Differing Mechanisms of Death Induction by Fluorinated Taxane SB-T-12854 in Breast Cancer Cells

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Abstract. *Background/Aim:* Classical taxanes are routinely used in cancer therapy. In this study, mechanisms involved in death induction by the novel fluorine-containing taxane SB-T-12854 were investigated. *Materials and Methods:* We employed breast cancer SK-BR-3, MCF-7 and T47D cell lines to assess activation of individual caspases, changes in the expression of proteins of the Bcl-2 family, and the release of pro-apoptotic factors from mitochondria into the cytosol after SB-T-12854 treatment. *Results:* Caspase-2, -8, and -9 were activated in SK-BR-3 and MCF-7 cells. Only caspase-8 was activated in T47D cells. Caspase-7 and -6 were activated in all tested cells while caspase-3 was activated only in SK-BR-3 cells. Pro-apoptotic Bad protein seems to be important for cell death induction in all tested cells. Anti-apoptotic Bcl-2 and pro-apoptotic Bim, Bok, Bid and Bik seem to be also associated with cell death induction in some of the tested cells. The mitochondrial apoptotic pathway was significantly activated in association with the release of cytochrome *c* and Smac from mitochondria, but only in SK-BR-3 cells, not in MCF-7 and T47D cells. *Conclusion:* Cell death induced by SB-T-12854, in the tested breast cancer cells, differs regarding activation of caspases, changes in levels of pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family and activation of the mitochondrial apoptotic pathway.

Taxanes are well known mitotic poisons used in the treatment of solid tumors. Currently two taxanes, *i.e.* paclitaxel (Taxol[®]) and docetaxel (Taxotere[®]) are routinely used in chemotherapy of breast, ovary, lung, and other cancers (1-3). Taxanes bind tubulin dimers and block de-

polymerization of microtubules (4). The hyperpolymerization of microtubules blocks the cell cycle in the G₂/M interphase and leads to mitotic arrest (5, 6). Subsequently, mitotic arrest usually results in apoptosis induction. However, other alternative pathways leading to cell death can be induced in cells treated with taxanes (7-10).

Pro-apoptotic Bax, Bak and Bok proteins of the Bcl-2 family form channels in the outer mitochondrial membrane and thus enable the release of cytochrome *c* into the cytosol (11). The level of Bax was found to increase, and the level of Bak was observed to decrease as well as to increase after taxane application (11-13). The role of the Bok protein remains somewhat unclear. Similarly, the levels of anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2 and Bcl-xL (they prevent formation of Bax/Bak channels in mitochondrial membrane), have been reported to be increased as well as decreased after taxane application (11, 12, 14, 15). In addition, apoptosis induced by taxanes is usually associated with Bcl-2 phosphorylation (16). Pro-apoptotic BH3-only proteins of the Bcl-2 family, that suppress the activity of anti-apoptotic Bcl-2 proteins, can effectively induce the intrinsic mitochondrial pathway of apoptosis induction (17-19). There are reports showing Bim protein to be directly involved in cell death induction by taxanes (20-21). Furthermore, some roles for Bad, Bik and Puma in cell death induction by taxanes in breast cancer cells have been reported (11, 17, 22).

Cytochrome *c* and Smac protein release from mitochondria are fundamentally important for the activation of the mitochondrial apoptotic pathway and activation of caspases. The release of cytochrome *c* and/or Smac occurs *in vitro* and *in vivo* in cancer cells treated with taxanes (23-25). On the other hand, cell death induced by taxanes in cancer cells can also be realized without release of cytochrome *c* (24).

Concerning caspases, the activation of caspase-8, the main initiator caspase of the extrinsic pathway of apoptosis induction, and caspase-9, the main initiator caspase of the intrinsic mitochondrial pathway of apoptosis induction, have been observed in various cell types after taxane treatment (7, 15, 26, 27). Recently, several groups including ours have

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reported that caspase-2, a highly conservative protease involved in cell death induction by different stimuli (28), appears to play an important role in taxane-induced cell death (29-31). Executioner caspase-3, -7 and -6 have also been activated after taxane application in various types of cancer cells (7, 12, 32). Caspase-3 probably plays the most important role here, while caspase-6 and -7 seem to have minor roles (23, 32).

