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Study of the Efficacy of Cysteine Protease Inhibitors Alone or Combined with Praziquantel as Chemotherapy for Mice *Schistosomiasis mansoni*

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***Abstract*—**This study was designed for assessment of 3 types of Cysteine protease inhibitors (CPIs) fluromethylketone (FMK), vinyl sulfone (VS) and sodium nitro prussid (SNP), to define which of them is the best for curing *S. mansoni* infection in mice? *In vitro*, treated *S. mansoni* adult worms recorded a mortality rate after 1 hr of exposure to 500 ppm of FMK, VS and SNP as 75, 70 and 60%, respectively. FMK+PZQ treatment recorded the maximum reduction in worm burden (97.2% at 5 wk PI). VS treatment alone or combined with PZQ increases IgM, total IgG, IgG2 and IgG4 levels. In EM study, the completely implanted spines were reported in the degenerated tegument of adult worms in all groups treated with CPIs. VS+PZQ Treatment increased Igs levels but, its effect was different on worm reduction. So, it is not enough to eliminate the infection and FMK+PZQ considered the antischistosomicidal drug of choice.

***Keywords*—**Praziquantel, fluromethylketone, vinyl sulfone, sodium nitro prussid.

I. Introduction

SCHISTOSOMIASIS is a common parasitic disease, in developing countries with a tropical or subtropical climate [1], [2]. Young children are especially vulnerable to infection because of their hygiene and play habits, and the symptoms are quite harmful to them, impairing learning ability and physical development, and even sometimes causing death [3].

The tegument coats the worm and acts as a physical barrier to host antibodies and complement. The tegument of schistosomula and adult worms is intriguing. It includes a single multinucleated cytoplasmic layer (syncytium) that covers the entire worm and is linked to underlying nucleated cell bodies by cytoplasmic connections that span the musculature [4].

New initiatives are aiming to reduce the global burden of schistosomiasis [5]. This can be achieved through health education [6], snail eradication and chemotherapy, the latter is considered as the most effective in this respect [7]. Treatment of schistosomiasis has two main arms; to prevent or reduce further tissue damage in infected individuals and to reduce egg excretion and consequently transmission in community [8]. Chemotherapy is the most effective method for the short term control, but it is alone insufficient to control human pathology [9]. Thus, research has to be continued towards the development of a new antischistosomal drug that is efficacious against all human schistosome species, exhibits high levels of activity against adult and Juvenile stages of parasites, has a good safety profile and is reasonably priced [10], [11].

Proteases or proteolytic enzymes catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteases were first organized as clans and families by Rawlings and Barrett [12]. CPIs are medically important and they are possible targets for therapy. Proteases are involved in many important biological processes including protein turnover, blood coagulation, complement activation hormone processing [13], and cancer cell invasion [14], thus, they are frequently chosen as targets for drug design and discovery. CPIs block parasite replication and differentiation, providing an alternative to traditional therapy in drug-resistant organism [15].

This study was designed for assessment of 3 types of CPIs (FMK, VS and SNP) to define, which of them is the best choice? The experiments aimed to define the protective power of each inhibitor alone or combined with PZQ to increase its efficacy for curing *S. mansoni* infection in mice.

II. Materials and Methods

Animals - Male C57BL/6 strain, albino mice, 6 to 8 wk old (18 ± 20 gm), clean from parasitic infection, were obtained from Schistosome Biological Supply Centre (SBSC), Theodor Bilharz Research Institute (TBRI). Animals were maintained under standard laboratory care (controlled temperature and light environment); and were given filtered drinking water and balanced diet. Mice were infected with 100 cercariae of the Egyptian strain of *S. mansoni* supplied from SBSC, TBRI by tail immersion method [16]. Animals' experiments were conducted in the animal unit according to the internationally valid guidelines and ethical conditions.

Experimental Procedures

In vitro Studies

To study the effect of the 3 CPIs on the viability and motility of different life cycle stages of *S. mansoni* (cercariae and adult worms).

- Different concentrations of each CPI (62.5, 125, 250 and 500 ppm) were added individually in Petri dishes and incubated at 37ºC for 24hr.
- In parallel, 50 *S. mansoni* cercariae and adult worms were cultured in 10ml dechlorinated tap water and then incubated at 37ºC for 24hr (serves as negative control).

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- The motility, viability and mortality of cercariae or adult worms were recorded using stercomicroscope (Zeisis, West Germany) at ½, 1, 1½, 2, 3 and 24 hr of incubation.

