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Sensitization to gimatecan-induced apoptosis by tumor necrosis factor-related apoptosis inducing ligand in prostate carcinoma cells

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TRAIL, TNF-related apoptosis inducing ligand
TPT, topotecan
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siRNA, small interfering RNA

ABSTRACT

Since the intrinsic resistance of prostate carcinoma likely reflects a low susceptibility to drug-induced apoptosis, in this study we explored the possibility of sensitizing prostate carcinoma cells to apoptosis by combination of TRAIL with camptothecins. Indeed, these agents are known to activate different pathways of apoptosis. Topotecan- and gimatecan induced moderate up-regulation of TRAIL-R1 and -R2 which resulted in a different cell response to the combination in androgen-independent cells (DU-145 and PC-3). In DU-145 cells apoptosis was increased by lower TRAIL concentrations and was earlier than in PC-3 cells, as shown using Annexin V-binding assay. The relative resistance of PC-3 cells to drug-induced apoptosis was associated with constitutive Akt activation, higher levels of cFLIP-L and Bcl-2, and lower levels of Bax. The different expression/activation of apoptosis-related factors appears to influence the sensitization of prostate carcinoma cells by TRAIL. Potentiation of camptothecin-induced apoptosis by TRAIL appears dependent on cooperation between extrinsic and intrinsic pathways, as documented by loss of the sensitization to apoptosis following reduction of caspase 8 after small interfering RNA transfection. The efficacy of the approach may be critically dependent on the intrinsic susceptibility to apoptosis of different tumors. These observations support that the activation of multiple signals could enhance apoptotic response and suggest the therapeutic interest of the TRAIL/camptothecin combination.

1. Introduction

Camptothecins, the clinically available DNA topoisomerase I inhibitors, are antitumor agents characterized by a wide spectrum of activity [1,2]. The development of lipophilic analogs has led recently to the identification of a novel 7-substituted camptothecin (ST1481, gimatecan), which appears a promising drug for the treatment of solid tumors. Cellular studies documented a high cytotoxic potency in several human tumor cell lines and drug capability to overcome

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multidrug resistance mediated by different transporters [3,4]. Preclinical evaluation of the antitumor efficacy in human tumor xenografts indicated potent antitumor activity and improved pharmacological profile as compared to topotecan (TPT) [3]. In particular, ST1481 exhibited an excellent activity against prostate carcinoma cells.

Prostate cancer has few treatment options and advanced disease has no cure, because hormone-refractory prostate cancer is a chemotherapy-resistant disease [5]. Resistance of prostate cancer cells to traditional chemotherapy stimulates the efforts to develop new agents or novel regimens which may be effective against resistant cells. The intrinsic resistance of prostate carcinoma to the conventional cytotoxic agents likely reflects a reduced susceptibility to drug-induced apoptosis. A number of studies have reported that various cytotoxic agents, in particular DNA damaging agents, could up-regulate cell death receptors [6,7]. This evidence supports the potential therapeutic potential of the combination of cytotoxic agents and TRAIL (TNF-related apoptosis inducing ligand). TRAIL is known to trigger programmed cell death upon binding and activation of the two major receptors (TRAIL-R1 and -R2) which transduce a signal that activates caspase 8, thereby starting a cascade of events leading to apoptosis [6,8]. It has been documented that TRAIL uses a pathway similar to the one used by the Fas/CD95 receptor [9]. In addition to TRAIL-R1 and -R2, two other receptors have been described, TRAIL-R3 and TRAIL-R4, which cannot mediate an apoptotic signal as they act as decoy receptors, and are largely expressed on normal cells, likely to protect cells from apoptosis [10]. For this reason, TRAIL is able to induce apoptosis in cancer cells but not in normal cells and therefore, in contrast to other apoptosis inducing ligands, may have the potential to be used with other apoptosis inducing agents. Thus, TRAIL is a potential candidate for therapies aimed at lowering the threshold for an apoptotic response [6,8]. The present study was designed to explore the possibility of sensitizing prostate carcinoma cells to drug-induced apoptosis by combined exposure to DNA topoisomerase I inhibitors and TRAIL, using cell lines characterized by low sensitivity to TRAIL-mediated apoptosis and by a different pattern of expression of factors involved in regulation of apoptosis [11].

The results show that exposure to TPT or ST1481 resulted in mild up-regulation of the expression of TRAIL-R1 and -R2 and in an increased apoptotic response in different cell systems, but the ability of TRAIL to sensitize cells to the camptothecins was not closely related to the level of up-regulation of TRAIL receptors. Our results suggest that potentiation of camptothecin-induced apoptosis by TRAIL is dependent not only on TRAIL receptor expression, but likely involves differential activation of apoptosis-related factors and cooperation between different apoptotic pathways.

2. Materials and methods

2.1. Drugs and reagents

TPT, kindly supplied by Smith-Kline Beecham Pharmaceuticals (King of Prussia, PA), was primarily dissolved in sterile distilled water and diluted in saline. ST1481 (gimatecan, 7-butoxyiminomethylcamptothecin) [12], obtained from Sigma–Tau (Pomezia, Italy) was dissolved in dimethyl-sulfoxide and working solutions were prepared in sterile distilled water. Soluble recombinant TRAIL was purchased from Alexis (Alexis Corporation Ltd., UK).

