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Article

Ortho-nitrobenzyl derivatives as potential anti-schistosomal agents

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In the search for new anti-schistosomal agents, a series of fifteen *ortho*-nitrobenzyl derivatives was assayed *in vitro* against both the schistosomulum (somule) and adult forms of *Schistosoma mansoni*. Compounds **8** and **12** showed significant activity against somules at low micromolar concentrations, but none was active against adults. The SAR demonstrated that the compounds most active against the parasite were mutagenic to the human cell line RKO-AS45-1 only at concentrations 10- to 40-fold higher than the worm-killing dose. Given their electrophilicity, compounds were also screened as inhibitors of the *S. mansoni* cysteine protease (cathepsin B1) *in vitro*. Amides **5** and **15** exhibited a modest inhibition activity with values of 55.7 and 50.6 % at 100 μM, respectively. The nitrobenzyl compounds evaluated in this work can be regarded as hits in the search for more active and safe anti-schistosomal agents.

Keywords: Nitro-aromatic. *Schistosoma mansoni*/ anti-schistosomal activity. Cathepsin B1. Mutagenicity

INTRODUCTION

Schistosomiasis is a neglected tropical disease caused by a flatworm of the genus *Schistosoma*. The major species involved are *S. haematobium,* which causes the urogenital form of the disease, and *S. mansoni* and *S. japonicum*, which are responsible for the intestinal disease (Colley *et al.*, 2014). An estimated 240 million people worldwide are infected by *Schistosoma* parasites and more than 700 million live in endemic areas (WHO, 2016a).

Praziquantel, a pyrazino-isoquinoline derivative (Figure 1), is registered as an essential medicine by the World Health Organization (WHO, 2016b) and is the first choice therapy for schistosomiasis. It is active against all schistosome species and is safe and inexpensive. However, concerns over the possible development of resistance to praziquantel due to its large-scale use, motivates the search of new anti-schistosomal agents (Wang, Wang, Liang, 2012; Caffrey, 2015).

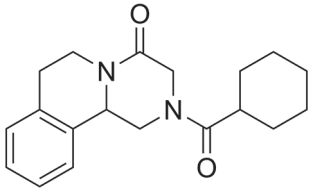

FIGURE 1 – Chemical structure of praziquantel.

Oxamniquine is a pro-drug that is activated by a *Schistosoma* sulfotransferase enzyme (Pica-Mattoccia *et al*., 2006). It is only clinically useful against *S. mansoni* infections (Caffrey, 2007; Axton, Garnett, 1976). The activated derivative is a nitro-aromatic compound that displays a good leaving group (sulfate) at the benzylic position. This product dissociates forming an electrophilic agent, which alkylates the parasite DNA (Abdul-Ghani *et al*., 2009) (Figure 2).

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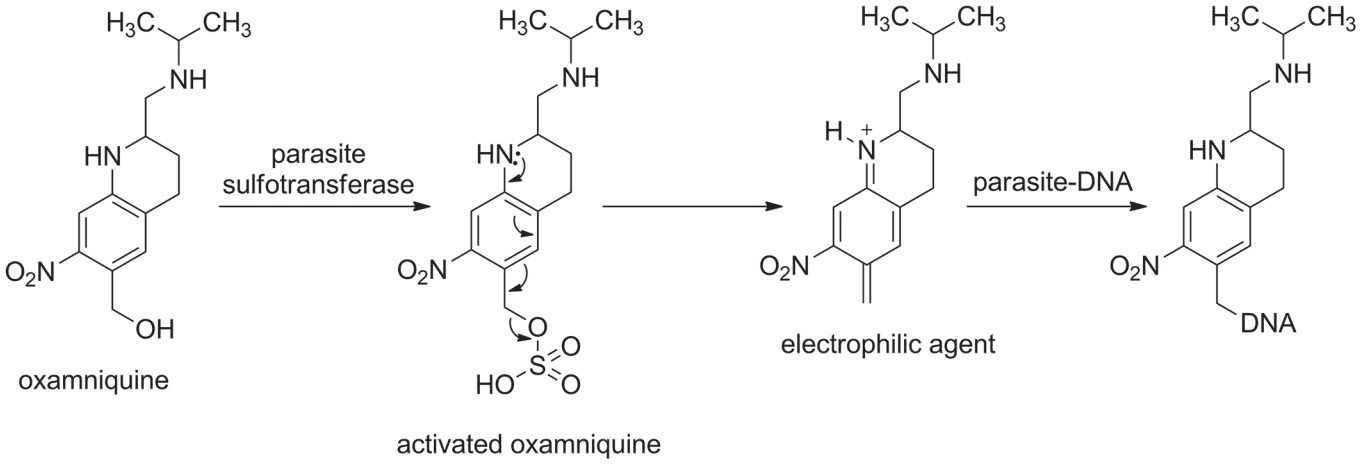

FIGURE 2 – Oxamniquine mechanism of action.

