A novel mechanism of action of the fumagillin analog, TNP-470, in the B16F10 murine melanoma cell line

Marcin Okrój[a], Wojciech Kamysz[d], Ewa M. Slominska[b], Andrzej Mysliwski[c] and Jacek Bigda[a]

TNP-470, a semisynthetic derivative of fumagillin, is an acknowledged angiogenesis inhibitor, presently undergoing clinical trials. It exerts an anti-proliferative effect directed against endothelial cells. This effect is known to be based on cell cycle inhibition effected by the p53/p21 pathway. We observed short-term toxicity of TNP-470 in the B16F10 murine melanoma cell line *in vitro* and investigated the mechanism of action. Cell death occurred as soon as 2 h after the addition of TNP-470, without typical apoptotic features. The toxic effect could be modulated and it depended on the type of culture medium or supplementation with anti-oxidants. Addition of *N*-acetylcysteine protected B16F10 cells from TNP-470-induced death and inhibited an increase in the generation of reactive oxygen species (ROS), which are detected by the 2′,7′-dichlorodihydrofluorescein diacetate probe. We conclude that TNP-470 can induce intracellular generation of ROS, which act toxically inside B16F10 cells. One may suggest that this novel activity of TNP-470 might be beneficial in some cases, but it could also be responsible for some undesirable side-effects. The possibility of its modulation gives a prospect for controlling the action of this potential drug and probably its derivatives. *Anti-Cancer Drugs* 16:817–823 © 2005 Lippincott Williams & Wilkins.

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[a]Department of Cell Biology, Intercollegiate Faculty of Biotechnology, Departments of [b]Biochemistry and, [c]Histology, Faculty of Medicine and [d]Department of Physical Chemistry, Faculty of Pharmacy, Medical University of Gdansk, Gdansk, Poland.

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Correspondence to J. Bigda, Department of Cell Biology, Medical University of Gdansk, ul. Debinki 1, 80-211 Gdansk, Poland.
Tel: +48 58 349 1438; fax: +48 58 349 1445;
e-mail: jjbigd@amg-gda.pl

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Introduction

During the past decade a variety of exogenous inhibitors of angiogenesis have been discovered. Some of them can be useful in cancer therapy, since tumor growth is dependent on new blood vessel formation. Currently, several inhibitors of angiogenesis are undergoing clinical trials (reviewed in [1,2]). Fumagillin and related molecules belong to this group of exogenous anti-angiogenic factors. TNP-470 is a semisynthetic derivative of fumagillin, characterized by a stronger cytostatic effect on endothelial cells and lower toxicity compared with the parental molecule [3]. TNP-470, similarly to fumagillin, acts directly on endothelial cells, inhibiting their cell cycle progression and proliferation [3–6]. Depending on the dose, the action of TNP-470 on endothelial cells can have cytostatic or cytotoxic features. A cytotoxic effect on human umbilical vein endothelial cells (HUVECs) was observed at a concentration of 75 μM, while a cytostatic effect could be detected at nanomolar concentrations [7]. Zhang *et al.* reported that TNP-470 inhibited S phase entry in HUVECs by the p53/p21-dependent pathway [5]. This phenomenon was also correlated with hypophosphorylation of Rb protein and a decreased activity of cyclin E-dependent kinases [8]. The molecular target of TNP-470 is methionine aminopeptidase type 2 (MetAP-2)—an enzyme responsible for cleavage of N-terminal methionine from newly synthesized proteins. The epoxide group of TNP-470 binds covalently histidine 231 of MetAP-2, blocking the proteolytic activity of the enzyme [9,10]. However, the exact mechanism of cytotoxic activity of TNP-470 is still unknown. Some investigations also revealed the effects of TNP-470 on non-endothelial cells. An inhibition of DNA synthesis and changed expression of cyclin-dependent kinases, cyclin D and p27 protein was observed in vascular smooth muscle cells [11,12]. Micromolar concentrations of TNP-470 caused growth inhibition of cell lines derived from choriocarcinoma, ovarian cancer and uterine endometrial cancer [13]. Sedlakova *et al.* studied TNP-470's toxicity for several human leukemia, myeloma, ovarian and breast cancer cell lines. The IC_{50}s for these cell lines varied between 5 and 25 μM, and in one case exceeded 40 μM [14]. However, there is no information about a possible mechanism of cytocidal activity of TNP-470. In the present study we investigated the effect of TNP-470 on the survival of B16F10 murine melanoma cells *in vitro* and proposed a mechanism that could be responsible for the rapid cell death observed after TNP-470 treatment.

