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Cell Death Induced by Novel Fluorinated Taxanes in Drug-Sensitive and Drug-Resistant Cancer Cells

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Abstract

The aim of this study is to compare the effects of new fluorinated taxanes SB-T-12851, SB-T-12852, SB-T-12853, and SB-T-12854 with those of the classical taxane, paclitaxel, and novel non-fluorinated taxane SB-T-1216 on cancer cells. Paclitaxel-sensitive MDA-MB-435 and paclitaxel-resistant NCI/ADR-RES human cancer cell lines were used. Cell growth and survival evaluation, colorimetric assessment of caspases activities, flow cytometric analyses of the cell cycle and the assessment of mitochondrial membrane potential, reactive oxygen species (ROS) and the release of cytochrome c from mitochondria were studied. All fluorinated taxanes examined have similar effects on cell growth and survival. For MDA-MB-435 cells, the C_{50} values of SB-T-12851, SB-T-12852, SB-T-12853 and SB-T-12854 were 3 nM, 4 nM, 3 nM and 5 nM, respectively. For paclitaxel-resistant NCI/ADR-RES cells, the C_{50} values of SB-T-12851, SB-T-12852, SB-T-12853, and SB-T-12854 were 20 nM, 20 nM, 10 nM and 10 nM, respectively. Selected fluorinated taxanes, SB-T-12853 and SB-T-12854, at the cell death-inducing concentrations (30 nM for MDA-MB-435 and 300 nM for NCI/ADR-RES) were shown to activate significantly caspase-3, caspase-2 and caspase-9, as well as caspase-8 in lesser extent. Cell death was associated with significant accumulation of cells in the G_2/M phase. Cytochrome c was not found to be released from mitochondria and other mitochondrial functions were not significantly impaired. The new fluorinated taxanes appear to use the same or very similar mechanisms of cell death induction as compared with SB-T-1216 and paclitaxel. New fluorinated and non-fluorinated taxanes are more effective against drug-resistant cancer cells than paclitaxel. Therefore, new generation taxanes, either non-fluorinated or fluorinated, are excellent candidates for further and detailed studies.

Keywords

Cell death; Paclitaxel; Taxanes; Fluorinated Taxanes; Cancer Cells; Caspases

Introduction

Taxanes represent a family of well known anticancer drugs currently used for treatment of patients with several types of cancer, including breast, ovarian, head and neck, lung, and prostate cancer (Galletti et al. 2007, Choy 2001). Paclitaxel, originally isolated from the bark of the Pacific Yew (*Taxus brevifolia*), has been shown to possess anticancer activity *in vitro* in 1971 by Wall and coworkers (Wani et al., 1971). In 1992, the Food and Drug Administration (FDA) of the U.S. approved paclitaxel (Taxol ®) to be used in clinical practice for the treatment of breast cancer (Ferlini et al., 2003). Later, paclitaxel was followed by the semisynthetic taxane, docetaxel, and other taxane analogs.

Our understanding of taxane action on the molecular level comes predominantly from studies employing paclitaxel (Chien and Moasser, 2008). Paclitaxel reversibly binds to the β -tubulin subunit of microtubules. β -Tubulin together with α -tubulin forms heterodimers, basic units of microtubules (Orr et al., 2003). Microtubules, one of the major components of cytoskeleton, are involved in a wide range of cellular functions including cell motility, intracellular trafficking, maintenance of cell shape, and assembly of the mitotic spindle (Orr et al., 2003; Tuszynski et al., 1997). Binding of paclitaxel to β -tubulin affects inherent dynamic instability of microtubules by accelerating the polymerization of tubulin and inhibiting microtubule depolymerization (Xiao et al., 2006), making microtubules unusually stable and resistant to depolymerization (Schiff and Horwitz, 1981). Thus, the interaction of paclitaxel with microtubules results in the formation of microtubule bundles in interphase cells and in the formation of asters instead of regular mitotic spindles during mitosis. In this way, taxanes are supposed to block progression through the M phase of the cell cycle (Jordan et al., 2002; Sackett and Fojo, 1997) and cause cell death. However, the causal relationship of the mitotic arrest induced by taxanes to the induction of cell death is unclear (Aoudjit and Vuori, 2001; Ehrlichová et al., 2005a; Fan, 1999).

