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Research Article

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Synthesis, Biological Evaluation, and Structure–activity Relationship of Clonazepam, Meclonazepam, and 1,4-Benzodiazepine Compounds with Schistosomicidal Activity

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The inherent morbidity and mortality caused by schistosomiasis is a serious public health problem in developing countries. Praziquantel is the only drug in therapeutic use, leading to a permanent risk of parasite resistance. In search for new schistosomicidal drugs, meclonazepam, the 3-methyl-derivative of clonazepam, is still considered an interesting lead-candidate because it has a proven schistosomicidal effect in humans but adverse effects on the central nervous system did not allow its clinical use. Herein, the synthesis, *in vitro* biological evaluation, and molecular modeling of clonazepam, meclonazepam, and analogues are reported to establish the first structure–activity relationship for schistosomicidal benzodiazepines. Our findings indicate that the amide moiety [N_1H-C_2(=O)] is the principal pharmacophoric unit of 1,4-benzodiazepine schistosomicidal compounds and that substitution on the amide nitrogen atom (N_1 position) is not tolerated.

Key words: 1,4-benzodiazepine, clonazepam, meclonazepam, pharmacophoric unit, schistosomiasis, structure–activity relationship

Abbreviations: IC_{50}, concentration to kill half the population of worms; *M*, concave 1,4-benzodiazepine boat conformation; *P*, convex 1,4-benzodiazepine boat conformation; E_{HOMO}, energy of the highest occupied molecular orbital; E_{LUMO}, energy of the lowest unoccupied molecular orbital; μ, dipole moment; MEP, molecular electrostatic potential; ADMET, absorption, distribution, metabolism, elimination, and toxicity.

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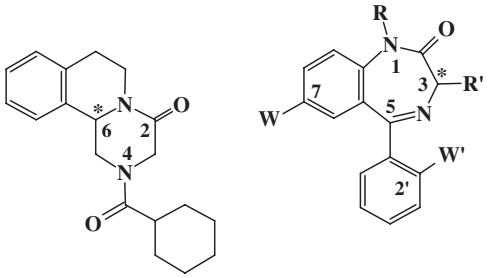
Schistosomiasis is a neglected disease whose death rate has been estimated at 280 000 a year, with other 200 millions infected and 800 millions at risk, especially in tropical and subtropical areas (1–3). Praziquantel (**1**, Figure 1) is the only drug in therapeutic use, leading to a permanent risk of parasite resistance (4). The schistosomicidal effect observed with 1,4-benzodiazepine compounds like the anxiolytic/antiepileptic drug clonazepam (**2**, Figure 1) led to an extensive screening that resulted in the identification of meclonazepam (**3** or Ro 11-3128, Figure 1) as an interesting schistosomicidal lead-candidate ([5, Stöhler, apud Pica-Mattoccia *et al.* 6]). However, adverse effects such as impairment of psychomotor functions, sedation, and ataxia prevented the therapeutic use of this 3-methyl-derivative of clonazepam (7).

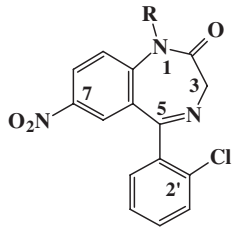
As clonazepam (**2**) and meclonazepam (**3**) are effective against *Schistosoma mansoni* and *S. haematobium*, like praziquantel (**1**), but not against *S. japonicum* (Stöhler, apud Bennett [8]), they have been proposed to act by a similar mode of action as praziquantel, even though their exact molecular target is still a matter of debate and could be different (6,9). Although the large intra-worm calcium influx caused by praziquantel (**1**) has been associated with muscular contraction and surface disruption of the worm, there is now controversy regarding its role in the parasite death (10). The benzodiazepine binding sites present in adult *S. mansoni* have also been discarded as putative molecular targets for meclonazepam and praziquantel, at least for their effects on worm musculature (11).