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Materials and methods

Cells

B16F10 (murine melanoma) cells were kindly provided by Dr Ewa Augustin (Technical University of Gdansk, Poland). The primary culture of Ab Bomirski hamster melanoma cells was established as described elsewhere [15]. HEK293 (human embryonic kidney) cells were obtained from the Department of Molecular Pharmacology (Medical University of Hannover, Germany). HeLa (human cervix carcinoma) and A9 (murine fibroblast) cells were obtained from the Weizmann Institute (Rehovot, Israel). H5V (murine heart endothelial) cells were kindly provided by Dr Annunciata Vecchi (Mario Negri Institute, Milan, Italy). B16F10 and Ab cells were cultured in F10 HAM medium (Gibco, Paisley, UK), HEK293, HeLa, A9 and H5V cells were cultured in DMEM medium (Biomed, Lublin, Poland). The media were supplemented with 10% fetal bovine serum (Gibco) and 100 μg/ml streptomycin/100 μg/ml penicillin (both from Polfa, Warsaw, Poland). The cultures were kept in a humidified atmosphere of 5% CO_2 at 37°C and passaged with 0.25% trypsin (Biomed).

Chemicals

N-Acetylcysteine (NAC) was purchased from Sigma (St Louis, Missouri, USA). TNP-470 [*O*-(chloroacetyl-carbamoyl) fumagillol] was generously provided by Dr H. Toguchi (Takeda Industries, Osaka, Japan). L-Tyrosine was purchased from Sigma. Pan-specific caspase inhibitor z-VAD-fmk was purchased from Calbiochem (San Diego, California, USA).

Cytotoxic assays

Cells were plated onto 96-well plates (2×10^4 cells/well) in an appropriate medium (F10 or DMEM) and cultured overnight. The next day the medium was removed and, where indicated, the cells were pre-incubated for 2 h with appropriate substances suspended in culture medium. Afterwards, the medium was changed to that containing TNP-470 and appropriate substances. After further incubation, the medium was removed and a solution containing Neutral red (Sigma-Aldrich, Munich, Germany) (1:50 v/v) was added. The cells were incubated for 30 min, washed with phosphate-buffered saline (PBS) and lysed with Sorensen buffer (8.04 g of sodium citrate and 20 mM HCl in 50% methanol). Absorbance at 540 nm was measured with a Multiscan 340 microplate reader (Labsystems, Helsinki, Finland).

Reactive oxygen species (ROS) detection

B16F10 cells (5×10^5 cells/sample) were incubated with 50 μM of TNP-470 with or without 2.5 mM NAC for various time periods. Fifteen minutes before cytometry analysis, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, Oregon, USA) was added to the final concentration of 20 μM. Data were collected using a Coulter Epics XL flow cytometer (Coulter-Beckman, Little Chalfont, UK) and processed with WinMidi 2.8 software.

Trypan blue exclusion assay

Cells were cultured in 96-well plates (2×10^4 cells/well) and treated with various concentrations of TNP-470. Following the incubation, the medium was removed and a 0.4% solution of Trypan blue (Cambrex, Baltimore, Maryland, USA) was added. After 5 min the percentage of stained cells was calculated under a light microscope. Three independent measurements were performed for each concentration of TNP-470 and for each time point.

Comparison of total free sulfhydryl group content

Assessment of total free sulfhydryl groups was performed according to method described by Riddles *et al.* [16] with modifications. B16F10 cells (5×10^5 per sample) were incubated in F10 medium in the absence or presence of TNP-470. Afterwards the cells were collected, centrifuged, washed in PBS, suspended in a lysis buffer (20 mM EDTA in redistilled water), frozen in liquid nitrogen and thawed immediately. Total protein content was assessed for every sample by Bradford's method. 5,5′-Dithio-bis-(2-nitrobenzoic acid) was then added to the final concentration of 0.35 mM and samples were incubated at 25°C for 30 min. Absorbance of the solution was measured at 412 nm.