It is generally believed that anticancer agents, including taxanes, induce apoptosis through the intrinsic mitochondrial pathway via the release of proapoptotic proteins such as cytochrome c from the mitochondria into the cytosol (Bhalla, 2003; Liao et al., 2008). It seems that paclitaxel-induced cell death pathways differ according to the origin of cancer cell (Mhaidat et al., 2007; Shi et al., 2008). After paclitaxel treatment, involvement of mitochondrial pathway of apoptosis with the activation of caspase-9 and caspase-3 has been reported in several breast cancer lines (Ehrlichová et al., 2005a; Fowler et al., 2000; Friedrich et al., 2001; Kottke et al., 1999; Razandi et al., 2000) as well as in cancer cells of another origin (Mhaidat et al., 2007). On the other hand, paclitaxel has been described to induce cell death independently of activation of caspase-9 and caspase-3 in breast cancer cells (Ofir et al., 2002). Studies of paclitaxel-induced apoptosis in the cancer cells of non-breast origin have shown involvement of other pathways such as death-receptor independent activation of caspase-8 (Von Haefen et al., 2003) or death-receptor independent activation of caspase-10 (Park et al., 2004).

In clinical practice, paclitaxel and docetaxel are taxanes routinely used. They are known as classical taxanes. Paclitaxel used as a single agent is associated with 30 % to 50 % response rates for advanced cancers of the breast and ovary, and 20 % to 40 % response rates for advanced cancers of the lung, head and neck, esophagus, and prostate (Chien and Moasser 2008; Mekhail and Markman, 2002; Rowinsky, 1997). Unfortunately, cellular resistance, either primary or secondary, represents a serious limitation to the clinical use of classical taxanes. Therefore, there is an intensive need to elucidate the molecular mechanisms of taxane resistance and to develop new generation of taxanes that would be effective against tumors resistant to the treatment with classical taxanes.

Examples of such taxanes are SB-T-1216 (Ojima et al., 1996), and fluorinated taxanes SB-T-12851, SB-T-12852, SB-T-12853, SB-T-12854 synthesized by Ojima et al. at Stony Brook (Pepe et al., 2009). These taxanes are anticipated to be more effective than classical taxanes, especially against paclitaxel-resistant cancer cells. Indeed, SB-T-1216 has been already shown to be more effective against the drug-resistant breast cancer cells in comparison with paclitaxel (Ehrlichová et al., 2005b).

In the present *in vitro* study, we focused on fluorinated taxanes SB-T-12851, SB-T-12852, SB-T-12853 and SB-T-12854. The main aim of the study was to compare their effect on human cancer cells with that of non-fluorinated classical taxane, paclitaxel, and non-fluorinated novel taxane, SB-T-1216. Employing two cancer cell lines, which differ significantly in their sensitivity to paclitaxel (paclitaxel-sensitive MDA-MB-435 cells and highly paclitaxel-resistant NCI/ADR-RES cells), we found that cell death-inducing effect of fluorinated taxanes is comparable with that of previously studied non-fluorinated taxane, SB-T-1216 (Kovář et al., 2009). However, these fluorinated taxanes are significantly more effective than classical taxane, paclitaxel, especially against paclitaxel-resistant cells. Cell death induced by all taxanes examined in MDA-MB-435 and NCI/ADR-RES cells was accompanied by the activation of caspase-3, caspase-9, caspase-2 and caspase-8, but was not associated with increased level of reactive oxygen species (ROS), mitochondrial membrane potential collapse and cytochrome c release from mitochondria.

Materials and Methods

Materials

Paclitaxel was obtained from Sigma-Aldrich (St. Louis, MO, USA). Novel taxane, SB-T-1216 (Ojima et al., 1996), and new fluorinated taxanes (Pepe et al., 2009), SB-T-12851, SB-T-12852, SB-T-12853, SB-T-12854 (Fig. 1) were synthesized by Prof. I. Ojima (Stony Brook, NY, USA). All taxanes were dissolved in DMSO (tissue culture quality) to obtain 10 mM stock solutions.