2.2. Cell lines and cell sensitivity to drugs

The human androgen-independent prostate carcinoma cell lines PC-3 and DU-145 were used in this study. The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Invitrogen Italia, San Giuliano Milanese, Italy). The cell sensitivity to antitumor agents was measured by using a growth-inhibition assay based on cell counting [13]. Exponentially growing cells were harvested and seeded in duplicate into six-well plates (3.5 cm diameter) and exposed to drug 24 h later. After 24 h of drug incubation, the medium was replaced with fresh medium. Cells were harvested 48 h later for counting with a cell counter. IC_{50} is defined as the drug concentration producing 50% decrease of cell growth.

2.3. Expression of TRAIL receptors

The expression of TRAIL receptors was measured by flow cytometry. Cells were seeded in 24-well plates, and 24 h later, they were exposed to the drug for 24 h. Cells were then harvested and incubated for 30 min at 4 °C with 1 μg of biotinilated anti-human TRAIL-R1 or -R2 antibody (R&D Systems, Minneapolis, MN, USA), washed twice with PBS and incubated in PBS containing 2 μl of streptavidin PE or FITC (BD Pharmingen, Becton-Dickinson, Mountain View, CA). Cells were then washed and samples were immediately used for flow cytometric analysis (FACStar Plus, Becton-Dickinson). Data were analysed using Cell Quest (Becton-Dickinson).

2.4. Apoptosis measurement

The percentage of apoptotic cells was analysed by flow cytometry, using Annexin V-binding assay (Bender MedSystem, Austria) [14]. Since apoptotic cells tend to detach from the culture plate and to float in the medium, the analysis was performed on non-adherent and adherent cells together. Briefly, PC-3 or DU-145 cells were seeded in 24-well plates and exposed to ST1481 or TPT for 24 h. Twenty-four hours later, TRAIL was added to medium for 24 h and cells were harvested at the end of incubation with TRAIL for the Annexin-V binding assay. For analysis of delayed apoptosis induction, PC-3 cells were exposed to ST1481 alone for 24 h and harvested 120 h after drug removal, or to a 24 h sequential treatment of camptothecin followed by 10 ng/ml TRAIL for additional 24 h after ST1481 removal. Culture plates were incubated for 96 h after TRAIL removal and cells were then harvested for Annexin V-binding assay. Floating cells were collected from the medium by centrifugation, while non-enzymatic cell dissociation solution (Sigma, St Louis, MO) was used for adherent cells. Total cells were processed for Annexin V-binding following manufacturer's instructions. Fluorescence was measured by flow cytometry using a Facstar Plus and data analysed with a specific software (Cell Quest, Becton-Dickinson).

2.5. Small interfering RNA duplexes and transfections

A small interfering RNA (siRNA) duplex targeting caspase 8 (StealthTM, Invitrogen; sequence: 5-CCUGAAGGAGCUGCUCUUCCGAAUU-3′) was used in the present study. Preliminary experiments were performed to define optimal siRNA concentrations and exposure times. The efficiency of down-regulation of target expression was monitored by Western blotting. For apoptosis experiments, cells were seeded in 12-well plates in triplicate (80,000 cells/well) and transfected 24 h later for 20 h with specific siRNA or control siRNA using oligofectamine (Invitrogen) following the manufacturer protocol, with minor modifications. The ratio between oligofectamine and serum-free medium was 1:100 and the concentration of siRNA was 10 nM. At the end of transfection, medium was replaced with fresh medium and 5 h later cells were incubated for 24 h with ST1481. Upon drug removal, TRAIL (1 ng/ml) was added for 24 h and cells were then harvested for Annexin-V binding assay. Three independent experiments were performed with triplicate samples, and in each experiment we monitored the decrease of caspase 8 before drug treatment and at the end of TRAIL exposure by Western blotting.

For growth inhibition assays, DU-145 cells were seeded in 12-well plates in triplicate and 24 h later they were transfected with control siRNA or caspase 8 siRNA as detailed above. Five h after removal of the transfection mix, cells were exposed to the ST1481 for 24 h and, after drug removal, 1 ng/ml TRAIL was added for 24 h. At the end of incubation with TRAIL cells were counted using a cell counter. Two independent experiments were performed.

2.6. Western blot analysis

Western blot analysis was carried out as described previously [15]. Briefly, samples were fractionated by SDS-PAGE and blotted on nitrocellulose sheets. Blots were preblocked in PBS containing 5% (w/v) dried non fat milk and then incubated overnight at 4 °C with antibodies to caspase 8 (BD Pharmingen), caspase 9 (Oncogene Research Products, Boston, MA), Phopho-Akt/Ser473 (Cell Signaling Technology, Beverly, MA), PKBα/Akt (Transduction Laboratories, Becton-Dickinson), Bax (BD Pharmingen), Bcl-2 (Dako, Glostrup, Denmark), Bid (Cell Signaling Technology), Bak (Sigma), FLIP (Santa Cruz Biotechnology Inc.). A rabbit anti-actin antibody (Sigma Chemicals Co., St. Louis, MO) was used as control for loading. Antibody binding to blots was detected by chemiluminescence (Amersham Pharmacia Biotech., Cologno Monzese, Italy). For all Western blot analyses at least three independent experiments were performed.

