TAUROLIDINE AND OXIDATIVE STRESS:
A RATIONALE FOR LOCAL TREATMENT OF MESOTHELIOMA

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ABSTRACT
Malignant mesothelioma is an asbestos-related, aggressive tumor, resistant to most anticancer therapies. Akt is a key mediator of mesothelioma cell survival and chemoresistance. **Question of the study:** the mechanism by which Taurolidine, a known synthetic compound with antimicrobial and antineoplastic properties, leads to mesothelioma cell death.

**Material and Methods:** Apoptosis was studied by Annexin V binding, cell cycle analysis, caspase 8 activation, PARP cleavage and TUNEL. Oxidative stress was measured by nitrites production and DNA oxidative damage. Protein expression and phosphorylation were evaluated by immunoprecipitation and immunoblotting.

**Results:** Taurolidine induces cell death of mesothelioma cells, but not of non-neoplastic human mesothelial cells. After Taurolidine treatment of mesothelioma cells Akt but not Erk 1/2 activity is inhibited in time- and dose-dependent manner. PP1α and PP2A are activated several hours after drug addition. Apoptosis induced by Taurolidine is driven by oxidative stress and cell exposure to sulphhydryl donors, as Glutathione-monoethylster (GSH) and L-N-acetylcysteine (L-NAC), significantly reduced pro-apoptotic effects and Akt inhibition. Conversely, expression of constitutively activated Akt did not affect cytotoxicity elicited by Taurolidine, which retained its ability to inhibit the kinase.

**Answer to the question:** Taurolidine induces mesothelioma cell death via oxidative stress, accompanied by inhibition of Akt signalling. This provides a promising molecular rationale for Taurolidine as local treatment of malignant mesothelioma.
INTRODUCTION

Malignant Mesothelioma is an asbestos-related malignant tumour. Due to its biological aggressiveness, this cancer is constantly fatal, except in rare less advanced cases, with a median survival of 12.6 months[1].

The continuing increase in mesothelioma incidence has been associated with the widespread use of asbestos in the past century even if genetic predisposition may render some individuals more susceptible [2]. Moreover, SV40 present in polio vaccines distributed in many countries behaves as co-carcinogen, causing malignant transformation of human mesothelial cells. [3], through an Akt-dependent survival signalling.[4, 5].

Taurolidine (TN), trade name for bis(1,1-dioxoperhydro-1,2,4-thiodiazinyl-4)-methane, is an antibacterial drug originally synthesized in 1970 with activity against a broad spectrum of microorganisms and has been used as a safe lavage antibiotic to prevent bacterial infection in patients after abdominal surgery[6].

Over the last years in vitro evidences highlighted the role of TN as pro-apoptotic and anti-angiogenic agent and as an inhibitor of protein biosynthesis[7, 8]. Some of these in vitro effects were also observed in brain tumour cells, whereas normal cells were unaffected[9]. Moreover, intraperitoneal administration of TN in experimental rats inhibited the growth of injected ovarian and colon cancer cells[10]. Safety and efficacy of intracavitary administration of TN, along with a prolonged i.v. administration has been proposed to treat human tumours [11] and indicated that this drug is of potential help for therapy of tumours with prevalent local spreading as peritoneal and pleural mesothelioma.
The importance of Akt pathway in maintaining anti-apoptotic survival signals, responsible for onset and progression of mesothelioma has been demonstrated [4, 12] and very recently we showed that addressing Akt with pharmacological agents is a promising approach also for mesothelioma [13, 14].

Oxidative stress generated by reactive oxygen species (ROS) has been indicated both as a negative regulator of the survival factor Akt in human leukaemia cells [15] and as one of the effects elicited by TN in glioma cells, leading to suppression of VEGF production and to cell death.[16]

We show here that TN specifically targets mesothelioma cells, but not non-neoplastic human mesothelial cells (HMC) and that its pro-apoptotic mechanism is mostly driven by oxidative stress and inhibition of Akt activity.
METHODS

