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In vitro biological evaluation of biguanides and dihydrotriazines against *Brugia malayi* and folate reversal studies

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ABSTRACT

Dihydrofolate reductase (DHFR) is a well-known target for antibacterial and anticancer therapy. DHFR inhibitors are useful for protozoan parasites, but are yet to be explored against metazoan species; hence the present work was designed to evaluate the efficacy of DHFR inhibitors against filariasis, one of the major neglected tropical diseases. Molecules from our in-house library of synthetic antifolate agents (biguanide and dihydrotriazine derivatives) were evaluated along with the antimalarial drug pyrimethamine and the antibacterial drug trimethoprim in an *in vitro* model against *Brugia malayi* microfilariae (Mf). Three biguanides and two dihydrotriazines were more potent than trimethoprim and pyrimethamine against *B. malayi* Mf. Trimethoprim, pyrimethamine and four of the five compounds active against Mf were also active against adult worms. To probe the mechanism of action of the compounds, reversal of activity of active compounds by folic acid and folinic acid was studied. In conclusion, DHFR inhibitors could be used as leads for new antifilarial drugs.

1. Introduction

Filariasis is a parasitic disease and is one of the leading causes of morbidity in endemic areas; over 40 million persons worldwide suffer from gross physical manifestations of lymphatic filariasis (Heidi, 2000). Currently available drugs such as diethyl carbamazine (DEC) along with ivermectin and albendazole continue to be the mainstay in the treatment of filariasis in spite of various limitations. The adverse reactions and the development of resistance against the existing antifilarial drugs necessitate the development of new antifilarial agents. Predictably, WHO has emphasized the need for novel drugs in this area (http://apps.who.int/tdr/svc/diseases/lymphatic-filariasis).

Gupta and Srivastava (2005) have exhaustively reviewed possible biochemical targets including myriad metabolic pathways, the micro-tubular system, several receptors and channels as well as immuno-modulation, for exploring potential antifilarial therapeutics. Several of imidazoles, coumarins, indoles, quinolones, aminoquinolines and triazines have been reported as prospective antifilarial agents (Tripathi et al., 2006). In an earlier report, some herbal extracts rich in polyphenolics (mainly flavonoids and coumarins) also showed promising results against this parasite (Sahare et al., 2008). Interestingly, some polyphenolic compounds are found to inhibit dihydrofolate reductase (DHFR) (Navarro-Perán et al., 2005).

DHFR catalyzes the crucial reaction of conversion of folic acid to dihydro and tetrahydro folic acids (cofactor involved in one carbon donation in purine and pyrimidine de-novo synthesis) using NADPH as cofactor. DHFR has been successfully explored as a target for bacterial and protozoal infections, but it is yet to be fully explored against metazoans (Gangjee et al., 2007). The structure of the enzyme varies subtly from species to species, allowing selective drug design (Blaney et al., 1984). Interestingly, DEC is reported to inhibit a number of enzymes involved in folate metabolism (Gupta and Srivastava, 2005). Suramin is also reported to inhibit DHFR enzyme of the related species *Onchocerca volvulus* and NADP dependent 10-formyl-H^4 folate dehydrogenase of *Brugia pahangi* (Gupta and Srivastava, 2005).

With this background, clinically used antagonists of folates such as the antibacterial trimethoprim and antimalarial pyrimethamine, were evaluated against *Brugia malayi* microfilariae (Mf). Promising results from this screening prompted us to test our in-house antifolate library of biguanide and dihydrotriazine derivatives against *B. malayi* Mf and adult worms.

2. Materials and methods

2.1. Compounds

All commercial chemicals were obtained from commercial source (Himedia laboratories Pvt. Ltd., Mumbai). All other com-

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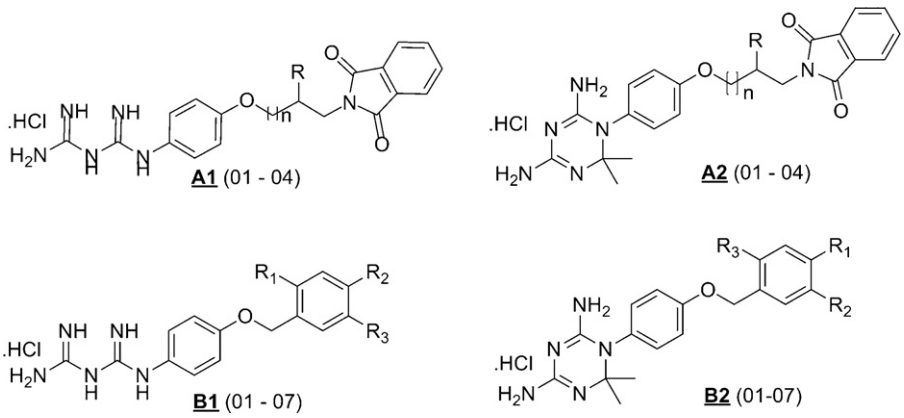

Fig. 1. General structures of synthesized compounds used in this study.

pounds evaluated were synthesized (Bag et al., 2009) in our laboratory (Fig. 1, Tables 1a and 1b) and the structures and purity of the compounds were established using spectroscopic methods (IR, ^{1}H NMR, ^{13}C NMR, MS analysis and chromatography). The use of animals for the study was approved by Institutional Animal Ethical Committee, which follows the Committee for the Purpose of Control and Supervision on Experiments on Animals, India norms (CPCSEA, India).