There is an abundance of data concerning cell death induction by novel taxanes (6, 7, 24, 31, 32). However, the precise roles of some components of the apoptosis-inducing pathways (Bcl-family proteins, mitochondria, and caspases) have not yet been elucidated.

Because innate or acquired resistance of cancer cells to clinically used taxanes remains a problem in chemotherapy (33), novel taxanes have been developed to overcome resistance (34-37). Some of these novel taxanes have proven to be significantly more effective than the classical taxanes in cell death induction in resistant cancer cells (7, 38).

We previously reported that novel fluorine-containing taxanes are effective in cell death induction in cancer cells as well as in cancer cells resistant to paclitaxel (7). In the present study, we showed that the novel fluorine-containing taxane SB-T-12854 (39) is effective in cell death induction in tested breast cancer cell lines SK-BR-3, MCF-7 and T47D. However, mechanisms of cell death induction seem to differ in these cells based on the observed changes in the expression of the Bcl-2 family proteins, the release of pro-apoptotic factors from mitochondria, and activation of caspases.

Materials and Methods

Materials. SB-T-12854 (39) was synthesized in the laboratory of Professor Iwao Ojima at the Institute of Chemical Biology and Drug Discovery (Stony Brook, NY, USA). For the structures of SB-T-12854 and classical paclitaxel see Figure 1. The taxane was dissolved in DMSO (tissue culture quality) to obtain a 1 mM stock solution. For western blot analysis, the following primary antibodies were used: rabbit monoclonal antibody against Bid (ab32060) and Bok (ab186745) from Abcam (Cambridge, UK), rabbit polyclonal antibody against Bad (#9292), Bax (#5023), Bik (#4592), and Bok (#4521), rabbit monoclonal antibody against Bcl-xL (#2764) and Bim (#2933), rabbit polyclonal antibody against cleaved caspase-3 (#9661), cleaved caspase-6 (#9761) and cleaved caspase-7 (#9491), rabbit monoclonal antibody against cleaved caspase-8 (#9496), rabbit polyclonal antibody against cleaved caspase-9 (#9505), rabbit polyclonal antibody against COX IV (#4844) and cytochrome *c* (#4272) and mouse monoclonal antibody against Smac/Diablo (#2954) from Cell Signaling Technology (Danvers, MA, USA), mouse monoclonal antibody against Bax (sc-7480) and Bcl-2 (sc-7382) from Santa Cruz Biotechnology, INC. (Dallas, TE, USA), rat monoclonal antibody against caspase-2 (ALX-804-356-C100) from Enzo Life Sciences (Farmingdale, NY, USA), and mouse monoclonal antibody against actin (AC-40, A3853) from Sigma-Aldrich.

Cells and culture conditions. Human breast carcinoma cell lines SK-BR-3, MCF-7, and T47D were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), the National Cancer Institute (Frederick, MD, USA), and the European Collection of Cell Cultures (ECCC, Porton Down, Salisbury, UK), respectively. SK-BR-3 adenoma cells are without functional p53, with functional caspase-3, and they overexpress Her2/Neu receptor. MCF-7 adenoma cells are with functional p53, without functional caspase-3 (40), and they carry progesterone and estrogen receptors. T47D ductal cells are without functional p53, with functional caspase-3, and they carry progesterone and estrogen receptors. These cell lines represent cells with differing status of key molecules and thus it helps elucidate various mechanisms involved in cell death induction. The basic medium was RPMI 1640 (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) (41). The culture medium consisted of basic medium supplemented with 10% heat-inactivated fetal bovine serum (Biocrom AG, Berlin, Germany). The cells were maintained in a culture medium at 37°C in a humidified atmosphere of 5% CO₂ in air. For experiments, taxanes were diluted in culture medium to achieve the required concentrations. Culture medium without taxane was used as controls.

Assessment of cell growth and survival. Cells were harvested and seeded at 20×10³ cells/100 µl of culture medium into wells of 96-well plastic plates. After a 24-h pre-incubation period, allowing cells to attach, the culture medium was replaced by either culture medium without taxane (control) or medium with tested taxane at the desired concentrations. Cell growth and survival were evaluated 96 h after taxane application. The number of living cells was determined using a hemocytometer after staining with trypan blue.

