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# Comparison of the rapid pro-apoptotic effect of trans-ß-nitrostyrenes with delayed apoptosis induced by the standard agent 5-fluorouracil in colon cancer cells

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**Abstract** Trans-ß-nitrostyrene (TBNS) has been reported to be a potent inhibitor of protein phosphatases PTB1 and PP2A and to display a pro-apoptotic effect even in multidrug resistant tumour cells. Here we compared the anti-tumour potential of TBNS with 5-fluorouracil (5-FU) as the standard chemotherapeutic agent for colorectal cancer in LoVo cells. Resistance to 5-FU based therapy might be a consequence of 5-FU's delayed effect requiring long-term effective concentrations in the tumour tissue. Thus, alternatives like platin containing drugs with a more rapid effect have been introduced recently.

Compared to 5-FU TBNS displayed a faster cytotoxic and pro-apoptotic effect. A 50% decrease in viability was observed already after 8 h with TBNS while 5-FU displayed no significant effect before 48 h. DNA fragmentation and caspase-3 assays confirmed the more rapid apoptotic effect of TBNS. Since apoptosis affects individual cells these results about a rapidly induced apoptosis were further studied on a single cell level in microscopic assays of caspase-3 and caspase-8 activation.

Adducts of trans-ß-nitrostyrene displayed an anti-tumour effect comparable to TBNS which suggests the possibility of creating adducts with optimised tissue targeting. Finally, the calculation of a drug combination index displayed a synergistic effect for the combination of TBNS and 5-FU in Lovo as well as in HT-29 and HCT116 colon cancer cells.

**Keywords** Trans-ß-nitrostyrene · 5-Fluorouracil · Drug-induced apoptosis · Caspase-3 activation · DNA fragmentation · LoVo adenocarcinoma cells

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## Introduction

Almost 1 million patients are diagnosed with colorectal cancer yearly, and half a million deaths occur from this neoplasm annually worldwide [1]. Since the 1990s fluorouracil plus leucovorin has been the standard therapy regimen as adjuvant chemotherapy, particularly in stage III colon cancer [2]. But even with this treatment the results of chemotherapy remain unsatisfactory. Standard agents used for colon cancer produce objective response rates of $<40\%$ [3]. Especially patients with advanced colorectal cancer that is refractory to 5-FU based therapy have a very poor prognosis [4].

An established principle of chemotherapy for neoplasia is that a multidrug strategy is frequently superior to single agents. This concept is based on the view that resistance to any single agent could be overcome by using multiple agents with distinct mechanisms of action. An alternative rationale for using combinations of anti-neoplastic agents is the potential additive or synergistic cytotoxicity they could engender [5].

Resistance to 5-FU based chemotherapy, especially acquired resistance, might be a potential problem of 5-FU's delayed effect requiring long-term effective concentrations in the tumour tissue [6–9]. Thus, the therapeutic regime was changed for a better response rate from bolus to prolonged infusions of 5-FU. In addition capecitabine, an oral prodrug of 5-FU, was introduced recently to achieve more consistent

drug levels [10, 11]. Furthermore alternatives like platin containing drugs, e.g. oxaliplatin, or DNA-topoisomerase-I inhibitors, e.g. irinotecan, with a more rapid effect and a distinct mechanism of action were added to the therapy concept [12–15].

Addition of these substances to the combination used in chemotherapy of colon cancer has increased the primary response rate but unfortunately the rate of acquired resistance as well [16]. Thus, there is still a need to develop new chemotherapeutic agents that could be added to the 5-FU based therapy or that otherwise act in a completely different way and could be given to refractory patients.

Apoptosis was found to be the main mechanism of the cytotoxic effect of chemotherapeutic agents [17, 18]. Characteristic features of apoptosis are viability loss, DNA fragmentation and membrane blebbing accompanied by sustained membrane integrity that limits the loss of intracellular enzymes and avoids damage to the surrounding cells in contrast to necrotic cell death [19, 20]. The caspase cascade plays a central role in the process of apoptosis [21, 22]. There is an extrinsic signalling way mediated by death receptor and caspase-8 [23–25] and an intrinsic mitochondrial way mediated by release of cytochrome *c* and activation of caspase-9 [26–28]. Both signalling cascades activate caspase-3 as the key downstream enzyme [29].

Reversible phosphorylation of proteins on tyrosine, threonine and serine residues is one of the key mechanisms in cellular signal transduction [30]. Inhibitors of protein phosphatases have been shown in various reports to be cytotoxic and display an anti-tumour potential [31–33]. A mitochondrial pool of PP2A regulates the phosphorylation status and activity of Bcl-2 family members. PP2A inhibition induces Bcl-2 hyperphosphorylation, thus inhibiting its anti-apoptotic activity and enhancing the apoptotic death pathway [34–37]. Moreover activated caspase-3 cleaves the regulatory A$\alpha$ subunit of PP2A, increasing its activity and effecting a change in the phosphorylation state of the cell. Thus, there is a link between caspases and signal transduction pathways [38].

The blister beetle toxin cantharidin is a natural inhibitor of phosphatases 1 and 2A and this ability correlates well with its anti-cancer activity [39, 40]. Treatment with norcantharidin results in an up-regulation of CD95 receptor and CD95 ligand on the cell surface of human colon cancer cells [41]. Furthermore several analogues of cantharidin have been described as potential chemotherapeutic agents preferentially affecting colon cancer cells [42, 43].

The limited availability of natural inhibitors of protein phosphatases urged for the investigation of synthetic inhibitors. It has been reported that trans-ß-nitrostyrene (TBNS) is a potent inhibitor of protein phosphatases PTB1 [44] and PP2A [45] and displays an associated pro-apoptotic effect even in some multidrug resistant tumour cells [45, 46].

In the present study we examined viability loss and apoptosis parameters like DNA fragmentation, caspase-3 and -8 activity after treatment of LoVo colon cancer cells with the experimental substance trans-ß-nitrostyrene in comparison with the standard agent 5-fluorouracil. Finally, we evaluated the effect of TBNS in combination with 5-FU by median effect analysis to determine the potential of TBNS as a synergistic addition to a 5-FU based therapy in LoVo, HT-29 and HCT116 colon cancer cells.

## Materials and methods

### Materials

Trans-ß-Nitrostyrene was from Lancaster (Morecambe, UK), 5-fluorouracil from Merck (Darmstadt, Germany), cisplatin from Sigma-Aldrich (Deisenhofen, Germany) and cantharidic acid from Biomol (Hamburg, Germany). Cell culture reagents were from Ccpro (Neustadt, Germany) and foetal calf serum from Biochrom (Berlin, Germany). The caspase-3 substrate Z-DEVD-AFC was obtained from Bachem (Weil am Rhein, Germany). Nitrostyrene derivatives and adducts were synthesized as previously described [46].

### Cell culture

LoVo colon adenocarcinoma cells [47] were maintained as monolayer in Dulbecco's modified Eagle's medium supplemented with 8% foetal calf serum, streptomycin (100 mg/l), penicillin (100000 U/l) and amphotericin B (250 $\mu$g/l) at 37°C in 5% $CO_2$.

### Transfection

For stable transfection with plasmid DNA the calcium phosphate-mediated transfer technique [48] was used. The day before treatment $2 \times 10^5$ LoVo cells were cultured in 1.5 ml DMEM per well of a 6-well plate. The medium was changed 2 h before the transfection procedure. $H_2O$ and $CaCl_2$ (2.5 M) were added to 10 $\mu$g DNA and the mixture carefully transferred into double concentrated HBS (HEPES buffered saline; 50 mM HEPES in 280 mM NaCl) so that a fine precipitate appeared. After 30 min 150 $\mu$l of the mixture were added to each well and the cells were incubated for 16 h at 37°C. For the shock procedure medium was sucked off, the cells were washed with 1 ml PBS and then 0.5 ml glycerol (20%) shocking solution was added for exactly 2 min. The cells were washed again with PBS and incubated with 1.5 ml DMEM for 24 h until the selection with 600 $\mu$g G418 per ml medium was started. Plasmids used were pCaspase-Sensor and pDsRed2-Bid from Clontech (Heidelberg, Germany).

