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DUAL INHIBITION OF TOPOISOMERASES ENHANCES APOPTOSIS IN MELANOMA CELLS

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The cytotoxicity of topoisomerase I inhibiting camptothecin, topoisomerase II inhibiting etoposide and their combination were investigated in wild type p53 Bowes and mutant p53 SK-MEL-28 melanoma cell lines during 24h. A combination of camptothecin and etoposide (1 μg/ml + 10 μg/ml) proved to be efficient in both types of cell lines, although mutant p53 cells exhibited a higher resistance. Further studies proved that in Bowes cells, a combination of drugs induced p53-dependent mitochondrial apoptosis characterized by activation of caspases-8 and -2, -9 and -3 with some concurrent involvement of oxidative stress. In SK-MEL-28 cells, apoptosis was found to be mediated via increased oxidative stress, activation of stress kinases such as p38 and SAPK/JNK and mitochondrial dysfunction without significant involvement of p53 and its transactivated target genes. These results demonstrate efficiency of dual inhibition of topoisomerases in melanoma cells with functional as well as mutant p53 and point out at possible further investigation of this modality in preclinical and clinical oncology.

Key words: topoisomerases, camptothecin, etoposide, apoptosis, melanoma, mitochondria

Malignant melanoma belongs to the class of aggressive neoplastic diseases which often metastasize at early stages and show high resistance to chemotherapy. The causes of this resistance are not entirely understood; however, they include upregulated cell survival and proliferation signaling such as Ras/Raf/MEK/ERK pathway, increased activity of specific drug efflux mechanisms, inactivation of senescence-regulating elements; i.e. $p16^{IKN4a}$/Rb (retinoblastoma protein) [1] and defects in cell death-apoptosis regulating cascades [2].

In particular, defective proapoptic signaling and failure to execute cell death seems to be of critical importance in malignant melanocytes since most cytostatic drugs exert their activity by inducing apoptosis. Previous studies carried out both on fresh melanoma isolates or established melanoma cell lines revealed that in advanced melanoma stochastic mutations or epigenetic silencing affect both external as well as internal apoptic pathways [3]. External or death receptor-initiated pathway has been reported by some research groups to be downregulated or specifically blocked [4]. In mitochondrial pathway, several biochemical mediators have been found to be overexpressed in melanoma cells such as antiapoptic Bcl-2 and related proteins [5] or inhibitors of apoptosis including survivin, livin, c-IAP, XIAP and Mcl-1 [6]. Conversely, proapoptic regulatory molecules such as Bax or Apaf-1 have been found suppressed or deleted in melanoma [7, 8]. In addition, although p53, which plays a critical role in regulation of apoptosis, is typically wild-type in melanoma cells, several research groups reported its defective activation and signaling along with other aberrant mechanisms which are responsible for resistance of malignant melanoma to DNA-damage induced apoptosis [9, 10, 11].

DNA topoisomerase inhibitors which target topoisomerase I, topoisomerase IIα and topoisomerase IIβ are among the most effective and widely used anticancer drugs in the clinical practice where they are employed in the treatment regimens of many solid tumors as well as hematological malignancies [12, 13]. Although generally efficient in inducing apoptosis mainly via promoting DNA strand breaks and activating p53-dependent mitochondrial apoptosis, their efficiency even in combination with other cytotoxic drugs in case of advanced melanoma remains very limited. Besides their pharmacology, the main reasons of this failure seem to be the decreased expression and activity of topoisomerases [14], perturbations in p53-mediated response to DNA damage as well the aberrant intrinsic apoptotic pathway signaling [15]. Still, antineoplastic activity of these drugs is rather complex and as suggested by

some reports; it appears that they may activate several parallel pathways leading to cell cycle arrest and apoptosis [13, 16]. This multivariate effect on cell signaling may be especially true in case of a combined inhibition of both topoisomerase I and II in melanoma cells.

To address this issue, we wanted to explore in detail cytotoxicity and proapoptic mechanisms of combined administration of topoisomerase I inhibiting camptothecin (CPT) and topoisomerase II inhibiting etoposide (ETO) in wild-type p53 melanoma cell line Bowes and in mutant p53 melanoma cell line SK-MEL-28 [17] while paying close attention to changes in intracellular signaling and mechanisms behind initiation and execution of apoptosis.

Materials and methods

Chemicals. Etoposide; camptothecin; Triton-X; 2´-7´- dichlorodihydrofluorescein diacetate, JC-1, 3-[(3-ch olamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS); dithiotreitol (DTT), dimethylsulfoxide (DMSO), propidium iodide, N-acetylcysteine (NAC), JNK-specific inhibitor SP600125 and β-actin were purchased from Sigma-Aldrich (Prague, Czech Republic). Primary antibodies against p53, cleaved Bid, Bax, Bcl-2 and Bcl-XL were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Secondary antibodies were from Alexis Corporation (Lausen, Switzerland). p53-specific inhibitor Pifithrin and p38-specific inhibitor SB 203580 were acquired from Calbiochem (EMD Biosciences, Inc., La Jolla, Ca, USA). Caspase-8 inhibitor was from ICN Biomedicals Inc. (Irvine, USA). All other chemicals were of highest analytical grade.

Cell culture and treatment. Wild-type p53 melanoma cell line Bowes (ATCC, No. CRL – 9607, Manassas, United States) and mutant p53 melanoma cell line SK-MEL-28 (ATCC,, No. HTB-72, Manassas, United States) were cultivated in DMEM (Sigma-Aldrich, Prague, Czech Republic) with 10 % fetal bovine serum (Gibco, Czech Republic), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were kept in an incubator at 37 °C and 5 % CO_2 atmosphere and were passaged two times a week using 0.05 % trypsin/EDTA upon reaching 90% confluence. Only mycoplasma-free cells were used for experiments.

CPT and ETO were dissolved in DMSO to the final stock concentration of 10 mM. The working concentrations of both antineoplastics were achieved by diluting the stock solution in treatment medium. The working concentrations of employed pharmacological inhibitors were achieved by diluting their stock solutions in treatment medium and were as follows: NAC (antioxidant, 1 mM – added to cells 24h prior to topoisomerase inhibitors exposure), Pifithrin (p53 inhibitor, 30 μM – added to cells 24h prior to CPT+ETO treatment), SP600125 (JNK inhibitor, 10 μM - supplemented to cells 30 min before exposure to CPT+ETO), SB203580 (p38 inhibitor, 10 μM - supplemented to cells 30 min before exposure to CPT+ETO) and z-IETD-fmk (caspase-8 inhibitor, 10 μM - added to cells simultaneously with CPT+ETO).

