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Synthesis of New 4-Oxo-2-Thioxo-1,2,3,4-Tetrahydropyrimidine Derivatives with an Incorporated Thiazolidinone Moiety and Testing Their Possible Serine Protease and Cercarial Elastase Inhibitory Effects with a Possible Prospective to Block Penetration of *Schistosoma mansoni* Cercariae into the Mice Skin

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5-Substituted 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine were synthesized by interaction of 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonylhydrazide with some aldehydes to give the corresponding Schiff-bases, which after cyclization gave corresponding thiazolidinones. For some of the thiazolidinones, Mannich bases reaction was carried out. All the derivatives were tested for their possible inhibitory effect on *Schistosoma mansoni* cercarial elastase (CE). Only, *N*'-(4-methylbenzyledine)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonylhydrazide was found to have potent inhibitory effect on the CE activity with IC_{50} = 264 μM. Upon its use as a paint for mice tails before infection with S. mansoni cercariae, the compound formulated in jojoba oil caused a significant reduction (93%; P-value = 0.0002) in the worm burden. IgG & IgM in mice sera were measured by using several *S. mansoni* antigens by ELISA. Sera from treated infected mice (TIM) 2, 4, and 6 weeks (W) post infection (PI) showed 1.2 folds lower, 1.2 folds higher, 1.7 folds lower IgM reactivity against soluble cercarial antigenic preparation (CAP), respectively, when compared with sera collected from infected untreated mice (IUM). Sera from TIM 2, 4, and 6WPI showed 1.3, 1.6, and 1.7 folds higher IgG reactivity, respectively against CAP than the IgG reactivity from IUM. Sera from TIM 2, 4 and 6WPI showed 1.5, 1.2 folds lower and 1.4 folds higher IgM reactivity, respectively against soluble worm antigenic preparation (SWAP) when compared with sera collected from IUM. Sera from TIM 2, 4, and 6WPI showed 1.4, 1 folds lower and 1 fold higher IgG reactivity, respectivley to SWAP when compared with sera from IUM. Sera from TIM 2, 4, and 6WPI had generaly lower IgM and IgG reactivities against soluble egg antigen (SEA) when compared with sera from IUM.

Key words: *N*'-(4-Methylbenzylidene)-4-oxo-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-sulfonylhydrazide, *Schistosoma mansoni*, Cercariae, Elastase, IgM, IgG

INTRODUCTION

The initial step in infection of humans by schistosome parasites is penetration of cercariae, the infective stage of the parasite into the host skin (Stirewalt and Dorsey, 1974; McKerrow *et al.*, 1991). Attempts to develop a topical formulation to prevent such step date backs to more than 30 years (Pellegrino, 1967). More recently, niclosamide, a cercaricidal compound was tested in field for its ability to prevent such infections (Abu-Elyazeed *et al.*, 1993; Podgore *et al.*, 1994). Some efficacy of niclosamide in preventing the infection caused by *Schistosoma mansoni*, but not by *S. haematobium*was reported, but this was insufficient to warrant a recommendation for its widespread use.

Penetration of cercariae into the hsot skin is facilitated by secretion of a potent serine protease, cercarial elastase (CE), from the preacetabular glands of cercariae (Bahgat *et al.*, 2001; Ruppel *et al.*, 2004). In previous work, we

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have defined the substrate specificity of this enzyme, kinetically characterized its activity in terms of Michaelis-Menten constants (K_m), maximum reaction velocity (V_{max}) and inhibitory coefficients (K_i) of several known serine protease inhibitors (Bahgat and Ruppel, 2002) and have also confirmed the potency of these inhibitors by direct biochemical assays.

Earlier studies have demonstrated that irreversible tetrapeptide chloromethyl ketone inhibitors blocked cercarial invasion *in vitro*, when applied to skin as a dimethyl sulfoxide (DMSO) solution (Cohen *et al.*, 1991). In addition to new information on the biochemical basis of cercarial invasion, there have been significant advances in the development of lotions to optimize delivery of drugs into the skin (Berti and Lipsky, 1995; Misoire and Bucks, 1997; Niemiec *et al.*, 1997; Goldenberg, 1996; Fox, 1996).

Lim *et al.,* (1999) showed that both peptide-based, irreversible inhibitors and non-peptide reversible inhibitors can block 97-100% of cercarial invasion *in vitro*, when applied in the form of different topical delivery formulations. When the optimal inhibitor formulation was applied to tails of BALB/c mice followed by infection with 120 cercariae by tail immersion, 80% reduction in worm burden and a 92% reduction in egg burden were achieved.

Alkyl [sulfonyl(oxy)] uracils (**1-2**), dihydrouracil derivatives, were found to be efficient and time-dependent inhibitors of leukocyte elastase and they formed acyl-enzymes that exhibited variable hydrolytic stability which appeared to be dependent on the nature of the R1 group (believed to be accommodated at the primary specificity site, S1, Groutas *et al.*, 1994). When some hexahydroimidazo[1,2-c]pyrimidine derivatives (HIPs) were tested for their anti-inflammatory effects on mouse-paw edema, they exerted a dose-dependent inhibition on paw swelling with significant reduction in leukocyte elastase activity (Vidal *et al.*, 2001). Recently, several pyrimidine derivatives have been reported for their potency as serine protease, selective factor Xa. inhibitors (He *et al.*, 2000).

Continuous oral and intravenous infusion of nifedipine (calcium channel blocker pyrimidine derivative) at a dose of 5.91 ± 0.53 micrograms/kg body weight per hour to patients undergoing cardiopulmonary bypass caused significantly lower levels of granulocyte elastase by forming alpha-proteinase inhibitor complex (Riegel *et al.*, 1988).

Chymase, chymotrypsin-like serine protease, possesses a wide variety of actions, including promotion of angiotensin II production and histamine release from mast cells. Replacement of the Val-Pro unit of the peptidic chymase inhibitor, Val-Pro-Phe-CF_3 with a (5-amino-6-oxo-2-phenyl-1,6-dihydro-1-pyrimidinyl)acetyl moiety and after studying structure-activity relationship revealed that phenyl substitution at the 2-position of the pyrimidinone ring results in protease inhibitory function (K_i = 0.0506 mmol) which is far more potent than the parent peptidic inhibitor, Val-Pro-Phe-C_{F3} (Akahoshi *et al.*, 2001). The structure, structure-activity relationships, pharmacokinetics, and inhibitory mechanism of pyrimidinone derivatives on the chymase have been recently reported (Akahoshi, 2003).

A variety of sulfonamides were found to function as either growth inhibitors for microbacterium tuberculosis or as carcinostatic agents (Abdel Hamid and Fathalla, 1993; Fathalla, 1992, 1999; Fathalla *et al.*, 2000).

