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Tetrahydro-2 *H*-1,3,5-thiadiazine-5-(4-pyridylcarboxamide)-2-thione derivatives as prodrugs for isoniazid; synthesis, investigations and *in vitro* antituberculous activity

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3-Substituted-5-(4-pyridylcarboxamido)tetrahydro-2 *H*-1,3,5-thiadiazine-2-thione derivatives **5a–e** were synthesized as prodrugs for isoniazid (INH) to overcome the resistance developed with its therapeutic use. These prodrugs revealed higher lipophilicity compared with INH. Their degradation kinetics were studied *in vitro* using aqueous buffer solutions of pH values 1.2 and 7.4 was well as biological media of human plasma and rat liver homogenate at 37 °C. They were more stable toward enzymatic degradation in biological than in chemical media. Release of INH from these derivatives was detected as a result of both chemical and enzymatic hydrolysis by HPLC. The antimycobacterial activity of the synthesized compounds and INH was tested *in vitro* against human type of *Mycobacterium tuberculosis.* They exhibited a greater antitubercular activity than the parent drug. This result is considered as an indicator for an improved permeation of the synthesized prodrugs through mycobacterial cell membranes relative to INH.

1. Introduction

Resistance of *Mycobacterium tuberculosis* strains to antimycobacterial agents is an increasing problem worldwide [1–8]. Drug-resistant strains of *Mycobacterium tuberculosis* can be transmitted by an infected individual, or resistance can be acquired during therapy for drug-susceptible disease [9]. On the other hand, in spite of toxicity on repeated dosing isoniazid (INH) is still considered to be a first line drug for the chemotherapy of tuberculosis [10–12]. Patients with disease caused by an isoniazid-sensitive strain of the tubercle bacillus should receive the drug if they can tolerate it. Recently it was suggested that the mechanism of resistance to INH is related to a failure of the drug to penetrate or to be taken up by the microorganisms [13]. Fortunate, pharmacokinetic properties and cellular permeability of a drug can be modulated by derivatization to bioreversable forms of this drug, namely prodrugs [14, 15]. Moreover, resistance development to a drug can be also obviated through prodrug approach [16, 17].

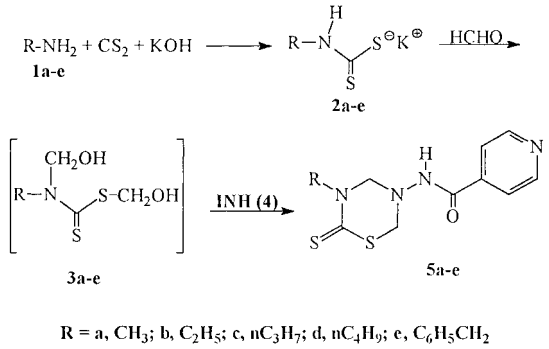
Tetrahydro-2 *H*-1,3,5-thiadiazine-2-thione derivatives were found to insinuate the criteria of the prodrug approach that impart the desirable physicochemical properties to attached drugs, and liberate the parent drugs through chemical or enzymatic degradation [18–21]. Accordingly, the current work describes the incorporation of isoniazid in a tetrahydro-2 *H*-1,3,5-thiadiazine-2-thione moiety (THTT). *In vitro* degradation kinetics of these synthesized prodrugs in aqueous buffer solutions and biological media of human plasma and rat liver homogenates were investigated. Furthermore, the *in vitro* antituberculous activity of the target compounds in comparison with the parent drug was also studied.

2. Investigations, results and discussion

2.1. Chemistry

The target compounds **5a–e** were synthesized in a two step reaction. In the first step of the reaction dithiocarbamic acid salts **2a–e** were formed by reacting the primary amines **1a–e** with carbon disulfide and potassium hydroxide. However, the second step of the reaction involved addition of formaline to the previously formed **2a–e** to afford compounds **3a–e** (*in situ*). This was followed by progressive addition of INH solution in ethanolic phosphate buffer (pH 7.8) to provide the designed derivatives **5a–e** (Scheme and Table 1). Structures of these synthesized compounds were verified on the bases of spectral and elemental methods of analyses.

