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ORIGINAL PAPER

Synthesis and antimicrobial activity of sulphamethoxazole-based ureas and imidazolidine-2,4,5-triones

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Progression of drug resistance among bacterial and fungal pathogens justifies the development of novel antimicrobial agents. Thus, a series of novel sulphamethoxazole-based ureas and imidazolidine-2,4,5-triones have been designed and synthesised. The urea derivatives were obtained by the reaction of sulphamethoxazole and isocyanates, and their cyclisation to imidazolidine-2,4,5-triones was performed via oxalyl chloride. All synthesised derivatives were evaluated in vitro to determine their activity against gram-positive and gram-negative bacteria, fungi, *Mycobacterium tuberculosis*, and atypical mycobacteria and their cytotoxicity. The growth of mycobacteria was inhibited within the range of 4–1000 μM and *M. tuberculosis* was the least-susceptible strain. 4-(3-Heptylureido)-*N*-(5-methylisoxazol-3-yl)benzenesulphonamide was identified as the most promising compound because it exhibited the highest activity against atypical mycobacteria at minimum inhibitory concentrations, from 4 μM, and with acceptable toxicity (selectivity indices for *M. avium* and *M. kansasii* higher than 16 and 62.5, respectively). Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, were inhibited at concentrations starting from 125 μM, whereas the investigated derivatives exhibited almost no antifungal potency and activity against gram-negative species.

Keywords: antimicrobial activity, antimycobacterial activity, imidazolidine-2,4,5-triones, sulphamethoxazole, ureas

Introduction

The alarming progression of drug resistance among human pathogens and the inappropriate use or misuse of antimicrobial chemotherapeutics justifies the development of novel antimicrobial agents. Therapeutic difficulties and resistance-related complications have been reported for many bacterial species, including *Mycobacterium tuberculosis* (*M. tuberculosis*), nontuberculous (atypical) mycobacteria, and gram-positive and gram-negative bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) or *Enterococcus* sp. Research studies should provide new antimicrobial molecules; also, the modification of known drugs represents an effective approach in drug design.

Sulphonamides have been widely used for the ther-

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apy of both gram-positive and gram-negative bacterial infections; moreover, these derivatives were used for the treatment of tuberculosis (TB) in the early 1950s but were later abandoned mostly for their toxicity and the accessibility of novel more efficient drugs. More recently, these derivatives were considered useless for the treatment of TB but concomitantly useful for the therapy of some nontuberculous mycobacterial infections. Based on the findings that both drug-susceptible and drug-resistant *M. tuberculosis* are susceptible in vitro to clinically achievable concentrations of sulphamethoxazole (SMX) and its combination with trimethoprim, this molecule has "resurrected" as a potential antimycobacterial agent with potential use in the treatment of multidrug-resistant TB- and HIV-co-infected patients and infections with nontuberculous mycobacteria (Alsaad et al., 2013; Ameen & Drancourt, 2013; Huang et al., 2012; Krátký et al., 2012). Although SMX is active against *M. tuberculosis*, further studies for the identification of more active and lipid-soluble sulphonamides preferably with improved pharmacokinetic and pharmacodynamic profiles are necessary (Huang et al., 2012).

Several sulphonamide-based derivatives have recently been designed and evaluated as potential antimycobacterial agents. Some of them show activity against various bacterial strains (Agertt et al., 2013; Desai et al., 2011; Krátký et al., 2012; North et al., 2013; Sukdolak et al., 2005; Thomas et al., 2011). Sulphonamides generally act as structural analogues of 4-aminobenzoic acid and therefore competitively inhibit dihydropteroate synthase thereby disrupting folate biosynthesis. More recently, these drugs have been found to inhibit various β-carbonic anhydrases, including those from *Mycobacterium* (Maresca et al., 2013; Minakuchi et al., 2009; Yun et al., 2012).

Based on these findings and on the fact that a wide range of urea derivatives were found to share antimycobacterial activity in vitro (Brown et al., 2011; North et al., 2013), sulphamethoxazole-based ureas and their cyclic homologues have been designed, synthesised, and evaluated as potential antimicrobial agents.

Experimental

General

All reagents and solvents were purchased from Sigma–Aldrich (Darmstadt, Germany) or Penta Chemicals (Prague, Czech Republic) and were used as received. The reactions and purity of the products were monitored by thin-layer chromatography using ethyl acetate/hexane ($\varphi_r = 2 : 1$) as the eluent; the plates were coated with 0.2 mm Merck 60 F254 silica gel (Darmstadt, Germany) and were visualised by UV irradiation (254 nm). The melting points were determined using a Büchi melting point B-540 apparatus (Büchi, Flawil, Switzerland) with open capillaries, and the reported values are uncorrected.

Elemental analysis was performed with an automatic Fisons EA 1110 CHNS-O CE microanalyzer (Fisons, Milano, Italy). Infrared spectra (ATR) were recorded on a Nicolet 6700 FTIR spectrometer in the range of 400–4000 cm^{-1}. NMR spectra (500 MHz for ^1H and 125 MHz for ^{13}C) were measured in DMSO-d_6 or THF-d_8 at ambient temperature using a Varian VNMR S500 instrument (Varian, Palo Alto, CA, USA) or a Varian Mercury-Vxbb 300 (300 MHz for ^1H and 75.5 MHz for ^{13}C; Varian, Palo Alto, CA, USA). Tetramethylsilane was used as the internal standard.

The calculated log$P$ values (Clog$P$) were determined using the CS ChemOffice Ultra version 12.0 program (CambridgeSoft, Cambridge, MA, USA).

General procedure for the synthesis of ureas II

Sulphamethoxazole (SMX, *I*) (2 mmol) was dissolved in dry acetonitrile (MeCN, 6 mL) and appropriate isocyanate (2.1 mmol) was added in one portion. The solution was heated under reflux for 3.5 h and then stirred at ambient temperature for 8 h. The resulting crystals were filtered, washed with a small amount of MeCN and dried. The product was recrystallised from ethyl acetate (EtOAc) if necessary.