Cells and culture conditions

The human breast carcinoma cell lines MDA-MB-435 and NCI/ADR-RES were obtained from National Cancer Institute at Frederick (MD, USA). The cells were maintained in a culture medium at 37°C in a humidified atmosphere of 5 % CO₂ in air. The culture medium represents a basic medium supplemented with 10 % heat-inactivated fetal bovine serum (Biocrom AG, Berlin, Germany). The basic medium was RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing extra L-glutamine (300 µg/ml), sodium pyruvate (110 µg/ml), HEPES (15 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml), as described previously (Musílková and Kovář, 2001).

Assessment of cell growth and survival

Cells were harvested and seeded at 20×10^3 cells/100 µl of the culture medium into the wells of a 96-well plastic plate. After 24-h preincubation period allowing cells to attach, the culture medium was replaced by the culture medium without a taxane (control) or with a taxane at desired concentrations. Cell growth and survival were evaluated after 96 h of incubation. The number of living cells was determined by hemocytometer counting after staining with trypan blue (Kovář et al., 2001).

Cell cycle analysis

Cells (approximately 500×10^3 cells per sample) were seeded. After 24-h preincubation period allowing cells to attach, the culture medium was replaced by the culture medium without a taxane (control) or with a taxane at death-inducing concentrations. After 12-h and

24-h incubation, the cells were harvested by low-speed centrifugation and fixed in cold 70 % ethanol overnight at 4 °C. Fixed cells were washed with PBS, stained with propidium iodide solution (40 µg/ml propidium iodide and 100 µg/ml RNase in PBS) for 45 min and the fluorescence was measured on a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Measurement of caspase-3, caspase-9, caspase-2 and caspase-8 activities

Commercial colorimetric Caspase-3 Assay Kit, Caspase-8 Assay Kit and Caspase-9 Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) as well as solutions from a Caspase-3 Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) combined with the chromogenic caspase-2 substrate and caspase-2 inhibitor (Alexis Biochemicals, Lausen, Switzerland) were used for the assessment of caspase-2, caspase-3, caspase-8 and caspase-9 activities. Cells were seeded at 200×10^3 cells/ml of culture medium (approximately 20×10^6 cells per sample). Taxanes were applied after 24-h preincubation as described above (see “*Cell cycle analysis*”). After 24-h incubation, cells were harvested by low-speed centrifugation, washed twice with PBS and lysed. The total protein concentration was determined by the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Assays were performed in 96-well plates according to the manufacturer's instructions and 100 µg of total protein per sample was analyzed. The sample absorbance was measured at 405 nm using a Sunrise ELISA Reader (Tecan, Maennedorf, Switzerland).

Flow cytometric analysis of the mitochondrial membrane potential ($\Delta\psi_m$)

Cells (approximately 500×10^3 cells per sample) were seeded and taxanes were applied after 24-h preincubation as described above (see “*Cell cycle analysis*”). After 24-h incubation, the cells were harvested by low-speed centrifugation and resuspended in PBS. The $\Delta\psi_m$ was measured as described previously (Koc et al., 2005). Cells were kept on ice and 20 nM 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] from Molecular Probes (Eugene, OR, USA) was added. After 20 min of incubation at 37 °C cells were again kept on ice. As a negative control, aliquots of cells were incubated in the presence of 100 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore causing a complete disruption of the $\Delta\psi_m$. The fluorescence was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Flow cytometric analysis of reactive oxygen species (ROS) level

The ROS level was measured by dihydroethidine probe (HE) according to the protocol described by Castedo et al. 2002) with minor modification. Briefly, cells (approximately 500×10^3 cells per sample) were seeded and taxanes were applied after 24-h preincubation as described above (see “*Cell cycle analysis*”). After 24-h incubation, the cells were harvested by low-speed centrifugation and resuspended in the culture medium. Cells were kept on ice and 5 µM dihydroethidium (Sigma, St. Louis, MO, USA) was added. After 30 min of incubation at 37 °C, cells were again kept on ice. The fluorescence was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Flow cytometric analysis of cytochrome c release

