage, for example by cytotoxic drugs or oxidative stress, and is characterized by the permeabilization of the mitochondrial outer membrane. Mitochondrial outer membrane results in cytochrome c release into the cytosol, which in turn effectively activates the effector caspases and induces the apoptotic machinery. This pathway is regulated by the proapoptotic (e.g. Bax, Bak, Bok, Bid, Bim, Noxa) and antiapoptotic (e.g. Bcl-2, Bcl-xL, Bcl-W, Mcl-1, A1) Bcl-2 family members [25]. The antiapoptotic Bcl-2 proteins can inhibit the proapoptotic proteins Bax and Bak and the BH3-only proteins Bad, Bik, Bid, Bim, Puma and Noxa. The BH3-only protein Noxa acts as a proapoptotic agent by interacting with antiapoptotic Bcl-2 proteins, preferentially Mcl-1 [26], and has been reported to be induced upon p53 activation, DNA damage and UV radiation [27]. Mcl-1 is an antiapoptotic Bcl-2 protein that interacts with the proapoptotic Bcl-2 family members Bak as well as BH3-only proteins [28]. Noxa releases Bak by interacting with Mcl-1 and subsequent displacement, thereby allowing Bak to form homo-oligomers with Bax leading to mitochondrial membrane permeabilization and release of cytochrome c, which results in caspase activation and finally leads to apoptosis, as shown by Willis *et al.* [28]. Mcl-1 protein is degraded by the proteasome [29], but binding of Noxa to Mcl-1 can also promote Mcl-1 degradation through a proteasome-dependent mechanism [28]. In summary, the Bcl-2 family of proteins functions as a 'life/death switch' determining whether or not the stress apoptotic pathway is activated.

In light of the molecular interplay between the proapoptotic and antiapoptotic pathways, the combination of a proteasome inhibitor that acts as an NF-κB inhibitor with anticancer drugs, such as camptothecin, doxorubicin or paclitaxel, may be a promising approach for cancer treatment, including those that do not respond to conventional chemotherapy alone, such as pancreatic cancer. Preliminary studies have shed some light on the effect of proteasome inhibitors with anticancer drugs (camptothecin, doxorubicin, etoposide) [7,15,30], but a systematic study comparing the effect of a proteasome inhibitor with different anticancer drugs in the same tumour indication is missing.

Hence, in this study, we report that combining the proteasome inhibitor MG-132 with the anticancer agent camptothecin enhances cytotoxicity because of an apoptotic response in pancreatic cancer cells, especially in MIA PaCa-2 cells. In contrast, combining MG-132 and doxorubicin resulted only in reduced apoptosis, whereas the combination of MG-132 and paclitaxel showed no effect on apoptosis. NF-κB is not involved in the enhanced cytotoxicity and sensitizing effect towards apoptosis. Hence, the proteasome inhibitory property of MG-132 is here directed towards proteins that are important in the apoptotic pathway. Consequently, caspase-3/7 activation turned out to be crucial. Moreover, we provide evidence that the balance between the proapoptotic protein Noxa and the antiapoptotic protein Mcl-1 appears to be responsible for this effect.

Materials and methods

Cell culture and reagents

The human pancreatic carcinoma cell lines MIA PaCa-2, AsPC-1 and MDAPanc-3 were kindly provided by Dr

Ralph Graeser (Tumour Biology Center, Freiburg, Germany). The MIA PaCa-2 and AsPC-1 cells were maintained in Roswell Park Memorial Institute 1640 medium, MDAPanc-3 cells in a mixture consisting of equal amounts of F12 Kaighn's and Dulbecco's Modified Eagle's Medium (all from Gibco BRL, Karlsruhe, Germany) in humidified atmosphere at 37°C and 5% CO_2. All media were supplemented with 10% fetal bovine serum (Sigma, Steinheim, Germany) and 1% penicillin and streptomycin (Roche Diagnostics, Mannheim, Germany). TNF-α was purchased from Pepro Tech (London, UK), and MG-132, actinomycin D and the pan-caspase-inhibitor Q-VD-OPh were purchased from Axxora (Lörrach, Germany). The anticancer drugs doxorubicin and paclitaxel were from Yic-Vic, Hongkong, and camptothecin from Hande Tech Development, Inc. (Houston, Texas, USA).

MTT assay

Cytotoxicity studies were conducted in 96-well plates (12 000 cells/well: MIA PaCa-2 and MDAPanc-3; 15 000 cells/well: AsPC-1). Cells were exposed to different stimuli for the times indicated. Afterwards, the medium was removed, cells were treated with 200 µl of a mixture consisting of equal amounts of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) solution [5.0 mg/ml in sterile phosphate-buffered saline (PBS)] and the respective culture media (final MTT concentration: 2.5 mg/ml) and incubated for 2 h at 37°C. The supernatant was removed and 100 µl of extraction buffer (20% SDS in water with 50% dimethylformamide) was added and incubated overnight. Colour development was measured with a microplate reader at 595 nm. Sample values were referred to untreated cells, and cytotoxicity was calculated as percentage of cell kill compared with untreated controls.

Cell death detection enzyme-linked immunosorbent assay

DNA fragmentation was measured by the cell death detection enzyme-linked immunosorbent assay (ELISA)^{plus} Kit (Roche Diagnostics) according to the manufacturer's instructions. Cells were seeded in 96-well plates (5000 cells/well) and incubated with different stimuli for the times indicated. After cell lysis, histone–DNA complexes were detected in the supernatant by ELISA. Measured values were referred to untreated cells and changes expressed as the factor by which they had increased.

DEVDase assay

For measuring the activity of the executioner caspases-3/7, the fluorogenic DEVDase assay was performed. A total of 750 000 cells per well were incubated with different stimuli for the indicated times. After washing and removing the cells by scraping in ice-cold PBS, the cell suspension was centrifuged at 4°C and 1600 rpm for 5 min, followed by resuspension of cells in 50 µl homogenization buffer. The caspase-3 activity assay was performed exactly as described in [31], using the caspase-3 substrate acetyl-Asp–Glu–Val–Asp-7-amino-4-methyl coumarin (Axxora) at a concentration of 200 nmol/l. Relative fluorescence unit values were calculated as the ratio of the average rate of the fluorescence increase and protein concentration as determined by the Bradford assay (Biorad, Munich, Germany). Relative fluorescence unit values were referred to untreated cells and are given as fold increase.

Preparation of nuclear and total cell lysates

For preparation of total cell lysates for western blot analysis, 2 × 750 000 cells were washed and afterwards scraped in ice-cold PBS. The cell suspension was centrifuged at 4°C at 1600 rpm for 5 min. The supernatant was removed, and for Noxa protein detection cells were resuspended in 140 µl of lysis buffer containing 50 mmol/l Tris-HCl, 250 mmol/l NaCl, 5 mmol/l EDTA, 50 mmol/l NaF, 0.5% Nonidet-P40, phosphatase inhibitor PhosStop and Complete protease inhibitor cocktail (both from Roche Diagnostics), shaken for 20 min at 4°C and centrifuged for 10 min at full speed. For Mcl-1 protein detection, cells were lysed in 100 µl lysis buffer containing 20 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l EDTA, 1% Triton X-100, 1 mmol/l Na_3VO_4, 20 mmol/l Na_3PO_4, 10 mmol/l NaF, 3 mmol/l β-glycerophosphate, 5 mmol/l sodium pyrophosphate, 100 µmol/l MG-132, phosphatase inhibitor PhosStop and Complete protease inhibitor cocktail, shaken for 15 min at 4°C and centrifuged for 15 min at full speed. The supernatant was collected and stored at −20°C.

To prepare nuclear extracts for the NF-κB electrophoretic mobility shift assay (EMSA), 2 × 750 000 cells per sample were washed, scraped, centrifuged and resuspended in 400 µl buffer A [10 mmol/l of *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid (HEPES), 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l ethylene glycol tetra-acetic acid] supplemented with 17 µg/ml of aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mmol/l phenylmethanesulfonylfluoride (PMSF) and 1 mmol/l dithiothreitol (DTT). After incubation on ice for 15 min, 25 µl Nonidet P-40 10% was added, and the sample was vortexed for 10 s. After centrifugation, the supernatant (cytoplasm) was collected and the nuclear residue was resuspended and shaken for 30 min in 50 µl buffer C (20 mmol/l HEPES, 0.4 mol/l NaCl, 1 mmol/l EDTA, 1 mmol/l ethylene glycol tetra-acetic acid) supplemented with 17 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mmol/l PMSF and 1 mmol/l DTT. After centrifugation, the supernatant was collected and stored at −80°C. Protein concentrations were determined using the Bradford assay.