In vivo Study

Drugs - The animals were giving 500 mg/kg bwt of PZQ [17], [18] while, the FMK, VS [19] and SNP [20] were administered orally in a dose of 50 mg/kg bwt /mouse.

Experimental design - 450 Albino mice were infected individually with 100 *S. manasoni* cercariae and divided into groups.

Group A: Mice were infected and left untreated as infected control.

Group B: Mice were treated with PZQ for 2 consecutive days at 3 and 5 wk PI.

Group C: Mice were treated with FMK for 14 consecutive days at 3 and 5 wk PI.

Group D: Mice were treated with VS for 14 consecutive days at 3 and 5wk PI.

Group E: Mice were treated with SNP for 14 consecutive days at 3 and 5 wk PI.

Group F: Mice were treated with FMK for 14 consecutive days and PZQ for 2 consecutive days at 3 and 5wk PI.

Group G: Mice were treated with VS for 14 consecutive days and PZQ for 2 consecutive days at 3 and 5wk PI.

Group H: Mice were treated with SNP for 14 consecutive days and PZQ for 2 consecutive days at 3 and 5 wk PI.

Parasitological Studies

Determination of the worm burden by hepatic and mesenteric perfusion - All animal groups were sacrificed at 8 wk PI and the mice were assayed for worm burden count according to the method of Yoles et al. [21].

Immunological studies

Determination of different immunoglobulin(Igs) isotypes levels in serum of different treated groups by using enzyme-linked immunoasorbent assay (ELISA) - ELISA has become the most common immunoassay used in research laboratories for its great sensitivity, specificity and safety in measuring antigens or antibodies. It was first introduced by Engvall and Perlman [22] and used to evaluate levels of anti-schistosome Igs. Serum of normal and treated mice was collected for ELISA test. Wells of the polysterene 96-well microtitre plates (Combridges, MA, USA) were coated with 100 µg SEA (given by immunology lab TBRI, Giza, Egypt) in coating buffer. Peroxidase-labeled affinity purified antibodies to mouse IgM and IgG absorbed with human serum produced in goat (Kirkegaard and Perry laboratories Inc. Pharmingen (San Diego, CA, USA). The absorbance was read spectrophotometrically at 405nm using microplate ELISA reader (Lab syst., Helsiniki, Finland).

Electron microscope (EM) studies - The collected worms were postifixed in 2% osmium tetraoxide, dehydrated with ascending concentrations of alcohol and embedded in epoxy resin according to the technique of Grimaud et al. [23]. Semi-thin and utra-thin sections were cut with a leika ultra microtome. Ultra-thin sections were contrasted with uranyl acetate and lead citrate stains then examined by Phillips (EM 208 Electron Microscope).

III. Results

A. In vitro Study

Effect of CPIs on the S. mansoni Life Cycle Stages

Cercaria - 500 ppm concentration of FMK, VS and SNP CPIs recorded the highest mortality rate on *S. mansoni* cercariae after 1hr exposure (75, 60 and 50) and 2 hr (90, 80 and 85) for FMK, VS and SNP, respectively. 100% mortality rate was obtained after 3hr exposure for all 3 types of CPIs inhibitors (data not shown).

Adult worms - Treated adult *S. mansoni* worms recorded higher mortality rate after 1 and 2 hr of exposure to the same concentration (500 ppm) of FMK, VS and SNP as 75%, 70%, 60% and 90%, 90%, 80%, respectively.

FMK inhibitor recorded the highest mortality rate even after only ½ hr of exposure for both cercariae and adult worms (data not shown).

B. In vivo Study

Worm burden and distribution - The three CPIs treatment of *S. mansoni* infected mice were significantly ($P<0.001$) less effective on worm burden than PZQ treatment (Table I). It was noticed that PZQ has a great effect when administered at late stage of infection at 5 wk PI on the worm burden percent reduction (PR).

On the other hand, treatment using combination of CPIs and PZQ at 3 wk PI, worm burden was reduced by 77% on using FMK ($P<0.001$) (group F), 75% in the case of VS ($P<0.001$) (group G) and 72% with SNP ($P<0.001$) (group H) in comparison to the infected untreated control group.

While treatment of infected mice with CPIs combined with PZQ recorded a highly significant PR of worm burden ($P<0.001$) by 97% on using FMK (group F) at 5 wk PI, 95% in the case of VS ($P<0.001$) (group G) and 91% on using SNP (group H). CPIs, when administrated in combinations with PZQ, showed a nonsignificant difference referring to PZQ alone. Except, for FMK combined with PZQ, (group F) had a significant ($P<0.05$) higher PR more than mice received PZQ treatment alone.