3. Results

3.1. Cell sensitivity to DNA topoisomerase I inhibitors and to TRAIL

The sensitivity of prostate carcinoma PC-3 and DU-145 cells to the topoisomerase I inhibitors TPT and ST1481 was examined using the growth-inhibition assay after 24 h drug exposure

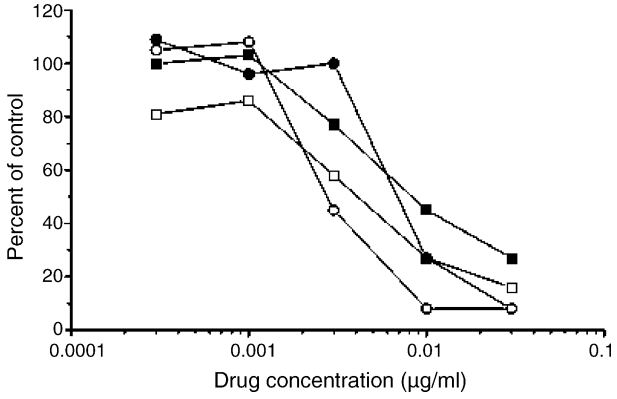

Fig. 1 – Cellular sensitivity of prostate carcinoma cells to camptothecins. Cell sensitivity was assessed by growth-inhibition assay after 24 h drug exposure to topotecan (TPT) or ST1481. Cells were treated 24 h after seeding and harvested 48 h after the end of drug exposure and counted using a cell counter. Values from a representative experiment (of 4) are shown. Symbols: closed squares, PC-3 cells treated with TPT; open squares, PC-3 cells treated with ST1481; closed circles, DU-145 cells treated with TPT; open squares, DU-145 cells treated with ST1481.

(Fig. 1). The cell lines exhibited a similar sensitivity to TPT, the IC_{50} values being 0.0089 ± 0.02 and 0.011 ± 0.002 μg/ml for PC-3 and DU-145 cells, respectively. ST1481 was substantially more potent as IC_{50} values were significantly lower than those observed for TPT in both cell lines (0.0026 ± 0.0002 μg/ml for PC-3 cells and 0.0034 ± 0.0002 μg/ml for DU-145 cells; ANOVA: $p < 0.05$). Under the same treatment conditions, equitoxic concentrations (IC_{80}) of both camptothecins induced a lower level of apoptosis in PC-3 than in DU-145 cells (around 10% in PC-3, 60% in DU-145, see below). This observation indicates that the antiproliferative activity was more pronounced in PC-3 than in DU-145 cells.

An analysis of cell sensitivity to TRAIL using the growth inhibition assay after 24 h exposure indicated no significant antiproliferative effect when cells were challenged with TRAIL concentrations ranging between 1 and 1000 ng/ml. Low TRAIL concentrations (1 and 10 ng/ml) were totally inactive, and a marginal inhibition of cell proliferation (36%) was observed in DU-145 cells treated with 1000 ng/ml TRAIL. At the same concentrations growth inhibition was around 20% in PC-3 cells.

3.2. Expression of TRAIL receptors

PC-3 and DU-145 cells expressed both TRAIL-R1 and TRAIL-R2 as detected by flow cytometry. In DU-145 cells, the basal expression of the receptors was about two-fold higher than that observed in PC-3 cells (Fig. 2A). The analysis of expression of TRAIL receptors after 24 h exposure to TPT or ST1481 indicated a moderate up-regulation of TRAIL-R2 in PC-3 and DU-145 cell lines (Fig. 2B). However, a differential modulation of TRAIL-R1 was found in the two cell systems, because drug treatment produced up-regulation only in PC-3 cells, whereas the drug effect was marginal in DU-145 cells. In both cell lines,

the modulation was detectable 24 h following drug removal, whereas no modulation could be detected after earlier exposure times (e.g., 6 h, not shown).

3.3. Apoptosis induction by combined treatment with TRAIL and camptothecins

As exposure to DNA topoisomerase I inhibitors resulted in a variable up-regulation of TRAIL receptors in the studied cell lines, we explored the possibility of increasing cell death induced by ST1481 by sequential TRAIL exposure. For this purpose, the cell systems were exposed for 24 h to drug concentrations corresponding to IC_{50} or IC_{80} and, after drug removal, 1 ng/ml TRAIL was added to the culture medium for 24 h. An analysis of apoptosis induction by Annexin-V binding assay indicated that addition of TRAIL to DU-145 cells pretreated with ST1481 resulted in a significant increase of drug-induced apoptosis (ANOVA, $p < 0.05$, when comparing the effect of ST1481 alone versus ST1481 plus TRAIL, Fig. 3). The sensitization was already evident at the lower concentration of ST1481. A different result was found in PC-3 cells in which no increase in apoptosis was observed upon exposure to the same TRAIL concentration (1 ng/ml, not shown). However, sensitization to apoptosis induced by camptothecins in PC-3 cells was observed using an increased concentration of TRAIL (10 ng/ml). Under these conditions, TRAIL increased camptothecin-induced apoptosis (ANOVA, $p < 0.05$ when comparing the effect of camptothecins versus camptothecin plus TRAIL) and the highest level of induced apoptosis observed was around 40%.