Nitro-aromatic compounds, such as fexinidazole, benznidazole, and niclosamide, (Figure 3) are known for their pharmacological potential and our group has investigated the biological activities of a series of *ortho*-nitrobenzyl derivatives that act as alkylating agents (Lopes *et al*., 2015; Lopes *et al*., 2011; Soares *et al*., 2010).

Based on the need for new anti-schistosomal agents, we evaluated the schistosomicidal activity of nitro-aromatic compounds, analogs of "activated" oxamniquine, against *S. mansoni in vitro*. These compounds would be active *per se*, without the requirement for sulfotransferase activation, as proposed in Figure 4. Evidence suggests that oxamniquine-resistant schistosomes do not express the enzyme sulfotransferase and therefore cannot convert the oxamniquine into its active form (Valentim *et al*., 2013; Pica-Mattoccia *et al*., 2006). Therefore, compounds that are active per se could be an alternative in the treatment of oxamniquine-resistant *S. mansoni* infections.

Based on their electrophilic characteristics, compounds were also assessed as possible inhibitors of *S. mansoni* cathepsin B1 (SmCB1), as this enzyme bears

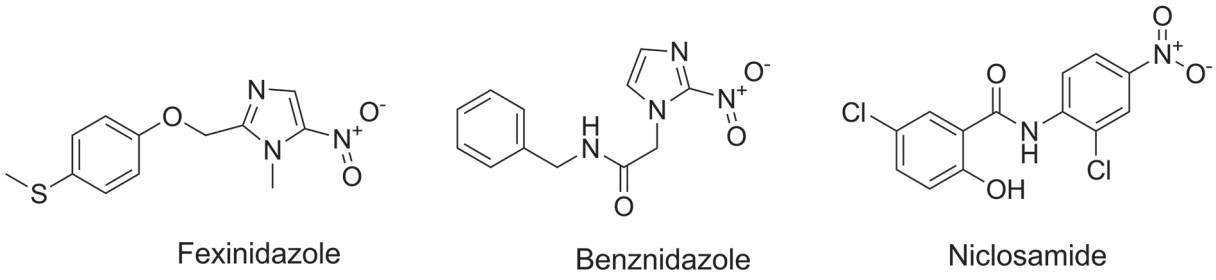

FIGURE 3 – Examples of nitro-aromatic drugs.

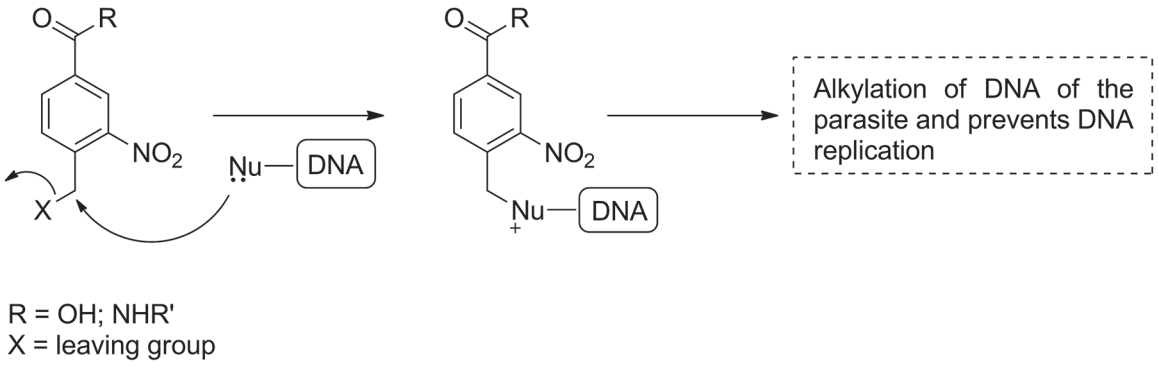

FIGURE 4 – Mechanism of action proposed for the ortho-nitrobenzyl derivatives synthesized.

a nucleophilic cysteine residue in its active site (Jílková *et al*., 2011; Klinkert *et al*., 1989). SmCB1 is an abundant cysteine protease in the parasite (Caffrey *et al*., 1997; Caffrey, Ruppel, 1997), contributes to the digestion of hemoglobin in the schistosome gut (Caffrey *et al.,* 2004; Sajid *et al*., 2003) and is an attractive drug target (Jílková *et al*., 2011; Abdulla *et al*., 2007).

