Abstract. Chemically active molecules, such as reactive oxygen species (ROS), are prone to induce cellular damage by oxidative stress and this could be exploited as a strategy to kill malignant cells. In this study, we evaluated the antitumor activity of a new compound, N’-formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide (FBZC) by assessing its pro-oxidant effects on breast cancer in vitro. Oxidative stress, generated by FBZC, was characterized by measuring reactive species and antioxidant enzymes and markers. Results showed that the cytotoxic effects of FBZC on MCF7 breast cancer cells (half inhibitory concentration of 5.4 μg/ml), were partially reversed by the addition of regular antioxidants. FBZC induced ROS and lipid peroxidation, together with a significant inhibition of superoxide dismutase, glutathione reductase and total glutathione levels as well as increases in catalase and glutathione-S-transferase activities, in an acute fast response. Thus, the antitumor effects of FBZC could be related to oxidative deregulation due to a combination of induction of ROS generation and inhibition of key antioxidant enzymes.

Breast cancer represents the most commonly diagnosed malignancy, after skin cancer, and the second leading cause of cancer-related death among women (1). New alternative therapies for this disease have emerged, however, most of them are associated with significant side-effects, high economic costs and reduced quality of life (2); thus the development of novel alternatives for this malignancy is a major priority.

Benzothiazole compounds have demonstrated highly selective antitumor activities in vitro (3, 4). Several such derivatives have been synthesized as pro-drugs and have already been introduced in clinical trials (5). On the other hand, nitrothiophene compounds are also known as potential cytotoxics, representing promising new leading analogs against cancer, possibly by inducing DNA damage (6). Both chemical structures have shown antitumor activities by inducing generation of reactive oxygen species (ROS) and apoptosis, involving nucleophilic attack by intracellular thiols (7, 8).

ROS are molecules characterized by their chemically active and unstable properties, being able to induce cellular damage. In this context, they could be useful due to their ability to kill tumor cells, which are susceptible to oxidative stress (9). Thus, we evaluated the potential antitumor activity of a new compound, N’-formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide (FBZC), which combines a benzothiazole and a 5-nitrothiophene in the same molecule, in a human breast cancer cell line by induction of oxidative stress and by the ensuing antioxidant response.

Materials and Methods

Synthesis of FBZC. The synthesis and physicochemical evaluations of FBZC (Figure 1) were performed according to Charris et al. (10). The melting point was determined on a Thomas micro hot stage apparatus and infrared spectra as KBr pellets on a Shimadzu model 470 spectrophotometer. The 1H-nuclear magnetic resonance, spectrum was recorded using a Jeol Eclipse 270 (270 MHz) spectrometer using dimethyl sulfoxide (DMSO)-d6, and was reported in ppm downfield from the residual DMSO. Elemental analyses were performed on a Perkin Elmer 2400 CHN analyzer, results were within ± 0.4% of the predicted values for all
Figure 1. Chemical structure of N'-formyl-2-(5-nitrothiopen-2-yl) benzothiazole-6-carboxyhydrazide (FBZC).

compounds. Chemical reagents were obtained from Aldrich Chemical Co, St. Louis, Mo, USA. All solvents were distilled and dried in the usual manner.

Cell cultures. Breast adenocarcinoma cells (MCF7) and non-tumor retinal pigment epithelium cells (RPE) were obtained from the European Collection of Cell Cultures, Salisbury Wiltshire, UK. Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (FBS), L-glutamine 1%, penicillin (50 units/mL) and streptomycin (50 μg/mL), in a humidified atmosphere of 95% air and 5% CO2 at 37˚C. Cell culture media were from HyClone Laboratories, Inc. South Logan, UT, USA.

Time and dose-effects of FBZC on cell viability. A total of 5×10^3 MCF7 or 5×10^3 RPE cells were cultured in a 96-well microtiter plate containing 0.1 ml of RPMI growth media/well for 24 h. Cells were incubated with the compounds dissolved in dimethylsulfoxide (DMSO)/medium 0.02% (5-100 μg/mL, 72 h) and were evaluated for their cytotoxicity (11). The half inhibitory concentration (IC50) values obtained were defined as the concentration of tested compounds resulting in a 50% reduction of the cell viability compared to vehicle-treated cells. Further evaluations of FBZC were performed using its IC50 value.

