In Vitro Study on the Protoscolicidal Effect of Synthesized Novel Azole Derivatives on Protoscoleces of Cystic Echinococcosis

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ABSTRACT

Objective: Echinococcosis is a zoonotic parasitic infection caused by Echinococcus granulosus sensu lato (E. granulosus s.l.) with world-wide distribution. In human, E. granulosus is found in the larval form, while the adult form is usually found in dogs. Surgery is still the treatment of choice for treatment of CE. However, chemotherapy using drugs like Albendazole, Mebendazole and Flubendazole has been used for a long time. There is still no agreement on their usage because of their high adverse effects, long-term therapy and low response. Using the new and novel drugs on protoscoleces is always interesting.

Materials and Methods: We applied in vitro protoscolicidal effect of a group of azole derivatives on protoscoleces of CE cyst. Sheep hepatic cysts were collected from Shiraz abattoir and transferred to the parasitology department at Shiraz medical school, where protoscoleces were separated aseptically for protoscolicidal studies. The protoscoleces were incubated against 7 new synthesized azole derivatives using LC50 of the compounds.

Results: Our results showed that ligand no.5 with a concentration of 50 µg/ml and 10 µg/ml, and the ligand no.3 in concentration of 50µg/ml had the most protoscolicidal effects. The lowest protoscolicidal effect was observed for ligand no.7 with 44.30±22.1 and 53.46±28.63 percent destroying effect, respectively.

Conclusions: Regarding the high protoscolicidal effect of the ligands no. 3 and 5, these compounds have in vitro protoscolicidal effects. Further ex vivo and, in vivo investigations on these compounds is suggested.

Keywords: In vitro, protoscolicidal, novel azole derivatives, Cystic echinococcosis. CE cyst
considered as the only therapeutic method for the disease despite out breaking, mortality and high recurrence rate (3). However, there are four different management modalities: percutaneous therapy, surgery, chemotherapy, and observation without intervention (watch and wait) (2). Since the discovery of Thiabendazole in 1961, different benzimidazoles have been applied for treatment of the disease. Three combinations of benzimidazoles including Albendazole, Flubendazole and Mebendazole have been applied more. The common benzimidazoles drugs stop parasite growth but do not kill the parasite. To compensate this therapeutic shortfall, new treatment alternatives are urgently needed (4,5). Mebendazole was the first combination of benzimidazoles that was applied for treatment of the disease, but its absorption from the bowel is weak. Moreover for effective results, great dose is needed for a long time duration (5). Albendazole has a better absorption with stronger effects. This drug prevents from absorbing the glucose by parasite; but it has no effect on glucose of blood concentration (5). Chemotherapy remains as an unsolved dilemma in curing the disease, as the effective drug on CE cyst, has not been found yet (5).

Many heterocyclic derivatives including azole derivatives have been synthesized as important substrates to develop novel therapeutic agents against different diseases. Azoles are a class of five-membered nitrogen-containing heterocyclic systems that may have at least one other non-carbon atom(s) such as nitrogen, sulfur, or oxygen. Azoles such as imidazole and triazole analogs as well as their fused derivatives with benzene rings such as benzimidazoles and benzotriazoles have attracted considerable attention in drug discovery and medicinal chemistry. Up to now, there has been an increase in the number of antifungal drugs available with various structures and scaffolds. However, their clinical value has been limited by the emergence of drug resistance, high risk of toxicity, insufficiencies in their antifungal activity as well as undesirable side effects. Azole derivatives are the most extensively studied class of antifungal agents due to their high therapeutic index, a broad spectrum of action, good bioavailability, chemical stability, and favorable safety profile (6). However, the anti-parasitic effects of these compounds including protoscolicidal effect of these compounds have not been investigated.

Regarding the above mentioned items, the present study was designed to investigate in vitro effect of synthesized novel azole derivatives on protoscoleces of cystic echinococcosis. To this end, the different azole derivatives including benzotriazole, benzimidazole, imidazole, 2-phenylimidazole, and succinimide were used to synthesize ligands 1-7. These azoles were selected since they are present in the structure of many drug exhibiting different chemotherapeutic properties.