However, as these nitrobenzyl derivatives act by DNA alkylation, studies to assess their mutagenicity were performed for a better comprehension of the possible genotoxic effects of these compounds on human host cells.

MATERIAL AND METHODS

Synthesis

The synthesis and characterization of the compounds **1-15** were previously described by us (Lopes *et al*., 2015). The compounds were synthesized in 1 to 3 steps from commercially available starting materials (*p*-toluic acid, 4-(bromomethyl)benzoic acid or 4-(chloromethyl)benzoic acid) (Figure 5).

In vitro screening of *S. mansoni* somules and adult worms

Maintenance of the *S. mansoni* life cycle, preparation of somules and adult worms, compound storage, and treatment of somules and adult worms were as described (Abdulla *et al*., 2009; Colley, Wikel, 1974; Duvall, DeWitt, 1967). Somules were cultured for four days in the presence of compounds at three concentrations, 0.1, 1.0 and 10 μM. Adults (approximately 5 pairs) were cultured for two days in the presence of 5 μM compound. We employ simple descriptors to describe the observable effects of compounds on the parasite (changes in shape, motility and general appearance; Table I) (Rojo-Arreola *et al*., 2014; Abdulla *et al*., 2009). To convert these observations into a partially quantitative output in order to facilitate relative comparisons of compound effects, each descriptor is awarded a score of 1 up to a maximum score of 4 (Table I; Fonseca *et al*., 2015; Long *et al*., 2016). Evidence of degeneracy or death was awarded the maximum score of 4. Death was adjudicated as the lack of parasite movement over a continuous 30 sec viewing period after tilting of the plate in order to agitate the parasite. For adults, damage to the tegument (outer surface) was awarded a score of 4 on the understanding that surface damage is lethal to the parasite *in vivo* (Andrews *et al*., 1983).

Enzymatic assays against the cathepsin B1 of *Schistosoma mansoni* (SmCB1)

The recombinant SmCB1 was expressed and purified as previously described (Sajid *et al*., 2003). The enzyme activity was measured by monitoring the cleavage of a fluorogenic substrate, Z-Phe-Arg-aminomethylcoumarin (Z-FR-AMC), with 340/440 nm excitation/emission filters, in a microplate reader Synergy 2 (Biotek®) from the Center of Flow Cytometry and Fluorimetry at the Biochemistry and Immunology Department (UFMG). To determine the initial reaction rates, fluorescence was monitored at 25 °C, for 20 minutes at 12s intervals at 340/440 nm excitation/emission, using BioTek's Gen5™ Reader Control and Data Analysis Software. Assays were performed using 20 μM substrate and 8 nM SmCB1 in sodium acetate buffer 0.1 M, pH 5.5, containing 0.01% Triton X-100 and 4 mM β-mercaptoethanol. In all assays, a 1% DMSO negative control and a 100nM E-64 positive control (E-64 is an inhibitor of cysteine proteases like SmCB1) were employed. Enzyme inhibition was calculated based on initial rates compared to the DMSO control. Compound stock solutions were prepared in DMSO. 1 μL of the 20 mM DMSO stock solution was added to each well, to a total volume of 200 μL in buffer assay, resulting in a final concentration of 100 μM of the compound and 1% DMSO in the assay. All compounds were soluble at the screening concentration and conditions. Compounds were screened with or without pre-incubation with the enzyme, for 10 min with the enzyme at room temperature. All assays were performed in at least two independent experiments, each in triplicate.

Cytokinesis-Block Micronucleus (CBMN) Assay

To assess the potential of the nitrobenzyl compounds **4**, **8** and **12** to induce chromosomal mutations *in vitro*, the cytokinesis–block micronucleus assay (CBMN) was performed in the RKO-AS45-1 (ATCC CRL-2577) human cell line. The procedures were carried out as described by Fenech (2007), with adaptations (Gontijo *et al*., 2015). Briefly, the cells were seeded in 24-well plates (2.5 x 10^5 cells/well) and maintained at 37 °C in a humid atmosphere with 5% CO_2. After 24 hours, the cells were washed twice with PBS and the treatments were performed in culture medium without serum for three hours. Each treatment was performed in triplicate and three independent experiments for each compound were carried out in this study. Four different concentrations (5, 10, 20 e 40 μM) of the compounds **4**, **8** and **12**, diluted in culture medium, were evaluated. The negative control

