Synthesis and Antimicrobial and Nitric Oxide Synthase Inhibitory Activities of Novel Isothiourea Derivatives

Zygmunt Kazimierczuk¹, Małgorzata Chalimoniuk², Agnieszka Ewa Laudy³, Rosa Moo-Puc⁴, Roberto Cedillo-Rivera⁴, Bohdan Jerzy Starosciak³, and Stanislaw J. Chrapusta¹

Department of ¹Experimental Pharmacology, Polish Academy of Sciences, 02-106 Warsaw, Poland, ²Cellular Signaling, Mossakowski Medical Research Center, Polish Academy of Sciences, 02-106 Warsaw, Poland, ³Department of Pharmaceutical Microbiology, Medical University of Warsaw, 02-007 Warsaw, Poland, and ⁴Unidad Interinstitucional de Investigación Clínica y Epidemiológica, Facultad de Medicina, UADY/Instituto Mexicano del Seguro Social, Mérida, Yuc., México

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The reaction of substituted benzylhalides, or of halomethyl derivatives of thiophene or furane, with thiourea or its derivatives yielded the respective isothioureas as hydrohalide salts. The products (a total of 17, including 16 novel compounds) were tested for activity against five Gram-positive and nine Gram-negative bacterial strains, six yeast species and two protozoan species. The most active against Gram-positive bacteria were S-(2,4-dinitrobenzyl)isothiourea hydrochloride (MIC range for four out of five strains tested: 12.5-25 µg/mL) and S-(2,3,4,5,6-pentabromobenzyl)isothiourea hydrobromide (MIC range: 12.5-50 µg/mL). The lowest MICs of novel isothioureas for yeast and Gram-negative bacteria ranged between 50 and 100 µg/mL. Nine novel isothioureas showed appreciable genotoxicity in the Bacillus subtilis 'rec-assay' test, the most potent being S-2-(5-nitrofuran-2-ylmethyl)isothiourea and S-(2-nitrobenzyl)isothiourea. At 10 µM concentration, S-(3,4-dichlorobenzyl)isothiourea hydrochloride and S-(2,3,4,5,6-pentabromobenzyl)isothiourea hydrobromide inhibited Ca²⁺/calmodulin-dependent (non-inducible) nitric oxide synthase activity in normal rat brain homogenates stronger (p < 0.05) than the reference drug 7-nitroindazole (by 78, 76 and 60%, respectively); ten other new isothiourea derivatives significantly inhibited the activity to a lower extent (by 28-60%). These results extend the list of promising isothioureas with substantial activity in vitro and suggest that an in-depth study of toxicity, antimicrobial properties in vivo and nitric oxide synthase isoform selectivity of selected novel compounds is warranted.

Key words: Isothiourea derivative, Synthesis, Antibacterial activity, Antifungal activity, Anti-protozoal activity, Nitric oxide synthase

INTRODUCTION

Isothioureas are a class of amphiphilic compounds carrying a highly basic isothiourea group of pKb ≈ 10. At physiological pH (pH = 7), these compounds exist in a protonated (cationic) form that may be of importance for their specific biological effects. Their synthesis and isolation is simple due to their poor solubility in reaction medium. On the other hand, in solid state, they form salts of usually good water solubility, which makes them particularly attractive for microbiology studies.

Isothioureas are highly interesting compounds from a pharmaceutical/pharmacological point of view as well. The 'parental' unsubstituted thiourea (thiocarbamide) is toxic and is a known carcinogen (see http://msds.chem.ox.ac.uk/TH/thiourea.html). However, a rapidly growing body of evidence demonstrates multiple potentially beneficial biological activities of its derivatives. Some of these compounds show potential as prodrugs of the alcohol deterrent agent cyanamide (Shirota et al., 1997), inhibitors of HIV capsid assembly (Li et al., 2009b), blockers of the CXCR4 chemokine receptors with potential for preventing HIV infection...
of target cells (Thoma et al., 2008), anticaner drugs (for review see Li et al., 2009a), hypoglycaemic drugs (Zhang et al., 2009), calcium blockers with potential neuroprotectant and cognition enhancer capability (Perlovich et al., 2009), anti-HCV agents with high selectivity index (Kang et al., 2009), and proapoptotic agents with substantial toxicity toward human glioblastoma cells in vitro (Kaminska et al., 2009). However, most attention is being given to these drugs as nitric oxide synthase (NOS) inhibitors with variable NOS isoform selectivity (Szabo et al., 1994; Southan et al., 1995; Handy et al., 1996; Wang et al., 1998; Paquay et al., 1999; Ijuin et al., 2005; Jin et al., 2009) and antimicrobial agents.