2.2. Preparation and collection of B. malayi Mf and adult worms

B. malayi were established and maintained in jirds (*Meriones unguiculatus*), mastomys (*Mastomys natelansis*) and mosquitoes (*Aedes aegypti*) using standard methods (Sanger et al., 1981). Mf were obtained by lavage of the peritoneal cavities of jirds with intraperitoneal filarial infection of 3 months or more duration. Similarly, adult worms of *B. malayi* were obtained from the peritoneal cavities of jirds, 4–6 months after infection with larval stage (L_3).

Table 1a
Biological activity of the compounds against microfilariae (Mf).

Number	Mol ID	N	R	Mol wt.	Activity*
1.	**A1-01**	0	H	402.83	6.33
2.	**A1-02**	1	H	416.86	7.00
3.	**A1-03**	2	H	430.88	11.67
4.	**A2-01**	0	H	442.899	5.67
5.	**A2-02**	1	H	456.925	8.00
6.	**A2-03**	2	H	470.952	8.00
7.	**A2-04**	1	OH	472.925	5.33

Table 1b
Biological activity of the compounds against microfilariae (Mf).

Number	Mol ID	R_1	R_2	R_3	Mol wt.	Activity*
1.	**B1-01**	H	H	H	319.789	10.33
2.	**B1-02**	Cl	H	H	354.234	85.33
3.	**B1-03**	H	H	Cl	354.234	99.67
4.	**B1-04**	Cl	Cl	H	388.679	100
5.	**B1-05**	H	Br	H	398.685	65.67
6.	**B1-06**	H	H	OCH_3	349.815	41.33
7.	**B1-07**	H	Ph	H	395.885	100
8.	**B2-01**	H	H	H	359.853	2.33
9.	**B2-02**	Cl	H	H	394.298	6.00
10.	**B2-03**	H	H	Cl	394.298	8.33
11.	**B2-04**	Cl	Cl	H	428.743	99.00
12.	**B2-05**	H	Br	H	438.749	8.33
13.	**B2-06**	H	H	OCH_3	389.879	8.67
14.	**B2-07**	H	Ph	H	435.949	99.00

* Expressed as % loss of motility at 20 μg/ml after 48 h. Trimethoprim and pyrimethamine showed 100% loss of motility at this concentration. DMSO, RPMI medium (negative controls) showed activity of 3.33 and 3.0 respectively.

The Mf and adult worms were washed with RPMI 1640 medium (containing 20 μg/ml gentamycin, 100 μg/ml penicillin, 100 μg/ml streptomycin; plated on sterile plastic petri-dishes and incubated at 37 °C for 1 h to remove peritoneal exudate cells from jirds'. The Mf were collected from petri-dishes, washed with RPMI 1640 medium and used for *in vitro* experiments (Chandrasekhar et al., 1984; Ash and Riley, 1970).

2.3. In vitro screening of DHFR inhibitors for antifilarial activity

Each of the compounds was dissolved in 1 ml of DMSO to make up the stock concentration of 2 mg/ml. The compounds were further diluted in suitable solvents to obtain the desired final concentration of 20 μg/ml in sterile 24 well culture plates (Nunc, Denmark) containing 900 μl of RPMI media. The highest concentration of DMSO used along with compound was 1%. Blank readings were taken with 1% DMSO. Approximately 100 Mf in 100 μl of RPMI media were introduced into the wells. The plates were incubated at 37 °C with 5% CO_2 for 48 h. Mf motility was assessed by microscopy after 48 h; the observations were recorded as the percentage of non-motile Mf in each well. The conditions for assay procedure were pre-standardized in our laboratory (Sahare et al., 2008). Each experiment was repeated thrice to check the reproducibility (Tables 1a and 1b).

The same study was also carried out with adult worms. However, due to the paucity of adult worms and based on the CPCSEA norms for sacrificing animals to procure adult worms, only compounds which showed good efficacy in Mf were used. Briefly, two worms (1 male and 1 female) were incubated in 1 ml of the medium alone and in medium containing 20 μg/ml of each of the compounds. The plates containing parasites were incubated at 37 °C with 5% CO_2 for 48 h. Motility was assessed by microscopy after 48 h, wherein the adult worm viability was assessed visually by direct microscopic observations using Nikon inverted microscope, Diaphot, TMD and the observations were scored as: (−) inactive or dead; (+) less active; and (++) highly active (Table 1c). Each compound was tested in triplicate.

2.4. Reversal of the antifolate activity of compounds by folic acid and folinic acid

In an attempt to understand the mechanism of action of these compounds folic acid and folinic acid reversal studies were carried out. Folic acid solution was freshly prepared and the Mf were pre-incubated with the final dose ranging from 10 to 100 μM for 1 h (Kinyanjui et al., 1999). Further, the dose at which 100% loss of motility was achieved by the test compound, was added to the wells and the 24 well culture plates were incubated in an atmosphere of