General procedure for the synthesis of imidazolidine-2,4,5-triones III

Oxalyl chloride (1.2 equivalent) was added in one portion to sulphonamide-based urea (*II*, 0.75 mmol) dissolved or suspended in dry THF (4 mL) and the mixture was heated under reflux for 2 h. After cooling, the crystallisation was initiated by an addition of hexane. The resulting crystals were filtered, washed with a small amount of dichloromethane and recrystallised from EtOAc if necessary.

Biological activity testing

The in vitro antimycobacterial activity of sulphonamide derivatives against *M. tuberculosis* 331/88 ($H_{37}Rv$; dilution of this strain was 10^{-3}), *M. avium* 330/88 (dilution of 10^{-5}), and two strains of *M. kansasii*, namely 235/80 (dilution of 10^{-4}) and the clinically isolated strain 6509/96 (dilution of 10^{-5}) was evaluated using a previously described method (Krátký et al., 2013). The following concentrations (in μM) were used: 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1. MIC is the lowest concentration at which complete inhibition of mycobacterial growth was observed. Isoniazid (INH) and SMX were chosen as the reference compounds. The tested compounds were prepared in quadruplicates and the determination was repeated twice.

The in vitro antibacterial activity of *II* and *III* was assayed against eight gram-positive and

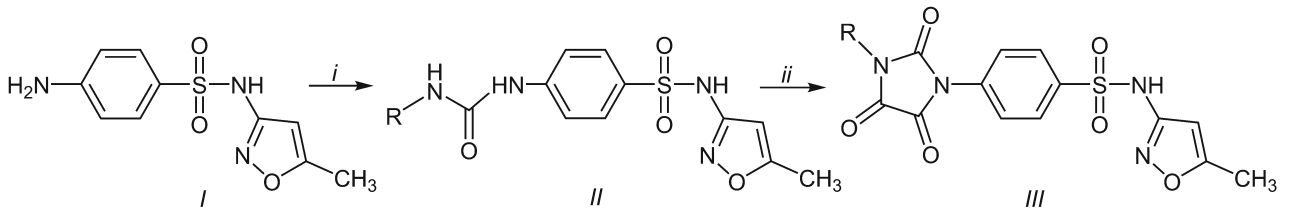

Fig. 1. Synthesis of ureas *II* and imidazolidine-2,4,5-triones *III*. Reaction conditions: *i*) isocyanate (R—N═C═O; for R, see Table 1), MeCN, reflux, 3.5 h, ambient temperature, 8 h; *ii*) oxalyl chloride, THF, reflux, 2 h.

gram-negative strains: *Staphylococcus aureus* CCM 4516/08, methicillin-resistant *Staphylococcus aureus* H 5996/08 (MRSA), *Staphylococcus epidermidis* H 6966/08, *Enterococcus* sp. J 14365/08; *Escherichia coli* CCM 4517, *Klebsiella pneumoniae* D 11750/08, ESBL-positive *Klebsiella pneumoniae* J 14368/08, and *Pseudomonas aeruginosa* CCM 1961.

The microdilution broth method with Mueller-Hinton broth was used. The tested compounds were dissolved in DMSO to final concentrations in the range of 500–0.49 μM. Bacitracin (BAC) and *I* were used as the comparative drugs. The minimum inhibitory concentrations were assayed as 95 % (IC_{95}) or higher reduction of growth compared with the control. The measurement was repeated twice. The method used for this determination was previously described by Krátký et al. (2013).

The in vitro antifungal activity of *II* and *III* was evaluated against four *Candida* strains (*Candida albicans* ATCC 44859, *Candida tropicalis* 156, *Candida krusei* E28, *Candida glabrata* 20/I), *Trichosporon asahii* 1188 and three filamentous fungi (*Aspergillus fumigatus* 231, *Absidia corymbifera* 272, *Trichophyton mentagrophytes* 445). The microdilution broth method was used in RPMI 1640 with glutamine. Fluconazole (FLU) was used as the reference drug. MICs were assayed as 80 % (IC_{80}) or higher reduction of growth compared with the control; for filamentous fungi, MICs are expressed as the IC_{50} values. The MIC determination was repeated twice using a method previously described by Krátký and Vinšová (2012).

Cytotoxicity of *II* and *III* in the human hepatocellular liver carcinoma cell line HepG2 (passage 33–39; ECACC, Salisbury, UK) was determined using a standard colorimetric method that involves measuring the reduction of a tetrazolium salt (CellTiter(R) 96 AQueous One Solution Assay, Promega G3580, Madison, WI, USA).

The cells were routinely cultured in Eagle's minimum essential media supplemented with 10 % of foetal bovine serum, 1 % of L-glutamine solution, and a non-essential amino acid solution. The investigated compounds were dissolved in a very small amount of DMSO, and a small volume of this solution was added to the cell culture. The tested compounds were prepared in triplicates at concentrations in the range of 0.1–2000 μM. The following types of controls were included: determination of 100 % viability and 0 % viability (cells were treated with a 10 % DMSO solution), no cell control, control for the determination of possible interactions of the tested compounds with the reagents, control of the incubation medium settings, and control of the DMSO toxicity. The results are expressed as the inhibitory concentration reducing the cell viability to 50 % of the maximal (control) viability (IC_{50}). IC_{50} for each of the tested substances was calculated using the GraphPad Prism software (version 5.02; GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2010. This method is described in detail in a previous report (Krátký et al., 2013).

Results and discussion

SMX-based ureas *II* were prepared from sulphonamide *I* and commercially available isocyanates (with different substituents: aryl, alkyl, cycloalkyl, arylalkyl) (Fig. 1) by heating under reflux for 3.5 h in dry acetonitrile in satisfactory yields (72–95 %) except for the adamantan-1-yl urea *IIe*, where the yield was only 40 %. However, this yield is significantly higher than 16 % obtained by the synthetic procedure reported previously by North et al. (2013).

The synthesised ureas *II* were used for cyclisation to form imidazolidine-2,4,5-triones *III* in the next step (Fig. 1) applying oxalyl chloride (Pejchal et al., 2011) in dry tetrahydrofuran. The yields were in the range of 47–95 %; the adamantyl derivative *IIIe* provided the lowest rate of conversion. Characterisation and spectral data of compounds *II* and *III* are summarised in Tables 1 and 2.