MTT viability assay

Cells (100 $\mu$l; $1 \times 10^4$ cells/well) were plated in 96-well plates and cultured for 24 h in complete medium. Then 50 $\mu$l medium with three times the concentration of the various final drug concentrations was added for 8, 24 and 48 h. For the exposure time experiments cells ($1 \times 10^4$ cells/well in 100 $\mu$l) were treated for 2, 8 and 24 h with 50 $\mu$M TBNS or 100 $\mu$M 5-FU. The medium was removed and substituted with complete drug-free medium and viability was analysed after 72 h. After addition of 10 $\mu$l MTT solution (5 mg/ml; Sigma, Taufkirchen, Germany) cells were incubated for 2 h. Then 100 $\mu$l 20% SDS in 0.02 N HCl was added and after 18 h of incubation MTT conversion was measured at 550 nm using a Molecular Devices UV max microplate reader [49].

Median drug effect analysis

For median drug effect analysis of the two-drug combination of TBNS and 5-FU we used serial concentrations of TBNS and 5-FU at a fixed ratio (1:2). In MTT assays LoVo (7500 cells/well), HT-29 (7500 cells/well) and HCT116 (4000 cells/well) colon cancer cells were tested. TBNS and 5-FU were added alone or together for 2 h (TBNS) or 24 h (5-FU) respectively and incubation continued thereafter in fresh medium. Finally after 72 h viability was determined. The combined synergistic, additive or antagonistic effect of the two-drug combination was analysed with the median drug effect analysis according to the method of [50]. Combination index (CI) values were expressed at each fraction affected ($F_a$). CI $<$ 1 indicates synergism, CI $=$ 1 indicates additivity, and CI $>$ 1 indicates antagonism of the interaction. The linear regression coefficient was greater than 0.95 in each case.

Analysis of DNA fragmentation

The day before treatment $7.5 \times 10^5$ cells per well were cultured in a 6-well plate. After 24 h of treatment with various drug concentrations the DNA fragments were isolated and semiquantitatively evaluated according to the protocol described in [51]. After neutral lysis of the cells with SDS, proteins and genomic DNA were precipitated with a CsCl solution and the DNA fragments in the supernatant were isolated by using GFX columns (Pharmacia, Erlangen, Germany). DNA fragments were separated by agarose gel electrophoresis on a 1.6% NuSieve gel (FMC BioProducts, Rockland, USA). Ten microliters of sample were mixed with 3 $\mu$l loading buffer containing orange G and loaded on the gels (Gibco Life Tech, Eggenstein, Germany). Gels were run for about 40 min at 90 V, stained with ethidiumbromide and documented with the Biodoc system (Biometra, Göttingen, Germany) after transillumination at 312 nm. The intensity of the DNA-ladder pattern was scored visually by 4 independent observers on a scale ranging from 0 to 4. The resulting mean values were calculated from these individual evaluations [51].

Caspase-3 assay

For biochemical analyses of caspase-3 activity $1.5 \times 10^4$ cells were cultured on glass slides in 150 $\mu$l medium per well of a 12-well Flexiperm device (Vivascience, Göttingen, Germany). After 24 h medium was changed and treatment started. After the indicated periods of time the cells were separated from the medium by centrifugation at 250 g for 10 min. The supernatants were sucked off and cells were lysed on ice for 15 min in 95 $\mu$l of lysis buffer (10 mM Hepes, 1% NP-40, 0.01 mM Digitonin, 2.6 mM Na-EDTA, 1 mM AEBSF and 10 mM DTT). Then 90 $\mu$l of the lysed cells were transferred to a new 96-well plate, frozen in liquid nitrogen and stored until further use at $-20^\circ$C. After thawing on ice 10 $\mu$l assay buffer (lysis buffer with a final concentration of 25 $\mu$M z-DEVD-AFC) were added to each well. The enzymatic reaction was run at 37°C. The released amount of AFC was repeatedly measured with a fluorometer (Packard Bioscience, Boston, USA) at $E_x = 440$ nm and $E_m = 515$ nm. From the linear part of the increase in fluorescence the enzymatic activity was calculated and expressed relative to the activity of the untreated controls.

For studying the involvement of upstream caspases in caspase-3 (DEVDase) activation, LoVO cells (200000/well) were plated on a 24 well plate. Cells were pre-incubated for 1 h with pancaspase inhibitor VAD-fmk, caspase-8 inhibitor IETD-fmk and casapse-9 inhibitor LEHD-fmk, each at 50 $\mu$M. Thereafter, cells were treated with TBNS (50 $\mu$M) for 6 h and DEVDase activity measured in the lysate as described above.

Immunoblots

LoVo cells ($1.5 \times 10^6$) were plated on 10 cm plates the day before the experiments. After incubation with or without TBNS at a concentration of 50 $\mu$M cells were harvested and lysed for 45 min on ice in a buffer containing 50 mM Tris, 150 mM NaCl, 0.1 % Triton X-100, 2 mM EDTA, 1 mM AEBSF, 1 mM $Na_3VO_4$ and 50 mM NaF. Thereafter, protein was measured in a sample with the bicinchonic acid method. Proteins were separated on 15% (Bcl-$X_L$) or 12% (pJNK) SDS-polyacrylamide gels. Each lane contained 20 $\mu$g of protein. Blotted proteins were probed with monoclonal antibodies to either Bcl-$X_L$ or a phosphospecific antibody towards JNK1 and 2 detecting phosphorylation on Thr183 and Tyr185 in these kinases. Both antibodies were from Cell Signaling (Danvers, USA) and used according to the company's

recommendations. Bands were visualized with the ECL reagent (GE Healthcare, Freiburg, Germany).

Caspase-3 sensor fluorescence assay on a single cell level

To visualize the caspase-3 activity in individual LoVo cells, these cells were stably transfected with the vector pCaspase-Sensor (Clontech, Heidelberg, Germany). The activated caspase-3 cleaves the cytosolic fusion protein and the EYFP part translocates into the nucleus.

The day before treatment $1.5 \times 10^4$ cells were cultured on a glass slide in 150 $\mu$l medium per well of a 12-well Flexiperm device. After the indicated period of time the cells were fixated on the slide with 4% paraformaldehyde for 30 min and washed twice with PBS. When the sample was dry 25 $\mu$l Mobiglow mounting medium (Mobitec, Göttingen, Germany) was applied and the slide was sealed with a cover slip. For visualisation an Axiovert 200 inverse fluorescence microscope (Zeiss, Göttingen, Germany) was used with a 40 × oil immersion objective (overall enlargement 400 × ) and a special filter set for GFP mutants with $E_x = 470/20$ nm and $E_m = 505$–$530$ nm. Pictures were taken with a CCD camera (Hamamatsu, Herrsching, Germany) and analysed with the image processing software Openlab 3.1. (Improvision, Coventry, UK). To quantify the amount of marked nuclei 10 fields of vision were screened and labelled nuclei counted. Results were presented relative to the untreated controls.

Red-Bid cleavage as caspase-8 activation assay on a single cell level

For visualisation of caspase-8 like activity LoVo cells were stably transfected with the vector pDsRed2-Bid (Clontech, Heidelberg, Germany). After activation of caspase-8 the red marked cleaved Bid translocates from the cytosol into the mitochondria. The evaluation procedure was similar to that described above, except that a filter set with $E_x = 546/12$ nm and $E_m = 590$ nm was used.

## Results

Time-dependence of drug induced cell death in LoVo cells

In MTT viability assays all tested agents at 50 $\mu$M induced a time dependent loss of viability (Fig. 1(A)). In contrast to 5-FU TBNS decreased tumour cell viability already after 8 h with 48% remaining viable cells, whereas 5-FU did not significantly decrease viability before 48 h (58% remaining viable cells). Since platin derivatives like oxaliplatin are used in the second line treatment or experimentally as addition to the first line treatment of colon cancer, we also tested cisplatin (Cis) in LoVo cells. In the MTT test cisplatin at 50 $\mu$M decreased viability after 24 h with 29% surviving cells, a level comparable to that observed with TBNS.

