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Original Research

Antileishmanial activity of 5-nitroindazole derivatives

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Abstract

Background: Currently, there is no safe and effective vaccine against leishmaniasis and existing therapies are inadequate due to high toxicity, cost and decreased efficacy caused by the emergence of resistant parasite strains. Some indazole derivatives have shown *in vitro* and *in vivo* activity against *Trichomonas vaginalis* and *Trypanosoma cruzi*. On that basis, 20 indazole derivatives were tested *in vitro* against *Leishmania amazonensis*.

Objective: To evaluate the *in vitro* activity of twenty 2-benzyl-5-nitroindazolin-3-one derivatives against *L. amazonensis*.

Design: For the selection of promising compounds, it is necessary to evaluate the indicators for *in vitro* activity. For this aim, a battery of studies for antileishmanial activity and cytotoxicity were implemented. These results enabled the determination of the substituents in the indazole derivatives responsible for activity and selectivity, through the analysis of the structure–activity relationship (SAR).

Methods: *In vitro* cytotoxicity against mouse peritoneal macrophages and growth inhibitory activity in promastigotes were evaluated for 20 compounds. Compounds that showed adequate selectivity were tested against intracellular amastigotes. The SAR from the results in promastigotes was represented using the SARANEA software.

Results: Eight compounds showed selectivity index >10% and 50% inhibitory concentration <1 μM against the promastigote stage. Against intracellular amastigotes, four were as active as Amphotericin B. The best results were obtained for 2-(benzyl-2,3-dihydro-5-nitro-3-oxoindazol-1-yl) ethyl acetate, with 50% inhibitory concentration of 0.46 ± 0.01 μM against amastigotes and a selectivity index of 875. The SAR study showed the positive effect on the selectivity of the hydrophilic fragments substituted in position 1 of 2-benzyl-5- nitroindazolin-3-one, which played a key role in improving the selectivity profile of this series of compounds.

Conclusion: 2-bencyl-5-nitroindazolin-3-one derivatives showed selective and potent *in vitro* activity, supporting further investigations on this family of compounds as potential antileishmanial hits.

Keywords: amastigote, 2-bencyl-5-nitroindazolin-3-one, indazole, leishmaniasis, promastigote

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Introduction

Leishmaniasis is an endemic disease present in 98 countries across Asia, Africa, America and the Mediterranean region.[1] A total of 12–15 million people are currently infected worldwide and 350 million are at risk of acquiring the disease. Every year, 1–2 million new cases of leishmaniasis are reported, and nearly 70,000 deaths occur due to this disease.[2]

The term leishmaniasis embodies a group of illnesses provoked by flagellate protozoan parasites

of the *Trypanosomatidae* family, *Kinetoplastida* class and *Leishmania* genus.[3] There are 18 *Leishmania* species pathogenic for humans.[4] Natural transmission is mediated by vectors of the diptera *Psychodidae* family, *Lutzomya* genus in the American continent and *Phlebotomus* in Europe, Asia and Africa.[5]

The parasite exhibits two morphological forms in its life cycle: the extracellular elongated and flagellated promastigotes, which occur in the insect vector and the intracellular, oval and non-motile amastigotes that reside primarily in macrophages of the vertebrate host.[6]

The most effective control measure available for leishmaniasis is chemotherapy. Pentavalent antimonials are the first-line antileishmanial chemotherapy in most countries. The second-line drugs are pentamidine and Amphotericin B (AmB). Miltefosine, the most recently approved antileishmanial drug, was formerly used as an anticancer agent. Miltefosine has shown clinical efficacy, but its potential teratogenicity is a concern for the treatment of pregnant women.[7] In general, the use of available drugs is limited due to severe side effects, high cost, declining efficacy and the long treatment times.[8] Therefore, new drugs against leishmaniasis that improve the shortcomings of the existing therapeutic options are required.

In vitro and in silico studies have demonstrated the potential of indazole derivatives as antiprotozoal agents, especially against *Trichomonas vaginalis*.[9] In the later research, structural optimization was oriented towards anti-parasite activity against *T. vaginalis* and, afterward, anti-*Trypanosoma cruzi* activity. Improved water solubility and reduced cytotoxicity for mammalian cells were also achieved with the optimized indazole compounds.[10–13] Growth inhibition, parasite immobility, cell lysis and disturbances of the mitochondria and other cytoplasmic organelles have been observed in parasites exposed to these test compounds.[10,14,15]

Based on previous reports of the anti-protozoan activity of indazole compounds, a group of twenty 2-bencyl-5-nitroindazolin-3-one derivatives was tested *in vitro* against *Leishmania amazonensis*.