Electrophoretic mobility shift assay

Nuclear extracts were prepared as described above. Equal amounts of protein were added to a reaction mixture

containing 20 µg bovine serum albumin, 2 µg poly(deoxy-inosinic-deoxycytidylic) acid (from Roche Diagnostics), 2 µl buffer D+ (20 mmol/l of HEPES, pH 7.9, 20% glycerol, 100 mmol/l KCl, 0.5 mmol/l EDTA, 0.25% NP-40, 2 mmol/l DTT, 0.1% PMSF), 4 µl buffer F (20% Ficoll 400, 100 mmol/l HEPES, 300 mmol/l KCl, 10 mmol/l DTT, 0.1% PMSF) and 100 000 cpm (Cerenkov) of a ^{33}P-labelled oligonucleotide for NF-κB and was made up to a final volume of 20 µl with distilled water. NF-κB oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; Promega, Mannheim, Germany) was labelled using [γ-^{33}P]-ATP (3000 Ci/mmol, Amersham Biosciences, Freiburg, Germany) and a T4 polynucleotide kinase (New England Biolabs, Frankfurt, Germany). After 25 min of incubation at room temperature, the samples were resolved through nondenaturing 6% polyacrylamide gel electrophoresis. Thereafter, the dried gel was exposed to an Imaging Plate (BAS-MS 2340; Fujifilm Life Science, Stamford, Connecticut, USA) overnight, which was finally analysed using an FLA-3000 (Fujifilm).

Western blotting

After protein determination of total cell lysates, 20–70 µg of protein was separated by SDS-polyacrylamide gel electrophoresis (12 or 15% gels) and transferred on to a 0.45 µm or 0.2 µm pore size polyvinylidene fluoride membrane (Roche Diagnostics resp. Biorad). Blots were blocked with 5% nonfat milk powder in 0.1% tris-buffered saline solution with Tween at room temperature. Primary antibodies were incubated overnight at 4°C at the following dilutions: anti-Noxa (Imgenex, San Diego, California, USA) 1.5 µg/ml in 1% nonfat milk powder; anti-Mcl-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) 1 : 1000 in 3% nonfat milk powder; and anti-actin (MP Biomedicals, Heidelberg, Germany) 1 : 10 000 in 1% bovine serum albumin. Horseradish peroxidase-labelled secondary antibodies [Jackson ImmunoResearch Laboratories (Newmarket, Suffolk, UK) or Cell Signaling (Frankfurt, Germany)] were incubated at room temperature for 1 h. Proteins were visualized by enhanced chemiluminescene (Amersham Bioscience) using LumiImager and LumiAnalyst Software (Roche Diagnostics).

Results

Human pancreatic cancer cell lines respond with different sensitivities to combination treatment with MG-132 and anticancer agents

To find new strategies to overcome chemoresistance in cancer chemotherapy, we studied the cytotoxic effect of combined treatment with the proteasome inhibitor MG-132 and the anticancer agents camptothecin, doxorubicin and paclitaxel against pancreatic cancer cells. The three pancreatic carcinoma cell lines AsPC-1, MDAPanc-3 and MIA PaCa-2 were exposed to different concentrations of a constant 1 : 1 ratio of MG-132 and either camptothecin, doxorubicin or paclitaxel. After 24 h, cytotoxicity was measured with the MTT assay, but no enhanced cytotoxic effect was detectable in any tested cell line when using the 1 : 1 combinations compared with use of single drugs (data not shown).

Considering that a five-fold excess of MG-132 compared with camptothecin has recently been shown to increase cytotoxicity in human retinoblastoma Y70 cells [32], we opted for the same strategy. Thus, pancreatic cancer cells were exposed to concentrations of a 5 : 1 mixture of MG-132 and camptothecin, MG-132 and doxorubicin, and MG-132 and paclitaxel for 12, 16 and 24 h, respectively. In MIA PaCa-2, the highest cytotoxicity and best enhanced effect was achieved by a combined treatment with MG-132 and camptothecin after 16 h of incubation (see Fig. 1a and Supporting information Fig. S1 for data after 12 and 24 h). This enhanced effect was additive, dose dependent and most distinct at 2.5 µmol/l MG-132 with 0.5 µmol/l of camptothecin. No further increase was observed at higher concentrations (Fig. 1a). Therefore, the incubation time of 16 h was chosen for testing the other combinations, that is, MG-132 and doxorubicin or paclitaxel, in MIA PaCa-2 cells. These cells showed either no or a relatively low sensitivity to doxorubicin (1 µmol/l had no effect; 2 µmol/l resulted in 35% cell kill) or to MG-132 (5 µmol/l induced 39% cell kill; Fig. 1b). In contrast, the combination of MG-132 (5 µmol/l) and doxorubicin (1 µmol/l) synergistically potentiated the cytotoxic effect, and cytotoxicity increased up to 66% compared with treatment with single agents (Fig. 1b). A similar effect was observed with 10 µmol/l of MG-132 and 2 µmol/l of doxorubicin (Fig. 1b). As shown in Fig. 1c, paclitaxel alone was more potent in this cell line and already induced 46% cytotoxicity at 0.016 µmol/l, but cell death could not be increased significantly by combination treatment with MG-132 (62% cytotoxicity).

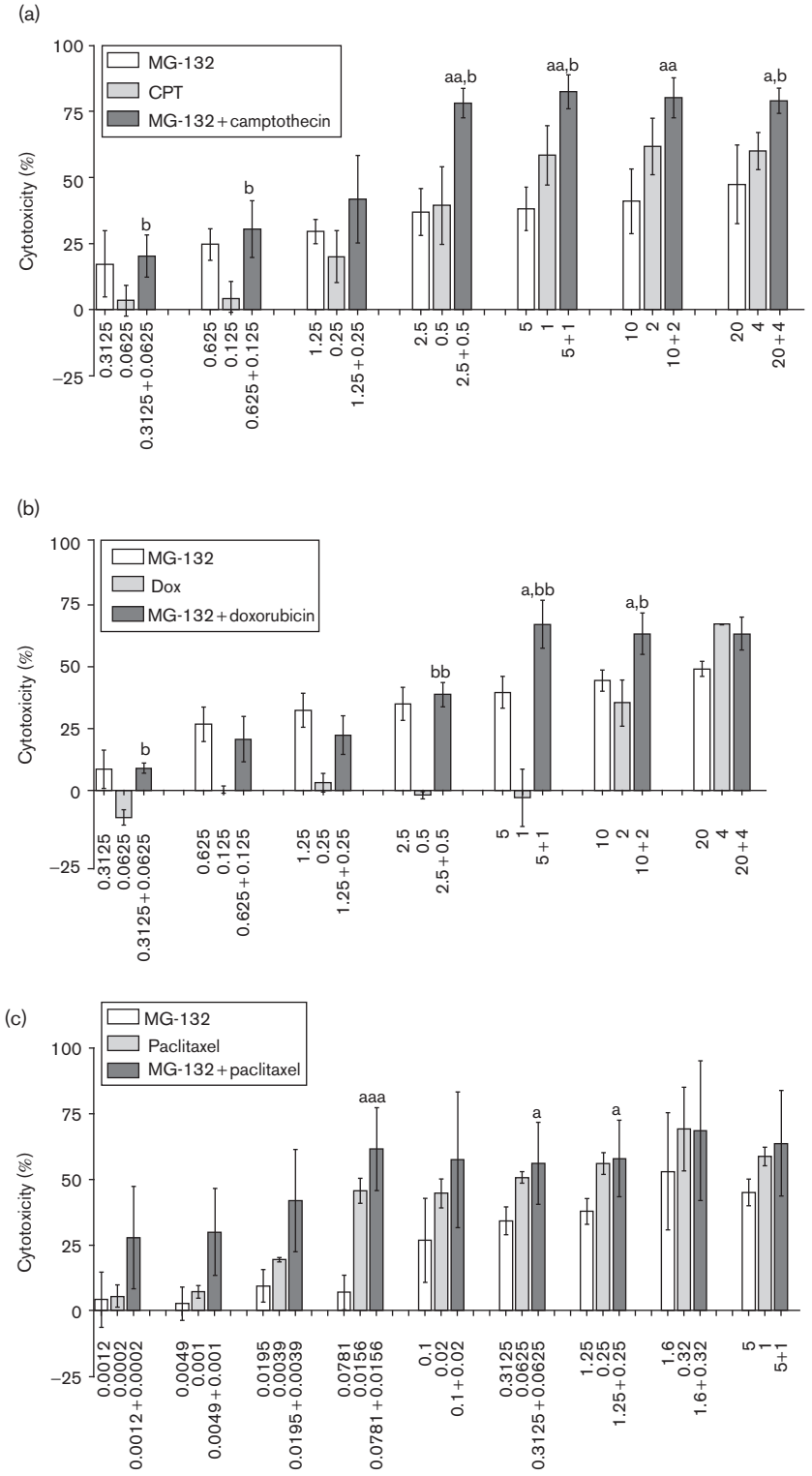
The sensitivity of the other pancreatic cancer cell lines MDAPanc-3 and AsPC-1 to the compounds was rather low, as shown in Fig. 2. A moderately enhanced cytotoxic effect upon combined treatment occurred in MDAPanc-3 cells only after 16 h of incubation with MG-132 and camptothecin (the effect was additive) and to a lesser extent with MG-132 and doxorubicin (the effect was synergistic; Fig. 2a and b). In AsPC-1 cells, treatment with MG-132 combined with any of the anticancer drugs did not result in enhanced cytotoxicity (Fig. 2c and d).