So, the aim of the present work was to prepare uracil-5-sulfonhydrazid, related Schiff bases, their cyclized form with thioglycolic acid and the Mannich form of some of them and to test their possible inhibitory effect on the cercarial serine protease activity.

MATERIALS AND METHODS

All melting points are uncorrected and were determined in capillary tube on a melting point microscope (Boetius; Great Britain Stuart Scientific Co. LTD.). IR spectra were recorded on an infrared spectrophotometer PU9712 (Beckman; WI, Madison, U.S.A.) by employing KBr discs. ^{1}H-NMR spectra were obtained on Japanese, JoelEX270-MHZ spectrometer by using TMS as internal standard. Mass spectra were recorded on Mass spectrometer (SSQ7000; Califonia, U.S.A.) at 70 ev and all reactions were followed and checked by T.L.C. by using chloroform/methanol (3:1) as mobile phase and the respective spots were examined under UV lamp.

Preparation of the starting compound 1 and the derivatives (2a-d)

In the present work, 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonyl hydrazide (**1**) was prepared through the reaction of 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonylchloride with hydrazine hydrate (99%), as previously described (Fathalla, 2001). Preparation of the Schiff-bases of uracil-5-sulfonhydrazide (**2a-d**), *N*'-(4-nitrobenzylidene)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonylhydrazide (**2a**), *N*'-(4-methyl benzylidene)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonylhydrazide (**2b**), *N*'-(4-furylmethylene)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonyl hydrazide (**2c**), 4-oxo-2-thioxo-*N*'-(3,4,5-trimethoxybenzylidene)-1,2,3,4-tetrahydropyrimidine-5-sulfonylhydrazide (**2d**) was carried out as reported previously (Fathalla, 2001). General method: a mixture of **1** (0.01 mol) and the calculated amount of the aldehydes, namely 4-nitrobenzaldehyde, 4-methylbenzaldehyde, furfural, and/or 3,4,5-trimethoxybenzaldehyde in 25 mL of absolute ethanol was stirred at room temperature for two days. The reaction mixture was cooled and the precipitate formed was filtered and air-dried. The product was recrystallized from the proper solvent to form the title

compounds **2a-d**, respectively.

Preparation of thiazolidinones (3a-d)

The thiazolidinones, *N*-[2-(4-Nitrophenyl)-4-oxo-1,3-thiazolidin-3-yl]-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonamide (**3a**), *N*-[2-(4-methylphenyl)-4-oxo-1,3-thiazolidin-3-yl]-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonamide (**3b**), *N*-[2-(2-furyl)-4-oxo-1,3-thiazolidin-3-yl]-4-oxo-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-sulfonamide (**3c**), 4-oxo-*N*-[4-oxo-2-(3,4,5-trimethoxy-phenyl)-1,3-thiazolidin-3-yl]-4-oxo-2-thioxo-1,2,3,4-tetrahyropyrimidine-5-sulfonamide (**3d**) were prepared as per the following general method. General method: Compounds **2a-d** (0.01 mol) were dissolved in dry benzene (25 mL), and thioglycolic acid (0.15 mol) in 10 mL of benzene was added and then the mixture was refluxed on a water bath for 8-12 h. The formed product was filtered off and recrystallized from the proper solvent to form **3a-d** (Fathalla, 2001). The physical and analytical data are provided in Tables I and II.

Mannich reaction for some of the thiazolidinones derivatives

4-Oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonic acid [5-(4-methylpiperazin-1-ylmethyl)-4-oxo-2-*p*-nitrophenylthiazolidin-3-yl]-amide (**4a**), 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonic acid [5-(4-methylpiperazin-1-ylmethyl)-4-oxo-2-*p*-tolylthiazolidin-3-yl]-amide (**4b**), 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonic acid (5-diethylaminomethyl-4-oxo-*p*-tolylthiazolidin-3-yl)-amide (**5b**), 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonic acid (5-diethylaminomethyl-2-furan-2-yl-4-oxo-thiazolidin-3-yl)-amide (**5c**), 4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonic acid (2-furan-2-yl-5-morpholine-4-yl-methyl-4-oxo-thiazolidin-3-yl)-amide (**6c**), and 4-oxo-2-thoxo-1,2,3,4-tetrahydropyrimidine-5-sulfonic acid [5-morpholin-4-ylmethyl-4-oxo-2-(3,4,5-trimethoxy-phenyl)-thiazolidin-3-yl]amide (**6d**) were prepared as per the following general method. General method: A mixture of 0.5 g (0.005 mol) of paraformaldehyde and 15 mL (0.01 mol) of *N*-methylpiperazine and diethyl amine or morpholine in 25 mL of absolute ethanol was refluxed for 30 min untill complete solubility of paraformaldehyde was achieved. A warmed solution of 2.5 g (0.02 mol) of pyrimidine in 30 mL of ethanol was subsequently added to the reaction mixture. The whole mixture was refluxed for 6-9 h and left at room temperature under stirring for three days, and after that the volatile material was evaporated. The dry residue was extracted with chloroform to form **4a-b** or **5b-c** and/or **6c-d** with a yield of 72, 75, and 80%, respectively. The structures of **2a-d**, **3a-d**, **4a-b**, **5b-c**, and **6c-d**, were confirmed by their elemental analysis, IR, ^{1}H-NMR, and mass spectra data.

Table I. Analysis and data of compounds **2a-d**, **3a-d**, **4a-b**, **5b-c**, and **6c-d.**

Comp. No.	m.p. °C Solv.	Yield %	Molecular Formula (M.W.)	Analysis Calcd./found % C	H	N
2a	251-3 DMF/W	67	$C_{11}H_9N_5O_5S_2$ (355.32)	37.18 37.13	2.55 2.51	19.71 19.69
2b	242-4 DMF/W	70	$C_{12}H_{12}N_4O_3S_2$ (324.38)	44.43 44.41	3.73 3.70	17.27 17.24
2c	301-3 DMF/W	65	$C_9H_8N_4O_4S_2$ (300.32)	35.99 35.95	2.69 2.65	18.66 18.62
2d	285-7 DMF/W	72	$C_{14}H_{16}N_4O_6S_2$ (400.43)	41.99 41.95	4.03 3.98	13.99 13.94
3a	270-2 E	73	$C_{13}H_{11}N5O6S_3$ (429.46)	36.36 36.34	2.58 2.53	16.31 16.28
3b	275-7 E	70	$C_{14}H_{14}N_4O_4S_3$ (398.48)	42.20 42.16	3.54 3.51	14.06 14.03
3c	>300 E	71	$C_{11}H_{10}N_4O_5S_3$ (374.42)	35.29 35.26	2.69 2.66	14.96 14.94
3d	>300 E	70	$C_{16}H_{18}N_4O_7S_3$ (474.54)	40.50 40.46	3.28 3.24	11.81 11.75
4a	284-6 A	65	$C_{19}H_{23}N_7O_6S_3$ (541.63)	42.13 42.08	4.28 4.25	18.10 18.06
4b	251-3 A	65	$C_{20}H_{26}N_6O_4S_3$ (510.66)	47.04 47.01	5.13 5.09	16.46 16.43
5b	>300 A	70	$C_{19}H_{25}N_5O_4S_3$ (483.63)	47.19 47.16	5.21 5.17	14.48 14.44
5c	>300 A	65	$C_{16}H_{21}N_5O_5S_3$ (459.57)	41.82 41.80	4.61 4.57	15.24 15.21
6c	>300 A	75	$C_{16}H_{19}N_5O_6S_3$ (473.55)	40.58 40.55	4.04 4.02	14.79 14.76
6d	>300 A	72	$C_{21}H_{27}N_5O_8S_3$ (573.67)	43.97 43.94	4.47 4.44	12.21 12.19

