Benzimidazole and aminoalcohol derivatives show in vitro anthelmintic activity against Trichuris muris and Heligmosomoides polygyrus

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Abstract

Background: Infections by gastrointestinal nematodes cause significant economic losses and disease in both human and animals worldwide. The discovery of novel anthelmintic drugs is crucial for maintaining control of these parasitic infections.

Methods: For this purpose, the aim of the present study was to evaluate the potential anthelmintic activity of three series of compounds against the gastrointestinal nematodes *Trichuris muris* and *Heligmosomoides polygyrus in vitro*. The compounds tested were derivatives of benzimidazole, lipidic aminoalcohols and diamines. A primary screening was performed to select those compounds with an ability to inhibit *T. muris* L_1 motility by more than 90% at a single concentration of 100 μM, and then, their respective IC_{50} values were calculated. Those compounds with IC_{50} lower than 10 μM were also tested against the adult stage of *T. muris* and *H. polygyrus* at a single concentration of 10μM.

Results: Of the 41 initial compounds screened, only compounds AO14, BZ6 and BZ12 had IC_{50} values lower than 10 μM on *T. muris* L_1 assay, showing IC_{50} values of 3.30, 8.89 and 4.17 μM, respectively. However, only two of them displayed activity against the adult stage of the parasites: BZ12 killed 81% of adults of *T. muris* (IC_{50} of 8.1 μM) and 53% of *H. polygyrus* while BZ6 killed 100% of *H. polygyrus* adults (IC_{50} of 5.3 μM) but only 17% of *T. muris.*

Conclusions: BZ6 and BZ12 could be considered as potential candidates for further *in vivo* efficacy testing.

Introduction

Soil transmitted helminths (STHs) are a group of human parasitic nematodes that affect around 1.5 billion of the world's population causing substantial disease and disability [1]. These infections are more common in people living in low and middle-income countries, in areas with poor access to adequate drinking water, sanitation and hygiene [2]. Of particular importance are infections produced by roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*), and hookworms (*Necator americanus* or *Ancylostoma duodenale*) [3].

According to the latest studies, global incidence to *T. trichiura* infections is estimated to range between 450 million to one billion active cases [4]. In animals, the presence of helminth infections has an important economic impact directly derived from reductions in productive yields associated with weight gain, milk production and wool quality [5]. In Europe, the annual economic losses caused by helminth infections in livestock have been estimated at 1.8 billion Euros, while those produced only by resistance to anthelmintics in the group of gastrointestinal nematodes reaches approximately 38 million Euros annually [6].

The main control of these infections in humans is based on preventive chemotherapy, employing large-scale administration of anthelmintic drugs to populations at risk. Currently, there are five drugs on the

World Health Organization (WHO) model list of essential medicines that are recommended for the treatment of soil-transmitted helminths: albendazole (ABZ), mebendazole (MBZ), levamisole (LEV), pyrantel pamoate (PYR) and ivermectin (IVM) [7]. However, the administration of these drugs is not always highly effective, reflecting low cure rates especially against trichuriasis when a single oral dose is administered [8, 9]. Moreover, the efficacy of these compounds has been notably decreasing over time, showing egg reduction rates falling from 72.6% in 1995 to 43.3% in 2005 [10].

Additionally, in gastrointestinal nematodes infecting livestock, multiple cases of rapid resistance development have been reported in all continents as a consequence of the abusive use of anthelmintics, mainly benzimidazoles (e.g. ABZ) and macrocyclic lactones (e.g. IVM) [11–13].

Considering the spread of anthelmintic resistance in animals and the low efficacy of some benzimidazoles against certain STHs in humans, it is clear that there is an urgent need to search for new drugs to control helminth infections.Therefore, the present study is focused on the determination of the potential *in vitro* anthelmintic activity of a series of synthetic compounds from different chemical families including benzimidazoles (BZ), lipidic diamines (AA) and aminoalcohols (AO) using two rodent models of gastrointestinal nematodes: *Trichuris muris* and *Heligmosomoides polygyrus* [14, 15]. For this purpose, a total of 41 compounds (15 BZ, 11 AA and 15 AO) were evaluated against two different stages of the parasites, the first stage larvae 1 (L_1) and adult forms.

