Biomedicine & Pharmacotherapy 99 (2018) 570–575

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# A single dose of the antineoplastics hydroxyurea or cisplatin has praziquantel-like effects on *Schistosoma mansoni* worms and host mouse liver

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ARTICLE INFO

*Keywords:*
Antischistosomal antineoplastic
Schistosoma stem cells
Neoblast
Egg-induced liver granuloma
Hydroxyurea
Cisplatin

ABSTRACT

Increasing resistance to praziquantel, the only available antischistosomal drug, is always developed by schistosomes. The recent description of stem cell-like neoblasts in schistosomes led to the idea of applying antineoplastic drugs as antischistosomal agents that may inhibit stem cell divisions and retard worm regeneration. Here, we explored the *in vitro* and *in vivo* effect of some antineoplastic drugs on *S. mansoni* worm and the host mouse liver. *S. mansoni* worms' viability was tested after exposure to either praziquantel or one of the antitumor drugs (hydroxyurea, cisplatin, methotrexate, and colchicine) *in vitro* for 24 and 48 h. The effect of two of them (hydroxyurea and cisplatin) on worm burden, tegument ultrastructure, and host liver structure and function was tested *in vivo* in *S. mansoni*-infected mouse model. All drugs affected variably the worm burden *in vitro*. Hydroxyurea and cisplatin, like praziquantel, damaged the worm tegument, reduced worm burden, and viable schistosome eggs, decreased anti-schistosome IgG, reduced egg-induced hepatic granuloma size and cellularity, restored liver organization and improved liver function as represented by serum alanine aminotransferase and albumin. In conclusions, a single dose of hydroxyurea and cisplatin had anti-schistosome effects and may offer a safe promising alternative to control of schistosomiasis. A direct link between antitumor drugs and inhibition of schistosome neoblasts remains to be proven.

## 1. Introduction

After vigorous control activities of the Egyptian government and the lower rate of prevalence of Schistosoma, the follow-up outcome was not as positive as imagined before. The prevalence of schistosomiasis in one recent study [1] was found to be 29% in some villages, with infection peaked to 50.8% in the young age group. *Schistosoma mansoni* was detected in 31 districts out of 35 in five governorates in the Nile Delta [2]. Internationally, the situation is not better. Approximately 779 million people live at risk of infection, and 230 million are infected [3]. Schistosomiasis is killing about 280,000 people every year in Africa and represents the second most common, after malaria, parasitic disease [4]. Thus, after 40 years of continuous treatment with Praziquantel, the only available anti-schistosomal medication, transmission of Schistosoma remains continuous and uninterrupted. In fact, the presence of only one drug against schistosomiasis is completely unsatisfactory, especially if it is known that immature worms survive praziquantel [5] and that emerging resistance to praziquantel is always developed by schistosomes [6], so that the treatment fails as reported in many instances [7]. This failure has been shown in Senegalese, Kenyan and Egyptian schistosoma-infected patients [8–10]. This inefficacy of praziquantel calls continuous efforts to develop alternative anti-schistosome drugs.

The blood-dwelling fluke of the genus Schistosoma can survive and lay eggs for decades in human. Adult schistosomes could modulate their growth, as a response to host immune signals [11], and could regenerate damaged tissues after exposure to sub-lethal doses of praziquantel [12]. These experiments revealed the flexibility and plasticity of schistosomes. In fact, the longevity of schistosomes, despite time and host immune attacks, reflects a powerful regenerative system. This regeneration may occur as a result of proliferation and differentiation of stem cells.

Recently, Collins et al. [13] described proliferating cells scattered throughout the parasite body that are akin to the previously described stem-cell-like neoblasts in planarians. It seems that these stem cells are essential to the longevity of Schistosoma in the host [14]. Antineoplastic drugs are drugs that prevent or inhibit cell maturation and proliferation. Because stem cells are always proliferating for self-renewal or differentiation, then antineoplastic drugs may represent a powerful defense against them. An antineoplastic agent may be an alternative drug against Schistosomiasis, targeting stem cell proliferation and differentiation in schistosome worms, and preventing the

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https://doi.org/10.1016/j.biopha.2018.01.098
Received 4 January 2018; Received in revised form 23 January 2018; Accepted 24 January 2018

regeneration of the parasite. Prevention of regeneration will expose the worm to the host attacks. This may lead to the eradication of the worm.