Cell proliferation - DNA synthesis. The rate of DNA synthesis in treated and control cells was estimated by means of measurement of bromodeoxyuridine (BrdU) incorporation into growing DNA strands using Cell proliferation ELISA BrdU kit (Roche s.r.o, Prague, Czech Republic). Cells at a concentration of 6,000 cells/well in 200 μl of DMEM containing 10 % fetal bovine serum were seeded in black 96-well microtiter plates, with the first column of wells representing blank. The cells were allowed to settle overnight at 37 °C and in 5 % CO_2. Next, cultures were treated according to protocol supplied by manufacturer and 2-24h before the end of incubation BrdU was added (10 μM final concentration, Sigma-Aldrich, Prague, Czech Republic). At the end of incubation period, medium was removed and the cells were fixed (30 min, RT) and labeled with anti-BrdU. After incubation (60 min, RT) and three washing cycles, luminol substrate was added and chemiluminescence was measured using a multiplate reader TECAN SpectraFluor Plus (TECAN Austria GmbH, Grödig, Austria). For all measurement, integration time was 1000 ms and gain 130. The results in relative light units (RLU) were expressed as a percentage of control.

Detection of apoptosis. Apoptosis in melanoma cells treated with CPT, ETO or their combination was quantitated using propidium iodide method. Cells were harvested with 0.1% trypsin, rinsed with PBS (5 min) and fixed in 70% ethanol at 4°C for 24h. After rinsing, cells were resuspended in a solution containing 0.1% Triton X-100, 50 μg/ml RNaseA and 0.5 μg/ml propidium iodide. Following the incubation (30 min, 25°C, dark room), the proportion of apoptotic cells with hypodiploid DNA in the examined cell samples was determined using a flow cytometer Cell Lab Quanta™ SC (Beckman Coulter Inc. Brea, CA, USA).

Measurement of oxidative stress. Generation of hydrogen peroxide and/or hydroxyl radical was monitored by intracellular conversion of 2´-7´- dichlorodihydrofluorescein diacetate (DFCH/DA – Sigma-Aldrich, Prague, Czech Republic) into a fluorescent product dichlorofluorescein (DCF). Melanoma cells were seeded into cultivation flasks and cultivated to 75% confluence at 37 °C and 5 % CO_2. After exposure to the tested chemicals control and treated cells were detached by a cell scraper and collected by centrifugation (50 x g, 5 min, 4 °C – JOUAN M21, Trigon, Prague, Czech Republic). The cells were resuspended in DMEM (pH adjusted to 7.2) and 5 μM DFCH/DA was added (5 min, 37 °C). Changes in the fluorescence intensity (485 nm excitation; 538 nm emission) were measured by Shimadzu UV - Visible Spectrophotometer UV – 1601 (SHIMADZU DEUTSCHLAND GmbH, Duisburg, Germany). The data were expressed as a percentage of fluorescence intensity increase per 10^6 cells.

Measurement of DNA damage – comet assay. DNA damage in treated cells was determined by alkali single-cell gel electrophoresis (comet assay). This assay is based on analysis of labile DNA damage sites where DNA forms characteristic tails – comets. Control and treated melanoma cells were at particular time intervals harvested by 0.25% trypsin, centrifuged

for 5 min at 1,500 rpm at 4 °C, suspended in 0.6% agarose and mounted to microscopic slides. Next, cells were lysed in cooled lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma, 1 % Triton X-100 and 10 % DMSO) for 1.5 h at 4 °C. After rinsing in TRIS, cells were allowed to unwind DNA in alkali buffer for 30 min. Electrophoresis was performed at 25 V and 300 mA for 30 min. After neutralizing slides and draining them, cells were stained with 100 µl ethidium bromide (Sigma-Aldrich, Prague, Czech Republic). One hundred cells from three independent samples were scored for tail migration intensity.

ELISA assay of p38 and JNK activities. Treated and control cells were harvested and collected by centrifugation. Whole cell extracts were prepared and p38 and stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) activities were measured using ELISA kits (Sigma-Aldrich, St. Luis, MO, USA and Calbiochem, San Diego, CA, USA) specific for total and phospho-p38 ($pTpY^{180/182}$) and total and phospho-JNK ($pTpY^{183/183}$) according to manufacturer's instructions. The assays were performed in 96-well plate format and samples were read against standard curves obtained from p38 and phospho-p38 and JNK and phospho-JNK standards. Results were normalized to micrograms of protein in the cell extract and expressed as the ratio of phospho to total kinase in the same sample. The results of were expressed as percentage of control values.

Mitochondrial transmembrane potential (Δψm) analysis. Changes in Δψm in CPT+ETO-treated melanoma cells were monitored by flow cytometry analysis of JC-1 dye staining using a flow cytometer (Cell Lab Quanta™ SC - Beckman Coulter Inc. Brea, CA, USA). Mitochondrial transmembrane potential changes were indicated as an increase in fluorescence intensity at 528 nm and data were expressed as a percentage of control.

Caspase activity assays. Combination (CPT+ETO)-treated and control cultures at 6, 12, and 24h were harvested by centrifugation (600 g, 5 min, JOUAN MR 22, Trigon, Prague, Czech Republic) and lysed on ice for 20 min in a lysis buffer containing 50 mM HEPES, 5 mM CHAPS and 5 mM DTT. The lysates were centrifuged at 14,000 g, 10 min, 4°C, and the supernatants were collected and stored at -80°C. The enzyme activity was measured in a 96-well microplate using a kinetic fluorometric method based on the hydrolysis of the fluorogenic caspase-specific substrate (VDVAD-AFC for caspase-2, Ac-LEHD-AFC for caspase-9, IETD-AFC for caspase-8 and DEVD-AFC for caspase-3, 37°C, 1h) by individual caspases. Specific inhibitors of caspase-2, caspase-9, caspase-8 and caspase-3 were used to confirm the specificity of the cleavage reaction. Fluorescence was recorded at 460/40 nm after excitation at 360/40 nm using TECAN SpectraFluor Plus (TECAN Austria GmbH, Grödig, Austria). Results are shown as fold increase in activity relative to untreated cells.