To investigate whether drug-induced apoptosis was a delayed event in PC-3 cells, cells were exposed to ST1481 or to a sequential treatment with ST1481 followed by 10 ng/ml TRAIL for 24 h and then they were incubated for 96 h after TRAIL removal in drug-free medium. Under these conditions, we found a marked level of ST1481-induced apoptosis that was further increased by TRAIL addition (ANOVA, $p < 0.05$; Table 1).

3.4. Modulation of apoptosis-related proteins

To gain insights into the cellular bases of differential apoptotic response of the two hormone-independent cell systems (DU-145 and PC-3) exhibiting quite a different susceptibility to apoptosis, we performed a biochemical analysis of activation of selected apoptosis-related proteins. Caspase 8 was investigated because it participates in triggering receptor-mediated apoptosis [9]. Caspase 8 activation (evidenced by the appearance of the proteolitically cleaved 40/36 doublet and 23 kDa subunit) was detected in DU-145 cells exposed to cytotoxic drug concentrations (Fig. 4A). Activation was enhanced by combination with TRAIL. No activation of caspase 8 was observed in PC-3 cells exposed to 1 ng/ml TRAIL alone or in combination with the studied camptothecins (not shown). As shown in Fig. 4B, a detectable activation of caspase 8 was found in PC-3 cells following exposure to 10 ng/ml TRAIL alone

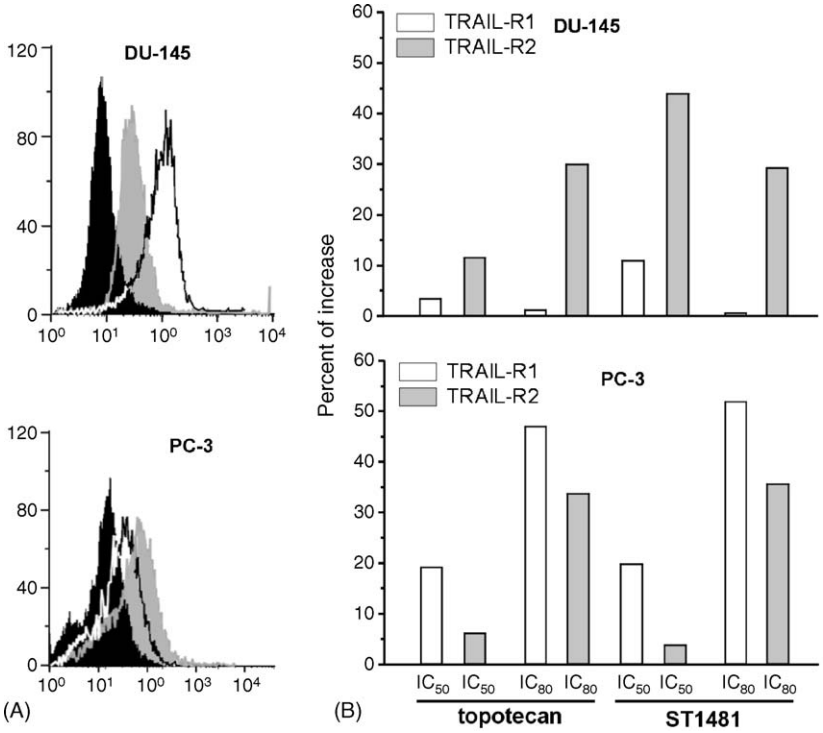

Fig. 2 – Expression of TRAIL receptors in untreated and drug-treated DU-145 and PC-3 cells. The levels of expression of TRAIL-R1 and -R2 were examined by flow cytometry. (A) Basal levels are shown. Black histograms refer to isotypic controls, light grey histograms refer to TRAIL-R1 and black line histograms refer to TRAIL-R2. (B) Cells were exposed to cytotoxic concentrations of topotecan (TPT) or ST1481 for 24 h. A representative experiment (out of 5) is shown. Results on modulation of TRAIL receptors expression are expressed as ratio between increase in mean fluorescence intensity of treated cells and untreated cells normalised for the increase of the two respective isotypic controls.