Preparation of cell lysates. Cells at desired concentrations were seeded in Petri dishes or culture flasks and taxane was added after a 24-h pre-incubation period. After the incubation period, cells were harvested by low-speed centrifugation (2,000 rpm, 9 min, 4°C), washed in PBS and centrifuged again. Cell pellets were stored at -80°C. Next, pellets were re-suspended in RIPA buffer (Sigma Aldrich, St. Louis, MO, USA) containing a 1% mixture of protease inhibitors (P8340, Sigma Aldrich). Protein lysates were centrifuged (14,000 rpm, 20 min, 4°C) and the supernatants containing proteins were stored at -80°C (24).

Cell fractionation. Cells (approximately 3.6×10⁶ cells per sample) were seeded into Petri dishes or culture flasks and taxanes were added after a 24-h pre-incubation. After the incubation period, cells were harvested by low-speed centrifugation (2,000 rpm, 9 min, 4°C), washed in PBS and centrifuged again. Cell pellets were re-suspended in a specific lysis buffer (75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, and 1% mixture of protease inhibitors P8340 from Sigma Aldrich, St. Louis, MO, USA) containing 0.635 mM digitonin D141 (Sigma-Aldrich) and vortexed for 60 s. Lysates were then centrifuged (14,000 rpm, 1 min, 4°C) and supernatants (containing cytosolic fractions) were removed and stored at -80°C. The specific lysis buffer described above, but containing 6.35 mM digitonin D141 (Sigma-Aldrich), was added to the pellets and suspensions were mixed for 60 s and centrifuged (14,000 rpm, 1 min, 4°C). After centrifugation, supernatants (containing mitochondrial fractions) were stored at -80°C. Cell fractions were analyzed using western blot.

Figure 1. Chemical structures of paclitaxel and SB-T-12854.

Western blot analysis. Concentration of proteins in cell lysates and cell fractions were assessed using the BCA Protein Assay Reagent from Pierce (Thermo Fisher Scientific, Rockford, IL, USA). Depending on protein concentrations, cell lysates and cell fractions were diluted in RIPA buffer to the gel loading concentration of proteins (2 µg/µl), mixed with equal volumes of sample buffer (0.125 M Tris/HCl pH 6.8, 10% glycerol, 4% SDS, 0.25 M DTT) and heated for 5-7 min at 110°C. Protein samples were separated using protein electrophoresis (Bio-Rad, Hercules, CA, USA). Proteins separated by SDS-PAGE were blotted onto a 0.2 µm PROTRAN BA 83 nitrocellulose membrane (Whatman-Schleicher and Schuell, Maidstone, UK) for 3 h at 0.25 A, using a MiniProtean II blotting apparatus (Bio-Rad). The membrane was blocked with 5% non-fat dry milk or 5% BSA in TBS for 15-20 min and incubated with the primary antibody at 4°C overnight. After the

incubation, the membrane was washed three times (5-10 min) with TBS containing 0.1% Tween-20. Then it was incubated for 1-2 h with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterward, the membrane was washed (as described above) and the chemiluminescence signal was detected using Supersignal reagents from Pierce (Thermo Fisher Scientific) and a CCD device (Carestream).

Results

Effect of fluorinated taxane SB-T-12854 on growth and survival.

We assessed the effect of fluorine-containing taxane SB-T-12854 (Figure 1) on cell growth and survival in breast cancer SK-BR-3, MCF-7 and T47D cells (see Materials and Method section) at wide range of concentrations (0.1-3,000 nM). In SK-BR-3 cells, SB-T-12854 induced cell death at a concentration of 30 nM and higher concentrations. C_{50} (concentration of taxane resulting in 50% of living cells relative to controls after 96 h of incubation) for SK-BR-3 cells was determined to be approximately 4 nM. In MCF-7 cells, the taxane also induced cell death at a concentration of 30 nM and higher concentrations. C_{50} for MCF-7 cells was also about 4 nM. Similar data were obtained with T47D cells. SB-T-12854 induced cell death at a concentration ≥ 30 nM and the C_{50} was approximately 5 nM (Figure 2).

In further experiments, we used the death inducing concentration, *i.e.* the lowest concentration with near maximum death-inducing effect, 100 nM of SB-T-12854 for SK-BR-3 cells and 600 nM for MCF-7 cells. For T47D cells, we used 300 nM because concentrations with a near maximum death-inducing effect were extremely high (Figure 2).

Effect of SB-T-12854 on activation of caspases.

