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Isothiazole dioxide derivative **6n** inhibits vascular smooth muscle cell proliferation and protein farnesylation

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Abstract

Isothiazole dioxides have been shown to inhibit *Trypanosoma brucei* protein farnesyltransferase (PFTase) in isolated enzyme, but elicited only a minor effect on mammalian PFTase. In the present study we have evaluated the effect of 3-diethylamino-4-(4-methoxyphenyl)-isothiazole 1,1-dioxides with different substituents at C5, on rat PFTase and protein geranylgeranyltransferase-I (PGGTase-I) with the final aims to improve the potency against mammalian PFTase and to identify new compounds with antiproliferative properties. For these purposes, in vitro and cell culture models have been utilized. The results showed that isothiazole dioxides with C4–C5 double bond and sulfaryl substituted at the C5 position but none of the dihydro-derivatives, were able to inhibit in vitro PFTase in a concentration dependent manner (IC_{50} ranging from 8.56 to 1015 μM). Among those, compound **6n** (C5; methyl-S) displayed 500-fold higher inhibitory potency on PFTase than PGGTase-I. Compound **6n** was shown to affect rat smooth muscle cell (SMC) proliferation at concentrations similar (IC_{50} = 61.4 μM) to those required to inhibit [^{3}H]-farnesol incorporation into cellular proteins (−44.1% at 100 μM). Finally, compound **6n** interferes with rat SMC proliferation by blocking the progression of G0/G1 phase without inducing apoptosis, as assessed by [^{3}H]-thymidine incorporation assay and flow cytometry analysis. Taken together, we described a new PFTase inhibitor containing the isothiazole dioxide moiety that affects mammalian protein farnesylation and SMC proliferation by inhibiting G0/G1 phase of the cell cycle.

Keywords: Cell proliferation; Farnesylation; Farnesyl transferase inhibitor; Smooth muscle cell; Farnesol; Atherosclerosis

1. Introduction

Proliferation of smooth muscle cells (SMCs) in the arterial wall in response to vascular injury is an important pathogenetic factor of vascular disorders such as atherosclerosis and restenosis after angioplasty [1]. A pharmacological approach to reduce this vascular response is to target intracellular signaling pathways that regulate cell proliferation during the progression of lesion development [2]. Among them, low molecular weight GTP-binding proteins, modified by lipid moieties, farnesol and geranylgeraniol, i.e. prenylated proteins, have gained attention for the development of antiproliferative agents [3,4]. The attached lipid is required for proper functioning of the proteins by mediating membrane association and specific protein–protein interactions [5]. Indeed, activation of farnesylated Ras has been demonstrated to promote cell proliferation in both transformed and primary cell lines [6,7]. The importance of H-Ras in SMC proliferation in response to vascular injury has been shown by the adenoviral delivery of a dominant negative mutant

Abbreviations: FCS, fetal calf serum; FOH, farnesol; FPP, farnesyl pyrophoshate; GGOH, geranylgeraniol; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; MEM, minimum essential medium; MVA, mevalonate; PBS, phosphate buffered saline; PFTase, protein farnesyltransferase; PGGTase, protein geranylgeranyltransferase; PMSF, phenylmethylsulphonylfluoride; SMC, smooth muscle cell

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of H-Ras, which effectively inhibits SMC accumulation after balloon injury of rat carotid artery [8,9]. Moreover, farnesyl thiosalicylic acid, a potent competitive inhibitor of the enzyme prenylated protein methyltransferase (PPMTase), which methylates the carboxyl-terminal *S*-prenylcysteine of Ras, has been shown to significantly reduce the development of lesions of ApoE-deficient mice [10]. More specifically, mice with reduced expression of the intracellular linker protein Grb2 in the intracellular signaling pathway mediated by Ras, are resistant to the development of neointima formation in response to vascular injury [11]. Therefore, selective inhibition of Ras proteins may represent an important therapeutic target to control SMC proliferation in vascular diseases.

There are few potential approaches for inhibiting Ras and Ras-related GTP-binding proteins. The best target so far identified, is to inhibit the formation of thioether linkages between the C_1 atom of farnesyl or geranylgeranyl isoprenoid lipids and cysteine residues at or near the carboxy-terminus of the proteins [5,12]. To date, three classes of enzymes, protein farnesyltransferase (PFTase) and protein geranylgeranyltransferases (PGGTase-I and II), have been identified in mammals to catalyze protein prenylation process [12,13]. The protein substrates include Ras, Rho, Rab, other Ras-related small GTP-binding proteins, γ-subunits of heterotrimeric G-proteins and nuclear laminins [5,14].