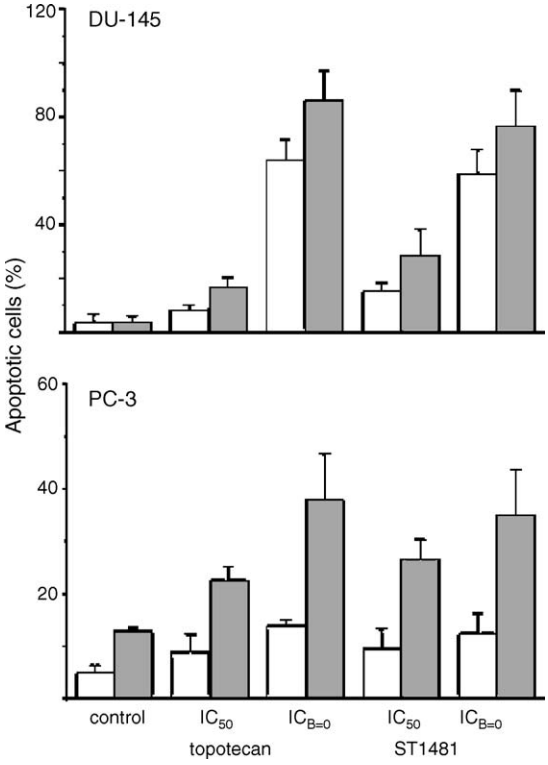

Fig. 3 – Induction of apoptosis in prostate carcinoma cells exposed to camptothecins and TRAIL as detected by Annexin V-binding assay. Cells were exposed to the drug for 24 h (IC_{50} or IC_{80}) and, after drug removal, TRAIL (1 ng/ml for DU-145, 10 ng/ml for PC-3 cells; grey bars) was added for 24 h. Values are the mean (±S.D.) of six independent experiments.

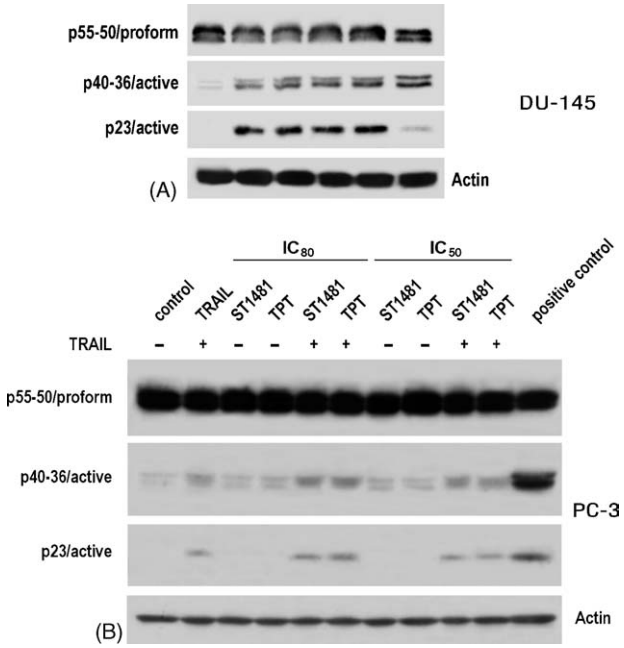

Fig. 4 – Western blot analysis of activation of caspase 8 in prostate carcinoma cells. (A) DU-145 cells were exposed to the ST1481 or topotecan (TPT; IC_{80}) for 24 h or to a sequential treatment with the camptothecin followed by TRAIL (1 ng/ml) for additional 24 h after removal of the camptothecin. (B) PC-3 cells were treated as indicated above using a higher TRAIL concentration (10 ng/ml). Cells were harvested at the end of TRAIL exposure and then processed for Western blot analysis. Control loading is shown by actin.

or in combination with ST1481 or TPT. Thus, activation of caspase 8 paralleled apoptosis induction (Fig. 3).

To examine the involvement of caspase 8 in apoptosis of DU-145 cells undergoing combined treatments, we used a siRNA approach to specifically inhibit the expression of caspase 8. Transfection of the siRNA duplexes targeting caspase 8 markedly reduced its protein level (Fig. 5A). The effect was evident at the end of transfection (0 h) and persisted at least for additional 48 h. The level of caspase 8 was not affected by the control siRNA. After exposure to ST1481, the sensitization to drug-induced apoptosis by TRAIL was significantly attenuated in the caspase 8 siRNA-transfected cells as compared to control siRNA transfected cells (Fig. 5B, ANOVA, $p < 0.05$). Cell growth inhibition assays indicated that caspase 8 was required for potentiation of ST1481 cytotoxicity. Indeed, a significant increase in inhibition of cell growth was observed only in DU-145 cells transfected with control siRNA (Table 2).

Exposure of DU-145 cells to camptothecins resulted in up-regulation of Bax expression that was more marked at the IC_{80} in combination with TRAIL (Fig. 6A). Bak levels were increased in treated cells. Disappearence of the full length 22 kDa form of Bid (suggesting truncation of Bid) was observed in the combination of IC_{80} of ST1481 or TPT with TRAIL ando also at the IC_{50} for ST1481.

Under the same experimental conditions, caspase 9 appeared to be activated in treated DU-145 cells, as shown by decrease of the p46/48 proenzyme level in cells exposed to DNA topoisomerase I inhibitors alone or in combination with TRAIL (Fig. 6B and C). Activation of caspase 9 was dose-dependent as observed for caspase 8. No marked activation of caspase 9 was found in PC-3 cells following exposure to 10 ng/ml TRAIL alone or in combination with ST1481 or TPT (Fig. 6D).