It was the aim of the present study to investigate whether antineoplastic drugs have a damaging effect on schistosome worms *in vitro* and *in vivo* in infected mice and to compare this effect to that of praziquantel.

## 2. Material and methods

### 2.1. In vitro culture of Schistosoma mansoni worms

All *in vitro* studies were conducted in sterile conditions under the laminar flow hood in tissue culture lab. Different antineoplastic drugs, in addition to Praziquantel, were added to DMEM culture medium (HyClone) supplemented with 10% fetal bovine serum (FBS, HyClone), 100 U/ml penicillin, 40 µg/ml Gentamicin sulfate, and 40 µg/ml Fluconazole. *In vitro* experiments were performed in 24-well flat-bottom culture plates. All drugs were prepared in different concentrations ranging from 1 to 100 µg/ml for Praziquantel, 10 to 200 µg/ml for Cisplatin, 1 to 100 µg/ml for Hydroxyurea, 0.01 to 100 µmol/L for Methotrexate, and 0.1 to 0.5 µmol/L for Colchicine. Collected worms were incubated at 37 °C, and 5% CO2, and scored for viability 24 and 48 h post incubation.

### 2.2. Animals

All animals received humane care. Study protocols comply with the institution's guidelines and animal research laws, and animal experiments have been conducted in accordance with the internationally valid ethical conditions and guidelines. The study protocols comply with Damietta University institution's guidelines. Female CD1 Swiss albino mice (Weight 20 g ± 2 g) bred and maintained at the Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Egypt, were used in this study. The procedure of mice infection by subcutaneous injection of schistosomal cercariae were performed also in SBSC at TBRI. Every mouse was injected subcutaneously with 100 ± 10 *Schistosoma mansoni* cercariae as described by Oliver & Stirewalt [15].

### 2.3. Animal groups

Mice were maintained on normal chow and drinking water, *ad libitum*, throughout experiments, and divided into 5 groups: 1) normal mice, non-infected negative control group (C−); 2) *S. mansoni* cercaria -infected mice, non-treated, positive control group (C+); 3) Praziquantel (PZQ)-treated *S. mansoni*-infected mice, in which animals were orally administered 0.1 ml of PZQ (600 mg/kg body weight) as one dose on day 42 post infection [16]; 4) Cisplatin (CP)-treated *S. mansoni*-infected mice, in which animals were i.p injected with 8 mg/kg/day Cisplatin for 3 days started on day 42 post infection. [17]; and 5) Hydroxyurea (HU)-treated *S. mansoni*-infected mice, in which infected mice were i.p injected with 100 mg/kg/day Hydroxyurea for 3 days. Injections started on day 42 post infection with *S. mansoni* [18]. Two weeks after the last dose, perfusion of the hepatic portal vein was performed in different groups to collect worms.

### 2.4. Parasite acquisition by hepatic portal vein perfusion

Hepatic portal vein perfusion infected mice was carried out to obtain adult *Schistosoma mansoni* worms. This process was performed 6–7 weeks post-infection for *in vitro* experiments and Two weeks after the last dose in *in vivo* experiments. The method of perfusion was carried out according to Smithers & Terry [19]. The infected mouse was anesthetized and dipped after two minutes in water and fixed on the perfusion plate from its limbs. Abdomen and diaphragm were cut by scissors and forceps to expose the internal organs and blood vessels. The 21-gauge perfusion needle has been inserted for a distance of about 1 cm through the chest ribs into the ascending aorta. The blood vessels and organs were washed with the citrate buffer saline (0.85% NaCl and 1.5% Na-Citrate). The citrate buffer was used also for the perfusion process. A small cut was made in the hepatic portal vein to collect the perfusate. The citrate buffer saline perfusate, together with the blood containing worms, has been collected. Worms were collected, washed, counted, examined and processed for histology and ultrastructural examinations.