Starting SMX (*I*), ureas *II* and imidazolidine-2,4,5-triones *III* were evaluated for their in vitro antimycobacterial activity against *M. tuberculosis* 331/88 ($H_{37}Rv$), *M. avium* 330/88 and two strains of *M. kansasii*, namely 235/80 and the clinical isolate 6509/96. The first-line antimycobacterial drug isoniazid was employed as the reference compound.

SMX and its derivatives, *II* and *III*, showed antimycobacterial activity with MICs of 4 μM or higher (Table 3); none of the compounds was completely inactive. *M. tuberculosis* showed the lowest susceptibility to the novel molecules (MICs $\geq$ 125 μM; the best result was obtained with 4-(trifluoromethyl)phenyl urea *IIh*), whereas *M. kansasii*, with MICs $\geq$ 4 μM, was

Table 1. Characterisation data of newly prepared compounds

Compound	R	Formula	M_r	w_i(calc.)/% w_i(found)/%			Yield	M.p.
				C	H	N	%	°C
IIa	cyclohexyl	$C_{17}H_{22}N_4O_4S$	378.45	53.95 53.79	5.86 5.88	14.80 14.89	73	210–212.5
IIb	phenyl	$C_{17}H_{16}N_4O_4S$	372.40	54.83 54.92	4.33 4.51	15.04 14.99	90	217–218.5
IIc	benzyl	$C_{18}H_{18}N_4O_4S$	386.42	55.95 55.87	4.70 5.00	14.50 14.64	91	225.5–228
IId	phenethyl	$C_{19}H_{20}N_4O_4S$	400.45	56.99 56.88	5.03 4.90	13.99 14.12	76	239–241
IIe	adamantan-1-yl	$C_{21}H_{26}N_4O_4S$	430.52	58.59 58.63	6.09 6.00	13.01 13.20	40	224.5–226
IIf	propyl	$C_{14}H_{18}N_4O_4S$	338.38	49.69 49.74	5.36 5.21	15.56 15.69	92	235–237
IIg	heptyl	$C_{18}H_{26}N_4O_4S$	394.49	54.80 54.67	6.64 6.80	14.20 14.00	72	219–220.5
IIh	4-CF_3-phenyl	$C_{18}H_{15}F_3N_4O_4S$	440.40	49.09 49.16	3.43 3.25	12.72 12.83	85	246–247
IIi	4-*tert*-butylphenyl	$C_{21}H_{24}N_4O_4S$	428.50	58.86 59.02	5.65 5.54	13.07 12.96	84	221.5–224
IIIa	cyclohexyl	$C_{19}H_{20}N_4O_6S$	432.45	52.77 53.00	4.66 4.59	12.96 12.86	74	200.5–203
IIIb	phenyl	$C_{19}H_{14}N_4O_6S$	426.40	53.52 53.48	3.31 3.47	13.14 12.99	87	236–237.5
IIIc	benzyl	$C_{20}H_{16}N_4O_6S$	440.43	54.54 54.65	3.66 3.70	12.72 12.73	95	208.5–210.5
IIId	phenethyl	$C_{21}H_{18}N_4O_6S$	454.46	55.50 55.37	3.99 3.86	12.33 12.19	83	206–207
IIIe	adamantan-1-yl	$C_{23}H_{24}N_4O_6S$	484.52	57.01 56.91	4.99 5.12	11.56 11.70	47	206.5–208.5
IIIf	propyl	$C_{16}H_{16}N_4O_6S$	392.39	48.97 49.11	4.11 4.02	14.28 14.41	80	198–200.5
IIIg	heptyl	$C_{20}H_{24}N_4O_6S$	448.49	53.56 53.44	5.39 5.47	12.49 12.60	73	147.5–148.5
IIIh	4-CF_3-phenyl	$C_{20}H_{13}F_3N_4O_6S$	494.40	48.59 49.33	2.65 2.51	11.33 11.36	86	216–218.5
IIIi	4-*tert*-butylphenyl	$C_{23}H_{22}N_4O_6S$	482.51	57.25 57.42	4.60 4.74	11.61 11.58	87	223–226

shown to be the most susceptible species. *M. avium* was inhibited at concentrations of at least 32 μM. All nontuberculous mycobacteria were suppressed most effectively by 4-(3-heptylureido)-*N*-(5-methylisoxazol-3-yl)benzenesulphonamide (*IIg*) with MICs of 4–16 μM for *M. kansasii* and 32–62.5 for *M. avium*. Propylurea *IIf* was inactive against *M. tuberculosis* and the adamantyl moiety containing imidazolidine *IIIe* was inactive against *M. avium*.

INH is much more active against *M. tuberculosis* than the SMX-based derivatives; two derivatives (SMX and heptylurea *IIg*) exhibited activity against the *M. kansasii* isolate 6509/96 comparable to that of INH. In contrast, all imidazolidine-2,4,5-triones *III* and five ureas (*IId*, *IIe*, *IIg*, *IIh*, *IIi*) showed higher activity against *M. kansasii* 235/80 than INH, and eight compounds (*IId*, *IIg*, *IIh*, *IIIa*, *IIId*, *IIIf*, *IIIg*, *IIIh*) exhibited lower MICs for *M. avium*. None of the ureas *II* or imidazolidine-2,4,5-triones *III* exceeded significantly the activity of the parent drug SMX against the studied mycobacterial strains, and only heptylurea *IIg* showed equal or slightly higher activity against nontuberculous strains.