Several classes of PFTase inhibitors with antiproliferative activity have been described, and a subset are undergoing to clinical trials [15,16]. Rationally designed direct PFTase inhibitors include at least three categories, namely farnesyl pyrophosphate (FPP) mimetic inhibitors which occupy the FPP site of PFTase, CAAX peptide mimetic inhibitors which block the binding site for the C-terminal CAAX tetrapeptide, and bisubstrate analogues which are designed to occupy both sites simultaneously [16]. Moreover, a series of double inhibitor of PFTase and PGGTase-I, as well as specific PGGTase inhibitors, have been shown a potent antiproliferative effect on SMC [17–19].

A class of compounds, containing the isothiazole dioxide moiety, that significantly inhibit *Trypanosoma brucei* PFTase activity in vitro but only marginally mammalian PFTase, has been recently described [20]. Mammalian and *T. brucei* PFTase show only a 21% and 38% of identity for the α-subunit and β-subunit, respectively, and overlapped but distinct substrate specificities have been seen with these enzymes [21]. Rat PFTase shows preference for Ser, Met, or Gln at the X-position of the C-terminal CAAX containing protein substrates, while *T. brucei* PFTase prefers Met or Gln but not Ser [21]. Although the interaction mode of isothiazole dioxides with the PFTase is unknown, in the present study we explored various substitutions at C5 of the isothiazole ring to improve potency of isothiazole dioxides against mammalian PFTase. 3-Diethylamino-4-(4-methoxyphenyl)-isothiazole 1,1-dioxides with different C5 substituents were therefore tested on rat PFTase, and PGGTase-I activity in vitro, incorporation of [^{3}H]-Farnesol (FOH) into cellular protein, as related to rat SMC proliferation.

2. Materials and methods

2.1. Materials

Eagle's MEM, trypsin ethylendiaminetetraacetate, penicillin (10,000 U ml^{-1}), streptomycin (10 mg ml^{-1}), tricine buffer (1 M, pH 7.4) and nonessential amino acid solution (100×), fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, CA, USA). Disposable culture flasks and Petri dishes were from Corning Glassworks (Oneonta, New York), and filters were from Millipore (Billerica, MA,USA). [6-^{3}H]-Thymidine, sodium salt (2 Ci mM^{-1}) was from Amersham (Cologno Monzese, Milan, Italy), and molecular weight protein standards from BIO-RAD Laboratories (Hercules, CA, USA). Isoton II was purchased from Instrumentation Laboratories (Milan, Italy). All-*trans* FOH, was purchased from SIGMA (Milan, Italy). SDS, TEMED, ammonium persulfate, glycine, and acrylamide solution (30% T, 2.6% C) were obtained from BIO-RAD Laboratories (Hercules, CA, USA). All-*trans* [1-^{3}H]-FOH (15–20 Ci mM^{-1}), all-*trans* [^{3}H]-farnesyl pyrophosphate (FPP) (20 Ci mM^{-1}) and all-*trans* [^{3}H]-geranylgeranyl pyrophosphate (GGPP) (20 Ci mM^{-1}) were from American Radiolabeled Chemicals (St. Louis, MD, USA). Avidin-agarose was from Pierce (Woburn, MA, USA). Cytox-Dye was purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA). Simvastatin in its lactone form (Merck, Sharp & Dohme Research Laboratories) was dissolved in 0.1 M NaOH to give the active form, and the pH was adjusted to 7.4 by adding 0.1 M HCl. The solution was sterilized by filtration.

Compounds **5a–c** [22]; **6a** and **b** [23]; **6c–g** [24]; **6h** [25]; **6i–p** [22]; were synthesized according to the methods described (Table 1).

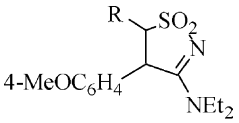
2.2. Cell proliferation and DNA synthesis

SMC were cultured from the intimal-medial layers of aorta of male Sprague–Dawley rats as previously described [26]. Cell proliferation was evaluated by cell counting with a Coulter Counter model ZM (Coulter Instruments) after trypsinization of the monolayers [27], and DNA synthesis was estimated by nuclear incorporation of [^{3}H]-thymidine [26].