Immunoblotting. Treated and control melanoma cells were harvested at different time intervals with trypsin, washed with PBS and centrifuged (1000 rpm, 5 min, 4 °C). The resulting pelet was resuspended in 5 ml of ice-cold lysis buffer (137 mM NaCl, 10 % glycerol, 1 % n-octyl-β-D-glucopyranoside, 50 mM NaF, 20 mM Tris, 1 mM sodium orthovanadate, Complete TMMini). The whole cell lysates were boiled for 5 min/95 °C in SDS sample buffer (Tris-HCl pH 6.81, 2-mercaptoethanol, 10 % glycerol, SDS, 0.1 % bromphenol blue) and thereafter they were loaded onto a 12% SDS/polyacrylamide gel. Each lysate contained equal amount of protein (30 µg) as determined by BCA assay. After electrophoresis, proteins were transferred to a PVDF membrane (100 V, 60 min) and incubated at 25 °C for 1.5h with a solution containing 5% nonfat dry milk, 10 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, and 0.1% Tween 20 (TBST). Membranes were incubated with primary antibodies (mouse anti-p53, 1:500, rabbit anti-cleaved Bid, 1: 750, rabbit anti-Bax, 1:500, rabbit anti-Bcl-2, 1: 750, rabbit anti-Bcl-XL, 1:600 and monoclonal mouse anti-β-actin, 1:100, all Cell Signaling Technology, Inc., Danvers, MA, USA) at 4 °C overnight followed by five 6 min washes in TBST. Next, the blots were incubated with secondary peroxidase-conjugated antibodies (1:1000, 1h, 25 °C), washed with TBST and the signal was developed with a chemiluminescence (ECL) detection kit (Boehringer Mannheim-Roche, Basel, Switzerland).

Statistics. Statistical analysis was carried out with a statistical program GraphPad Prism 4 (GraphPad Software, Inc. San Diego, USA). We used one-way Anova test with Dunnett's post test for multiple comparisons. Results were compared with control samples, and means were considered significant if $P<0.05$.

Results

Effect of topoisomerase inhibitors on DNA synthesis and apoptosis of melanoma cells.

CPT as well as ETO had a concentration-dependent inhibitory effect on proliferation of Bowes and SK-MEL-28 melanoma cells. In both wild-type p53 Bowes and mutant type p53 SK-Mel-28 cells, CPT proved to be more cytotoxic than ETO, and, predictably, SK-MEL-28 cells showed higher resistance to both topoisomerase inhibitors used (Fig. 1A, B). To determine whether a combination of CPT and ETO would show enhanced antiproliferative and cytotoxic effects, melanoma cells were exposed to a combination of both chemicals and their effect on DNA synthesis was measured. Fig. 1C and D shows that the most potent in both cells lines was a combination of CPT (1 µg/ml) and ETO (10 µg/ml), which inhibited cell proliferation in mutant p53 cells too. Since the cytotoxicity and inhibition of cell proliferation are often associated with induction of cell death, the rate of apoptosis was next determined in both melanoma cells lines exposed to a range of topoisomerase inhibitor combinations. As predicted, the most effective cytotoxic drug combination also proved to be the most potent apoptosis-inducer; however, it was still significantly less effective in mutant SK-MEL-28 cells (Fig. 2). To study mechanisms whereby this cytotoxic combination of topoisomerase inhibitors stimulates apoptosis and to investigate observed differences in p53 status-differing melanoma cells, further experiments were conducted.

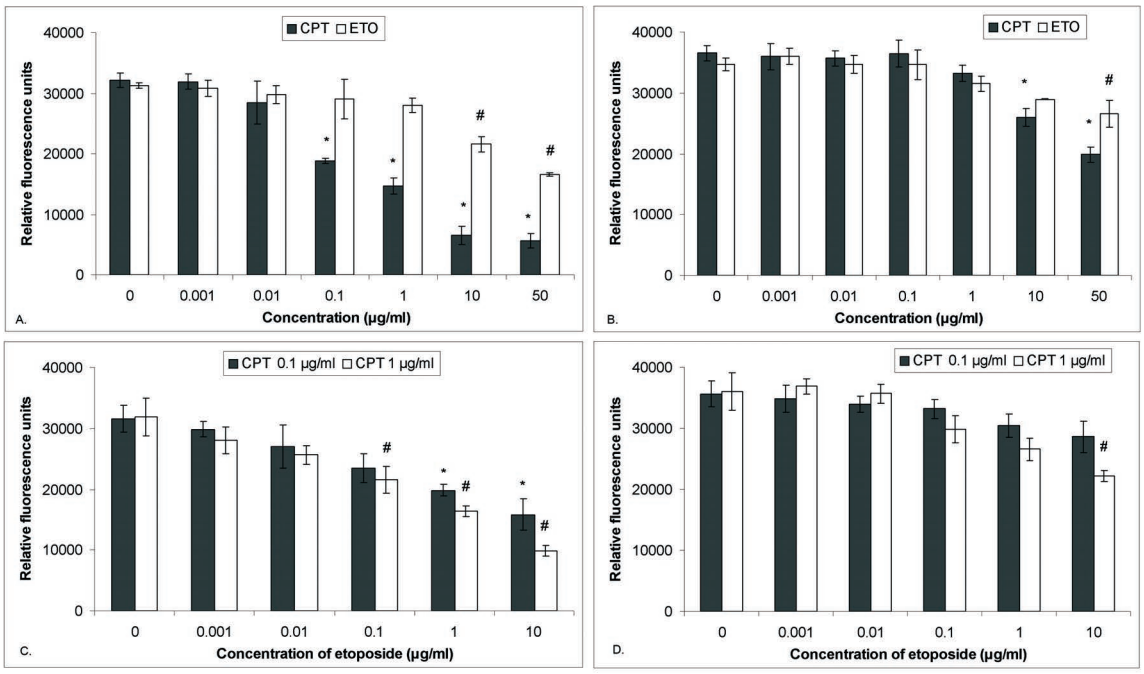


Fig. 1. Effect of campthotecin (CPT), etoposide (ETO) and their combination on DNA synthesis of human melanoma cells Bowes (wild-type p53) and SK-MEL-28 (mutant p53) during 24h as measured by Cell proliferation ELISA BrdU assay. (A and C) Bowes cells, (B and D) SK-MEL-28 cells. Data represent the mean ± SD of three different experiments. *significantly lower than Bowes control cultures; # significantly lower than SK-MEL-28 control cultures with one way-Anova test and Dunnet´s post test for multiple comparisons.