Materials and methods

Compounds

Twenty 2-benzyl-5-nitroindazolin-3-one derivatives (Table 1) were synthesized and purified to over 95%.[12,13,16] The compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mg/mL and an additional dilution was made in culture media to reach a maximum concentration depending on the type of assay.

Parasite culture

L. amazonensis (MHOM/BR/77/LTB0016 strain) promastigotes were cultivated in Schneider's insect medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (200 µg/mL penicillin and 200 µg/mL streptomycin). Cultures were incubated at 26°C and passages were conducted every 3–4 days to maintain parasites in exponential multiplication.

Macrophages

Macrophages were obtained by washing the peritoneal cavity of BALB/c mice using ice-cold RPMI culture medium. Once collected, the cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated fetal bovine serum at 33°C and 5% CO_2 atmosphere.

Reference drug

AmB deoxycholate was used as positive control. It was dissolved to 2 mg/mL in sterile distilled water and conserved at 4°C until use.

In vitro studies

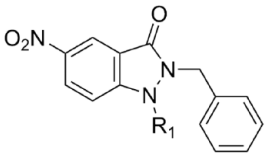
Cytotoxicity in mouse peritoneal macrophages. From a cell suspension of macrophages obtained by peritoneal lavage and adjusted to 3×10^5 macrophages/mL, 200 µL were seeded per well in a 96-well plate and incubated at 33°C and 5% CO_2 for 2h. Subsequently, non-adhered cells were discarded and the monolayer was washed with phosphate-buffered saline (PBS). Finally, 200 µL of medium with the test compounds were added. A maximum concentration of 650 µM was evaluated and 8 serial dilutions at a 1:2.5 ratio were made, each concentration was tested

in quadruplicate. After 48h of incubation, 20µL of 3mM resazurin were added. Cell viability was measured by fluorescence after 4h of incubation in a plate reader (SUMA, UltraMicroanalytical System, Cuba). The 50% cytotoxic concentration (CC_{50}) was calculated by a non-linear fit of the corresponding concentration-effect curves. Each compound was tested in at least three independent trials.

Susceptibility assay in promastigotes. In 96-well plates, 198µL of a culture of promastigotes in exponential growth phase were seeded at a density of 10^5 promastigotes/mL, 2µL of each compound dissolved in DMSO were added so that the final concentrations ranged between 120 and 0.2µM. Every concentration of the test compounds was assessed in quadruplicate. Four wells with culture media and cells without any test compound (negative controls) and four with AmB at 5µM (positive control) were included in every culture plate.[17] Also, a solvent negative control containing culture medium, parasites and DMSO at a concentration of 0.5% was included. Minimum Parasiticidal Concentration (MPC) and Mean Inhibitory Concentration (IC_{50}) were taken as indicators of in vitro activity after 72h incubation. The incubation time selected was in correspondence with the growth pattern of the *Leishmania* strain used in the research. MPC was estimated as the geometric mean (four replicas) of the concentrations that cause total inhibition of the movement of the promastigotes and was obtained by observation under an inverted microscope. Parasite growth was measured by adding 20µL per well of 3mM resazurin and incubating for 18 additional hours. The 50% inhibitory concentration (IC_{50}) values were estimated by non-linear fitting to the E_{max} sigmoid curve. The result was expressed as the mean ± standard deviation for each concentration. In addition, the effect of different concentrations of the most active compounds was determined at 24, 48 and 72h using a Neubauer hemocytometry chamber. To establish statistically significant differences with the control group ($p < 0.05$), Kruskal–Wallis and post hoc multiple comparison tests (STATISTICA 10 software package, version 10.0; StatSoft Inc: Tulsa, USA, 2010) were used.

Intracellular amastigotes assay. The activity on intracellular amastigotes was studied in 24-well culture plates with coverslips and RPMI culture medium.

Table 1. Indazole derivatives of 2-bencyl-5-nitroindazolin-3-one.

