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2,4-Diaminotriazines as anti-infective agents

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Triazines are scaffolds of interest to medicinal chemists as they have a wide variety of pharmacological activities. Molecules with the triazine moiety have been explored extensively for their anti-infective potential. In this paper, we report a series of 2,4-diaminotriazine derivatives which have been designed and synthesized to explore their DHFR inhibiting potential in various micro-organisms. These molecules have not only been evaluated for their anti-tuberculosis activity, but also for their *E. coli, S. aureus* and their anti-leishmanial potential. The series has moderate activity vs *E. coli* and *S. aureus*, low activity against tuberculosis and good potential for leishmaniasis, from the preliminary results.

Keywords: 2,4-Diaminotriazine, tuberculosis, leishmaniasis, cytotoxicity.

Introduction

The triazine scaffold is present in drugs like lamotrigine, melarsomine, altretamine, almitrine, etc. for diverse therapeutic indications. In addition to these, molecules containing the triazine moiety have shown diverse pharmacological actions for potential use as anticancer[1], antibacterial[2–4], anti-fungal[2–4], anti-tuberculosis[5], anti-viral[6], anti-malarial[7], anti-leishmanial[8], anti-inflammatory[9,10], anti-depressant[10], anti-arthritic[10], analgesic[10], antihistaminic[10] etc.[9–11] agents. Our research group has been actively involved in exploring the triazine class of compounds as dihydrofolate reductase (DHFR) inhibitors of *Mycobacterium tuberculosis* (*Mtb*)[12–15], as well as for their potential against various opportunistic micro-organisms[16,17] and in Alzheimer's Disease (unpublished work).

In continuation with our previous work, in this paper we report a series of novel 2,4-diaminotriazines derivatives which were synthesized and evaluated for their potency against *Mycobacterium tuberculosis* H37Rv strain, *E. coli, S. aureus* as well as promastigotes of *Leishmania donovani.* The cytotoxicity of some potent molecules was evaluated against the human embryonic kidney (HEK 293) cell line.

Experimental

All chemicals, reagents and materials used for synthesis and biological evaluation were purchased from SD Fine Chem Ltd. (India), and Alfa Aesar (India), Spectrochem Ltd. (India), Hi-Media Ltd. (India), Sigma Aldrich (India), Borosil (India), Tarsons (India) and Eppendorf (India). Melting points were determined on a Hally Instruments melting point apparatus. Infrared (IR) spectra were recorded on a Perkin-Elmer Spectrum-two infrared spectrometer with KBr disks. High-performance liquid chromatography (HPLC) analysis was carried out using a JASCO-LC 4000 instrument (JASCO). The mobile phase used for HPLC was acetonitrile/water = 80:20 and the column used was 30 cm, 10 μ C18. ^{1}H NMR spectra were recorded on an Agilent 400 MR instrument.

Synthesis:

The synthesis of the diaminotriazine derivatives was carried out according to the reactions depicted in Fig. 1. In step

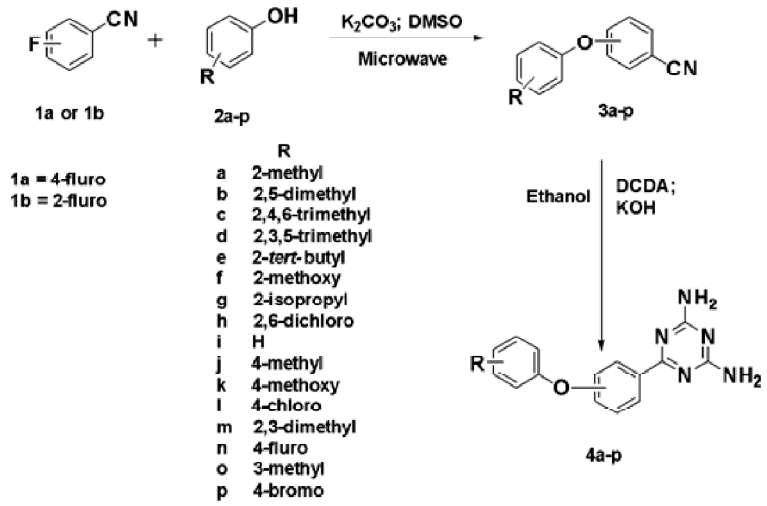

Fig. 1. Reaction scheme for synthesis of 2,4-diaminotriazine derivatives (**4a-4p**).