In view of the urgent need for alternative schistosomicidal agents and taken into account the schistosomicidal activity of clonazepam (**2**) and meclonazepam (**3**), the objective of present work was to establish a structure–activity relationship for 1,4-benzodiazepine compounds with schistosomicidal activity. Six N_1-modified derivatives of clonazepam were synthesized [Figure 1: methyl (**4**), allyl (**5**), benzyl (**6**), ethyloxycarbonylmethyl (**7**), methyloxycarbonylmethyl (**8**),

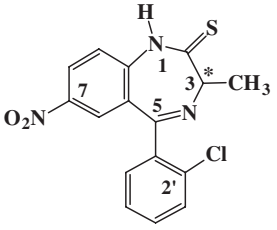


Praziquantel (1)

R	R'	X	W	W'	
H	H	O	NO_2	Cl	Clonazepam (2)
H	CH_3	O	NO_2	Cl	Meclonazepam (3)
CH_3	H	O	Cl	H	Diazepam (10)
CH_3	H	O	NO_2	F	Flunitrazepam (11)



R	
CH_3	LASSBio-1252 (*N*-metilclonazepam) (4)
$CH_2CH{=}CH_2$	LASSBio-1253 (5)
CH_2Ph	LASSBio-1254 (6)
CH_2CO_2Et	LASSBio-1117 (7)
CH_2CO_2Me	LASSBio-1255 (8)
CH_2CO_2H	LASSBio-1132 (9)



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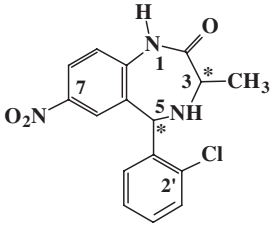

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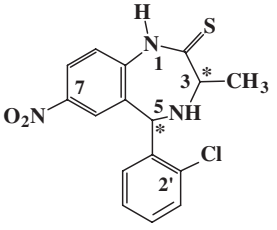

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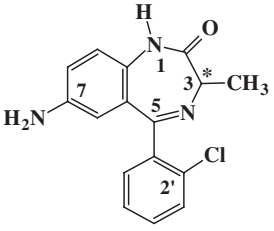

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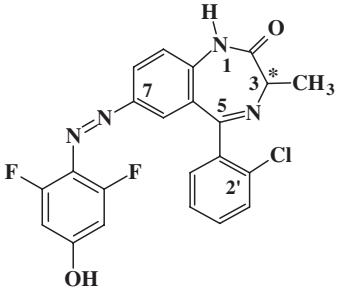

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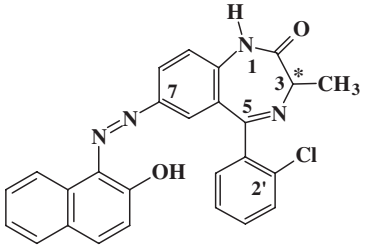


(17)

Figure 1: Praziquantel (**1**), clonazepam (**2**), meclonazepam (**3**), and other 1,4-benzodiazepine compounds (**4**–**17**).

and respective carboxymethyl (**9**)] and incorporated in our theoretical studies together with two classical 1,4-benzodiazepine drugs [diazepam (**10**) and flunitrazepam (**11**)] and some analogues of meclonazepam reported by Mahajan *et al.* (12), (**12**–**17**; Figure 1).

Methods and Materials

Chemistry

General procedure for the preparation of (*E*)-5-(2-chlorophenyl)-7-nitro-1*H*-benzo[*e*](1,4)diazepin-2(3*H*)-one *N*-1-alkylated derivatives (**4**–**9**)

3.17 mmol of clonazepam (**2**) and 0.5 g of KF-Al_2O_3 (40% w/t) in 100 mL of acetone were added to 3.8 mmol of the appropriated alkyl halides. The suspension was stirred at reflux for 24 h. The clonazepam *N*-1-alkylated analogues (**4**–**9**) were isolated by filtration followed by addition of 50 mL water and extraction with dichloromethane (5 × 50 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was purified by column chromatography on silica gel (CH_2Cl_2; CH_2Cl_2: MeOH 0.5%; and CH_2Cl_2: MeOH 1%). Yields 26–36%. The products were identified by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra, infrared (IR) spectra, melting point, and elemental microanalyses.

Biological assays

Clonazepam (**2**), meclonazepam (**3**, *S*-(+)-enantiomer), diazepam (**10**), flunitrazepam (**11**), and praziquantel (**1**, *R,S*-(±)-praziquantel) were evaluated according to their short-term effect on worm motility (13) and schistosomicidal effect estimated using the standard operating procedures proposed by TDR-WHO (14).