Scheme



R = a, CH_3; b, C_2H_5; c, nC_3H_7; d, nC_4H_9; e, $C_6H_5CH_2$

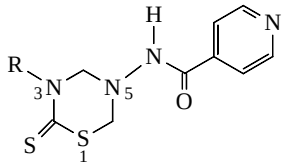
The most differentiating stretching bands in IR spectra of the target compounds **5a–e** are: stretching absorption for the amidic NH at the range of 3340–3445 cm^{-1} (due to the electron withdrawing effect of the adjacent ring nitrogen), aliphatic C–H stretching around 2855–3016 cm^{-1}, aromatic C–H stretching around 3040–3125 cm^{-1}, amidic C=O stretching at 1660–1666 cm^{-1}, pyridine C=N stretching at 1485–1496 cm^{-1}, and C=S stretching around 1163–1249 cm^{-1}.

With the exception of the N^3-substituents of the THTT moiety the ^1H NMR resonance of the remaining sites of the protons of the synthesized derivatives is almost superimposable (Table 2).

2.2. Lipophilicity

Lipophilicity of the synthesized derivatives **5a–e** and the parent compound, INH, is expressed in terms of their log P values. These values were computed with a routine method called calculated log P (Clog P) contained in a

Table 1: Some physicochemical data of the synthesized 3-alkyl or benzyl-5-(4-pyridylcarboxamido)tetrahydro-2*H*-1,3,5-thiadiazine-2-thione derivatives 5a–e



Compd.	R	Yield (%)	Formula[a]	M.P. (°C)	Clog P[b]
5a	CH_3	43	$C_{10}H_{12}N4OS_2$	209	1.244
5b	C_2H_5	47	$C_{11}H_{14}N4OS_2$	192	1.773
5c	C_3H_7	60	$C_{12}H_{16}N4OS_2$	151	2.302
5d	C_4H_9	68	$C_{13}H_{18}N4OS_2$	122	2.831
5e	$C_6H_5CH_2$	75	$C_{16}H_{16}N4OS_2$	205	2.884

[a] Elemental analyses for C, H, N, S are within ±0.5%
[b] Clog P value for INH is −0.708 (reported −0.700) [22]

PC-software package described in the experimental section. Computation of the log P is based on the fragment method developed by Leo [22].

As shown in Table 1, the lipophilicity of the synthesized prodrugs **5a–e** increased remarkably compared with the parent drug, INH. This may be rendering them more capable of penetrating various biomembranes [23], consequently improving their permeation properties through mycobacterial cell membranes [24]. In other words, the increase in lipophilicity of the synthesized derivatives probably enhances their bioavilability to the requested site of action. This in turn participates in overcoming the resistance developed from the failure of the drug to penetrate the microorganisms.

2.3. Kinetic measurements

The degradation kinetics of the synthesized prodrugs **5a–e** were studied in aqueous buffer solutions of pH 1.2 considered as simulated gastric fluid (SGF), and 7.4, physiological pH, at 37 °C. At constant pH and temperature disappearance of the tested compounds displayed strict first order kinetics reactions over several half-lives (Table 3) and all reactions proceeded to completion. Liberation of INH from these prodrugs was almost quantitative as was confirmed by HPLC.

Table 3: Chemical and enzymatic degradation kinetic data of the synthesized THTT derivatives at 37 °C

Compd.	$K \times 10^3$ min^{-1} ($t_{1/2}$, h)			
	pH		Enzyme system	
	1.2	7.4	plasma	liver homogenate
5a	8.3586 (1.4)	15.9970 (0.72)	1.2822 (9.00)	0.9954 (11.00)
5b	4.2837 (2.7)	15.8991 (0.73)	3.1921 (3.62)	2.7979 (4.13)
5c	1.6007 (7.2)	11.1013 (1.04)	7.9878 (1.44)	4.3065 (2.68)
5d	1.5662 (7.4)	10.5147 (1.10)	6.3838 (1.80)	7.1159 (1.62)
5e	1.4342 (8.1)	4.9720 (2.30)	7.2365 (1.59)	7.2091 (1.60)

The rate data obtained for the various derivatives (Table 3), revealed that as a general pattern these prodrugs are quite stable in acidic media (SGF) compared with that investigated at pH 7.4. This observation is consistent with our previously reported THTT derivatives [20, 21], however, the INH derivatives **5a–e** are much more labile for chemical degradation. This liability may be attributed to the electronic effect of the pyridinecarboxamide moiety attached to N-5 of the THTT moiety. Furthermore, the degradation rates of the synthesized derivatives was affected by the variation of N-3 substituents on the THTT moiety. The 3-benzyl derivative **5e** is more stable in the investigated buffer solutions than the 3-alkyl derivatives **5a–d**. Stability of those derivatives was found to increase with prolongation of their alkyl chain.