The investigation on the influence of N^3-substitution on the activity for *M. tuberculosis* revealed that the highest activity is conferred by a longer alkyl (*IIg* vs. *IIf*) and 4-substituted phenyl (*IIi* and especially *IIh*) vs. unsubstituted phenyl *IIb*. Hydrogenation of the phenyl ring (*IIa* vs. *IIb*) and the insertion of a methylene group between nitrogen and phenyl (*IIb* vs. *IIc*) did not affect the MIC values, but the derivative with a two-carbon-long linker showed enhanced activity (*IIb* vs. *IId*). Similar results were obtained for *M. avium* with the exception of *IIh*, which is not significantly more active than *IIb*. A longer alkyl (*IIg*) and 4-substituted phenyl (*IIh*, *IIi*) were also the best sub-

Table 2. Spectral data of newly prepared compounds

Compound	Spectral data
IIa	IR, $\tilde{\nu}$/cm^{-1}: 3356 (N—H), 1677 (C═O) ^{1}H NMR (300 MHz, DMSO-d_6), δ: 11.20 (1H, s, SO_2NH), 8.79 (1H, s, N^{1}H ureido), 7.67 (2H, d, J = 8.9 Hz, H2, H6), 7.52 (2H, d, J = 8.9 Hz, H3, H5), 6.26 (1H, d, J = 7.8 Hz, N^{3}H ureido), 6.10 (1H, s, isoxazole), 3.51–3.38 (1H, m, H1′), 2.28 (3H, s, CH_3), 1.82–1.46 (5H, m, H2′, H3′, H4′, H5′, H6′), 1.36–1.07 (5H, m, H2′, H3′, H4′, H5′, H6′) ^{13}C NMR (75.5 MHz, DMSO-d_6), δ: 170.58, 158.12, 154.24, 145.50, 130.97, 128.53, 117.31, 95.77, 48.14, 33.17, 25.62, 24.72, 12.49
IIb	IR, $\tilde{\nu}$/cm^{-1}: 3401 (N—H), 1696 (C═O) ^{1}H NMR (500 MHz, DMSO-d_6), δ: 11.26 (1H, s, SO_2NH), 9.14 (1H, s, N^{1}H ureido), 8.80 (1H, s, N^{3}H ureido), 7.75 (2H, d, J = 8.8 Hz, H2, H6), 7.63 (2H, d, J = 8.9 Hz, H3, H5), 7.45 (2H, d, J = 7.7 Hz, H2′, H6′), 7.28 (2H, t, J = 7.9 Hz, H3′, H5′), 6.99 (1H, t, J = 7.4 Hz, H4′), 6.12 (1H, s, isoxazole), 2.29 (3H, s, CH_3) ^{13}C NMR (125 MHz, DMSO-d_6), δ: 170.71, 158.15, 152.64, 144.80, 139.68, 132.04, 129.35, 128.66, 122.84, 118.99, 118.19, 95.89, 12.57
IIc	IR, $\tilde{\nu}$/cm^{-1}: 3367 (N—H), 1671 (C═O) ^{1}H NMR (300 MHz, DMSO-d_6), δ: 11.22 (1H, s, SO_2NH), 9.09 (1H, s, N^{1}H ureido), 7.74–7.66 (2H, m, H2, H6), 7.61–7.54 (2H, m, H3, H5), 7.36–7.18 (5H, m, H2′, H3′, H4′, H5′, H6′), 6.82 (1H, t, J = 5.9 Hz, N^{3}H ureido), 6.11 (1H, s, isoxazole), 4.29 (2H, d, J = 5.8 Hz, CH_2), 2.28 (3H, s, CH_3) ^{13}C NMR (75.5 MHz, DMSO-d_6), δ: 170.61, 158.09, 155.13, 145.44, 140.39, 131.15, 128.76, 128.54, 127.55, 127.23, 117.55, 95.77, 43.18, 12.49
IId	IR, $\tilde{\nu}$/cm^{-1}: 3371 (N—H), 1657 (C═O) ^{1}H NMR (300 MHz, DMSO-d_6), δ: 11.21 (1H, s, SO_2NH), 9.00 (1H, s, N^{1}H ureido), 7.72–7.66 (2H, m, H2, H6), 7.58–7.52 (2H, m, H3, H5), 7.33–7.16 (5H, m, H2′, H3′, H4′, H5′, H6′), 6.31 (1H, t, J = 5.7 Hz, N^{3}H ureido), 6.11 (1H, s, isoxazole), 3.34 (2H, q, J = 6.6 Hz, CH_2NH) 2.74 (2H, t, J = 7.1 Hz, PhCH_2), 2.28 (3H, s, CH_3) ^{13}C NMR (75.5 MHz, DMSO-d_6), δ: 170.36, 157.86, 154.77, 145.24, 139.58, 130.79, 128.86, 128.58, 128.29, 126.31, 117.17, 95.53, 40.81, 35.81, 12.25