We used western blot analysis in order to assess the activation of caspase-2, -8, -9, -3, -7, and -6 after application of SB-T-12854 at the death-inducing concentration in tested cell lines. Caspase activation was assessed by detecting the cleaved forms of caspases (see Materials and Methods section). Cleaved caspase-2, cleaved caspase-8 and cleaved caspase-9 appeared in significant amounts 36 h after taxane treatment in SK-BR-3 cells. Lower levels of cleaved caspase-8 and -9 were observed after only 24 h. As for executioner caspases, we detected high levels of cleaved caspase-3 and -7 after 36 h. Lower levels of cleaved caspase-3 and -7 were also observed after 24 h. Similarly, cleaved caspase-6 was detected 36 h after taxane treatment (Figure 3). In MCF-7 cells, the level of cleaved caspase-2 significantly increased 36 h after SB-T-12854 application. However, after 48 h the level substantially decreased. Cleaved caspase-8 was present after 36 as well as 48 h. Cleaved caspase-9 was detected between 24 h and 48 h after taxane application. Since there is not functional caspase-3 in MCF-7 cells (40), cleaved caspase-3 was not detected. Low levels of cleaved caspase-

Figure 2. Effect of SB-T-12854 (0.1-3,000 nM) on growth and survival of SK-BR-3, MCF-7, and T47D cells. Control cells (C) were incubated without SB-T-12854. The cells were seeded at 20×10^3 cells/100 μ l of medium per well (dotted line). The number of living cells was determined after 96 h of incubation (see Materials and Methods section). Each column represents the mean of two separate cultures $\pm$ SEM.

7 were seen after 24 h with a significant increase after 36 h. However, the level of cleaved caspase-7 decreased 48 h after taxane application. Cleaved caspase-6 was detected 36 h after taxane treatment with its subsequent decrease in cleaved caspase-6 level (Figure 3). A significant increase of the level of cleaved caspase-2 was not detected in T47D cells

after SB-T-12854 application. Concerning caspase-8, the level of cleaved caspase-8 increased between 72 h and 120 h after taxane application. Nearly undetectable levels of cleaved caspase-9 were observed between 48 h and 120 h after taxane treatment. Interestingly, only low levels of cleaved caspase-3 were detected after the treatment. Concerning other executioner caspases, levels of cleaved caspase-6 as well as caspase-7 were significantly increased 120 h after SB-T-12854 application (Figure 3).

To summarize, all tested initiator and executioner caspases were activated in SK-BR-3 cells within 36 h. The same was true for MCF-7 cells with the exception of the non-functional caspase-3. In T47D cells, only the activation of caspase-8, -7 and -6 was observed.

Effect of SB-T-12854 on levels of proteins of the Bcl-2 family. We measured changes in levels of proteins of the Bcl-2 family in cells after taxane treatment (see Materials and Methods section). The level of pro-apoptotic (channel forming) Bax protein did not change due to SB-T-12854 application, while the level of Bok protein significantly decreased 36 h after taxane application in SK-BR-3 cells (Figure 4A). The level of anti-apoptotic Bcl-2 protein significantly increased 12 h after taxane application. Most of proteins seemed to be phosphorylated (phosphorylated form is presented by the upper band of the double band observed). On the other hand, the level of anti-apoptotic Bcl-xL protein did not change significantly (Figure 4B). Concerning the BH3-only pro-apoptotic proteins of the Bcl-2 family, the level of Bad protein started to increase 12 h and peaked 24 h after taxane application. After 36 h a decrease was seen. The level of Bid protein decreased significantly 36 h after taxane treatment. The level of Bik seemed to increase 12 h after taxane treatment and then decreased 24 h after treatment. Bim level decreased significantly 24 h after treating with taxane (Figure 4C). In MCF-7 cells, SB-T-12854 did not significantly affect the level of Bax protein. However, the level of Bok protein increased significantly 36 h after taxane application with a subsequent decrease (Figure 4A). The level of anti-apoptotic Bcl-2 started to increase 12 h after taxane application. At the same time some of Bcl-2 was phosphorylated. Interestingly, Bcl-2 became significantly dephosphorylated 36 h after application. The level of Bcl-xL did not change significantly after taxane treatment (Figure 4B). As to the BH3-only proteins, the level of Bad seemed to be slightly increased 24 h after treatment. We did not observe any change in the level of Bid. Concerning Bik level, it appeared to increase 12 h after treatment. The level of Bim protein increased 12-24 h and decreased 36 h after taxane treatment (Figure 4C). There were no significant changes in the level of Bax and Bok proteins in T47D cells after SB-T-12854 application (Figure 4A). The level of anti-apoptotic Bcl-2 was found to decrease from 72 h to 96 h after taxane application. Phosphorylation of