Commercial InnoCyte™ Flow Cytometric Cytochrome c Release Kit (Merck, Darmstadt, Germany) was used for the assessment of cytochrome c release from mitochondria. Cells (approximately 3×10^6 cells per sample) were seeded and taxanes were applied after 24-h preincubation as described above (see “*Cell cycle analysis*”). After 24-h incubation, the cells were harvested by low-speed centrifugation and resuspended in PBS. The cells were permeabilized with Permeabilization Buffer, then fixed with 8 % paraformaldehyde in PBS and repeatedly washed. Blocking Buffer was added and the cells were incubated. After the

addition of the primary antibody against cytochrome c, the cells were again incubated and washed. The cells were subsequently incubated with the secondary anti-IgG FITC antibody. After washing the cells were resuspended and the fluorescence was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Cell fractionation and Western blot analysis of cytochrome c level

Cells (approximately 20×10^6 cells per sample) were seeded and taxanes were applied after 24-h preincubation as described above (see “*Cell cycle analysis*”). After 24-h incubation, the cells were harvested by low-speed centrifugation and resuspended in PBS. Cell fractionation was performed as described previously (Ehrlichová et al., 2005a).

Western blot analysis of the levels of cytochrome c was carried out with some modifications as described in detail previously (Ehrlichová et al., 2005a). Proteins separated by SDS-PAGE were blotted onto 0.2 μ m nitrocellulose membrane for 2 h at 0.25 A using MiniProtein II blotting apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5 % non-fat milk in TBS for 15 min. Tween-20 (0.1 %) in TBS was used for washing. The washed membrane was incubated with the primary rabbit polyclonal antibody #4272 against human cytochrome c (Cell Signaling, Danvers, MA, USA). After the incubation (overnight, 4 °C), the washed membrane was incubated for 1 h with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The horseradish peroxidase-conjugated secondary antibody was detected by enhanced chemiluminescence using the Supersignal reagent from Pierce (Rockford, IL) and LAS 1000 CCD device (Fuji). As a control of proper fractionation, the primary rabbit polyclonal antibody #4844 (Cell Signaling, Danvers, MA, USA) against the integral mitochondrial protein cytochrome c oxidase IV (COX IV) was used.

Results

Effects of taxanes on cancer cell growth and survival

The effects of SB-T-12851, SB-T-12852, SB-T-12853 and SB-T-12854 on the growth and survival of drug-sensitive MDA-MB-435 cells (Ehrlichová et al., 2005a) was examined at a wide range of concentrations, i.e., 0.03–3,000 nM. The effects of all four fluorinated taxanes examined was similar. These fluorinated taxanes induced the death of MDA-MB-435 cells within 96 h of incubation at 10 nM or concentrations higher than 10 nM. The C_{50} values (concentration of taxane resulting in 50 % of living cells in comparison with the control after 96 h of incubation) of SB-T-12851, SB-T-12852, SB-T-12853 and SB-T-12854 were 3 nM, 4 nM, 3 nM and 5 nM, respectively (Fig. 2).

In the case of drug-resistant NCI/ADR-RES cells (Ehrlichová et al., 2005a), the effects of fluorinated taxanes were also examined at a wide range of concentrations, i.e., 0.1–10,000 nM. Again, the effects of all four fluorinated taxanes examined were more or less similar. These taxanes induced death of NCI/ADR-RES cells within 96 h of incubation at 30 nM or concentrations higher than 30 nM. The C_{50} values of SB-T-12851, SB-T-12852, SB-T-12853 and SB-T-12854 were 20 nM, 20 nM, 10 nM and 10 nM, respectively (Fig. 2). These data show that about 3–6-fold higher concentrations of fluorinated taxanes are required to induce the death of drug-resistant NCI/ADR-RES cells than that of drug-sensitive MDA-MB-435 cells.