Molecular modeling studies

Calculation of the physicochemical properties and stereoelectronic surfaces was performed using the SPARTAN'08 software.[a] The MMFF94 force field (15) was employed to construct the molecular structures of *R*-(−)-praziquantel (**1**), clonazepam (**2**), *R*-(−)-meclonazepam and *S*-(+)-meclonazepam (**3**), the N_1-modified clonazepam analogues (**4**–**9**), diazepam (**10**), flunitrazepam (**11**), and meclonazepam-related compounds (**12**–**17**) (12). Similar results were obtained using the semiempirical methods PM3 (16) and AM1 (17), being this last one chosen for further procedures. The *P* (convex) conformations and *M* (concave) conformations were considered. Pharmacokinetics and toxicological properties (ADMET) were estimated using the VOLSURF+ (version 1.0.4) software.[b]

Results and Discussion

Chemistry

The synthesis of derivatives **4**–**9** was performed using clonazepam (**2**) as starting material and exploring a chemoselective *N*-alkylation using KF/Al_2O_3 in acetone, at room temperature, in the presence of the appropriate alkyl halides (18,19). Ester **7** was used as intermediate for the synthesis of compounds **8** and **9** in a two-step sequence: transesterification and hydrolysis by general base catalysis.

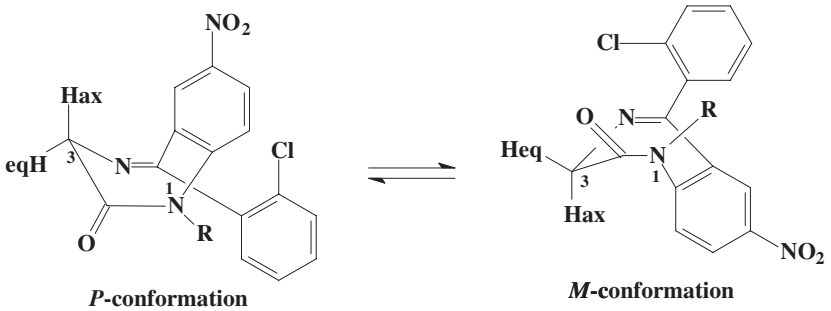
Usually, the 1,4-benzodiazepine system exists in a 7-membered boat conformation with the hydrogens at C_3 in axial and equatorial positions so that conformational enantiomers (*M* and *P* conformers) can exist, as reported for diazepam and analogues (Figure 2) (20,21). The increase in the size of the N_1 substituent in the 1,4-benzodiazepine system will increase the inversion barrier, while smaller substituents prevent resolution of these compounds at room temperature because of the lower racemization barrier (20,22).

The analysis of ^1H NMR spectra (300 MHz, $DMSOd_6$ or $CDCl_3$, 25 °C) of N_1-modified clonazepam analogues, where the hydrogen present in the N_1 atom of clonazepam (**2**) was substituted by methyl (**4**), allyl (**5**), benzyl (**6**), and carboxymethylene groups (**7**–**9**), revealed the presence of two doublet signals (J_{HeqHax} = 10.8 Hz) relative to the methylene (C_3) hydrogens in the equatorial and axial positions of the 1,4-benzodiazepine system, indicating that these compounds exist as conformational enantiomers. On the other hand, the ^1H NMR spectra (300 MHz, $DMSOd_6$ or $CDCl_3$, 25 °C) of clonazepam revealed the presence of one broad signal at 4.31 ppm relative to C_3-protons (Hax and Heq), suggesting that the inversion barrier is not high enough to resolve the methylene in the equatorial and axial positions, so that the interconversion of the seven-membered boat, through the ring flipping, is very fast on the NMR time scale at room temperature.

Biological assays

Short-term effect on worm motility

In control conditions, the worms exhibited a variety of spontaneous body movements, including small and fast generalized shortening and lengthening of the body and propagating body waves along the anterior–posterior axis, similar to peristaltic waves (13). In this model, praziquantel (**1**), clonazepam (**2**), and meclonazepam (**3**) lead to a spastic paralysis followed by a rapid reduction in worm's body area: approximately 30% after 1-min exposure to 1 μM praziquantel and 10 μM meclonazepam and 20% with 10 μM clonazepam (11). On the other hand, the N_1-modified clonazepam analogues (**4**–**9**) and the benzodiazepine drugs, diazepam (**10**) and flunitrazepam (**11**), did not alter the motility and general aspect of the worms during the 30-min period of observation after exposition to 10 μM of the compounds.