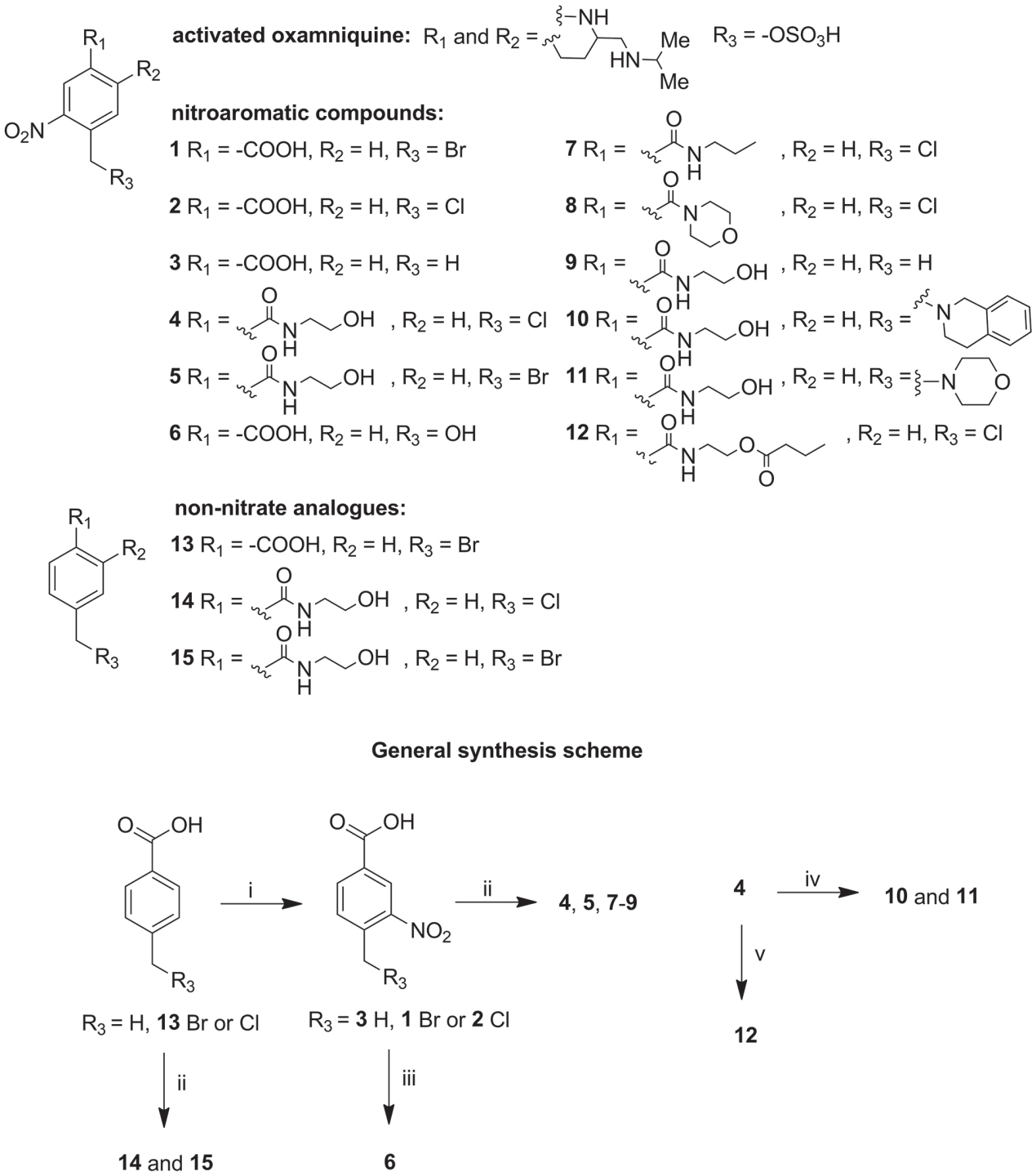

FIGURE 5 – Synthesized nitro-aromatic compounds and non-nitrate analogs and general synthesis scheme. Reagents and conditions: (i) fuming HNO3, 0 ºC, 51-87%; (ii) EDC, NHS, dichloromethane, rt or 0 ºC, then corresponding amines, 22-74%; (iii) $NaHCO_3$ (aq), 80 ºC, 86%; (iv) tetrahydroisoquinoline (56%) or morpholine (64%), acetone, rt,; (v) butyric acid, EDC, DMAP, acetone, rt, 87%.