DNA damage and oxidative stress induced by a combination of topoisomerase inhibitors. A combination of CPT (1 μg/ml) and ETO (10 μg/ml) induced marked changes in DNA integrity of melanoma cells Bowes and SK-MEL-28 already at 4h of treatment. In time, DNA damage increased, reaching maximum levels at 24h of treatment, irrespective of cell line

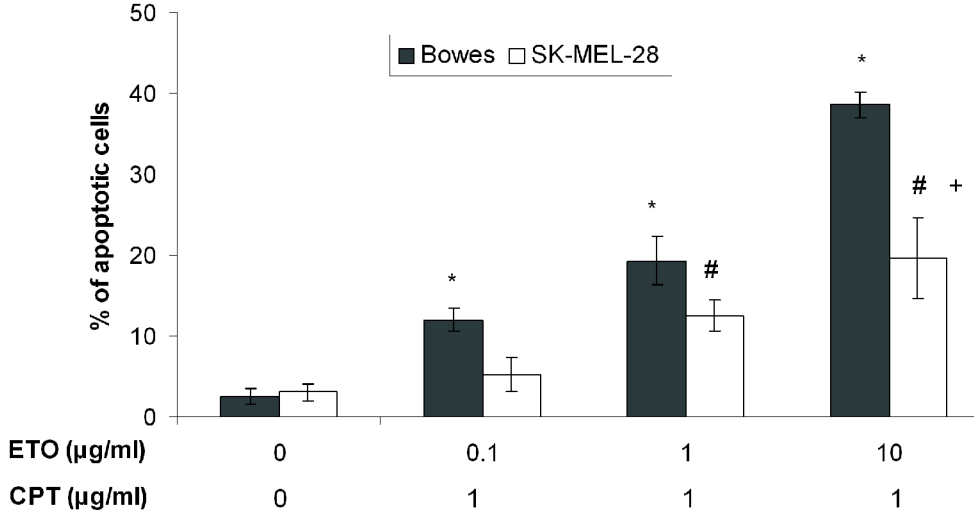
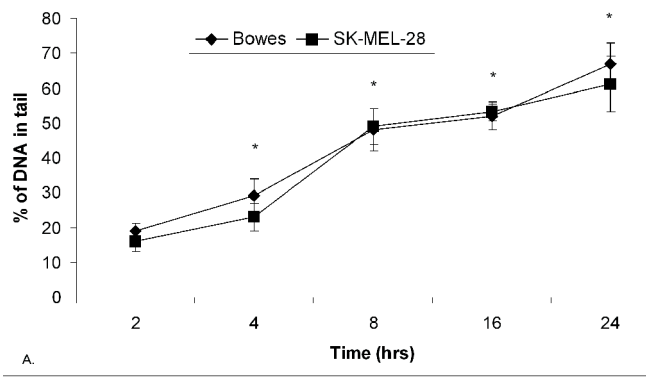
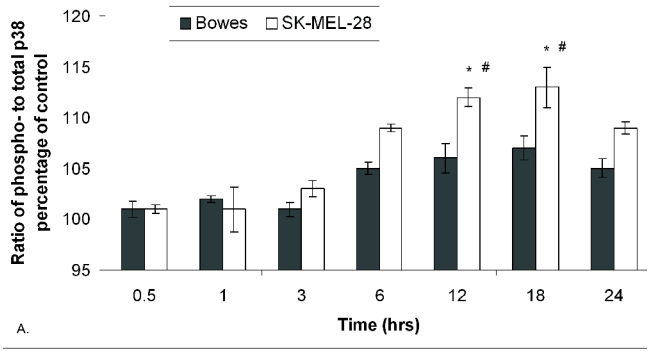
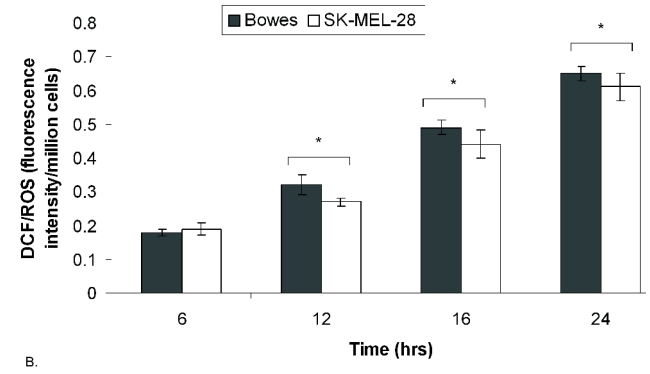


Fig. 2. Apoptosis of human melanoma cells Bowes (wild-type p53) and SK-MEL-28 (mutant p53) after a combination treatment with camptothecin (CPT) and etoposide (ETO). Control and treated cultures were at individual time intervals harvested, processed and apoptosis was measured using flow cytometry as described in Materials and methods section. Data represent the mean ± SD of three different experiments. *significantly lower than Bowes control cultures; # significantly lower than SK-MEL-28 control cultures; + significantly lower that in Bowes cells treated with the same concentrations of ETO and CPT at the same time interval with one way-Anova test and Dunnet´s post test for multiple comparisons.







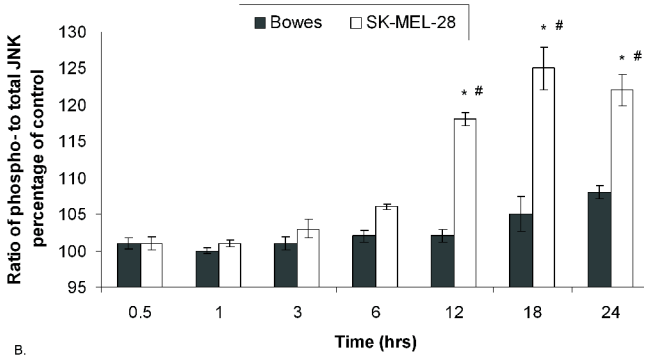


Fig. 3. DNA damage and oxidative stress in wild-type p53 Bowes and mutant p53 SK-MEL-28 melanoma cells treated with a combination of campthotecin (CPT) – 1 μg/ml and etoposide (ETO) 10 μg/ml during 24h. Data represent the mean ± SD of three different experiments. (A) DNA damage as measured by Comet assay (Materials and methods section), * significantly different than control cultures where DNA found in tail at all treatment intervals was always lower than 5% with one way-Anova test and Dunnet´s post test for multiple comparisons. (B) oxidative stress determined by flow cytometry as specified in Materials and methods section, * significantly different than control cultures where fluorescent intensity at all treatment intervals was always below 0.1 with one way-Anova test and Dunnet´s post test for multiple comparisons.