Table 1 – Delayed apoptosis induction in PC-3 cells exposed to ST1481 or to ST1481 in combination with TRAIL[a]

	Apoptosis (%)
Control	3.6 ± 0.6
TRAIL	3.2 ± 0.4
ST1481 (IC_{50})	70.3 ± 0.3
ST1481 (IC_{80})	76.2 ± 3.6
ST1481 (IC_{50}) + TRAIL	84.5 ± 0.9
ST1481 (IC_{80}) + TRAIL	90.0 ± 1.0

[a] Cell susceptibility to apoptosis was assessed by Annexin-V binding assay. Cells were exposed to ST1481 for 24 h or to a sequential treatment with the camptothecin followed by TRAIL (10 ng/ml) for additional 24 h after removal of ST1481; cells were harvested 96 h after TRAIL removal. Values are the mean (±S.D.) of three independent experiments.

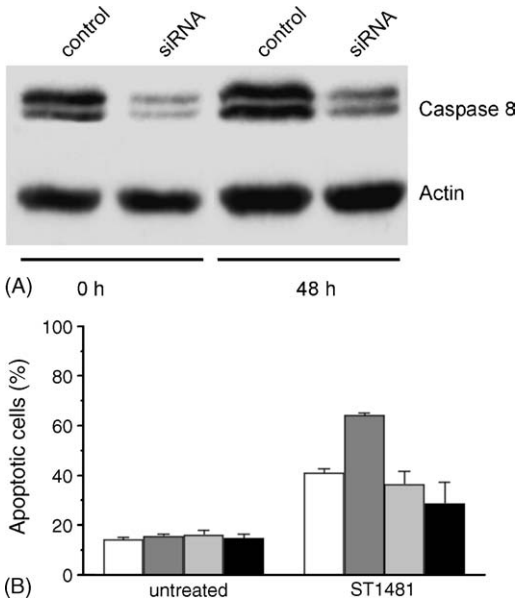

Fig. 5 – Effect of transfection of caspase 8 siRNA in DU-145 cells. (A) Western blot analysis of samples prepared from cells incubated for 24 h with a caspase 8 targeting siRNA or a control siRNA. Harvesting was at the end of incubation (0 h) and 48 h later. Control loading is shown by actin. (B) Induction of apoptosis in DU-145 cells exposed to ST1481 and TRAIL after transfection of caspase 8 or control siRNAs as detected by Annexin V-binding assay. Cells were exposed to the drug for 24 h (IC_{50}) and, after drug removal, 1 ng/ml TRAIL was added for 24 h. White columns, cells incubated with control siRNA; grey columns, control cells plus TRAIL; light grey columns, cells incubated with caspase 8 siRNA; black columns, cells incubated with caspase 8 siRNA plus TRAIL. Values are the mean (±S.D.) of three independent experiments.

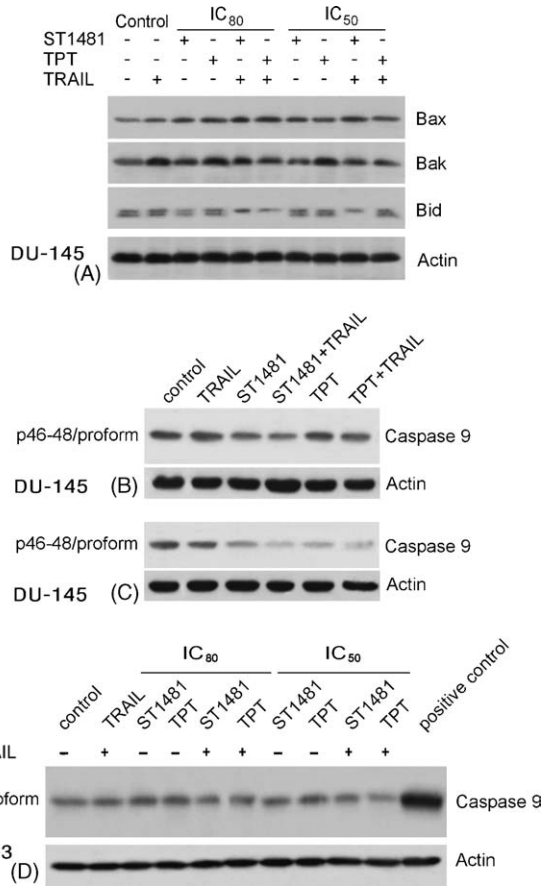

Fig. 6 – Western blot analysis of apoptosis-related proteins. (A) DU-145 cells were exposed to camptothecins (ST1481 or topotecan, TPT) for 24 h or to a sequential treatment with the camptothecin followed by TRAIL (1 ng/ml) for additional 24 h after removal of the camptothecin. (B) DU-145 cells were treated as indicated in (A) with IC_{50} concentrations. (C) DU-145 cells were treated as indicated in (A) with IC_{80} concentrations. (D) PC-3 cells were treated as indicated above using a higher TRAIL concentration (10 ng/ml). Cells were harvested at the end of TRAIL exposure and then processed for Western blot analysis. Control loading is shown by actin.