Figure 3. Effect of SB-T-12854 on the activation of caspase-2, -8, -9, -3, -6 and -7 in SK-BR-3, MCF-7, and T47D cells. After incubation period with the death-inducing concentration of SB-T-12854 (0-36 h and 100 nM for SK-BR-3 cells, 0-48 h and 600 nM for MCF-7 cells, 48-120 h and 300 nM for T47D cells), levels of cleaved forms of caspases were determined using western blot analysis and relevant antibodies (see Materials and Methods section). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.

Bcl-2, which was detected in SK-BR-3 and MCF-7 cells, was not observed in T47D cells. The level of Bcl-xL did not seem to be significantly affected by taxane treatment (Figure 4B). Concerning the BH3-only proteins, the level of Bad protein started to increase 48 h after taxane application. The level of Bid protein seemed to decrease 72 h after taxane application. There was nearly no effect of the taxane on the level of Bik protein. The level of Bim protein was found to increase 48 h after SB-T-12854 treatment (Figure 4C).

In summary, higher levels of Bcl-2 together with its phosphorylation were detected in SK-BR-3 and MCF-7 after SB-T-12854 application. However, there was no up-regulation and phosphorylation of Bcl-2 in T47D cells. The level of Bad increased in all cell lines after application. The most conspicuous effect was observed in SK-BR-3 cells. Finally, the levels of Bok and Bim seemed to change significantly, but the changes differed in the individual tested cell lines.

Effect of SB-T-12854 on cytochrome c and Smac release from mitochondria. To clarify the involvement of mitochondria in apoptosis induction by SB-T-12854 in the tested cell lines, the release of cytochrome *c* and Smac protein from mitochondria into the cytosol was assessed using western blot analysis after cell fractionation (see Materials and Methods section). As expected, cytochrome *c* and Smac were detected in mitochondrial fractions in control SK-BR-3, MCF-7 as well as T47D cells. For SK-BR-3 cells, cytochrome *c* and Smac, in significant amounts, were detected in the cytosolic fraction 36 h after SB-T-12854 treatment at death-inducing concentration. We observed small amounts of cytochrome *c* in the cytosolic fraction of MCF-7 cells 36-48 h after taxane treatment, albeit it was not a significant release. We did not detect any release of cytochrome *c* or Smac into the cytosol of T47D cells after taxane treatment (Figure 5).

Figure 4. Effect of SB-T-12854 on the level of proteins of the Bcl-2 family in SK-BR-3, MCF-7, and T47D cells, i.e. (A) proapoptotic proteins of the Bax subfamily, (B) antiapoptotic proteins of the Bcl-2 subfamily and (C) proapoptotic proteins of the BH3-only subfamily. After the incubation period with the death-inducing concentration of SB-T-12854 (0-36 h and 100 nM for SK-BR-3 cells, 0-48 h and 600 nM for MCF-7 cells, 24-96 h and 300 nM for T47D), levels of the proteins were determined using western blot analysis and relevant antibodies (see Materials and Methods section). Actin levels were used to confirm equal protein loading. The data shown were obtained in one representative experiment of two independent experiments.

Thus, the mitochondrial pathway seems to play an important role in apoptosis induction in SK-BR-3 cells, but not in MCF-7 and T47D cells.

Discussion

In our previous studies on the mechanisms of cell death induction by taxanes in breast cancer cells, we reported that

during cell death induction by taxanes caspase-2 was involved as the apical caspase and its activation results in the mutual activation of caspase-3 and -7 (31, 32). In the present study we investigated molecular mechanisms of cell death induction by novel fluorinated taxane SB-T-12854 (39) in breast cancer cells. We used SK-BR-3 and T47D cells (without functional p53) and MCF-7 cells (without functional caspase-3) (see Materials and Methods section).