Material And Methods

Chemical compounds

Compounds belonging to three different chemical families, namely 2-aminoalkan-1-ol (AO), alkane-1,2-diamine (AA) and 2-phenylbenzimidazoles (BZ), were synthesized by previously reported procedures [16–18] (structures summarized in Tables 1-3). Stock solutions (10 mM) of these compounds were prepared in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich®) while the final dilutions were made with distilled water in order to maintain a maximum concentration of 1% (v/v) DMSO in the well. All compounds were stored at 4ºC pending use. To perform all the *in vitro* assays, levamisole (LEV) was used as positive control (Sigma-Aldrich®) and 1% DMSO as negative one.

Animals and parasites

The complete life cycles of *T. muris* and *H. polygyrus* are maintained at Swiss TPH (Swiss Tropical and Public Health Institute). Experiments were approved by national and cantonal Swiss authorities (permission No. 2070). Three-week-old female mice (NMRI for *H. polygyrus* and C57BL/6N for *T. muris*) were allowed to acclimatize to the new environment for one week before use. During the acclimatization period, from day two after arrival the animals received 0.25 mg/L dexamethasone (Sigma-Aldrich®) in the drinking water in order to immunosuppress them and to facilitate parasite

establishment. During the experiment, mice were kept at 22°C, 50% humidity, with a 12-h light/dark cycle and water and rodent food (KLIBA NAFAG, Switzerland) was available *ad libitum* according to Swiss Animal Welfare guidelines. Mice were infected orally with 200 embryonated *T. muris* eggs or 90 *H. polygyrus* L_3 stage.

Drug discovery strategy

To investigate the activity against *T. muris*, 41 compounds were first subjected to a primary screen to assess their ability to inhibit the motility of *T. muris* at the L_1 stage at a single concentration of 100 μM. Only compounds with the ability to inhibit the motility of more than 90% of larvae (activity higher than 90%), progressed to subsequent dose-response evaluation to estimate their half maximal inhibitory concentration (IC_{50}) value. Then, those with IC_{50} values lower than 10 μM were tested on the adult stage of *T. muris* and *H. polygyrus* following a similar protocol as mentioned above: initial screening at 10 μM and estimation of IC_{50} values of those compounds with activities higher than 80%.

In vitro assays

Evaluation of anthelmintic activity on T. muris L_1s

The assay was performed according to Wimmersberger et al, (2013). Briefly, unembryonated eggs were collected from faeces of infected mice. After storing the eggs for three months at room temperature in a dark box, hatching occurred by incubation with 10^7-10^8 cells/mL of *Escherichia coli* BL-21 stock. Approximately, 40 L_1 per well were placed into a 96-well plate and incubated for 24 hours at 37 ºC and 5% v/v CO_2 atmosphere, in the presence of 100 μL RPMI-1640 medium supplemented with antibiotic mixture (12.5 μg/mL amphotericin B, 500 U/mL penicillin and 500 μg/mL streptomycin) and 100 μM of the compound to be tested. As a positive control, (LEV) at a final concentration of 50 μM was used (Sigma-Aldrich) while wells with medium and 1% DMSO served as a negative control. Each compound was tested in duplicate and the assay was repeated on a different day. After 24 hours incubation, the viability of the larvae was determined by using a binary scale that discriminates live from dead larvae: “0”= no sign of motion = dead and “1” = motion observed = alive. The percentage of dead larvae was established for each well. To stimulate the movement of all live larvae, 100 μl of hot water (≈80 °C) was added to each well following previous protocols [19]. Each larvae was observed for 3-5 seconds. Compounds that showed a dead effect higher than 90% at 100 μM were selected to determine their IC_{50}. For this, L_1s were incubated with at least six different concentrations of the compound ranging from 100 to 0.41 μM (serial dilutions 1:3).