Thin-layer chromatography (TLC)

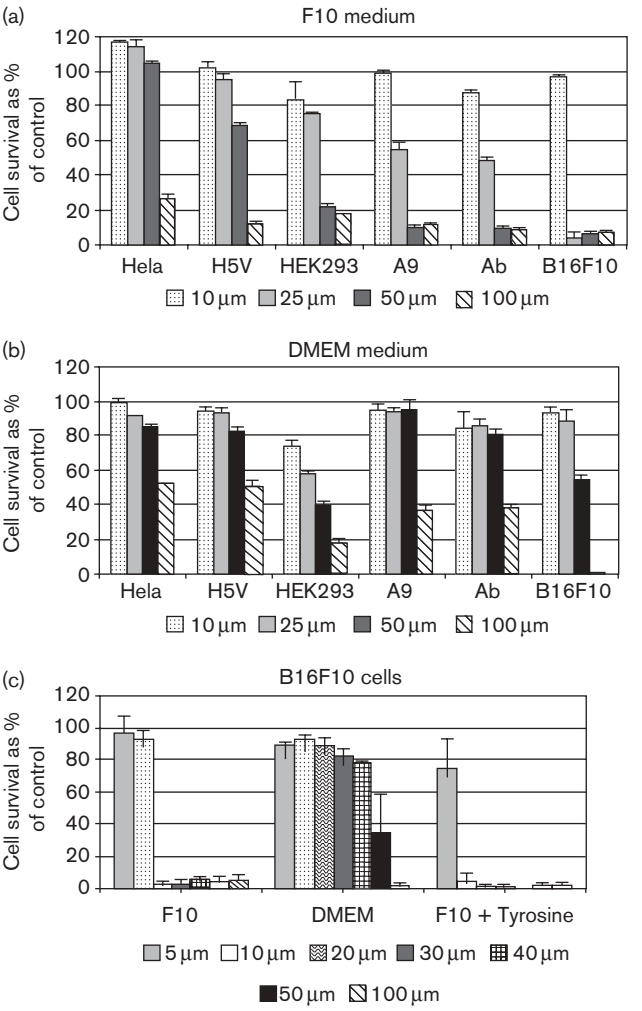
Chromatography was developed on the surface of silica plate Kieselgel 60 (Merck, Darmstadt, Germany). After loading, the sample was dried and the plate was placed onto the chromatography chamber filled with hexane:ethyl acetate 2:1 (v:v). After development, the plate was dried again and stained in an iodium-saturated chamber.

Results

Among the few cell lines analyzed, we found that B16F10 murine melanoma cells were especially vulnerable to TNP-470's cytotoxicity (Fig. 1a and b). A higher toxicity of TNP-470 was observed when the cells were incubated in F10 medium rather than in DMEM. We chose B16F10 cells as a model for further investigation of the mechanism of the cytocidal effect of TNP-470. TNP-470, at a concentration of 20 μM, was completely toxic for the cells incubated in F10 medium, while the concentration of 10 μM was well tolerated. The same toxic effect was achieved at concentrations above 50 μM, when cells were incubated in DMEM medium. B16F10 cells produce melanin if the culture medium is supplemented with an appropriate L-tyrosine concentration [17,18]. This phenomenon was also observed in our cells cultured in DMEM medium. The production of melanin is impaired in F10 medium because of insufficient L-tyrosine content. The supplementation of this amino