In summary, MIA PaCa-2 cells turned out to be the most suitable cell line and MG-132/camptothecin and MG-132/doxorubicin the most promising combinations for studying the effect of overcoming chemotherapy resistance in more detail.

Potentiation of camptothecin-induced but not of doxorubicin-induced apoptosis by MG-132

To determine whether cytotoxicity was due to apoptosis, cell lines were incubated with MG-132 and camptothecin,

Fig. 1



The combination of MG-132 with camptothecin or doxorubicin, but not with paclitaxel, induces increased cell death compared with single-compound treatment in the MIA PaCA-2 cancer cell line; (a) MG-132/camptothecin; (b) MG-132/doxorubicin; (c) MG-132/paclitaxel, concentration in μmol/l. Cells were incubated with different concentrations of the indicated compounds or mixtures for 16 h. Cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay and expressed as percentage cytotoxicity compared with untreated cells. Data represent means of at least three independent experiments ± SD. Statistical significance was expressed as follows: a: MG-132 vs. combination, b: anticancer drug vs. combination; one letter (a or b) means $P < 0.05$, two letters (aa or bb) $P < 0.01$ and three letters (aaa) $P < 0.001$.

doxorubicin or paclitaxel for 16 h, and apoptosis-associated DNA fragmentation was quantified by ELISA. Interestingly, 1 μmol/l of camptothecin alone induced apoptosis in MIA PaCa-2 cells (4.2-fold compared with the untreated control), but MG-132 at a concentration of 5 μmol/l alone failed to do so (1.3-fold), whereas a significant increased apoptotic effect was induced by combining both drugs (eight-fold; Fig. 3a). In MDAPanc-3 cells, only a slight increase occurred on combined treatment (Fig. 3b), and no apoptosis was detectable in AsPC-1 cells in response to any drug (data not shown). Moreover, studies were undertaken to elucidate whether the ratio of MG-132: camptothecin (5 : 1) was optimal. As shown in Fig. 3c, enhanced apoptosis was detectable at a three-fold excess of MG-132, whereas no significant further increase was achieved at ratios between 3 : 1 and 12 : 1.

Interestingly, exposing MIA PaCa-2 to MG-132 and doxorubicin induced no apoptotic effect, although an enhanced cytotoxic effect was obtained compared with single-agent treatment (compare Fig. 4a and Fig. 1b). Doxorubicin alone led to a strong apoptotic response (6.5-fold at a concentration of 2 μmol/l compared with untreated cells), although the cytotoxic activity was comparably weak (35%). In MDAPanc-3 cells, none of the substances or combinations induced apoptosis (Fig. 4b).

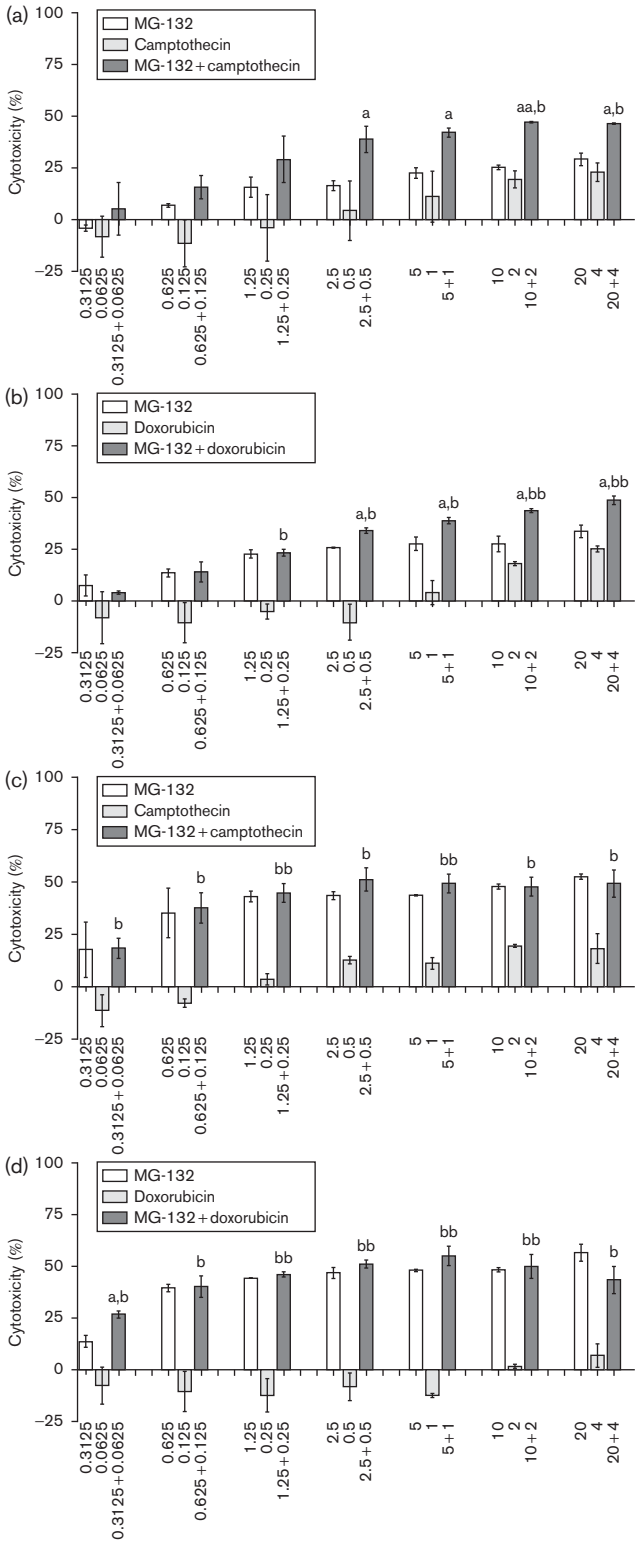
Paclitaxel and MG-132 together did not induce apoptosis in a distinct manner in MIA PaCa-2 cells (Fig. 4c), although 0.32 μmol/l of paclitaxel alone led to a marginal, but significantly higher, apoptosis level compared with 1.6 μmol/l of MG-132 or the combination. Treating MDAPanc-3 cells with MG-132 and paclitaxel did not result in an enhanced apoptotic effect (data not shown).

In summary, it was shown for the first time that combining MG-132 and camptothecin led to significantly enhanced apoptosis induction in MIA PaCa-2 cells, whereas combining MG-132 and doxorubicin conversely diminished the apoptotic response in the same cell line, although cytotoxicity was increased.