Thus, the effects of fluorinated taxanes examined resembles that of the previously studied novel non-fluorinated taxane, SB-T-1216. About 3-fold higher concentrations of SB-T-1216 were also required to induce the death of drug-resistant NCI/ADR-RES cells than that of drug-sensitive MDA-MB-435 cells (Kovář et al., 2009). On the other hand, an approximately 300-fold higher concentration of classical taxane, paclitaxel, was required to

induce the death of drug-resistant NCI/ADR-RES cells than that of drug-sensitive MDA-MB-435 cells (Ehrlichová et al., 2005a).

On the basis of obtained data, we selected 30 nM and 300 nM as the cell death-inducing concentrations (i.e. the lowest concentrations with full death-inducing effect) of fluorinated taxanes for drug-sensitive MDA-MB-435 cells and drug-resistant NCI/ADR-RES cells, respectively. These concentrations were used in further experiments, comparing the effect of fluorinated taxanes and that of previously studied non-fluorinated novel taxane, SB-T-1216 (Kovář et al., 2009) as well as the effect of paclitaxel (Ehrlichová et al., 2005a). Under the same experimental conditions, 30 nM and 300 nM were also used as the death-inducing concentrations of SB-T-126 for drug-sensitive MDA-MB-435 and drug-resistant NCI/ADR-RES cells, respectively. In the case of paclitaxel, 30 nM concentration for the drug-sensitive cells and 3,000 nM concentration for the drug-resistant cells were used as reported previously (Ehrlichová et al., 2005a).

Effect of taxanes on the cell cycle

Flow cytometric analysis, after propidium iodide staining, showed that the application of selected fluorinated taxanes, SB-T-12853 and SB-T-12854, at the cell death-inducing concentration (30 nM) led to a significant accumulation of drug-sensitive MDA-MB-435 cells in the G₂/M phase of the cell cycle after 12 h of incubation. After 24 h of incubation, nearly total accumulation of the cells in the G₂/M phase was observed. A similar pattern was found for non-fluorinated novel taxane, SB-T-1216, as well as paclitaxel at the cell death-inducing concentration (30 nM) (Fig. 3).

The application of the two fluorinated taxanes at the cell death-inducing concentration (300 nM) also resulted in a significant accumulation of drug-resistant NCI/ADR-RES cells in the G₂/M phase similarly to the application of SB-T-1216 and paclitaxel at the cell death-inducing concentration of 300 nM and 3,000 nM, respectively. Again, after 24 h of incubation with all taxanes examined, nearly total accumulation of the cells in the G₂/M phase was observed as compared with the number of cells in G₁ phase. However, in the case of paclitaxel at 3,000 nM concentration, a significant accumulation of hypodiploid cells/particles was also detected (Fig. 4).

Effect of taxanes on the activity of caspases

The colorimetric assay employed showed that after 24 h of incubation with fluorinated taxanes SB-T-12853 and SB-T-12854 as well as non-fluorinated taxane, SB-T-1216, and paclitaxel at the cell death-inducing concentration (30 nM), the activity of caspase-3 increased approximately 2 to 3-fold in drug-sensitive MDA-MB-435 cells. However, in the case of drug-resistant NCI/ADR-RES cells after the incubation with the fluorinated taxanes SB-T-12853 and SB-T-12854 at the cell death-inducing concentrations (300 nM) as well as SB-T-1216 (300 nM) and paclitaxel (3,000 nM), 8 to 10-fold increase in activity was observed (Fig. 5).

With regard to caspase-9 activity, nearly zero or zero activity was detected in control for both cancer cell lines. However, a certain increase in caspase-9 activity was observed after the incubation of taxanes at the cell death-inducing concentrations. When comparing absolute values, the activity was 3.5 to 11-fold higher in drug-resistant NCI/ADR-RES cells than that in drug-sensitive MDA-MB-435 cells (Fig. 5).