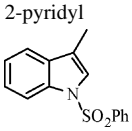
2.3. Cell cycle analysis

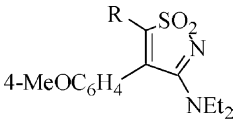
Flow cytometry was utilized to analyze cell cycle distribution. Cells were trypsinized and centrifuged for 5 min at 1000 rpm. Pellets were resuspended in 0.5 ml of permeabilizing buffer of Cytox Dye (0.5 μM in 100 mM Tris

Table 1
Isothiazole dioxide derivatives

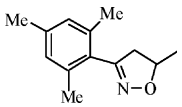


5a-5c

Compound	R	Reference
5a	Methyl-S	[22]
5b	$C_6H_{11}S$	[22]
5c	Phenyl-S-	[22]
6a	H	[23]
6b	Methyl-	[23]
6c	Phenyl-	[24]
6d	Phenylethinyl-	[24]
6f	2-pyridyl	[24]
6g		[24]



6a-6p

Compound	R	Reference
6h		[25]
6i	Phenyl-S-	[22]
6l	2-Pyridyl-S	[22]
6m	4-Methyl-phenyl-S	[22]
6n	Methyl-S	[22]
6o	Farnesyl-S	[22]
6p	Perillyl-S-	[22]

pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ 0.1% NP-40). Samples were placed in the dark for 30 min and the fluorescence of individual nuclei was measured. Nuclear Cytox Dye fluorescence signal was recorded on the FL2 channel of a FACS scan flow cytometer (Becton Dickinson) and analyzed with CellQuest software. The number of cells in G0/G1, S and G2/M phases was expressed as percentages of total events (10,000 cells) [19].

2.4. Labelling of proteins with [^{3}H]-FOH and SDS-PAGE analysis

The SMC prenylated proteins were labeled by incubating cell monolayers with [^{3}H]-FOH (50 μCi) and simvastatin (3 μM), in the presence or absence of indicated concentrations of the compound for 24 h. Cell monolayers were scraped into 1.5 ml of PBS containing 1 mM PMSF. After centrifugation at 14000 rpm for 3 min, lipids were extracted with cold acetone followed by chloroform/methanol (2:1). The delipidated samples were solubilized in 3% SDS, 62.5 mM Tris–HCl, pH 6.8, and an aliquot (40 μg protein) was analyzed by 12.5% SDS-PAGE. The gel was treated with Amplify (Amersham, Cologno Monzese, Milan, Italy) and exposed to Kodak X-Omat-AR film at −80 °C for 6 weeks. Fluorographic signals were analyzed by densitometric scanning using Quantity One software (BIO-RAD) [14].

2.5. Assays for inhibition of PFTase and PGGTase-I

Recombinant rat PFTase and PGGTase-I were produced in Sf9 insect cells and purified as described [28]. The standard reaction mixture for PFTase assay contains 0.75 μM (0.3 μCi) [^{3}H]-FPP and 5 μM Ras-CVIM, in a total volume of 20 μl containing 30 mM potassium phosphate, 1 mM $MgCl_2$, 20 μM $ZnCl_2$, pH 7.7. PGGTase-I assay was carried out using 0.75 μM (0.3 μCi) [^{3}H]-GGPP and 5 μM H-Ras-CVLL as substrates. Stock solutions of the compounds were prepared in DMSO, and 1-μl aliquot was added to the reaction mixture (final concentration of DMSO was 5%). Reaction was initiated by adding PFTase or PGGTase-I. After incubation at 30 °C for 15 min, the reaction was stopped by adding 200 μl of 10% HCl in ethanol. The amount of the prenylated protein product was determined by the glass fiber filter method as previously described [29].

2.6. Statistical analysis

Experimental data are expressed as mean ± S.D. The effects of the tested compounds versus control on the different parameters were analyzed by two-tailed Student's *t*-test for unpaired data. The concentration of compounds required to inhibit 50% of cell proliferation (IC_{50}) was calculated by linear regression analysis of the logarithm of the concentration.