Fig. 4. Time course of p38 and stress-activated protein kinase/Jun-amino-terminal kinase SAPK/JNK activation in wild-type p53 Bowes and mutant p53 SK-MEL-28 melanoma cells treated with a combination of campthotecin (CPT) – 1 μg/ml and etoposide (ETO) 10 μg/ml during 24h. Data represent the mean ± SD of three different experiments. Cells were harvested and (A) p38 activity or (B) SAPK/JNK activity were measured in cells lysates using ELISA assay (Materials and methods section).*significantly higher than SK-MEL-28 control cultures; # significantly higher than Bowes cultures at the same treatment interval with one way-Anova test and Dunnet´s post test for multiple comparisons.

studied (Fig. 3A). Time-course studies of oxidative stress in melanoma cells revealed that compared to DNA damaging, drug-induced generation of free oxygen species first became significant only at 12h of treatment and increased in time, again with no difference between cell lines studied (Fig. 3B).

A combination of topoisomerase inhibitors stimulates changes in stress kinases activity. Since the initiation of apoptosis in tumor cells is known to proceed via pathways involving oxidative stress and activation of the specific stress kinases, their involvement was determined in the present model during 24h of exposure. The results of these experiments show that the exposure of melanoma cells to a CPT+ETO combination (1 μg/ml +10 μg/ml) resulted in a time-dependent activation of both p38 and SAPK/JNK although their increased activity was detectable in SK-MEL-28 cells only. Conversely, no significant changes p38 and SAPK/JNK activities were recorded in Bowes cells throughout all treatment intervals although a slight elevation in their activities was observed at later treatment periods (Fig. 4A and B).

Inhibition of topoisomerases activates mitochondrial and caspase-dependent apoptosis. Concerning DNA damaging, prooxidative and stress effects of topoisomerase inhibition in melanoma cells, in the next step contribution of the established mediators of apoptosis was studied in the present model. It is shown that in wild-type p53 Bowes cells, treatment with CPT+ETO combination increased abundance of p53 as well as Bax proteins as early as at 8h of treatment. Approximately at the same time frame, Bid underwent cleavage and the expression of antiapoptic Bcl-2 and Bcl-XL proteins decreased. These changes preceded any significant loss of mitochondrial

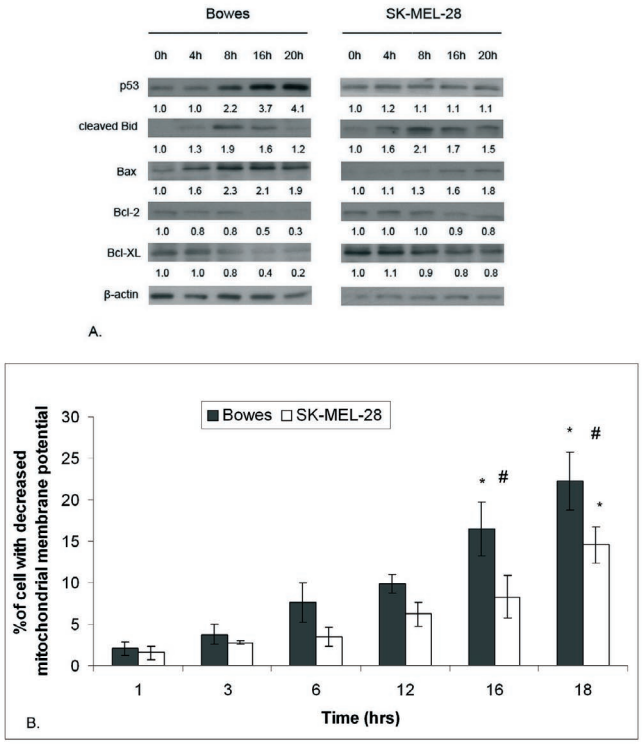


Fig. 5. Changes in expression of proapoptic and antiapoptic proteins and mitochondrial membrane potential in wild-type p53 Bowes and mutant p53 SK-MEL-28 melanoma cells treated with a combination of campthotecin (CPT) – 1 μg/ml and etoposide (ETO) 10 μg/ml during 24h. (A) Western blot analysis of p53, Bid, Bax, Bcl-2 and Bcl-XL expression in treated cells as described in Materials and methods section. Results were normalized to β-actin loading and relative quantifications of protein expression were measured using GelQuant Ver 2.7 software (DNR Bio-Imaging Systems, Jerusalem, Israel). (B) Mitochondrial membrane potential in treated cells was determined by using JC-1 staining and its quantification with help of flow cytometry (Materials and methods section). Data represent the mean ± SD of three different experiments.*significantly higher than at the beginning of treatment for each cell line; # significantly higher than SK-MEL-28 cultures at the same treatment interval with one way-Anova test and Dunnet´s post test for multiple comparisons.

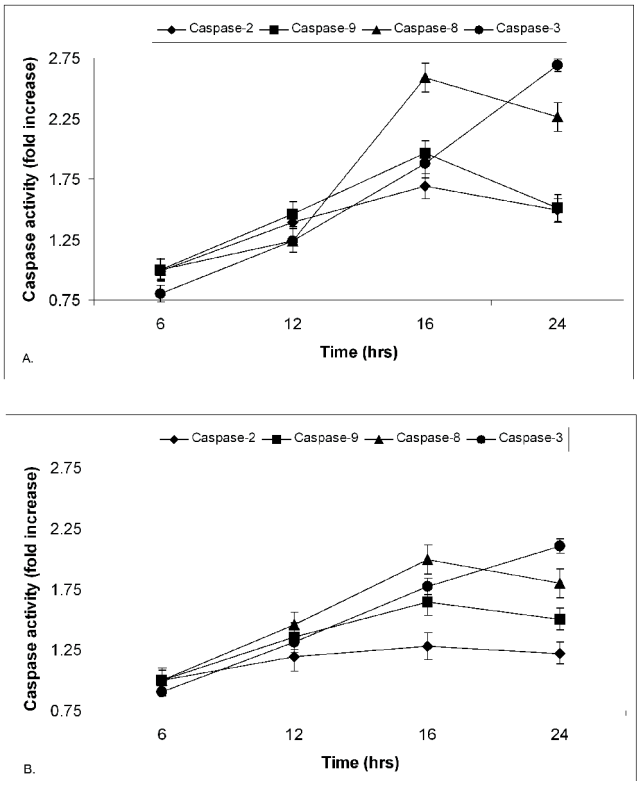


Fig. 6. The effect of a combination of campthotecin (CPT) – 1 μg/ml and etoposide (ETO) 10 μg/ml treatment on caspase-2, -9, -8, and -3 activities in (A) wild type p53 Bowes and (B) mutant p53 SK-MEL-28 melanoma cells during 24h of cultivation. Cells were lysed and caspase activities were determined in a 96-well microplate using a kinetic fluorometric method as described in Materials and methods section. Results represent means ±SD of three experiments.