### 2.5. Mice intestinal schistosome egg picture

Mice intestines were analyzed for the presence and morphology of Schistosome eggs. Number and percentages of live mature, dead and immature eggs were calculated in the small intestine of mice of all infected groups. In each intestinal segment, the calculations were performed in a total of 25 eggs/segment. Eggs were classified according to Pellegrino et al. [20] as follows: (1) Viable immature (1st–4th stages) and mature eggs; (2) Dead immature (eggs with retracted embryo, semitransparent, darkened, or granular eggs) and dead mature eggs (calcified eggs, those with disintegrating miracidia, or eggs with retracted miracidia); (3) Shells and (4) Granulomas

### 2.6. Measurement of egg-induced hepatic granuloma size

Livers from host mice of different groups have been processed for histological examination. Granuloma size was studied according to Ohmae et al. [21] in livers stained with hematoxylin and eosin stain. The diameter of a single liver egg granuloma in liver sections of each mouse was light microscopically measured. Aggregated granuloma were not considered for this measurement. Percentage of change between control and treated groups was calculated according to the following equation:

$$percentage\ of\ change = \frac{Test\ \text{measurement} - Control\ mean}{Control\ mean} \times 100$$

### 2.7. Biochemical analysis

Albumin, alanine aminotransferase (ALT) and antischistosomal IgG were tested in mice serum of each group using commercial kits from local suppliers.

### 2.8. Statistical analysis

Values were expressed as arithmetic mean ± SEM. The difference between different groups was estimated by ANOVA. Whenever ANOVA gave significance, Student's *t*-test was applied as a *post hoc* to differentiate between every two groups. A p-value of $< .05$ between two groups was considered as significant.

## 3. Results

### 3.1. Antineoplastic drugs reduce the viability of S. mansoni worms in vitro

First, we thought to conduct some *in vitro* experimental trials to determine the effect of some anti-cancer drugs (Cisplatin, Methotrexate, Hydroxyurea, and Colchicine) on *S. mansoni* worm burden. For this purpose, freshly excised normal worms were cultured at 37 °C and 5% $CO_2$ in DMEM supplemented with 10% FCS and the necessary antibiotics and antifungal agents. Also, we applied a wide range of concentrations of different drugs. A group of worms was treated similarly with a wide range of concentrations of praziquantel to observe the similarity/dissimilarity trend between praziquantel and other antineoplastic drugs. The numbers of viable worms were recorded after 24 and 48 h of culture, and the survival rate of the *schistosoma mansoni*