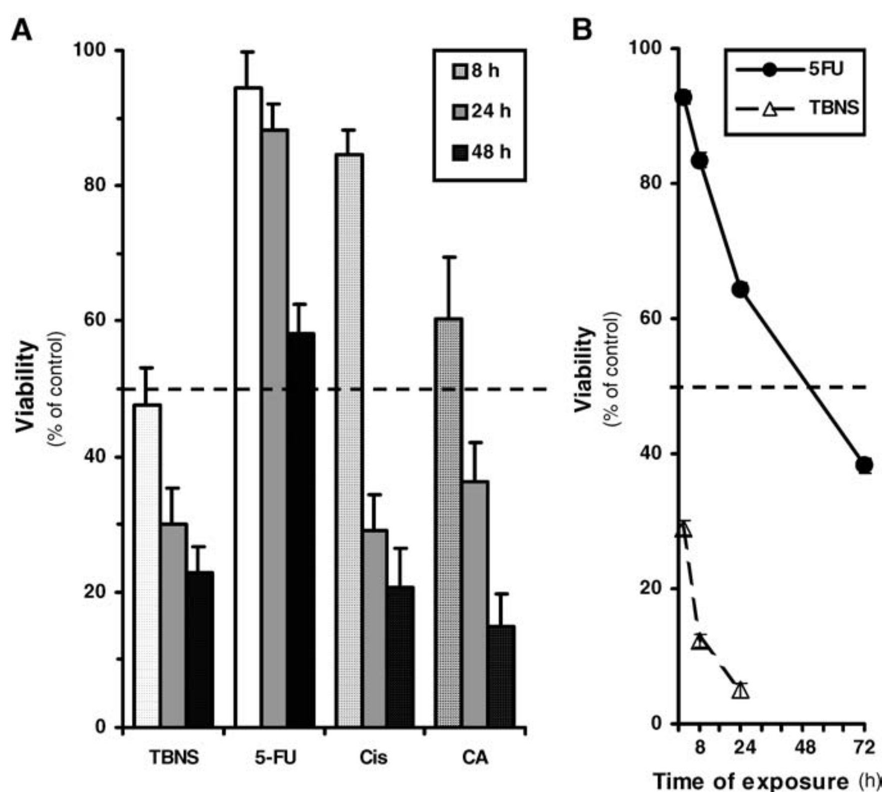



**Fig. 1** Viability of LoVo cells. (A) Time-dependent effect of trans-ß-nitrostyrene (50 $\mu$M), 5-fluorouracil (50 $\mu$M) and cisplatin (50 $\mu$M) and cantharidic acid (50 $\mu$M) on viability of LoVo colon cancer cells after 8, 24 and 48 h. Each bar represents the mean + SD ($n$ = 3). (B) Different exposure times (2, 8, 24 and 72 h) to trans-ß-nitrostyrene (50 $\mu$M) and 5-fluorouracil (100 $\mu$M) and their long-term effect after 72 h on viability of LoVo cells. The broken lines indicate 50% viability loss

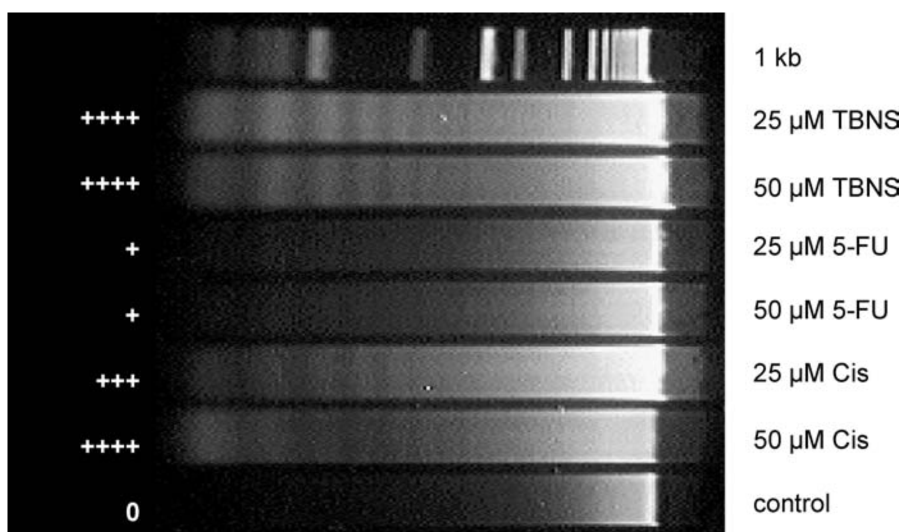



**Fig. 2** DNA fragmentation induced by trans-ß-nitrostyrene, 5-fluorouracil and cisplatin after a 24 h treatment. Representative gel out of three. Semi-quantitative evaluation of fragmentation intensity was evaluated as described under methods and scores are given on the left side

The natural phosphatase inhibitor cantharidic acid decreased viability after 8 and 24 h in a range comparable to TBNS, whereas after 48 h with 15% remaining viable cells it seemed to be slightly more effective when compared to TBNS.

To determine the role of exposure time and to compare the efficacy cells were exposed to TBNS and 5-FU for 2, 8, 24 and 72 h and viability was analysed in MTT-tests after 72 h (Fig. 1(B)). Already a 2 h treatment with TBNS (50 $\mu$M) drastically decreased viability after 72 h with 30% remaining viable cells, whereas 5-FU (100 $\mu$M) required a 24 h exposure to reach a significant decrease of viable cells (65% remaining viable cells) and only the permanent treatment for the whole 72 h decreased tumour cell viability in a comparable range to TBNS with 48% surviving cells.

## DNA fragmentation and biochemical caspase-3 assays as apoptosis markers on a multi-cellular level

To verify whether there was apoptosis or necrosis involved in this process of cell death we examined apoptosis specific DNA fragmentation (Fig. 2). The fragmentation of DNA in discrete bands of about 200 bp or multiples of it is a common marker of drug-induced apoptosis [52]. Treatment (24 h) with TBNS (25 and 50 $\mu$M) resulted in distinct DNA ladders, while after 24 h 5-FU treated cells revealed only a slight difference to the control cells. As expected from the viability test, after 24 h cisplatin displayed distinct DNA ladders with both concentrations (25 + 50 $\mu$M) albeit intensity appeared somewhat less than in TBNS treated cells.

Apoptosis specific DNA fragmentation is the result of the preceding activation of caspases, especially of downstream effector caspases like caspase-3 [21]. Thus, the time-dependent activation of caspase-3 was initially tested in a standard fluorometric assay using the substrate z-DEVD-AFC (Fig. 3). The effects of TBNS and 5-FU were compared after 8, 16, 24 and 48 h. At 50 $\mu$M TBNS already induced a 5.5-fold increase of caspase-3 activity after 8 h, the maximum effect was reached after 16 h while after 24 or 48 h caspase-3 activity declined. On the other hand, treat-

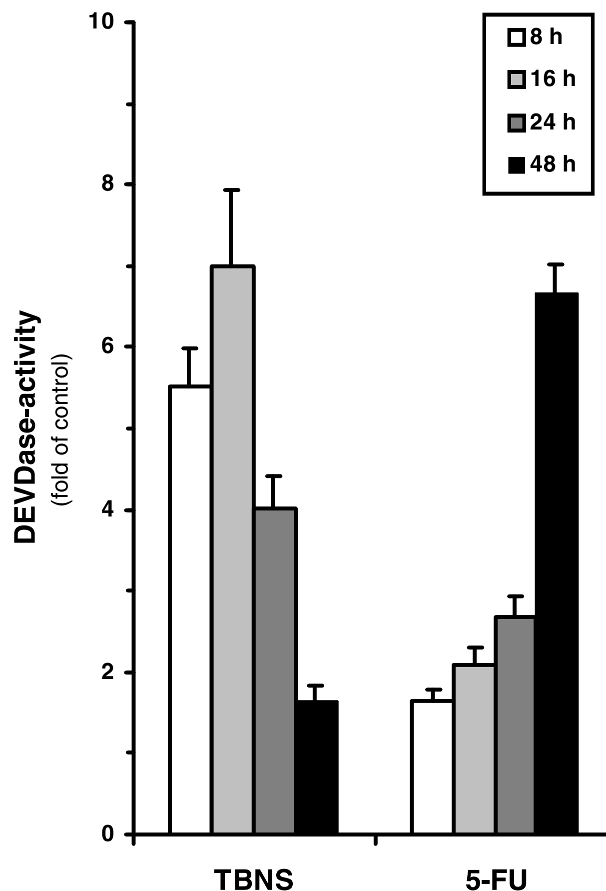
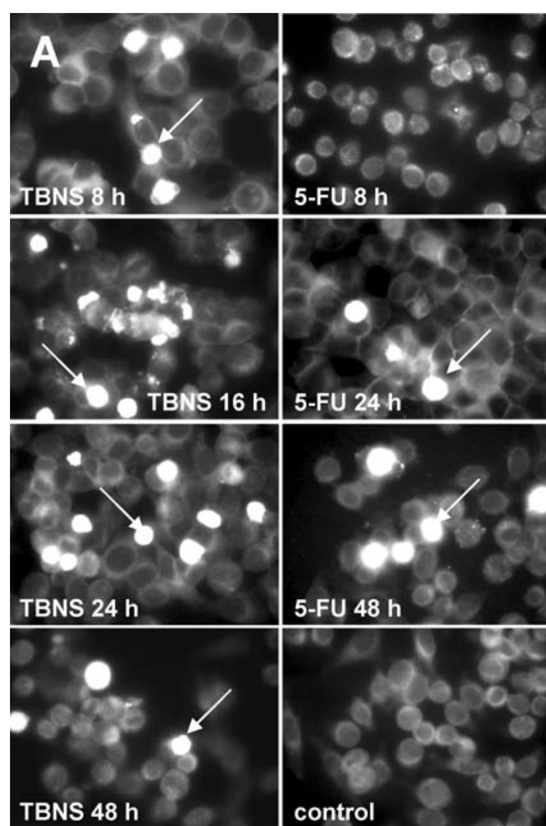