Effect of antioxidants on cytotoxicity of FBZC. A total of 5×10^3 MCF7 cells cultured for 24 hours in a 96-well microtiter plate containing 0.1 mL of RPMI growth medium/well were incubated with vehicle, FBZC IC50, N-acetyl-cysteine (0.5 mM), or TROLOX (0.1 mM) in different incubation combinations (37˚C for 72 h). Cells were then incubated with Alamar Blue (37˚C for 4 h) and resorufin was measured at 570/600 nm. The results are expressed as the percentage of cell viability compared to that of the vehicle control.

Intracellular ROS levels. The determination of intracellular ROS after FBZC incubations over time was measured with the non-fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), as previously described (12).

Sample preparation. For the determination of lipid peroxidation, enzyme activities and total glutathione, 2×10^6 MCF7 cells were treated either with vehicle or FBZC at IC50 (37˚C for 3, 6, 12 and 24 h), cells were trypsinized and then 1×10^6 they were suspended in PBS and centrifuged (1200 xg for 5 min). Samples were subjected to different protocols, depending to the enzyme activity/metabolite determinations.

Lipid peroxidation assay. To determine lipid peroxidation by the compound, we followed the procedure previously described (13), measuring the production of thiobarbituric reactive oxygen species (TBARs) at 535/580 nm.

Activities of superoxide dismutase (SOD) and catalase. SOD activity was measured according to McCord and Fridovich (14), which tracks the reduction of cytochrome c at 550 nm. The catalase activity was measured according to Aebi (15), which tracks the reduction of H2O2 at 240 nm.

Determination of glutathione level, glutathione reductase (GR) and glutathione-S-transferase (GST) activities. To determine the total glutathione level, we followed the procedure previously described by Tietze (16). The absorbance at 412 nm was registered at every 20 s for 5 min due to the formation of the reduced product, thionitrobenzene. To determine GR activity we followed a procedure previously described (17) and the decrease of the NADPH levels, at 340 nm, was recorded. For the GST activity, we followed the procedure previously described by Rice et al. (18), measuring the accumulation of 5-(2,4-dinitrobenzyl) glutathione at 340 nm.

Determination of dehydrogenase enzyme activities. To determine the activities of glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), we followed a procedure described by Tian et al. (19), measuring the fluorescence produced by the conversion of NADP* to NADPH + H+ by either G6PDH or 6PGDH.

Protein levels of samples were determined according to Bradford (20). Data are presented as the average of four independent experiments (n=4) and tested for statistical significance using unpaired t-tests for specific group comparisons, assuming 95% confidence limits, using the GraphPad Prism 4.02 software for Windows, San Diego, CA, USA.

Results

FBZC (Figure 1) was synthesized and evaluated for its potential antineoplastic activity in vitro.

The ability of FBZC to induce cytotoxic effects on the human breast tumor cell line MCF7 is presented in Table I. FBZC was more than 20-fold more specific to the tumor cell line than to non-tumor cells. The results also highlighted the more toxic and specific effect of this compound compared to cisplatin and doxorubicin. † p<0.05 compared to results for RPE cells.

Table 1. Cytotoxic effect of N'-formyl-2-(5-nitrothiopen-2-yl) benzothiazole-6-carboxyhydrazide (FBZC) compared with cisplatin and doxorubicin. Results are expressed as the mean±SEM of the IC50 values in four independent experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MCF7 IC50 (μg/mL)</th>
<th>RPE IC50 (μg/mL)</th>
<th>Specificity (RPE/MCF7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBZC</td>
<td>5.4±1.7*** †</td>
<td>113.8±5.71</td>
<td>21.07</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>7.19±0.47</td>
<td>7.6±0.01</td>
<td>1.06</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>62.11±3.5</td>
<td>5.18±0.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>

IC50: Half inhibitory concentration; ***p<0.001 compared to doxorubicin. †p<0.05 compared to results for RPE cells.
those of regular antineoplastics cisplatin and doxorubicin. The toxic effects on MCF7 cells were partially reversed by the presence of two functionally unrelated antioxidants suggesting that oxidative stress might be responsible for the cytotoxicity of FBZC (Figure 2).