A = acetic acid, DMF/W = dimethylformamide/water, E = absolute ethanol.

Preparation of cercarial secretions (CSs)

The secretions of schistosome cercariae were prepared as per the previously described procedure (Bahgat *et al.*, 2001) and were used as source for the cercarial elastase (serine protease) activity. To obtain cercariae, the infected snails were exposed to light for 1 h and were then gently poured into petri dishes (15 cm diameter) that were previously painted with linoleic acid (0.9 g/mL) and air dried. A total of 10.000 cercariae were used per dish. The plates were incubated at 37°C for 30 min. During this incubation, cercariae release the contents of acetabular glands while trying to penetrate into the linoleic acid and transforming into schistosomula. All the water containing

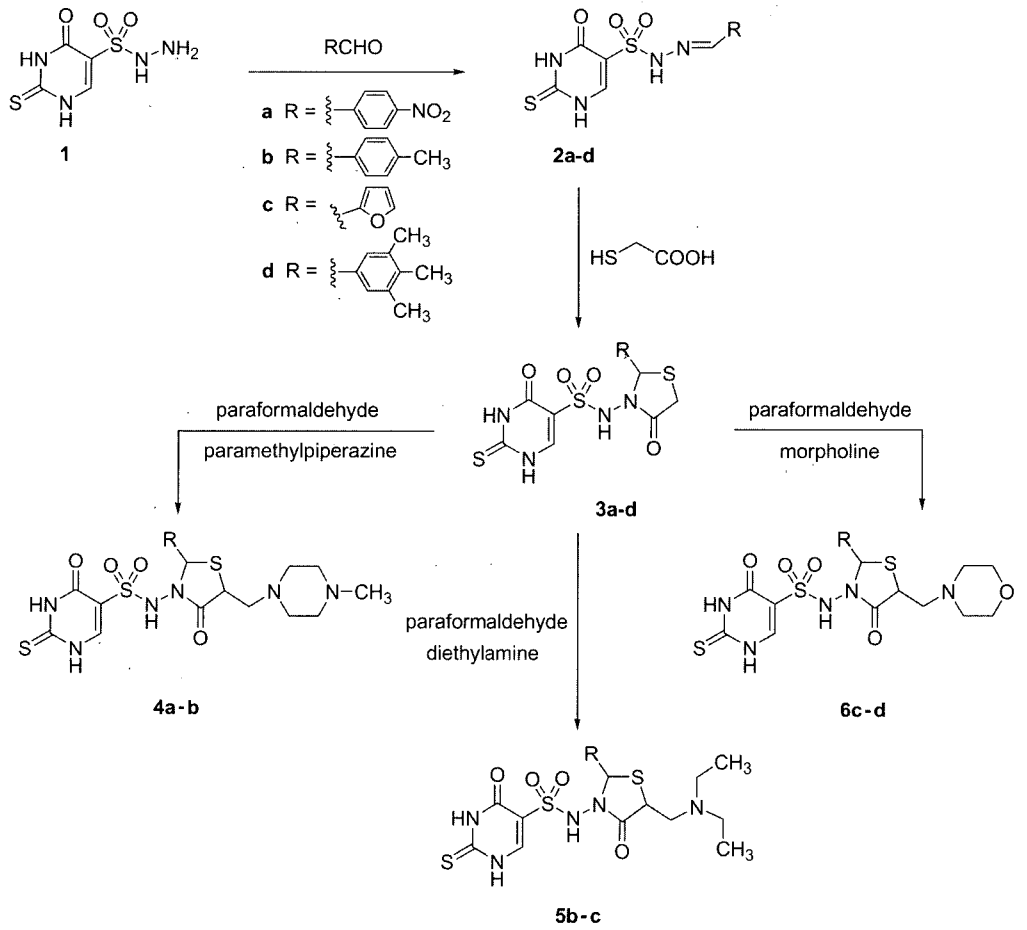
Table II. IR, mass and ^{1}H-NMR spectral data of the newly synthesized compounds