The activity of caspase-2 increased approximately 2 to 3-fold in drug-sensitive MDA-MB-435 cells after the incubation of all taxanes examined at the cell death-inducing concentrations. In drug-resistant NCI/ADR-RES cells, the increase in caspase-2 activity was much more pronounced, i.e. 14 to 20-fold (Fig. 5).

We observed only slight increase in caspase-8 activity for both drug-sensitive MDA-MB-435 cells and drug-resistant NCI/ADR-RES cells after the incubation of taxanes at the cell death-inducing concentrations. The increase was 30–70 % for the sensitive cells and 40–60 % for the resistant cells (Fig. 5).

Effect of taxanes on cytochrome c release

Flow cytometric analysis showed that after 24 h of incubation of taxanes, SB-T-12853 and SB-T-12854, as well as non-fluorinated novel taxane SB-T-1216 and paclitaxel, at the cell death-inducing concentration (30 nM), cytochrome c was not significantly released from the mitochondria in drug-sensitive MDA-MB-435 cells. Similar data were obtained with SB-T-12853 and SB-T-12854 (300 nM) as well SB-T-1216 (300 nM) and paclitaxel (3,000 nM) at the cell death-inducing concentrations in drug-resistant NCI/ADR-RES cells (Fig. 6).

These results were confirmed by Western blot analysis after cell fractionation. In both drug-sensitive MDA-MB-435 cells and drug-resistant NCI/ADR-RES cells after 24-h incubation of taxanes at the cell death-inducing concentrations, cytochrome c was detected in the mitochondrial fraction, but not in the cytosolic fraction. As a control of proper fractionation, we performed Western blot analysis of the integral mitochondrial protein cytochrome c oxidase IV (COX IV) for both mitochondrial and cytosolic fractions. COX IV was only detected in the mitochondrial fraction of both cancer cell lines (data not shown).

Effect of taxanes on mitochondrial functions

Employing flow cytometric analysis after staining cells with DiOC₆(3), we assessed the effect of taxanes at the cell death-inducing concentrations on the mitochondrial membrane potential ($\Delta\psi_m$) in both drug-sensitive MDA-MB-435 cells and drug-resistant NCI/ADR-RES cells. With regard to the effect of 24-h incubation of fluorinated taxanes, SB-T-12853 and SB-T-12854, as well as non-fluorinated taxane, SB-T-1216, and paclitaxel at the cell death-inducing concentrations in both drug-sensitive MDA-MB-435 cells and drug-resistant NCI/ADR-RES cells, we only detected a minor population of cells with decreased mitochondrial membrane potential ($\Delta\psi_m$) when compared with the control cells (Fig. 7).

Employing flow cytometric analysis using dihydroethidine probe, we also examined the effect of the taxanes at the death-inducing concentrations on reactive oxygen species (ROS) production. We did not detect any significant increase of ROS level in both drug-sensitive MDA-MB-435 and drug-resistant NCI/ADR-RES cells (Fig. 8).

Discussion

The aim of the present study was to compare the cell death-inducing effects of new fluorinated taxanes SB-T-12851, SB-T-12852, SB-T-12853, SB-T-12854, on human cancer cells with that of classical taxane, paclitaxel, and novel non-fluorinated taxane, SB-T-1216. We employed two human cancer cell lines, MDA-MB-435 and NCI/ADR-RES. Cancer cell line MDA-MB-435 has been found to be sensitive to paclitaxel in comparison with NCI/ADR-RES cell line, which has been shown to be highly resistant to paclitaxel (Ehrlichová et al., 2005a). Both cell lines were originally classified as cancer cell lines of breast cancer origin. However, recently their origin has been widely questioned (Ellison et al., 2002; Wang et al., 2006) and their origins remain unclear. Nevertheless, they are still excellent models to study the effects of taxanes.