Measurement of different Igs isotypes - Tables (II and III) summarize the data of all measured Igs to different mice groups, as mean ± standard deviation (M ± SD) and difference percentage (% Diff.) were calculated referring to the results of infected control (group A). Infection causes a significant ($P<0.001$) slight increase in IgM level (33.3% and 35.7%), remarkable increase in IgG2 level (49.1% and 46.1%) and IgG4 level (50% and 52.5%). While there is a vigorous increase only in total IgG level (78.6% and 72.9%) in both infected untreated groups of mice (only administrated saline at 3 or 5 wk PI, respectively).

TABLE I
EFFECT OF CPI (FMK, VS AND SNP) TREATMENT OF *S. MANSONI* INFECTED MICE ALONE OR IN COMBINATION WITH PZQ ON THE WORM BURDEN AT 3 AND 5 WK PI

Mice Groups		Worm burden			
		3 wk		**5 wk**	
		Mean ± SD	**PR**	**Mean ± SD**	**PR**
A	Infected control	25.20±0.44		25.60±1.40	
B	PZQ	8.30±0.81***	67%	2.80±0.21***	89%
C	FMK	12.6±1.20***#	50%	9.00±0.11***(###)	65%
D	VS	13.9±0.19***##	45%	10.20±0.17***(###)	60%
E	SNP	14.60±1.15***###	42%	11.00±0.13***(###)	57%
F	FMK and PZQ	5.80±0.08***	77%	0.80±0.16***(#)	97%
G	VS and PZQ	6.30±0.12***	75%	1.20±0.20***	95%
H	SNP and PZQ	7.10±0.16***	72%	2.30±00.19***	91%

Significance referred to control (T-test), # Significance referred to PZQ (Chi^2- test), N=30

TABLE II
DETERMINATION OF DIFFERENT SERUM IMMUNOGLOBULINS ISOTYPES OF *S. MANSONI* INFECTED MICE TREATED WITH FMK, VS AND SNP ALONE OR IN COMBINATION WITH PZQ AT 3 WK PI

Mice Groups		Immunoglobulin Isotypes							
		IgM		**Total IgG**		**IgG2**		**IgG4**	
		M±SD	**%Diff**	**M±SD**	**%Diff**	**M±SD**	**%Diff**	**M±SD**	**%Diff**
	Control (Uninfected)	0.34±0.23		0.31±0.09		0.29±0.18		0.32±0.03	
A	Infected control	0.51±0.20###	33.3	1.45±0.22###	78.6	0.57±0.13###	49.1	0.62±0.23###	50
B	PZQ	0.69±0.23	26.1	1.85±0.14	21.6	0.99±0.19	42.4	1.25±0.24	50.4
C	FMK	0.73±0.19***	30.1	1.74±0.27***	16.7	0.91±0.23***	37.3	1.01±0.18***	38.6
D	VS	0.82±0.27***	37.8	1.63±0.13***	11.1	0.98±0.13***	41.8	1.52±0.29***	59.2
E	SNP	0.55±0.19-	7.2	1.53±0.17***	5.2	0.67±0.30-	14.9	1.36±0.22***	54.4
F	FMK and PZQ	0.74±0.20***	31.1	1.91±0.12***	24.1	1.11±0.23***	48.6	1.46±0.21***	57.5
G	VS and PZQ	1.01±0.21***	49	1.83±0.23***	20.8	1.50±0.24***	62	1.72±0.24***	63.9
H	SNP and PZQ	0.71±0.25***	28.2	1.52±0.10-	4.6	0.81±0.30**	29.6	1.26±0.22***	50.7

*Significance referred to control (T-test), # Significance referred to PZQ (Chi^2-test), N=30.

TABLE III
DETERMINATION OF DIFFERENT SERUM IMMUNOGLOBULINS ISOTYPES OF *S. MANSONI* INFECTED MICE TREATED WITH FMK, VS AND SNP ALONE OR IN COMBINATION WITH PZQ AT 5WK PI