Caspase-3/7 activation is involved in the enhanced apoptotic effect of MG-132 and camptothecin

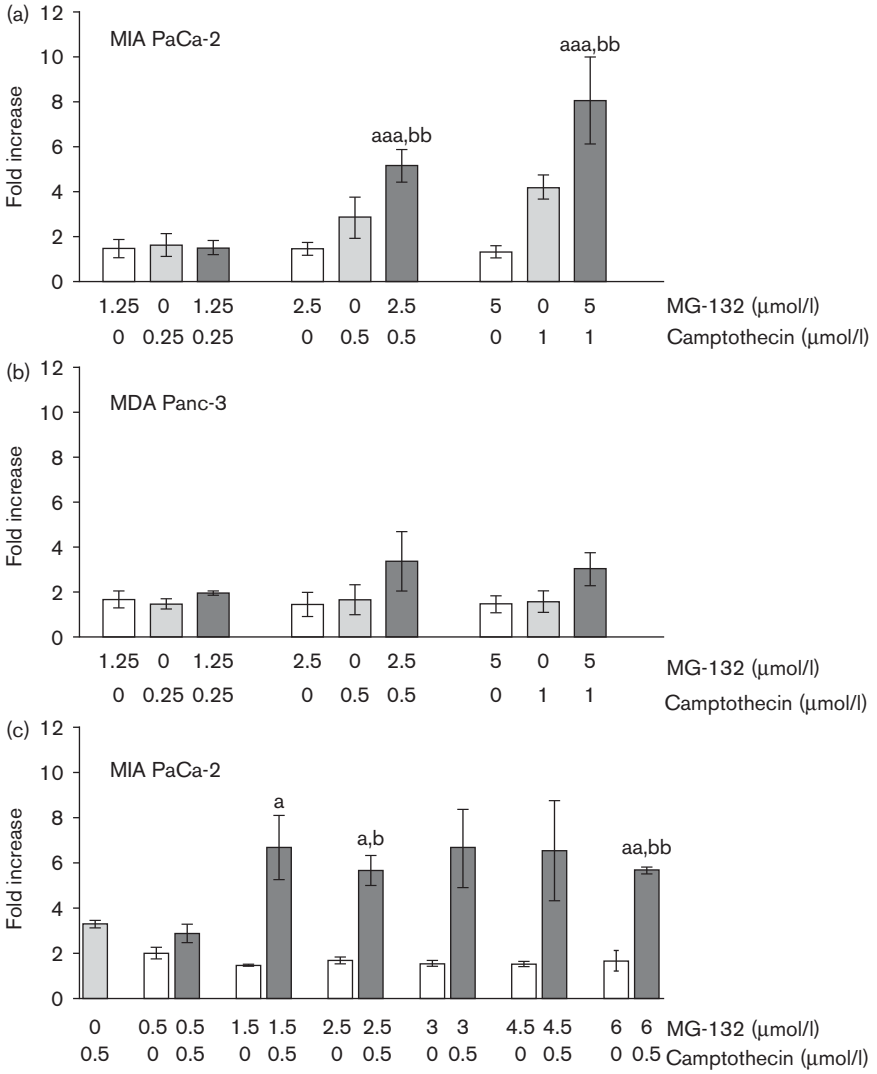
To confirm the enhanced apoptotic effect of MG-132 and camptothecin in MIA PaCa-2 cells detected in the apoptosis-associated DNA fragmentation assay, caspase-3/7 activation was measured in the DEVDase assay.

Fig. 2



Low cytotoxicity upon treatment with combinations of MG-132 and the anticancer drugs camptothecin and doxorubicin in MDAPanc-3 (a and b) and AsPC-1 (c and d) pancreatic cancer cell lines. MDAPanc-3 and AsPC-1 cells were incubated with different concentrations (in μmol/l) of the indicated compounds or mixtures for 16 h. Cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide viability assay and expressed as percentage cytotoxicity compared with untreated cells. Data represent means of at least three independent experiments ± SD. Statistical significance was expressed as follows: a: MG-132 vs. combination, b: anticancer drug vs. combination; one letter (a or b) means $P < 0.05$, two letters (aa or bb) $P < 0.01$.

Fig. 3

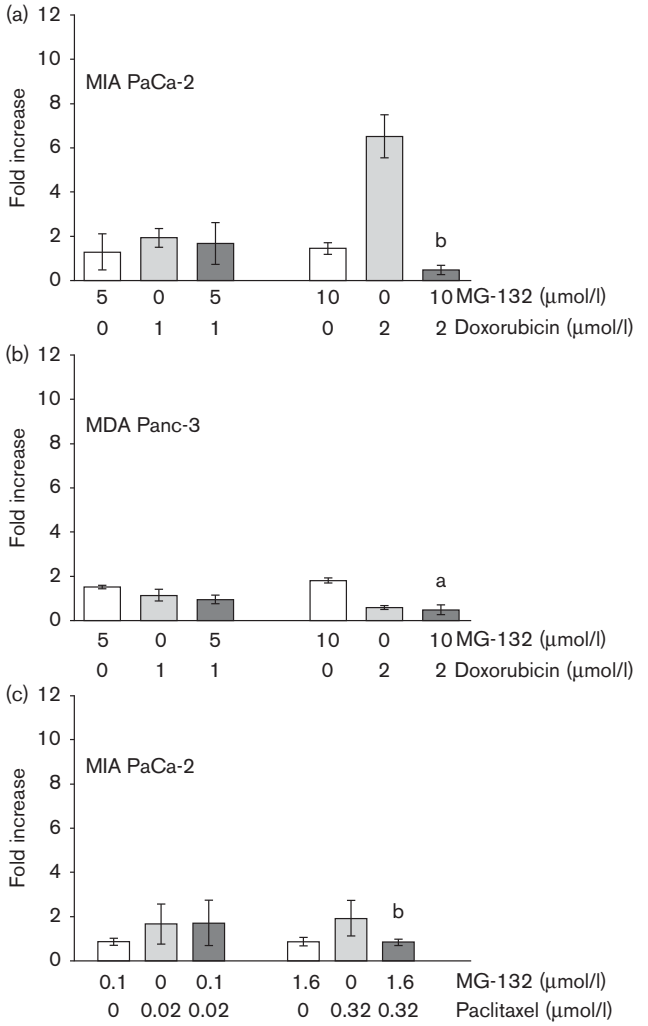


Enhanced apoptotic effect after MG-132 and camptothecin occurred only in MIA PaCa-2 after 16 h and a five-fold excess of MG-132. MIA PaCa-2 (a) and MDAPanc-3 (b) cells were incubated with the indicated concentrations of MG-132, camptothecin and mixtures for 16 h. In MIA PaCa-2, different ratios from 1 : 1 to 12 : 1 of MG-132 to camptothecin (c) were examined. Apoptotic cells were determined using Cell Death Detection ELISA^{Plus} and changes were expressed by the factor by which apoptosis increased when compared with untreated cells. Data represent means of at least three independent experiments ± SD. Statistical significance was expressed as follows: a: MG-132 vs. combination, b: camptothecin vs. combination; one letter (a or b) means $P<0.05$, two letters (aa or bb) $P<0.01$ and three letters (aaa) $P<0.001$.

Indeed, time-dependency experiments showed maximal effector caspase activation upon combined treatment after a 16-h incubation time compared with single treatment in the case of MG-132 and camptothecin (Fig. 5a). Although 1.25 μmol/l of MG-132 and 0.25 μmol/l of camptothecin, a combination that only showed a marginal apoptotic effect, did not enhance caspase-3/7 activation, higher concentrations (5 μmol/l of MG-132 and 1 μmol/l of camptothecin) strongly increased caspase activation compared with each agent alone (5.6-fold increase compared with no increase for MG-132 or a 2.4-fold increase for camptothecin alone; Fig. 5b). Pre-incubation with QVD-OPh, a pan-caspase inhibitor, reduced cytotoxicity in the MTT assay only upon combined treatment with MG-132 and camptothecin, although cell death was not totally blocked (Fig. 6). Neither the cytotoxicity of MG-132 nor that of camptothecin was influenced by the pan-caspase inhibitor. These results as well as the results from the apoptosis ELISA clearly suggest that the enhanced cell death of MG-132 and camptothecin results from increased apoptosis mediated by caspase-dependent apoptotic cell death.