Evaluation of anthelmintic activity on H. polygyrus and T. muris adult worms

The assay was performed according to Karpstein et al, (2019). Briefly, *H. polygyrus* and *T. muris* adults were collected from the cecum (*T. muris*) and the colon (*H. polygyrus*) of the animals at two (*H. polygyrus*) and seven weeks (*T. muris*) post-infection, respectively. Three to four adult worms were placed in each well of a 24-well plate and exposed to the test compounds at 10 µM final concentration in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin in a final volume of 2.5 ml. The medium for *H. polygyrus* was supplemented with 12.5 µg/mL amphotericin B and the medium for *T. muris* with 5 % fetal calf serum (iFCS, 100 U/ml). Wells containing 1% (v/v) DMSO in water served as negative control. Worms were incubated at 37 °C and 5% CO_2 up to 72 h, after which the drug effect was evaluated using a phenotypic readout. The assay was conducted in duplicate and repeated twice at different days. The condition of the worms was microscopically evaluated based on their phenotype, using a viability scale ranging from 3 to 0 (3: good motility; 2: low motility; 1: very low motility; and 0: death). In the case adult worms did not move enough for a clear scoring, they were stimulated with 500 µL hot water (≈80 °C) [20]. Therefore, the effect of the compounds was expressed by the percentage of dead larvae, considering dead those with a score of 0, and live those with a score ranging from 1 to 3. Compounds with efficacies greater than 80% at 10 µM were then selected for IC_{50} determination (1:4 serial dilutions ranging from 10 to 0.039 µM).

Data analysis

IC_{50} values of both assays were calculated based on median effect principle, using the CompuSyn software (CompuSyn, version 3.0.1). These values were defined as the concentration of a drug required to decrease the mean worm's motility by 50%. The "r" value is the linear correlation coefficient of the median-effect plot; it illustrates the goodness of fit, and thus, the accuracy of the IC_{50} value.

Cytotoxicity assays and Selectivity Indexes

Cytotoxicity assays for most compounds were previously carried out in previous study on two different cell lines, the human colorectal adenocarcinoma Caco-2 (ATCC® HTB-37™) and the human hepatocarcinoma HepG2 (ATCC® HB-8065™) using the Alamar Blue staining method, in order to estimate their toxicity [18,21]. In the current study, the cytotoxicity of only two compounds, AO14 and AO15, were performed following the previous protocols mentioned above.

Selectivity indexes (SIs) were calculated by dividing the CC_{50} values obtained in the cytotoxicity assays by the IC_{50} values of the *in vitro* assays. The greater the SI value, the more selective the compound is inhibiting *T. muris* activity and the less inhibiting mammalian cell growth (general cytotoxicity).

Results

Tables 1 to 3 display the basic scaffold of the different class of compounds tested and the results of the *in vitro* assays performed against *T. muris* L_1, along with cytotoxicity data and SIs. AO and AA compounds are arranged according to the type and size of substituents present on R^1, R^2 and R^3, and to the length (n) of the alkylside-chain. BZ compounds are distributed in first place (R^1) by the type of substituents at position C-5 (C-6) of the benzimidazole system and second (R^2) by the substituents on the 2-phenyl ring.

Four AO derivatives (AO5, AO11, AO14 and AO15) displayed activities higher than 90% in the initial screening at 100 μM against L_1 *T. muris*, but only AO14 showed an IC_{50} lower than 10 μM. IC_{50} values of the other three compounds were 25.6, 17.5 and 46.0 μM, respectively (Table 1). For AA derivatives, only the compound AA18 reached an activity higher than 90% (93.55%) in the initial screening showing an IC_{50} value of 21.9 μM (Table 2), while five BZs (BZ1, BZ2, BZ6, BZ12 and BZ13) reached activities higher than 90% at 100 μM. IC_{50} values in the tested BZs were higher than 15 μM except for BZ12 and BZ6 with values of 8.89 and 4.17 μM, respectively against L_1 *T. muris* (Table 3).

Regarding SIs obtained on L_1 assays, only five compounds belonging to the three families tested (AO14, AA18, BZ2, BZ6 and BZ12) obtained values higher than one on both Caco2 and HepG2 cells lines, with BZ6 reaching the highest SI values, 5.41 in Caco2 cells and 4.21 in HepG2 cells.

The assay performed with AO14, BZ12 and BZ6 against *T. muris* and *H. polygyrus* adults (Table 4) at a fixed concentration of 10 μM showed that BZ12 had an effect of 81% and 53% on adults of *T. muris* and *H. polygyrus* after 72 hours of exposure, respectively. BZ6 killed 100% of *H. polygyrus* adults at this concentration while it had an activity of 17% against *T. muris*. On the other hand, AO14 did not reach an efficacy higher than 23% against any parasite. IC_{50} values were calculated on those compounds with activities higher than 80% at a concentration of 10 μM, therefore, BZ12 showed an IC_{50} value of 8.1 μM on *T. muris* adults while BZ6 showed a lower IC_{50} of 5.3 μM on *H. polygyrus* adults. In the case of the SIs estimated on the adult stage, BZ6 displayed the highest SIs on both cell lines (4.3 in Caco2 and 3.1 in HepG2 cells), while BZ12 showed values closer to one (1.5 in Caco2 and 1.8 in HepG2 cells).