**Fig. 3** Time-dependent activation of DEVDase (caspase-3 like activity) by trans-ß-nitrostyrene (50 $\mu$M) and 5-fluorouracil (100 $\mu$M) after 8, 16, 24, und 48 h. Each bar represents the mean + SD ($n$ = 3)



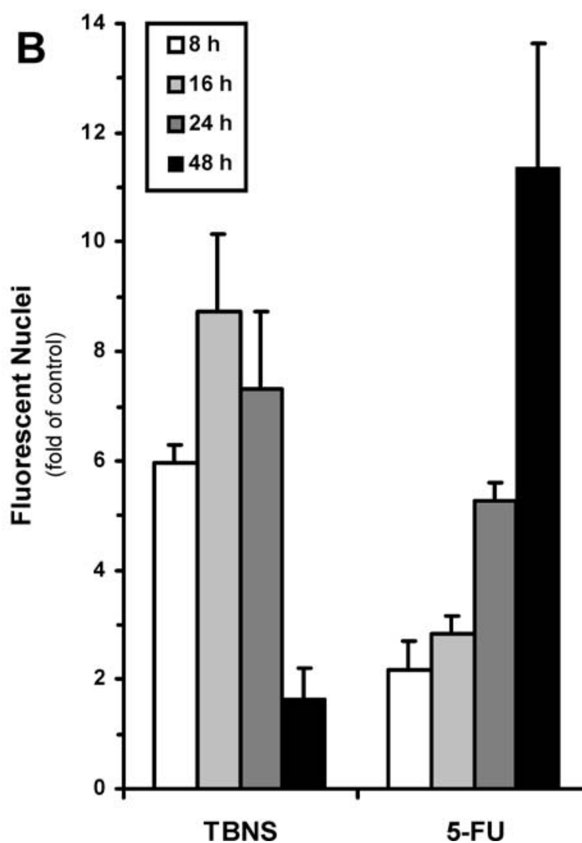




**Fig. 4** Detection of caspase-3 activation by microscopic fluorescence assay after treatment with trans-ß-nitrostyrene (50 $\mu$M) and 5-fluorouracil (100 $\mu$M). (A) Labelled nuclei are marked with an arrow. (B) Time-dependent quantification of fluorescent nuclei. Each bar represents the mean + SD ($n = 3$)

ment with 100 $\mu$M 5-FU resulted only in a 2.5-fold increase of z-DEVD-AFC cleavage after 24 h while finally at 48 h a comparable and prominent 6.5-fold increase was detected.

## Comparison of drug-induced caspase-3 activation on a single cell level

Since drug-induced apoptosis is a specific process affecting individual cells we established a microscopic caspase-3 assay using pCaspaseSensor transfected LoVo cells to examine caspase-3 activity on the individual cell level. The activated caspase-3 cleaves the cytosolic fusion protein and the EYFP part was translocated into the nucleus. Thus, individual labelled apoptotic cells can be detected and counted with an inverse fluorescence microscope (Fig. 4(A)). As expected already after 8 h at 50 $\mu$M TBNS displayed a significant activation of caspase-3 which could be detected by labelled nuclei. This activity increased after 16 and 24 h while it declined after 48 h. Treatment with 5-FU even at 100 $\mu$M revealed no significant change after 8 h compared to control cells. Here a significant increase of caspase-3 activity was detected after 24 h while an effect comparable to the maximal TBNS effect was observed after 48 h.

To quantify the amount of cells with activated caspase-3 10 fields of vision were screened and labelled nuclei counted (Fig. 4(B)). The effects of TBNS and 5-FU were compared after 8, 16, 24 and 48 h. At 50 $\mu$M TBNS induced a 6-fold increase of caspase-3 activity already after 8 h, while the maximum was detected after 16 h. After 48 h the activity declined at all concentrations. Whereas 5-FU displayed no significant increase of caspase-3 activity after 8 and 16 h, after 24 h a 5-fold increase at 100 $\mu$M was detected. After 48 h 5-FU finally reached an 11-fold increase of caspase-3 activity at 100 $\mu$M which was comparable to the maximal effect observed with TBNS at 100 $\mu$M already after 16 h (data not shown).

## Early aspects of TBNS induced apoptosis including evaluation of caspase-8 activation by TBNS and 5-FU on a single cell level

Caspase-8 is a central part in the extrinsic signalling way of apoptosis mediated by death receptors. Therefore, we also established a microscopic caspase-8 assay using pDsRed2-Bid transfected LoVo cells to verify whether caspase-8 might be involved in apoptosis induced by trans-ß-nitrostyrene and

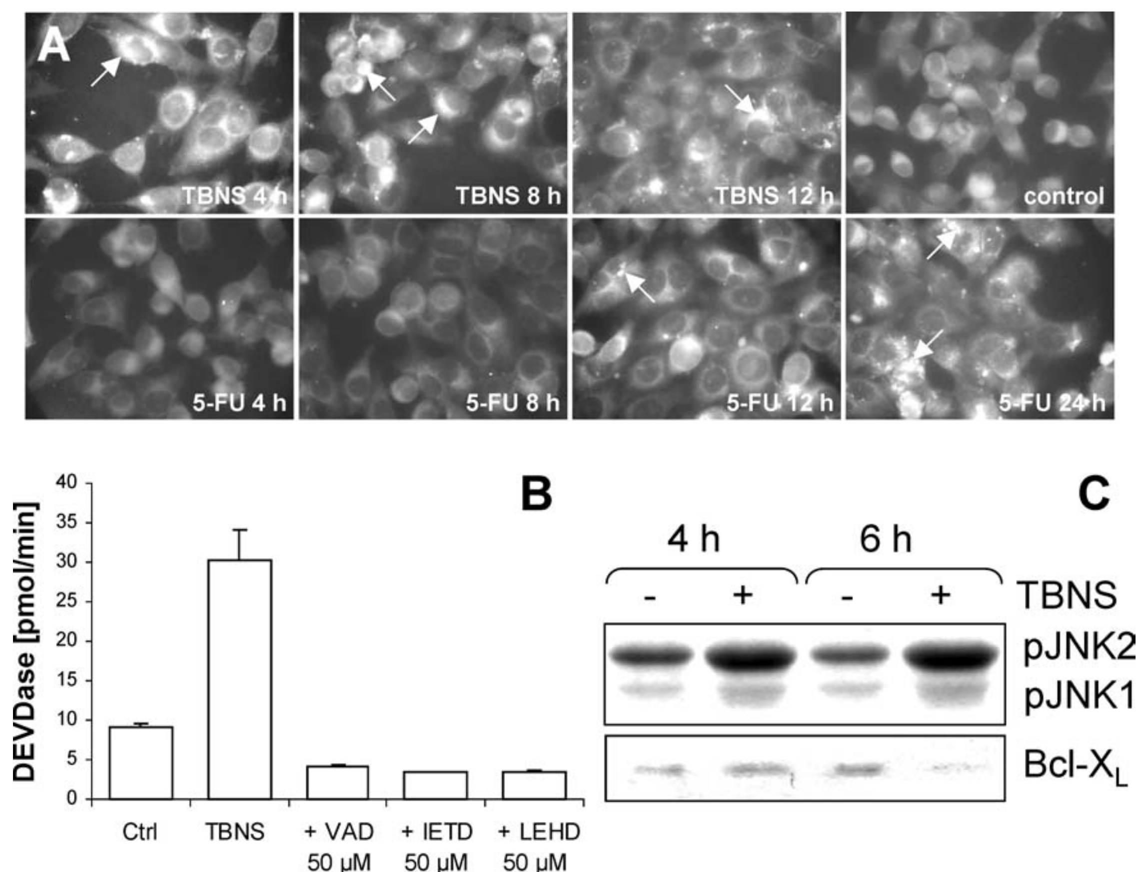



**Fig. 5** Early aspects of TBNS induced apoptosis. (A) Time-dependent detection of caspase-8 activation after treatment of LoVo cells with trans-ß-nitrostyrene (50 $\mu$M) and 5-fluorouracil (100 $\mu$M). Mitochondrial translocation of cleaved Bid-marker-protein is labelled with an arrow. (B) Pre-incubation of LoVo cells with specific caspase inhibitors can prevent caspase-3 (DEVDase) activation after 6 h of TBNS treatment. Each bar represents the mean + SEM ($n = 3$). (C) Detection of increased phosphorylation of JNK1 and JNK2 after 4 and 6 h of incubation with TBNS (50 $\mu$M) and respective immunoreactivity of Bcl-$X_L$ in LoVo cells