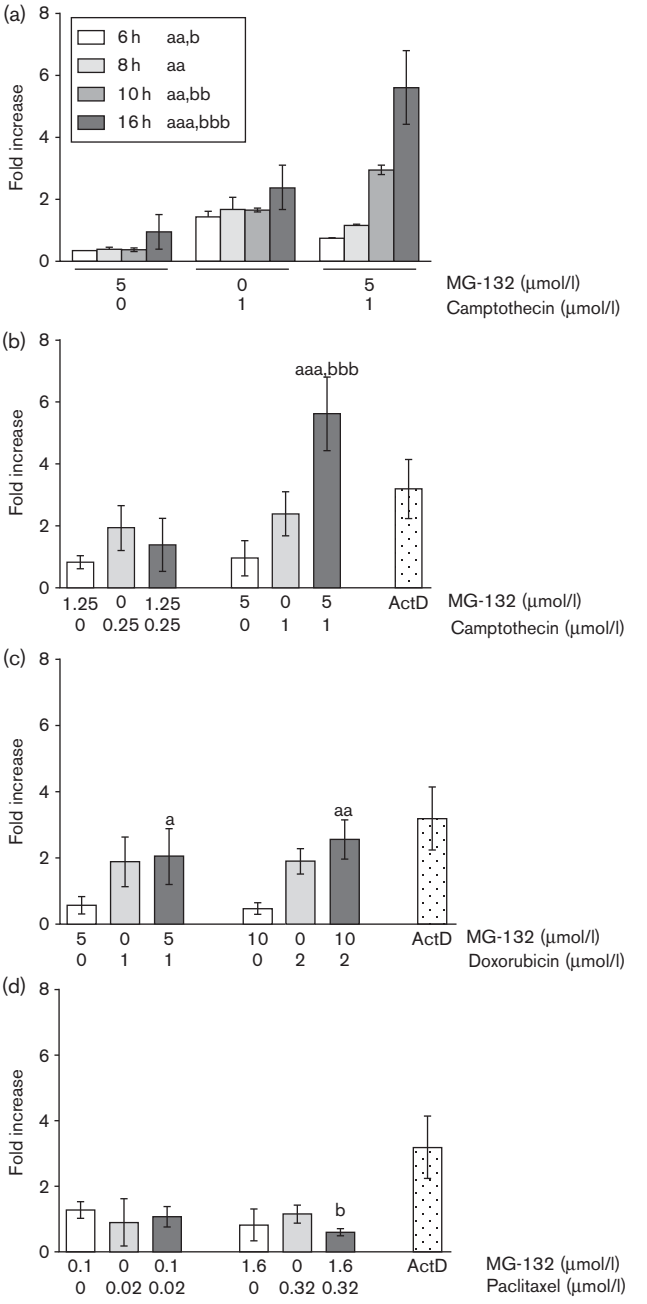
Neither the combination of MG-132 with doxorubicin (Fig. 5c) nor that of MG-132 with paclitaxel (Fig. 5d)

Fig. 4



Combining MG-132 with doxorubicin or paclitaxel does not lead to elevated apoptosis levels. MIA PaCa-2 (a) and MDA Panc-3 (b) cells were incubated with the indicated concentrations of MG-132 and doxorubicin for 16 h. Furthermore, MIA PaCa-2 cells were incubated with MG-132 and paclitaxel for 16 h (c). Apoptosis was determined using Cell Death Detection ELISA^{Plus} and expressed as fold increase compared with untreated cells. Data represent means of at least three independent experiments ± SD. Statistical significance was expressed as follows: a: MG-132 vs. combination, b: anticancer drug vs. combination; $P<0.05$.

Fig. 5



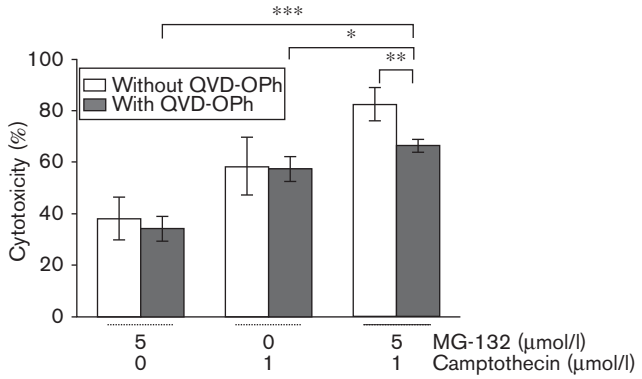
Caspase-3/-7 activity is enhanced upon treatment with MG-132/camptothecin but not with MG-132/doxorubicin or MG-132/paclitaxel in MIA PaCa-2 cells. MIA PaCa-2 cells were incubated for different times with 5 μmol/l of MG-132, 1 μmol/l of camptothecin and mixtures thereof (a). Two different concentrations of the combination MG-132 and camptothecin (1.25 μmol/l of MG-132 and 0.25 μmol/l of camptothecin; 5 μmol/l of MG-132 and 1 μmol/l of camptothecin) were used treating MIA PaCa-2 cells for 16 h (b). MIA PaCa-2 cells were incubated with MG-132, doxorubicin and combinations thereof (c) or with MG-132, paclitaxel and mixtures thereof (d) for 16 h. Cell extracts were studied in the DEVDase assay for activation of caspase-3/-7. Actinomycin D (ActD; 4 μmol/l) was used as positive control. Data represent means ± SD of at least three independent experiments. Statistical significance is indicated as follows: a: MG-132 vs. combination, b: anticancer drug vs. combination; one letter (a or b) means $P<0.05$, two letters (aa) $P<0.01$ and three letters (aaa or bbb) $P<0.001$.

caused a comparable effect on caspase activation, confirming the above-mentioned results on apoptosis levels for the particular combination of camptothecin and MG-132.

The NF-κB pathway is not involved in the enhanced cytotoxicity

It is well known that most tumour cells show a high basal NF-κB expression level [19] that contributes to chemotherapy resistance. Therefore, blocking NF-κB is an effective approach to improving chemotherapy against tumour cells. MIA PaCa-2 cells are known for their

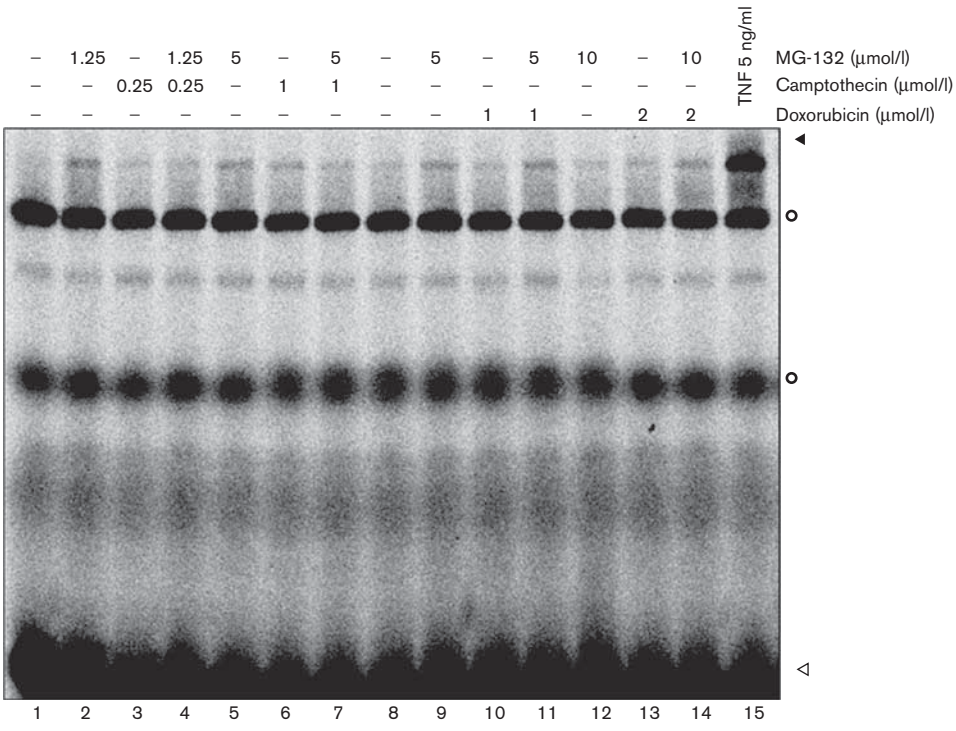
Fig. 6



Increased cytotoxic effect of combined MG-132 and camptothecin is caspase dependent in MIA PaCa-2 cells. MIA PaCa-2 cells were treated with 25 μmol/l of the pan-caspase inhibitor QVD-OPh 30 min before incubation with the single compounds and the combination thereof for 16 h. Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data represent means of at least three independent experiments ± SD. The *P* values are presented as follows: $*P<0.05$, $**P<0.01$, $***P<0.001$.