3. Results

3.1. Different C5 substitutions in isothiazole dioxide ring modulated the inhibitory potency against PFTase and PGGTase-I

It has been previously demonstrated that isothiazole dioxides are able to affect *T. brucei* PFTase activity in

vitro but elicited only a minor effect on mammalian PFTase action [20]. In the present study, to improve the ability of isothiazole compounds to inhibit mammalian PFTase we had chemically modified the 3-diethylamino-4-(4-methoxyphenyl)-isothiazole 1,1-dioxides in position C5 with different substituents, with the final aim to identify new compounds with antiproliferative properties. The number of varied substitutions explored in the five-membered ring resulted in the utilization of different chemistry, and the synthetic pathways for the synthesis of 5-substituted-3-amino-4-arylisothiazole 1,1-dioxides (series 6) and their 4,5-dihydroderivatives (series 5) have been previously reported (Table 1) [22–25].

In the first set of experiments we evaluated the effect of different synthesized compounds on the activity of recombinant rat PFTase and its closely related enzyme, PGGTase-I that catalyze the attachment of geranylgeranyl moiety. Recombinant enzymes were incubated with a single 100 μM concentration of different compounds, except for compounds **6a** and **6d** where 5 and 2 μM concentrations were used due to their limited solubility in aqueous solution. Compounds **6i**, **6l**, **6n**, and **6p** showed significant antagonist effect on rat PFTase with 43.5–66.0% inhibition at 100 μM, and only a minor effect on PGGTase-I (16-27% reduction) (Table 2). In contrast, compound **6b** was more selective for PGGTase-I, leading to 55.5% inhibition at 100 μM and only 27.0% antagonist activity on PFTase (Table 2). Thus, a single substitution on C5 of 3-diethylamino-4-(4-methoxyphenyl)-isothiazole 1,1-dioxides could alter the antagonist activity on PFTase and the selectivity versus the PGGTase-I. The presence of an S atom as a linker between the isothiazole moiety and the substituent appears to significantly increase the inhibitory activity versus the PFTase. Indeed, compounds **6i**, **6l**, and **6n** were much more potent inhibitors than the corresponding compounds lacking the S atom (**6c**, **6f**, and **6b**) (Table 2). Moreover, the presence of C4–C5 double bond was required for the inhibitory action of isothiazole dioxides, as demonstrated by the direct comparison between **6n** and **6i** versus their respective dihydroderivatives compounds **5a** and **5c** (Tables 1 and 2). Finally, IC_{50} value calculated for compounds **6i**, **6l**, **6n**, and **6p**, clearly showed that **6n** was the most potent compound to inhibit PFTase activity (IC_{50} = 8.56 μM), with almost 500-fold higher inhibitory activity on PFTase compared to PGGTase-I (IC_{50} on PGGTase-I = 3971 μM) (Table 3).

Table 2
Inhibitory effect of Isothiazole Dioxides compounds on rat PFTase and PGGTase-I

Compound	% Inhibition PFTase (μM)	% Inhibition PGGTase-I (μM)
5a	4.8 (100)	35.8 (100)
5b	5.6 (100)	19.6 (100)
5c	5.1 (100)	17.2 (100)
6a	0 (5)	11.8 (5)
6b	27 (100)	55.5 (100)
6c	0 (100)	25.6 (100)
6d	2.1 (2)	7.1 (2)
6f	2.7 (100)	6.7 (100)
6g	4.5 (100)	24.7 (100)
6h	3.2 (5)	15.6 (5)
6i	43.5 (100)	16 (100)
6l	50.5 (100)	27 (100)
6m	4.5 (100)	24.7 (100)
6n	66 (100)	23 (500)
6o	0 (100)	28.2 (100)
6p	48 (100)	21 (100)

Rat PFTase or PGGTase-I (20 ng protein) was incubated at 30 °C for 20 min with 0.75 μM (0.3 μCi) [^{3}H]-FPP/5 μM RAS-CVIM or 0.75 μM (0.3 μCi) [^{3}H]-GGPP/5 μM H-Ras-CVLL, respectively. Compounds were tested at indicated concentrations shown in parentheses for inhibition of rat PFTase and PGGTase-I reactions as described in Section 2.