FBZC increased the production of ROS, and lipid peroxidation, by TBARs, rose in a time-dependent manner and, consequently induced alteration in the normal oxidative status of cells (Figure 3).

Cells are well-equipped with a complete and integrated antioxidant defence system. SOD and catalase represent the first line of defence against ROS. FBZC inhibited SOD activity by approximately 25% and induced a non-monotonous effect on catalase due to a strong stimulatory response observed at the first 12 h of incubation; but its activity faded at 24 h (Table II). This complex response illustrated the importance to perform time-dependent studies with this compound.

The GSH redox cycle is an important system for the maintenance of the normal redox status in cells and for their protection from potential oxidants (21). FBZC caused a strong and rapid decrease in GR activity over time, especially at short incubation times, where a near 80% inhibition was observed. Total glutathione levels also decreased in parallel, then were no different from the control at 12 h, and at 24 h they were strongly impaired to about 30% that of control levels. GST, which is an enzyme that uses glutathione as a substrate to detoxify xenobiotics, was significantly activated, by approximately 30%, at short incubation times, but afterwards its action was inhibited until cells eventually died (Table II).

In neoplastic cells one of the main routes for the utilization of carbohydrates is the pentose phosphate pathway (PPP) (22), with its particularly important oxidative branch, which generates NADPH to be used as a reducing agent in the antioxidant cycles that counteract the deleterious effects of free radicals. FBZC deregulated the enzymes of the oxidative branch of the PPP (Table II).

**Discussion**

In spite of the availability of a diverse group of antineoplastic agents for use in the treatment of breast cancer, unwanted effects have been reported, while their efficacy is also often less than optimal (2). Thus, the discovery of new compounds is of high priority. Benzothiazoles are heterocyclic analogs with *in vitro* antineoplastic activity towards some types of human cancer.
cells (23). It has been described that the mechanisms of action of this chemical moiety could be related to oxidative stress and by activation of the apoptosis machinery (24). On the other hand, nitrothiophenes have also demonstrated different biological activities, such as potential antitumor and antibiotic actions, possibly involving nucleophilic attack by intracellular thiols and DNA damage (25).

Cellular macromolecules such as proteins, lipids and DNA are prone to alterations and damage by ROS and oxidative stress and these events could be exploited for the development of potential antitumor compounds as ROS inducers or as inhibitors of different antioxidant enzymes. Abietyl-isothiocyanate (ABITC) and (−)-epigallocatechin-3-gallate (EGCG) are potential antitumor agents that induce ROS generation and subsequent apoptosis (26, 27). In cancer cells, the oxidative stress induced by compounds is superimposed on the intrinsic stress, resulting in a more potent ROS-mediated cytotoxic process that preferentially inhibits tumor cell proliferation or kills these cells (28). In this context, and in the search for new anticancer compounds, a benzothiazole derivative that is conjugated with reactive metabolites, including lipid peroxidation products and FBZC, to detoxify them through electrophilic agents in a reaction catalyzed by endogenous GST. Indeed, the strong inhibition of GR by this compound was particularly striking due to its magnitude (around 80%) and rapid onset. Such inhibition impairs the GSH redox cycle, where glutathione disulfide is reconverted to GSH by GR. The oxidation of GSH by H2O2 or lipid hydroperoxides is catalyzed by glutathione peroxidases, while GSH can also be conjugated with electrophilic agents in a reaction catalyzed by endogenous GST. Indeed, the strong inhibition of GR may have impaired the detoxification of H2O2 and of lipid hydroperoxides by glutathione peroxidase, due to the lack of GSH. This effect could be worsened by the strong increase in GST activity observed at 3 h, since the available GSH would be conjugated with reactive metabolites, including lipid peroxidation products and FBZC, to detoxify them through GST. These results are in concordance with those for other azole compounds, since thiazole and benzothiazole induced activity of and are detoxified by GST (32).