5-fluorouracil. After activation of caspase-8 the red marked cleaved Bid translocates from the cytosol into the mitochondria and thus the area around the nuclei will be labelled. We compared the effects of TBNS at 50 $\mu$M and 5-FU at 100 $\mu$M after 4, 8, 12 and 24 h (Fig. 5(A)). An activation of caspase-8 could be detected with TBNS as well as with 5-FU. But again there was a difference in the kinetics between both substances as has been expected from the previous results. Already after 4 h TBNS treated cells displayed a beginning translocation of Bid into the mitochondria, this translocation increased after 8 and 12 h whereas it seemed to decline after 24 h. In contrast, 5-FU revealed no significant change after 4 and 8 h. After 12 h a first beginning translocation was detected that increased after 24 h.

In addition, the involvements of upstream caspases-8 and -9 in TBNS action were shown by the use of specific caspase inhibitors (Fig. 5(B)). The pre-incubation of LoVo cells with 50 $\mu$M of pancaspase inhibitor VAD-fmk, caspase-8 inhibitor IETD-fmk and caspase-9 inhibitor LEHD-fmk completely prevented the raise of caspase-3 (DEVDase) activity induced by treatment of cells with 50 $\mu$M TBNS for 6 h.

The incubation of LoVo cells with 50 $\mu$M TBNS for 4 and 6 h revealed at these early time points an increased phosphorylation of stress activated kinases JNK1 and JNK2. At the later time point this was accompanied by a decrease in immunoreactivity for the anti-apoptotic protein Bcl-$X_L$ (Fig. 5(C)).

## Cytotoxic potential of TBNS adducts and derivatives

Furthermore we tested different nitrostyrene derivatives and adducts in MTT viability assays to assess their cytotoxic potential (Fig. 6). Like TBNS its cystein adduct (NS-Cys), its ascorbic acid adduct (NS-Asc) and its 2,4,6 triamino-pyrimidine adduct (TAP-NS) displayed a concentration-dependent anti-tumour effect comparable to trans-ß-nitrostyrene with only a slight decrease in potency. All adducts decreased tumour cell viability with 35% remaining viable cells at 50 $\mu$M after 24 h. Changes in the side chain with respect to differences in cytotoxic potency revealed that 2-methyl-trans-ß-nitrostyrene (2-$CH_3$-NS) displayed a somewhat higher potency (25% remaining viable

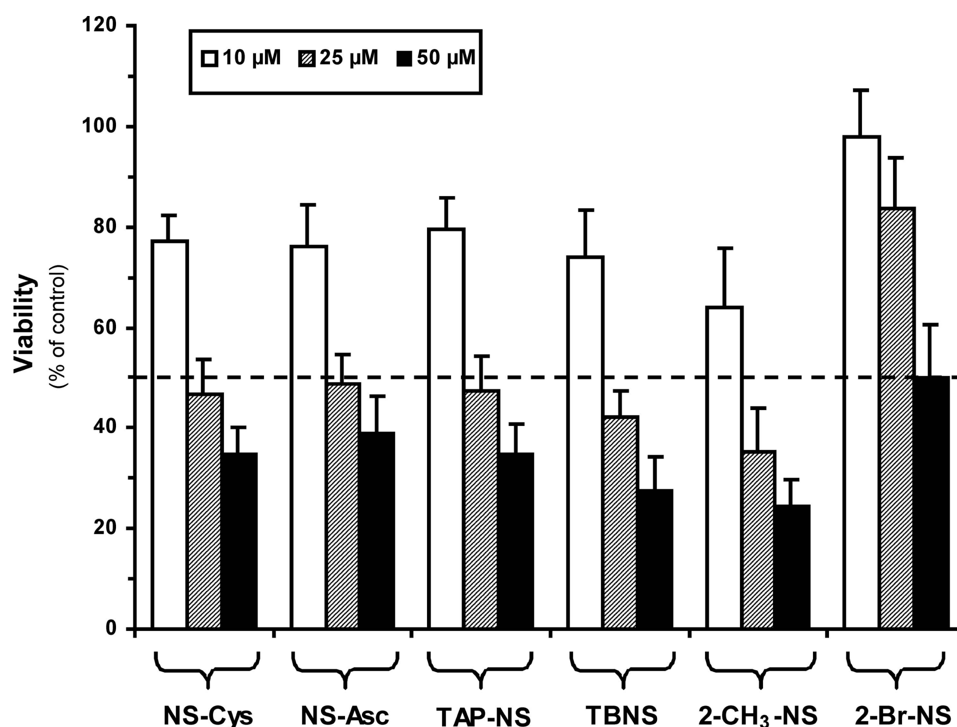



**Fig. 6** Concentration-dependent cytotoxic effect after 24 h treatment of LoVo cells with trans-ß-nitrostyrene (TBNS) in comparison to its adducts with cystein, ascorbic acid and 2,4,6-triamino-pyrimidine and the derivatives 2-methyl- and 2-bromo-nitrostyrene at 10 $\mu$M, 25 $\mu$M, 50 $\mu$M. Each bar represents the mean + SD ($n = 3$). The broken line indicates 50% viability loss

cells at 50 $\mu$M) and 2-bromo-trans-ß-nitrostyrene (2-Br-NS) a decreased potency in cytotoxicity (50% of cells remaining viable at 50 $\mu$M) when compared to TBNS.

Evaluation of the combination of TBNS and 5-FU by median effect analysis

Finally we tested the combination of TBNS and 5-FU in MTT viablitiy tests (Fig. 7(A)). The cells were treated with 10 $\mu$M TBNS and 20 $\mu$M 5-FU, thus one fifth of the concentrations previously used. After 72 h TBNS and 5-FU decreased viability only slightly to a comparable degree (about 75% remaining viable cells) whereas the combination of TBNS and 5-FU at a 1:2 ratio reduced viability of the LoVo cells by 70%, which apparently was more than an additive effect.

Figure 7(B) shows the subsequent median effect analysis of TBNS in combination with 5-FU at a 1:2 ratio assuming mutually non-exclusive interaction. Simultaneous exposure of LoVo cells to TBNS and 5-FU yielded CI values of less than 1 up to $F_a < 0.8$ and greater than 1 for $F_a > 0.8$. The results indicate that there is a marked synergistic effect for the combination of the two drugs over a cytotoxic range when less than 80% of the cells are affected ($F_a > 0.8$), an agonistic effect for $F_a = 0.8$ and a moderate antagonistic effect for $F_a > 0.8$. In experiments with HT-29 and HCT116 colon cancer cells the combination of TBNS and 5-FU also displayed synergism (Fig. 7(B)). Thus, three colon cancer cell lines differing in many genetic aspects but all with a low sensitivity towards 5-FU [7] responded rapidly to the new agent and displayed synergism in a median effect analysis when TBNS and 5-FU were used in combination.

## Discussion

Colorectal cancer is the second leading cause of death from cancer in western countries [53]. Despite recent additions to the armoury of chemotherapeutic agents for colorectal cancer treatment, the results of chemotherapy remain unsatisfactory. 5-Fluorouracil (5-FU) still represents the cornerstone of treatment and resistance to its actions is a major obstacle to successful chemotherapy. This resistance seems to be a potential problem of 5-FU's delayed effect requiring the continuous presence of effective concentrations in the tumour tissue [6–9]. Therefore alternatives like platin containing drugs, e.g. oxaliplatin, with a more rapid effect [12, 13, 54] or oral fluoropyrimidines, e.g. capecitabine, providing a more constant drug level [11, 55], have been introduced recently. Thereby the primary response rate has increased but unfortunately the rate of an acquired resistance increased as well [16]. Thus, new active agents reaching a faster accumulation in the tumour tissue and showing less resistance than 5-FU are still urgently required.