Cell cultures

We used two human mesothelial non-neoplastic cell lines (HMC and MET5A) and two established human malignant mesothelioma cell lines (MMB and MMP). As controls we used human dermal fibroblast (HDF) and human lung carcinoma (A549). Primary HMC were obtained from patients with congestive heart failure and cultured in Ham’s F12 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS; Life Technologies, Rockville, MD, USA). MET5A were purchased from ATCC and cultured in Medium 199 (Sigma-Aldrich) supplemented with 10% FBS. MMB and MMP cells were derived from pleural effusions of malignant mesothelioma patients and cultured in Ham’s F12 medium supplemented with 10% FBS. HDF were obtained from a healthy donor and cultured in Ham’s F12 medium supplemented with 10% FBS. A549 were purchased from ATCC and cultured in Ham’s F12 medium supplemented with 10% FBS. Cells were grown at 37°C in a 5% CO₂-humified atmosphere. MyrAkt-MMB were obtained by transfecting MMB cells with Addgene plasmid 9008 (pcDNA3 myr-HA-Akt1), using lipofectamine 2000. Transfectants were selected by G418 for 3 weeks.

Chemicals

Glutathione reduced ethyl ester (GSH), N-Acetyl-L-cysteine (L-NAC) and Rapamycin were purchased from Sigma-Aldrich.

Cytofluorimetric analysis of apoptosis
Subconfluent cells were exposed to TN 100 µM (Taurolin; Geistlich Pharma, Wollhusen, Switzerland) or CD95-activating antibody 100 ng/ml (clone CH11; Upstate Biotechnology, Lake Placid, NY, USA). After 24 hours incubation, cells were harvested in binding buffer (10 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) stained in the dark for 10 minutes with 5 µl of FITC-labelled Annexin V (Alexis, Lausanne, Switzerland), washed with binding buffer then stained with 1 µg/ml propidium iodide (Sigma-Aldrich). We analyzed 5,000 events per sample. Apoptotic cells were positive to Annexin V staining only and late apoptotic the cells positive to both Annexin V and Propidium iodide staining.

**TUNEL analysis**

Apoptosis was evaluated by TUNEL analysis (DeadEnd™ Colorimetric TUNEL system; Promega, Madison, WI, USA), following treatment for 24 hours with TN 100 µM alone and in presence of 10 mM Glutathione mono-ethylester (GSH) or 10 mM L-N-Acetyl-cysteine (L-NAC). In brief, sub-confluent cell cultures were exposed to medium supplemented with 2% FBS according to different treatments for 24 hours and fixed in 10% buffered formalin. Biotin-dU positive nuclei were counted on 10 fields with at least 100 cells in the same slide.

**Cell Cycle analysis**

Cells were synchronized by 0.1 µg/ml Colcemyd (Sigma-Aldrich) treatment for 24 hours, and then kept in normal medium for 4 days before analysis. After treatment with TN 100 µM for 6 hours, cells were washed in PBS, fixed in Ethanol and stained for 30
minutes at room temperature with 50 µg/ml Propidium Iodide (PI; Sigma-Aldrich) in PBS containing RNAse. 10,000 events per sample were analyzed by flow cytometry.

**Cytotoxicity and DNA adducts**

Cells were treated for different times and at different drug concentrations in presence of 2% FBS. Cytotoxicity was assessed by MTT assay, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) and performed in quadruplicate, as previously described[17]. Normalized viability percentages were obtained according to the ratio: (A_{570} mean values of extracts from exposed samples / A_{570} mean values of extracts from control cell samples)x100. DNA adducts were evaluated by HPLC on DNA extracts from cells treated with TN 100 µM for 16 hours and are expressed as amount of 8-OHdG per 10^5 dG, as previously described[18].

**Nitrite production**

Cells were cultured in medium containing 2% FBS and then stimulated with TN 100 µM in medium containing 2% FBS. Nitrite production was determined by the Griess Reagent System (Promega).