R = H (2, Clonazepam)
R = CH_3 (4, LASSBio-1252)
R = $CH_2CH = CH_2$ (5, LASSBio-1253)
R = CH_2Ph (6, LASSBio-1254)
R = CH_2CO_2Et (7, LASSBio-1117)
R = CH_2CO_2Me (8, LASSBio-1255)
R = CH_2CO_2H (9, LASSBio-1132)

Figure 2: 7-membered boat conformation of the 1,4-benzodiazepine system.

Schistosomicidal effect

The *in vitro* schistosomicidal effect of praziquantel (**1**), clonazepam (**2**), and meclonazepam (**3**) was confirmed because all worms died after 5-day exposition at 1 μM, 20, and 5 μM, respectively. As shown in Figure 3, the concentrations killing half the population of worms (IC_{50}) after the standard 5-day period of observation were estimated at 0.4, 10, and 3 μM, for praziquantel (**1**), clonazepam (**2**), and meclonazepam (**3**), respectively. In the same conditions, the N_1-modified clonazepam analogues (**4**–**9**) had no effect, even after a 5-day exposition to 50 μM (Table 1).

Molecular modeling studies

The conformational analysis employing the AM1 semiempirical method (17) revealed that all 1,4-benzodiazepine compounds present preferentially the *M* conformation, although the relatively low-energy barrier between *M* and *P* conformers (*ca.* 1–5 kcal/mol) should allow the free interconversion among them (23). In addition, the methyl group (3-position) of meclonazepam (**3**) exhibits a lower steric effect on the amide [N_1H-C_2(=O)] and imine (N_4=C_5) moieties of the 1,4-diazepine ring when it is located at the equatorial position (*i.e.*, *S*-(+)-enantiomer) in the *M* conformation, the result that might explain the major antiparasitic effect reported for this enantiomer (4,6) but also its sedative effect (24). In addition, we have previously reported (11) an atomic overlapping between the amide moiety of the *M* conformation of *S*-(+)-meclonazepam (**3**) and the most active *R*-(−)-praziquantel (**1**), in which the carbonyl carbon of the isoquinoline-4-one (2-position, Figure 1) was considered to be essential for the schistosomicidal effect (25). This observation claims our attention in reason of experimental data, indicating a similar mode of action between these compounds (6,9).

To challenge the importance of the amide moiety [N_1H-C_2(=O)] as a pharmacophoric unit as well as to establish a structure–activity relationship for 1,4-benzodiazepine compounds with schistosomicidal effect, we analyzed the influence of the chemical functions and substituent groups present in the 1,4-benzodiazepine system of clonazepam (**2**), meclonazepam (**3**), their analogue and derivative compounds (**4**–**17**). Only the *M* conformation will be considered from here, based on preliminary results.

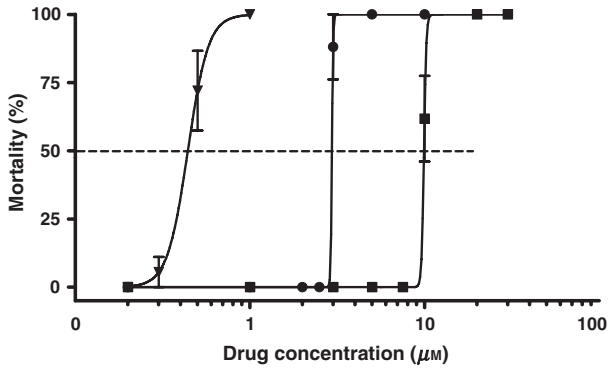


Figure 3: Concentration-effect curve for the schistosomicidal effect of praziquantel (**1**), clonazepam (**2**), and meclonazepam (**3**). Adult male worms were exposed to different concentrations of *R,S*-(±)-praziquantel (▼), clonazepam (■), or *S*-(+)-meclonazepam (●) during 5 days in culture medium. At the end of this period, the worms were observed individually with an inverted microscope to determine the number of dead parasites. Mortality (%) corresponds here to the mean values (percent of dead worms) obtained from 3–6 different experiments with about 9–12 worms each.