1, various phenols (**2a-2p)** (10 mmol) were condensed with 2 or 4-fluorobenzonitrile (**1a** or **1b)** (1 g, 8.25 mmol) in 5 mL of dimethyl sulfoxide (DMSO) with potassium carbonate (K_2CO_3) (1.7 g, 12.4 mmol) as a base, using microwave irradiation (5 cycles at power 120 W, temperature 180°C for 20 min with an intermittent cooling cycle). The completion of reactions was monitored by TLC. After completion, the reaction mass was poured into 20 mL of ice water and extracted thrice with 10 mL of ethyl acetate (EtOAc). The combined organic layer was treated with brine (15 mL), dried over sodium sulfate, and concentrated *in vacuo* to obtain derivatives **3a-3p**. In step 2, dicyandiamide (DCDA) (0.50 g, 5.95 mmol) and potassium hydroxide (KOH) (0.42 g, 7.5 mmol) were added to the solution of **3a-3p** (5 mmol) in EtOH (5 mL). The resultant reaction mixture was heated under reflux in an oil bath for 18–24 h. After completion of the reaction, as monitored by TLC, the precipitated solid was filtered, washed with EtOH (5 mL) followed by hot water (25 mL), and dried to afford the corresponding diaminotriazine derivatives **4a-4p** in 40–60% yield. Purification, when needed, was carried out by treatment with suitable solvents. The synthesized compounds were suitably characterized.

Biological evaluation:

These compounds were tested for their anti-tuberculosis activity against *Mycobacterium tuberculosis* H37Rv using Microtiter Alamar Blue Assay (MABA) and the two-fold dilution technique. *Mt* H37Rv cultures in Middlebrook 7H9 broth with 10% OADC (0.1% casitone, 0.5% glycerol, supplemented oleic acid, albumin, dextrose, and catalase) having OD_{590} 1.0 were diluted 1:20, of which 100 μL culture was used as inoculum for screening. Stock solutions of compounds were prepared at 50 μg/mL and serial dilution was performed in 96-well microtiter plates. All the sampling was performed in duplicate. Assay results were compared against the TB drugs, rifampicin (Rif), ethambutol and isoniazid (Inh). Humidity was maintained by adding water to peripheral wells. Plates were subsequently incubated at 37°C for a week. After the incubation period, 30 μL of Alamar blue solution was added to each well, and the plate was re-incubated for 12 h. Growth was indicated by a colour change from blue to pink, the lowest concentration of compound that did not change colour recorded as its MIC value.

The synthesized derivatives were tested for their antibacterial activity against the Gram-positive *S. aureus* and the Gram-negative *E. coli* using two-fold dilution technique of the resazurin microtiter assay (REMA). Assay results were compared against the antibiotic drugs kanamycin and streptomycin. Around 1×10^4 cells per well of bacterial culture were incubated along with the different concentrations of the test compounds at 37°C for 24 h in an incubator. Then 30 μL of

0.2% of resazurin solution was added to each well, and the plate was re-incubated for 12 h. Growth was indicated by a colour change from blue to pink.

The compounds were tested for their anti-leishmanial potential against promastigotes of *Leishmania donovani*. In a 96-well microtiter plate, 3×10^6 promastigotes of *Leishmania donovani* per well were cultured in RPMI 1640 (Gibco) and allowed to multiply for 24 h in the medium alone (control group), in solvent (another control group) or in the presence of 100 μg/mL concentrations of the test compounds, at 25°C. After incubation, 5 μL of 20 mg/L solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added to each well, and further incubated for 3 h in the incubator. Then the plates were spun at 1500 g for 5 mins, and the supernatant was replaced with 100 μL of dimethyl sulfoxide. The plates were finally read at 570 nm. The percent growth of the microbes was monitored at 100 μg/mL concentration of each drug. From this, percent growth inhibition was calculated.

The seven molecules (**4b**, **4c**, **4h**, **4k**, **4l**, **4n**, **4p**) which had shown good anti-leishmanial activity were evaluated for their toxicity on human embryonic kidney (HEK 293) cell lines. Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% of foetal bovine serum was used as the growth medium. Around 1×10^4 cells per well were seeded in a 96-well plate and incubated in a CO_2 incubator with 5% CO_2, at 30°C for 24 h, to allow the cells to adhere to the surface of the plate. Serial dilutions of the compounds (0–100 μg/mL) were prepared in DMEM. After 24 h of incubation, supernatant media was replaced with fresh DMEM medium containing serially diluted compounds and further incubated in the CO_2 incubator at 30°C for 24 h. The cytotoxic effect of compounds was estimated by measuring the metabolic activity by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. A 5 μL of 20 mg/L solution of MTT was added to each well, and further incubated for 3 h. Then the plates were spun at 1500 g for 5 mins, and the supernatant was replaced with 100 μL of dimethyl sulfoxide. The plates were finally read at 570 nm.