SB-T-12854 induced cells death in breast cancer cell lines examined (Figure 2). The taxane has also been shown to be effective in cell death induction in other cancer cell lines (7, 42). The effect of SB-T-12854 on the growth and survival of breast cancer cells was similar to the effect of previously studied novel non-fluorinated taxane SB-T-1216 (31, 32). Compared to SB-T-1216, the fluorine atoms in the structure of SB-T-12854 are supposed to increase the resistance of the taxane molecule to metabolism (42). Interestingly, both taxoids SB-T-12854 and SB-T-1216 were able to overcome the acquired resistance of breast cancer cells to paclitaxel (7, 43). Thus, SB-T-12854 seems to be a promising agent for induction of cell death in sensitive as well as resistant breast cancer cells.

Initiator and executioner caspases have been found to be activated in many types of cancer cell lines after the application of classical as well as novel taxanes (7, 12, 27, 31, 44). Activation of individual caspases seems to be cell type and also applied taxane specific. Therefore, we focused on the activation of all initiator and executioner caspases, except of caspase-10. In this study, we found that SB-T-12854 activated initiator caspase-8, caspase-9 and caspase-2 in SK-BR-3 and MCF-7 cells (Figure 3). Previously we have reported the activation of caspase-2 in breast cancer cells after taxane application, but this activation was not associated with PIDDosome formation (31). We detected a significant release of cytochrome *c* into the cytosol only in SK-BR-3 cells, but not in MCF-7 cells after SB-T-12854 treatment. Therefore, we suggest that activation of caspase-9 in MCF-7 cells could result from direct activation by caspase-2 (32). Interestingly, caspase-8 was the only initiator caspase activated significantly in T47D cells (see Figure 3). Concerning the mechanism of activation of caspase-8, it seems different from the previously described non-receptor activation of caspase-8 by caspase-3 (26) or by caspase-2 (32) since we did not observe significant activation of caspase-3 or caspase-2 in T47D cells. It seems that the role of caspase-8 in apoptosis induction after taxane treatment in T47D cells is more important than that in SK-BR-3 and MCF-7 cells as discussed previously (32).

Caspase-3 was significantly activated only in SK-BR-3 cells, taking into account the fact that there is not functional caspase-3 in MCF-7 cells. On the other hand, caspase-7 and caspase-6 were activated in all tested cells. The activation of executioner caspases has obviously been described after taxane application previously (12, 26, 32, 44) and caspase-3 was usually the most important cell death inducer. Caspase-3 is known as the executioner caspase functioning upstream of caspase-7 and caspase-6. This fact led to a question. How are caspase-7 and caspase-6 activated in MCF-7 and T47D cells? It is possible that initiator caspases, such as caspase-2 or caspase-8, are somehow involved. It seems that the activation of caspases induced by SB-T-12854 in SK-BR-3 and MCF-7 cells follows

Figure 5. Effect of SB-T-12854 on the release of cytochrome *c* and Smac protein from mitochondria in SK-BR-3, MCF-7, and T47D cells. After incubation period with the death-inducing concentration of SB-T-12854 (0-36 h and 100 nM for SK-BR-3 cells, 0-48 h and 600 nM for MCF-7 cells, 24-72 h and 300 nM for T47D cells), levels of cytochrome *c* and Smac were determined in mitochondrial (M) and cytosolic (C) fractions using western blot analysis and relevant antibodies (see Materials and Methods section). Cells incubated in taxane-free medium for 24 h (Control) were used as control for T47D cells. COX IV (integral mitochondrial protein) level was used to confirm proper fractionation. The data shown were obtained in one representative experiment of two independent experiments.

a previously proposed scheme (32). The activation of caspases seems to differ in T47D cells. In particular, caspase-8 may play a more important role here (see above).

Proteins of the Bcl-2 family are known to play an important role in taxane-induced cell death in many types of cancer cells (12, 21, 22, 45). Concerning proteins of the Bcl-2 family, we decided to test channel-forming Bax protein and less studied Bok protein. Anti-apoptotic Bcl-2 and Bcl-xL proteins were selected according to our preliminary data. Selected Bad, Bid, Bik and Bim proteins from the BH3-only subfamily had been previously reported to have potential functions in cell death induction by taxanes. As for the channel forming pro-apoptotic proteins of this family, Bok expression increased after SB-T-12854 treatment in MCF-7 cells, showing a possible role in cell death induction in these cells (Figure 4A). As far as we know, there are no data in the literature demonstrating the role of Bok in taxane-induced cell death in breast cancer cells. Interestingly, Bok has been reported to be important in cell death induction after blocking the cell cycle (46), which results from taxane treatment. However, there was no significant increase in Bok levels in SK-BR-3 and T47D cells and thus Bok does not seem to play a key role in cell death induction by SB-T-12854 in breast cancer cells.

