Benzo[b]thiophenesulphonamide 1,1-dioxide derivatives inhibit tNOX activity in a redox state-dependent manner

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Benzo[b]thiophenesulphonamide 1,1-dioxide (BTS) derivatives are strong cytotoxic agents that induce reactive oxygen species (ROS) overproduction and apoptosis in tumour cells. Although the precise origin of BTS-induced ROS is not known, a clear correlation between their cytotoxic effect and ability to inhibit a tumour-associated NADH oxidase (tNOX) activity of the plasma membrane has been described. To analyse the putative implication of tNOX in BTS-induced ROS generation, in this work we have synthesised and tested a new BTS derivative, the 6-[N-(2-phenylethyl)]benzo[b]thiophenesulphonamide 1,1-dioxide. According to its high lipophility, this compound showed a strong cytotoxic activity against a panel of six human tumour cell lines, including two human leukaemia (K-562 and CCRF-CEM) and four human solid tumours (HT-29, HTB54, HeLa and MEL-AC). We also tested the ability of this compound to inhibit the tNOX activity and we found an absolute dependence of this inhibition on the redox state of the tNOX: while under reducing conditions, that is, 100 mM GSH, the drug inhibits strongly the NOX activity with an EC50 of about 0.1 nM, under oxidising conditions, there is no effect of the drug or just a slight stimulation of activity.

Keywords: benzo[b]thiophenesulphonamides; antineoplastic drugs; ECTO-NOX; tumour-associated NADH oxidase; redox state

ECTO-NOX are cell surface-associated and growth-related protein oxidases that exhibit two different activities, protein disulphide-thiol interchange and hydroquinone (or NADH) oxidation, that alternate to yield oscillatory patterns (Morre and Morré, 2003). Two forms of ECTO-NOX have been detected in sera of cancer patients (Wang et al, 2003): a widely distributed constitutive NOX (CNOX) of the mammalian cell surface that has a period length of 24 min (Sedlak et al, 2001) and is resistant to inhibition by quinone site inhibitors (Chueh et al, 2002a) like the vainilloid capsaicin or the antitumour sulphonylurea N-[4-(methylphenylsulphonyl)-N'-4-(chlorophenyl)urea (LY181984), and a tumour-associated NOX (tNOX) with a period length of 22 min (Chueh et al, 2002a) that is inhibited by capsaicin (Morré et al, 1997a) or LY181984 (Morré and Reust, 1997b) and is low or absent from sera of individuals not diagnosed as having cancer (Morré et al, 1997a). The tNOX protein is also specifically inhibited in HeLa and human mammary adenocarcinoma cells by (−)-epigallocatechin-3-gallate (EGCg) (Morré et al, 2000), the principal catechin of green tea; EGCg also inhibited growth of transformed cells in culture. Since this action appears to result from an effect on regulation of cell cycle progression and induction of apoptosis (Ahmad et al, 1997, 2000, 2002; Gupta et al, 2000) rather than from an unspecific antioxidant function (Salucci et al, 2002), tNOX protein has been proposed as the molecular target on cancer cells to explain their specific inhibition of growth by EGCg (Morré et al, 2000).

The putative implication of tNOX in BTS-induced ROS generation and the fact that some enzymes related with ROS
control such as the glutathione reductase and the glutathione S-transferase present a hydrophobic pocket near their active site (Karplus and Schulz, 1989; Chern et al., 2000) led us to synthesise and analyse new derivatives of the benzothiophene 1,1-dioxide carrying hydrophobic substituents of different length and grade of flexibility on the sulphonamide group and, in some cases, a clear correlation between the lipophilicity (log P) and the cytotoxic effect of these compounds was observed (Villar et al., 2004). Here we describe the synthesis and cytotoxic activity of 6-[N-(2-phenylethyl)]benzo[b]thiophenesulphonamide 1,1-dioxide (BTS-2), a new BTS derivative with increased flexibility, high lipophilicity (log P = 2.82) and a predicted low toxicity for its putative metabolites, and we show its ability to specifically inhibit the tNOX activity and the absolute dependence of this inhibition on the redox state of the tNOX.

MATERIALS AND METHODS

Chemistry

Benzo[b]thiophenesulphonamide 1,1-dioxide (BTS-1) was prepared as previously described (Martínez-Merino et al., 2000).