The impairment of the GSH-dependent H2O2 elimination by inhibition of GR could be compensated by the strong (about 3-fold) and rapid up-regulation of catalase, induced by FBZC. Since in MCF-7 cells, glutathione peroxidases and catalase contribute together, equally, for the elimination of

### Table II. Effect of N'-formyl-2-(5-nitrothiophen-2-yl)benzothiazole-6-carbohydrazide (FBZC) (5.4 μg/mL) on different antioxidant parameters over time, in breast cancer MCF7 cells. Results are expressed as the mean±SEM of four independent experiments.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTR</td>
<td>FBZC</td>
<td>CTR</td>
<td>FBZC</td>
</tr>
<tr>
<td>SOD (U/mL*mg)</td>
<td>0.66±0.01</td>
<td>0.48±0.01***</td>
<td>0.66±0.01</td>
<td>0.48±0.02**</td>
</tr>
<tr>
<td>G6PDH (U/mL*mg)</td>
<td>4.91±0.10</td>
<td>1.17±0.12***</td>
<td>4.81±0.30</td>
<td>1.04±0.16***</td>
</tr>
<tr>
<td>GR (U/mg)</td>
<td>0.66±0.01</td>
<td>0.84±0.01***</td>
<td>0.67±0.01</td>
<td>0.79±0.01***</td>
</tr>
<tr>
<td>GSH+GSSG (nM/mg)</td>
<td>70.96±0.94</td>
<td>67.08±1.05**</td>
<td>78.01±1.11</td>
<td>70.68±0.94**</td>
</tr>
<tr>
<td>GST (U/mg)</td>
<td>3.41±0.11</td>
<td>4.12±0.18*</td>
<td>3.40±0.15</td>
<td>3.70±0.22</td>
</tr>
<tr>
<td>CAT (U/mg*100)</td>
<td>0.70±0.03</td>
<td>0.61±0.01*</td>
<td>0.74±0.02</td>
<td>0.78±0.04</td>
</tr>
<tr>
<td>SOD: Superoxide dismutase</td>
<td>CAT: catalase</td>
<td>GST: glutathione-S-transferase</td>
<td>GSH: reduced glutathione</td>
<td>GSSG: oxidized glutathione</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01 and *** p<0.001 compared to vehicle control (CTR).
H$_2$O$_2$ (33), it is probable that a 3-fold up-regulation of catalase not only compensates for the impairment of the GSH system, but it may result from an adaptive response triggered by an increased production of H$_2$O$_2$, caused by FBZC. The near three-fold increase in the DCFH measurement of ROS, observed after 2 h incubation with FBZC, further supports this notion. Thus, the inhibition of antioxidant enzymes combined with the increase in the production of ROS may justify the highly cytotoxic effect of FBZC on MCF-7 cells.

The up-regulation of catalase triggered by FBZC illustrates an adaptive response in which cells respond to oxidative stress by inducing antioxidant defences. In this regard, it is interesting to note that SOD was inhibited by FBZC in a steady fashion throughout the incubation. In this case, the adaptive response was unable to counteract this inhibition. This may be particularly important since inhibition of SOD causes accumulation of cellular superoxide anion and perhydroxyl, leading to oxidative damage of mitochondrial membranes, inducing the release of cytochrome c from mitochondria, thus causing apoptosis of tumor cells (34). In addition, SOD activity is related to a decrease of breast tumor cell growth and proliferation both in vitro and in vivo (35); thus, the inhibitory activity of FBZC on this enzyme, observed here, may be relevant for its potential antitumor action.

In conclusion, FBZC showed pro-oxidative effects on MCF7 cells by increasing ROS and TBARs, along with causing inhibition of SOD and GR. These responses led to a remarkable deregulation of the cell antioxidant profile which triggered oxidative stress, leading to subsequent cell death. The multiple target effects of this compound could explain its superior selective toxicity towards tumor cells, compared to regular antineoplastics in vitro, underlying its potential as a new antitumor agent.

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References