IIf	IR, $\tilde{\nu}$/cm^{-1}: 3368 (N—H), 1668 (C═O) ^{1}H NMR (300 MHz, DMSO-d_6), δ: 11.20 (1H, s, SO_2NH), 8.92 (1H, s, N^{1}H ureido), 7.71–7.65 (2H, m, H2, H6), 7.58–7.51 (2H, m, H3, H5), 6.33 (1H, t, J = 5.7 Hz, N^{3}H ureido), 6.10 (1H, s, isoxazole), 3.03 (2H, q, J = 6.7 Hz, CH_2NH), 2.28 (3H, s, isoxazole-CH_3), 1.42 (2H, sext, J = 7.3 Hz, CH_2), 0.85 (3H, t, J = 7.4 Hz, CH_2CH_3) ^{13}C NMR (75.5 MHz, DMSO-d_6), δ: 170.35, 157.86, 154.83, 145.33, 130.70, 128.27, 117.13, 95.53, 41.05, 23.02, 12.24, 11.47
IIg	IR, $\tilde{\nu}$/cm^{-1}: 3378 (N—H), 1681 (C═O) ^{1}H NMR (300 MHz, DMSO-d_6), δ: 11.20 (1H, s, SO_2NH), 8.91 (1H, s, N^{1}H ureido), 7.71–7.65 (2H, m, H2, H6), 7.57–7.51 (2H, m, H3, H5), 6.30 (1H, t, J = 5.7 Hz, N^{3}H ureido), 6.10 (1H, s, isoxazole), 3.06 (2H, q, J = 6.5 Hz, CH_2NH), 2.28 (3H, s, isoxazole-CH_3), 1.40 (2H, quint, J = 6.9 Hz, CH_2CH$_2$NH), 1.31–1.20 (8H, m), 0.83 (3H, t, J = 6.9 Hz, CH_2CH_3) ^{13}C NMR (75.5 MHz, DMSO-d_6), δ: 170.58, 158.10, 155.04, 145.57, 130.93, 128.51, 117.36, 95.77, 39.48, 31.70, 30.01, 28.87, 26.74, 22.50, 14.39, 12.49
IIh	IR, $\tilde{\nu}$/cm^{-1}: 3345 (N—H), 1698 (C═O) ^{1}H NMR (500 MHz, DMSO-d_6), δ: 11.28 (1H, s, SO_2NH), 9.27 (1H, s, N^{3}H ureido), 9.23 (1H, s, N^{1}H ureido), 7.79–7.75 (2H, m, H2, H6), 7.68–7.61 (6H, m, H3, H5, H2′, H3′, H5′, H6′), 6.13 (1H, s, isoxazole), 2.29 (3H, s, CH_3) ^{13}C NMR (125 MHz, DMSO-d_6), δ: 170.40, 157.79, 152.15, 144.07, 143.15, 132.16, 128.33, 126.30 (q, J = 3.8 Hz), 124.66 (d, J = 271.1 Hz), 122.43 (q, J = 32.0 Hz), 118.35, 118.17, 95.56, 12.23
IIi	IR, $\tilde{\nu}$/cm^{-1}: 3351 (N—H), 1698 (C═O) ^{1}H NMR (300 MHz, DMSO-d_6), δ: 11.25 (1H, s, SO_2NH), 9.09 (1H, s, N^{1}H ureido), 8.72 (1H, s, N^{3}H ureido), 7.77–7.71 (2H, m, H2, H6), 7.65–7.58 (2H, m, H3, H5), 7.39–7.27 (4H, m, H2′, H3′, H5′, H6′), 6.12 (1H, s, isoxazole), 2.29 (3H, s, isoxazole-CH_3), 1.25 (9H, s, CH_3 of *tert*-butyl) ^{13}C NMR (75.5 MHz, DMSO-d_6), δ: 170.36, 157.80, 152.30, 144.82, 144.53, 136.68, 131.55, 128.30, 125.61, 118.49, 117.75, 95.53, 34.09, 31.40, 12.23
IIIa	IR, $\tilde{\nu}$/cm^{-1}: 1732 (C═O) ^{1}H NMR (300 MHz, THF-d_8), δ: 10.41 (1H, s, SO_2NH), 8.00–7.94 (2H, m, H2, H6), 7.77–7.73 (2H, m, H3, H5), 6.25 (1H, s, isoxazole), 4.09 (1H, tt, J = 12.3 Hz, J = 3.7 Hz, H1′), 2.30 (3H, s, CH_3), 1.91–1.64 (5H, m, H2′, H3′, H4′, H5′, H6′), 1.47–1.16 (5H, m, H2′, H3′, H4′, H5′, H6′) ^{13}C NMR (75.5 MHz, THF-d_8), δ: 171.19, 158.71, 156.65, 155.88, 153.32, 140.53, 136.17, 128.51, 126.31, 95.98, 53.12, 30.30, 26.62, 25.98, 12.25
IIIb	IR, $\tilde{\nu}$/cm^{-1}: 1738 (C═O) ^{1}H NMR (500 MHz, THF-d_8), δ: 11.61 (1H, s, SO_2NH), 8.08–8.05 (2H, m, H2, H6), 7.74–7.70 (2H, m, H3, H5), 7.57 (2H, t, J = 7.6 Hz, H3′, H5′), 7.49 (1H, t, J = 7.6 Hz, H4′), 7.44 (2H, d, J = 7.6 Hz, H2′, H6′), 6.17 (1H, s, isoxazole), 2.30 (3H, s, CH_3) ^{13}C NMR (125 MHz, THF-d_8), δ: 170.74, 157.49, 156.05, 155.86, 152.15, 139.34, 134.77, 130.51, 129.46, 129.17, 128.14, 127.19, 126.79, 95.66, 12.26