Comp. No.	IR cm^{-1} (KBr)	Mass. (R.I)	^{1}H-NMR (DMSO-d_6), ppm.
2a	3335,3206 (NH), 3066 (C-H aromatic), 1673(CO of thiouracil), 1346 (NO_2), 1322, 1215 (-N-SO_2-) 1200(-SO_2-) and 837 for (C-N).	355.3	7.6,7.9 (4H, dd, aromatic), 8.3 (1H, s, of uracil), 8.9 (1H,s,-N=CH) ,11, 11.4,11.6(3H,s, 3NH exchange -able with D_2O).
2b	3318,3217(NH), 3118 (C-H aromatic), 1680 (CO of thiouracil), 1327, 1262 (-N-SO_2-) and 1200 for (-SO_2-).	324.4	2.3(3H, s, CH_3), 6.3 - 7.2 (4H,dd, aromatic), 8.3 (1H,s, of uracil), 8.8 (1H, s, -N=CH), 10.1,11.2,11.4 (3H, s, 3NH, exchangeable with D_2O).
2c	3201 (NH), 3050(C-H aromatic), 1675(CO of thiouracil), 1345, 1327 (-N-SO_2-) and 1200 for (-SO_2-), 835(C-N).	300.3	6.2,6.5,7.1 (3H,s, C=CH), 8.3 (1H, s, of uracil), 9 (1H, s, N=CH), 10.3 -11,7 (3H,b, 3NH, exchangeable with D_2O).
2d	3305 (NH), 3089, 3035(C-H aromatic), 1690(CO of thiouracil), 1345, 1327(-N-SO_2-) and 1200 for (-SO_2-).	400.5	3.4,3.6,3.7 (9H, s, 3 OCH_3), 7.2 (2H, dd, aromatic), 8.3 (1H,s, of uracil), 8.9 (1H,s, -N=CH), 10.5 -11.7 (3H,b, 3NH. exchangeable with D_2O).
3a	3410 (NH), 3130, 2987 (C-H aromatic), 1735 (CO), 1672 (CO of thiouracil), 1342 (NO_2), 1320, 1235 (-N-SO_2-), and 1200 for (-SO_2-) 835(C-N).	429.4	4.5(2H,s, CH_2), 6.1, 7.2(4H, dd, aromatic), 8.3 (1H, s, of uracil), 8.9 (1H,s, -N-CH), 10.5, 10.7, 11.2 (3H, s, 3NH, exchangeable with D_2O).
3b	3340 (NH), 3230, 3076 (C-H aromatic), 1715 (CO), 1695 (CO of thiouracil), 1323, 1220 (-N-SO_2-) and 1200 for (-SO_2-).	398.5	2.4(3H,s, CH_3), 4.3(2H,s, CH_2), 7.1,7.5(4H,dd, aromatic), 8.3 (1H,s, of uracil), 9.0 (1H, s, -N-CH), 10.3 -11.4 (3H, b, 3NH, exchangeable with D_2O).
3c	3330 (NH), 3227, 3136 (C-H aromatic), 1721 (CO), 1695 (CO of thiouracil), 1323, 1222 (-N-SO_2-), 1200 (-SO_2-).	374.4	3.9 (2H, s, CH_2), 6.2, 6.5, 7.1 (3H, s, -C=CH), 8.3 (1H,s, of uracil), 9.1 (1H,s, -N-CH), 10.1, 10.5,10.7, (3H,s, 3NH, exchangeable with D_2O).
3d	3343 (NH), 3223, 3078 (C-H aromatic), 1724 (CO), 1675 (CO of thiouracil), 1325, 1220 (-N-SO_2-), and 1200 for (-SO_2-).	474.5	3.1,3.4,3.6 (9H, s, 3 OCH_3), 4.2-4.6(2H,s, CH_2), 7.2 (2H, dd, aromatic), 8.3 (1H,s, of uracil), 8.9 (1H,s, -N-CH), 10.5 - 11.7 (3H,b, 3NH, exchange able with D_2O).
4a	3335 (NH), 3219, 3059 (C-H aromatic), 1723 (CO),1712, 1697 (2 CO of uracil), 1345(NO_2), 1322, 1215 (-N-SO_2-), 1200 for (-SO_2-) and 835 for (C-N).	541.6	3.1-3.5(7H, m, NCH_3, CH_2-N-CH_2), 3.6, 3.8 (4H, s, CH_2-N-CH_2), 4.4 (2H, s, CH_2), 5.8(1H, s, -C-CH), 6.2, 7.4 (4H,dd, aromatic), 8.3(1H, s, of uracil), 8.9 (1H, s, -N-CH), 10.5, 10.7, 11.2 (3H,s, 3NH, exchange -able with D_2O).
4b	3335 (NH), 3219, 3059 (C-H aromatic), 1723 (CO), 1692 (CO of thiouracil), 1322, 1215 (-N-SO_2-) and 1200 for (-SO_2-).	510.7	2.3(3H,s, CH_3), 3.1 - 3.5 (7H, m, NCH_3, CH_2-N-CH_2), 3.6, 3.8 (4H,s, CH_2-N-CH_2), 4.5 (2H, s, CH_2), 6.1 (1H, s, C-CH), 7.1, 7.5 (4H, dd, aromatic), 8.3 (1H, s, of uracil), 9 (1H, s, -N-CH), 10.3 -11.4 (3H, b, 3NH, exchangeable with D_2O).
5b	3225 (NH), 3120, 3059 (C-H aromatic), 1723 (CO), 1687 (CO of thiouracil), 1322, 1215 (-N-SO_2-), 1200 (-SO_2-) and 837 for (C-N).	496.5	1.1,1.2(6H,t, 2CH_3), 2.4 (3H, s, CH_3), 4.2, 4.5 (4H,q, 2 CH_2), 4.3 (2H, s, CH_2), 6(1H,s, -C-H), 7.1, 7.5 (4H, dd, aromatic), 8.3(1H, s, of uracil), 9.0 (1H, s, -N-CH), 10.3 - 11.2 (3H, b, 3NH, exchangeable with D_2O).
5c	3235(NH), 3219, 3059 (C-H aromatic), 1723 (CO), 1697 (CO of thiouracil), 1322, 1215 (-N-SO_2-), 1200 (-SO_2-) and 837 for (C-N).	459.6	1.1,1.3(6H, t, 2CH_3), 2.3 (3H, s, CH_3) 4.2, 4.5 (4H, q, 2 CH_2), 4.5 (2H, s, CH_2), 6.0 (1H, s, C-CH), 6.2, 6.5, 7.1 (3H, s, C=CH), 8.3 (1H, s, of uracil), 9.1 (1H, s, -N-CH), 10.1, 10.5,10.9, (3H,s, 3 NH, exchangeable with D_2O).
6c	3225 (NH), 3219, 3159 (C-H aromatic), 1710 (CO), 1691 (CO of thiouracil), 1320, 1218 (-N-SO_2-), 1200 (-SO_2-) and 838 for (C-N).	473.5	2.3,3.1(4H,s, CH_2-O-CH_2), 3.8,4.2 (4H,s, CH_2-N-CH_2), 4.6 (2H, s, CH_2) 6(1H,s, C-CH), 6.2, 6.9 (2H,s, CH= CH), 7.4 (1H, s, CH = CH), 8.3 (1H, s, of uracil), 8.7 (1H, s, -N-CH), 10.3, 10.6, 11,3 (3H, 3NH, exchangeable with D_2O).
6d	3232 (NH), 3119, 3057 (C-H aromatic), 1723 (CO), 1697 (CO of thiouracil), 322, 1215 (-N-SO_2-), 1200 (-SO_2-) and 837 for (C-N).	573.7	2.3, 3.1 (4H, s, CH_2-O-CH_2), 3.2, 3.4,3.6 (9H, s, 3OCH_3), 4.7 (2H, s, CH_2) 6 (1H, s, C-CH), 6.1, 7.2 (2H, m, aromatic), 6.3 - 7.4 (6H, s, CH_2-N-CH_2), 8.3 (1H, s, of uracil), 8.9 (1H, s, -N-CH), 10,2, 10.6, 11.4 (3H, s, 3NH, exchangeable with D_2O).

cercarial materials were collected in 15 mL Falcon tubes and larvae were sedimented on ice. The supernatant containing CSs was spun down for 2 min at 3000 g to eliminate cercarial debris, then concentrated by lyophilization and was then reconstituted with water to one tenth of its original volume. The protein content was measured by employing BCA test.