Discussion

In recent years, the number of new anthelmintics compounds introduced into the market to control the infections produced by gastrointestinal nematodes has been limited, mainly due to economic difficulties in the development and marketing of new drugs [22]. Only four drugs have entered the market the last two decades: emodepside [23], monepantel [24], derquantel [25] and tribendimidine [26]. There is therefore a clear need to develop novel anthelmintic drugs for the control of these parasitic worms in humans and

farm animals. One of the approaches proposed to alleviate the severe scarcity of anthelmintics is the synthesis of new derivatives of known drugs. Although BZ resistance is present in many gastrointestinal species infecting livestock, the synthesis of novel BZ derivatives can lead to compounds with improved properties such as better solubility and pharmacokinetic profile, resulting in increased effectiveness [27]. Some promising compounds, such as tenvermectin [28], diisopropylphenyl-imidazole [29], and mebendazole nitrate [30], have been developed in recent years following this approach.

Based on these assumptions, in the present study, a total of 15 AO and 11 AA derivatives, both structurally related to sphingosine, and 15 benzimidazole derivatives were tested against L_1 of *T. muris* and adult stages of *T. muris* and *H. polygyrus*. The anthelmintic activity of most of these compounds were previously tested *in vitro* against the gastrointestinal nematode infecting sheep *Teladorsagia circumcincta* [18,21] and some of them were also tested against *Leishmania sp*. [31,32], *Trypanosoma sp*. [17,33] and *Strongyloides venezuelensis* [34].

The L_1 assay has proven to be a good tool to screen new potential candidate compounds before carrying out adult motility assays, the *in vitro* assay of choice, which is more expensive, labor-intensive, time-consuming and it requires the use of live animals [19]. Moreover, the results obtained with the motility assay based on L_1 seem to correspond to the findings observed with adult *T. muris* [35]. However, some studies showed that L_1 appears to be more sensitive to drugs than older stages of *T. muris* [36,37], which can facilitate the discarding compounds with no activity.

In the present study 10 out of the 41 compounds tested showed activity higher than 90% against the L_1 stage of *T. muris* at 100 µM, and only three namely AO14, BZ12 and BZ6, reached an IC_{50} lower than 10 µM. The screening performed at a single final concentration of 10 µM on adults showed that only BZ12 and BZ6 had significant activity against the adult stage of *T. muris* and *H. polygyrus*, respectively.

Comparing the results obtained with these derivatives to the previous study carried out against *T. circumcincta,* it reveals that of the 10 compounds screened at 100 µM that showed more than 90% activity against *T. muris* L_1, six (BZ1, BZ2, BZ6, AO11, AO15 and AA18) of them also showed ovicidal activity against *T. circumcincta* eggs, but only BZ6 reached an IC_{50} value lower than 10 µM (IC_{50}= 6.54 µM). In the case of *T. circumcincta* L_1, four of them (AO5, AO11, AA18 and BZ6) reached IC_{50} values below 10 µM (IC_{50} for AO5=2.87 µM, IC_{50} for AO11=1.21 µM, IC_{50} for AA18=6.29 µM and IC_{50} for BZ6=5.01 µM) and only AO5 and AO11 showed IC_{50} values below 10 µM (IC_{50} for AO5=5.55 µM, IC_{50} for AO11=4.58 µM) against *T. circumcincta* L_3. Some of the compounds that have not shown activity in the L_1 *T. muris* assay, had shown activity against other parasite models such as *Trypanosoma brucei* (compounds AO4 and AA19 with IC_{50} values close to 0.5 µM) and *Leishmania sp.* (compounds AA25 and AA26). This is also the case of the study carried out on *S. venezuelensis* L_3, in which compounds AO6, AA18, AA19, AA24 and AA25 showed activity against this nematode (IC_{50} values ranging from 31.9±0.5 µM to 39.1±4.7 µM), but only compound AA18 showed activity in the current study, against L_1 of *T. muris*.