group was treated with culture medium without serum, and a positive control group was established by treatment with methyl methanesulphonate (MMS - 400 µM). After completing the treatments, cells were washed twice with PBS and fresh complete medium containing cytochalasin-B (3.0 µg/mL) was added for 24 h. Next, the cells were processed, fixed and the slides were stained with DAPI (4',6-diamidino-2-phenylindole – 1 µg/mL) diluted in PBS. The microscopic analysis was performed with a fluorescent microscope (Zeiss, Axioscope A1)

with an excitation filter of 365 nm and a barrier filter of 445/450 nm. One thousand binucleated cells with a well-preserved cytoplasm were analyzed for each treatment in a blind test. Cells containing 1–3 micronuclei were scored (Gomes *et al*., 2011). The criterion for the identification of micronuclei (MNs) was according to a previous report (Titenko-Holland *et al.*, 1997). For statistical analysis, ANOVA was performed followed by the Tukey post-test with a significance level of 0.05.

Nuclear Division Index (NDI)

The influence of the synthetic compounds on cell proliferation was assessed by calculating the nuclear division index (NDI) in RKO-AS45-1. The same slides prepared for the CBMN assay were used, and 300 cells with a well-preserved cytoplasm were counted using fluorescence microscopy, as described above. The NDI was calculated according to Fenech (2007) and Eastmond and Tucker (1989), using the equation:

$$NDI = \frac{(M1 + 2(M2) + 3(M3) + 4(M4))}{N}$$

where M1–M4 are the numbers of cells with 1, 2, 3 and 4 nuclei, respectively, and N is the total number of counted cells. For statistical analysis, ANOVA was performed followed by the Tukey post-test with a significance level of 0.05.

RESULTS AND DISCUSSION

Compounds **1-15** were synthesized according to previously reported procedures (Lopes *et al*., 2015) based on variations at the R_1, R_2 and R_3 positions of sulfate-oxamniquine (Figure 5). We tested the effect of different substituents at the benzylic ($-R_3$) position because it is expected that the mechanism of action is by DNA alkylation. Analogues without the nitro group (**13**, **14** and **15**) were also assessed in order to evaluate the importance of this group for schistosomicidal activity.

Compounds were screened for *in vitro* schistosomicidal activity against somules and adult *S. mansoni* as described above. The data for active compounds are presented in Table I.

Three compounds (**4**, **8** and **12**) elicited progressive and deleterious phenotypic alterations in *S. mansoni* somules: compounds **8** and **12** were the most potent. Both compounds caused degeneration of the parasites within 24 h at 10 and 1 μM. These changes were eventually lethal. Effects (darkening of the parasite) were also noted at 0.1 μM on the third day. As a comparator, oxamniquine induced less pronounced degenerative changes within 24 h at 10 and 1 μM. Effects (slowing and darkening of the parasite) were also noted at 0.1 μM on the third day.

Taking into account that the mode of action of these compounds is by alkylation, it would be expected that compounds bearing a good leaving group, such as bromine and chlorine, to be the most potent. However, all compounds containing a bromine substituent were inactive. This can be explained by the lower stability of the bromo benzyl group in the assay medium. Thus, our results indicate that the chlorine leaving group shows the best apparent trade-off between activity and stability. As expected, compounds without a leaving group at the benzylic position (**3**, **6**, **9**, **10** and **11**) showed no activity.

In contrast to the result obtained for compound **4**, the corresponding non-nitrated analog **14** did not display

TABLE I – Descriptors[a] and severity scores[b] for the responses of *S. mansoni* somules and adults to nitrobenzyl compounds **4**, **8** and **12**

	Somule responses (and severity scores) on the indicated day												**Adult *S. mansoni* responses (and severity scores) at 5 μM on the indicated day**	
	0.1 μM				**1.0 μM**				**10.0 μM**					
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**
4	ne (0)	ne (0)	ne (0)	ne (0)	ne (0)	ne (0)	ne (0)	ne (0)	ne (0)	deg (4)	**D** (4)	**D** (4)	ne (0)	ne (0)
8	ne (0)	ne (0)	dk (1)	dk (1)	R, dk (2)	R, dk (2)	deg (4)	deg (4)	deg (4)	**D** (4)	**D** (4)	**D** (4)	ne (0)	ne (0)
12	ne (0)	ne (0)	dk (1)	dk (1)	R, dk (2)	deg (4)	**D** (4)	**D** (4)	deg (4)	**D** (4)	**D** (4)	**D** (4)	ne (0)	ne (0)
Oxa	ne (0)	ne (0)	S, dk (2)	n.t.	R (1)	dk (1)	R, deg (4)	n.t.	R (1)	dk (1)	R, deg (4)	n.t.	ne (0)	S, dk (2)