Results and discussion

In our previous study[12] on diaminotriazines which were designed as DHFR inhibitors for *Mtb*, the position of the linker and the substitutions at the terminal phenyl ring were shown to play a significant role. In order to further explore the significance of the substitutions at position-2 of the terminal phenyl ring against *Mtb,* derivatives **4a-4h** were synthesized. On the other hand, derivatives **4i-4p** were designed and synthesized to explore the role of molecular shape in *Mtb* activity.

The biological evaluation results (Table 1) showed that the smaller electron withdrawing as well as electron donating groups like methyl, methoxy, chloro did not lead to any significant activity against *Mtb.* Only the bulkier tri-methyl substituted compounds (**4c** and **4d**), 2-tertiary butyl and 4-bromo derivatives (**4e** and **4p**) were moderately active against *Mtb.*

The compounds were evaluated against one Gram-positive and one Gram-negative bacterial strains to explore their broad-spectrum potential. The preliminary results showed that the derivatives **4d**, **4m** and **4n** had moderate activity (MIC values 62.5 μg/mL) while the derivatives **4c**, **4e**, **4p** had very good activity, with MIC of **4e** being <7.81 μg/mL, against *E. coli.* Molecules **4b**, **4c**, **4d** and **4e** were active against the Gram-positive bacteria *S. aureus*, with compound **4e** being extremely potent with MIC value less than 7.81 μg/mL.

Since these molecules showed activity in multiple bacteria, these molecules were further explored for leishmaniasis. It was seen that the molecules **4b**, **4h**, **4k**, **4l**, **4n** and **4p** showed 55–60% growth inhibition of *Leishmania donovani* promastigotes at a concentration of 100 μg/mL (Table 1). Molecules in which the triazine ring was linked to the phenoxy ring at the *ortho* position were more active than the core with the *para* substitution. Also, methoxy and halogen substitutions at the terminal phenyl ring were better than methyl substitution.

The cytotoxicity data of the seven molecules (**4b**, **4c**, **4h**, **4k**, **4l**, **4n**, **4p**) on the HEK 293 cell lines has been represented in Fig. 2. All the compounds were non-toxic at lower concentration i.e. 12.5 μg/mL. Compounds **4h**, **4k**, **4n** were

Table 1. Biological evaluation data for the synthesized compounds

Compound code	Starting material for synthesis	MIC against *Mtb* H37Rv (μg/mL)[a]	MIC agianst *E. coli* (μg/mL)[b]	MIC against *S. aureus* (μg/mL)[b]	% GI[c] of promastigotes of *Leishmania donovani*
4a	**1a**	>25	>125	>125	20
4b	**1a**	>25	>125	31.25	55
4c	**1a**	12.5	15.625	31.25	20
4d	**1a**	25	62.5	31.25	35
4e	**1a**	12.5	<7.81	<7.81	18
4f	**1a**	>25	>125	>125	19
4g	**1a**	>25	>125	>125	33
4h	**1a**	>25	125	>125	55
4i	**1b**	>25	>125	>125	15
4j	**1b**	>25	>125	>125	13
4k	**1b**	>25	>125	>125	55
4l	**1b**	>25	>125	>125	60
4m	**1b**	>25	62.5	>125	5
4n	**1b**	>25	62.5	>125	55
4o	**1b**	>25	125	>125	10
4p	**1b**	25	15.625	>125	56
Isoniazid	–	0.1	–	–	–
Rifampicin	–	0.2	–	–	–
Ethambutol	–	1.56	–	–	–
Streptomycin	–	–	<7.81	<7.81	–
Kanamycin	–	–	15.625	<7.81	–

[a]The highest concentration tested was 25 μg/mL. [b]The highest concentration tested was 125 μg/mL while the lowest concentration tested was 7.8 μg/mL. [c]% GI is the % Growth Inhibition, which was assessed at the concentration of 100 μg/mL of the molecules.

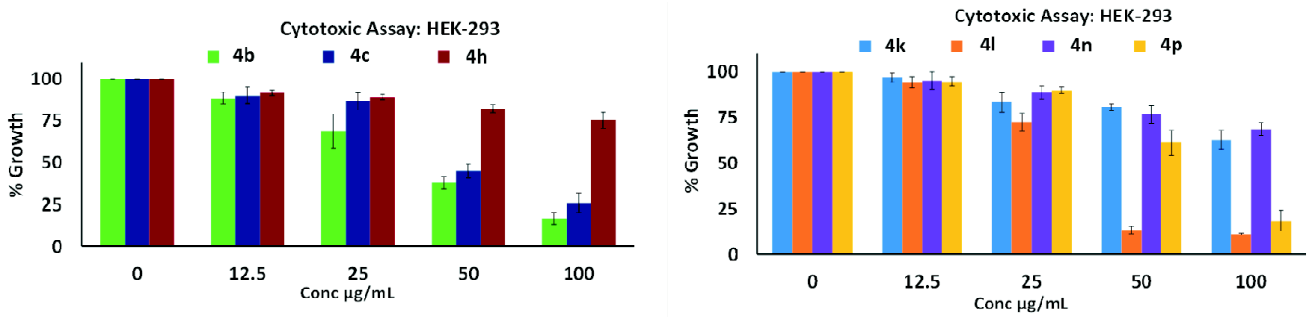

Fig. 2. Comparision of growth of promastigotes of *Leishmania donovani* on addition of test compounds w.r.t to control.