MATERIALS AND METHODS

Sheep liver cyst samples were prepared from slaughterhouse in Shiraz and transferred to the Helminthology research laboratory at Shiraz Medical School for further works. In the laboratory, surface of the cysts were sterilized by heat. Then the cysts were immediately aspirated with 10 or 20 ml syringe. The cysts were opened by sterilized blade of surgery and the contents including protoscoleces were aspirated by syringe. The contents of the cysts were transferred into 50ml falcon tubes. The tubes were putted in the laboratory for sedimentation of the protoscoleces. The liquid were poured out and the protoscoleces were washed using sterilized PBS. The viability of protoscoleces was determined as the percent of viable protoscoleces to the total protoscoleces in 1ml of the CE cyst fluid, using eosin stain 0.1% (0.1mg eosin in 100ml distilled water) (7). For staining, 10μl of protoscoleces containing solution was used with 10μl of eosin solution and checked by microscope (X100 and X400). Dead protoscoleces were stained while live protoscoleces were remained unstained (Figure 1). The viability of protoscoleces was monitored, before starting the protoscolicidal study and, only the batches with viability of more than 85% were used for the tests. For investigation the effects of ligands on protoscoleces, three concentrations of 5, 10 and 50 microgram per ml solution in DMSO (as a solvent media for chemical agents) were used. For each set of experiment cell culture microplates were used where one ml of DMSO solutions with the mentioned concentration of ligands, plus 100 to 200 protoscoleces were placed in an incubator with 37°C with 5 percent CO₂. RPMI containing 10 percent FCS and DMSO with 1:1000 concentration were used as the negative control. Albendazole drug with 50 microgram concentration per ml were used as positive control. A total of seven ligands were used as are follows:

1. 1-[2-(1H-Benz[d][1,2,3]triazole-1-yl)-1-phenylethylidene]-2-phenylhydrazine
2. 1-[2-(1H-Benz[d]imidazol-1-yl)-1-phenylethylidene]-2-phenylhydrazine
3. 1-[2-(1H-Benz[d]imidazol-1-yl)-1-(4-methoxyphenyl) ethylidene]-2-phenylhydrazine
4. 1-[2-(1H-Imidazole-1-yl)-1-phenylethylidene]-2-phenylhydrazine
5. 1-[2-(1H-Imidazole-1-yl)-1-(4-methoxyphenyl) ethylidene]-2-phenylhydrazine
6. 1-Phenyl-2-[1-phenyl-2-(2-phenyl-1H-imidazole-1-yl) ethylidene]hydrazine
7. 3-[2-Phenyl-2-(phenylhydrazono)ethyl]-imidazolidine-2,4-dione.

To investigate the effects of ligands on protoscoleces, the viability of protoscoleces were checked under invert microscope using eosin method (7).

**RESULTS**

The protoscolicidal effects of tested ligands, are as follows:

Ligand number 1: protoscolicidal effects of ligand number 1, in three concentrations of 5, 10 and 50 microgram per ml, compared with positive and negative controls. Albendazole, had 100 percent protoscolicidal effect on protoscoleces at thirteenth day post exposure, while ligand number 1, had such effect at twenty eight days post exposure. Mean percentage of protoscolicidal effect in concentration of 50, 10 and 5 microgram per ml was 66.1±22.5, 53.6±17.8 and 62.9±22.9 about 28 days post exposure, respectively. The mean percentage of protoscolicidal effect of Albendazole with similar times of exposure was 80.2±26.8. (Figure 2). Ligand number 2: ligand number 2 applied in the same concentration 5, 10 and 50 microgram per ml. The mean percentage of its protoscolicidal effect in concentration of 50 microgram per ml was 68.5±27.9, 73.7±26.8 and 74.3±25.7 percent during 29th days post exposure respectively while this rate for Albendazole was 81.7±25.9 percent (Figure 3). Ligand number 3: ligand number 3 applied in concentration 5, 10 and 50 microgram per ml. In concentration 50 microgram per ml, this ligand had 100 percentages protoscolicidal effect on protoscoleces in twenty third days post exposure. While in the concentration of 5 and 10 microgram per ml, had 100 percentages protoscolicidal effect on 18th and 19th day. While a 100 percentage protoscolicidal effect was observed with Albendazole in 13th day post exposure. The mean protoscolicidal effect for this ligand in the concentrations of 5, 10 and 50 micro-gram per ml was 63.0±18.1 in concentration of 10 microgram per ml was 60.6±20.4 and in concentration of 5 microgram per ml was 60.8±18.6 percent during 28 days. While mean protoscolicidal effect of Albendazole during the same time was 79.9±26.9 percent. (Figure 3). Ligand number 3: ligand number 3 applied in concentration 5, 10 and 50 microgram per ml. In concentration of 50 microgram per ml of this ligand on fifth day, a 100 percent destroying effect on protoscoleces was observed. Comparing to Albendazole as the positive control it had faster protoscolicidal effect. The mean of protoscolicidal effect of this ligand in 50 microgram per ml, during 9 days, was 81.5±26.8 percent and in 5 and 10 microgram per ml was 63.2±28.4 and 62.6±26.2 percent, respectively (Figure 4). Ligand number 4: ligand number 4 used in concentrations 5, 10 and 50 microgram per ml. A 100 percentage protoscolicidal effect of this ligand was observed in concentration 50 microgram per ml in 8th day post exposure which was similar to positive control. The mean of protoscolicidal effect of this ligand in concentrations of 5, 10 and 50 microgram per ml was 73.8±32, 67.7±26 and 67.1±25.1 percent, respectively while this rate for Albendazole was 81±6±22.3 percent (Figure 5). Ligand number 5: ligand number 5 also investigated for the same three concentrations 5, 10 and 50 microgram per ml. In concentration 10, 50 microgram per ml, this ligand was compared to Albendazole as the positive control, had faster protoscolicidal effect such that in ninth day post exposure 100 percentages protoscolicidal effect was observed. While Albendazole in thirteenth day, had such effect. The mean protoscolicidal effect of this ligand in the concentration 50 microgram per ml, during 29th days post exposure was 85.6±27 percent and in the concentration 10 microgram per ml was 81.7±25 percent (Figure 6). Ligand number 6: ligand number 6 was also investigated in 3 concentrations 5, 10 and 50 microgram per ml. In concentration 50 microgram per ml, this ligand had 100 percentages protoscolicidal effect on protoscoleces in twenty thirteenth days post exposure. While in concentrations of 5 and 10 microgram per ml had 100 percentages protoscolecid effect on 18th and 19th day. While a 100 percentage protoscolicidal effect of Albendazole as the positive control was observed on 13th day. The mean protoscolicidal effect for this ligand in the concentrations of 5, 10 and 50 micro-gram per ml was 68.5±27.9, 73.7±28.1 and 74.3±25.7 percent, respectively while this rate for Albendazole was 81.7±25.9 percent (Figure 7). Ligand no. 7: The protoscolicidal effect of this ligand was also investigated in three concentrations of 5, 10 and 50 microgram per ml. In concentration of 50 microgram per ml, a 100 percent protoscolicidal effect for this ligand was observed in 23rd day post exposure while in concentration of 10 microgram per ml in 28th day and in concentration 5 microgram per ml in 26th day. A 100 percentage protoscolicidal effect was observed with Albendazole in 13th day post exposure. The mean protoscolicidal effect for this ligand in the concentrations of 5, 10 and 50 micro-gram per ml was 67.9±26.8, 44.3±22.0 and 53.4±28.6 percent, respectively; while this rate for Albendazole was 81.7±25.9 percent (Figure 8). The mean protoscolicidal effects of the ligands in different concentrations is shown in Table 1.
Table 1: The mean rate of protoscolicidal effects of the ligands in different concentrations