3.2. The inhibition of [^{3}H]-farnesol incorporation into cellular proteins by compound **6n** correlated with the antiproliferative effect

To study whether the ability of compound **6n** to interfere with PFTase activity in vitro was maintained also in cultured cells and to further investigate the importance of the S atom as linker, the incorporation of [^{3}H]-FOH into smooth muscle cellular proteins was evaluated in the presence or absence of compound **6n** and the corresponding homolog lacking the S atom, compound **6b**. In each experiment, simvastatin was added to block endogenous mevalonate synthesis and to increase the specific radioactivity of the [^{3}H]-FOH for efficient radiolabeling of proteins [14]. As shown in Fig. 1, major labeled proteins were those with molecular weights of 21 kDa (mostly small GTP-binding proteins), and 45 kDa protein [14]. Compound **6n** that have sulfanyl substitutions at C5, significantly reduced [^{3}H]-FOH incorporation into 21 kDa proteins by 44.1%, while compound **6b** was ineffective (Fig. 1A and B). Therefore, the inhibitory action of compounds **6n** and **6b** observed on PFTase activity in vitro (Table 2) was confirmed on [^{3}H]-FOH incorporation in cultured cells (Fig. 1A and B).

It is well established that PFTase inhibitors potently inhibit both tumour cells and SMC growth in culture [18,19,30]. Therefore, to test whether the inhibitory effect

Table 3
Concentration-dependent inhibitory activity of compounds 6i, 6l, 6n and 6p on rat PFTase and PGGTase-I

Compound	PFTase IC_{50} (μM)	PGGTase-I IC_{50} (μM)	PGGTase-I/PFTase
6i	774	1401	1.81
6l	119	455	3.82
6n	8.56	3971	463.9
6p	1015	6953	6.85

For determination of IC_{50}, dose dependent inhibition assays were performed as described in Table 2, and the IC_{50} values were calculated by linear regression analysis of the logarithm of the concentration. Selectivity of the inhibitory action is indicated by the ratio between the IC_{50} of PGGTase-I and PFTase.

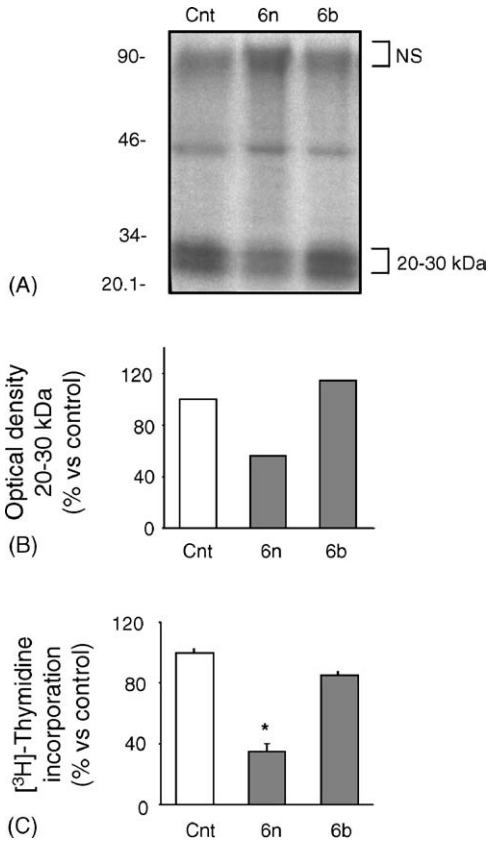


Fig. 1. Effect of compound **6n** and **6b** on [^{3}H]-FOH and [^{3}H]-thymidine incorporation into proteins of rat SMCs. (A) Cells were seeded at a density of 9×10^5/60 mm Ø dish and incubated with Eagle's MEM supplemented with 10% FCS; 24 h later, the medium was changed with one containing 0.4% FCS to stop cell growth and the cultures were incubated for 120 h. At this time, the medium was replaced with one containing 10% FCS, [^{3}H]-FOH (50 μCi), and simvastatin (3 μM), in the presence or absence of 100 μM of tested compounds. After 20 h, at 37 °C, cell pellets were analyzed by SDS-PAGE and fluorographed. NS: not specific. (B) Densytometric analysis of 21 kDa labelled proteins. (C) For the evaluation of [^{3}H]-thymidine incorporation, cells were treated as indicated for panel A, in the absence of simvastatin. Cells were labelled with [^{3}H]-thymidine for 2 h at the end of the incubation with indicated compounds. Each bar represents the mean ± S.D. of triplicate dishes. The mean value for control experiments (without inhibitor) was $156 \times 10^3 \pm 3.70 \times 10^3$ dpm mg prot^{-1}. Inhibitor vs. control: $^{*}p < 0.01$ (Student's *t*-test). The data are representative of two replicate experiments.

of compound **6n** on [^{3}H]-FOH incorporation correlates with their antiproliferative activity, we measured [^{3}H]-thymidine incorporation in rat SMC under the same experimental conditions. As shown in Fig. 1C, compound **6n**, which effectively inhibited protein farnesylation, strongly reduced DNA synthesis by 65.1%. In contrast, its respective compound lacking the S atom (compound **6b**) showed lower inhibitory action (−14.6%). These data demonstrated a significant correspondence of the degree of inhibition of compound **6n** on the metabolisms of protein farnesylation and DNA synthesis.