In the present study we compared the anti-tumour effect and the potential of apoptosis induction of trans-ß-nitrostyrene (TBNS) with the standard 5-fluorouracil in LoVo colon cancer cells. Trans-ß-nitrostyrene induced in

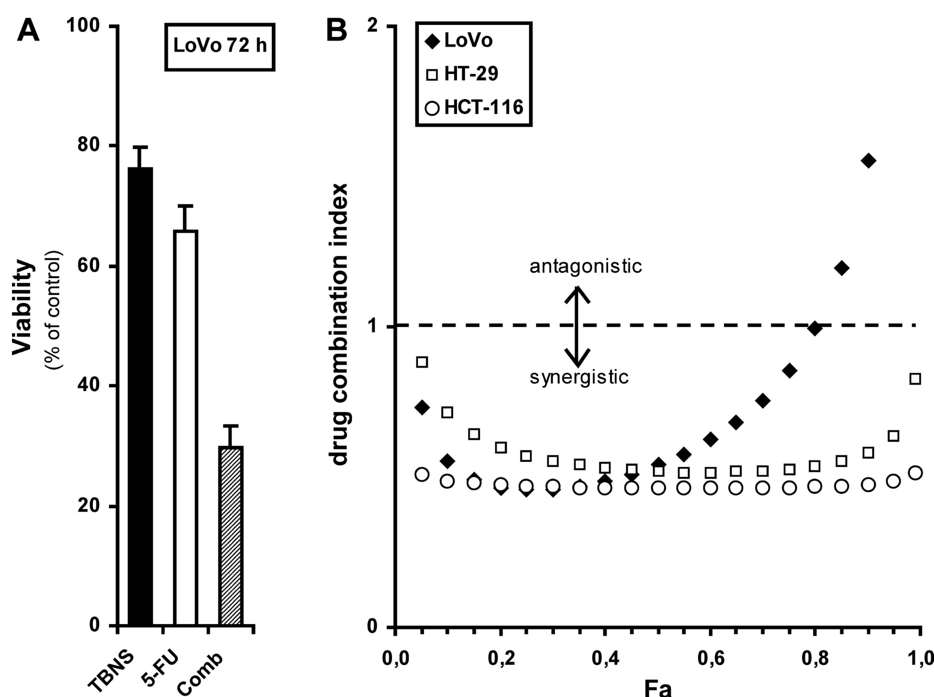



**Fig. 7** Viability of LoVo cells after 72 h exposure to trans-ß-nitrostyrene (10 $\mu$M), 5-fluorouracil (20 $\mu$M) and the combination of both agents (A). Median effect analysis of the interaction between TBNS and 5-FU (B). LoVo (filled diamonds), HT-29 (open squares) and HCT116 (open circles) colon cancer cells were exposed to TBNS (2 h) and 5-FU (24 h) as well as the combination of both agents at a ratio of 1:2. Viability was determined after 72 h. CI is plotted as a function of the fraction of cells affected by the cytotoxic effect (Fa). CI $>$ 1 indicates antagonism, CI $=$ 1 indicates additivity and CI $<$ 1 indicates synergism

LoVo cells a much faster time-dependent loss in viability than 5-fluorouracil. Moreover we could show that this faster onset of action is translated into improved efficacy of TBNS compared to 5-FU. Already a 2 h treatment with TBNS decreased viability after 72 h with 30% remaining viable cells, whereas cells exposed to 5-FU required the whole 72 h treatment to reach a significant decrease of viable cells (40% remaining viable cells). Thus, exposure time towards TBNS could be much shorter than towards 5-FU. This behaviour is in good agreement with the high calculated octanol/water partition coefficient of TBNS of about 200 [56] which suggest a rapid and extensive intracellular accumulation of TBNS.

As expected from previous results in rat pituitary $GH_3$ tumour cells [46] and in multidrug resistant HIT hamster B-cells [45] TBNS behaved as a potent inducer of apoptosis in LoVo colon cancer cells as well. We could confirm our conclusions from the MTT viability tests with DNA fragmentation data as well as with biochemical caspase-3 assays where trans-ß-nitrostyrene displayed much faster apoptotic kinetics when compared to 5-fluorouracil.

These observations about a late anti-tumour effect of 5-fluorouracil have also been reported [57], where 5-FU at 20 $\mu$M displayed still 50% remaining viable LoVo cells after 72 h. LoVo cells appeared to be less responsive towards 5-FU compared to SW620 and DLD-1 colon cancer cells. LoVo cells displayed a lack of G2/M block and a loss of *S* phase whereas SW620 and DLD-1 cells showed a relatively higher sensitivity with a prolonged *S* phase and a G2/M block. Accumulation in G1 phase and a significantly decreased proportion of *S* phase after treatment with 5-FU in colon cancer cells was described in [58] as well, here it was postulated that 5-FU might act dose-dependently via two different pathways: A G1/S phase cell cycle arrest and apoptosis at 1000 ng/ml and a G2/M phase cell cycle arrest with mitotic catastrophe at 100 ng/ml.

Cantharidic acid has been shown to be a selective inhibitor of ser/thr phosphatases 1 and 2A [59], an effect which correlated well with the induction of cell death in tumour cells [60]. The observation of a loss in LoVo cell viability by cantharidic acid comparable to TBNS confirmed data of [61]. Cantharidic acid treatment at 25 $\mu$M for 24 h (72 h) resulted in 35% (85%) dead cells even in a doxorubicin-resistant LoVo cell line displaying the MDR-phenotype [61].

Since apoptosis is a specific phenomenon of individual cells [17, 18] and caspase-3 is one of the key enzymes in this process [21, 22, 29] we established a microscopic caspase-3 assay using pCaspaseSensor transfected LoVo cells to examine caspase-3 activity in each individual cell. In this microscopic caspase-3 assay we could confirm and supplement our results from the biochemical caspase-3 assay with an earlier activation of caspase-3 by trans-ß-nitrostyrene compared to 5-fluorouracil in LoVo cells. Especially at earlier time points (8 h) and at lower concentrations (data not shown) the new microscopic assay appeared to be more sensitive because it enabled us to detect the alterations in each affected cell.

The early activation of caspase-3 by TBNS confirmed the results of [46], where a prominent increase of caspase-3 activity after 6 h at 25 $\mu$M TBNS in $GH_3$ cells has been reported.

Our observations about a later activation of caspase-3 by 5-FU compared to TBNS in colon cancer cells are consistent with the reports of [62]. These authors reported a significant increase of caspase-3 activity after 48 h in COLO 201 cells. The observation that the Fas system might not be involved in this activation of caspase-3 was suggested by the results of [63] which revealed an activation of caspase-3 and 8 in LoVo and WiDr cells without participation of the Fas signalling way.

To clarify whether caspase-8 is involved in the induction of apoptosis by 5-FU and TBNS we also established a microscopic caspase-8 assay using pDsRed2-Bid transfected LoVo cells. The Bid protein is a pro-apoptotic member of the Bcl-2 family and functions as a bridge between the intrinsic and extrinsic signalling way of apoptosis [64, 65]. After cleavage by activated caspase-8 the Bid fusion protein translocates from the cytosol into the mitochondria and thus the area around the nuclei becomes labelled. For both agents we could show that caspase-8 and Bid were involved in the induction of apoptosis in a time-dependent pattern. In accordance with its more rapid kinetics TBNS displayed an activation of caspase-8 already after 4 h whereas 5-FU revealed a first beginning translocation after 12 h.

It has been described that inhibition of the mitochondrial pool of PP2A induces Bcl-2 hyperphosphorylation, inhibiting its anti-apoptotic activity and enhancing the apoptotic death pathway [34–37]. In addition, inhibition of PP1 and PP2A resulted in an upregulation of CD95 receptor and CD95 ligand on the cell surface of human colon cancer cells [41]. Thus TBNS might activate caspase-8 via mitochondrial PP2A inhibition and enhancing the intrinsic pathway by inhibition of Bcl-2 and activation of Bid. Additional results obtained by using specific caspase inhibitors revealed the involvement of upstream caspases-8 and -9 in the activation of downstream caspase-3 (DEVDase) early in TBNS action (Fig. 5(B)). In addition, increased phosphorylation and thus activation of stress-activated kinases JNK1 and JNK2 could be detected early in TBNS treated LoVo cells (Fig. 5(C)). Such activation has been reported during apoptosis induction by PP2A inhibitors norcantharidin [66] and cantharidic acid [67] as well as with a synthetic inhibitor of PP2C [67]. Activation of stress-activated kinase JNK was associated with some forms of drug-induced apoptosis [68]. Functional aspects of the role of activated JNK in apoptosis were provided by the results from [69]. These authors described the affection of anti-apoptotic members of the Bcl-2 family by JNK activation in mitochondrion-dependent apoptosis. In the present paper increased phosphorylation of JNK1 and JNK2 was observed after 4 and 6 h of TBNS treatment and was accompanied by a decrease of the level of anti-apoptotic Bcl-$X_L$ after 6 h. A down-regulation of anti-apoptotic members of the Bcl-2 family has been reported to be important in apoptosis induced by protein kinase inhibitors in B-cell chronic lymphatic leukaemia cells [70].