[a]Descriptors: ne = no effect, dk = dark, R = shape altered, deg = degenerating, **D** = death, S = slow, n.t. = not tested. [b]Each descriptor is awarded a 'severity score' of 1 (in parentheses) and these are added up to the maximum score of 4. The descriptors 'deg' and '**D**' are automatically awarded a score of 4. **Oxa** = oxamniquine.

activity, suggesting a positive influence of nitro group on reactivity and activity. The electron-withdrawing character of this group may be associated with an increased electrophilicity of the benzylic carbon thereby promoting alkylation.

Regarding the substituent at R_1, compounds having a carboxylic acid group showed no activity against *S. mansoni* somules whereas amides containing a hydrogen bond acceptor atom in the side chain showed relevant activity. The morpholine-amide **8** was more potent than ethanolamide (**4**) and propylamide (**7**) analogs. Esterification of the hydroxyl group of **4** with butyric acid resulted in significant increase of activity.

These findings are in agreement with our previous studies (Lopes *et al.*, 2015) which have indicated the importance of the presence of a good leaving group at the benzylic position for the antiproliferative activity these compounds on different human cancer cell lines (IC_{50} ranging from 1.3 to 83.4 μM). However, unlike the results presented here, compounds bearing a benzyl bromide were cytotoxic for cancer cells independent of the presence of the nitro group.

In summary, the results found in the present study indicate that: a) the presence of a leaving group at the benzylic position is essential for the antishistosomal activity; b) the presence of a nitro group is also indispensable for the activity; c) compounds containing an amide group are more active than those containing a carboxylic acid group; d) compounds bearing a hydrogen bond acceptor atom in the side chain display higher activity. These assumptions are illustrated in Figure 6.

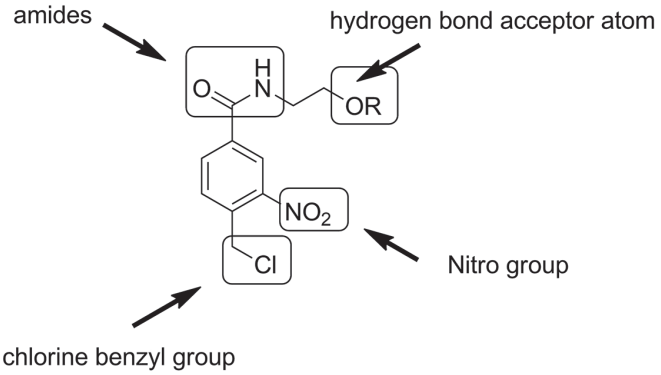

FIGURE 6 – Key functional groups important for anti-schistosomal activity.

Despite the results obtained in the somule assay, none of the compounds showed activity against the adult worm. In contrast, oxamniquine induced degenerative changes in adults on the second day of the incubation (Table I). The lack of activity may be due to an inability of the compounds to cross the adult parasite tegument (surface) and/or differences in expression of potential targets. Structural differences in the outer membrane of larval and adult stages are known (Hockley, McLaren, 1973). Also, differences in transcriptome expression profiles between developmental stages of the parasite, including between somules and adults are well documented (Liu *et al*., 2014; Liu *et al*., 2006; Verjovski-Almeida *et al*., 2003). Modifications of the physicochemical properties of compounds could be performed in an attempt to enhance their membrane permeability.

Alkylating agents are potentially carcinogenic compounds that can introduce lesions at DNA nucleophilic centers (Sobol *et al*., 2007) and can cause clastogenic effects and, consequently micronuclei formation. Micronuclei are small, extranuclear chromatin bodies surrounded by a nuclear envelope, which arise in dividing cells from acentric chromosome fragments or whole chromosomes. They result in chromosomal alterations that have been transmitted to daughter cells and are frequently used as an endpoint in genotoxicity testing (Hintzchea *et al*., 2017). OECD guidelines (OECD, 2016) suggest the use of the micronucleus assay to assess the risks associated with potential clastogenic/aneugenic compounds and the FDA recommends the micronucleus assay in the test battery for genotoxicity testing of Pharmaceuticals (FDA, 2016).