The synthesis of BTS-2 was carried out by the usual methods described for the synthesis of sulphonamide derivatives (Villar et al., 2004), that is, through the treatment of the sulphonyl chloride derivative with ammonia or amines (Scheme 1). The chlorosulphonyl derivative was obtained from the 6-aminothieno[b]thiophene 1,1-dioxide by the Meerwein’s method (Meerwein et al., 1957) (treatment of diazonium salts with sulphonyl chloride in the presence of cuprous chloride), and then treated with phenethylamine to give the BTS-2 (28.1% yield). The previous amine derivative was produced by reduction of 6-nitrobenzo[b]thiophene 1,1-dioxide, and the last one was synthesised according to procedures previously published (Challenger and Clapham, 1948) (60% yield). The oxidation of benzo[b]thiophene was carried out with 30% hydrogen peroxide.

Cell culture

American Type Culture Collection (ATCC, Manassas, VA) or European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, UK) provided human tumour cell lines. Six cell lines were used: two human leukaemia (K-562 and CCRF-CEM) and four human solid tumours, one colon carcinoma (HT-29), one lung carcinoma (HTB54), one cervix epitheloid carcinoma (HeLa) and one melanoma (MEL-AC). MEL-AC cells were kindly provided by Dr Natalia López-Moratalla (Universidad de Navarra, Pamplona, Spain) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 10 mM HEPES buffer (pH 7.4).

Cytotoxicity assay

The cytotoxic effect of each substance was tested at five different doses between 0.01 and 100 μM. Each substance was initially dissolved in DMSO at a concentration of 0.1 μM, and serial dilutions were prepared using culture medium. The plates with cells from the different lines, to which media containing the substance under test were added, were incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO₂. Cytotoxicity was then determined by a colorimetric microassay based on the use of MTT (Mosmann, 1983). Results are expressed as GI₅₀, concentration that reduced by 50% the growth of treated cells with respect to untreated controls.

Protein isolation and determination

Cell membrane NOX activity (Cellex) was released in soluble form from HeLa cells (whole cells) as previously described (Del Castillo-Olivares et al., 1998).

Proteins were determined by the bicinchoninic acid/copper assay (Smith et al., 1985) obtained from Pierce using bovine serum albumin as standard.

NADH oxidase activity measurement

NADH oxidase activity was determined as the disappearance of NADH at 340 nm in a reaction mixture containing 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit any potential mitochondrial oxidase activity and 150 μM NADH at 37°C and continuous stirring (Wang et al., 2003). Assays were initiated by addition of NADH. Absorbance was recorded for 1 min after adding NADH and a millimolar extinction coefficient of 6.22 was used to determine specific activity.

RESULTS

The cytotoxic activity of BTS-1 and BTS-2 (Figure 1) was tested against a panel of human tumour cell lines, including cervix epitheloid carcinoma (HeLa), lymphocytic leukaemia (CCRF-CEM), myelocytic leukaemia (K-562), melanoma (MEL-AC), cervical carcinoma (HTB54), one cervix epitheloid carcinoma (HeLa) and one melanoma (MEL-AC). MEL-AC cells were kindly provided by Dr Markus Nabholz (ISREC, Epalinges, Switzerland). Cells were grown in RPMI 1640 medium (Life Technologies, Barcelona, Spain) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 10 mM HEPES buffer (pH 7.4).

Figure 1 Structure of BTS used in this work.

Scheme 1: i: Acetic acid, H₂O₂ 30% (v:v⁻¹), reflux, 30 min; ii: nitric acid 100%; iii: Fe/CINH₄, ethyl alcohol/water 50%; iv: NaNO₂, HCl (ac); SO₂/CuCl₂, acetic acid; v: CH₂Cl₂; triethylamine.
human colon carcinoma (HT-29) and lung carcinoma (HTB-54), and also against normal HLFs growing in culture. Cells were grown in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 μg ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 10 mM HEPES buffer (pH 7.4). The cytotoxic effect of each substance was tested at five different doses between 0.01 and 100 μM. The plates with cells from the different lines, to which media containing the substance under test were added, were incubated for 3 days and cell survival was then determined by an MTT-based colorimetric assay (Mosmann, 1983). Results are expressed as GI₅₀ concentration that reduced by 50% the growth of treated cells with respect to untreated controls. As shown in Table 1, growth of every cell line was clearly affected by the tested compounds, and is worth noting that according to its higher lipophilicity, BTS-2 was more cytotoxic against each one of the tested cell lines than the lead compound (BTS-1). Moreover, activity of BTS-2 was in the same order of magnitude as commercial doxorubicin against K-562 tumour cells and normal lung fibroblasts growing in culture. Figure 2 shows curves with the original data from which the GI₅₀ values for BTS-1 and BTS-2 were calculated.