Quantitative assay of the serine proteinase activity by using chromogenic substrate

The serine protease activity in CSs was quantitatively assessed by using L-1195 (Boc-Val-Leu-Gly-Arg-PNA), a known substrate for trypsin like serine proteinases (Bahgat and Ruppel, 2002). The proteolysis of the covalent bond formed between the arginine amino acid and the *p*-nitroanilide group by the cercarial elastase (CE) results in the



Scheme 1. Compounds **2a-d**, **3a-d**, **4a-b**, **5b-c**, and **6c-d**

release of yellow colored *p*-nitroaniline that was quantified by measuring the absorbance at 405 nm using microtiterplate reader (TECAN, SUNRISE REMOTE CONTROL, Groedig, Austria). The intensity of the yellow color is directly proportional to the enzyme activity. Stock solution of the substrate at a concentration of 10 mg/mL was prepared in dimethylsulfoxide (DMSO) and was then diluted with substrate buffer(30 mM tris containing 60 mM NaCl and 0.05 % NaN_3) to the desired final concentration of optimal pH.

Inhibition of the CE

Inhibitory effect of the different pyrimidine derivatives (prepared in the present work) on *S. mansoni* CE was investigated. All the derivatives were found to be completely soluble in DMSO/ethanol mixture (1/1). Stock solutions of the pyrimidine derivatives of interest (10 mg/mL) were prepared. For performing inhibition assays, 50 μL CSs were preincubated with 10 μL of each of the serial dilutions of different pyrimidine derivatives for 10 min before adding the substrate L-1195. Similar volumes of the solvents (DMSO/ethanol) were mixed with the CSs as means of negative controls.

Using the pyrimidine derivative 2b to block invasion of mice skin by *S. mansoni* cercariae based on its CE inhibitory effect

The pyrimidine derivative (**2b**; *N*'-(4-methylbenzylidene)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonylhydrazide) that showed *in vitro* CE inhibitory effect was further tried at its most potent inhibitory concentration activity to block invasion of mice skin by cercariae of *S. mansoni*. For this purpose, pathogen free female Swiss Albino mice weighing 18-20 gm at age 6-12 weeks were employed. Animals were fed on standard rodents chew, supplied with water and maintained at 23°C ambient temperature. The inhibitor was dissolved in DMSO/ethanol (1/1), mixed with jojoba oil and used to paint the tails of 5 mice. Control group of 5 mice whose tails were painted with the oil mixed with the solvents without inhibitor were included. Each mouse received 80 cercariae by tail immersion method (Oliver and Stire Walt, 1952).

Separation of mouse sera

Sera were collected from the treated infected and untreated infected mice after 2, 4, and 6 weeks of post

infection. Sera were also collected from uninfected mice to be used as means of negative control. For different mice groups (treated infected, control infected, and control uninfected), available sera for IgG and IgM measurements at the 3 time points were at least from 3 individual mice. Sera missing at one or more time points were excluded either for being hemolysed or not enough to be included or the bleeding was not successful at one or more time point because of some technical problem.

Assessments of worm recovery

Seven weeks post infection, treated and untreated infected mice were perfused. This means that mice where dissected and the worms were recovered by perfusion through their hepatic portal veins. The seven weeks are known to be the time at which adult parasite worms are mature, pairing, and laying eggs.

Recovery of adult worms was performed through hepatic portal vein (Duvall and De Witt, 1961). Total worm counts in intestine and liver was determined. Protection was assessed as percentage reduction in worm counts according to the following formula:

$$P = (C-T)/C \times 100$$

P: Percentage reduction in worm counts in liver and intestine.
C: Mean worm in control infected mice (tail painted with oil containing the solvent without compound **2b**).
T: Mean worm count in pre-tail painted mice with the oil containing compound **2b** before infection with *S. mansoni* cercariae.

Enzyme linked immunosorbent assay (ELISA)

The assay was performed according to the method of Bahgat *et al.* (2001) to determine levels of IgG & IgM in sera of different mice groups against antigens derived from early and late stages of the *S. mansoni* parasite. Plates were coated with three different schistosome antigens (100 μL/well); (1) cercarial antigenic preparation (CAP; early stage antigen) at a concentration of 25 μg/mL, (2) soluble worm antigen preparation (SWAP; late stage antigen) at a concentration of 50 μg/mL and (3) soluble egg antigen (SEA; late stage antigen) at a concentrated dose of 25 μg/mL. After coating with the respective antigens, the plates were incubated at room temperature overnight. Plates were washed thrice with PBS containing 0.05% Tween20 (PBST) and antigen free sites were blocked against non specific binding by using 200 μL of PBST containing 1% BSA (PBST-BSA)/well, and was then left at 37°C for 1 h. After washings three times, 100 μL /well of diluted sera (1:100 in PBST-BSA) was added and incubated at 37°C for 2 h. Peroxidase labeled anti-mouse IgG or IgM conjugates was added at a dilution of 1:500 and 1:5000, respectively in PBST-BSA and incubated at 37°C for 1 h. *O*-phenylenediamindihydrochloride (OPD) substrate was used as substrate for visualization of the antigen antibody binding in the presence of H_2O_2. To avoid an increase in the background of the enzyme substrate reaction, 2N HCl was used as a stopper for the reaction and change in optical density was measured at λmax of 490 nm in a micro-well plate reader ELISA reader (TECAN, SUNRISE REMOTE CONTROL, Groedig, Austria).

RESULTS

Inhibition of the CE activity of *S. mansoni* cercariae by employing pyrimidine derivative 2b

All the prepared pyrimidine derivatives were tested *in vitro* for their CE inhibitory effect. Only compound **2b** (*N*'-(4-methylbenzylidene)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonylhydrazide) exerted a potent inhibitory effect on CE when serial concentrations of it were tested (Fig. 1) and 100% inhibition was obtained at a concentration of 600 μM. The IC_{50} (inhibitor concentration that abolishes 50% of the elastase activity) of the compound was observed to be 264 μM.

Perfusion results of mice treated with the pyrimidine derivative

The perfusion results of mice whose tails were painted with formulated compound **2b** in jojoba oil are presented in Table III. The worm burden results of treated, tail painted mice differed significantly (P-value = 0.0002) from the control mice, with a reduction of 93% in the recovered worms from the treated mice in comparison to the control ones. This significant reduction in the worm burden also led to granuloma free liver which reflects a complete disappearance of the pathological consequences of schistosomiasis infection due to almost complete absence of the source of eggs, the worms.