Table 2. (continued)

Compound	Spectral data
IIIc	IR, $\tilde{\nu}$/cm^{-1}: 1734 (C═O) ^{1}H NMR (300 MHz, THF-d_8), δ: 11.60 (1H, s, SO_2NH), 8.05–7.99 (2H, m, H2, H6), 7.70–7.64 (2H, m, H3, H5), 7.45–7.26 (5H, m, H2′, H3′, H4′, H5′, H6′), 6.16 (1H, s, isoxazole), 4.78 (2H, s, CH_2), 2.29 (3H, s, CH_3) ^{13}C NMR (75.5 MHz, THF-d_8), δ: 170.74, 157.50, 157.08, 156.06, 153.09, 139.05, 135.27, 134.89, 128.63, 128.01, 127.86, 127.02, 95.63, 42.28, 12.27
IIId	IR, $\tilde{\nu}$/cm^{-1}: 1736 (C═O) ^{1}H NMR (300 MHz, THF-d_8), δ: 11.60 (1H, s, SO_2NH), 8.06–8.00 (2H, m, H2, H6), 7.68–7.62 (2H, m, H3, H5), 7.36–7.20 (5H, m, H2′, H3′, H4′, H5′, H6′), 6.16 (1H, s, isoxazole), 3.79 (2H, t, J = 7.8 Hz, CH_2N) 2.82 (2H, t, J = 7.8 Hz, PhCH_2), 2.30 (3H, s, CH_3) ^{13}C NMR (75.5 MHz, THF-d_8), δ: 170.72, 157.47, 156.78, 156.01, 152.84, 139.11, 137.96, 134.79, 128.83, 128.74, 128.03, 126.96, 126.80, 95.63, 40.16, 33.46, 12.25
IIIe	IR, $\tilde{\nu}$/cm^{-1}: 1730 (C═O) ^{1}H NMR (500 MHz, THF-d_8), δ: 10.44 (1H, s, SO_2NH), 7.97–7.94 (2H, m, H2, H6), 7.68–7.64 (2H, m, H3, H5), 6.24 (1H, s, isoxazole), 2.49 (6H, m, CCH_2CH) 2.30 (3H, s, CH_3), 2.16–2.10 (3H, m, CH), 1.81–1.68 (6H, m, CHCH_2CH) ^{13}C NMR (125 MHz, THF-d_8), δ: 171.15, 158.72, 157.38, 155.37, 153.67, 140.57, 136.04, 128.41, 126.70, 95.99, 62.46, 40.83, 36.89, 30.94, 12.25
IIIf	IR, $\tilde{\nu}$/cm^{-1}: 1732 (C═O) ^{1}H NMR (300 MHz, DMSO-d_6), δ: 11.56 (1H, s, SO_2NH), 8.04–7.99 (2H, m, H2, H6), 7.68–7.62 (2H, m, H3, H5), 6.16 (1H, s, isoxazole), 3.53 (2H, t, J = 7.0 Hz, CH_2N), 2.30 (3H, s, isoxazole-CH_3), 1.62 (2H, sext, J = 7.3 Hz, CH_2), 0.91 (3H, t, J = 7.4 Hz, CH_2CH_3) ^{13}C NMR (75.5 MHz, DMSO-d_6), δ: 170.97, 157.77, 157.43, 156.40, 153.44, 139.29, 135.18, 128.25, 127.24, 95.89, 40.91, 21.30, 12.52, 11.59
IIIg	IR, $\tilde{\nu}$/cm^{-1}: 1736 (C═O) ^{1}H NMR (500 MHz, DMSO-d_6), δ: 11.58 (1H, s, SO_2NH), 8.03–8.00 (2H, m, H2, H6), 7.67–7.63 (2H, m, H3, H5), 6.15 (1H, s, isoxazole), 3.55 (2H, q, J = 7.1 Hz, CH_2NH), 2.30 (3H, s, isoxazole-CH_3), 1.59 (2H, quint, J = 7.1 Hz, CH_2CH$_2$NH), 1.36–1.18 (8H, m), 0.85 (3H, t, J = 6.7 Hz, CH_2CH_3) ^{13}C NMR (125 MHz, DMSO-d_6), δ: 170.70, 157.49, 157.11, 156.14, 153.14, 138.92, 134.92, 127.98, 126.95, 95.62, 38.96, 31.31, 28.41, 27.57, 26.15, 22.22, 14.06, 12.25
IIIh	IR, $\tilde{\nu}$/cm^{-1}: 1746 (C═O) ^{1}H NMR (500 MHz, THF-d_8), δ: 10.48 (1H, s, SO_2NH), 8.05–8.01 (2H, m, H2, H6), 7.87 (2H, d, J = 8.5 Hz, H3′, H5′), 7.79–7.73 (4H, m, H3, H5, H2′, H6′), 6.25 (1H, s, isoxazole), 2.30 (3H, s, CH_3) ^{13}C NMR (125 MHz, THF-d_8), δ: 171.26, 160.45, 158.67, 155.44, 152.28, 141.20, 135.69, 130.82 (q, J = 32.7 Hz), 128.76, 127.12 (q, J = 3.7 Hz), 126.96, 126.74, 124.93 (d, J = 272.0 Hz), 118.87, 118.55, 95.99, 12.26
IIIi	IR, $\tilde{\nu}$/cm^{-1}: 1736 (C═O) ^{1}H NMR (500 MHz, DMSO-d_6), δ: 11.61 (1H, s, SO_2NH), 8.06 (2H, d, J = 8.3 Hz, H2, H6), 7.71 (2H, d, J = 8.4 Hz, H3, H5), 7.59 (2H, d, J = 8.3 Hz, H3′, H5′), 7.36 (2H, d, J = 8.1 Hz, H2′, H6′), 6.17 (1H, s, isoxazole), 2.30 (3H, s, isoxazole-CH_3), 1.32 (9H, s, CH_3 of *tert*-butyl) ^{13}C NMR (125 MHz, DMSO-d_6), δ: 170.72, 157.48, 156.18, 155.90, 152.23, 151.77, 139.30, 134.79, 128.11, 127.88, 127.18, 126.39, 126.26, 95.65, 34.73, 31.18, 12.25

stitution patterns for the inhibition of *M. kansasii*. In this case, the cyclohexyl (*IIa*) and benzyl (*IIc*) derivatives showed somewhat lower activity than phenylurea *IIb*, and phenethyl *IId* and adamantyl urea *IIe* exhibited slightly lower MICs.

However, replacement of the 4-amino group of SMX with an R-ureido moiety decreased predominantly the antimycobacterial activity of the parent compound, especially against the *M. tuberculosis* strain, which can be explained by weaker interaction with the target structure(s). Additionally, Clog$P$ values are not sufficiently satisfactory predictors of antimicrobial activity. Similar results were obtained for sulphonamide–phtalimide hybrids by Akgün et al. (2012).

Another goal of this study was to describe the influence of the cyclisation of ureas *II* to imidazolidine-2,4,5-triones *III* on their antimycobacterial activity. The findings are not uniform. In general, cyclisation of more efficient ureas produced derivatives with similar (*IIg* or *IIi* for *M. tuberculosis* and *M. avium* and *IId* for atypical mycobacteria) or higher (*IIg* or *IIi* for *M. kansasii*, *IId* for *M. tuberculosis*, and *IIh* for all strains) MIC values. In contrast, the modification of ureas *II* with weaker antimycobacterial activity resulted in products *III* with enhanced in vitro efficacy, particularly for nontuberculous strains (*IIa* and *IIf* for all strains and *IIb* and *IIc* for *M. kansasii*). Analysis of the remaining compounds and strains revealed no significant differences between imidazolidine-2,4,5-triones and ureas.