It has been previously shown that BTS-1 and some related diarylsulphonylureas (DSU) are able to inhibit a tumour cell-specific NOX activity present in the membrane of CCRF-CEM cells (Alonso et al., 2001). As we had said before, until now tNOX activity is the only cell target proposed to explain the specific inhibition of growth of cancer cells induced by the antitumour sulphonylurea LY181984 (Morre´ et al., 1995a, b) and the green tea catechin EGCg (Morre´ et al., 2000). Since BTS-2 was designed on the basis of experimental results obtained with BTS-1 and other BTS that are structurally related with DSU, we decided to further analyse the action of BTS-2 on tNOX activity. For these purpose, we performed standard NOX assays using either a preparation released at pH 5 from HeLa cells obtained from Cellex Biosciences

| Compound | HeLa | CCRF-CEM | K-562 | MEL-AC | HT-29 | HTB-54 | HLF | log P |
|----------|------|----------|-------|--------|-------|--------|-----|-------|
| BTS-1    | 2.2  | 2.86     | 29.41 | 7.14   | 8.55  | 5.89   | 5.04 | -0.03 |
| BTS-2    | 0.24 | 0.1      | 0.07  | 0.81   | 2.52  | 0.23   | 0.04 | 2.82  |
| Dx       | 0.02 | 0.03     | 0.02  | 0.01   | ND    | ND     | 0.01 | ND    |

Dx: doxorubicin; ND: not determined.

Figure 2 Determination of the cytotoxic effect of (A) BTS-1 and (B) BTS-2. Cells were incubated in the presence of every compound at the indicated concentration for 72 h. Cytotoxicity was then determined by a colorimetric microassay based on the use of MTT (Mosmann, 1983). Values represent means ± s.d. derived from three independent experiments each performed in quadruplicate.

Figure 3 Dose-dependent inhibition by BTS-2 of NOX activity of (A) HeLa cells and (B) Cellex preparations in the presence of 100 mM GSH (solid symbols). Assay was based on spectrophotometric measurement of NADH disappearance at 340 nm. The compound was dissolved in ethanol and compared to solvent alone (open symbols).
(Minneapolis, USA), which is a mixture of NOXs (Del Castillo-Olivares et al., 1998), or from intact HeLa cells. NADH is an impermeant substrate and, therefore, can be used to measure cell surface NADH activity with whole cells. Results with isolated plasma membrane and with whole cells were similar. NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm. The compound was dissolved in ethanol and compared to solvent alone. As shown in Figure 3, in the presence of reducing conditions, that is, 100 μM GSH, the drug inhibited strongly the NOX activity of both the intact HeLa (Figure 3A) and Cellex (Figure 3B) preparations with an EC₅₀ of about 0.1 nM. The figure also shows small stimulations by ethanol (chemical hormesis) that are not unusual for the cell surface NOX activities (Morre´, 2000). Although both the Cellex and intact HeLa preparations contain a cancer-specific and drug-inhibited tNOX and a constitutive and drug-resistant NOX, the data show that only tNOX is being inhibited, and indicate that this inhibition, which is dose dependent, is almost complete at 0.1 μM. Otherwise, the residual NOX activity corresponding to CNOX would approach zero at the high drug concentration. In agreement with this, NOX activities of plasma membranes purified from soybean (Glycine max), which contain only CNOX, were unaffected by BTS-2 over the concentration range 10⁻¹¹–10⁻⁵ M (N = 4) (not shown).

The effect of the drug on the NOX activity under oxidising conditions is analysed in Figure 4. As can be seen, when the oxidising conditions were provided by treatment of HeLa cells with hydrogen peroxide (Figure 4A), the derivative inhibited only at the highest concentration of 10 μM. Moreover, with Cellex preparations (Figure 4B), stimulation was recorded at 10 μM. As with hydrogen peroxide, when the assays were performed in the presence of 100 μM GSSG (Figure 4C and D), we could see no inhibition of the NOX activity, only a slight stimulation. Thus, it can be concluded that while under reducing conditions BTS-2 strongly inhibits tNOX, under oxidising conditions there is no effect of the drug or just a slight stimulation of activity. The absolute dependency of inhibition on redox state of tNOX has also been described with the antitumour sulphonylureas (Morre´ et al., 1998).