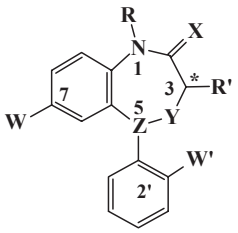
Amide moiety [N_1H-C_2(=O)]

1 Identical HOMO distribution (predominance in the chlorophenyl substituent group at 5-position) and molecular electrostatic potential surfaces [composed by four negative potential regions located on the amide nitrogen atom (N_1), carbonyl oxygen atom (C_2(=O)), imine nitrogen atom (N_4), and oxygen atoms of the nitro group (7-position)] (data not shown) are observed in clonazepam (**2**) and meclonazepam (**3**). Analyses of the correspondent MEPs also show similar electron distribution such as a low electron density on the region of the amide carbonyl carbon (**2**, Figure 4B) and (**3**, Figure 4C). Likewise, a low electron density is observed on the amide region of *R*-(−)-praziquantel, especially at the amide carbonyl carbon C_2 (**1**, Figure 4A). The identification of electron density regions in a molecule allows proposing specific points able to establish important electrostatic interactions in the process of drug-receptor recognition therefore contributing to elucidate structure–activity relationships and to recognize pharmacophoric features (26–28);

2 The analysis of the six inactive N_1-modified clonazepam analogues (**4**–**9**) showed increase in the HOMO distribution and the size and number of negative potential regions on the 1,4-benzodiazepine system (data not shown). The enrichment of the electron density occurred especially on the amide subunit and was independent of the nature (size and poorer or richer inductive effect) of the alkyl substituent. This is illustrated by the decrease in the intensity and extension of the blue color (partially replaced by a weak green color) in the correspondent region of the MEP of LASSBio-1252 (**4**, Figure 4D), in comparison with the same region of clonazepam (**2**, Figure 4B). Similar electron density distributions were observed with diazepam (**10**) and flunitrazepam (**11,** Figure 4E), two N_1-methyl 1,4-benzodiazepine drugs with no schistosomicidal effect (Table 1);

3 The replacement of the carbonyl oxygen atom of meclonazepam (**3**) by a more electropositive, polarizable, and isoster sulfur atom, generating its 2-thiocarbonyl analogue (**12**) (12), produced a comparable electrophilic profile (**12**, Figure 4F) of the amide moiety (**3**, Figure 4C), compatible with the reported similar schistosomicidal activities (IC_{50} of 1.12 and 1.01 μM, respectively) (Table 1). However, such replacement (O → S) could modify pharmacokinetics properties that are putatively unfavorable for the druggability. Indeed, predictions using VolSurf+ indicated: (i) decrease in metabolic stability (MetStab = 57.48 (**3**) to 46.97% (**12**), suggesting a higher sensibility to CYP3A4, a major enzyme in clonazepam metabolism [29]); (ii) decrease in water solubility [LgS7.5 = −4.28 (**3**) to −4.63 (**12**)]; (iii) increase in lipophilicity [LOGP n-Oct = LgD7.5 = 3.063 (**3**) to 3.553 (**12**)]; and (iv) increase in the ability to cross the blood brain barrier [LgBB = −0.19 (**3**) versus −0.30 (**12**)], a key drawback for the clinical use of meclonazepam as a schistosomicidal drug;

Table 1: Schistosomicidal activity and physicochemical properties of 1,4-benzodiazepine compounds