acid up to the level which is present in DMEM did not increase the survival of B16F10 cells treated with TNP-470 and even decreased it (Fig. 1c). Using the Trypan blue exclusion assay we analyzed at what time point after exposure to TNP-470 the B16F10 cells exhibited membrane permeability (Fig. 2a). Permeability did not increase significantly compared with the control cells for the first 60 min. After 120 min of exposure to 50 or 100 μM TNP-470, the majority of the cells were permeable, while a negligible increase of permeability was observed for 25 μM. We also analyzed 24 h survival of B16F10 cells when TNP-470 was removed from the environment after a certain time period. At a concentration of 25 μM, TNP-470 was able to induce death of more than 80% of cells when incubated with the cells for 2 h, but at 50 and 100 μM concentrations for only 30 min of incubation were enough to induce a serious or complete cytocidal effect. Thus, a short incubation with a high dose of the drug, and its subsequent removal before the cell membrane became permeable, did not prevent cell death (Fig. 2b). B16F10 cells treated with TNP-470 exhibited some of the apoptotic features like an internucleosomal DNA degradation pattern and chromatin condensation, while other markers such as elevated phosphatidylserine exposure, cell shrinkage, loss of mitochondrial potential and detachment from the surface could not be detected (data not shown). Additionally, application of the pan-specific caspase inhibitor z-VAD-fmk did not save B16F10 cells from TNP-470-induced death (data not shown). We did not see any difference in survival between cells pre-treated with inhibitor and control cells. We sought the cause of the differential B16F10 cell survival in F10 and DMEM medium. One suggestion was that it could stem from different levels of anti-oxidants in these culture media. The hypothesis of participation of oxidative stress in the toxic effect induced by TNP-470 was indicated by analysis of the content of total free sulfhydryl groups in cells treated with the angiogenesis inhibitor. The ratio between the total amount of free sulfhydryl groups and the total amount of cellular protein in cells treated with 25 or 50 μM of TNP-470 for 1 h (F10 medium) was significantly decreased to 80.6 (± 5.5) (SD) and 73.16% (± 7.48) of that in control cells, respectively.

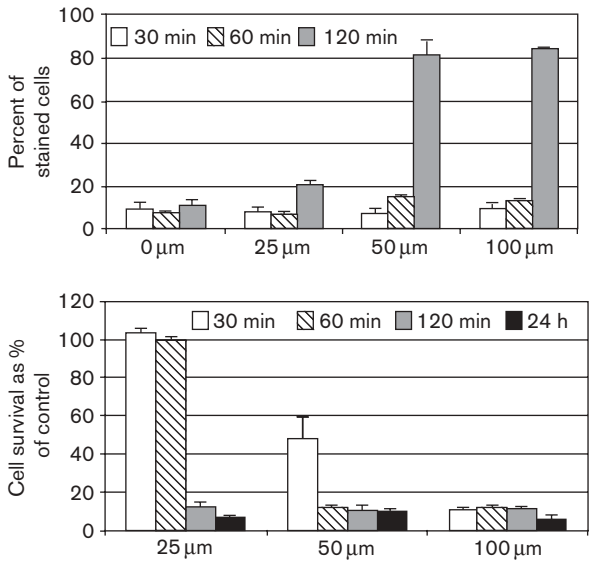
Fig. 1



Cytotoxic assay was performed for 24 h. Every sample was run in triplicate. (a) Results obtained for cells cultured in F10 medium. (b) Results obtained for cells cultured in DMEM medium. (c) Detailed results for B16F10 cells cultured in F10, DMEM and F10 medium supplemented with L-tyrosine up to a concentration of 72 mg/l, respectively. The figure presents the average data obtained in four independent experiments. Error bars represent SD.

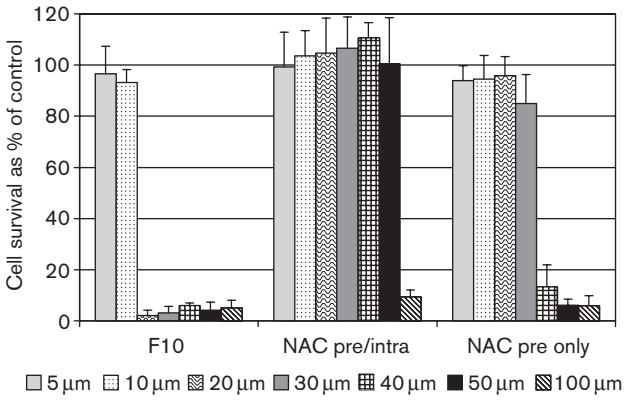
Therefore we checked if addition of an anti-oxidant and free radical scavenger (NAC) to F10 medium could decrease TNP-470 toxicity for B16F10 cells. Pre-incubation of B16F10 cells with 2.5 mM NAC and subsequent co-incubation with TNP-470 resulted in complete survival at even 50 μM of TNP-470. The protective effect of NAC was also achieved when only pre-incubation was performed and in this case the cells survived at 30 μM of TNP-470 (Fig. 3). To confirm the biological nature of the protective effect of NAC, we checked if a chemical reaction between NAC and TNP-470 could take place in F10 medium. TNP-470 and NAC were loaded onto TLC plates at the same molar ratio as in *in vitro* experiments and some lanes were overloaded in order to visualize any potential byproducts. Only at the highest load was a negligible spot of a byproduct visible. Our results showed that such a reaction may take place, but its efficiency is minimal (Fig. 4). Investigating the mechanism of the rapid cell death induced by TNP-470 we checked if, following TNP-470 treatment, the formation of ROS takes place. We analyzed the cytocidal effect of TNP-470 at a concentration of 50 μM in order to find out if the time course of ROS formation would correlate with the observed cell damage and if the survival of the cells after NAC addition would correlate