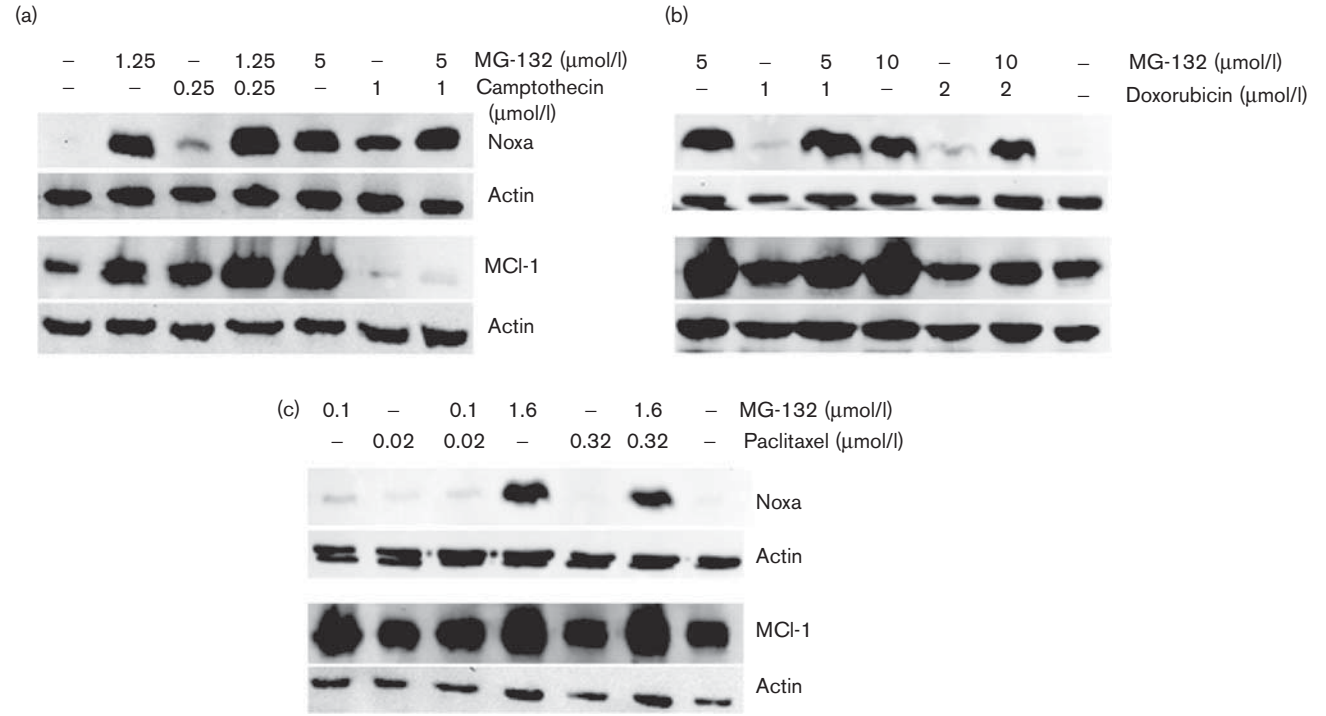
constitutive NF-κB activity [19]. To analyse whether the NF-κB pathway is involved in our experimental setting, NF-κB DNA binding activity was determined under various conditions by EMSA studies. Cells were left untreated or stimulated with TNF-α (5 ng/ml) for 30 min, and NF-κB DNA binding was determined by EMSA. No NF-κB activity was obtained in nonstimulated cells (Fig. 7, lane 1) but could be induced upon TNF-α stimulation (Fig. 7, lane 15). To determine whether NF-κB was activated upon camptothecin treatment and whether this could be prevented by MG-132, MIA PaCa-2 cells were incubated at the indicated concentrations for 3 h (Fig. 7). Only a very slight induction of NF-κB activity was observed upon camptothecin, doxorubicin or MG-132 treatment, indicating that the enhancing effect could probably not be explained by inhibition of NF-κB activity by MG-132. Taken together, these results indicate that, in contrast to previous reports, the pancreatic cell line MIA PaCa-2 does not reveal constitutively active NF-κB, nor is this pathway involved in the increased apoptotic response after treatment with MG-132, camptothecin or doxorubicin.

Fig. 7



Nuclear factor (NF-κB) is not constitutively expressed in MIA PaCa-2 and not significantly activated after treatment with camptothecin or doxorubicin. Lane 1 shows unstimulated control cells, lane 15 shows cells treated with 5 ng/ml of TNF-α for 30 min In the other lanes, cells were treated for 3 h with the indicated concentrations of the compounds and compound combinations. Equal amounts of protein from nuclear cell extracts were analysed for NF-κB DNA binding activity in an electrophoretic mobility shift assay (EMSA). ◀ NF-κB-DNA-complex, ○ unspecific activity binding to the probe, ◁ unbound oligonucleotide. The EMSA results shown are representative of two independent experiments.

Fig. 8



Noxa/Mcl-1 balance decides the cytotoxic response in MIA PaCa-2 cells. Anti-Noxa and anti-Mcl-1 western blot analyses of total cell extracts of MIA PaCa-2 after 16 h of incubation with MG-132 and camptothecin (a), MG-132 and doxorubicin (b) or MG-132 and paclitaxel (c) were performed. One representative experiment from two independent experiments is shown and actin was used as the loading control.

Noxa/Mcl-1 balance plays a key role in the MG-132-mediated enhancing cytotoxic effect towards camptothecin

Noxa is an important player in camptothecin-mediated apoptosis, and the Noxa/Mcl-1 ratio correlates with the amount of apoptosis induction [33]. To further investigate whether these two Bcl-2 family member proteins are also involved in the observed apoptotic effect of combined MG-132 and camptothecin treatment, both proteins were monitored using western blotting. MIA PaCa-2 cells were incubated with the indicated concentrations of MG-132 and camptothecin or doxorubicin and paclitaxel, respectively, at a ratio of 5 : 1. As shown in Fig. 8, MG-132 treatment increased the Noxa protein levels, presumably by inhibiting its degradation by the proteasome. Camptothecin, known to induce Noxa protein expression [33], also increased Noxa levels in MIA PaCa-2 cells in a dose-dependent manner, but to a lesser extent. Combining both drugs, the Noxa protein level was influenced in a very similar range as by MG-132 alone. Hence, the enhanced cytotoxic effect cannot be explained by elevated Noxa level alone. In addition, combined treatment with 5 μmol/l of MG-132/1 μmol/l of camptothecin and 1.25 μmol/l of MG-132/0.25 μmol/l of camptothecin gave approximately the same level of Noxa expression, but only the higher concentrations resulted in enhanced cytotoxicity. In a next step, Mcl-1 levels were investigated. Stimulation with either MG-132 (1.25 and 5 μmol/l) or camptothecin (0.25 μmol/l) alone resulted in upregulation of Mcl-1, whereas 1 μmol/l of camptothecin diminished Mcl-1 levels below the level of untreated cells. This result was also obtained with the combination that resulted in enhanced apoptosis, that is, 5 μmol/l of MG-132 and 1 μmol/l of camptothecin, but not with the lower concentrations of MG-132/camptothecin of 1.25 μmol/l/ 0.25 μmol/l (Fig. 8a). Consequently, it can be concluded that increased Noxa expression and decreased Mcl-1 expression upon 5 μmol/l of MG-132 and 1 μmol/l of camptothecin treatment results in a specific ratio of Noxa to Mcl-1 and finally leads to enhanced cytotoxicity because of increased apoptosis. To substantiate this theory, Noxa and Mcl-1 levels were also determined after MG-132 and doxorubicin treatment (demonstrated in Fig. 8b), a combination that had shown no increased apoptosis. As expected, Noxa levels were strongly induced by MG-132 and its combination with doxorubicin but only marginally upon doxorubicin treatment alone. Of note, Mcl-1 levels increased after treatment with MG-132 and doxorubicin at both concentrations. Consequently, the ratio between Noxa and Mcl-1 was shifted towards the antiapoptotic protein Mcl-1, and concomitantly no apoptosis induction occurred. Similar results were obtained after combined treatment of MIA PaCa-2 cells with MG-132 and paclitaxel (Fig. 8c).

In this combination, the concentrations of paclitaxel and the low concentration of MG-132 (0.1 μmol/l) did not show any effect on Noxa expression, whereas the higher concentration of MG-132 (1.6 μmol/l) and the combination of both increased Noxa expression. Mcl-1 was increased on all treatments, the largest increase being observed in the combination of paclitaxel with MG-132, as well as MG-132 alone. Hence, the level of the antiapoptotic protein predominated.