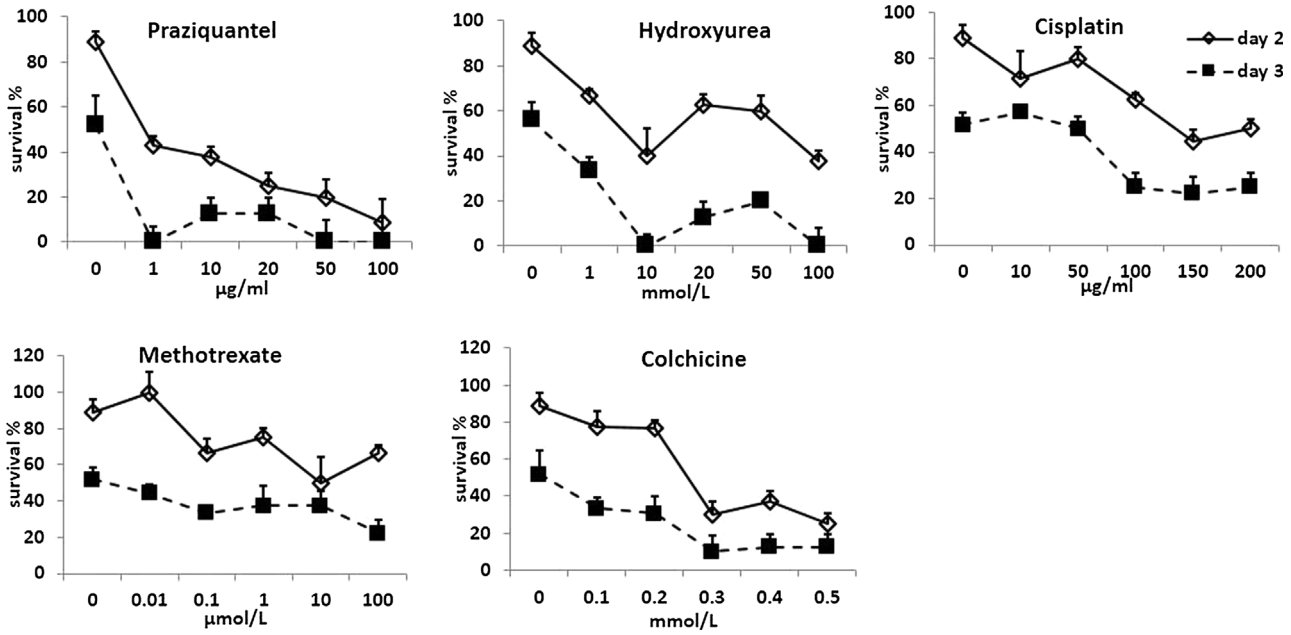


**Fig. 1.** Antineoplastic drugs reduce viability of *Schistosoma mansoni* worms *in vitro*: worms were incubated for 24 (day 2) and 48 h (day 3) with different concentrations of 4 antineoplastic drugs and praziquantel and their survival rate (%) was calculated in comparison to control worms. Data are presented as mean ± SEM of a number of experiments (N) = 3. Statistical analysis: except for both methotrexate groups, p was &. 05 within the same treatment group (ANOVA).

worms was calculated in response to different drugs (Fig. 1). The efficacy of treatment by praziquantel and the tested antineoplastic drugs was demonstrated as reductions in viability of the treated worms after 24 h (day 2) and, especially, after 48 h (day 3), compared with that in the non-treated control groups. The survival rate of the treated worms decreased gradually with the increase of drug concentration. All drugs affected the worm burden *in vitro*. However, the deleterious effect was variable in response to different drugs. These *in vitro* viability results, however, cannot be compared to each other, due to the variable nature, concentrations and biologic actions of different drugs. Within the range of applied concentrations, the collected data revealed that some antineoplastic had minor *in vitro* effects, as methotrexate; some had moderate effect as colchicine and Cisplatin; and others kill adult schistosomes, similar to praziquantel, as Hydroxyurea.

### 3.2. Hydroxyurea and cisplatin reduce worm burden, viable eggs and immunogenicity of S. mansoni

Second, we treated schistosome-infected mice with either Hydroxyurea, Cisplatin or praziquantel. Blood serum and liver samples were obtained; livers were perfused and worms were recovered from them, counted and examined; the oogram was examined in the intestine; worm samples were prepared for transmission electron microscope and liver samples for histological examination; sera were analyzed for liver function and anti-schistosome IgG content.

Egg maturation was evaluated in a rectal oogram (Fig. 2A), where several stages (immature, mature, and dead eggs) were counted. Data were collected 2 weeks after the last treatment dose. The results are presented as mean ± SEM of percentages, considering the summation of all 3 egg categories (living, dead and immature) as 100%. Analysis of variance between different groups indicated a significant difference ($p < .00001$). Both treatments with Hydroxyurea and Cisplatin significantly reduced the number of living ova, while the number of dead eggs significantly increased (*t*-test, $p < .05$). The number of immature eggs was also non-significantly more in both treatments after 1 and 2 weeks.

Mean worm burdens of treated mice with Cisplatin, Hydroxyurea or Praziquantel were compared to those of untreated mice. The mean number of recovered worms is illustrated in Fig. 2B. Generally, all treatments reduced similarly the recovered worm number. Analysis of variance (ANOVA) indicated that between groups a statistically significant difference was observed ($p = .00002$). The effect of antineoplastic drugs was similar to that of praziquantel.

Schistosoma antibodies to soluble egg antigen have been estimated in sera of all groups (Fig. 2C). IgG titer was high in the positive control group (480 ± 75.4), compared to the negative control group (96 ± 14.6). This difference was significant ($p = .0012$, *t*-test). Treatment with different drugs significantly lowered the IgG titer, as compared with the positive control titer. Values were 208 ± 44.6, 176 ± 35.8, and 144 ± 16.9 for Praziquantel, Hydroxyurea and Cisplatin groups, respectively. No statistically significant differences have been observed between healthy controls and treated groups. The variance between all of those groups was significant and had a P-value of. 00054.

Taken together, the previous data reveal a reduction in the severity of schistosomiasis in mice treated with the antineoplastic Cisplatin and Hydroxyurea.