We showed previously that novel non-fluorinated taxane, SB-T-1216, was significantly more effective than classical taxanes, paclitaxel and docetaxel, particularly against drug-resistant cells (Ehrlichová et al., 2005b; Kovář et al., 2009). Thus, SB-T-1216 may represent a potentially powerful agent for the treatment of drug-resistant cancers. In the current

experiments we compared previously studied taxanes, paclitaxel and SB-T-1216, with new fluorinated taxanes, SB-T-12851, SB-T-12852, SB-T-12853, and SB-T-12854. Paclitaxel and SB-T-1216 were used as standards. Such design of the study allowed us to compare different taxanes under the same experimental conditions and also to assess the effects of fluorine incorporation to taxane molecules (e.g., SB-T-12854 vs. SB-T-1216, see Fig. 1) on cell growth and survival, cell cycle progression, activation of caspases and mitochondrial functions.

All fluorinated taxanes examined, SB-T-12851, SB-T-12852, SB-T-12853 and SB-T-12854, have similar effects on cell growth and survival of the cancer cell lines employed. Generally, the effects of fluorinated taxanes resemble that of previously studied non-fluorinated taxane, SB-T-1216. Thus, our data confirmed that novel taxanes are more effective on the inhibition of cancer cell growth and survival than paclitaxel, particularly against drug-resistant cells.

We detected significant activation of the key executioner, caspase-3, and also the activation of initiator caspase-9, representing the mitochondrial pathway for apoptosis induction, in both drug-sensitive MDA-MB-435 and drug-resistant NCI/ADR-RES cells when cell death was induced by all taxanes. Such result may suggest the involvement of the mitochondrial pathway in the induction of apoptosis by taxanes. Therefore, we also investigated other mitochondrial functions.

It has been described that an intrinsic mitochondrial pathway is involved in apoptosis induction by taxanes (Bhalla, 2003; Ehrlichová et al., 2005a; Fowler et al., 2000; Friedrich et al., 2001; Kottke et al., 1999; Liao et al., 2008; Razandi et al., 2000), although other apoptotic pathways were reported as well (Mhaidat et al., 2007; Ofir et al., 2002; Shi et al., 2008). In this study, we found that cytochrome c was not released from mitochondria during apoptosis induction by taxanes. However, in our previous study (Ehrlichová et al., 2005a) we detected cytochrome c release when apoptosis was induced by paclitaxel at a very high concentration (3,000 nM) in drug-resistant cells. The difference could be ascribed to the use of different paclitaxel preparation, but we must wait for further investigation.

We also did not detect any significant change in the mitochondrial membrane potential $\Delta\psi_m$. Collapse of $\Delta\psi_m$ is anticipated when the mitochondrial pathway is involved. Increased ROS production was not detected as well in this study. Generation of ROS was described previously as a result of paclitaxel treatment (Alexandre et al., 2007). However, it seems that ROS does not play a significant role in apoptosis induced by taxanes for both cancer cell lines.

Thus, it seems that the mitochondrial pathway is not the predominant pathway of apoptosis induction by taxanes in MDA-MB-435 and NCI/ADR-RES cells. The activation of caspase-9 may just represent a side pathway.

Significant activation of caspase-2 and a certain activation of caspase-8 were also found in both cell lines. Caspase-2 has a unique position among caspases. It is suggested that caspase-2 is the most relevant caspase in the apoptotic cascade when apoptosis is induced by DNA damage and cytotoxic stress (Lassus et al., 2002; Zhivotovsky and Orrenius, 2005). Several studies demonstrated caspase-2 activation in various cancer cell lines after apoptosis induction by taxanes (Mhaidat et al., 2007; Wang et al., 2004). Thus caspase-2 may be a major player in apoptosis induction. Concerning caspase-8 activation, it suggests that the death receptor pathway of caspase activation could also be somehow involved in cell death induced by taxanes. The activation of caspase-8 was shown during taxane-induced apoptosis in lymphoma and melanoma cells (Mhaidat et al., 2007; Wang et al., 2004).

Considering the activation of all caspases examined in this study, the functional sequence of the activation of individual caspases remains to be elucidated. We hypothesize that the key event of the induction of apoptosis by taxanes in the two cancer cell lines used for the study is the activation of caspase-2. Caspase-2 may act as the most relevant caspase that is responsible for the activation of all other caspases, including caspase-9.