Since PC-3 cells are known to lack functional PTEN protein and to exhibit constitutive activation of Akt [16,17], we examined basal Akt expression and activation which can be evidenced by phosphorylation at Ser473 in PC-3 and DU-145 cells. Indeed, whereas the basal amount of the inactive kinase was similar in the two cell systems, activating phosphorylation at Ser473 was evident only in PC-3 cells (Fig. 7A). Under the same

Table 2 – Cell sensitivity of DU-145 cells to ST1481 and TRAIL after transfection of caspase 8 or control siRNAs as detected by growth inhibition assay[a]

ST1481 (μg/ml)	Percent of cell growth (mean ± S.D.)			
	Control siRNA + TRAIL		Caspase 8 siRNA + TRAIL	
0.001	109 ± 8.1	78.0 ± 4.1[b]	79.2 ± 3.2	81.0 ± 1.3
0.003	79.0 ± 5.0	59.0 ± 3.0[b]	78.6 ± 1.0	76.1 ± 1.9
0.0045	19.3 ± 0.8	12.4 ± 0.19[b]	26.0 ± 0.3	24.0 ± 0.3
0.006	12.9 ± 0.8	5.9 ± 0.48[b]	18.6 ± 2.5	15.2 ± 1.9

[a] DU-145 cells were seeded and 24 h later they were transfected for 20 h with control siRNA or caspase 8 siRNA. Cells were then exposed to the ST1481 for 24 h and, after drug removal, 1 ng/ml TRAIL was added for 24 h. At the end of incubation with TRAIL cells were counted using a cell counter.

[b] $p < 0.01$ vs. control siRNA without TRAIL by ANOVA.

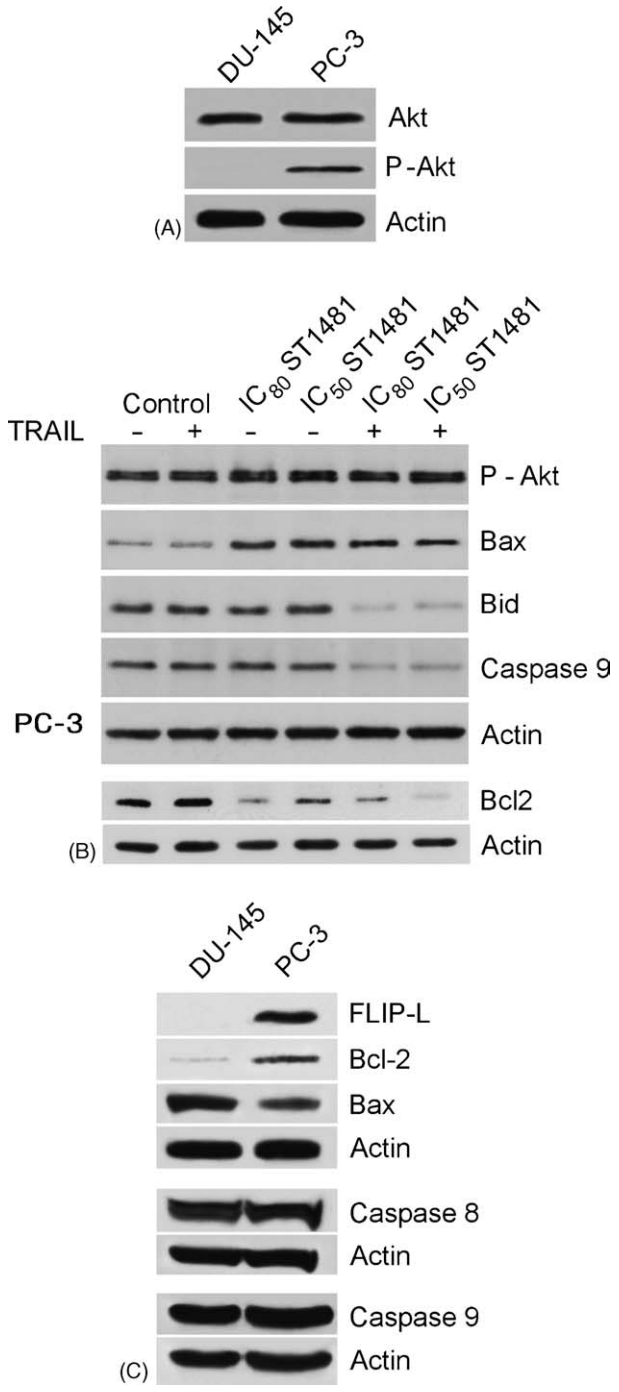

Fig. 7 – Levels of apoptosis-related proteins in prostate carcinoma cells. (A) Exponentially growing cells were harvested for Western blot analysis. Akt, total Akt; P-Akt, Akt phoshorylated at Ser^{473}. (B) PC-3 cells were exposed to ST1481 (IC_{50} or IC_{80}) for 24 h or to a sequential treatment with the camptothecin followed by TRAIL (10 ng/ml) for additional 24 h after removal of ST1481; cells were then processed for Western blot analysis 96 h after TRAIL removal. Caspase 9, p46–48 proform. (C) Exponentially growing cells were harvested for Western blot analysis. Caspase 8, p55–50 proform; caspase 9, p46–48 proform. Control loading is shown by actin.

experimental conditions used for analysis of delayed drug-induced apoptosis (96 h after TRAIL removal), no modulation of Akt phosphorylation was found (Fig. 7B). Exposure of cells to ST1481 induced up-regulation of the proapoptotic protein Bax not affected by addition of TRAIL, whereas Bcl-2 was down-regulated in camptothecin-treated cells both in the presence of TRAIL and without TRAIL. A marked activation of caspase 9 as well as down-regulation of full length Bid were evident in cells treated with both ST1481 and TRAIL. PC-3 cells expressed higher levels of cFLIP-L and Bcl-2 than DU-145 cells and lower levels of Bax (Fig. 7C). Basal expression of caspase 8 and 9 was not different in the two cell systems.