### 3.3. Hydroxyurea and cisplatin destroy schistosome tegument

At the ultra-structural level, transmission electron microscopic examination of *S. mansoni* recovered from infected mice showed erosion, necrosis and severe damages of the tegument surface, abnormal dropped spines from tegument surface, vacuolization of the subtegumental cells and disorganization of muscle layers after treatment with either Praziquantel, Cisplatin or Hydroxyurea (Fig. 3). The effect of the 3 treatments was more or less similar. Moreover, we have recovered worms 1 and 2 weeks after the last treatment dose and found similarities in Cisplatin and Hydroxyurea groups after 1 week (Fig. 3C and E) and 2 weeks (Fig. 3D and F).

### 3.4. Hydroxyurea and cisplatin improve livers of S. mansoni infected mice

The histological investigation of hepatic tissue sections of *S. mansoni* infected mice revealed that schistosome eggs caused severe peri-ovular large granulomatous inflammatory response located within medium-sized hepatic parenchymal space, as indicated by inflammatory cell infiltration as well as sinusoidal dilatation. Histological liver section

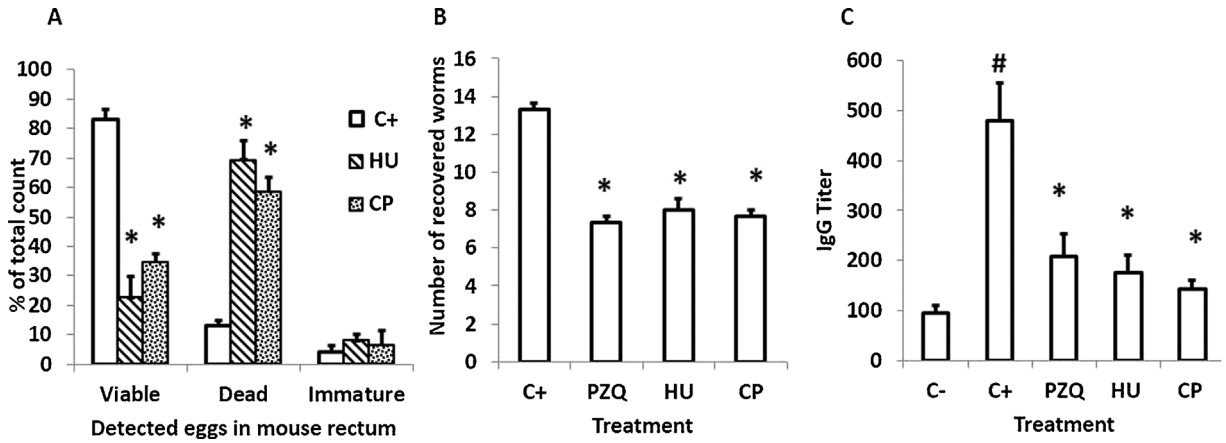


**Fig. 2.** Treatment of *S. mansoni*–infected mice with the antineoplastic Hydroxyurea or Cisplatin reduces antigenicity of Schistosoma. Data are collected two weeks after the last treatment dose. A. The total count of detected eggs in mouse rectum (N = 4). Values are presented as percentages, considering the total count as 100%. B. The number of recovered worms in untreated and treated groups (N = 6). C. The IgG Titer in different groups (N = 6). Statistical analysis: Data are presented as mean ± SEM. Statistical analysis: ANOVA indicated a significantly difference between different groups in all 3 subfigures (p &. 0006); *t*-test to compare each 2 groups: * = significantly different from the corresponding control value, # = significantly higher than the value of negative control group.

also revealed peri-portal infiltration (Fig. 4A). Granulomas were marked by concentric fibrosis with many fibroblasts encircling the viable trapped eggs. They were surrounded by inflammatory cells and collagenous fibers. Liver section also revealed disorganization of hepatic lobular structure, interstitial congestion, degenerated hepatocytes, mild canalicular cholestasis and sinusoidal dilatation as well as dilated portal vein.