**Immunoblotting**

After drug treatment subconfluent cells were lysed in L-buffer (2.5% SDS, Tris-HCl 250 mM pH 7.4) and 40 µg of total cell lysates were loaded in reducing conditions. After separation on SDS-PAGE and transfer to nitrocellulose filter (Protran; S&S, Dassel, Germany), filters were probed with Phospho-Akt (Ser^473), Phospho-p70 S6 Kinase (Thr^389), Phospho-p38 MAPK (Thr^{180}/Tyr^{182}), Phospho-SAPK/JNK
(Thr\(^{183}/\)Tyr\(^{185}\)) Phospho-PTEN (Ser\(^{380}/\)Thr\(^{382/383}\)), Phospho-PP1α (Thr\(^{320}\)), Akt, p70 S6 Kinase, p38, SAPK/JNK, PTEN, PP1α, PP2A and Caspase-8 antibodies (all from Cell Signaling Technology, Beverly, MA, USA), Phospho-Erk 1/2 (Thr\(^{183}/\)Tyr\(^{185}\)) and α-Tubulin antibodies (both from Sigma-Aldrich), Phospho-PP2A (Tyr\(^{307}\)) and PARP-1 antibodies (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The signal was detected by the enhanced SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

**Immunoprecipitation**

For immunoprecipitation, after drug treatment total cellular proteins were extracted by RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS) containing protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM Na\(_3\)VO\(_4\), 2 mM NaF). For co-immunoprecipitation, after drug treatment cells were lysed in Solubilization buffer (20 mM Tris-HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 10% glycerol, 1% Triton X-100) with protease and phosphatase inhibitors. 500 µg aliquots of clarified cell lysates were incubated with 1 µg of antibody immobilized on protein-A-sepharose 4B packed beads (GE Healthcare, Piscataway, NY, USA) for 2 hours at 4°C. After extensive washes with lysis buffer, precipitated proteins were loaded in reducing conditions as described above. Filters were probed with Met and PDGFR-β antibodies (both from Santa Cruz Biotechnology), phospho-tyrosine, p85, p110α and nitro-tyrosine antibodies (all from Upstate Biotechnology), Hsp-90 (form BD Biosciences, San Jose, CA, USA).
Statistics

Data from cytotoxicity, apoptosis and cell cycle cytofluorimetric analysis, nitrite production and DNA adducts are expressed as mean ± Standard Error (SE) of at least three independent experiments. Statistical differences were evaluated by analysis of variance (ANOVA), followed by Tukey’s HSD. Values from TUNEL assay are expressed as percentages of positive nuclei over total counted. Statistical analysis was performed by Fisher’s Exact test. In all statistical evaluation the significance threshold was specified in the text. All statistical tests were two-sided and calculated using Origin software (Microcal Software, Northampton, MA, USA).

RESULTS

Taurolidine is cytotoxic and pro-apoptotic in mesothelioma cells

Primary mesothelial (HMC), immortalized mesothelial (Met-5A) and mesothelioma cells (MMB and MMP) were treated with different concentrations of Taurolidine (TN), ranging from 25 µM to 150 µM, for 16 hours in low serum. Normal dermal fibroblasts and lung carcinoma cells were also treated as controls. A significant cytotoxic effect was observed at concentrations above 50 µM in MMB, MMP and A549 neoplastic cells, compared with non neoplastic cells (p<0.001). MMB cells were more sensitive to TN, while non-neoplastic cells, mesothelial or not in origin, displayed negligible cytotoxicity upon the same TN treatment (Fig. 1 A). The cytotoxic effect induced by TN 100 µM was time-dependent starting after 5 hours treatment (p<0.001) (Fig. 1 B). TN-dependent programmed cell death was also examined. Mesothelioma cells, labelled with Propidium Iodide and Annexin V, were analyzed for apoptosis by flow cytometry.
in the presence of TN. Moreover, given that Fas takes part of cell death pathways in mesothelioma [19], apoptosis has been verified also following agonistic CD95 antibodies (CH-11). A significant number of apoptotic cells was observed in mesothelioma cells with 100 µM TN for 24 hours (p<0.001). However, apoptosis induced in MMB cells was by far higher than that induced in MMP cells (Fig. 2 A). These results were confirmed by cell cycle analysis (Fig. 2 B) revealing a significant increase in sub-G1 population in MMB (p<0.001) and MMP (p<0.005) cells treated with TN. Moreover, treatment with TN induced a significant decrease in G2-M sub-population in both mesothelioma cells compared to untreated cells (p<0.001) (Table 1).