non-toxic even at higher concentrations of 50 μg/mL. But at 100 μg/mL all the compounds, especially **4b**, **4c**, **4l** and **4p** were seen to inhibit the cell growth.

Conclusions

The results indicate that the compounds with more hydrophobicity like **4e** are potent against the Gram-positive and Gram-negative bacilli and can be further modified to improve their potency. While designing such hydrophobic compounds, the solubility of the molecules should also be taken into consideration.

Unlike our previous work, these molecules do not show potent anti-tuberculosis activity.

The preliminary anti-leishmanial study data has indicated that these molecules have good potential for anti-leishmanial activity. Further exploration to find molecules to differentiate activity and toxicity on leishmaniasis is ongoing in our laboratory.

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Supporting Information

Spectral characterization data for the representative molecules is mentioned below.

*6-(4-(o-Tolyloxy)phenyl)-1,3,5-triazine-2,4-diamine (**4a**):* White solid; yield 85%; m.p. 238°C. IR (KBr): υ_{max} 3396, 3296, 3189, 1644, 1611, 1395, 1253, 1111 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ (ppm): 8.2–8.3 (d, 2H, ArH), 7.05–7.27 (m, 4H, ArH), 6.8 (s, 2H, ArH), 6.7 (s, 4H, D_2O exchange, NH_2), 2.1 (s, 3H, CH_3); ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm): 172 (1C), 167 (2C), 160 (1C), 154 (1C), 131–132 (2C), 130 (2C), 127(1C), 125 (1C), 122 (1C), 120 (1C), 118 (2C), 17 (1C). MS: 294.3 [M+1]. HPLC: retention time (RT): 3.5 min.

*6-(4-(2-(Tert-butyl)phenoxy) phenyl)-1,3,5-triazine-2,4-diamine (**4e**):* White solid; yield 60%; m.p. 265°C. IR (KBr): υ_{max} 3500, 3480, 3313, 3145, 1669, 1626, 1542, 1391, 1234, cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ (ppm): 8.22 (d, 2H, ArH), 7.38 (m, 1H, ArH), 6.89–7.14 (m, 5H, ArH), 6.7 (s, 4H, D_2O exchange, NH_2), 1.3 (s, 9H, CH_3); ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm): 170 (2C), 168 (1C), 160 (1C), 155 (1C), 141(1C), 132 (2C), 130 (1C), 128 (2C), 125 (1C), 122 (1C), 118 (2C), 34 (1C), 30 (3C). MS: 336.3 [M+1]. HPLC: retention time (RT): 4.7 min.

*6-(2-Phenoxyphenyl)-1,3,5-triazine-2,4-diamine (**4i**):* White solid; yield 85%; m.p. 200°C. IR (KBr): υ_{max} 3476, 3426, 3319, 3132, 1623, 1532, 1382, 1221 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ (ppm): 7.58 (d, 1H, ArH), 7.39 (t, 1H, ArH), 7.21 (m, 3H, ArH), 6.91 (m, 4H, ArH), 6.64 (s, 4H, D_2O exchange, NH_2); ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm): 172 (1C), 167 (2C), 158 (1C), 154 (1C), 131–132 (2C), 130 (3C), 124–122(3C), 118 (2C), MS: 279.2 $[M]^+$. HPLC: retention time (RT): 3.3 min.

*6-(2-(p-Tolyloxy)phenyl)-1,3,5-triazine-2,4-diamine (**8j**):* White solid; yield 85%; m.p. 220°C. IR (KBr): υ_{max} 3522, 3493, 3418, 3310, 3120, 1632, 1605, 1540, 1242, 1221 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): δ (ppm): 7.55 (d, 2H, ArH), 7.36 (t, 1H, ArH), 7.15 (t, 1H, ArH), 6.76–7.06 (d, 5H, ArH), 6.6 (s, 4H, NH_2), 2.2 (s, 3H, CH_3); ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm): 172 (1C), 167 (2C), 155 (2C), 131–132 (2C), 130 (3C), 122 (1C), 120 (1C), 118 (3C), 20 (1C). MS: 293.3 $[M]^+$. HPLC: retention time (RT): 3.5 min.

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