Taurolidine and oxidative stress: a rationale for local treatment of mesothelioma

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ABSTRACT: Malignant mesothelioma is an asbestos-related, aggressive tumour, resistant to most anticancer therapies. Akt is a key mediator of mesothelioma cell survival and chemoresistance. This study aimed to clarify the mechanism by which taurolidine (TN), a known synthetic compound with antimicrobial and antineoplastic properties, leads to mesothelioma cell death.

Apoptosis was studied by annexin V binding, cell cycle analysis, caspase-8 activation, poly(ADP-ribose) polymerase (PARP) cleavage and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL). Oxidative stress was measured by nitrite production and DNA oxidative damage. Protein expression and phosphorylation were evaluated by immunoprecipitation and immunoblotting.

TN induces cell death of mesothelioma cells, but not of non-neoplastic human mesothelial cells. After TN treatment of mesothelioma cells, Akt but not extracellular signal-regulated kinase (Erk) 1/2 activity is inhibited in a time- and dose-dependent manner. Protein phosphatase (PP)1 α and PP2A are activated several hours after drug addition. Apoptosis induced by TN is driven by oxidative stress and cell exposure to sulfhydryl donors, such as glutathione monoethylester and L-N-acetylcysteine, significantly reduced pro-apoptotic effects and Akt inhibition. Conversely, expression of constitutively activated Akt did not affect cytotoxicity elicited by TN, which retained its ability to inhibit the kinase.

TN induces mesothelioma cell death *via* oxidative stress, accompanied by inhibition of Akt signalling. This provides a promising molecular rationale for TN as local treatment of malignant mesothelioma.

KEYWORDS: Akt, apoptosis, malignant mesothelioma, oxidative stress, taurolidine

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 a co-carcinogen, causing malignant transformation of human mesothelial cells (HMCs) [3] through Akt-dependent survival signalling [4, 5].

Taurolidine (TN), or bis(1,1-dioxoperhydro-1,2,4-thiodiazinyl-4)-methane, is an antibacterial drug originally synthesised 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 recent years, *in vitro* evidence has highlighted the role of TN as a 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 indicates that this drug is of potential help for therapy of tumours with prevalent local spreading, such as peritoneal and pleural mesothelioma.

The importance of the Akt pathway in maintaining anti-apoptotic survival signals, responsible

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for onset and progression of mesothelioma, has been demonstrated [4, 12] and very recently we showed that addressing Akt with pharmacological agents is also a promising approach for mesothelioma [13, 14].

Oxidative stress generated by reactive oxygen species 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 HMCs, 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 HMCs were obtained from patients with congestive heart failure and cultured in Ham's F12 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Rockville, MD, USA). MET5A were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), 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-humidified 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 mono-ethylester (GSH), *N*-acetyl-L-cysteine (L-NAC) and rapamycin were purchased from Sigma-Aldrich.

Cytofluorimetric analysis of apoptosis

Subconfluent cells were exposed to 100 µM TN (Taurolin®; Geistlich Pharma, Wolhusen, Switzerland) or CD95-activating antibody 100 ng·mL⁻¹ (clone CH11; Upstate Biotechnology, Lake Placid, NY, USA). After 24-h 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 min with 5 µL of fluorescein isothiocyanate-labelled annexin V (Alexis, Lausanne, Switzerland), washed with binding buffer and then stained with 1 µg·mL⁻¹ propidium iodide (Sigma-Aldrich). We analysed 5,000 events per sample. Apoptotic cells were positive to annexin V staining only and late apoptotic cells were positive to both annexin V and propidium iodide staining.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling analysis

Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) analysis (DeadEnd™ Colorimetric TUNEL System; Promega, Madison, WI, USA) following treatment for 24 h with 100 µM TN alone and in the presence of 10 mM GSH or 10 mM L-NAC. In brief, subconfluent cell cultures were exposed to medium supplemented with 2% FBS according to different treatments for 24 h and fixed in 10% buffered formalin. Biotin-deoxyuridine (dU)-positive nuclei were counted on 10 fields with at least 100 cells in the same slide.

Cell cycle analysis

Cells were synchronised by 0.1 µg·mL⁻¹ colcemid (Sigma-Aldrich) treatment for 24 h, and then kept in normal medium for 4 days before analysis. After treatment with 100 µM TN for 6 h, cells were washed in phosphate-buffered saline, fixed in ethanol and stained for 30 min at room temperature with 50 µg·mL⁻¹ propidium iodide (Sigma-Aldrich) in phosphate-buffered saline containing RNase. 10,000 events per sample were analysed by flow cytometry.