Based on the results obtained in this study, new fluorinated taxanes appear to use the same or a very similar mechanisms of cell death induction to exert their anticancer effects *in vitro* as compared with non-fluorinated taxane, SB-T-1216, and the classical taxane, paclitaxel. Fluorinated taxanes and non-fluorinated taxane examined in our experiments are significantly more effective against drug-resistant cancer cells than paclitaxel. Therefore, new generation taxanes, either non-fluorinated or fluorinated, are excellent candidates for further and detailed studies. These taxanes may provide a good treatment option for cancer patients refractory to the treatment with classical taxanes in the future.

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Figure 1. Chemical structures of tested taxanes

Figure 2. Effect of SB-T-12851, SB-T-12852, SB-T-12853 and SB-T-12854 (0.1 – 1000 nM) on the growth and survival of MDA-MB-435 and NCI/ADR-RES cells

Control cells (C) were incubated without taxane. The cells were seeded at 20×10^3 cells/100 μ l of medium in the well. The number of cells of the inoculum is shown as a dotted line. The number of living cells was determined after 96 h of incubation (see “Materials and Methods”). Each column represents the mean of 8 separate cultures $\pm$ SEM.

Figure 3. Effect of paclitaxel, SB-T-1216, SB-T-12853 and SB-T-12854 at death-inducing concentrations on the DNA histogram of sensitive MDA-MB-435 cells
Control cells were incubated without taxane. After the incubation period (12, 24 h), the cells were stained with propidium iodide (see “Materials and Methods”) and analyzed by flow cytometry.

Figure 4. Effect of paclitaxel, SB-T-1216, SB-T-12853 and SB-T-12854 at death-inducing concentrations on the DNA histogram of resistant NCI/ADR-RES cells
Control cells were incubated without taxane. After the incubation period (12, 24 h), the cells were stained with propidium iodide (see “Materials and Methods”) and analyzed by flow cytometry.

Figure 5. Effect of paclitaxel, SB-T-1216, SB-T-12853 and SB-T-12854 at death-inducing concentrations on the activity of caspase-3, -9, -2 and -8 in sensitive MDA-MB-435 and resistant NCI/ADR-RES cells

Control cells were incubated without taxane. After 24 h of incubation, the activity of caspases was measured as the absorbance of the cleaved product of a respective chromogenic substrate at 405 nm, employing a commercial colorimetric kit (see “Materials and Methods”). Each column represents the mean of 2 experimental values $\pm$ SEM.

* $P < 0.05$, ** $P < 0.01$ when comparing with control values.

Figure 6. Effect of paclitaxel, SB-T-1216, SB-T-12853 and SB-T-12854 at death-inducing concentrations on cytochrome c release from mitochondria in sensitive MDA-MB-435 and resistant NCI/ADR-RES cells

Control cells were incubated without taxane. After 24 h of incubation, the release of cytochrome c was assessed by flow cytometric analysis of cells, employing a commercial cytochrome c release kit (see “Materials and Methods”). Cells without staining are shown (blank areas).

Figure 7. Effect of paclitaxel, SB-T-1216, SB-T-12853 and SB-T-12854 at death-inducing concentrations on the mitochondrial membrane potential ($\Delta\Psi_m$) in sensitive MDA-MB-435 and resistant NCI/ADR-RES cells

Control cells were incubated without taxane. After 24 h of incubation, the mitochondrial membrane potential was assessed by flow cytometric analysis of cells after staining with 20 nM [DiOC6(3)] (see “Materials and Methods”). The collapse of $\Delta\Psi_m$ caused by the treatment of cells with 100 μ M CCCP is shown (blank areas).

Figure 8. Effect of paclitaxel, SB-T-1216, SB-T-12853 and SB-T-12854 at death-inducing concentrations on reactive oxygen species (ROS) production in sensitive MDA-MB-435 and resistant NCI/ADR-RES cells

Control cells were incubated without taxane. After 24 h of incubation, the ROS production was assessed by flow cytometric analysis of cells after staining with 5 μ M dihydroethidium (see “Materials and Methods”). The analysis of cells without staining is shown (blank areas).