Thus, compared to previous studies, BZ6 seems to be the only compound reaching IC_{50} values below 10 μM in both eggs and L_1 of *T. circumcincta* (IC_{50}=6.54 μM in eggs and IC_{50}=5.01 μM in L_1) and also in L_1 of *T. muris* (IC_{50}= 4.17 μM), with values quite close to each other). However, BZ6 did not have any affect against the adult stage of *T. muris* at a concentration of 10 μM (17.2% of activity) but it was effective against *H. polygyrus* adults (100% of activity) displaying an IC_{50} of 5.3 μM. On the other hand, BZ12 did not produce effect against any of the stages of *T. circumcincta*, eggs, L_1 or L_3, but it showed activity against *T muris* L_1 with an IC_{50} of 8.89 μM. Moreover, this BZ12 reached an efficacy of 53.3 and 81.7% on the adult stage of *H. polygyrus* and *T. muris* at 10 μM, respectively, presenting an IC_{50} of 8.1 μM in the latter.

In terms of the relationship between the structure and efficacy of the compounds and focusing on the benzimidazoles, the only group of compounds that has shown significant efficacy on the adult stage of the parasites in this study, we can observe that the presence of a mild basic group as the–NH_2 group on R_1 (BZ15) did not induce any measurable effect on the nematode viability, while the combinations of 5-Me–4'-OMe/Cl (BZ1 and BZ2), 5-Cl – 4'-Cl (BZ6), 5-NO_2 – 4'-Cl/diMe (BZ12 and BZ13) produce a deadly effect higher than 90% on the initial screening of *T. muris* L_1. Regarding to substituent present on the B-phenyl ring (R^2), the 4'-Cl^- is required for the anthelmintic effect since all compounds with this substituent at this position showed anthelmintic activity on *T. muris* L_1 (BZ2, BZ6 and BZ12), including here the two most potent compounds (BZ6 and BZ12), while double substitutions on this ring, as those 3'-$NO_2$4'-OMe (BZ4, BZ9 and BZ14) or 3'-NH_2 4'-OMe (BZ10), led to inactivity. However, a di-substitution in position 2´ and 6´ with electron donating groups such as 2´,6´-diMe in addition to a polar group in ring A as 5-NO_2 (BZ13), gave good anthelmintic inhibitory activity in *T. muris* L_1 (99.40 inhibition at 100 μM), although its IC_{50} was higher than 10 μM.

Comparing the adult motility assay results of from the present study with previous experiments using the marketed human drugs (ABZ, MBZ, LEV, PYR and IVM), it can be observed that the IC_{50} values obtained are much lower (8.1 μM for BZ12): BZ compounds and IVM showed a lack of activity on adult *T. muris*, while LEV and PYR shown IC_{50} values around 68 and 57 μM, respectively [19].

All compounds tested against L_1 had a possible toxic potential as their SIs were very close to one, except for BZ6 and AO14, which reached values greater than four in both cell lines. Regarding to the SIs obtained in the adult assays, although in any case they were greater than one, BZ6 seems to be a safer candidate than BZ12, as it had SI values of 4.3 for Caco2 cells and 3.1 for HepG2 cells.

Conclusions

The present study identified compounds BZ12 and BZ6 as hits for future studies that should include the evaluation of their pharmacological properties (absorption, distribution, metabolism, metabolism, excretion and toxicity; ADMET) as wells as *in vivo* efficacy and safety experiments with animals.

Moreover, it would be interesting to evaluate the activity of the compounds against other different species of gastrointestinal nematodes to test their potential spectrum of activity.

Abbreviations

AA: diamine; ABZ: albendazole; ADMET: absorption, distribution, metabolism, excretion and toxicity; AO: aminoalcohol; BZ: benzimidazole; CC_{50}: cytotoxic concentration 50; IC_{50}: inhibitory concentration 50; IVM: ivermectin; LEV: levamisole; MBZ: mebendazole; PYR: pyrantel pamoate; SI: selectivity index.

Declarations

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- ## Availability of data and materials

The datasets supporting the conclusions of this article are included within the article text and additional files.

- ## Authors’ contributions

Conceptualization, MMV, RBF and JK; methodology, EVG, CH, MAB, NE, JDV, VCG; drafting the manuscript EVG; funding acquisition MMV, RBF, EDO; experimental design, EVG and MMV. All authors read and approved the final manuscript.

- ## Ethics approval and consent to participate

Experiments were approved by national and cantonal Swiss authorities (permission No. 2070).

- ## Competing interests

The authors declare that they have no competing interests.

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