Compounds *I*–*III* were investigated in vitro against four gram-positive bacterial strains, namely *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Enterococcus* sp., and four gram-negative strains, namely *Es-*

Table 3. Antimycobacterial activity of SMX, INH, ureas *II* and imidazolidine-2,4,5-triones *III*

Compound	R	MIC/μM[a]										Clog*P*
		M. tuberculosis 331/88		*M. avium* 330/88		*M. kansasii* 235/80			*M. kansasii* 6509/96			
		14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d	
IIa	cyclohexyl	1000	1000	1000	1000	500	1000	1000	250	500	1000	2.03
IIIa		500	500	250	250	32	62.5	125	32	62.5	62.5	2.01
IIb	phenyl	1000	1000	500	1000	250	500	500	250	500	500	2.14
IIIb		1000	1000	1000	1000	250	250	500	62.5	125	250	2.13
IIc	benzyl	1000	1000	1000	1000	500	1000	1000	250	500	1000	2.21
IIIc		500	> 500	500	> 500	125	250	500	62.5	125	250	2.20
IId	phenethyl	250	250	250	500	125	125	125	125	250	250	2.49
IIId		500	1000	250	500	125	125	125	125	250	250	2.48
IIe	adamantan-1-yl	500	500	1000	1000	125	250	250	125	250	250	2.31
IIIe		500	> 500	> 500	> 500	125	250	250	250	500	500	2.29
IIf	propyl	> 1000	> 1000	1000	1000	500	1000	1000	250	500	500	1.31
IIIf		**250**	**250**	250	250	32	62.5	62.5	32	62.5	125	1.29
IIg	heptyl	**250**	500	**32**	**62.5**	**4**	**8**	16	**4**	**4**	**8**	2.97
IIIg		**250**	**250**	**62.5**	125	32	62.5	125	62.5	125	250	2.96
IIh	4-CF_3-phenyl	**125**	**125**	**62.5**	125	16	62.5	125	16	32	62.5	3.07
IIIh		500	500	250	250	62.5	125	125	62.5	125	250	3.05
IIi	4-*tert*-butylphenyl	**250**	**250**	500	500	32	62.5	62.5	16	32	32	3.85
IIIi		**250**	500	500	500	62.5	125	250	125	250	500	3.83
SMX	–	32	32	32	62.5	8	16	16	4	4	4	0.86
INH	–	0.5	1	> 250	> 250	> 250	> 250	> 250	4	8	8	–

a) One or two of the best MIC value(s) for each strain are shown in bold.

cherichia coli, *Klebsiella pneumoniae*, ESBL-positive *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Bacitracin (BAC) was used as the comparative drug (Table 4).

None of the derivatives inhibited the growth of *P. aeruginosa* and *K. pneumoniae* at concentrations of 500 μM or lower, whereas *E. coli* was inhibited by two ureas (*IIf* and *IIi*) at this concentration. Gram-positive cocci were more susceptible, i.e., they were inhibited by some derivatives with MICs starting from 125 μM. *S. aureus* exhibited the highest susceptibility with no marked difference between methicillin-susceptible and MRSA strains.

Five ureas (*IIa–IIe*) and three imidazolidine-2,4,5-triones (*IIIb*, *IIIc*, *IIIg*) did not exhibit any antibacterial activity at the investigated concentrations. In contrast, *IIg* showed the highest in vitro potency (MICs against *S. aureus* of 125–250 μM), which is comparable or slightly superior to that of the parent SMX. However, none of *II* and *III* was proved to be superior to BAC or to produce low micromolar MICs. In most cases, imidazolidine-2,4,5-triones *III* showed better antibacterial properties than the parent ureas *II* (*IIa* vs. *IIIa*, *IId* vs. *IIId*, *IIf* vs. *IIIf*, and *IIi* vs. *IIi*), while two ureas were superior to *III* (*IIg* vs. *IIIg* and *IIh* vs. *IIIh*), indicating that neither of the two ureido hydrogens is essential for this action.

Antifungal properties of SMX derivatives were evaluated in vitro against eight species. However, the investigated sulphonamides showed almost no antifungal activity, except for 3-heptyl derivatives *IIg* and *IIIg*. Urea *IIg* inhibited *Absidia corymbifera* at 500 μM, and its cyclic analogue *IIIg* affected the growth of *Candida glabrata* and *C. krusei* with MICs of 250 μM and 500 μM, respectively.

Cytotoxicity of the tested compounds *I–III* was measured using the standard hepatic cell line HepG2 (Table 5). The used CellTiter 96 assay was based on the reduction of tetrazolium dye in living cells to formazan. The cytotoxicity is expressed as IC_{50}, i.e., the concentration reducing the viability of the cells to 50 % of the maximal viability. It was not possible to determine the exact IC_{50} value for three derivatives (*IIf*, *IIIf*, *IIg*) due to their limited solubility in the cell culture medium used in this experiment.

The experiments showed that the studied compounds can be divided into three groups. The first group includes compounds *IIe*, *IIh*, *IIi*, *IIIh*, and *IIIg*, which exhibit comparatively higher toxicity in HepG2 cells (IC_{50} in the range of 103.3–237.9 μM). The second group consists of *IIIa–IIId*, *IIIi*, *IIIe*, *IIb*, and *IId*, which have moderate toxicity (348.3–910.6 μM), and the third group includes molecules with low in vitro cytotoxicity (*I*, *IIa*, *IIc*, *IIf*, *IIg*, *IIIf* with IC_{50} > 1000 μM). Thus, the enhanced cytotoxicity of SMX derivatives is observed in the presence of 4-substituted phenyl (*IIh*, *IIIh*, *IIi*, *IIIi*) and adamantyl (*IIe*, *IIIe*) moieties, whereas the cyclohexyl-, benzyl-, phenethyl-, and alkyl-containing ureas showed comparatively milder IC_{50} values.