In addition, the two other pancreatic cancer cell lines AsPC-1 and MDAPanc-3 were treated with MG-132, camptothecin and the combination thereof under the same conditions as described for MIA PaCa-2 cells. In AsPC-1 cells, Noxa levels increased and those for Mcl-1 decreased upon treatment with camptothecin or the combination, but Mcl-1 did not decrease as much as observed in MIA PaCa-2 cells (Supporting information Fig. S2A). In MDAPanc-3 cells, Mcl-1 levels did not decrease, and Noxa and Mcl-1 levels were similar (data not shown, but available on request from the co-author). In summary, our results suggest that the ratio between Noxa and Mcl-1 is a critical parameter that influences the degree of cytotoxicity, but above all the induction of apoptosis, in MIA PaCa-2 cells when exposed to MG-132 in combination with camptothecin and doxorubicin.

Discussion

Because of the poor response of pancreatic cancer to conventional chemotherapy, various combination therapies with suitable modulators have been intensively investigated [2,34]. This study aimed to discover a combination that exhibits an increased cytotoxic effect compared with the single drugs and obtain insights into the underlying molecular mechanism. The anticancer agents camptothecin, doxorubicin and paclitaxel were combined individually with MG-132, a potent proteasome inhibitor that elicits an inhibition of NF-κB by preventing IκBα degradation. Three different pancreatic cancer cell lines were tested on the respective cytotoxic response. Some compounds that directly or indirectly inhibit NF-κB have already been shown to enhance cytotoxic effects against pancreatic cancer cells when studied either alone or in combination studies [15,16,23,30,35]. In our investigations, among the three tested cell lines, MIA PaCa-2 was the most promising one, showing the strongest cytotoxic effect when combining MG-132 and camptothecin at a ratio of 5 : 1 (i.e. 5 μmol/l : 1 μmol/l). The enhanced cell-killing effect resulted from apoptosis and increased caspase-3/7 activity. In contrast, the increased cytotoxicity of MG-132 and doxorubicin was induced by nonapoptotic-mediated cytotoxicity. The combination of doxorubicin and MG-132 even reduced the apoptotic effect. Similarly, a reduced apoptotic effect in the presence of doxorubicin was previously reported when monohydroxyethylrutoside and doxorubicin were combined [36].

Often, tumour cells develop intrinsic NF-κB activity, or treatment with anticancer drugs induces NF-κB activation resulting in a lack of response to chemotherapy. Interestingly, MIA PaCa-2 cells, although reported as constitutively expressing NF-κB [19], did not show any constitutive NF-κB activity, and anticancer agents did not activate NF-κB in our experiments. Accordingly, the enhanced cytotoxicity due to a sensitizing effect of apoptosis does not appear to be related to inhibition of NF-κB. This differs from other studies, in which replace a by an NF-κB-dependent mechanism was proven in human colorectal cancer cells [22,37]. We provide evidence that the decrease in protein level of Mcl-1, a member of the prosurvival Bcl-2 family, is the decisive step for the observed increased cell death. The balance between Noxa and Mcl-1 is hereby influenced in such a way that the concentration of Noxa exceeds that of Mcl-1 and results in enhanced apoptosis. The pivotal role of the Noxa/Mcl-1 balance in regulating susceptibility of cells to apoptosis has already been proven with camptothecin in HeLa cells [33]. Knockdown of antiapoptotic Mcl-1 increased and knockdown of proapoptotic Noxa decreased apoptosis induction upon camptothecin treatment in these cells, and the balance between these two proteins was a decisive factor for the apoptotic response. Mcl-1 overexpression can also account for developing chemotherapy resistance in pancreatic cancer [38] and may be a target for overcoming chemoresistance. Furthermore, it has been demonstrated that downregulation of Mcl-1 by RNA interference contributes to proteasome inhibition-induced apoptosis of myeloma cells, as shown with Bortezomib [39]. An enhanced chemosensitivity was also obtained when gemcitabine was used in pancreatic carcinoma cells stably expressing short hairpin RNA targeting Mcl-1 [40]. Interestingly, the response to Bortezomib not only depends on Mcl-1 but also on Noxa levels, which have been shown to be upregulated [39]. This is in agreement with our results, as we demonstrated elevated Noxa levels in response to MG-132 and anticancer agent treatment and varying protein levels of Mcl-1 according to the respective chemotherapeutic applied.

In our study, we observed pronounced decreased Mcl-1 levels only after combined treatment with MG-132 and camptothecin, the combination that increased apoptosis, whereas Noxa levels were increased. Therefore, we concluded that the ratio of Noxa and Mcl-1 is important for the beneficial apoptotic effect using the combination of MG-132 and camptothecin in MIA PaCa-2 cells at a drug ratio of 5 : 1. This observation is supported by the fact that upon treatment with MG-132 and doxorubicin or paclitaxel in MIA PaCa-2 cells, as well as on treatment with MG-132 and camptothecin in AsPC-1 and MDA-Panc-3 cells, no changes in the ratio of Noxa and Mcl-1 occurred, which could lead to a predominance of the antiapoptotic protein Mcl-1. Nevertheless, probably

other factors were additionally involved. Willis *et al.* [28] have shown that Bak not only interacts with Mcl-1 but also with Bcl-xL, and liberation of Bak from both Mcl-1 and Bcl-xL is required for apoptosis. Furthermore, Bcl-xL, Bax, Bcl-2 and Mcl-1 have been shown to be overexpressed in MIA PaCa-2 [38]. A sensitizing effect upon blockade of Bcl-xL and Bcl-2 has already been demonstrated with the combination of antimycin A3 and the camptothecin derivative SN-38 in a colon cancer cell line [41]. Interestingly, the increased growth inhibitory effect only occurred after treatment with camptothecin or its derivative, but not with doxorubicin. It was also reported that the combination of camptothecin-11 or Bortezomib, both of which lead to Noxa upregulation, with the small molecule inhibitor of Bcl-2/Bcl-xL, ABT-737, resulted in synergistic cytotoxicity and caspase activation in a human colorectal cancer cell line [42]. In addition, the combination of etoposide and ABT-737 sensitized human renal cell carcinoma cells, inducing apoptosis in which Noxa-dependent inactivation of Mcl-1 or A1 was involved [43].

The tumour suppressor protein p53 is another important substrate for proteasomal degradation. Mutations in p53 and loss of function are one of the most frequent genetic alterations in human tumours. Activated p53 leads to cell cycle arrest and promotes apoptosis. MIA PaCa-2 has been shown to contain mutant p53 [44]. Knockdown of mutant p53 has turned out to sensitize cells to anticancer drugs and to inhibit cell proliferation [45]. Of note, Lauricella *et al.* [32] and Cusack *et al.* [22] have shown that combining a proteasome inhibitor with camptothecin or a camptothecin derivative resulted in upregulation and stabilization of p53. It would be interesting to determine whether the p53 pathway is also involved in MG-132 and camptothecin-induced or doxorubicin-induced apoptosis in MIA PaCa-2 cells.

In summary, we have obtained the first valuable insights that a combination therapy of the proteasome inhibitor MG-132 with camptothecin at a fixed ratio, but not with doxorubicin, induces a synergistic apoptotic response in a pancreatic cell line. This may also have beneficial effects in the treatment of pancreatic cancer, which has, however, to be proven *in vivo*. Moreover, our results indicate that the ratio between Noxa and Mcl-1 is a pivotal factor that influences the efficacy of the combination of MG-132 with different anticancer agents. Consequently, these proteins might serve as possible biomarkers to predict the response to combined treatment strategies in pancreatic cancer.

Acknowledgements

Dr Mildred Scheel Stiftung der Deutschen Krebshilfe (German Cancer Aid).

Conflicts of interest

There are no conflicts of interest.