Cytotoxicity and DNA adducts

Cells were treated for different times and at different drug concentrations in the 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]. Normalised 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) × 100. DNA adducts were evaluated by high-performance liquid chromatography on DNA extracts from cells treated with 100 µM TN for 16 h and are expressed as amount of 8-hydroxy-2-deoxyguanosine per 10⁵ deoxyguanosines, as previously described [18].

Nitrite production

Cells were cultured in medium containing 2% FBS and then stimulated with 100 µM TN 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% sodium dodecyl sulfate (SDS), Tris-HCl 250 mM pH 7.4) and 40 µg of total cell lysates were loaded in reducing conditions. After separation on SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose filter (Protran; S&S, Dassel, Germany), filters were probed with phospho-Akt (Ser⁴⁷³), phospho-p70 S6 kinase (Thr³⁸⁹), phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), phospho-SAPK/c-Jun N-terminal kinase (JNK) (Thr¹⁸³/Tyr¹⁸⁵), phospho-PTEN (Ser³⁸⁰/Thr^{382/383}), phospho-protein phosphatase (PP)1α (Thr³²⁰), Akt, p70 S6 kinase, p38, SAPK/JNK, PTEN, PP1α, PP2A and caspase-8 antibodies (all from Cell Signaling Technology, Beverly, MA, USA), phospho-extracellular signal-regulated kinase (Erk) 1/2 (Thr¹⁸³/Tyr¹⁸⁵) and α-tubulin antibodies (both from Sigma-Aldrich), phospho-PP2A (Tyr³⁰⁷) and poly(ADP-ribose) polymerase (PARP)-1 antibodies (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The signal was

FIGURE 1. Taurolidine (TN) induces cell death in mesothelioma cells in a time- and dose-dependent manner. a) Viability (MTT) assay performed on human dermal fibroblast (●), human mesothelial cells (HMCs; ■), MET5A (▲), malignant mesothelioma cell lines MMB (△) and MMP (□), and A549 cells (○) treated with TN (25–150 μM), for 16 h in 2% fetal bovine serum medium. Filled symbols indicate non-neoplastic cells and empty symbols indicate neoplastic cells. The lineage of different cells are reported in the Methods section. b) Viability (MTT) assay performed at the indicated times on HMCs (■), and MMB (△) and MMP (□) cells treated with 100 μM TN.

TABLE 1		Cell cycle analysis of synchronised human mesothelial cells (HMCs) and malignant mesothelioma (MMB and MMP) cells upon treatment with 100 μM taurolidine (TN) for 6 h				
	TN	Cell cycle phase				
		Sub-G1	G1	S	G2-M	
HMC	-	7.42±1.25	56.67±4.02	8.82±1.23	27.32±2.43	
	+	6.38±1.43	58.21±3.98	9.28±1.76	26.21±2.76	
MMB	-	3.65±0.97	47.82±4.68	18.61±1.98	29.96±2.12	
	+	40.05±3.01	31.22±2.65	16.38±1.97	12.25±1.98	
MMP	-	4.25±1.12	57.28±4.12	16.34±2.05	26.32±2.87	
	+	7.91±1.23	53.84±3.01	20.42±2.87	18.01±1.96	

Data are presented as average percentage of cells in cell cycle phase±SE.

FIGURE 2. Taurolidine (TN) is pro-apoptotic in mesothelioma cells. a) Flow cytometry analysis of apoptosis in human mesothelial cells (HMCs), malignant mesothelioma MMB and MMP cells treated with 100 ng·mL⁻¹ CD95-activating antibody (■) or with 100 μM TN for 24 h in 2% fetal bovine serum (FBS) medium (□) after annexin V/propidium iodide (PI) labelling. □: untreated cells as compared with αCD95 (CD95 activating antibody)- and TN-treated cells. b) Representative picture of flow cytometry cell cycle analysis, performed as reported in the Methods section on HMCs, and MMB and MMP cells either treated or not treated with 100 μM TN for 6 h in 2% FBS. The percentages of cells in the different phases of the cell cycle are given in table 1. c) Immunoblotting analysis of poly(ADP-ribose) polymerase (PARP) cleavage and caspase-8 activation on total cell lysates of HMCs, and MMB and MMP cells treated with 150 μM TN for 6 h in 2% FBS.