Our results about an earlier caspase-8 activation compared to the activation of caspase-3 in colon cancer cells treated with 5-FU are in agreement with the results of [62] where an increase of caspase-8 activity was shown after 10 h, whereas an delayed activation of caspase-3 was observed after 48 h.

In addition, we tested different TBNS adducts and derivatives in viability assays. Although these agents had lost their double bond in the side chain which appeared to be necessary for phosphatase inhibition [44] by the adduct formation they still displayed an anti-tumour effect comparable to TBNS. Presumably this was achieved by time-dependent liberation of TBNS [H. J. Steinfelder—unpublished results]. This observation suggests the interesting opportunity to create certain adducts of TBNS as prodrug forms which by chemical synthesis strategies could result in an optimised tissue targeting of the agents. In this context [43] published some interesting data on derivatives of the natural phosphatase inhibitor cantharidin. These derivatives displayed a 10-fold higher anti-tumour potency in colon cancer cells when compared to other tumour forms supporting the idea that colon cancer cells might be especially sensitive towards phosphatase inhibitors with optimised tissue targeting.

Combination chemotherapy is a classical approach to improve chemotherapeutic efficacy in cancer patients compared to treatment with a single agent. Since resistance to any single agent could be overcome by using multiple agents with distinct mechanisms of action, they could engender a potential additive or synergistic cytotoxicity [5]. Thus, we also tested the combination of low concentrations of TBNS and 5-FU and observed a more than additive effect of the combination. This observation prompted us to perform a more detailed median effect analysis of TBNS and 5-FU combinations according to [50]. These experiments revealed that the simultaneous addition of TBNS together with 5-FU at a 1:2 ratio interacted synergistically ($CI < 1$) for $F_a < 0.8$ in LoVo cells. Synergistic effects ($CI < 1$) were also observed in HT-29 and HCT116 cells, two other colon cancer cell lines displaying reduced sensitivity towards 5-FU alone [7]. These observations suggest that the synergism between TBNS and 5-FU is independent of genetic differences between these lines, e.g. p53 functional status, functional bax content and deficiency or proficiency of DNA mismatch repair [7].

## Conclusion

The presented data reveal that TBNS behaves as a fast and potent pro-apoptotic agent in colon carcinoma cells. Compared to 5-FU TBNS displayed a much earlier induction of apoptosis as measured by DNA fragmentation and caspase-3 and

-8 assays on a single cell level. This faster kinetics with the requirement of a shorter exposure time might be an important advantage in comparison to 5-FU based chemotherapy and might also help to prevent the development of resistance towards 5-FU in a combinatory approach [6–9, 71]. Taken together, the possibility of creating similarly effective adducts of TBNS with various partners as prodrugs and the synergism of the two-drug combination of TBNS with 5-FU suggests a potential of nitrostyrene based agents in tumour therapy.

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## References

1. Stewart BW, Kleihues P (eds) (2003) World cancer report. IARC Press, Lyon, France
2. Van Cutsem E, Dicato M, Wils J et al (2002) Adjuvant treatment of colorectal cancer (current expert opinion derived from the third international conference: perspectives in colorectal cancer, Dublin, 2001). Eur J Cancer 38:1429–1436
3. Goker E, Gorlick R, Bertino JR (1998) Resistance mechanisms to antimetabolites. In: Pinedo HM, Giaccone G (eds) Drug resistance in the treatment of cancer. Cambridge University Press, Cambridge, MA, USA, pp 1–13
4. Yoshimatsu K, Kato H, Ishibashi K et al (2003) Second-line chemotherapy with low-dose CPT-11 and cisplatin for colorectal cancer resistant to 5-FU-based chemotherapy. Cancer Chemother Pharmacol 52(6):465–468
5. Cazin JL, Gosselin P, Cappelaere P et al (1992) Drug resistance in oncology: from concepts to applications editorial. J Cancer Res Clin Oncol 119:76–86
6. Gorlick R, Banerjee D (2002) Fluoropyrimidine resistance in colon cancer. Expert Rev Anticancer Ther 2:409–416
7. Violette S, Poulain L, Dussaulx E et al (2002) Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of Bcl-2 and Bcl-X(L) in addition to Bax and p53 status. Int J Cancer 98:498–504
8. Kuranaga N, Shinomiya N, Mochizuki H (2001) Long-term cultivation of colorectal carcinoma cells with anti-cancer drugs induces drug resistance and telomere elongation: an in vitro study. BMC Cancer 1:10
9. Iqbal S, Lenz HJ (2001) Determinants of prognosis and response to therapy in colorectal cancer. Curr Oncol Rep 3:102–108
10. Rougier PH, Paillot B, LaPlanche A (1997) 5-Fluorouracil (5-FU) continuous intravenous infusion compared with bolus administration. Final results of a randomised trial in metastatic colorectal cancer. Eur J Cancer 33:1789–1793
11. Pazdur R (1998) New agents for colorectal cancers: Oral fluorinated pyrimidines and oxaliplatin. In: Educational book, ASCO, 34th annual meeting, May 16–19. ASCO, Alexandria, Los Angeles, pp 301–310
12. Raymond E, Faivre S, Chaney S, Woynarowski J, Cvitkovic E (2002) Cellular and molecular pharmacology of oxaliplatin. Mol Cancer Ther 1:227–235
13. Arango D, Wilson AJ, Shi Q et al (2004) Molecular mechanisms of action and prediction of response to oxaliplatin in colorectal cancer cells. Br J Cancer 91:1931–1946
14. Guichard S, Cussac D, Hennebelle I, Bugat R, Canal P (1997) Sequence-dependent activity of the irinotecan-5FU combination in human colon-cancer model HT-29 in vitro and in vivo. Int J Cancer 73:729–734
15. Xu JM, Azzariti A, Tommasi S et al (2002) Combination of 5-fluorouracil and irinotecan on modulation of thymidylate synthase and topoisomerase I expression and cell cycle regulation in human colon cancer LoVo cells: clinical relevance. Clin Colorectal Cancer 2:182–188
16. Schmidt WM, Kalipciyan M, Dornstauder E et al (2004) Dissecting progressive stages of 5-fluorouracil resistance in vitro using RNA expression profiling. Int J Cancer 112:200–212
17. Lowe SW, Lin AW (2000) Apoptosis in cancer. Carcinogenesis 21:485–495
18. Johnstone RW, Ruefli AA, Lowe SW (2002) Apoptosis: a link between cancer genetics and chemotherapy. Cell 108:153–164
19. Arends MJ, Wyllie AH (1991) Apoptosis: mechanisms and roles in pathology. Int Rev Exp Path 32:223–354
20. Cho SG, Choi EJ (2002) Apoptotic signalling pathways: caspases and stress-activated protein kinases. J Biochem Mol Biol. 35:24–27
21. Chang HY, Yang X (2000) Proteases for cell suicide, functions and regulation of caspases. Microbiol Mol Biol Rev 64:821–846
22. Fuentes-Prior P, Salvesen GS (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J 384:201–232
23. Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME (1998) Apoptosis signalling by death receptors. Eur J Biochem 254:439–459
24. Bratton SB, MacFarlane M, Cain K, Cohen GM (2000) Protein complexes activate distinct caspases cascades in death receptor and stress-induced apoptosis. Exp Cell Res 256:27–33
25. Debatin KM, Krammer PH (2004) Death receptors in chemotherapy and cancer. Oncogene 23:2950–2966
26. Desagher S, Osen-Sand A, Nichols A et al (1999) Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome *c* release during apoptosis. J Cell Biol 144:891–901
27. Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770–776
28. Green DR, Kroemer G (2004) The pathophysiology of mitochondrial cell death. Science 305:626–629
29. Jänicke RU, Sprengart ML, Wati MR, Porter AG (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J Biol Chem 273:9357–9360
30. Alonso A, Sasin J, Bottini N et al (2004) Protein tyrosine phosphatases in the human genome. Cell 117:699–711
31. Kato Y, Fusetani N, Matsunaga S, Hashimoto K (1988) Calyculins, potent antitumour metabolites from the marine sponge Discodermia calyx: biological activities. Drugs Exp Clin Res 14:723–728
32. Kiguchi K, Glesne D, Chubb CH, Fujiki H, Huberman E (1994) Differential induction of apoptosis in human breast tumor cells by okadaic acid and related inhibitors of protein phosphatases 1 and 2A. Cell Growth Differ 5:995–1004
33. von Zezschwitz C, Vorwerk H, Tergau F, Steinfelder HJ (1997) Apoptosis induction by inhibitors of Ser/Thr phosphatases 1 and 2A is associated with transglutaminase activation in two different human epithelial tumour lines. FEBS Lett 413:147–151
34. Chen YH, Chen JC, Yin SC et al (2002) Effector mechanisms of norcantharidin-induced mitotic arrest and apoptosis in human hepatoma cell lines. Int J Cancer 100:158
35. Deng X, Ito T, Carr B, Mumby M, May WS (1998) Reversible phosphorylation of Bcl-2 following interleukin 3 or bryostatin 1 is