As schistosoma-infected mice were treated with the ordinary anti-schistosomal drug, Praziquantel, liver histology showed many peri-ovular granulomatous inflammatory response in the hepatic parenchyma. Peri-portal, centro-lobular and peri-lobular cellular infiltration, sinusoidal dilatation and degenerated and necrotic hepatocytes. However, it was noticeable that Granulomas' size was smaller (Fig. 4B).

In Hydroxyurea group, large areas of normal organization of hepatic lobular structure were detected although the hepatocytes appeared degenerated to a great extent and sometimes also necrotic (Fig. 4C). Small granulomatous inflammatory response was observed in hepatic parenchyma in response to the schistosomal ova. Granulomas seemed to have the least diameter among all schistosome-infected groups. Schistosomal ova were all dead.

The periovular granulomatous inflammatory response in the liver from Cisplatin-treated group (Fig. 4D) was characterized by small size, less fibrosis, and minor cellular infiltration. Almost all granulomas contained dead schistosome eggs. However, many hydropic degenerated and even necrotic hepatocytes as well as periportal and perilobular cellular infiltration and portal vessels dilatation have been appeared. This could be attributed to the hepatotoxicity of Cisplatin.

The mean diameters of liver egg granuloma in positive control and treated groups were calculated in liver sections (Fig. 4E). The results indicate that all treatments caused a decrease in the size of granuloma. Only the data for Cisplatin and Hydroxyurea were significantly lower than that of the control, and even than that of the praziquantel group, indicating an advantage of antineoplastic drugs. On the other side, there was also a differential effect of treatment of both antitumor drugs, since granulomas were significantly smaller in size in Hydroxyurea treated group than that of Cisplatin group.

To examine the liver function, alanine aminotransferase (ALT) and albumin were measured in mice infected with *S. mansoni* and treated

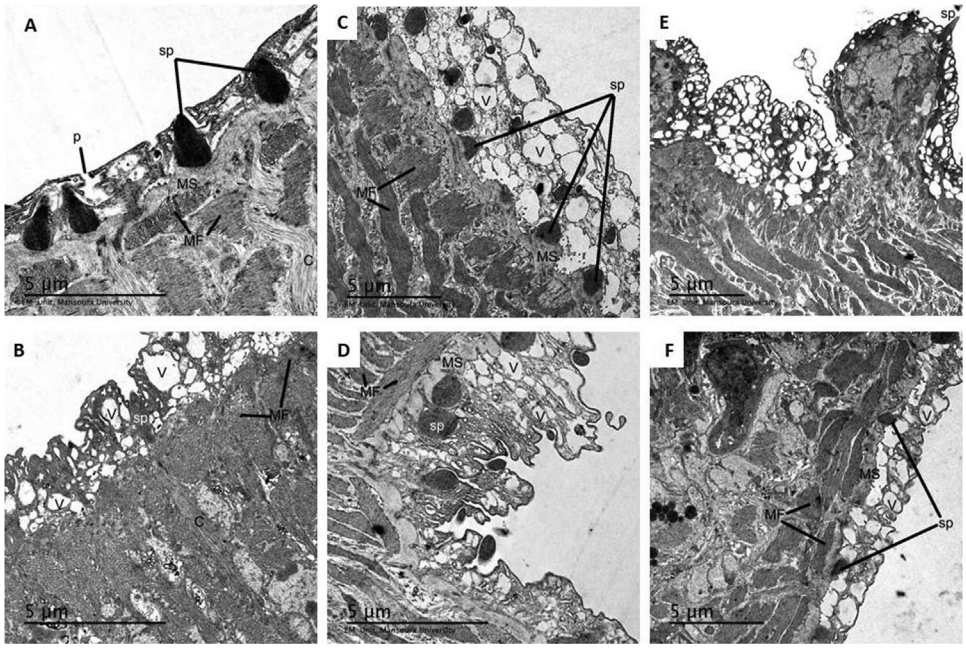


**Fig. 3.** Treatment with hydroxyurea or cisplatin disrupts schistosomal tegument: Ultra-structural examination of tegument surface of adult male *Schistosoma mansoni* after recovery from infected mice treated with praziquantel (B), hydroxyurea (C & E) or cisplatin (D & F), compared to the control (A) showed erosion of tegumental cells, abnormal spine positions in tegument surface (sp), presence of tremendous vacuolation (V) spreads everywhere in subtegumental area and swelling in longitudinal and circular muscle fibers (MF). The (c) stands for canals coming from tegumental cell and (MS) for matrix syncytium.