Humoral immune responses in mice whose tails were painted with compound 2b, the newly characterized serine protease inhibitor after infection with *S. mansoni* cercariae

The humoral, both primary and secondary immune responses against several antigens derived from different stages of *Schistosoma mansoni* life cycles were measured in sera obtained from mice treated with compound **2b** and then infected, infected untreated mice, and uninfected control mice. The antigens used were soluble cercarial antigenic preparation (CAP) that represents larval, early stage of antigen. The antigens derived from late stage, soluble adult worm antigenic preparation (SWAP) and soluble egg antigen (SEA) were used. The measurment

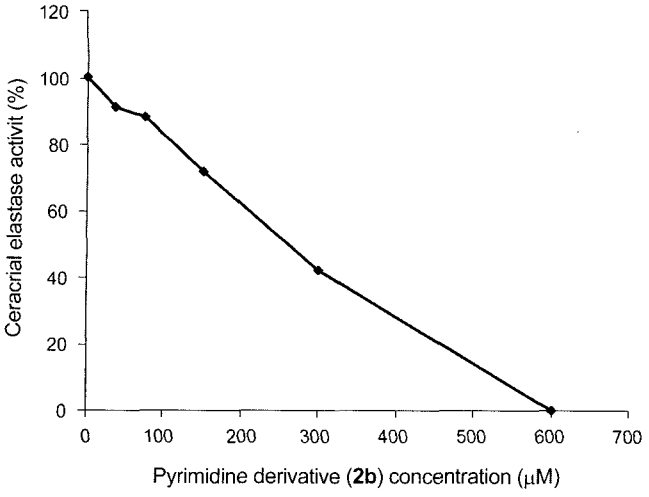


Fig. 1. Inhibition of elastase activity of *Schistosoma mansoni* cercariae upon using serial concentrations of the pyrimidine derivative. The pyrimidine derivative successfully inhibited the enzyme activity and 100% inhibition was achieved at 600 μM. The IC_{50} (inhibitor concentration that abolishes 50% of the elastase activity) of the compound was 264 μM.

Table III. Reduction in worm burden in tail painted mice with the compound **2b,** formulated in jojoba oil in comparison to control mice

Treated mice with compound **2b** formulated in jojoba oil			Control treated mice with DMSO /ethanol in jojoba oil		
Number of mice	Mean worm burden	St. dev.	Number of mice	Mean worm burden	St. dev.
5	1	0.8	5	14	2.64
Reduction in worm burden			93%		
P-value			0.0002, considered extremely significant		

of both IgM and IgG were carried out in sera collected after 2, 4, and 6 weeks (2, 4, and 6W) of post infection from both treated (TI) and untreated (I) mice. For different mice groups, available sera for IgG and IgM measurements at the 3 time points were at least from three individual mice. Sera missing at one or more time points were excluded either for being hemolysed or not enough to be included or the bleeding was not successful at one or more time point because of some technical problem.

Using CAP as an antigen in ELISA, the IgM response (Fig. 2) was found to be significantly higher ($P<0.05$) in sera from both treated infected (TI) and infected (I) untreated mice at different time points when compared with sera from uninfected control mice except among sera from 4 weeks infected untreated mice (4WI), where no significant difference was observed when compared with the control mice. Sera from treated infected mice (TIM) after 2, 4, and 6 weeks (W) post infection (PI) showed 1.2 folds lower, 1.2 folds higher, 1.7 folds lower IgM reactivity

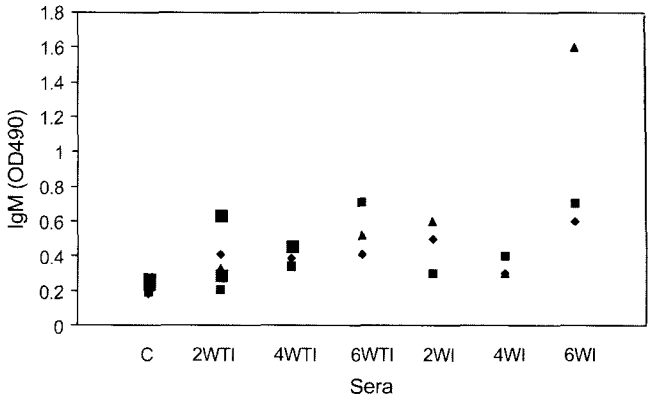


Fig. 2. Reactivity of IgM against *Schistosoma mansoni* soluble cercarial antigenic preparation (CAP) in sera from treated, tail-painted, mice with compound **2b** formulated in jojoba oil at different time points of post infection. The IgM response was significantly higher ($P<0.05$) in sera from both treated infected (TI) and infected (I) untreated mice at different time points when compared with sera from uninfected control mice, except among sera from 4 weeks infected untreated mice (4WI) where no significant difference was observed when compared with control mice. Sera collected from treated infected mice 2 weeks post infection (2WTI) showed 1.2 folds lower IgM reactivity when compared with sera collected from infected untreated mice at the same time point (2WI). At 4 weeks post infection, the treated infected mice (4WTI) showed 1.2 folds higher IgM reactivity to CAP than the infected untreated (4WI) ones. At 6 weeks, the IgM response in sera from treated infected mice was 1.7 folds lower than in those from untreated mice.

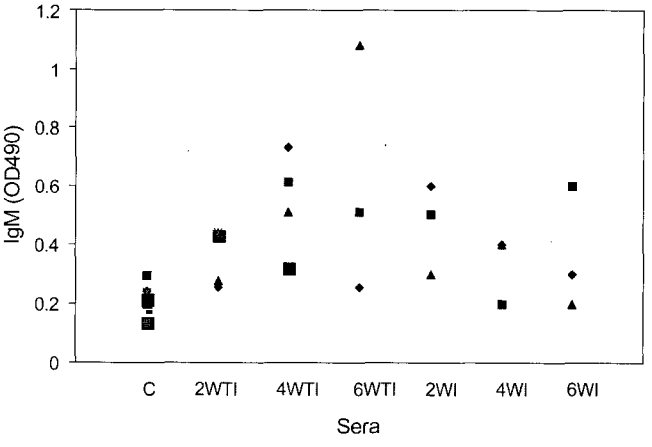


Fig. 3. Reactivity of IgG against *Schistosoma mansoni* soluble cercarial antigenic preparation (CAP) in sera from treated, tail-painted, mice with compound **2b** formulated in jojoba oil at different time points of post infection. Sera collected from treated infected mice 2 weeks post infection (2WTI) showed 1.3 folds higher IgG reactivity when compared with sera collected from infected untreated mice at the same time point (2WI). At 4 weeks post infection, the treated infected mice (4WTI) showed 1.6 folds higher IgG reactivity to CAP than the infected untreated (4WI) ones. At 6 weeks, the IgG response in sera from treated infected mice was 1.7 folds higher than in those from untreated mice.