In general, although the newly synthesised derivatives did not exhibit significant cytotoxicity, all modifications of the parent SMX (IC_{50} = 2622 μM) increased

Table 4. Antibacterial activity of SMX, ureas *II* and imidazolidine-2,4,5-triones *III*

Compound	R	MIC/μM[a]									
		S. aureus		MRSA		*S. epidermidis*		*Enterococcus* sp.		*E. coli*	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
IIa	cyclohexyl	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
IIIa		500	500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
IIb	phenyl	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
IIIb		> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
IIc	benzyl	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
IIIc		> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
IId	phenethyl	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
IIId		500	> 500	500	> 500	500	> 500	500	> 500	> 500	> 500
IIe	adamantan-1-yl	> 125	> 125	> 125	> 125	> 125	> 125	> 125	> 125	> 125	> 125
IIIe		500	500	500	500	500	> 500	**500**	> 500	> 500	> 500
IIf	propyl	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	**500**	> 500
IIIf		500	> 500	500	> 500	500	> 500	**500**	> 500	> 500	> 500
IIg	heptyl	**125**	**250**	**250**	**250**	**125**	**250**	**500**	> 500	> 500	> 500
IIIg		> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
IIh	4-CF_3-phenyl	**125**	500	**125**	250	> 500	> 500	> 500	> 500	> 500	> 500
IIIh		500	500	500	500	500	> 500	500	> 500	> 500	> 500
IIi	4-*tert*-butylphenyl	**250**	500	500	> 500	> 500	> 500	> 500	> 500	**500**	> 500
IIIi		**250**	500	**250**	500	**250**	500	**250**	> 500	> 500	> 500
SMX	–	250	500	62.5	250	250	500	> 500	> 500	> 500	> 500
BAC	–	15.62	31.25	15.62	31.25	15.62	31.25	31.25	31.25	> 500	> 500

a) One or two of the best MIC value(s) for each strain are shown in bold.

Table 5. Cytotoxicity and selectivity indices of SMX, ureas *II* and imidazolidine-2,4,5-triones *III*

Compound	R	IC_{50} HepG2/μM	Selectivity index[a]			
			M. tuberculosis	*M. avium*	*M. kansasii*	*Staphylococci*
IIa	cyclohexyl	1129.0	1.13	1.13	1.13–4.52	< 2.26
IIIa		375.2	0.75	1.50	3.00–11.73	≤ 0.75
IIb	phenyl	499.5	0.50	0.50–1.00	1.00–2.00	< 1.00
IIIb		667.0	0.67	0.67	1.33–10.67	< 1.33
IIc	benzyl	1263.0	1.26	1.26	1.26–5.05	< 2.53
IIIc		486.8	≤ 0.97	≤ 0.97	0.97–7.79	< 0.97
IId	phenethyl	910.6	3.64	1.82–3.64	3.64–7.28	< 1.82
IIId		867.2	0.87–1.73	1.73–3.47	3.47–6.94	≤ 1.73
IIe	adamantan-1-yl	114.5	0.23	0.11	0.46–0.92	< 0.92
IIIe		352.7	≤ 0.71	> 0.71	0.71–2.82	≤ 0.71
IIf	propyl	> 1500	nd	> 1.50	> 1.50–4.00	nd
IIIf		> 1000	> **4.00**	> 4.00	> 8.00–31.25	nd
IIg	heptyl	> 1000	> **2.00–4.00**	> **16.00–31.25**	> **62.50–250.00**	> **4.00–8.00**
IIIg		237.9	0.95	1.90–3.81	1.90–7.43	< 0.48
IIh	4-CF_3-phenyl	176.4	1.41	1.41–2.82	1.41–11.03	≤ 1.41
IIIh		181.3	0.36	0.73	0.73–2.90	≤ 0.36
IIi	4-*tert*-butylphenyl	103.3	0.41	0.21	1.65–3.23	≤ 0.41
IIIi		348.3	0.70–1.39	0.70	0.70–5.57	0.70–1.39
SMX	–	2622.0	81.94	41.95–81.94	163.88–655.50	5.24–41.95

a) The best selectivity index value (SI = IC_{50}/MIC) for each strain is shown in bold; nd = not determined (the IC_{50} and MIC values are not exactly known).

the cytotoxicity by up to one order of magnitude for the most toxic compounds.

The selectivity indices (SIs) of all sulphonamides for *M. tuberculosis*, atypical mycobacteria and *S. aureus* were calculated. SI is defined as the ratio of IC_{50} to MIC, and values higher than 10 indicate rather acceptable toxicity (based on the analogy of the therapeutic index). In some cases it was not possible to determine exact SI values due to the limited solubility of the compounds in the medium used for the cytotoxic-

ity assays (*IIf*, *IIIf*, *IIg*) or the anti(myco)bacterial assays. The analysis of *M. tuberculosis* revealed that the propyl and heptyl derivatives *IIIf* and *IIg* exhibit the best profiles, but their values are low. Similar results were found for *Staphylococci*. The unsatisfactory SIs are a consequence of low antibacterial activity; heptylurea *IIg* was found to be favourable with SI values higher than 4 or 8, but it was not possible to determine the exact value due to its limited solubility in the testing medium. *IIg* also exhibited the most convenient toxicity properties for atypical mycobacterial strains, i.e., its SI values for *M. avium* and for both strains of *M. kansasii* ranged from 16 to 31.25 and from 62.5 to 250, respectively.

Three of the ureas *II* displayed higher cytotoxicity than the corresponding imidazolidine-2,4,5-triones *III* (*IIb* vs. *IIIb*, *IIe* vs. *IIIe*, and *IIi* vs. *IIIi*), whereas the opposite relationship was found for four compound pairs: *IIa* vs. *IIIa*, *IIc* vs. *IIIc*, *IId* vs. *IIId*, and *IIg* vs. *IIIg* (urea *IIg* showed an IC_{50} value of more than 4.2-fold higher).

Conclusions

In this study, eighteen ureas and their corresponding imidazolidine-2,4,5-trione derivatives based on the parent molecule sulphamethoxazole were designed and synthesised. All of them underwent a set of in vitro biological tests to determine their potential as antimicrobial agents against four mycobacterial and eight bacterial and fungal strains with respect to their cytotoxicity. The highest activity was found against *M. kansasii*, whereas the fungi and gram-negative bacteria were the least-susceptible groups. The novel derivatives inhibited mostly the growth of both tuberculous and nontuberculous mycobacteria; however, most of the derivatives did not exceed the activity of the parent sulphonamide. There is no unambiguous influence of urea cyclisation on the biological activity: in some cases, imidazolidine-2,4,5-triones exhibited improved activity and decreased the cellular toxicity, but inverse results were obtained in other cases. Analysis of the selectivity indices revealed that one of the derivatives (heptylurea) provides values exceeding 10 for atypical mycobacteria.

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