A strong linear correlation is observed between the lipophilicity, Clog P, and the degradation rates of the investigated compounds at pH 1.2 ($r = -0.9285$, $n = 5$), while a fairly good linear relation existed with the degradation rate at pH 7.4 and Clog P ($r = -0.8834$). Thus, the enhancement of the lipophilicity of the tested compounds results in an enlargement of stability in the investigated buffer solutions. This may be attributed to the reduction of wetness of the lipophilic compounds with the aqueous buffer solutions [25].

Table 2: ^{1}N NMR spectral data of derivatives 5a–e

Compd.	Chemical shifts [a], δ ppm, (J = Hz)				
	4-CH_2, 6-CH_2	3-CH, 5-CH	2CH, 6-CH	N^5-NH-CO	N^3-R
5a	4.75 (4 H, bs)	7.8 (2 H, d, J = 7.75)	8.82 (2 H, d, J = 7.75)	10.75 (1 H, S)	3.5 (3 H, S, CH_3)
5b	4.75 (4 H, bs)	7.8 (2 H, d, J = 7.73)	8.83 (2 H, d, J = 7.72)	10.75 (1 H, S)	1.15 (3 H, t, J = 7.4, $CH_2\underline{CH_3}$), 4.08 (2 H, q, J = 7.5, $\underline{CH_2}CH_3$)
5c	4.75 (4 H, bs)	7.76 (2 H, d, J = 7.75)	8.76 (2 H, d, J = 7.75)	10.70 (1 H, S)	0.89 (3 H, t, J = 7.3, $CH_2CH_2\underline{CH_3}$), 1.71 (2 H, m, $CH_2\underline{CH_2}CH_3$), 3.95 (2 H, t, J = 7.3, $\underline{CH_2}CH_2CH_3$)
5d	4.72 (4 H, bs)	7.76 (2 H, d, J = 7.75)	8.76 (2 H, d, J = 7.75)	10.75 (1 H, S)	0.85 (3 H, t, J = 7.6, $CH_2(CH_2)_2\underline{CH_3}$), 1.1–1.5 (4 H, m, $CH_2\underline{(CH_2)_2}CH_3$), 4.1 (2 H, t, J = 7.3, $\underline{CH_2}(CH_2)_2CH_3$)
5e	4.78 (4 H, bs)	7.9 (2 H, d, J = 7.75)	8.92 (2 H, d, J = 7.75)	10.90 (1 H, S)	5.5 (2 H, s, $\underline{CH_2}C_6H_5$), 7.5 (5 H, s, $\underline{CH_2C_6H_5}$)

[a] In DMSO-d_6

The degradation rates of compounds **5a–e** were also investigated in 80% human plasma and 10% rat liver homogenate at 37 °C. These two different enzyme systems were selected in order to get information about the susceptibility of the synthesized derivatives toward enzymatic metabolism. Strict first oder kinetics were also observed with the enzyme systems used under the investigated conditions. It is obvious from Table 3 that the THTT derivatives **5a–e** are quite resistant to enzymatic compared with chemical degradation. Furthermore, most of the tested compounds are more susceptible to human plasma enzymes than to rat liver homogenate. It is worthy to note that contrary to chemical degradation the benzyl derivative **5e** is the most susceptible compound for enzymatic degradation in both of the investigated enzyme systems. The methyl derivative is the least susceptible one. Moreover, as was observed in chemical degradation reactions, a significant linear correlation is observed between the lipophilicity, Clog P, and the degradation rates of the investigated compounds in rat liver homogenate ($r = 0.9930$, $n = 5$). While a reasonably good linear relation existed with the degradation rate in human plasma and Clog P ($r = 0.8749$). Nevertheless, in the case of enzymatic degradation the increase in lipophilicity of the investigated compounds results in an augmentation of their susceptibility to enzymatic degradation. This may be owed to the enhancement of the averaged association with the enzyme(s) responsible for their degradation which depends mostly on lipophilicity [26].