3.3. Compound *6n* interfered with cell cycle progression in G0/G1 phase

Compound **6n** has been shown the strongest inhibitory effect on PFTase in vitro (Table 3), and most importantly, at similar concentrations inhibited PFTase activity, [^{3}H]-FOH incorporation into cellular proteins, and [^{3}H]-thymidine incorporation in rat SMC (Table 3, Fig. 1). We therefore, further investigated its antiproliferative properties. Compound **6n** inhibited SMC proliferation in a concentration-dependent manner with an IC_{50}-value of 61.4 μM (Fig. 2A), and caused a significant inhibitory effect on [^{3}H]-thymidine incorporation at 100 μM (Fig. 2B). To determine in which phase of the cell cycle compound **6n** exerted its antiproliferative action, we performed cell cycle analysis of quiescent and proliferating SMC after treatment with **6n** by flowcytometry. Incubation of rat SMC for 120 h with culture media containing 0.4% FCS led to the accumulation of the cells in G1 phase (90.9%) with only a small percentage in S phase (2.8%) (Fig. 3). As expected, after 20 h stimulation with 10% FCS we observed an increase in the percentage of cells in S phase (12.4%) and a reduction in G1 phase (65.4%) (Fig. 3). Incubation of SMC with 100 μM **6n** caused a significant accumulation of the cells in G1 phase, in a similar extent seen with 0.4% FCS, indicating a complete arrest of cell proliferation (Fig. 3). This effect strongly correlates with the inhibitory action observed on [^{3}H]-thymidine incorporation assay (compare Fig. 2B with Fig. 3). Importantly, we did not detect any significant increase in the percentage

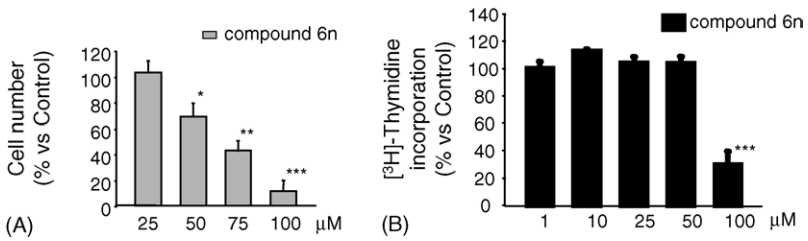


Fig. 2. Concentration-dependent effect of compound **6n** on proliferation of rat aortic SMC and [^{3}H]-thymidine incorporation. (A) Experimental conditions are as in Table 4. The mean value of cell number at time 0 was 463.0×10^3 ($\pm 14.2 \times 10^3$). Each bar represents the mean ± S.D. of triplicate dishes. The mean value for control experiment (without inhibitor) was 1072.3×10^3 ($\pm 57.5 \times 10^3$) cells/dish. Inhibitor vs. control: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t*-test). The data are representative of three replicate experiments with triplicate determinations. (B) Experimental conditions are as in Fig. 1. The mean value for control experiment (without inhibitor) was 18.5×10^3 ($\pm 3.6 \times 10^3$) dpm mg prot^{-1}. Inhibitor versus control: $^{***}p < 0.001$ (Student's *t*-test). The data are representative of three replicate experiments with triplicate determinations.

(A)

Incubation	<G1 (%)	G1 (%)	S (%)	G2/M (%)
0.4% FCS	0.7	90.9	2.8	5.6
10% FCS	4.8	65.4	12.4	17.3
6n 50 μM	5.1	65.8	10.7	18.9
6n 75 μM	5.6	66.8	10.5	17.2
6n 100 μM	3.6	88.3	2.9	5.2

(B)