Considering anti-apoptotic proteins of the Bcl-2 family, we observed increased levels of Bcl-2 in SK-BR-3 and MCF-7 cells after SB-T-12854 application. In addition, Bcl-2 was phosphorylated in both cell lines after taxane application. In T47D cells, the level of Bcl-2 decreased after SB-T-12854 treatment and there was no significant phosphorylation of Bcl-2 (Figure 4B). Increased level of Bcl-2 has been previously shown to be protective cells against the effect of taxanes (12). However, some contra-indicatory data exist concerning the role of Bcl-2 in taxane effect (14). We suggest that increased levels of Bcl-2 followed by its phosphorylation could represent some type of protective mechanism of SK-BR-3 and MCF-7 cells against taxane effect. The situation seems to be different in T47D cells, where decreasing levels of Bcl-2 after taxane application correlated with cell death induction (Figure 4B).

As for the BH3-only proteins of the Bcl-2 family, the level of Bad was found to be more or less transiently increased in all tested cells (Figure 4C). Apoptosis induction by taxanes has been shown to be associated with the pro-apoptotic activity of Bad protein (12). Furthermore, the involvement of Bad in the regulation of cell-cycle progress associated with cell death induction has also been demonstrated (22). We assume that Bad is, directly or indirectly, involved in cell death induction by SB-T-12854. The level of Bim was transiently higher in MCF-7 and T47D cells after SB-T-12854 application (Figure 4C). Bim protein can induce cell death by affecting Bcl-2 protein during cytoskeleton stress, however, recently other indirect mechanisms of cell death induced by Bim that were associated with mitotic aberrations

have been described (21, 45, 47). This could also be the case of tested MCF-7 and T47D cells. However, Bim levels were significantly decreased after taxane application in SK-BR-3 cells (Figure 4C). Similarly, a decrease of Bik and Bid levels was also found in SK-BR-3 cells after SB-T-12854 application. The relationship between the decrease and cell death induction remains unclear.

Previously we and other groups have reported that cell death dependent on as being well as independent of cytochrome *c* release from mitochondria into the cytosol. It means dependence on or independence from mitochondrial the apoptotic pathway (24, 44). Thus, we tested activation of this crucial pathway of apoptosis induction. Significant release of cytochrome *c* and Smac into the cytosol after SB-T-12854 application was only observed in SK-BR-3 cells (Figure 5). Thus, it is likely that caspase-9 is activated by an alternative mechanism unrelated to the classical mitochondrial apoptotic pathway in MCF-7 cells. We suggest that the mitochondrial apoptotic pathway is activated only in SK-BR-3 cells after taxane application and it is not required for cell death induction in MCF-7 and T47D cells.

We can conclude that the novel taxane SB-T-12854 induces cell death effectively in SK-BR-3, MCF-7 and T47D breast cancer cells. The activation of initiator caspase-2, -8, -9, in SK-BR-3 and MCF-7 cells and caspase-8 in T47D cells, as well as activation of executioner caspase-3 in SK-BR-3 cells and caspase-7, -6 in all tested cell lines were found to be associated with SB-T-12854 application. The pro-apoptotic Bad protein of the Bcl-2 family seems to be important for cell death induction by taxane SB-T-12854 in the tested cells. The anti-apoptotic Bcl-2 protein also seems to be associated with apoptosis induction in SK-BR-3 and MCF-7 cells and the pro-apoptotic Bim in MCF-7 and T47D cells. Similarly, the pro-apoptotic Bok in MCF-7 cells as well as the pro-apoptotic Bid and Bik in SK-BR-3 cells could be involved in apoptosis induction by SB-T-12854. The mitochondrial apoptotic pathway, after SB-T12854 application, was significantly activated in association with the release of cytochrome *c* and Smac from mitochondria, but only in SK-BR-3 cells, and not in MCF-7 and T47D cells.

In summary, cell death induced by SB-T-12854 in the tested breast cancer cells differs with regard to the activation of caspases, changes in levels of pro-apoptotic as well as anti-apoptotic proteins of the Bcl-2 family, and release of pro-apoptotic factors (cytochrome *c*, Smac) from mitochondria into the cytosol.

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