To achieve a molecular characterization of TN-induced apoptosis, PARP and Caspase-8 cleavage was determined by Western blotting on total cell lysates of HMC, MMB and MMP, after 6 hours treatment with 150 µM TN. In these conditions TN induced a clear-cut cleavage of PARP and of Caspase-8 proteins in mesothelioma cells, as shown by the appearance of lower molecular weight bands (Fig. 2 C). Apoptosis, PARP and Caspase 8 cleavage or cell cycle alterations were not observed in non-neoplastic HMC (Fig. 2).

We conclude that TN specifically induces apoptosis of mesothelioma cells, in time- and dose-dependent manner. The lack of effects on non-transformed mesothelial cells prompted us to verify the mechanism of TN targeting to mesothelioma cells.

**Taurolidine inactivates Akt and activates PP2A in mesothelioma cells**

TN has been reported to affect several intracellular pathways and to inhibit protein synthesis.[7] As we and others reported Akt as playing a key role in mesothelioma survival [4, 5, 12], we verified in mesothelioma cells the effect of TN on the activity of Akt and its downstream p70 S6 Kinase (p70\textsuperscript{S6K}) effector, a known regulator of
translation.. TN inhibited Akt and p70S6K phosphorylation only in mesothelioma cells (MMP and MMB), but not in non-neoplastic HMC. On the contrary, the specific mTor inhibitor Rapamycin was effective in blocking the downstream p70S6K in all cells examined (Fig. 3 A). To verify Akt phosphorylation in cells resistant or sensitive to TN treatment, we compared non-neoplastic, TN-resistant HMC with TN-sensitive MMP mesothelioma cells, displaying comparable levels of Akt protein. The inhibition of Akt activity by TN was dose-dependent, being evident at 50 µM and reaching the maximum at 150 µM, whereas in HMC Akt phosphorylation was totally unaffected (Fig. 3 B). The inhibitory effect of TN on Akt activity was also time-dependent, starting at 30 minutes and lasting up to eight hours (Fig. 3 C). To verify the specificity of TN signalling inhibition we compared also the activities of Erk 1/2, JNK and p38 by immunoblotting with phosphospecific antibodies on lysates of HMC and MMP cells, treated with TN with the same kinetics evaluated for Akt. Moreover, to get a better insight on the inhibitory effect induced by TN, we examined the activities of two protein phosphatases and of one lipid phosphatase, which have been reported to regulate Akt.[20] Therefore, the phosphorylation of residues critical for the activity of PP2A (Tyr307), PP1α (Thr320) and PTEN (Ser380, Thr382/383) phosphatases, all positively regulated by Ser/Thr or Tyr de-phosphorylation [21], was evaluated by immunoblotting with phosphospecific antibodies on non-neoplastic HMC and on MMP cells. Regarding Erk 1/2 activity, beside of a sharp increase induced after 30 minutes TN treatment in both cell types, no differences were observed thereafter, even upon prolonged (8 hours) cell exposure to the drug. Conversely, JNK and p38 phosphorylation was increased by TN treatment both in HMC and in MMP cells (Fig. 3 C). Analysis of the phosphatase activities revealed that PP2A became de-phosphorylated with a slower kinetics than that
of Akt, with a significant activation since the second hour from treatment, indicating a progressive activation in phosphohydrolase activity upon TN. On the contrary, PP1α showed a very late (8 hours) and weak activation upon TN, whereas PTEN activity did not vary at all (Fig. 3 C). Similar findings were found in MMB cell line (data not shown).

Moreover, we tested cytotoxicity elicited by TN on MMB cells expressing a myristoylated form of Akt (myrAkt-MMB), which is permanently localized to the plasma membrane and therefore constitutively active [22]. Surprisingly, no differences were observed between wild-type MMB and the same cells expressing active Akt in cell viability and in inhibiting Akt phosphorylation in dose-dependent manner (data not shown).

Altogether, these results indicate that TN exerts a specific, time- and dose-dependent inhibition on Akt activity only in mesothelioma cells, but not in normal mesothelial cells and that PP2A jointly with PP1α might be additionally involved in sustaining this process. The increase in JNK and p38 activities is also induced by TN treatment, but it is not specific for neoplastic cells.
Taurolidine pro-apoptotic activity on mesothelioma cells is mediated by oxidative stress

TN pro-apoptotic effect on glioma cells relied upon the generation of reactive O₂ intermediates [16] and oxidative stress can cause Akt inhibition in human leukaemia cells.[15] We verified if the mechanism of TN pro-apoptotic activity in mesothelioma cells may be the activation of an oxidative pathway and if this was responsible for TN biological effects.