Compound	R	X	R'	Y-Z	W	W'	IC_{50} (μM)	IC_{50} (μM)[a]	V (Å^3)	μ (D)	E_{HOMO} (ev)	E_{LUMO} (ev)
2	H	O	H	N=C	NO_2	Cl	10		281.87	0.86	−9.87	−1.43
3	H	O	CH_3	N=C	NO_2	Cl	3	1.12	300.28	1.15	−9.85	−1.40
4	CH_3	O	H	N=C	NO_2	Cl	>50		300.96	1.10	−9.84	−1.43
5	$CH_2CH=CH_2$	O	H	N=C	NO_2	Cl	>50		333.38	1.31	−9.82	−1.38
6	CH_2Ph	O	H	N=C	NO_2	Cl	>50		384.09	1.45	−9.82	−1.37
7	CH_2CO_2Et	O	H	N=C	NO_2	Cl	>50		368.33	2.18	−9.90	−1.44
8	CH_2CO_2Me	O	H	N=C	NO_2	Cl	>50		349.79	2.15	−9.91	−1.45
9	CH_2CO_2H	O	H	N=C	NO_2	Cl	>50		329.15	2.00	−9.41	−1.49
10	CH_3	O	H	N=C	Cl	H	>50		279.35	4.88	−9.16	−0.37
11	CH_3	O	H	N=C	NO_2	F	>50		292.77	0.86	−9.81	−1.42
12	H	S	CH_3	N=C	NO_2	Cl		1.01	309.08	0.45	−9.09	−1.67
13	H	O	CH_3	NH-CH_2	NO_2	Cl		3.92	304.55	4.43	−9.52	−1.08
14	H	S	CH_3	NH-CH_2	NO_2	Cl		13.67	312.84	3.68	−9.01	−1.58
15	H	S	CH_3	N=C	NH_2	Cl		>29	288.97	5.86	−8.52	−0.31
16	H	S	CH_3	N=C	N=N-(2″,6″-Di-F-4″-Phenol)	Cl		8.63	399.88	5.51	−9.05	−1.19
17	H	S	CH_3	N=C	N=N-(β-Naphtol)	Cl		>22	440.65	4.69	−8.46	−1.33

The physicochemical properties were calculated using the AM1 semiempirical method.

IC_{50}, Concentration to kill half the population of worms ([a]values determined by Mahajan *et al.* [12], expressed originally in μg/mL); V, Molecular volume; μ, Dipole moment; E_{HOMO}, Energy of the highest occupied molecular orbital; E_{LUMO}, Energy of the lowest unoccupied molecular orbital.

The *M* conformation with the 2′-halogem atom position oriented to the lower face of the plane of the 1,4-benzodiazepine system has been considered, except for compounds **13** and **14.**

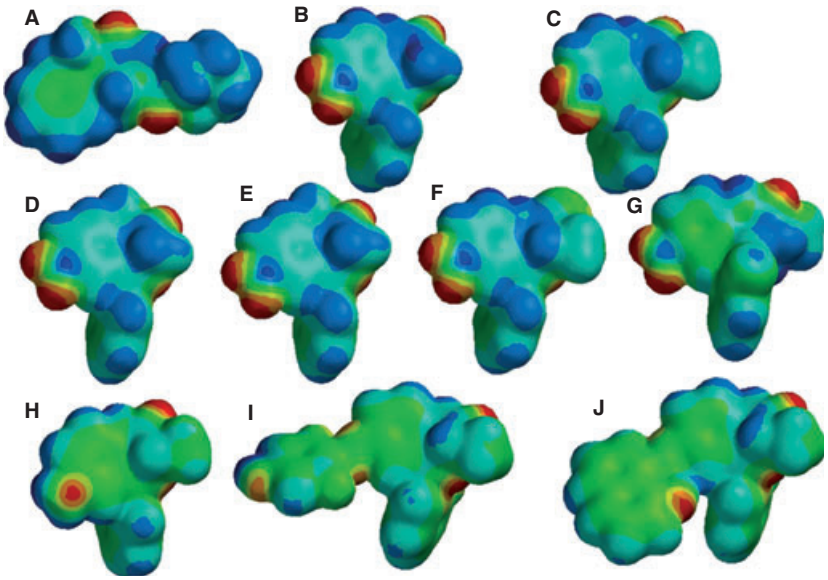


Figure 4: Molecular electrostatic potential maps (MEPs) of the minimum energy conformations of (**A**) *R*-(−)-praziquantel (**1**), (**B**) clonazepam (**2**), (**C**) *S*-(+)-meclonazepam (**3**), (**D**) LASSBio-1252 (**4**), (**E**) flunitrazepam (**11**), and the *S*-(+)-meclonazepam-related compounds: (**F**) 2-thiocarbonyl analogue (**12**), (**G**) imine-reduced bond derivative (**13**), (**H**) amine analogue (**15**), and the 7-azo derivatives: (**I**) 2″,6″-difluoro-4″-phenol (**16**) and (**J**) β-naphtol (**17**) calculated using the AM1 semiempirical method (Appendix S3).