CAP when compared to sera collected from infected untreated mice (IUM, Fig. 2, This result reflect the effect of treatment as it compares 1ry immune response among

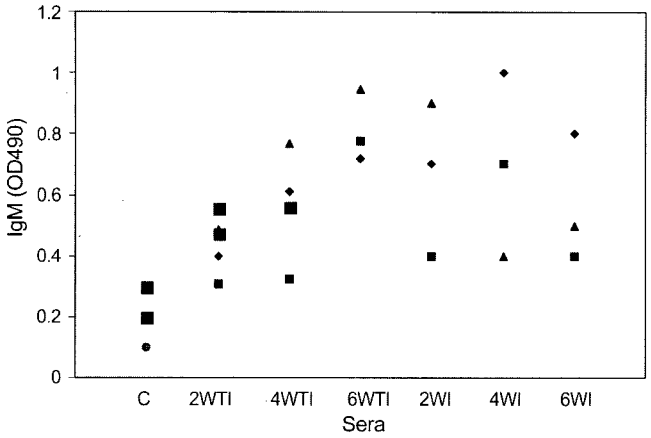


Fig. 4. Reactivity of IgM against *Schistosoma mansoni* soluble adult worm antigenic preparation (SWAP) in sera from treated, tail painted, mice with compound **2b** formulated in jojoba oil at different time points of post infection. Sera collected from treated infected mice 2 weeks post infection (2WTI) showed 1.5 folds lower IgM reactivity when compared with sera collected from infected untreated mice at the same time point (2WI). At 4 weeks post infection, the treated infected mice (4WTI) showed 1.2 folds lower IgM reactivity to SWAP than the infected untreated (4WI) ones. At 6 weeks, the IgM response in sera from treated infected mice was 1.4 folds higher than in those from untreated mice.

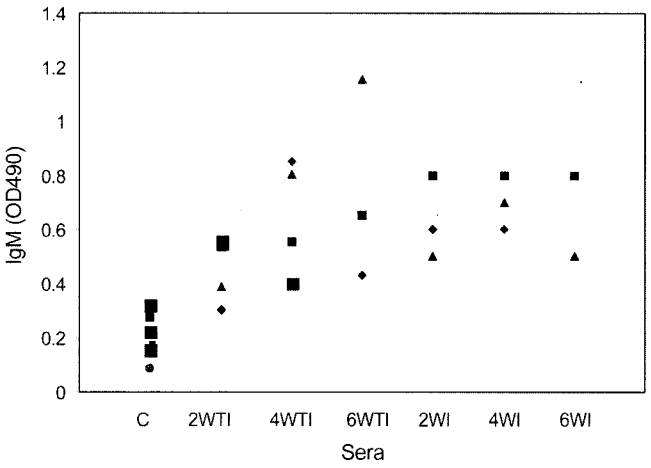


Fig. 5. Reactivity of IgG against *Schistosoma mansoni* soluble adult worm antigenic preparation (SWAP) in sera from treated, tail painted, mice with compound **2b** formulated in jojoba oil at different time points post infection. Sera collected from treated infected mice 2 weeks post infection (2WTI) showed 1.4 folds lower IgG reactivity when compared with sera collected from infected untreated mice at the same time point (2WI). At 4 weeks post infection, the treated infected mice (4WTI) showed 1 fold lower IgG reactivity to SWAP than the infected untreated (4WI) once. At 6 weeks, the IgG response in sera from treated infected mice was 1 fold higher than in those from untreated mice.

infected treated and infected untreated mice). Sera from TIM 2, 4, and 6WPI showed 1.3, 1.6, and 1.7 folds higher IgG reactivity, respectively against CAP than in those from IUM (Fig. 3).

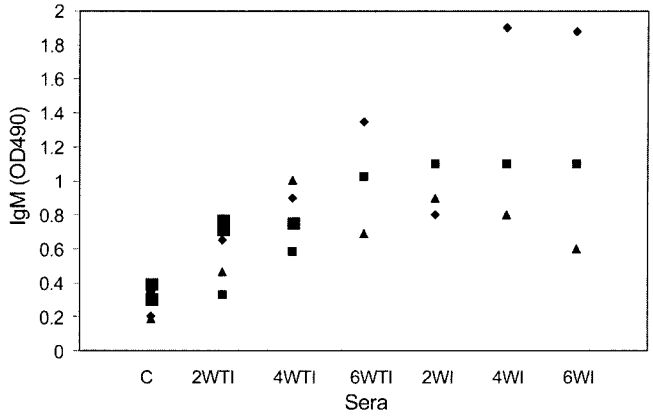


Fig. 6. Reactivity of IgM against *Schistosoma mansoni* soluble egg antigen (SEA) in sera from treated, tail-painted, mice with compound **2b** formulated in jojoba oil at different time points of post infection. Sera collected from treated infected mice 2 weeks post infection (2WTI) showed 1.6 folds lower IgM reactivity when compared with sera collected from infected untreated mice at the same time point (2WI). At 4 weeks post infection, the treated infected mice (4WTI) showed 1.6 folds lower IgM reactivity to SEA than the infected untreated (4WI) ones but the difference was not significant. At 6 weeks, the IgM response in sera from treated infected mice was 1.2 folds lower than in those from untreated mice.

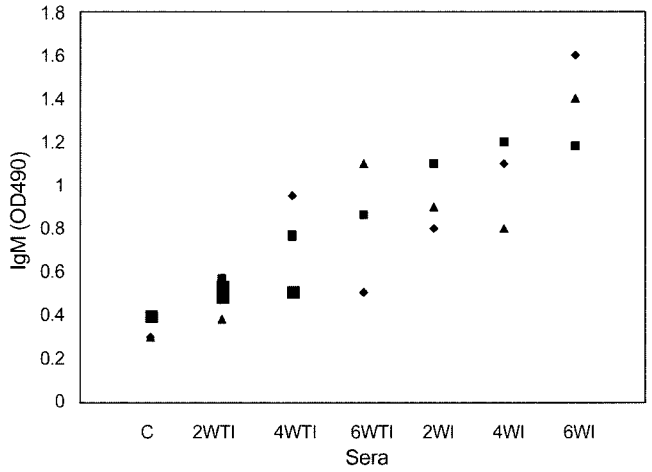


Fig. 7. Reactivity of IgG against *Schistosoma mansoni* soluble egg antigen (SEA) in sera from treated, tail-painted, mice with compound **2b** formulated in jojoba oil at different time points of post infection. Sera collected from treated infected mice 2 weeks post infection (2WTI) showed significantly, 1.9 folds lower (P-value<0.05) IgG reactivity when compared with sera collected from infected untreated mice at the same time point (2WI). At 4 weeks post infection, the treated infected mice (4WTI) showed 1.4 fold lower IgG reactivity to SEA than the infected untreated (4WI) once. At 6 weeks, the IgG response in sera from treated infected mice was 1.7 fold lower than in those from untreated mice.