Fig. 2



Cells were seeded onto 96-well plates. For each analyzed concentration of TNP-470 at a particular time point, six wells were used. Three of them were used for the Trypan blue exclusion assay (top graph), while three others were used for the 24 h cytotoxic assay (bottom graph). With regard to the cytotoxic assay, after an indicated incubation time the medium with TNP-470 was removed, cells were washed gently with PBS buffer and fresh F10 medium was added. The figure presents the data obtained from a representative experiment (out of five performed). Error bars represent SD within replicates in the experiment.

Fig. 3



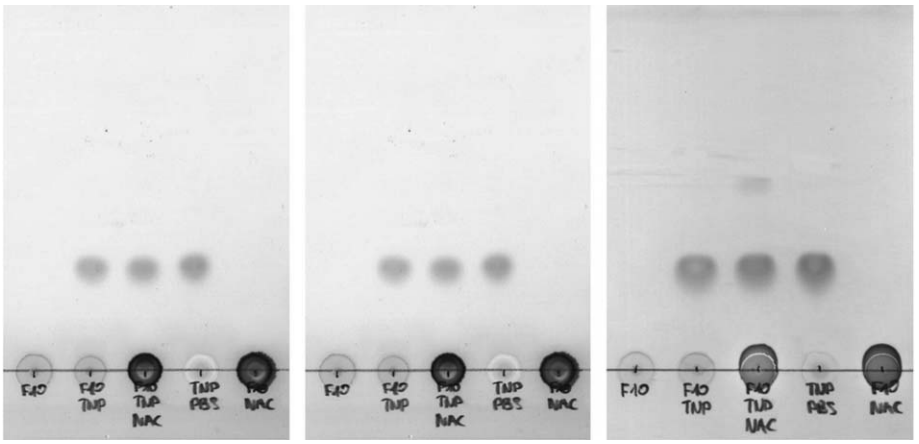
The test was performed for 24 h. B16F10 cells were maintained in F10 medium supplemented with 2.5 mM NAC for 2 h, washed gently with PBS buffer and subjected to TNP-470 in the presence of NAC ('NAC pre/intra') or in the absence of NAC ('NAC pre only'). Control cells were incubated in F10 medium with TNP-470. Every sample was run in triplicate. Data were collected from three independent experiments. Error bars represent SD.

with the ROS formation silencing effect. A marked ROS elevation was visible after 90 min of incubation with TNP-470, with a plateau after 150 min. NAC addition resulted in the inhibition of ROS formation (Fig. 5).