4 All these results (1 and 3) indicate that the nitrogen atom of the amide moiety [N_1H-C_2(=O)] should not be substituted and the respective carbonyl carbon region of 1,4-benzodiazepines should exhibit a low electron density to confer a schistosomicidal activity. This could suggest that the interaction of the amide moiety with the putative receptor might occur through electrostatic interaction (*e.g.,* hydrogen bond) or covalent interaction (*i.e.,* electrophylic center), perhaps with residues of specific parasite enzymes.

3-position

In apparent contradiction to this view, meclonazepam (**3**) was more potent (lower IC_{50}) than clonazepam (**2**) with respect to their schistosomicidal activity *in vitro* (Table 1), albeit the electron density at the carbonyl carbon of the amide moiety [C_2(=O)] is somewhat lower in clonazepam (Figure 4B) than in meclonazepam (Figure 4C). However, considering that there are no other steric or electronic differences between these two compounds, the higher antiparasitic effect of meclonazepam (**3**) might be attributed to the increment of a hydrophobic interaction point with the putative receptor.

Imine bond (N_4=C_5)

Reduction of the meclonazepam imine bond resulted in two compounds (**13** and **14**) (12) with a new chiral center at the carbon C_5 and a higher flexibility of the 1,4-diazepine ring. Increase in and delocalization of the negative potential on the nitrogen atom (4-position) (not shown) and increase in the electron density at the 2-oxo [C_2(=O)] (**13**, Figure 4G) and, in particular, at the 2-thio [C_2(=S)] carbon atoms were also observed. Both structural features may explain the decreased schistosomicidal activity reported for these 2-carbonyl (**13**; IC_{50} = 3.92 μM) and 2-thiocarbonyl (**14**; IC_{50} = 13.67 μM) dihydro derivatives in relation to their prototypes (IC_{50} of 1.12 and 1.01 μM, respectively, for **3** and **12**) (Table 1) and indicate that the imine bond should be preserved.

5-position

The phenyl ring and its halogen atom (2′-position) have no significant effect on the total electron density even in the face of the greater inductive electron-attracting property of chlorine to fluorine (30) as observed by comparing the MEPs of LASSBio-1252 (**4** (W′=Cl), Figure 4D) and flunitrazepam (**11** (W′=F), Figure 4E). The change in the halogen atom to the 4′-position decreased the electron density at the amide carbonyl carbon [C_2(=O)]. However, the inherent hydrophobic feature of the phenyl ring and its chlorine/halogen substituent atom might allow complementary interactions with the parasite receptor, contributing to the antiparasitic effect;

7-position

The contribution of the 7-substituent, commonly represented by the nitro group, to the antiparasitic activity was analyzed comparing meclonazepam (**3**, W=NO_2) and its amine analogue (**15**, W=NH_2) (12) which is less potent (higher IC_{50} value, Table 1). The loss of the biological activity can be related to the electron-attracting nature of the nitro group, decreasing the electron density at the amide carbonyl carbon (2-position) (**3**, Figure 4C) in comparison with the higher electron density effect promoted by the electron donor amine group (**15**, Figure 4H). This comparison was reinforced by a calculated meclonazepam analogue in which the nitro group was removed, resulting in an intermediary electron density, *i.e.,* higher than in **3** but lower than in **12** (data not shown). In addition, an electronic distribution similar to the one of meclonazepam (**3**) was observed when the nitro group was positioned at the 8-position, whereas a decrease in the electron density and steric effect was verified when moving to the 6- or 9-positions; Finally, the subsequent conversion of the amine (**15**) into diazo derivatives **16** and **17** (12) resulted in decrease in the schistosomicidal activity in relation to meclonazepam (**3**) (Table 1) and extension of the distribution of HOMO and increase in the electron density at the 1,4-benzodiazepine system, including that on the amide carbonyl carbon [C_2(=O)] region [(**16**, Figure 4I) and (**17,** Figure 4J)].

The physicochemical properties analyzed (V, μ, E_{HOMO} and E_{LUMO}) (Table 1) varied according to the atom/group substitution, although no correlation with the schistosomicidal properties was observed.

As a conclusion, we believe that the findings reported herein may be helpful for researchers interested to the design of new schistosomicidal compounds, an important but neglected topic.

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Notes

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Chemistry.

Appendix S2. Biological assays (13).

Appendix S3. Molecular modeling studies.

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