Release of INH by enzymatic degradation was also detected though in some what lower concentrations compared with chemical degradation. Chemical or enzymatic degradation was suggested to occur via ring cleavage at N-5 of the THTT moiety with the release of the compound of interest [19–21]. The detectable release of INH under both chemical and enzymatic degradation conditions supported strongly such a suggestion. Furthermore, liberation of the target compound from such derivatives fulfills the requirements of the prodrug approach.

2.4. Antituberculous activity

The synthesized compounds **5a–e** were tested for their antimycobacterial activity *in vitro* against human type of *Mycobacterium tuberculosis*, according to the protocol described in the experimental section. Results of the *in vitro* antitubercular activity are expressed as minimum inhibitory concentrations (MIC) and presented in Table 4. Rapid glance to the obtained results revealed that compounds **5b–e** exhibited a four fold higher activity against the tested *Mycobacterium tuberculosis* than IHN, while the methyl derivative **4a** was as active as INH. However, the prodrugs **5a–e** show greater antitubercular activity compared with INH when molar concentrations of the tested doses were considered.

Quite a good linear relation is also observed between the antitubercular activity of the investigated compounds (MIC) and their lipophilicity (Clog P), ($r = -0.836$, $n = 6$). This relation is a good indicator for the improvement of the premeation properties of the synthesized prodrugs toward mycobacterial cell membranes.

3. Experimental

3.1. Materials and aquipment

Isonicotinic acid hydrazine (INH) was a gift from Chemical Industries Development Co, CID, Cairo, Egypt. All other chemicals were of commercial grade except the HPLC solvents and the buffer reagents were of analytical grade.

Melting points were determined on an electrothermal melting point apparatus (Fa. Sturat Scientific, England), and were uncorrected. Precoated silica gel plates (Kieselgel 0.25 mm, 60G F254, Merk) were used for TLC. Chloroform/methanol (80 : 20) was used as developing solvent system; the spots were detected by UV light. Products were percolated by CC. The column was packed with silica gel 60 (particle size 0.063–0.2 mm) and eluted with the same TLC developing system.

IR spectra (KBr disc) were recorded on IR-470 Shimadzu spectrometer, Japan.

^{1}H NMR Spectra were scanned on a Varian EM-360 L NMR spectrometer (60 MHZ) USA, using DMSO-d_6 as a solvent. Chemical shifts are expressed in δ (ppm) relative to TMS as an internal standard. Elemental analyses were performed at the Department of Chemistry, Faculty of Science, Assiut University, Assiut, Egypt.

Refrigerated centrifuge; Minifuge 2-Haraeus-Type 41231-Germany, was used to obtain rat liver homogenate. pH Values were recorded on a Chekit micro pH meter (England) at room temperature.

A HPLC system consisting of a pump (Knauer HPLC pump 64, Germany), a variable-wavelength detector (Knauer), a reversed-phase HPLC column (stainless steel, 25 × 0.5 cm i.d., C-18 Eurospher 80) connected with a cartridge guard column, a Shimadzu C-R 6A chromatopac recording integrator, and a 20-µl injection loop was used. Mobile phase systems of Acetonitrile, water and 1% triethylamine were used. The ratio of acetonitrile/water was adjusted in order to give a retention time of 3.7 ∼ 6 min for the synthesized derivatives and 2.5 ∼ 4 min for INH. The column effluent was monitored at 265 nm and the flow rate was 1 ml/min. Quantitation of the eluted compounds was done from peak height measurements in relation to those of standards chromatographed under the same conditions.

Tests for antituberculous activity were performed at the Department of Microbiology, Faculty of Medicine, Assiut University, Assiut, Egypt.