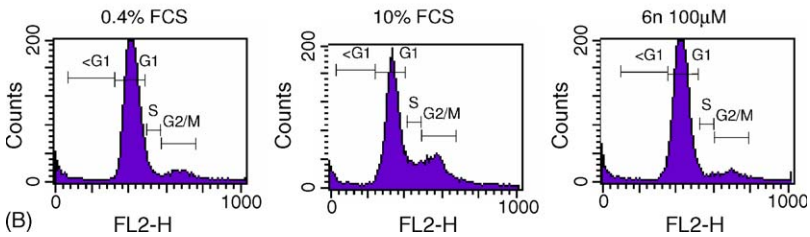


Fig. 3. Effect of compound **6n** on cell cycle of rat SMC. Experimental conditions are as in Fig. 1. (A) Table summarizing flow cytometry analysis of cell cycle performed in the presence of 0.4%, 10% FCS alone, or 10% FCS with reported concentrations of compound **6n**. (B) Representative flowcytometry analysis of SMC incubated with 100 μM of compound **6n**. The data are representative of three replicate experiments.

of sub G0/G1 cells even after incubation with 100 μM of compound **6n**, which indicates a specific interference with the progression of the G1 phase of the cell cycle without induction of apoptosis (Fig. 3). These results demonstrate a specific inhibitory action of compound **6n** on the progression of the G1 phase of the cell cycle and thereby proliferation of cultured rat SMC.

4. Discussion

In the present study, we explored the possibility to improve the potency of isothiazole dioxides as inhibitors of mammalian PFTase, which have been previously described as inhibitors of *T. brucei* PFTase [20]. We started from a basic structure (3-diethylamino-4-(4-methoxyphenyl)-isothiazole1,1-dioxides) and we examined the effects of different C5 substitutions on the inhibitory activity against rat PFTase and PGGTase-I. Our analysis demonstrated that selective changes of C5 substituents, and the presence or absence of C4–C5 double bond, led to compounds with different inhibitory activities on mammalian PFTase and PGGTase-I in vitro. Using this approach, we identified four compounds that effectively inhibited PFTase, with **6n** (C5; methyl-S) having the lowest IC_{50}-value, equal to 8.56 μM and 500-fold higher inhibitory potency on PFTase than PGGTase-I. Interestingly, a similar inhibitory potency (IC_{50} = 2.5 μM) has been observed by using the well-characterized PFTase inhibitor, SCH44342 [31], although other PFTase inhibitors with greater inhibitory potency have been described [32]. It is also important to notice that the effect of compound **6n** on protein farnesylation was confirmed in cultured primary SMC by measuring [^{3}H]-farnesol incorporation into cellular proteins. This effect directly demonstrated and confirmed the inhibitory activity of isothiazole dioxides on mammalian PFTase, not only on the recombinant enzyme but also in a cell-based assay. This is of particular interest, since several PFTase inhibitors frequently loose 2–3 logs of potency on their ability to inhibit Ras processing in whole cells due to poor cell permeability [30], although compounds effective at 0.01–1 μM in cultured cells have been previously identified [33].

By changing substituents at C5, we strongly improved the inhibitory action of isothiazole dioxides versus mammalian PFTase, compared to *T. brucei* PFTase [20]. In the previous report only one compound was shown to significantly inhibit rat PFTase (compound **6b**, −27% at 100 μM), with a strong inhibitory activity on rat PGGTase-I (−55.5% at 100 μM) [20]. By adding either phenyl, 2-pyridyl, perillyl, or methyl substituents at position C5, with an S atom as a linker between the isothiazole moiety and the substituent, we identified new inhibitors of PFTase with IC_{50} values equal to 774, 119, 8.56, and 1015 μM, respectively.

By cell counting, thymidine incorporation assay, and cell cycle analysis we demonstrated that compound **6n** elicited a concentration-dependent inhibitory action on rat SMC proliferation. Although the actual antiproliferative mechanism still needs to be determined, the inhibitory effects of compound **6n** on PFTase activity in vitro (IC_{50} = 8.56 μM), on proliferation of rat SMC (IC_{50} = 61.4 μM), and incorporation of [^{3}H]-farnesol into cellular proteins (inhibition of 55.9% at 100 μM) were elicited at a very similar concentration range, suggesting a direct relationship between these events.

More accurate analysis conducted on compound **6n** has determined that its antiproliferative action is elicited by a direct interference with the progression of the G0/G1 phase of the cell cycle. A specific block in G0/G1 phase has previously been demonstrated with other PFTase inhibitors in studies carried out with a variety of tumor cell lines [34],

and similar effect has been shown in vascular SMC [18,35,36]. The farnesyl pyrophosphate analogue, an inhibitor of PFTase, efficiently inhibited mammary artery SMC by interfering with early phase of the cell cycle, after stimulation with platelet derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) [36].