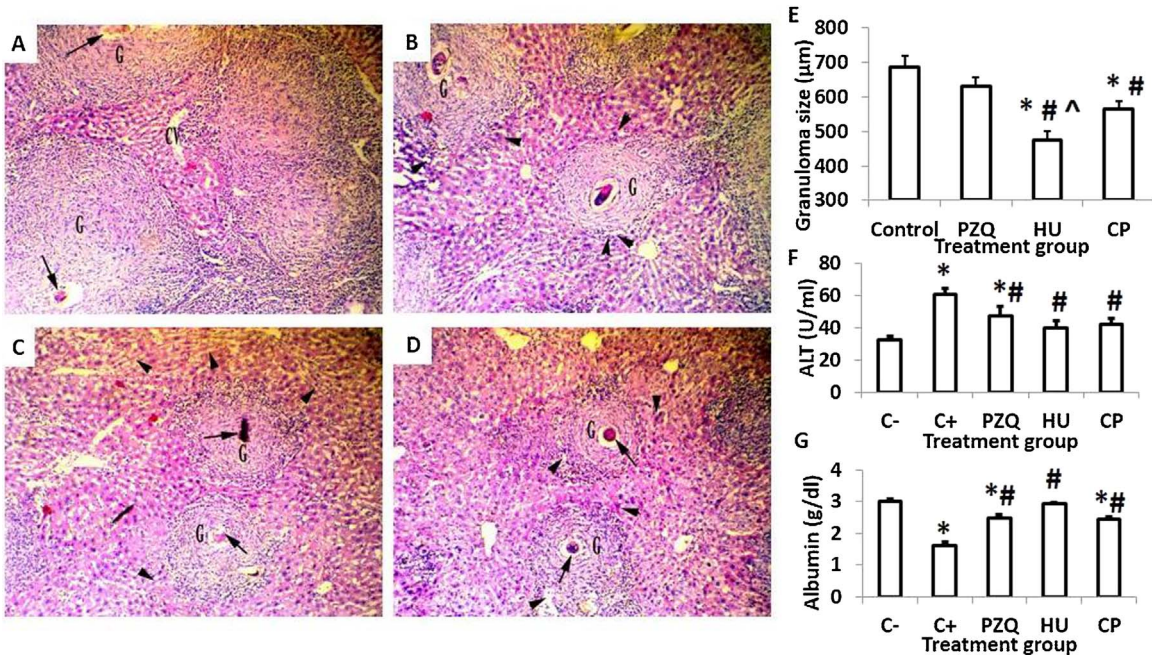


**Fig. 4.** Improvement of *S. mansoni*-infected mice liver after treatment with antineoplastic drugs. Histological sections (H & E) show reduction in schistosome eggs (arrows) – induced granuloma (G) size after treatment with praziquantel (B), hydroxyurea (C) or cisplatin (D) in comparison to untreated infected group (A). Arrowheads show degenerated or necrotic hepatocytes. The reduction of granuloma size of different groups is calculated (E). Liver function tests included serum ALT (F) and albumin (G). Data are mean ± SEM of N = 6. Statistical analysis: ANOVA indicated a significantly difference ($p < .05$) between different groups in E–G; *t*-test to compare each 2 groups: * = significantly different from the corresponding control value, # = significantly higher than the value of praziquantel group in E and infected control mice group in F and G, ^ = hydroxyurea value is different from cisplatin value ($p < .05$).

with either Praziquantel, Hydroxyurea or Cisplatin. ALT values showed a significant drop after drug treatments, especially with Hydroxyurea and Cisplatin. Analysis of variances indicated a significance between groups ($p = .00088$). ALT values of Hydroxyurea and Cisplatin were statistically similar to that of the control non-infected mice. Both of these ALT values were also significantly lower than that of the control positive group (the infected non-treated group), and even lower than that of praziquantel (Fig. 4F).

