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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