**DISCUSSION**

We have previously shown that BTS derivatives are strong cytotoxic agents that induce ROS overproduction and apoptosis

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**Figure 4** Lack of inhibition of the NOX activity by BTS-2 under oxidising conditions (solid symbols). (A) Normal NOX assays performed with intact HeLa cells in which the preparations were first reduced with 100 μM GSH for 10 min and then fully oxidised by addition of 0.03% hydrogen peroxide for 10 min. (B) As in (A) but with 50 ml of the Cellex preparation. (C) As in (A) but with assay in the presence of 100 μM GSSG. (D) As in (B) but with assay in the presence of 100 μM GSSG. The compounds were dissolved in ethanol and compared to solvent alone (open symbols). The ethanol concentration was 0.16% maximum.
in tumour cells (Alonso et al, 2003). We have also shown that while the B Ts derivatives that exhibit this cytotoxic activity are able to inhibit a NOX activity of the plasma membrane of leukaemia CCRF-CEM cells, those that lack cytotoxic activity also lack this inhibitory effect (Alonso et al, 2001), thus suggesting that ECTO-NOX proteins could provide a molecular target for the induction of ROS production by B Ts derivatives. Here we report that B Ts-2, a new B Ts derivative with high lipophilicity and improved cytotoxic activity, specifically inhibits the tNOX activity of HeLa cells, and we show that this inhibition is dependent on the redox state of tNOX.

A number of motifs within tNOX, including an NADH binding site, a quinone binding site and sites for protein disulphide-thiol interchange, account for the range of its functional activities (Chueh et al, 2002b). The drug response of NOX has been studied extensively with L181984 (Morré et al, 1998). Activity is inhibited or stimulated by this sulphonylurea depending on the redox environment of the protein. Ubiquinone competes with both L181984 binding and inhibition of enzymatic activities and so the drug, like capsaicin (Morré et al, 1995c), EGCg (Morré et al, 2000) and adriamycin (Morré et al, 1997c), which also inhibit activity, is considered to occupy the quinone binding site. Interestingly, while the normal CNOX isoform and tNOX share functional characteristics, CNOX appears to lack the quinone binding motif EEMTE (Chueh et al, 2002a), a fact that could explain why both enzymes differ primarily in their sensitivity to these drugs. Since we show here that under reducing conditions B Ts-2 inhibits strongly the NOX activity of both intact HeLa cells and Cellux preparations containing both NOX and CNOX, while ECTO-NOX activities of plasma membranes from soybean, which contain only CNOX, are unaffected by this drug, the possibility that B Ts derivatives exert their specific inhibitory action on tNOX activity through binding to the quinone site should be considered.

It has been described that the tNOX activity can be inhibited by thiol reagents such as the N-ethyl-maleimide (NEM) (Morré et al, 1995a,b). It has also been reported that compounds carrying the benzol[b]thiophene 1,1-dioxide nucleus, like BTS-1 and -2, are thiol reagents (Bordwell et al, 1954). For this reason, it is very interesting that the C2-C3 dihydro derivative of BTS-1, which was reported to be inactive both in the cytotoxicity and enzymatic assays (Alonso et al, 2001), cannot be considered a thiol reagent. Since this compound and BTS-1 have identical molecular sizes and capacities to interact through polar groups, hydrogen bonds and/ or hydrophobic interactions with putative receptor sites, activity of BTS-1 can be attributed to the C2-C3 double bond reactivity. In agreement with this, the C3-methyl derivative of BTS-1 shows no cytotoxic activity (Villard et al, 2004), a result that can be attributed to its impaired reactivity towards thiol groups. Thus, the possibility that B Ts derivatives exert their inhibitory action on tNOX activity by interacting with cysteines of the active sites for protein disulphide-thiol interchange should also be considered.

The fact that B Ts actions over cell growth are prevented by previous treatment of the cells with N-acetyl-cysteine (Alonso et al, 2003) and our finding that under oxidising conditions there is no effect of B Ts-2 on the tNOX activity also support this idea.

Interference with DNA and RNA is generally considered to account for the cytotoxicity of anthracycline antitumour drugs. However, it has been shown that doxorubicin is cytotoxic even without entering the cells (Tritto n and Yee, 1982), and inhibition of tNOX activity by doxorubicin (EC50 = 0.7 nm) has been described (Morré et al, 1997c; Hedges et al, 2003). Interestingly, when cell growth is inhibited, redox activities of the plasma membrane are also inhibited, and thus NOX activity of the plasma membrane has been suggested as a growth-related doxorubicin target at the surface of cancer cells (Kim et al, 2002; Hedges et al, 2003). For this reason, the fact that B Ts-2 acts in the same order of magnitude as doxorubicin against K-562 tumour cells and also against normal HLFs that should have a low or even absent tNOX activity rises the possibility of additional sites of action for this drug. Further experiments are required to clarify this fact.

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