Discussion

TNP-470 has been described as an anti-angiogenic agent, which inhibits the proliferation of endothelial cells [3–7]. It is clearly proved that a chemical interaction of the drug with MetAP-2 is responsible for endothelial cell cycle inhibition [9,10,19]. Some reports describe a similar anti-proliferative effect on several tumor cell lines [13]. It cannot be excluded that this activity is caused by the same mechanism, but it is difficult to judge and additional experiments should be conducted. Some studies also revealed a cytotoxic effect of TNP-470 on non-endothelial cells [11–14]. However, these results were obtained after 72 h of incubation with TNP-470, so we do not know anything about the kinetics as well as the mechanism of the process. In our study we analyzed the cytotoxic effect of TNP-470 which is very prompt and, as such, not dependent on cell cycle inhibition. Cell damage observed in B16F10 cells was rapid, and already after 2 h the cells exhibited Trypan blue uptake, permeability for propidium iodide (data not shown) and a decrease in cellular ATP level (data not shown). A short incubation with high doses of TNP-470 induced cell death and the removal of the drug after more than 30–60 min did not prevent cells from destruction. Thus, one can speculate that TNP-470 could be incorporated and stored inside the cell where accumulation of a certain critical concentration of the drug might induce fatal events. Such a hypothesis would be in agreement with the observed abrupt decrease in survival in cytotoxic assays when cells were incubated in F10 medium. Based on our results, it is difficult to judge if TNP-470 induces programmed cell death (PCD) or necrosis in B16F10 cells. Some markers of PCD were detected, while the other features could indicate necrosis [20–23]. It cannot be excluded that an apoptosis/necrosis switch took place in B16F10 cells treated with TNP-470. Apoptosis is an endoergic process and the depletion of available ATP facilitates the switch into necrosis [24]. Irrespective of that, the application of a pan-specific caspase inhibitor did not influence cell survival. Although the described markers of B16F10 cells damage could be detected relatively early, they do not seem to be the primary events caused by the action of TNP-470. They may be rather considered as the results of TNP-470-activated mechanisms. Trying to identify these primary mechanisms, we compared the composition of F10 and DMEM medium, as the efficiency of TNP-470 cytotoxicity was different in these media. Several differences in the concentration of substances important in respect of anti-oxidant potential were identified: piridoxine and folic acid [25], nicotinamide [26,27], thiamine [28], cysteine or cystine [29], methionine [30] and Fe^{2+} [31]. Moreover, the decreased content of free sulfhydryl groups observed by us in the TNP-470-treated cells could be one of the essential markers of oxidative stress [32]. In order to confirm that TNP-470 toxicity observed in our model was connected with ROS formation, we supplemented F10 medium with

Fig. 4



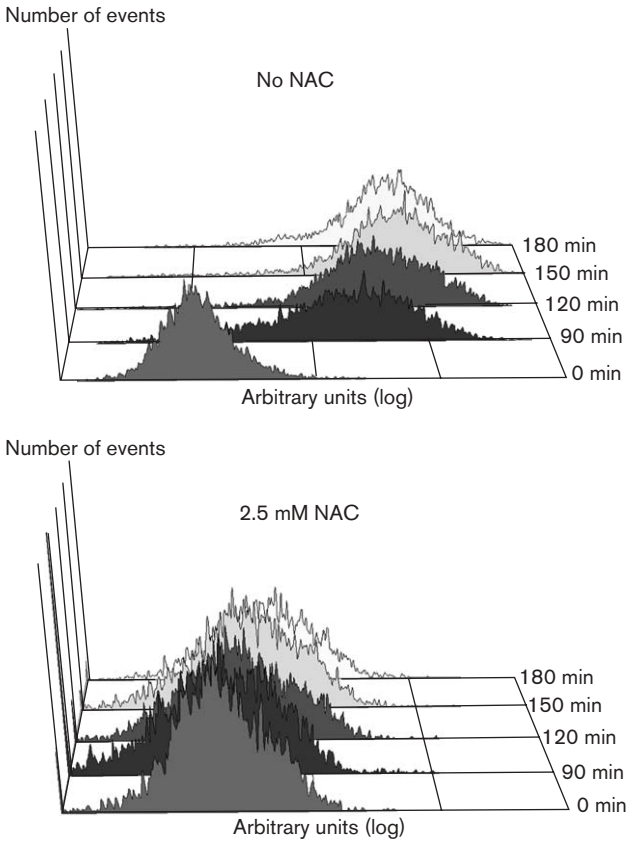
Silica plates were loaded in the following order (from left): F10 medium, F10 medium with TNP-470, F10 medium with TNP-470 and NAC, TNP-470 in PBS buffer (standard), and F10 medium with NAC. The mutual stoichiometric ratio of TNP-470 and NAC was identical to the cytotoxic assays, and the concentrations were 1 and 25 mM, respectively. The left plate was loaded with 5 μl of appropriate solutions, the middle plate with 20 μl and the right plate with 80 μl. Only at the highest load was a negligible spot of a byproduct visible.