3.2. Synthesis of 3-substituted-5-(4-pyridylcarboxamido)tetrahydro-2 H-1,3,5-thiadiazine-2-thione derivatives **5a–e**

Carbon disulfide (60 mmol) was added portionwise to a stirred mixture of the appropriate alkyl or benzylamine **1a–e** (10 mmol) and potassium hydroxide (20%, 10 mmol) in water (5 ml), stirring was continued at ambient temperature for 6 h. Formaldehyde solution (35%, 22 mmol) was added to the mixture and the stirring was continued for further 2 h. To the resulting clear solution, a solution of INH (10 mmol) in a mixture of phosphate buffer (pH 7.8, 5 ml) and ethanol (10 ml) was added portionwise during 15 min. After stirring for 6 h at ambient temperature, dilute hydrochloric acid (5%, 5 ml) was added and stirring was continued for further 1 h. The formed precipitate was collected by filtration, washed with methanol and dried. The crude product was crystallized from chloroform/methanol (1 : 1) to afford compounds **5a–e**. Yields, m.p.'s and physical data are given in Table 1. ^{1}H NMR data are given in Table 2.

3.3. Kinetic measurements

Degradation rates of the synthesized derivatives **5a–e** in aqueous isotonic phosphate buffer, pH 7.4, and simulated gastric fluid without enzyme (containing 0.02% w/v Tween® 80), pH 1.2, were determined at 37 °C. The ionic strength of the prepared buffer solutions was adjusted with KCl to $\mu = 0.5$.

The reactions were initiated by adding 250 µl of the stock methanolic solution of each derivative (1×10^{-3} molar solution) to 2.5 ml of preheated buffer solutions in screw-capped test tubes. At appropriate intervals samples were taken and chromatographed. The residual concentrations displayed a pseudo first order rate of hydrolysis.

Degradation studies in 80% human plasma containing isotonic phosphate buffer of pH 7.4 at 37 °C were done by adding appropriate amounts of the stock methanolic solution of derivatives to the plasma solution. The initial concentration was 1×10^{-5} M. At appropriate times samples of 50 µl were withdrawn and mixed with 50 µl of acetonitrile for deproteinization and centrifuged at 10^4 rpm for 5 min. The clear supernatant (20 µl) was analyzed by HPLC as described above.

Male wistar rat livers were homogenized with ice-cooled saline to give a concentration of 40% w/v, and were then centrifuged at 15000 rpm for 15 min. The supernatant was collected and stored at −40 °C until use. The homogenate was thawed 10 min before the experiments and diluted with saline to give a preparation of 10% w/v concentration. The hydrolysis studies in rat liver homogenate were performed as described for the 80% human plasma solution above. Results of the kinetic measurements are given in Table 3.

3.4. Calculation of log P values

The log P values of the synthesized derivatives as well as the parent compound, INH, were computed with a routine method called calculated log P (Clog P) contained in a PC-software package (MacLog P 2.0, BioByte Corp., Ca, USA). A representation of the molecular structure where hydrogens are omitted, or 'suppressed' (SMILES notation), is entered into the program, which computes the log P based on the fragment method developed by Leo [22]

Table 4: ***In vitro*** **antituberculous activity of the synthesized prodrugs, 5a–e, and the parent drug INH against** ***Mycobacterium tuberculosis***

Compd.	MIC (μg/ml)
5a	100
5b	25
5c	25
5d	25
5e	25
INH (**4**)	100

3.5. In vitro antituberculous activity

The synthesized prodrugs, **5a**–**e**, and the parent drug, INH, were solubilized in DMSO at a concentration of 10^3 μg/ml. Appropriate amounts of each compound were diluted with Lowenstein-Jensen media to give concentrations of 25, 50, 75 and 100 μg/ml of the growth media. The media containing different compounds were sterilized at 70 °C for 1 h in a hot air oven for three successive days. The sterilized media were then inoculated by human type of *Mycobacterium tuberculosis*, isolated from a tubercular patient and identified in our lab. The minimum inhibitory concentrations (MIC) were determined after incubation at 37 °C for six weeks. MIC was the lowest concentration of an antitubercular active compound (see the above concentrations), at which inhibition of *Mycobacterium tuberculosis* growth occurred. A control experiment was done in the same manner and the results are given in Table 4.

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