FIGURE 3. Taurolidine (TN) specifically inhibits the Akt pathway in mesothelioma cells. a) Immunoblotting analysis of the phosphorylation of Akt (P-Akt, Ser⁴⁷³), p70 S6 kinase (P-p70^{S6 kinase}, Thr³⁸⁹) on non-neoplastic human mesothelial cells (HMCs), on neoplastic malignant mesothelioma MMP and MMB cells treated with 200 nM of the mTOR inhibitor rapamycin (RA) or with 150 μ M TN for 30 min in 2% fetal bovine serum (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 (Ser⁴⁷³) on HMCs and MMP cells treated with between 50 μ M and 500 μ M of TN for 30 min 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 (Ser⁴⁷³), Erk 1/2 (Thr¹⁸³/Tyr¹⁸⁵), p38 (Thr¹⁸⁰/Tyr¹⁸²), JNK (Thr¹⁸³/Tyr¹⁸⁵), PP2A (Tyr³⁰⁷), PP1 α (Thr³²⁰) and PTEN (Ser³⁸⁰/Thr^{382/383}) on HMCs and MMP cells treated with 150 μ M TN for different times ranging from 30 min to 8 h in 2% FBS medium. The protein levels of the single effectors and of α -tubulin are also reported as loading controls.

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 μ g·mL⁻¹ aprotinin, 10 μ g·mL⁻¹ leupeptin, 10 μ g·mL⁻¹ pepstatin, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (1 mM Na₃VO₄, 2 mM NaF). For co-immunoprecipitation, after drug treatment, cells were lysed in solubilisation 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- μ g aliquots of clarified cell lysates were incubated with 1 μ g of antibody immobilised on protein-A-sepharose 4B packed beads (GE Healthcare, Piscataway, NY, USA) for 2 h at 4°C. After extensive washes with lysis buffer, precipitated proteins were loaded in reducing conditions as described above. Filters were probed with Met (hepatocyte growth factor receptor) and platelet-derived growth factor receptor (PDGFR)- β antibodies (both from Santa Cruz Biotechnology), phospho-tyrosine, p85, p110 α and nitro-tyrosine antibodies (all from Upstate Biotechnology), Hsp-90 (from 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 $\pm$ SE of at least three independent experiments. Statistical differences were evaluated by ANOVA, followed by Tukey's honestly significant difference test. 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

TN is cytotoxic and pro-apoptotic in mesothelioma cells

Primary mesothelial (HMC), immortalised mesothelial (Met-5A) and mesothelioma cells (MMB and MMP) were treated with different concentrations of TN, ranging from 25 μ M to 150 μ M, for 16 h in low serum. Normal dermal fibroblasts and lung carcinoma cells were also treated as controls. A significant cytotoxic effect was observed at concentrations >50 μ M 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, whether mesothelial or not in origin, displayed negligible cytotoxicity upon the same TN

treatment (fig. 1a). The cytotoxic effect induced by 100 μ M TN was time-dependent, starting after 5 h of treatment ($p < 0.001$) (fig. 1b).

TN-dependent programmed cell death was also examined. Mesothelioma cells, labelled with propidium iodide and annexin V, were analysed for apoptosis by flow cytometry in the presence of TN. Moreover, given that Fas takes part in cell death pathways in mesothelioma [19], apoptosis has also been verified following agonistic CD95 antibodies (CH-11). A significant number of apoptotic cells was observed in mesothelioma cells treated with 100 μ M TN for 24 h ($p < 0.001$). However, apoptosis induced in MMB cells was far higher than that induced in MMP cells (fig. 2a). These results were confirmed by cell cycle analysis (fig. 2b), which revealed a significant increase in the 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 subpopulation in both mesothelioma cells compared with untreated cells ($p < 0.001$) (table 1).

To achieve a molecular characterisation of TN-induced apoptosis, PARP and caspase-8 cleavage was determined by Western blotting on total cell lysates of HMCs, and MMB and MMP cells after 6-h treatment with 150 μ M TN. Under 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. 2c). Apoptosis, PARP and caspase-8 cleavage or cell cycle alterations were not observed in non-neoplastic HMCs (fig. 2).