As compounds **4**, **8** and **12** are active *per se* it was necessary to evaluate their genotoxicity on a mammalian cell line. The CBMN assay was carried out using the human cell line RKO-AS45-1 *(*ATCC CRL-2577*)* and results are shown in Figure 7. Compound **4** was mutagenic only in the highest concentration assessed (40 μM) and the NDI was not altered in any concentration assessed. Compound **8** also induced chromosomal mutations on human cell line at 40 μM but the NDI was altered at 20 and 40 μM, indicating a reduction in proliferation rate at these conditions. Compound **12** was able to induce DNA damages in concentrations ranging 10-40 μM but the NDI was reduced only at 20 and 40 μM.

These results showed that the *ortho*-nitrobenzyl halides really have a mutagenic effect, probably due to their alkylating potential. However, the concentrations needed to induce genotoxic effects in the human cell line used was much higher than those necessary to induced phenotypic alterations and death in *S. mansoni* (Table II). Furthermore, the alterations of the NDI observed to the compounds **8** and **12** showed that the human cells used responded to the damages induced by the compounds, probably arresting the cell cycle to promote the DNA repair. Unlike these compounds, studies indicate that

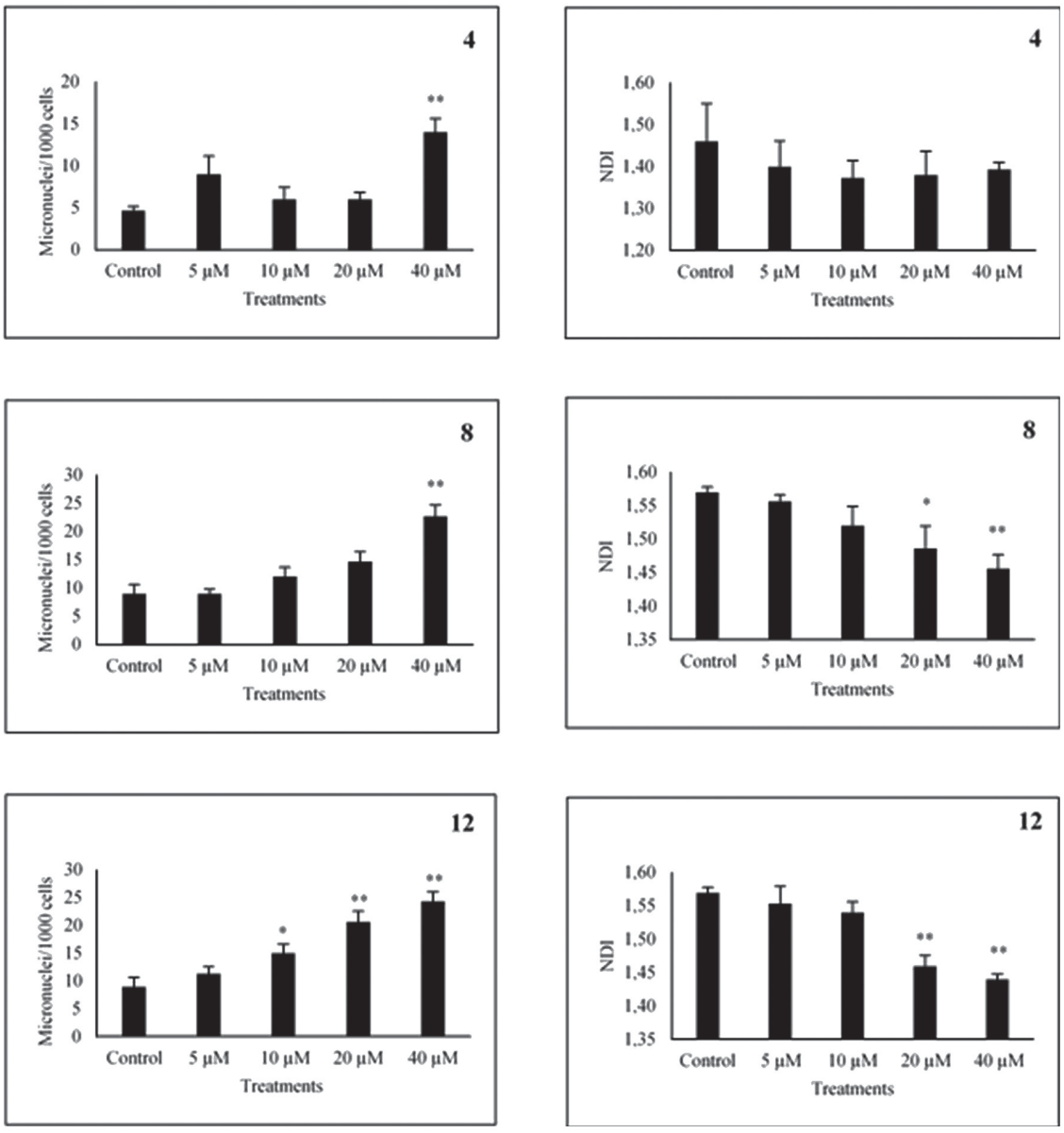

FIGURE 7 - Mean and standard deviation of the frequency of micronuclei in binucleated cells and nuclear division index (NDI) after the treatment of RKO-AS45-1 human cell line with the compounds **4**, **8** and **12** (incubation time = 3 hours). Three independent experiments were performed to each compound. *p<0.05 and **p<0.01 when compared with the control. MMS = Methyl methanesulfonate (400 µM).

the oxamniquine has low mutagenic potential (Ray *et al*. 1975), although there are reports of its mutagenic effect when tested on *Salmonella typhimurium* strain TA100 (Batzinger, Bueding, 1977; Dayan, Deguingand, Truzman, 1985).

Given the electrophilic nature of these derivatives and the nucleophilic character of catalytic cysteine in the protease SmCB1, these compounds were also evaluated against this enzyme, a pharmacological target for schistosomiasis (Jíková *et al*., 2011; Caffrey *et al*., 1997). In order to evaluate their ability to inhibit SmCB1,

TABLE II – *In vitro* effect of **4**, **8** and **12** against *S. mansoni* somules and the least concentration necessary to induce genotoxicity

Compound	Schistosomicidal activity (µM)*	Mutagenic Concentration (µM)
4	10.0	40.0
8	1.0	40.0