4. Discussion

The present results clearly support the ability of TRAIL to sensitize human prostate carcinoma cells to camptothecins. The potentiation of drug effects by TRAIL reflected an increased induction of apoptosis in androgen-independent (DU-145 and PC-3) cells, under conditions where TRAIL alone was substantially inactive as apoptosis inducer (Fig. 3). The two chosen androgen-refractory cell lines selected for this study exhibited a substantially different susceptibility to apoptosis (Fig. 3). Exposure of DU-145 or PC-3 cells to TRAIL per se was not sufficient to induce significant levels of apoptosis, in spite of expression of the death receptors TRAIL-R1 and -R2 (Fig. 2). Our study shows that the expression of TRAIL receptors could be enhanced by exposure to camptothecins in cells exhibiting low susceptibility to TRAIL-mediated apoptosis and that a slight increase of expression could be associated with a modulation of apoptotic response (Figs. 2 and 3). Since camptothecins are known to activate both extrinsic and intrinsic pathways of apoptosis [18], a plausible explanation of the synergistic interaction is an enhanced cooperation among multiple pathways to induce an apoptotic response. The evidence is supported by the finding that transfection of caspase 8 siRNA duplexes, which efficiently reduced caspase 8 levels, significantly attenuated the sensitization to ST1481-induced apoptosis by TRAIL in DU-145 cells (Fig. 5).

Thus, the two studied model systems displayed major differences in terms of activation of the apoptotic pathways in response to the camptothecins or to the combinations. In PC-3 cells, a slight induction of apoptosis was observed after exposure to equitoxic concentrations of camptothecins (Fig. 3). Since equitoxic concentrations of the drugs are referred to cell growth inhibition (Fig. 1), this finding indicates that the cytostatic activity of the camptothecins was more pronounced than its cytotoxic activity in PC-3 as compared with DU-145 cells. The apoptotic response was increased in both cell lines in the presence of TRAIL, but in PC-3 cells the effect required a substantially higher concentration of the ligand (Fig. 3). Moreover, caspase 8 was activated in PC-3 cells only in the presence of TRAIL (Fig. 4B). The low susceptibility of PC-3 cells to apoptosis could be related to the expression of proteins promoting survival and blocking apoptosis (Fig. 7A–C). Indeed, Akt was constitutively active due to PTEN loss [16,17] and we have documented that untreated PC-3 cells express phosphorylated Akt (Fig. 7A). The constitutive

activation of the protein is consistent with failure of treatment to activate caspase 9 when cells were harvested under the same experimental conditions of DU-145 cells (Fig. 6D), because the Akt-(PI3K) signalling pathway is known to be involved in negative regulation of caspase 9 [19].

A number of additional features of PC-3 cells could contribute to the relative resistance to apoptosis, including an increased level of the caspase 8 inhibitor cFLIP-L, the increased expression of anti-apoptotic factors and down-regulation of proapoptotic proteins (Fig. 7A and C). The stimulation of ST1481-induced apoptosis when cells were incubated in drug-free medium for longer time than DU-145 cells (delayed apoptosis) was associated with activation of caspase 9, Bid cleavage, and Bcl-2 down-regulation (Table 1 and Fig. 7B).

Taken together, the results support that activation of multiple proapoptotic signals could enhance the apoptotic response and that the potentiation of camptothecin-based chemotherapy by TRAIL could be of therapeutic interest in the development of novel combination strategies in the treatment of prostate cancer. However, the therapeutic potential of this approach could be limited by expression or activation of protective pathways. Indeed, the sensitivity to apoptotic stimuli and the pattern of activation of apoptosis by camptothecins was different in the studied prostate carcinoma cell systems. In DU-145 cells it appears to reflect efficient cooperation between extrinsic and intrinsic pathway. A slower activation of cell death occurred in PC-3 cells and was related to the expression of anti-apoptotic and survival factors. A synergistic effect between TRAIL and chemotherapeutic agents has been documented in other model systems and has been related to up-regulation of proapoptotic molecules or down-regulation of anti-apoptotic molecules [17,20]. Approaches aimed at enhancing the apoptotic response through inhibition of anti-apoptotic pathways may improve the efficacy of the combined TRAIL/camptothecin treatment. The evidence that TRAIL receptors are modulated by camptothecins may have implications for the design of therapeutic strategies involving the novel lipophilic camptothecin ST1481, which has been reported to be highly effective in the treatment of prostate carcinoma models [21].

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