<table>
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<tr>
<th>Ligand Number</th>
<th>Drug Concentration</th>
<th>Death Rate Mean</th>
<th>Standard Deviation</th>
<th>Test Number</th>
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<td>27.08</td>
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Figure 2. Protoscolicidal effect of ligand number 1 with concentrations of 5, 10 and 50 microgram per ml comparing to Albendazole

Figure 3. Protoscolicidal effect of ligand number 2 with concentrations of 5, 10 and 50 microgram per ml comparing to Albendazole

Figure 4. Protoscolicidal effect of ligand number 3 with concentrations of 5, 10 and 50 microgram per ml comparing to Albendazole

Figure 5. Protoscolicidal effect of ligand number 4 with concentrations of 5, 10 and 50 microgram per ml comparing to Albendazole
DISCUSSION

For many years, surgery was the only therapeutic method for CE cyst treatment (3). It is still the selective method for treating CE cyst (3,7). However surgery method has over 10 percent recurrence, 0-20 percent mortality and 25-40 percent out breaking (7). Chemotherapy remains as the unsolvable dilemma about CE, because less effective drug has been found for CE cyst (5). Derivatives of benzimidazoles like Albendazole and Mebendazole are commonly used for chemotherapy CE cyst (5). In cases of humans, high doses should be taken for a long time, therefore their side effects observing mostly (5). A study on the effects of flubendazole on protoscoleces showed similar results and showed that protoscoleces exposed to Flubendazole after 25 days, only 13.9±5.9 percent of protoscoleces were survived and after 13 days, all of protoscoleces were killed (8,9). In this research, in vitro effects of seven combination of novel azole derivatives that has been synthesized in chemistry department of Shiraz University (10,11), tested on protoscoleces Echinococcus granulosus. In comparison with effects of in vitro protoscolicidal drugs including Nitazoxanide (12), Praziquantel (13), Artemisinin and its derivatives (14), ligands had less destroying effects on protoscoleces. The effects of ligand no. 3 and 4, was similar to the results of the study by Walker et al. on Nitazoxanide (12), and Artemisinin and its derivatives (14); while in comparison with in vitro destroying effects of Flubendazole and Praziquantel (5,13), the ligand no. 3, had stronger destroying effects. Similar effects of Nitazoxanide drug scolicidal effects on protoscoleces, seems to be similar to the effect of ligand no. 5 with dose dependent effect (12). In comparison with in vitro destroying effects of Flubendazole (5) and Praziquantel (13) destroying effects of ligand no. 5 was stronger. In vitro destroying effects of ligand no. 6 was weaker in comparison with destroying effect of Nitazoxanide, Praziquantel and Artemisinin (12-14). In comparison with destroying effects of Flubendazole (5) that was 100 percent protoscolicidal effect on protoscoleces in 13th day, destroying effect of ligand no. 6 about used concentration was more stronger. Protoscolicidal effects of ligand no. 7, was similar to in vitro protoscolicidal effects of Flubendazole (5). While in comparison with in vitro effects of Praziquantel (13) Nitazoxanide (12), Artemisinin and its derivatives (14), destroying effects of ligand no. 7 was weaker. These chemicals have a higher protoscolicidal effect than natural ones (15-17). Although benzimidazoles have been the cornerstone of medical therapy since the late 1980s, many issues (e.g., duration of therapy) remain unresolved (18). Many other compounds have been tested experimentally without success. A synergy between benzimidazoles and other agents such as metformin seen in vitro, has been reported. However, still a great need for development of new chemotherapeutic agents both synthetic and natural is essential (18-21).

CONCLUSION

In conclusion, the results showed that compound no. 5 with a concentration of 50 µgr/ml and 10 µgr/ml and
compound no.3 in concentration of 50µgr/ml had the most protoscolicidal effects. The lowest protoscolicidal effect was observed for ligand no. 7 with 44.30±22.1 and 53.46±28.63 percent destroying effect, respectively. This study is the first in vitro protoscolicidal effects of these compounds, so, we suggest ex vivo and, in vivo studies on the ligands no. 3 and 5.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: This study was approved by the research ethics committee of the Shiraz University of Medical Sciences (ethical code: IR.SUMS.REC.1386. S3762).

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