The rate of oxygen free radical generation is changed by production of nitric oxide. We evaluated nitrite production by Griess assay, upon treatment of HMC, as well as of MMB and MMP mesothelioma cells with 100 µM TN for different times, from 30 minutes to 24 hours. We observed a time-dependent nitrite production, occurring early only in mesothelioma cells, where between 2 and 4 hours the differences with mesothelial cells became significant (p<0.001). In HMC cells only after 24 hours was observed a significant level of nitrite production, which was lower than that of mesothelioma cells (p<0.001) (Fig. 4 A). To verify the oxidative-stress induced by TN, we measured the amount of 8-hydroxy-2’ deoxy-guanosine (8-OH-dG) DNA adducts, as a consequence of intracellular reactive oxygen species (ROS) production.[23] Upon TN treatment, the fold increase of DNA adducts, normalized for 10⁵ 2’-deoxyguanosine molecules, was significantly higher in mesothelioma cells as compared to non-neoplastic mesothelial cells (p<0.001) (Fig. 4 B). These results confirm that mesothelioma cells are more sensitive to TN activity, which is mediated by oxidative stress.

Anti-oxidant agents has been widely used to prevent the effects elicited by oxidative stress in live cells.[24] We evaluated Akt phosphorylation in lysates from MMP cells
pre-treated with Glutathione mono-ethylester (GSH) 10 mM for 24 hours, before addition of TN 150 µM for 30 minutes. GSH did not affect Akt phosphorylation in absence of TN, whereas GSH cell pre-treatment completely prevented Akt inhibition by TN, suggesting that Akt dephosphorylation by TN is mediated via an oxidative response (Fig. 4 C). Similar results were obtained in a parallel experiment conducted with the anti-oxidant agent L- N-Acetylcysteine (L-NAC; Fig. 4 C). These anti-oxidant agents did not alter TN-induced JNK and p38 activation (data not shown).

Cell viability was examined by MTT assay in cells treated 24 hours with increasing concentrations of TN, after 24 hours pre-treatment with GSH 10 mM or L-NAC 10 mM. In these condition TN induced a dose-dependent decrease in cell viability, which is more pronounced than that observed after only 16 hours (see Fig. 1 A). Pre-exposure of the same cells to the anti-oxidant agents significantly inhibited the cytotoxic effect of TN, even at the highest concentration (p<0.001) (Fig. 4 D). The effect of antioxidants was evaluated also on apoptosis, using TUNEL assay on MMP cells. A higher number of Biotin-dU positive nuclei was observed upon cell exposure to TN 100 µM for 24 hours (14.1%) than in control non stimulated cells (3.1%). The concurrently treatment with GSH 10 mM or L-NAC 10 mM decreased the percentage of apoptotic nuclei to 2.6% and 3.1% respectively (Fig. 4 E). These differences were statistically significant (p<0.001). We obtained similar results for cell viability and apoptosis, with both anti-oxidant agents on the mesothelioma MMB cells (data not shown).

We conclude that the cytotoxic, pro-apoptotic effect of TN on mesothelioma cells, stems from oxidative stress, involves nitrite production and is reversed by general anti-oxidant agents.
DISCUSSION

Our results provide an explanation of the mechanisms underlying the anticancer effects exerted by TN on mesothelioma cells. Selective anticancer effects of TN has been focused [9, 25] and its role for mesothelioma treatment has been already hypothesized [26, 27]. The mechanism by which TN may provoke tumour cell death has been largely debated [8], but not clarified yet. Here we demonstrate that TN selectively induces mesothelioma cell death via oxidative stress and negatively affecting Akt activity.