membrane potential which started to decrease between 12-16h of treatment. On the other hand, in mutant p53 SK-MEL-28 cells no changes in p53 expression were observed following treatment with CPT-ETO combination. Although Bax abundance gradually increased and Bid underwent significant early cleavage, no significant drop in Bcl-2 and Bcl-XL expression was noted (Fig. 5). To correlate the observed phenomena with kinetics of activation of individual caspases in treated cells, specific activity assays of selected initiation (caspase-2, -8 and -9) and execution caspases (caspase-3) were carried out. The kinetic analyses revealed that with exception of caspase-2, which unlike in Bowes cells, did not appear to play a role in CPT+ETO-induced cell death in SK-Mel-28 cell line, all other caspases showed a substantial elevation in their activities between 12 and 16h of treatment. Still, the levels of activities of individual involved caspases were generally lower in mutant p53 SK-MEL-28 cells (Fig. 6).

Dual inhibition of topoisomerase induces cell-line specific proapoptic signaling. Since several mechanisms seemed to induce stress signaling in both melanoma cell lines after exposure to a combination of topoisomerase inhibitors, the relative contribution of these mechanisms to the observed cell demise was studied using a series of pharmacological inhibitors. In Bowes cells, only the use of p53-blocking chemical Pifithrin significantly reduced apoptosis while abrogation of oxidative stress with antioxidant NAC as well as inhibition of stress kinases p38 and JNK and caspase-8 did not succeed in marked prevention of induced cell death. Conversely, in SK-MEL-28 cells, the use of NAC significantly protected the cells against apoptosis and similarly marked protection was also achieved with inhibition of p38, JNK as well as caspase-8, suggesting the strong involvement of these mediators in topoisomerase inhibitors-stimulated cell death (Fig.7). As expected, suppression of caspase-3 activation almost completely abolished induced apoptosis in both cel lines (data not shown).

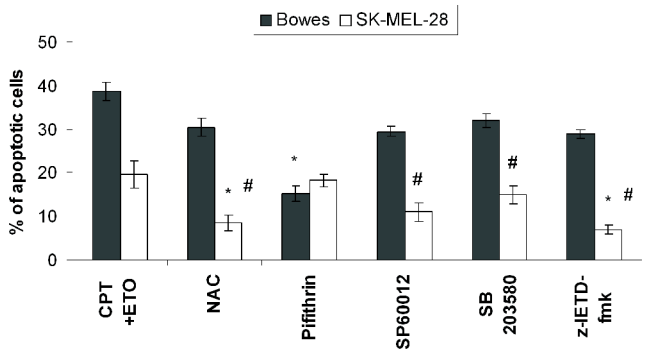


Fig. 7. Effects of inhibition of oxidative stress (N-acetylcysteine – NAC), p53 signaling (Pifithrin), activation of p38 (SB203580), activation of stress-activated protein kinase/Jun-amino-terminal kinase SAPK/JNK (SP60012) and activation of caspase-8 (caspase-8 inhibitor z-IETD-fmk) on camptothecin (CPT) - 1 μg/ml and etoposide (ETO) - 10 μg/ml-induced apoptosis in melanoma cells during 24h of treatment. Cells were preincubated or coincubated with the specified inhibitors, exposed to a combination of CPT+ETO and apoptosis was determined as described in Materials in methods section. Results represent means ± SD of at least three experiments. * significantly lower compared to cells treated with CPT+ETO only and # significantly lower compared to Bowes cultures treated with the same CPT+ETO combination and exposed to the same inhibitor with one way-Anova test and Dunnett's post test for multiple comparisons.

Discussion

Although advanced and metastasizing melanoma shows increased resistance towards chemotherapy and immunotherapy, in subsets of patients several drugs show certain efficiency [18]. To this end, topoisomerase inhibiting agents alone or in combination with other antineoplastics have been found to exert antiproliferative effects against malignant melanocytes [19, 20] despite the fact that selective resistance against them may develop in the same cells [21]. Since the expression of individual topoisomerases in melanoma may vary [14] in addition to often aberrant function of p53-dependent pathway which is mostly targeted by topoisomerase inhibitors [2], concerted suppression of topoisomerase I and II in the same cells may be perspective in enhancing cytotoxicity and proapoptic response while overcoming aforementioned obstacles.

Thus in the present work, we investigated antiproliferative and proapoptic activity of topoisomerase I inhibiting camptothecin and topoisomerase II inhibiting etoposide as single agents or in combination in wild-type p53 melanoma Bowes and mutant p53 melanoma SK-MEL-28 cells. We first demonstrated that both agents are cytotoxic in melanoma cells and their efficiency is lower in p53 mutant cells which generally agrees with earlier observations [22]. Moreover, when combined both topoisomerase inhibitors upon low concentration (10 μg/ml etoposide and 1 μg/ml camptothecin) synergized in their cytotoxicity, inducing a marked effect irrespective of p53 status in tested cells. In addition, the same combination of drugs was also shown to induce apoptosis despite the fact that its extent was significantly lower in mutant p53 cells. CPTs and etoposide have been shown to induce p53-dependent mitochondrial apoptosis, with strong involvement of caspases [19, 23, 24]. Still, p53-independent death has also been associated with topoisomerase inhibitors [25] and it appears that several other mechanisms are involved in both processes as individual agents might interfere with other stress signaling pathways and/or directly with some organelles such as mitochondria as has been demonstrated in human gastric cancer cells [26] and cervical cancer cells [27].