the free radical scavenger and glutathione precursor NAC [33,34]. Under such conditions an effective dose of TNP-470 which killed B16F10 cells was at least 3 times higher and comparable to that in DMEM medium. The protective effect of NAC did not stem from the chemical reaction between the anti-oxidant and TNP-470. Although both substances possess chemically reactive groups (thiol and epoxide, respectively), our TLC analysis revealed a negligible amount of a byproduct, which was visible only when TLC lanes were highly overloaded. Moreover, pre-incubation of B16F10 cells prior to TNP-470 treatment without further presence of NAC also resulted in a protective effect. We made an assumption that if ROS generation was responsible for the toxic effect of TNP-470 on B16F10 cells, they should be detectable relatively early after TNP-470 treatment and undetectable in cells which would survive TNP-470 administration. Cytometric analysis revealed that ROS formation is elevated inside TNP-470-treated cells. The time course of this effect and comparison with the Trypan blue uptake assay suggests that ROS formation might be an event proximal to permeabilization of the cell membrane and thus crucial for fatal damage of B16F10 cells treated with TNP-470. NAC, when added to F10 medium, protected B16F10 cells from the toxicity of TNP-470 applied at a dose up to 50 μM. We showed that under these conditions a massive occurrence of ROS did not take place and subsequently cells survived for 24 h.

We found some similarity in our results to the results of other groups studying the influence of ROS-generating agents on various cell lines. Kaminski *et al.* proved that menadione can trigger apoptosis as well as necrosis in 143B cells. Moreover, the co-existence of apoptosis and necrosis markers within one cell was reported [35,36]. Although some of the cells exhibited apoptosis features only, application of z-VAD-fmk was ineffective, but NAC could prevent cells from menadione-induced death. Bonfoco *et al.* studied the influence of peroxynitrite-generating agents on neuronal cells [37]. Mild doses of these agents resulted in the appearance of apoptosis features, while higher doses caused a necrotic phenotype. In the case of high doses, Trypan blue uptake was noticed after 30 min of stimulation.

In summary, we proved that TNP-470 possesses an additional property, which is the ability to induce cytotoxicity mediated by ROS formation. This effect can be modulated with anti-oxidants. Sometimes this activity can be beneficial if tumor cells are more vulnerable to the drug in comparison to normal cells. In such situations, TNP-470 would be more effective by acting not only as an anti-angiogenic agent, but also as a cytotoxic agent. This effect could probably explain the very advantageous results of the study of Mysliwski *et al.*, in which TNP-470 was administered peritumorally to Ab melanoma-bearing hamsters and the animals even showed a complete remission of the tumors [38]. Studies performed on the same model revealed that TNP-470 treatment does not result in long-term damage of the liver, kidney and lung blood vessels [39]. However, some undesirable side-effects were observed in the early phase of TNP-470 treatment and it is possible that the ROS-inducing activity of TNP-470 may be responsible for them. It is also possible that the ROS-inducing activity may have other implications. For example, neurotoxicity

Fig. 5



B16F10 cells were incubated with 50 μM of TNP-470 for the indicated periods of time in the presence (and following 2 h pre-incubation) of NAC (bottom histogram) and in the absence of NAC (top histogram). The fluorescent probe H_2DCFDA at the final concentration of 20 μM was added to each sample 15 min before each measurement. The intensity of fluorescence was measured with a flow cytometer and data were analyzed with WinMidi 2.8 software. Histograms represent results of a representative experiment (out of four performed).

was reported as a dose-limiting factor in phase I clinical trials with TNP-470. Cerebellar dysfunctions like ataxia, vertigo, dizziness and other neurological symptoms such as decreased concentration and short-term memory, depression or insomnia were observed [40,41]. Maybe one could relate these results to the fact that the central nervous system is especially vulnerable to ROS-induced damage, as the brain is characterized by high O_2 consumption, high lipid content and a relatively inefficient oxidation defense mechanism as compared to other tissues [42]. There are increasing numbers of reports suggesting the involvement of free radicals in a variety of pathological events and multistage neural disorders [43]. An investigation of TNP-470's influence on neuronal cells would be able to answer the question whether the same mechanism of toxicity takes place in these cells and if the application of particular anti-oxidants could prevent neurological side-effects *in vivo*.

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