Given the crucial role played by Akt signalling in human tumours and in development, progression and chemo-resistance of mesothelioma, previously shown by us [4, 5] and by others [12], we assessed whether TN could interfere with Akt activity. Our results clearly demonstrate that TN inhibits Akt signalling, as demonstrated by the relevant reduction of Akt and of downstream mTOR-dependent p70S6K activities, but not of Erk 1/2 activity. Following TN treatment, we observed in HMC and in mesothelioma cells a clear and progressive increase of p38 and Jnk activities, mostly involved in cell stress responses.[28]

On the other hand, sensitivity of mesothelioma cells to oxidative stress has been proposed as a potential therapeutic strategy [29], while others suggested a cytotoxic effect of TN on glioma cells via oxygen intermediates-dependent apoptosis.[16]. Others demonstrated that TN also exerts an oxidative stress on mesothelioma cells, with subsequent p53 activation and down-regulation of survivin, Bcl-2 and Mcl1 survival proteins and cell death. [27]

In the present study, we observed a specific and time-dependent nitrite production upon mesothelioma cell treatment with TN. Interestingly, either in MMB and in MMP cells
the kinetics of nitrite release is congruent with time-course of cytotoxicity caused by this drug. Moreover, also Akt inhibition occurs after TN treatment, following a similar trend. Altogether, these results lead us to conclude that TN induces oxidative stress and this scenario is confirmed by the significant increase of 8OH-dG adduct production, induced by TN in mesothelioma cells, compared to non-neoplastic mesothelial cells. This selectivity of TN action on mesothelioma cells is further assessed by restoring cell viability when TN treatment is accompanied by GSH or L-NAC. Both anti-oxidants rescue Akt activity inhibited by TN, highlighting the close association between TN-induced cytotoxicity and Akt de-phosphorylation.

In MMB cells expressing a myristoylated, constitutively active, Akt [22] TN was still able either to inhibit Akt phosphorylation and to induce cytotoxicity as in wild-type MMB cells. The fact the TN inhibited even constitutive activated Akt (although at higher concentrations) reinforces the specific effect of TN on this signalling. Interestingly, PP2A activity was selectively increased upon TN treatment only in MMP but not in HMC, with a peak starting two hours after drug exposure, whereas PP1α was activated very late and PTEN activity was not modified at all. The role of PP2A has been extensively studied as modulator of signalling kinases and as key regulator in human tumours.[30] Moreover, other studies underscored that PP2A can be specifically targeted by pharmacological agents [31] and that this phosphatase specifically inhibits Akt activity.[32] Our results reveal a difference in timing of Akt inhibition and PP2A activation. We can hypothesize either that very low PP2A activity, below the immunoblotting detection threshold, may be sufficient to inhibit Akt in the early phases of cell response to TN or - more likely - that PP2A and possibly of PP1α sustain the Akt inhibited status anyhow provoked by another yet unknown effector. It has been recently
suggested that the oxidative stress may influence Akt activity by nitration of p85 subunit of PI3K, leading to the impairment of the p85/p110 complex.[33] We did not observe in mesothelioma cells any detectable nitration of p85 regulatory subunit upon TN treatment (data not shown). On the other hand, other studies reported that nitration may stimulate receptor tyrosine kinases (RTK) leading to increase of Akt activity in rat fibroblasts.[34] However, we were not able to detect any modification of tyrosine phosphorylation levels of RTKs most commonly expressed in mesothelioma cells, like Met (Hepatocyte Growth Factor Receptor) and PDGFRβ (Platelet Derived Growth Factor receptor β) (data not shown). The discrepancy with our results may be due to cell lineage differences in responsiveness to oxidant agents. Our results strongly suggest that TN is acting on the mechanisms of Akt activation/inhibition more than on the upstream signalling. The chaperone heat-shock protein Hsp90 preserves Akt activity by preventing PP2A-mediated dephosphorylation.[35] In the conditions of TN-induced inhibition of Akt, we did not observe any difference in Hsp90 or PP2A association with the kinase (data not shown). We conclude that another yet unidentified mechanism underlies the observed effects of TN on Akt, at least one early event (already occurring after 30 minutes of treatment), which might contribute to the observed apoptosis due to oxidative stress.

Locally injected TN in mice with intra-peritoneal mesothelioma exerted anti-tumour effects [26], suggesting that mesothelioma may be cured with TN, through intra-pleural prolonged infusion. Our study provides a rationale for TN as a novel local treatment for malignant mesothelioma.
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REFERENCES


Table 1. Cell cycle analysis of synchronized HMC and MM cells upon treatment with 100 µM TN for 6 hours.