mediated by direct interaction with protein phosphatase 2A. J Biol Chem 273:34157
36. Haldar S, Jena N, Croce CM (1995) Inactivation of bcl-2 by phosphorylation. Proc Natl Acad Sci USA 92:4507
37. Ruvolo PP, Deng XM, Ito T, Carr BK, May WS (1999) Ceramide induces Bcl-2 dephosphorylation via a mechanism involving mitochondrial PP2A. J Biol Chem 274:20296
38. Santoro MF, Annand RR, Robertson MM et al (1998) Regulation of protein phosphatase 2A activity by caspase-3 during apoptosis. J Biol Chem 273:13119–13128
39. Li YM, Casida JE (1992) Cantharidin-binding protein: identification as protein phosphatase 2A. Proc Natl Acad Sci USA 89:11867–11870
40. Hart ME, Chamberlin AR, Walkom C, Sakoff JA, McCluskey A (2004) Modified norcantharidins; synthesis, protein phosphatases 1 and 2A inhibition, and anticancer activity. Bioorg Med Chem Lett. 14:1969–1973
41. Peng F, Wei YQ, Tian L et al (2002) Induction of apoptosis by norcantharidin in human colorectal carcinoma cell lines: involvement of the CD95 receptor/ligand. J Cancer Res Clin Oncol 128:223–230
42. McCluskey A, Ackland SP, Gardiner E, Walkom CC, Sakoff JA (2001) The inhibition of protein phosphatases 1 and 2A: a new target for rational anti-cancer drug design? Anticancer Drug Des 16:291–303
43. Sakoff JA, Ackland SP, Baldwin ML, Keane MA, McCluskey A (2002) Anticancer activity and protein phosphatase 1 and 2A inhibition of a new generation of cantharidin analogues. Invest New Drugs 20:1–11
44. Park J, Pei D (2004) Trans-B-Nitrostyrene Derivates as slow-binding inhibitors of protein tyrosine phosphatases. Biochemistry 43:15014–15021
45. Fathi AR, Krautheim A, Kaap S, Eger K, Steinfelder HJ (2000) Michael adducts of ascorbic acid as inhibitors of protein phosphatase 2A and inducers of apoptosis. Bioorg Med Chem Lett 10:1605–1608
46. Kaap S, Quentin I, Tamiru D, Shaheen M, Eger K, Steinfelder HJ (2003) Structure activity of the pro-apoptotic, anti-tumour effect of nitrostyrene adducts and related compounds. Biochem Pharmacol 65:603–610
47. Drewinko B, Romsdahl MM, Yang LY, Ahearn MJ, Drujillo JM (1976) Establishment of a human carcinoembryonic antigen-producing colon adenocarcinoma cell line. Cancer Res 36:467–475
48. Steinfelder HJ, Hauser P, Nakayama Y et al (1988) Thyrotropin H-releasing hormone regulation of human TSH $\cdot$ expression: role of pituitary-specific transcription factor (Pit/GHF-1) and potential interaction with a thyroid hormone-inhibitory element. Proc Acad Sci USA 88:3130–3133
49. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
50. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
51. Rustenbeck I, Krautheim A, Jörns A, Steinfelder HJ (2004) ß-Cell toxicity of ATP-sensitive $K^+$ channel-blocking insulin secretagogues. Biochem Pharmacol 67:1733–1741
52. Arends MJ, Morris RG, Wyllie AH (1990) Apoptosis. The role of the endonuclease. Am J Pathol 136:593–598
53. GLOBOCAN 2000 (2001) cancer incidence, mortality and prevalence worldwide, version 1.0. IARC CancerBase no. 5. Lyon. IARC Press, France
54. Andre T, Boni C, Mounedji-Boudiaf L et al (2004) Multicenter international study of Oxaliplatin/5-Fluorouracil/Leucovorin in the adjuvant treatment of colon cancer (MOSAIC) investigators: Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 350:2343–2351
55. Twelves C, Wong A, Nowacki MP et al (2005) Capecitabine as adjuvant treatment for stage III colon cancer. N Engl J Med 352:2696–2704
56. Milhazes N, Calheiros R, Marques MP et al (2006) Beta-nitrostyrene derivatives as potential antibacterial agents: a structure-property-activity relationship study. Bioorg Med Chem. 14:4078–4088
57. Tokunaga E, Oda S, Fukushima M, Maehara Y, Sugimachi K (2000) Differential growth inhibition by 5-fluorouracil in human colorectal carcinoma cell lines. Eur J Cancer 36: 1998–2006
58. Chen XX, Lai MD, Zhang YL, Huang Q (2002) Less cytotoxity to combination of 5-fluorouracil and cisplatin than 5-fluorouracil alone in human colon cancer cell lines. World J Gastroenterol 8:841–846
59. Honkanen RE (1993) Cantharidin, another natural toxin that inhibits the activity of serin/threonine protein phosphatases types 1 and 2A. FEBS Lett 330:283–286
60. Laidley CW, Cohen E, Casida JE (1997) Protein phosphatases in neuroblastoma cells: [$^3$H]cantharidin binding site in relation to cytotoxicity. J Pharmacol Exp Ther 280:1152–1158
61. Sieder S, Richter E, Becker K, Heins R, Steinfelder HJ (1999) Doxorubicin-resistant LoVo adenocarcinoma cells display resistance to apoptosis induction by some but not all inhibitors of ser/thr phosphatases 1 and 2A. Toxicology 134:109–115
62. Adachi Y, Taketani S, Oyaizu H, Ikebukuro K, Tokunaga R, Ikehara S (1999) Apoptosis of colorectal adenocarcinoma induced by 5-FU and/or IFN-gamma through caspase 3 and caspase 8. Int J Oncol 15:1191–1196
63. Backus HH, Wouters D, Ferreira CG et al (2003) Thymidylate synthase inhibition triggers apoptosis via caspases-8 and -9 in both wild-type and mutant p53 colon cancer cell lines. Eur J Cancer 39:1310–1317
64. Wang K, Yian XM, Chao DT, Milliman CL, Korsmeyer SJ (1996) BID: A novel BH3 domain only death agonist. Genes Dev 10:2859–2869
65. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132–1136
66. Huh JE, Kang KS, Chae C et al (2004) Roles of p38 and JNK mitogen-activated protein kinase pathways during cantharidin-induced apoptosis in U937 cells. Biochem Pharmacol 67:1811–1818
67. Kaap S, Brechlin P, Quentin I, Eger K, Steinfelder HJ (2004) Apoptosis by 6-O-palmitoyl-L-ascorbic acid coincides with JNK-phosphorylation and inhibition of $Mg^{2+}$ dependent phosphatase activity. Biochem Pharmacol 67:919–926
68. Guo YL, Baysal K, Kang B, Yang LJ, Williamson JR (1998) Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-a in rat mesangial cells. J Biol Chem 273:4027–4034
69. Schroeter H, Boyd CS, Ahmed R et al (2003) *c*-Jun N-terminal kinase (JNK)-mediated modulation of brain mitochondria function: new target proteins for JNK signalling in mitochondrion-dependent apoptosis. Biochem J 372:359–369
70. Kitada S, Zapata JK, Andreeff M, Reed JC (2000) Protein kinase inhibitors flavopiridol and 7-hydroxy-staurosporine down-regulate antiapoptosis proteins in B-cell chronic lymphocytic leukaemia. Blood 96:393–397
71. Arnould S, Hennebelle I, Canal P, Bugat R, Guichard S (2003) Cellular determinants of oxaliplatin sensitivity in colon cancer cell lines. Eur J Cancer 39:112–119