Inhibition of topoisomerases in tumor cells produces an essential DNA damaging and in our case we observed its early increase and time-dependency, suggesting the functionality of topoisomerases in both cell lines. Also, generation of reactive oxygen species (ROS) and resulting oxidative stress often accompanies disturbed DNA integrity in cells exposed to topoisomerase inhibitors [28, 29] and to this end our data indicating significantly elevated levels of oxidative stress in treated cells agree with this scenario. ROS may activate an entire array of stress signaling mediators including stress kinases p38 and SAPK/JNK [30, 31]. Thus in the next step the activities of these two kinases were studied. Surprisingly, in wild type p53 Bowes cells both p38 and SAPK/JNK did not seem to be activated by cytotoxic treatment contrary to mutant p53 cells where their significant activation was noted between 12 and 24h of exposure. This different activation profile of p38 and SAPK/JNK in melanoma cells points to relatively underexplored area in skin cancer treatment biology and may reflect variability in response to elevated ROS levels in malignant melanocytes due to varying tolerance to oxidative stress or defective signaling circuits [32]. On the other hand, the relationship between p53 status and individual kinase activities in treated melanoma cells may be more complex as there are reports indicating activation of ROS-p38/SAPK/JNK axis in wild type p53 cells only [33] as well as those documenting no difference between cells with varying p53 status [34].

p53 protein is an important transcription factor, acting in treated tumor cells as a stress responder and apoptosis regulator, with its activity being also related to ROS [35]. In malignant melanoma, p53 gene is relatively rarely mutated although its activation is often aberrant [10]. This is clearly seen from our data showing an increased expression of wild type p53 protein and concomitantly changed abundance of Bax, Bcl-2 and Bcl-XL proteins along with an early cleaved Bid following the treatment with topoisomerase inhibitors. Conversely, mutant p53 protein did not increase after the same treatment and besides cleaved Bid, other p53-transactivated proteins did show delayed and less effective response. Here it may be hypothesized that elevated ROS levels might acted as an adjunct to p53-dependent signaling in wild-type p53 Bowes cells whereas in mutant p53 SK-MEL-28 cells their role was p53-response suppressing as suggested by previous reports [35].

The presence of cleaved Bid, increased expression of proapoptic Bax and lower abundance of antiapoptotic Bcl-2 and

Bcl-XL proteins in treated melanoma cells prompted us to check whether observed signaling included mitochondria and if specific caspases were responsible for ultimate apoptic response. Accordingly, we detected late treatment-induced loss of mitochondrial membrane potential as well as a significant activation of major initiation and execution caspases in both cell lines. Still, in p53 mutant cells apoptosis was significantly lower than in wild type p53 cell line. Regarding our findings, this difference may be attributed to the following facts: firstly, topoisomerase inhibitors induced in Bowes cells p53-dependent response whose importance is further seen in case of pharmacological blockage of p53 activity with Pifithrin. Secondly, activation of caspase-2 which has recently been reported as p53-related [36] might have served as an amplifying factor (it was not detected in p53 mutant cells) as well as the active caspase-8-mediated Bid cleavage with its later mitochondrial translocation (data not shown). The role of ROS in these processes remains elusive as antioxidant NAC failed to provide substantial protection of Bowes cells against apoptosis. Conversely, in p53 mutant SK-MEL-28 cells ROS seemed to play a decisive role as seen by substantial inhibition of induced apoptosis by NAC. The implication of oxidative stress in proapoptic signaling in these cells was further underscored by abrogating the apoptosis via p38, SAPK/JNK and caspase-8 inhibition.

The results herein presented clearly show that dual inhibition of topoisomerase I and II with a combination of CPT and ETO has significant chemotherapeutic effects in malignant melanoma cells with differing p53 status. Still, both chemicals induce cytotoxicity and apoptosis via different mechanisms, with Bowes cells triggering p53-dependent mitochondrial apoptosis involving caspases and SK-MEL-28 cells activating mitochondrial and caspase-dependent apoptosis through ROS-mediated stress kinase signaling and possibly via other mechanisms. In summary, these findings draw attention to the efficiency of dual blocking of topoisomerases in inhibiting growth and inducing apoptosis in malignant melanoma cells both wild-type p53 and harboring mutation in p53 gene. This modality is therefore plausible for its further investigation in preclinical and clinical melanoma research.

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References

[1] GRAY-SCHOPFER V, WELLBROCK C, MARAIS R. Melanoma biology and new targeted therapy. Nature. 2007; 445: 851–857. doi:10.1038/nature05661

[2] GROSSMANN D, ALTIERI DC. Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. Cancer Metastasis Rev. 2001; 20: 3–11. doi:10.1023/A:1013123532723

[3] SOENGAS MS, LOWE SW. Apoptosis and melanoma chemoresistance. Oncogene. 2003; 22: 3138–3151. doi:10.1038/sj.onc.1206454

[4] IVANOV VN, BHOUMIK A, RONAI Z. Death receptors and melanoma resistance to apoptosis. Oncogene. 2003; 22: 3152–3161. doi:10.1038/sj.onc.1206456

[5] SELZER E, SCHLAGBAUER-WADL H, OKAMOTO I, PEHAMBERGER H, POTTER R et al. Expression of Bcl-2 family members in human melanocytes, in melanoma metastases and in melanoma cell lines. Melanoma Res. 1998; 8: 197–203. doi:10.1097/00008390-199806000-00001

[6] BOWEN AR, HANKS AN, ALLEN SM, ALEXANDER A, DIEDRICH MJ et al. Apoptosis regulators and responses in human melanocytic and keratinocytic cells. J Invest Dermatol. 2003; 120: 48–55. doi:10.1046/j.1523-1747.2003.12010.x

[7] SOENGAS MS, CAPODIECI P, POLSKY D, MORA J, ESTELLER M et al. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. Nature. 2001; 409: 207–211. doi:10.1038/35051606

[8] TANG L, TRON VA, REED JC, MAH KJ, KRAJEWSKA M et al. Expression of apoptosis regulators in cutaneous malignant melanoma. Clin Cancer Res. 1998; 4: 1865–1871.

[9] HU R, APLIN AE. Skp2 regulates G2/M progression in a p53-dependent manner. Mol Biol Cell. 2008; 19: 4602–4610. doi:10.1091/mbc.E07-11-1137

[10] SATYAMOORTHY K, CHEHAB NH, WATERMAN MJ, LIEN MC, EL-DEIRI WS et al. Aberrant regulation and function of wild-type p53 in radioresistant melanoma cells. Cell Growth Differ. 2000; 11: 467–474.

[11] YAMASHITA T, TOKINO T, TONOKI H, MORIUCHI T, JIN HY et al. Induction of apoptosis in melanoma cell lines by p53 and its related proteins. J Invest Dermatol. 2001; 117: 914–919. doi:10.1046/j.0022-202x.2001.01464.x

[12] ARUN B, FRENKEL EP. Topoisomerase I inhibition with topotecan: pharmacologic and clinical issues. Expert Opin Pharmacother. 2001; 2: 491–505. doi:10.1517/14656566.2.3.491

[13] HARTMANN JT, LIPP HP. Camptothecin and podophyllotoxin derivatives: inhibitors of topoisomerase I and II - mechanisms of action, pharmacokinetics and toxicity profile. Drug Saf. 2006; 29: 209–230.