We conclude that TN specifically induces apoptosis of mesothelioma cells in a 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.

TN 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 have 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^{S6K}) effector, a known regulator of translation. TN inhibited Akt and p70^{S6K} phosphorylation only in mesothelioma cells (MMP and MMB), and not in non-neoplastic HMCs. On the contrary, the specific inhibitor of mTor, rapamycin, was effective in blocking the downstream p70^{S6K} in all cells examined (fig. 3a). To verify Akt phosphorylation in cells resistant or sensitive to TN treatment, we compared non-neoplastic TN-resistant HMCs 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 μ M and reaching a maximum at 150 μ M, whereas in HMCs Akt phosphorylation was totally unaffected (fig. 3b). The inhibitory effect of TN on Akt activity was also time-dependent, starting at 30 min and lasting for up to 8 h (fig. 3c). To verify the specificity of TN signalling inhibition, we also compared the activities of Erk 1/2, JNK and p38 by immunoblotting with phosphospecific antibodies on lysates of HMCs and MMP cells treated with TN with the same kinetics evaluated for Akt. Moreover, to get a better insight into 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), PP1 α (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 HMCs and on MMP cells. Regarding Erk 1/2 activity, beside of a sharp increase induced after 30 min of TN treatment in both cell types, no differences were observed thereafter, even upon prolonged (8 h) cell exposure to the drug. Conversely, JNK and p38 phosphorylation was increased by TN treatment both in HMCs and in MMP cells (fig. 3c). Analysis of the phosphatase activities revealed that PP2A became de-phosphorylated with a slower kinetics to those of Akt, with a significant activation from the second hour of treatment, indicating a progressive activation in phosphohydrolase activity upon TN. On the contrary, PP1 α showed a very late (8 h) and weak activation upon treatment with TN, whereas PTEN activity did not vary at all (fig. 3c). Similar findings were found in the 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 localised 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 a 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, and not in normal mesothelial cells, and that PP2A jointly with PP1 α 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.

TN pro-apoptotic activity on mesothelioma cells is mediated by oxidative stress

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

The rate of oxygen free radical generation is changed by the production of nitric oxide. We evaluated nitrite production by

Griess assay, upon treatment of HMCs, as well as of MMB and MMP mesothelioma cells, with 100 μ M TN for different times ranging from 30 min to 24 h. We observed a time-dependent nitrite production occurring early and only in mesothelioma cells, where the differences with mesothelial cells became significant ($p < 0.001$) at between 2 and 4 h. In HMCs, only after 24 h was a significant level of nitrite production observed, which was lower than that of mesothelioma cells ($p < 0.001$) (fig. 4a). To verify the oxidative-stress induced by TN, we measured the amount of 8-hydroxy-2' deoxyguanosine (8-OHdG) DNA adducts, as a consequence of intracellular reactive oxygen species production [23]. Upon TN treatment, the fold increase of DNA adducts, normalised for 10⁵ 2'-deoxyguanosine molecules, was significantly higher in mesothelioma cells as compared with non-neoplastic mesothelial cells ($p < 0.001$) (fig. 4b). These results confirm that mesothelioma cells are more sensitive to TN activity, which is mediated by oxidative stress.

Antioxidant agents have 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 10 mM GSH for 24 h before the addition of 150 μ M TN for 30 min. GSH did not affect Akt phosphorylation in the 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. 4c). Similar results were obtained in a parallel experiment conducted using the antioxidant agent L-NAC (fig. 4c). These antioxidant agents did not alter TN-induced JNK and p38 activation (data not shown).

Cell viability was examined by MTT assay in cells treated for 24 h with increasing concentrations of TN, after 24 h of pre-treatment with 10 mM GSH or 10 mM L-NAC. Under these conditions, TN induced a dose-dependent decrease in cell viability, which is more pronounced than that observed after only 16 h (see fig. 1a). Pre-exposure of the same cells to the antioxidant agents significantly inhibited the cytotoxic effect of TN, even at the highest concentration ($p < 0.001$) (fig. 4d). The effect of antioxidants on apoptosis was also evaluated using TUNEL assay on MMP cells. A higher number (14.1%) of biotin-dU-positive nuclei was observed upon cell exposure to 100 μ M TN for 24 h than in control nonstimulated cells (3.1%). The concurrent treatment with 10 mM GSH or 10 mM L-NAC decreased the percentage of apoptotic nuclei to 2.6% and 3.1%, respectively (fig. 4e). These differences were statistically significant ($p < 0.001$). We obtained similar results for cell viability and apoptosis, with both antioxidant agents acting 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 antioxidant agents.