Mice Groups		Immunoglobulin Isotypes							
		IgM		**Total IgG**		**IgG2**		**IgG4**	
		M±SD	**%diff**	**M±SD**	**%diff**	**M±SD**	**%diff**	**M±SD**	**%diff**
	Control (uninfected)	0.27±0.22		0.32±0.12		0.21±0.18		0.29±0.23	
A	Infected control	0.42±0.19###	35.7	1.18±0.13###	72.9	0.39±0.12###	46.1	0.61±0.21###	52.5
B	PZQ	0.78±0.12	46.1	1.598±0.23	26.1	0.89±0.11	56.1	1.045±0.21	41.6
C	FMK	0.61±0.2***	31.1	1.43±0.22***	17.5	0.88±0.22***	55.6	0.92±0.19***	33.7
D	VS	0.80±0.21***	47.5	1.56±0.15***	24.4	0.71±0.19***	45.1	1.34±0.25***	54.5
E	SNP	0.46±0.19	8.6	1.36±0.18***	13.2	0.56±0.21***	30.4	1.12±0.19***	45.5
F	FMK and PZQ	0.89±0.23***	52.8	1.66±0.14***	29.3	0.91±0.19***	57.1	1.29±0.22***	52.7
G	VS and PZQ	0.98±0.21***	57.1	1.79±0.27***	34.1	1.38±0.24***	71.7	1.52±0.25***	59.9
H	SNP and PZQ	0.69±0.30***	39.1	1.48±0.10***	20.1	0.98±0.32***	60.2	1.06±0.232***	42.4

*Significance referred to control (T-test), # Significance referred to PZQ (Chi^2-test), N=30.

IgM level - It was clear that VS treatment stimulated an enhancement of primary immunization isotype (IgM) at 3 wk PI recording the highest significant (P<0.001) difference percentage alone or combined with PZQ (37.8% and 49%, respectively).

Also, when the mice treated at 5 wk PI, VS was the effective immune enhancer recording 47.5% and 57.1% at combination with PZQ, which recorded only 46.1% when administered alone as a nonsignificant elevation of IgM level, while FMK recorded a significant (P<0.001) increase when combined with PZQ (52.8%).

IgG2 level - The effect of CPIs was clear earlier at 3 wk PI on the IgG2 level. VS treatment alone recorded nearly the same increase in IgG2 secretion (41.8%) as that stimulated by PZQ (42.4%). The combination between VS and PZQ recorded the best result, it stimulated the IgG2 secretion recording a highly significant data (P<0.001), 62% at 3 wk PI and 71.7% at 5 wk PI.

IgG4 level - Earlier, at 3 wk PI treatment of PZQ recorded a moderate nonsignificant elevation of IgG4 level (50.4%) as a differential percentage referring to the data of infected untreated group.

On the other hand, VS again recorded a good indication as an immune-enhancer treatment at 3 wk PI, increasing significantly (P<0.001) the IgG4 level when administered individually (59.2%) or combined with PZQ (63.9%). Also, VS administration alone or combined with PZQ at 5 wk PI causes significant increase in IgG4 secretion (54.5 and 59.9, respectively).

FMK played a significant role when given to mice at 3 wk PI (P<0.001) on elevating IgG4 level only when combined with PZQ recording 57.5%. When mice were treated at 5 wk PI, FMK had a little effect on the IgG4 level when administered alone (33.7%), but it recorded a significant (P<0.001) high effect when given in combination with PZQ (52.7%).

SNP recorded a significant increase of IgG4 when administrated individually or combined with PZQ at 3 wk PI (54.4% and 50.7%, respectively). But, it recorded a moderate significant (P<0.001) increase of IgG4 when treatment was given at 5 wk PI individually and in combination with PZQ, (45.5% and 42.4% respectively).

EM studies - The tegument of untreated *S. mansoni* adult worm revealed pointed spine on normal tegument (Fig. 1 (a)) while, treatment with PZQ (group B) revealed partially implanted spine (Fig. 1 (b)). There was a completely implanted spine in degenerated tegument of the worms in all infected animals groups treated with CPIs (FMK, VS and SNP) alone (Figs. 1 (c)-(e)). On the other hand, infected mice treated with combination of CPIs and PZQ show degenerated spines and severe degenerated teguments figures (Figs. 1 (f)-(h)). Partially implanted and degenerated spines were noticed in tegument of *S. mansoni* adult worms of PZQ treated mice at 5 wk PI (group B) (Fig. 2 (a)). While, the degeneration extended to muscle layer of adult worm in all groups that orally treated with the CPIs alone (Figs. 1 (b)-(d)). Electron micrographs of tegument of *S. mansoni* adult worms in mice groups treated with CPIs in combination with PZQ at 5 wk PI (group F, G and H) were not available, because there was a complete eradication of adult worms in these groups.