It is also important to notice that the inhibitory action of compound **6n** on DNA synthesis is observed at slightly higher concentrations than those required to affect cell proliferation. This difference may be dependent from the incubation time utilized for thymidine incorporation and cell proliferation assay, 24 and 72 h, respectively. Interestingly, HMG-CoA reductase inhibitors, statins, that affect cell proliferation by inhibiting protein prenylation processes, have also shown to be effective on DNA synthesis at higher concentration ranges than those required to inhibit cell proliferation [37]. This effect may be dependent from the intracellular turnover of Ras, which has been estimated to be around 20–24 h [38].

Previously, we have also shown that the PFTase inhibitor, BZA-5B, and a double inhibitor of PFTase and PGGTase-I, perillic acid, affect SMC proliferation [18,35]. Interestingly, vascular SMC expressing a dominant negative farnesyl transferase α-subunit showed a reduced basal and insulin-stimulated proliferation levels, as measured by BrdU incorporation, further supporting the role of PFTase in the regulation of the early phase of the cell cycle [39]. More importantly, a selective inhibition of H-Ras by a genetic approach has been demonstrated to block very efficiently the neointimal formation in response to balloon injury, suggesting that pharmacological modulation of H-Ras signaling is sufficient to prevent vascular restenosis [9,40]. In addition, another member of Ras family, K-Ras, may undergo geranylgeranylation by PGGTase-I when PFTase is inhibited, a lipid modification that hampers, or even blocks, the activity of K-Ras [41]. Thus, several mechanisms could explain how a specific inhibition of PFTase may be effective to control SMC proliferation in response to vascular injury. On these basis, we are currently designing experiments for evaluating the effect of compound **6n** on intimal hyperplasia after perivascular manipulation of carotid artery [42]. These experiments will add further insights on the potential application of the pharmacological modulation of farnesylation process in preventing vascular restenosis. Compound **6n** will be also utilized as a pharmacological tool aimed at identifying which farnesylated protein is primarily involved in SMC proliferation by 2D SDS-PAGE analysis [14].

The interaction of isothiazole dioxides with PFTase still need to be determined, but it would be interesting to know whether these compounds are competing with the CAAX portion of prenylated proteins, with the farnesyl-pyrophosphate substrate, or with both [16]. Although the interaction mode of isothiazole dioxides with PFTase is unknown, we found significant structure-activity relationship for the potency of the isothiazole dioxide derivatives against mammalian PFTase. We observed in vitro, and confirmed for the methyl substitution (compounds **6n** and **6b**) in cultured cells, that the addition of an S atom as a linker between the isothiazole moiety and the substituent is required for the inhibitory activity on PFTase. PFTase contains an active site zinc ion that is necessary for the catalytic activity and peptide/protein binding [43,44]. The zinc ion directly coordinates the sulfur atom of the ''CAAX'' cystein residue [45]. It is tempting to speculate that S atom, only presents in effective isothiazole dioxide inhibitors of PFTase, may directly coordinate the zinc ion in the catalytic site of PFTase. This would also explain why compound **6n**, which has the smallest substituent in C5 (methyl-S) as compared to **6i**, **6l** and **6p**, elicited the strongest inhibitory activity in vitro [44]. Other zinc chelating inhibitors of PFTase have been previously described, showing a similar efficacy and potency in inhibiting the enzymatic activity in vitro [46]. Interestingly, the inhibitory activity of these chelating compounds was not simply due to the metal binding power but also to the affinity to the aromatic pocket in the β subunit of the PFTase [46]. Accordingly, we provided evidences that the structure of the isothiazole ring plays also an important role in the inhibitory effect on PFTase. Indeed, the 3-dialkylaminoisothiazole dioxides, such as **6n** and **6i**, were relatively effective inhibitors of rat PFTase, while the corresponding dihydroderivatives **5a** and **5c** were very poor (Tables 1 and 2), showing that the saturation of the C4–C5 double bond is detrimental for the inhibitory action, suggesting that the planarity of the isothiazole ring is crucial for enzyme interaction.

In conclusion, in the present report we described a new PFTase inhibitor compound **6n** containing the isothiazole dioxide moiety that effectively inhibits protein farnesylation and SMC proliferation by interfering with the G0/G1 phase of the cell cycle.

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