References

1 Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010; **60**:277–300.
2 Cascinu S, Verdecchia L, Valeri N, Berardi R, Scartozzi M. New target therapies in advanced pancreatic cancer. *Ann Oncol* 2006; **17 (Suppl 5):** v148–v152.
3 Rothenberg ML. Irinotecan (CPT-11): recent developments and future directions – colorectal cancer and beyond. *Oncologist* 2001; **6**:66–80.
4 Armand JP, Terret C, Couteau C, Rixe O. CPT-11: the European experience. *Ann N Y Acad Sci* 1996; **803**:282–291.
5 Woodward EJ, Twelves C. Scheduling of taxanes: a review. *Curr Clin Pharmacol* 2010; **5**:226–231.
6 Geffen DB, Man S. New drugs for the treatment of cancer, 1990–2001. *Isr Med Assoc J* 2002; **4**:1124–1131.
7 Desai SD, Li TK, Rodriguez-Bauman A, Rubin EH, Liu LF. Ubiquitin/26S proteasome-mediated degradation of topoisomerase I as a resistance mechanism to camptothecin in tumor cells. *Cancer Res* 2001; **61**: 5926–5932.
8 Li QY, Zu YG, Shi RZ, Yao LP. Review camptothecin: current perspectives. *Curr Med Chem* 2006; **13**:2021–2039.
9 Liu LF, Desai SD, Li TK, Mao Y, Sun M, Sim SP. Mechanism of action of camptothecin. *Ann N Y Acad Sci* 2000; **922**:1–10.
10 Sordet O, Khan QA, Kohn KW, Pommier Y. Apoptosis induced by topoisomerase inhibitors. *Curr Med Chem Anticancer Agents* 2003; **3**: 271–290.
11 Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol* 1999; **57**:727–741.
12 Ganansia-Leymarie V, Bischoff P, Bergerat JP, Holl V. Signal transduction pathways of taxanes-induced apoptosis. *Curr Med Chem Anticancer Agents* 2003; **3**:291–306.
13 Huang TT, Wuerzberger-Davis SM, Seufzer BJ, Shumway SD, Kurama T, Boothman DA, *et al.* NF-kappaB activation by camptothecin: a linkage between nuclear DNA damage and cytoplasmic signaling events. *J Biol Chem* 2000; **275**:9501–9509.
14 Wang W, Cassidy J, O'Brien V, Ryan KM, Collie-Duguid E. Mechanistic and predictive profiling of 5-fluorouracil resistance in human cancer cells. *Cancer Res* 2004; **64**:8167–8176.
15 Arlt A, Vorndamm J, Breitenbroich M, Folsch UR, Kalthoff H, Schmidt WE, *et al.* Inhibition of NF-kappaB sensitizes human pancreatic carcinoma cells to apoptosis induced by etoposide (VP16) or doxorubicin. *Oncogene* 2001; **20**:859–868.
16 Dong QG, Sclabas GM, Fujioka S, Schmidt C, Peng B, Wu T, *et al.* The function of multiple IkappaB: NF-kappaB complexes in the resistance of cancer cells to taxol-induced apoptosis. *Oncogene* 2002; **21**:6510–6519.
17 Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* 2000; **18**:621–663.
18 Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol* 2002; **3**:221–227.
19 Wang W, Abbruzzese JL, Evans DB, Larry L, Cleary KR, Chiao PJ. The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. *Clin Cancer Res* 1999; **5**: 119–127.
20 Patel NM, Nozaki S, Shortle NH, Bhat-Nakshatri P, Newton TR, Rice S, *et al.* Paclitaxel sensitivity of breast cancer cells with constitutively active NF-kappaB is enhanced by IkappaBalpha super-repressor and parthenolide. *Oncogene* 2000; **19**:4159–4169.
21 Rajkumar SV, Richardson PG, Hideshima T, Anderson KC. Proteasome inhibition as a novel therapeutic target in human cancer. *J Clin Oncol* 2005; **23**:630–639.
22 Cusack JC Jr, Liu R, Houston M, Abendroth K, Elliott PJ, Adams J, *et al.* Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: implications for systemic nuclear factor-kappaB inhibition. *Cancer Res* 2001; **61**:3535–3540.
23 Bold RJ, Virudachalam S, McConkey DJ. Chemosensitization of pancreatic cancer by inhibition of the 26S proteasome. *J Surg Res* 2001; **100**:11–17.
24 Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002; **2**:420–430.
25 Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008; **9**:47–59.
26 Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, *et al.* Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005; **17**:393–403.
27 Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000; **288**:1053–1058.

28 Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, *et al*. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* 2005; **19**:1294–1305.

29 Nencioni A, Hua F, Dillon CP, Yokoo R, Scheiermann C, Cardone MH, *et al*. Evidence for a protective role of Mcl-1 in proteasome inhibitor-induced apoptosis. *Blood* 2005; **105**:3255–3262.

30 Matsuo Y, Sawai H, Ochi N, Yasuda A, Sakamoto M, Takahashi H, *et al*. Proteasome inhibitor MG132 inhibits angiogenesis in pancreatic cancer by blocking NF-kappaB activity. *Dig Dis Sci* 2010; **55**:1167–1176.

31 Schmidt C, Fronza M, Goettert M, Geller F, Luik S, Flores EM, *et al*. Biological studies on Brazilian plants used in wound healing. *J Ethnopharmacol* 2009; **122**:523–532.

32 Lauricella M, Calvaruso G, Giuliano M, Carabillo M, Emanuele S, Vento R, *et al*. Synergistic cytotoxic interactions between sodium butyrate, MG132 and camptothecin in human retinoblastoma Y79 cells. *Tumour Biol* 2000; **21**:337–348.

33 Mei Y, Xie C, Xie W, Tian X, Li M, Wu M. Noxa/Mcl-1 balance regulates susceptibility of cells to camptothecin-induced apoptosis. *Neoplasia* 2007; **9**:871–881.

34 Holcomb B, Yip-Schneider M, Schmidt CM. The role of nuclear factor kappaB in pancreatic cancer and the clinical applications of targeted therapy. *Pancreas* 2008; **36**:225–235.

35 Shah SA, Potter MW, McDade TP, Ricciardi R, Perugini RA, Elliott PJ, *et al*. 26S proteasome inhibition induces apoptosis and limits growth of human pancreatic cancer. *J Cell Biochem* 2001; **82**:110–122.

36 Bruynzeel AM, bou El Hassan MA, Torun E, Bast A, Van d V, Kruyt FA. Caspase-dependent and -independent suppression of apoptosis by monoHER in doxorubicin treated cells. *Br J Cancer* 2007; **96**:450–456.

37 Guo J, Verma UN, Gaynor RB, Frenkel EP, Becerra CR. Enhanced chemosensitivity to irinotecan by RNA interference-mediated down-regulation of the nuclear factor-kappaB p65 subunit. *Clin Cancer Res* 2004; **10**:3333–3341.

38 Miyamoto Y, Hosotani R, Wada M, Lee JU, Koshiba T, Fujimoto K, *et al*. Immunohistochemical analysis of Bcl-2, Bax, Bcl-X, and Mcl-1 expression in pancreatic cancers. *Oncology* 1999; **56**:73–82.

39 Podar K, Gouill SL, Zhang J, Opferman JT, Zorn E, Tai YT, *et al*. A pivotal role for Mcl-1 in Bortezomib-induced apoptosis. *Oncogene* 2008; **27**: 721–731.

40 Wei SH, Dong K, Lin F, Wang X, Li B, Shen JJ, *et al*. Inducing apoptosis and enhancing chemosensitivity to gemcitabine via RNA interference targeting Mcl-1 gene in pancreatic carcinoma cell. *Cancer Chemother Pharmacol* 2008; **62**:1055–1064.

41 Guichard SM, Hua ML, Kang P, Macpherson JS, Jodrell DI. Short hairpin RNAs targeting Bcl-xL modulate senescence and apoptosis following SN-38 and irinotecan exposure in a colon cancer model. *Cancer Chemother Pharmacol* 2007; **60**:651–660.

42 Okumura K, Huang S, Sinicrope FA. Induction of Noxa sensitizes human colorectal cancer cells expressing Mcl-1 to the small-molecule Bcl-2/Bcl-xL inhibitor, ABT-737. *Clin Cancer Res* 2008; **14**:8132–8142.

43 Zall H, Weber A, Besch R, Zantl N, Hacker G. Chemotherapeutic drugs sensitize human renal cell carcinoma cells to ABT-737 by a mechanism involving the Noxa-dependent inactivation of Mcl-1 or A1. *Mol Cancer* 2010; **9**:164.

44 Yan W, Liu G, Scoumanne A, Chen X. Suppression of inhibitor of differentiation 2, a target of mutant p53, is required for gain-of-function mutations. *Cancer Res* 2008; **68**:6789–6796.

45 Bossi G, Lapi E, Strano S, Rinaldo C, Blandino G, Sacchi A. Mutant p53 gain of function: reduction of tumor malignancy of human cancer cell lines through abrogation of mutant p53 expression. *Oncogene* 2006; **25**: 304–309.