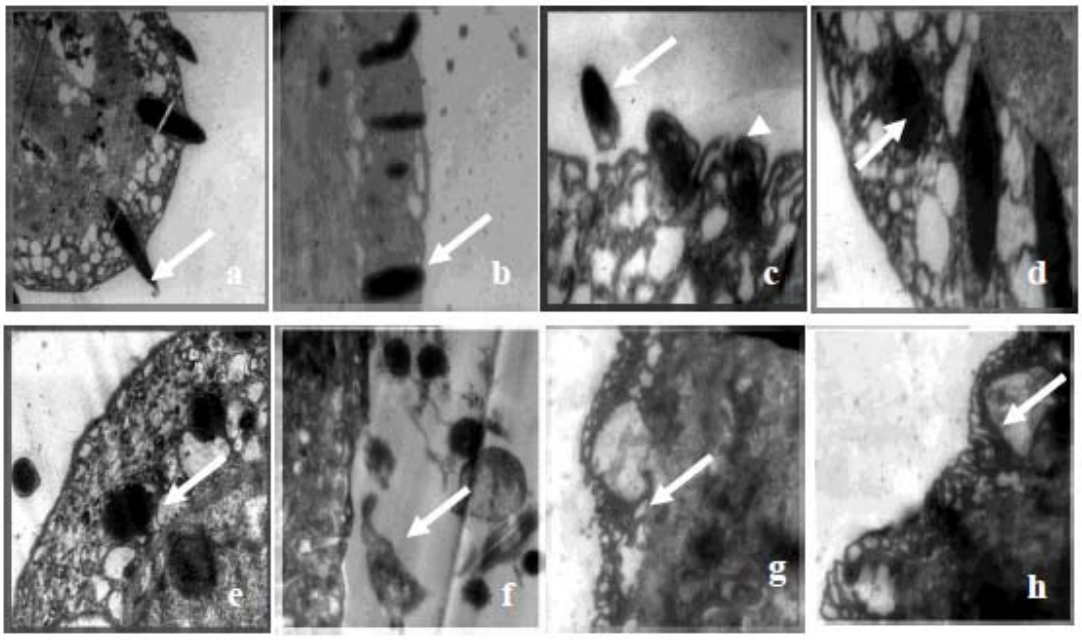

Fig. 1 Electron micrograph of *S. mansoni* worm tegument of (a) Infected untreated mice revealed pointed spines (arrow) (X1800); (b) PZQ treated mice showing partially implanted spines (arrow) 3 wk PI (X2000); (c) FMK treated mice showing detachment (arrow) and implanted spines in (arrow head) degenerated tegument 3 wk PI (X2000); (d) VS treated mice showing completely implanted spines (arrow) in degenerated tegument 3 wk PI (X2000); (e) SNP treated mice at 3 wk PI showing completely implanted spines (arrow) (X2000); (f) FMK & PZQ treated mice at 3 wk PI showing tegument degeneration (arrow) (X 8500); (g) VS & PZQ treated mice at 3 wk PI showing severely degenerated teguments and the spines couldn't be detected (arrow) (X35000); (h) SNP & PZQ at 3 wk PI showing completely degenerated spines within the teguments (arrow) (X35000)

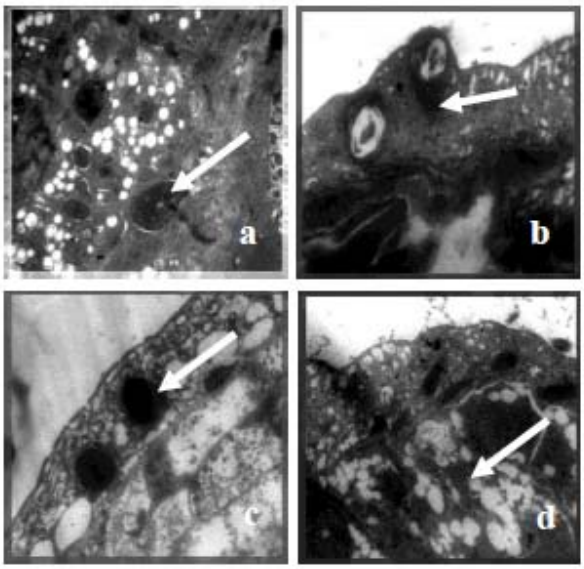

Fig. 2 Electron micrograph of *S. mansoni* adult worm tegument of (a) PZQ treated mice at 5 wk PI showing partially implanted and degenerated spines (arrow) (X2000); (b) FMK treated mice at 5 wk PI showing degeneration of tegument with completely implanted and degenerated spines (arrow) (X2000); (c) VS treated mice at 5 wk PI showing completely implanted spines (arrow) in the degenerated teguments and muscle layers (X2000); (d) SNP treated mice at 5 wk PI showing severally degenerated tegument and muscle layers with completely implanted and degenerated spines (arrow) (X2000)

IV. Discussion

Schistosomiasis is the second most significant parasitic disease in the world after malaria in terms of sosioeconomic and public health importance. It is estimated that 207 million people are infected in 74 countries and more than 779 million people are at risk of infection, with mortality estimated up to 280 thousands deaths annually in sub-Saharan Africa alone [24].