Sera from TIM 2, 4, and 6WPI showed 1.5, 1.2 folds lower and 1.4 folds higher IgM reactivity, respectively against SWAP when compared with sera collected from

IUM (Fig. 4). Sera from TIM 2, 4, and 6WPI showed 1.4, 1 fold lower and 1 fold higher IgG reactivity, respectively to SWAP when compared with sera from IUM (Fig. 5). Sera from TIM 2, 4, and 6WPI had genraly lower IgM and IgG reactivities agaisnt soluble egg antigen (SEA) when compared with sera from IUM (Fig. 6 and 7). The IgM reactivity was 1.6, 1.6, and 1.2 folds lower while for IgG it was 1.9, 1.4, and 1.7 folds lower in TIM than IUM at 2, 4, and 6WPI, respectively and the difference was significant (P-value<0.05) for IgG at 2WPI.

DISCUSSION

In this report, we describe a new serine protease inhibitor that is able to kill 100% of CE the most essential if not the only enzyme responsible for cercarial penetration for human host skin (review: Ruppel *et al.*, 2004). We have employed a cercarial elastase, a previously characterized enzyme as serine protease that could be inhibited by known serine protease inhibitors (Bahgat and Ruppel, 2002).

The protease inhibitory effect for our new pyrimidine derivative, *N'*-(4-methylbenzylidene)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-sulfonylhydrazide (**2b**) is not surprising in the light of previous reports demonstrating that previously characterized pyrimidine derivatives can block several serine proteases like nifedipine, alkyl [sulfonyl(oxy)] uracils **1-2**, dihydrouracil, hexahydroimidazo [1,2-*c*]pyrimidine derivative, that could block leukocyte elastase (Riegel *et al.*, 1988; Groutas *et al.*, 1994; He *et al.*, 2000), (5-amino-6-oxo-2-phenyl-1,6-dihydro-1-pyrimidinyl)-acetyl-Phe-CF_3 and pyrimidinone derivatives that showed chymase inhibitory effect (Akahoshi *et al.*, 2001; Akahoshi, 2003). However, a novel finding of our present report is that when compound **2b** was formulated in jojoba oil, it efficiently blocked the cercarial penetration as indicated by 93% reduction in worm burden and complete absence of granuloma caused in the liver by the eggs of the parasite, *Schistosoma*. Although the described serine protease inhibitor in this report and its effect to block cercarial penetration are new but the approach is a continuation of earlier trials carried out to develop protease inhibitory formulations that can successfully block cercarial penetration both *in vitro* and *in vivo* (Cohen *et al.*, 1991; Berti and Lipsky, 1995; Misoire and Bucks, 1997; Niemiec *et al.*, 1997; Goldenberg, 1996; Fox, 1996; Lim *et al.*, 1999).

The purpose of using antigens derived from different parasite stages was to figure out if blockage of cercarial pentration that was expected to take place upon treatment of mice by the pyrimidine derivative due to its remarkable serine protease inhibitory effect will have an effect on the immune response to such antigens.

Comparing the IgM reactivity with CAP in sera collected from treated infected mice at regular time intervals of post infection to its reactivity in sera collected from infected untreated mice at the same time points showed that sera collected from treated infected mice (2 weeks post infection (2WTI)) possessed 1.2 folds lower IgM reactivity when compared with sera collected from infected treated mice at the same time point (2WI). This could be due to the fact that treatment retarded the penetration of cercariae and as a result lowered the primary immune response particularly at such an early phase of post infection.

At 4 weeks post infection, the treated infected mice (4WTI) showed 1.2 folds higher IgM reactivity to CAP than the infected untreated (4WI) ones. This could be due to an enhanced primary immune response due to chemical attenuation of some of the cercariae upon treatment. Those cercariae may have penetrated the mice skin and stimulated an increase in the IgM immune response among the treated infected mice when compared with the untreated infected ones.

At 6 weeks, the IgM response to CAP in sera from treated infected mice was 1.7 folds lower than in those from untreated mice. This reflects that although treatment led to attenuation of some cercariae and may have caused enhancement of the IgM response 4 weeks post infection, this was very transient and the shelf life of this IgM resposne was so short that it dropped at 6W in the treated infected mice when compared with the untreated infected mice.

Upon conducting the similar comparison on the IgG level showed that sera collected from treated infected mice 2, 4, and 6 weeks post infection generally showed higher IgG reactivity when compared with sera collected from infected untreated mice at the same time point. This confirms our conclusion that treatment with our newly identified protease inhibitor caused attenuation of some of the penetrating cercariae which in turn stimulated the persistent IgG (secondary immune) resposne to cercarial antigens at different time intervals of post infection in comparison to the IgG reactivity in the infected untreated mice.

Measuring both IgM and IgG reactivities agaisnt SWAP in sera collected from treated infected mice at regular time intervals of post infection to its reactivity in sera collected from infected untreated mice at the same time points revealed that sera collected from treated infected mice 2 weeks and 4 weeks post infection had lower IgM and IgG reactivities to SWAP than the infected untreated ones at the same time points. This could be due to the fact that attenuation of the cercariae caused by skin treatment may have caused retardation in the development of cercaraie into schistosomulae and juvenile worms and as a result led to retardation of the developed IgM and IgG responses to the adult worm antigens among sera from treated mice

when compared with those of untreated ones. At 6 weeks, the juvenile worms whose development was retarded at early stages of infection may have started to accumulate and as a result it could have stimulated an accumulative higher IgM and IgG responses that was detected by worm antigens in sera from treated mice as compared with the untreated ones.

Both IgM and IgG reactivities to soluble egg antigen were generally lower in sera collected at different time intervals from treated infected mice when compared to their reactivities in sera collected from infected untreated mice at the same time points. This does makes a lot of sense, since we have previously shown in the perfusion results that the liver of the treated mice was free from granuloma, which means that there were no detectable eggs in their liver. Besides, the absence of the immune reactions with the egg antigens reflects the retardation of the sexual maturiry of the remaining developing worms (7%, see the curve of the perfusion results), if any was still their, coming from the attenuated penetrated cercaraie in case of treated infected mice.

All present investigation so far indicates that our newly described serine protease that has a pyrimidine nucleus can successfully block cercarial penetration due to its serine protease and cercarial elastase inhibitory effects, and this blockage could be traced not only by perfusion results but also by a drop in the immune reactions against antigens derived from late stages of the parasite.

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