DISCUSSION

Our results provide an explanation of the mechanisms underlying the anticancer effects exerted by TN on mesothelioma cells. The selective anticancer effect of TN has been focused on [9, 25] and its role for mesothelioma treatment has already been hypothesised [26, 27]. The mechanism by which TN may

FIGURE 4. Taurolidine (TN) action on mesothelioma cells is mediated by oxidative stress. a) Griess assay for nitrite micromolar determination in human mesothelial cells (HMCs; □), and malignant mesothelioma MMP (■) and MMB (■) cells treated with 100 μM TN at different times, ranging from 30 min to 24 h, in 2% fetal bovine serum (FBS) medium. b) 8-Hydroxy-2-deoxyguanosine (8-OHdG) adduct determination in HMCs and MMP and MMB cells treated with 100 μM TN for 16 h in 2% FBS. Data are reported as fold increase over the respective untreated sample. c) Immunoblotting analysis of phosphorylation of Akt (Ser⁴⁷³) in MMP cells in 2% FBS medium pre-treated for 24 h with 10 mM glutathione mono-ethylester (GSH) or with 10 mM L-N-acetyl-cysteine (L-NAC), respectively, and exposed to 150 μM TN for 30 min and to both TN and antioxidants (upper panel, TN+GSH and lower panel, TN+L-NAC). 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 ranging from 50 to 150 μM for 24 h or treated with TN at the same concentrations in presence of 10 mM GSH or L-NAC. e) terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) assay performed on MMP cells in 2% FBS medium, with 100 μM TN for 24 h, in either the 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 on the same slide.

provoke tumour cell death has been debated [8], but not yet clarified. We demonstrate herein that TN selectively induces mesothelioma cell death *via* oxidative stress and by negatively affecting Akt activity.

Given the crucial role played by Akt signalling in human tumours and in development, progression and chemo-resistance of mesothelioma, as 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 p70^{S6K} activities, but not of Erk 1/2 activity. Following TN treatment, we observed in HMCs and in mesothelioma cells a clear and progressive increase of p38 and JNK activities, mostly involved in cell stress responses [28].

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

In the present study, we observed the effects of specific and time-dependent nitrite production upon mesothelioma cell treatment with TN. Interestingly, in both MMB and MMP cells, the kinetics of nitrite release is congruent with the time-course of cytotoxicity caused by this drug. Moreover, Akt inhibition also 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 8-OHdG adduct production induced by TN in mesothelioma cells compared with non-neoplastic mesothelial cells. This selectivity of TN action on mesothelioma cells was then further assessed by restoring cell viability when TN treatment was accompanied by either GSH or L-NAC. Both antioxidants 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 to inhibit Akt phosphorylation and induce cytotoxicity, as in wild-type MMB cells. The fact that TN inhibited even constitutive activated Akt (albeit 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 HMCs, with a peak starting 2 h after drug exposure, whereas PP1 α was activated very late and PTEN activity was not modified at all. The role of PP2A has been extensively studied as a modulator of signalling kinases and as a key regulator in human tumours [30]. Moreover, other studies reinforced 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 hypothesise 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 PP1 α sustain the

Akt inhibited status provoked by another yet unknown effector. It has recently been suggested that oxidative stress may influence Akt activity by nitration of the p85 subunit of phosphoinositide 3-kinase, leading to the impairment of the p85/p110 complex [33]. We observed no detectable nitration of the p85 regulatory subunit in mesothelioma cells upon treatment with TN (data not shown). Conversely, other studies reported that nitration may stimulate receptor tyrosine kinases (RTKs) leading to increase of Akt activity in rat fibroblasts [34]. However, we were not able to detect any modification of tyrosine phosphorylation levels of those RTKs most commonly expressed in mesothelioma cells, such as Met and PDGFR 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]. Under the conditions of TN-induced inhibition of Akt, we observed no difference in Hsp90 or PP2A association with the kinase (data not shown). We conclude that another as yet unidentified mechanism underlies the observed effects of TN on Akt, comprising at least one early event (already occurring after 30 min of treatment), which might contribute to the observed apoptosis due to oxidative stress.

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

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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