Protease inhibitors are key components of human immune-deficiency virus therapy [25]. Also, CPIs have a demonstrated activity in a variety of parasitic models, for example, FMK had been shown to cure murine malaria [26], VS worked in animal models of Leishmaniasis and Chagas disease [27], and CPIs are reported to arrest or cure murine schistosomiasis [28].

So, this study was designed for assessment of 3 types of CPIs, to define which of them is the best choice? The experiments aimed to define the protective power of each inhibitor alone or combined with PZQ to increase its efficacy for curing *S. mansoni* infection in mice. The 3 CPIs included in this study were FMK, VS and SNP. Animals were separated into 2 control groups [normal healthy and infected untreated (group A)] and infected groups treated with PZQ (group B), FMK (group C), VS (group D), SNP (group E), FMK+PZQ (group F), VS+PZQ (group G) and SNP+PZQ (group H). Each group contains 2 subgroups according to the treatment schedule, treatments was at 3 or 5 wk PI. At 8 wk PI, all groups were going to evaluation of parasitological, immunological and histological parameters.

Olson et al. [29] studied the effect of FMK and VS on mouse malaria model. In studies with a FMK; they reported that orally administered morpholine urea – phenylalanine – homophenylalanine - fluoromethyl ketone delayed the progression of murine malaria. Also, in studies of a new series of VS, a set of related compounds demonstrated marked inhibition of falcipain and of parasite biological activities *in vitro.* One of these compounds (*N*-methyl piperazine urea-leucine-homophenylalanine-2-aphthalene VS) cured about 40% of mice when administered orally twice-a-day for four days.

Utzinger et al. [30], [31] found that the treatment of *S. japonicum* infected hamsters with single oral dose of ART (120 mg/kg) 42 days PI induced a reduction of total worm burden by only 33%. These results depicted are also quite comparable to that obtained by Xiao et al. [32] who recorded PR= 30-51% in 35-56 day old adult worms and Mahmoud and Botros [33] work which recorded PR= 57% of 6 wk old worms. The variation of these results could be attributed to the difference in the dose and route of treatment.

Helmy et al. [34] were inagreement with this study; they reported the assessment of anti-*Fasciola* activity of VS *in vitro*, against adult *F. gigantica* worms using a well-established culture medium by measurement of viability and motility. They recorded that, 300 ppm concentrations of VS causing immediate death of adult worms, while it exhibited minimum effective concentrations of 50 ppm after 12hr.

In another study, *S. mansoni* adult worms were treated with phenyl vinyl sulfone for incubation periods ranging from 0 to 12hr and its activity was assessed in terms of viability, motility and death of worms. VS exhibited a minimum effective concentration of 50 ppm after 12hr. 300 ppm concentrations were most potent causing immediate death of adult flukes *in vitro* [19].

Animals that were treated with FMK at doses of 100 or 200 mg/kg/day demonstrated antimalarial effects and not toxic but not full protective. These animals developed malaria, which

was eventually lethal, but the disease progressed more slowly in treated animals than in controls. Higher dose (300-400 mg/kg/day) had a considerably greater antimalarial efficacy but theses doses were not tolerated and developed anorexia [29].

Copo et al. [35] reported that the activity of secreted *T. foetus* CPIs in culture supernatants was decreased in the presence of VS inhibitors. Inhibitor K11777 reduced the *in vitro* cytopathogenic effects of *T. foetus* in bovine fetal trophoblast cells, which are relevant target cells since, this pathogen interferes with pregnancy. Therefore, VS inhibitor reduces several effects of *T. foetus*-secreted CPIs, including cytotoxicity on relevant target host cells and genital infection in a murine model.

The current study investigated three specifically designed CPIs that were selected to explore their role in treatment of *S. mansoni* infected mice. The *in vitro* study is considered as an indicator of the evaluation of the schistosomicidal properties of the tested component *in vitro.* The data revealed that, treated *S. mansoni* adult worms were died after 1hr of exposure to 500 ppm of FMK, VS and SNP by a mortality rate (75%, 70% and 60%, respectively), while the 3 CPIs gave 100% mortality after 3hr. at concentration 500 ppm with comparison to control group. Also, treated cercariae recorded 75%, 60% and 50% mortality after 1hr exposure (at concentration 500 ppm) to FMK, VS and SNP, respectively and mortality reached 100% after 3 hr upon exposure to the same inhibitors at the same concentration with comparison to control group. The mortality rate increases with increasing of both Lc and duration of exposure.