[14] LAGE H, HELMBACH H, DIETEL M, SCHADENDORF D. Modulation of DNA topoisomerase II activity and expression in melanoma cells with acquired drug resistance. Br J Cancer. 2000; 82: 488–491. doi:10.1054/bjoc.1999.0947

[15] HELMBACH H, KERN MA, ROSSMANN E, RENZ K, KISSEL C et al. Drug resistance towards etoposide and cisplatin in human melanoma cells is associated with drug-dependent apoptosis deficiency. J Invest Dermatol. 2002; 118: 923–932. doi:10.1046/j.1523-1747.2002.01786.x

[16] MONTECUCCO A, BIAMONTI G. Cellular response to etoposide treatment. Cancer Lett. 2007; 252: 9–18. doi:10.1016/j.canlet.2006.11.005

[17] IKEDIOBI ON, DAVIES H, BIGNELL G, EDKINS S, STEVENS C et al. Mutation analysis of 24 known cancer genes in the NCI-60 cell line set. Mol Cancer Ther. 2006; 5: 2606–2612. doi:10.1158/1535-7163.MCT-06-0433

[18] TARHINI AA, AGARWALA SS. Cutaneous melanoma: available therapy for metastatic disease. Dermatol Ther. 2006; 19: 19–25. doi:10.1111/j.1529-8019.2005.00052.x

[19] CICHOREK M, KOZLOWSKA K, BRYL E. The activity of caspases in spontaneous and camptothecin-induced death of

melanotic and amelanotic melanoma cell. Cancer Biol Ther. 2007; 6: 346–353.

[20] OPPERMANN M, GEILEN CC, FECKER LF, GILLISSEN B, DANIEL PT et al. Caspase-independent induction of apoptosis in human melanoma cells by the proapoptotic Bcl-2-related protein Nbk / Bik. Oncogene. 2005; 24: 7369–7380. doi:10.1038/sj.onc.1208890

[21] KISSEL CK, SCHADENDORF D, ROCKMANN H. The altered apoptotic pathways in cisplatin and etoposide-resistant melanoma cells are drug specific. Melanoma Res. 2006; 16: 527–535. doi:10.1097/CMR.0b013e3280103a7c

[22] LI G, TANG L, ZHOU X, TRON V, HO V. Chemotherapy-induced apoptosis in melanoma cells is p53 dependent. Melanoma Res. 1998; 8: 17–23. doi:10.1097/00008390-199802000-00004

[23] Li G, BUSH JA, HO VC. p53-dependent apoptosis in melanoma cells after treatment with camptothecin. J Invest Dermatol. 2000; 114: 514–519. doi:10.1046/j.1523-1747.2000.00867.x

[24] ZANON M, PIRIS A, BERSANI I, VEGETTI C, MOLLA A et al. Apoptosis protease activator protein-1 expression is dispensable for response of human melanoma cells to distinct proapoptotic agents. Cancer Res. 2004; 64: 7386–7394. doi:10.1158/0008-5472.CAN-04-1640

[25] MCDONALD AC, BROWN R. Induction of p53-dependent and p53-independent cellular responses by topoisomerase 1 inhibitors. Br J Cancer. 1998; 78: 745–751.

[26] LEE S, LEE HS, BAEK M, LEE DY, BANG YJ et al. MAPK signaling is involved in camptothecin-induced cell death. Mol Cells. 2002; 14: 348–354.

[27] FU X, WAN S, LYU YL, LIU LF, QI H. Etoposide induces ATM-dependent mitochondrial biogenesis through AMPK activation. PLoS ONE. 2008; 3: 1–10. doi:10.1371/journal.pone.0002009

[28] KARAWAJEW L, RHEIN P, CZERWONY G, LUDWIG WD. Stress-induced activation of the p53 tumor suppressor in leukemia cells and normal lymphocytes requires mitochondrial activity and reactive oxygen species. Blood. 2005; 105: 4767–4775. doi:10.1182/blood-2004-09-3428

[29] LI Y, RORY GOODWIN C, SANG Y, ROSEN EM, LATERRA J et al. Camptothecin and Fas receptor agonists synergistically induce medulloblastoma cell death: ROS-dependent mechanisms. Anticancer Drugs. 2009.

[30] NIIDA H, NAKANISHI M. DNA damage checkpoints in mammals. Mutagenesis. 2006; 21: 3–9. doi:10.1093/mutage/gei063

[31] VAN LAETHEM A, NYS K, VAN KELST S, CLAERHOUT S, ICHIJO H et al. Apoptosis signal regulating kinase-1 connects reactive oxygen species to p38 MAPK-induced mitochondrial apoptosis in UVB-irradiated human keratinocytes. Free Radic Biol Med. 2006; 41: 1361–1371. doi:10.1016/j.freeradbiomed.2006.07.007

[32] WITTGEN HG, VAN KEMPEN LC. Reactive oxygen species in melanoma and its therapeutic implications. Melanoma Res. 2007; 17: 400–409. doi:10.1097/CMR.0b013e3282f1d312

[33] SELIMOVIC D, HASSAN M, HAIKEL Y, HENGGE UR. Taxol-induced mitochondrial stress in melanoma cells is mediated by activation of c-Jun N-terminal kinase (JNK) and p38 pathways via uncoupling protein 2. Cell Signal. 2008; 20: 311–322. doi:10.1016/j.cellsig.2007.10.015

[34] GULATI AP, YANG YM, HARTER D, MUKHOPADHYAY A, AGGARWAL BB et al. Mutant human tumor suppressor p53 modulates the activation of mitogen-activated protein kinase and nuclear factor-kappaB, but not c-Jun N-terminal kinase and activated protein-1. Mol Carcinog. 2006; 45: 26–37. doi:10.1002/mc.20149

[35] LIU B, CHEN Y, ST CLAIR DK. ROS and p53: a versatile partnership. Free Radic Biol Med. 2008; 44: 1529–1535. doi:10.1016/j.freeradbiomed.2008.01.011

[36] ZHIVOTOVSKI B, ORRENIUS S. Caspase-2 function in response to DNA damage. Biochem Biophys Res Commun. 2005; 331: 859–867. doi:10.1016/j.bbrc.2005.03.191