First papers on antimicrobial activity of N,S-substituted isothioureas were published in late 1980s (Tait et al., 1989, 1990). Some of those compounds showed substantial activity against Gram-positive and Gram-negative bacteria, but not against fungi tested (Microsporum canis, Aspergillus niger, A. flavus and Candida albicans). The most potent substances in that series were S-(3,4,5-trichlorobenzyl)-isothiourea and its N,N'-tetramethyl congener. Recently, S-(3,4-dichlorobenzyl) isothiourea and S-(4-chlorobenzyl)isothiourea, but not their S-ethyl, S-nonyl and S-cyclohexyl analogs, were found to interfere with chromosome partitioning and induce spherical and spherical anucleate cells in Escherichia coli (Iwai et al., 2004). This effect likely results from their interaction with rod shape-determining proteins (Iwai et al., 2004). Most recently, S-(4-chlorobenzyl)isothiourea has been shown to have substantial activity against many strains of Pseudomonas aeruginosa and Burkholderia cepacia complex that are important pathogens in cystic fibrosis patients (Nicholson et al., 2009). Anti-protozoal activity of isothiourea derivatives has not been studied until now.

There are three NOS isoforms: an inducible enzyme (iNOS) and the constitutive (Ca²⁺/calmodulin-dependent) neuronal (nNOS) and endothelial (eNOS) isoforms, first two of which, and particularly iNOS, can cause NO overproduction and, hence, participate in the related tissular damage. In a contrast, eNOS has no known detrimental effect, but plays an important role in maintaining blood pressure and flow. Because of differing roles of these enzymes in physiology and pathology, there is an ongoing search for the respective selective inhibitors (Garvey et al., 1994; Szabo et al., 1994; Babu and Griffith, 1998; Salerno et al., 2002; Castano et al., 2008). This search included also a number of studies on isothiourea derivatives, of which two most investigated and active were, so far, ethylisothiourea and aminoethylisothiourea (Garvey et al., 1994; Southan et al., 1995; Shearer et al., 1997; Salerno et al., 2002; Xu et al., 2003; Paesano et al., 2005; Barocelli et al., 2006).

Below, we describe the synthesis of a series of new compounds of this class, and the results of our preliminary studies in vitro of their antibacterial, antifungal and anti-protozoal activities as well as inhibitory activity against Ca²⁺/calmodulin-dependent NOSs.

**MATERIALS AND METHODS**

**Chemistry**

All solvents and reagents were purchased from Sigma-Aldrich. Melting points (uncorr.) were measured in open capillary tubes in a Gallenkamp-5 melting-point apparatus. 1H-NMR spectra were measured with a Varian Gemini 200 MHz (or Varian UNITYplus 500 MHz) spectrometer at 298 °K in D₂O(DMSO), using tetramethylsilane as an internal standard. Flash chromatography was performed with Merck silica gel 60 (200-400 mesh). Elemental (C, H, N) analyses of the new compounds were within 0.4% of the respective theoretical values.

**Synthesis**

3,5-Dinitrobenzylisothiourea hydrochloride (Exemplary synthesis)

To a hot solution of thiourea (400 mg, 5.1 mmol) in anhydrous ethanol (20 mL) 3,5-dinitrobenzylchloride (1.08 g, 5 mmol) was added. The mixture was refluxed for 20 min and then the solvent was partially evaporated to a final volume of about 15 mL. This was left refrigerated overnight. The chromatographically pure crystals that formed (0.75 g, 78% yield) were filtered off and washed with a small volume of cold ethanol-ethyl ether mixture (1:1, v/v). For elemental analysis, a small amount of the product was recrystallized from ethanol.

**Biological studies in vitro**

**Antibacterial, antifungal and genotoxicity assays**

The microorganisms employed were as follows: (1) Gram-positive bacteria: Staphylococcus aureus ATCC 6538P, Staphylococcus aureus NCTC 4163, Enterococcus faecalis ATCC 29212, Bacillus subtilis ATCC 6633, Bacillus stearothermophilus ATCC 7953; (2) Gram-negative bacteria: Proteus vulgaris NCTC 4635, Escherichia coli ATCC 25922, E. coli NCTC 8196, Klebsiella pneumoniae ATCC 13883, Pseudomonas aeruginosa NCTC 6749, Stenotrophomonas maltophilia ATCC 1363, Acinetobacter baumannii 18006, Bordetella bronchiseptica ATCC 4617, Burkholderia...