Albumin level dropped significantly in the schistosome-infected group during the treatment period. ANOVA between groups was significant with P-value of. 00000047. Fig. 4G shows that albumin level of Hydroxyurea group was statistically similar to the healthy negative control group ($p = .3$, unpaired *t*-test) and significantly higher than that of the positive control group ($p = .0001$). Albumin levels of praziquantel and Cisplatin were still significantly lower than that of the negative control group, but significantly higher than that of the positive control group.

All of these results confirm the improvement of liver function in schistosome-infected mice after treatment with both antineoplastic drugs. Their effect can also be considered as equivalent or even advantageous to the effect of praziquantel.

## 4. Discussion

The idea of this work was to explore the ability of antineoplastic drugs, which stop or retard the mitotic activity, division or proliferation of any dividing cells, to target *S. mansoni* worms. Since stem cells in any organism are continuously dividing to regenerate damaged cells or to self-renew themselves to maintain the pool of the stem cells inside the body, then they are logically targets of antineoplastic drugs. Stem cells (neoblasts) were recently discovered, described and characterized in Schistosomes [13], and they are thought to be responsible for the regeneration of the parasite inside the host body [14]. Such regeneration could be responsible for the longevity of the worm, which could live for 30 years or even longer inside the host's body. In this long period, worms perform their main function, which is to lay eggs. These eggs are the causative factors of the pathogenicity of schistosomes. Therefore, the aim of the present work was to target the *Schistosoma mansoni* worms with some antineoplastic drugs, hoping they could render an effect on the viability and function of the worm, and consequently could offer an alternative drug for schistosomiasis. In fact, Pearce [22] has also imagined targeting schistosomes' neoblasts as a tool to enhance the effectiveness of drugs like praziquantel in the treatment of schistosomiasis. We aimed here to prove that the anticancer drug *per se* could be enough as anti-schistosome drug, based on its ability to prevent cell divisions of neoblasts and worm regeneration, leading to worm death.

We have exposed *S. mansoni* worms to a wide range of concentrations of several anti-mitotic (Hydroxyurea, Cisplatin, methotrexate, and colchicine) and also to a wide range of praziquantel concentrations *in vitro*. Then two drugs were selected (Hydroxyurea and Cisplatin) and, together with praziquantel, were tested *in vivo* in *S. mansoni* mouse model. All drugs affected the worm viability *in vitro*. The collected data revealed that some antineoplastic had minor *in vitro* effects, such as methotrexate, some had moderate effect as colchicine and Cisplatin, and others had a great effect, similar to praziquantel, as Hydroxyurea. However, published data about the *in vitro* effect of the other applied anticancer drugs are lacking. *In vivo*, Hydroxyurea and Cisplatin showed also praziquantel-like action: they damaged the worm tegument and reduced worm burden and viable schistosome eggs. They also improved the liver structure and function of the infected host, through reduction of the antigenicity, inflammatory response to the eggs, as presented by the reduction of granuloma size and cellularity. Thus, the applied antineoplastic drugs revealed similar or even better result, compared to the action of praziquantel.

It is obvious that hydroxyurea and cisplatin had anti-schistosome effects in *S. mansoni*–infected mice. However, it is unclear from the present work whether these effects refer to a direct toxic effect of these drugs on the worm or indirect *via* an anti-stem cell action. The inability of *Schistosoma mansoni* to regenerate after this damage due to

hydroxyurea/cisplatin drug-affected neoblast cannot be neglected. On the contrary, one could think that the drug praziquantel may also target schistosomal neoblasts. However, a confirmatory study remains to be done. This study depended on an expected anti-stem cell action of antineoplastic drugs against neoblasts of *Schistosoma mansoni*. Since these drugs have been proven to have a positive anti-schistosomal effect, a more detailed research has to be conducted, studying the changes in schistosome neoblasts themselves and the mechanism of action of these cells in response to such drugs. Also, a research of a direct effect of antineoplastic drugs on schistosomal neoblasts remains to be done.

Although these drugs are anticancer chemotherapies, and their side effects are well known in cancer therapy regimes, they will not represent such severity in schistosome treatment. This can be explained by the single dose method in this case, which will be effective and sufficient against schistosoma, but not against cancer. This single dose will not be expected to represent any risk to the patient.

## A conflict of interest declaration for all authors

The authors declare that there are no conflicts of interest.

## Acknowledgement

This work is supported by the Damietta University MSc grant to Eman Eldeeb.

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