12	1.0	10.0

*Concentration of compound at which the maximum severity score of 4 is achieved.

compounds **1-15** were screened *in vitro* at 100 μM with and without a pre-incubation period with the enzyme prior to addition of substrate (Table III). Most compounds were weak inhibitors of SmCB1 and appear to be slightly more effective when pre-incubated with the enzyme. Although *o*-nitrobenzyl derivatives present an electrophilic character and can potentially bind covalently to the cysteine residue, in order to do so they need to have sufficient affinity for enzyme active site. Thus, it is supposed that these compounds are not able to inhibit SmCB1 due to their poor complementarity to the enzyme's active site. Amides **5** and **15** showed the best results, with moderate inhibitory activity (55 and 50% respectively) with pre-incubation. These compounds have in common the ethanolamine chain and the bromine atom at the benzylic position. Further SAR studies are necessary to obtain compounds with more affinity and activity against SmCB1.

TABLE III – Inhibitory activity of **1-15** against SmCB1

Compound	% inhibition at 100 μM ± SD*	
	without incubation	with incubation
1	4.4 ± 1.6	24.3 ± 6.0
2	4.3 ± 0.8	0.0 ± 0.0
3	3.3 ± 3.8	1.1 ± 2.0
4	14.5 ± 2.4	30.5 ± 2.9
5	14.7 ± 0.0	55.7 ± 20.7
6	0.0 ± 0.0	17.3 ± 3.7
7	0.4 ±0 .6	27.4 ± 1.0
8	4.7 ± 4.8	26.5 ± 12.3
9	0.0 ±0 .0	4.6 ± 5.0
10	2.1 ± 1.0	44.5 ± 18.5
11	7.3 ± 2.9	11.1 ± 5.6
12	27.1 ± 0.4	19.9 ± 3.3
13	15.6 ± 1.8	26.5 ± 0.1
14	5.7 ± 1.0	16.5 ± 2.6
15	6.1 ± 0.2	50.6 ± 2.4
E64	55.4 ± 5.6[a]	82.5 ± 9.9[a]

*All reported percentages of inhibition were calculated based on DMSO controls, which were included in every assay and considered as 100% activity. SD = Standard Deviation. [a]Evaluated at 100nM.

CONCLUSION

In conclusion, the *ortho*-nitrobenzyl derivatives **8** and **12** showed significant activity against somules *in vitro*. A structure-activity relationship could be established with the identification of key functional groups of these alkylating agents. However, it was interesting to observe that they triggered the death of *S. mansoni* somules at lower concentrations than those that induced chromosomal mutations in human cells *in vitro.* Nevertheless, none of the compounds was active against the adult parasite *in vitro* and further modifications are needed to find analogs that display activity against both life stages of *S. mansoni*. None of the compounds efficiently inhibited SmCB1 *in vitro*.

ACKNOWLEDGEMENTS

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