Lescana et al. [36] evaluated the effect of intramuscular injection of artemether in mice experimentally infected with *S. mansoni*, at the time of infection, during schistosomula maturation and after the beginning of egg-laying. They found that a reduction in egg-laying and the number of worms recovered was observed in mice treated with artemether (50 or 100 mg/kg) on the 20th day PI. The decrease in the number of worms was more noticeable among *S. mansoni* females.

Similar findings have been reported by Neves et al. [37] and da Silva et al. [38]. They found that, LPSF-PT05 [3 -benzyl - 5 - (4 - chloro-arylazo) - 4- thioxo - imidazoldire - 2 - one], showed higher activity *in vitro* against adult *S. mansoni* worms with 100% mortality after 24hr of contact at all concentrations tested and the effect obtained showed a dose defendant relationship.

In another study, mated males and females of *S. mansoni* were kept in RPMI-1640 medium, along with penicillin/ streptomycin, in a 5% CO_2 and 37ºC incubator. The results of these tests showed schistosomicidal activity in the samples during the observation period, which lasted 72hr. The activity of artemisinin and artesunic acid in adult couples of the BH strain of *S. mansoni*, native of Belo Horizonte (-19°55'15"/-43°56'16"), Brazil, by means of *in vitro* tests has compared 22 hr after the addition of the two compounds, death was observed in all concentrations tested [39].

And this was in agreement with Gamboa and Rosenthal [40] and Olson et al. [29] where, they suggested that the marked potency of FMK against malaria parasite. FMK delayed malarian progression and cured about 40% of malaria infected mice when administered orally twice a day for 4 days.

Also, Engel et al. [27] reported that FMK CPI treatment rescued mice from the acute phase of a lethal experimental *T. cruzi* infection and cleared parasitemia in choronically infected mice without toxicity to the mammalian host. These results provided an important proof of concept for the development of CPIs as chemotherapy for a number of diseases including cancer cell invasion, inflammation and *schistosomiasis*.

Abdulla et al. [20] added that the significant reduction in parasite burden and pathology by this VS CPI validates *Schistosome* cysteine proteases as drug targets and offers the potential of a new direction for chemotherapy of human schistosomiasis.

Neves et al. [37], da Silva et al. [38] and Pitta et al. [41] reported that, oral doses of 100 mg/kg of the three formulations of LPSF-PT05 was used to treat *S. mansoni* infected mice, the average worm burden was significantly lower than in control group with a PR ranging from 19.8% to 70.5%.

Low doses of PZQ and oxamniquine against different development stages of *S. mansoni* showed only slightly higher PR of worm burden than those achieved with PZQ administrated alone at a curative dose [42].

The combination regimen showed the highest efficacy when were administrated at 5 wk PI resulted, worm burden reduction was 42%, which was marginally higher than that achieved with PZQ or oxamniquine monotherapy at either a curative dose [24].

The oral treatment of infected mice with 500 mg/kg PZQ for 2 consecutive days at 2 time intervals, 3 and 5 wk PI, worm burden was reduced by 67% and 89.2%, respectively. This indicates that PZQ has a maximum effect on the adult stage of *S. mansoni* worms which occur at 5 wk PI. As regards FMK, the reduction in the number of *S. mansoni* adult worms was started when administrated earlier at 3 wk PI. Worm burden was reduced by 50%, 45% and 42% on using FMK, VS and SNP respectively. The worm burden reduction was increased in treatment at 5 wk PI, where it was 65% on using FMK, 60% on using VS and 57% with SNP.

In agreement with others by using a combination between the tested CPI and PZQ at 5 wk PI, it was obvious that worm burden was highly reduced by 97% on using FMK, 95% in the case of VS and 91.1% on using SNP, respectively. It was clear that the combination between CPIs and PZQ has a promising effect on *S. mansoni* adult worms.

In another study, naïve mice were immunized intravenously with 10 ug of SEA in three doses at 2 days intervals 6 wk before infection. Three groups of mice were infected with 100 cercariae, and treated with PZQ, PTX or silymarin administered alone. Another 2 groups were treated with PZQ combined with PTX or silymarin. All treated animals and respective controls were sacrificed 12 wk PI [43]. They reported that PR in worm burden was associated with reduction in ova count and changes in oogram pattern which

were mainly due to PZQ treatment.