<table>
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Data are expressed as average percentage of cells in cell cycle phases ± SE.
FIGURE LEGENDS

Figure 1. TN induces cell death in mesothelioma cells in time- and dose-dependent manner. A) Viability (MTT) assay performed on ♦ HDF, ■ HMC, ▲ Met-5A, △ MMB, □ MMP, ◆ A549 cells treated with TN from 25 µM up to 150 µM, for 16 hours in 2% FBS medium. Black symbols: non-neoplastic cells, white symbols: neoplastic cells. The lineage of different cells are reported in Material and Methods; B) Viability (MTT) assay performed on ■ HMC, △ MMB, □ MMP cells treated with TN 100 µM for the indicated times.
Figure 2. TN is pro-apoptotic in mesothelioma cells. A) Flow cytometry analysis of apoptosis in HMC, MMB and MMP cells treated with 100 ng/ml CD95-activating antibody or with 100 µM TN, for 24 hours in 2% FBS medium, after Annexin V/Propidium Iodide labelling. B) Representative picture of flow cytometry cell cycle analysis, performed as reported in Material and Methods on HMC, MMB and MMP cells treated or not with 100 µM TN for 6 hours in 2% FBS. The percentages of cells in the different phases of the cell cycle are given in details in Table 1. C) Immunoblotting analysis of PARP cleavage and Caspase-8 activation on total cell lysates of HMC, MMB and MMP cells, treated with 150 µM TN for 6 hours in 2% FBS.
Figure 3. TN specifically inhibits the Akt pathway in mesothelioma cells. A) Immunoblotting analysis of the phosphorylation of Akt (P-Akt, Ser\textsuperscript{473}), p70 S6 Kinase (P-p70 S6 Kinase, Thr\textsuperscript{389}) on non-neoplastic HMC, on neoplastic MMP and MMB cells treated with 200 nM of the mTOR inhibitor Rapamycin or with 150 µM TN for 30 minutes in 2% FBS medium. The protein level of the single kinases and of α-tubulin are also reported as controls; B) Dose-response Immunoblotting assay of P-Akt (Ser\textsuperscript{473}) on
HMC and MMP cells treated with TN from 50 µM to 500 µM for 30 minutes in 2% FBS medium. Expression of Akt and α-tubulin are also reported as controls; C) Time-course Immunoblotting analysis of phosphorylation levels of Akt (Ser^473), Erk 1/2 (Thr^{183}/Tyr^{185}), p38 (Thr^{180}/Tyr^{182}), Jnk (Thr^{183}/Tyr^{185}), PP2A (Tyr^{307}), PP1α (Thr^{320}), PTEN (Ser^{380}/Thr^{382/383}) on HMC and MMP cells treated with 150 µM TN for different times from 30 minutes up to 8 hours in 2% FBS medium. The protein levels of the single effectors and of α-tubulin are also reported as loading controls.

Figure 4. TN action on mesothelioma cells is mediated by oxidative stress. A) Griess assay for nitrite micromolar determination in HMC, MMP and MMB cells treated with
100 µM TN at different times, from 30 minutes up to 24 hours, in 2% FBS medium (white bars = HMC; dashed bars = MMP; black bars = MMB); B) 8-OHdG adducts determination in HMC, MMP and MMB cells treated with 100 µM TN for 16 hours in 2%FBS. Data are reported as fold increase over the respective untreated sample; C) Immunoblotting analysis of phosphorylation of Akt (Ser<sup>473</sup>), in MMP cells in 2% FBS medium pretreated for 24 hours with 10 mM Glutathione mono-ethylester (GSH) or with 10 mM L-N-Acetyl-cysteine (L-NAC) respectively, exposed to 150 µM TN for 30 minutes and with both TN and anti-oxidant. Levels of Akt and α Tubulin proteins are reported as loading controls; D) Dose-response viability (MTT) assay, performed on MMP cells in 2% FBS medium, with TN from 50 to 150 µM for 24 hours or treated with TN at the same concentrations in presence of 10 mM GSH or L-NAC; E) TUNEL assay performed on MMP cells in 2% FBS medium, with TN 100 µM for 24 hours, in presence or absence of GSH 10 mM or L-NAC 10 mM. Percentages of apoptotic nuclei are shown, following counts on 10 fields with at least 100 cells in the same slide.
Figure 4