The present study measured different Igs isotypes levels. It was found that, *S. mansoni* infection itself causes a significant (P<0.001) slight increase in IgM level (33.3% and 35.7%), remarkable increase in IgG2 level (49.1% and 46.1%) and IgG4 level (50% and 52.5%), while there is a vigorous increase only in total IgG level (78.6% and 72.9%) in both infected untreated groups of mice only administered saline at 3 or 5 wk PI, respectively. VS treatment caused an enhancement of primary immunization isotype (IgM) at 3 wk PI recording the highest significant (P<0.001) difference percentage alone or combined with PZQ (37.8% and 49%, respectively). Also, combination between VS and PZQ was the best in stimulating the IgG2 secretion recording a highly significant data (P<0.001) (62% at 3 wk PI and 71.7% at 5 wk PI). Again, VS recorded a very good signs as immune-enhancer treatment increasing significantly (P<0.001) IgG4 level when administered individually (59.2%) or combined with PZQ (63.9%) at 3 wk PI. FMK played a significant role (P<0.001) on elevating IgG4 only when combined with PZQ at 3 wk PI recording 57.5%.

It is obvious that, treatment with VS combined with PZQ increased Igs levels when administered at both 3 and 5wk PI. But, its effect was different on the other measured parasitological parameter. Thus the CPIs, which enhanced the immune stimulatory function, were not enough to eliminate the infection. So, it is better to choose CPIs which has a direct effect on the parasite at its different life cycle stages.

These results were in agreement with Boctor and Peter [44]; they showed that total IgG of the *S. mansoni* infected patients was elevated in the range of two to three times above normal. The magnitude of increase differed markedly among the four subclasses of IgG. The IgG1, IgG2 and IgG3 concentrations were approximately two to four times higher than normal, whereas the IgG4 concentration was 20 times normal (9000 mg/*I*).

Rabia et al. [43] reported that, the elevation of Igs and cytokines levels were observed in *S. mansoni* infected mice treated with PZQ alone or combined with PTX or silymarin.

Nagy et al. [45] tested the subcutaneous injection of 50 µg protein of nucleo protein vaccine of *B. alexandrina* followed by another inoculation 15 days later. The data showed that total serum IgE and tumor necrosis factor-Alfa (TNF-α) were significantly increased.

In this study, as regards to the effect of the 3 inhibitors (FMK, VS and SNP) on worm tegument, a completely implanted spine was reported in the degenerated tegument in all animal groups treated earlier with CPIs alone at 3 wk PI. But, the degeneration extended to muscle layer in all groups that orally treated with the inhibitors alone at 5 wk PI. Meanwhile, only detachment of spines was observed in PZQ treated group at 5 wk PI. The results of CPIs treatment at 5 wk PI in combination with PZQ were not shown because all worms were nealy eradicated (PR>90%). The adult *S. mansoni* worms were found with completely damaged tegument and the muscle layer beneath. So it was difficult to be examined.

These results were confirmed by Helmy and his colleages [34]. They reported that the histological study of VS CPI *in vitro* on *Fasciola gigantia* worms showed that there was tegmental flattening, rupture of vesicle and spine loss. So, these results were in agreement with EM examination in the current study.

Neves et al. [37], da Silva et al. [38] and Pitta et al. [41] reported that EM observation of the derivative LPSF- PT05 revealed alteration in the teguments surface of *S. mansoni* worms with the formation of bubbles and peeling, indicating damage to cells; the magnitude of effect was directly relate to the duration of exposure (*in vitro*). These results support the findings in this study which confirm the highly antischistosomal effect of FMK on *S. mansoni* adult worm tegument.

Kerr et al. [46] reported the crystal structures of three major parasites cystein proteases, cruzain, falcipain-3 and the first reported structure of rhodesain, in complex with a class of potent, small molecule, CPIs, and the potential of VS, which are antiparasitic drugs.

In conclusion, treatment with PZQ in context with FMK, VS, or SNP as examples to CPIs resulted in significant reduction of parasitological parameters and raise of different Igs isotopes level.

Although, there is a great effect of VS treatment on the increasing the Igs level when administered in combination with PZQ at 3 or 5 wk PI. But, it has not the same highly positive parasitological effect on the worm burden pattern. The data of this study indicated that the treatment which has only a stimulatory enhancing effect on the immune response by increasing Igs level is not powerful enough to eradicate the parasitic infection. The optimum treatment should also has a direct action on the parasite itself at different life cycle stages. In conclusion, FMK with PZQ is considered as the anti-schistosomicidal drug of choice between three CPIs in this study. It indicates its safety and powerful efficacy to eradicate the *S. mansoni* infection with a promising percentage.

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