Scaffold	ID	R_1
	VA5-13L	CH_3
	VATR1	$(CH_2)_2$–CH_3
	VATR3	$(CH_2)_3$–CH_3
	VATR69	CH_2–$COOCH_3$
	VATR75	$(CH_2)_2OH$
	VATR79	$(CH_2)_2N(CH_3)_2$
	VATR80	$(CH_2)_2$–Pyrrolidine
	VATR81	$(CH_2)_2$–Piperidine
	VATR82	$(CH_2)_2$–$COOCH_3$
	VATR83	$(CH_2)_3$–$CONH_2$
	VATR87	$(CH_2)_2$–Br
	VATR89	$CH=CH_2$
	VATR92	CH_2–$CH=CH_2$
	VATR93	H_2C–$C\equiv CH$
	VATR99	$(CH_2)_3$–NH_2
	VATR100	$(CH_2)_3$–$NHCH_3$
	VATR101	$(CH_2)_3$–$N(CH_3)_2$
	VATR120	$(CH_2)_2$ NH_2
	VATR131	$(CH_2)_2$–$OCOCH_3$
	VATR136	$(CH_2)_2OCH_3$

1mL of cellular suspension (10^6cell/mL) was added to each well, the incubation temperature of the assay was in correspondence with the species-specific thermotolerance mechanism developed by the promastigotes of *Leishmania*.[18] After 2h at 33°C and 5% CO_2, non-adhered cells were eliminated by discarding culture medium and washing the monolayer with PBS to remove non-adherent cells. Fresh medium was added immediately afterwards and the attached macrophages were infected with 1mL of stationary phase promastigote culture (4×10^6 promastigotes/mL) in a parasite/macrophage ratio of 4:1. After a 4h incubation period, the culture medium was discarded to eliminate free promastigotes and then fresh culture medium and the test compounds were added to reach final concentrations of 10–0.1µM. Each concentration was tested in duplicate in at least three independent assays. The coverslips were removed after 48h, and the cultures developed on them were fixed with methanol and stained with Giemsa. The number of amastigotes in every 200 macrophages was determined by

microscopic examination under a light microscope (1500× magnification). The trial was considered valid when the Baseline Infectious Index in the untreated control groups was equal to or greater than 80%. Results were expressed as percent of reduction of the infection rate in comparison to controls.[19,20]

In silico *studies*

Structure–activity relationships

Network-like similarity graphs. The SARANEA[21] software was used for the study of the main Structure–Activity Relationship (SAR) keys in the activity of 2-bencyl-5-nitroindazolin-3-one derivatives on promastigotes and the cytotoxicity of these compounds against peritoneal macrophages. SARANEA is a freely available software implementing a graphical user interface to network similarity graphs (NSG) and NSG-based techniques for data mining.

The molecular structures (in SMILES format), the IC_{50} and CC_{50} values (in nanomolar units), and the corresponding individual (dIC_{50} and dCC_{50}) and overall (GD) desirability values are provided in Supplemental Table 1S. The definitions of dIC_{50}, dCC_{50} and GD are provided below. It is important to remark that overall desirability has a similar interpretation as selectivity (CC_{50}/IC_{50}); therefore, they will both be treated in the same way.

The input to SARANEA software were MACCS (Molecular ACCess System) fingerprints previously calculated using the CDK software[21,22] and the SMIILES strings of the compounds.

The dataset of 20 compounds was represented through NSG, where each molecule is shown as a node, and nodes were connected by edges if the structural similarity exceeded a Tanimoto coefficient of 0.85. To visualize the distribution of the selectivity or the overall desirability, nodes were coded by applying a continuous spectrum from green (lowest) to red (highest). In addition, the largest nodes reflect molecules with high discontinuity scores, which means that the compound is involved in the formation of selectivity cliffs.[21] Selectivity cliffs in the data set are formed by similar compounds with significant differences in selectivity. In NSG, combinations of large green and red nodes linked by an edge are markers of selectivity cliffs, which can be easily recognized in this way.

To define the overall desirability function (GD), each individual response value (IC_{50} and CC_{50}) was transformed into the corresponding desirability score (*di*). Desirability values are bound to the range [0–1].[21,23] A desirability score of 1 represents the compound with the most desirable property.

The desirability scores for activity (IC_{50}) were calculated with a minimization desirability function $\left(di = \frac{Yi - YS}{YI - YS}^{s} \right)$ while the desirability scores for cytotoxicity (CC_{50}) were computed with a maximization one $\left(di = \frac{Yi - YI}{YS - YI}^{s} \right)$ In these equations *Yl* and *YS* are the lowest and highest values of the property (either activity or cytotoxicity), while *Yi* is the value of the property for the *i*th compound.

The extreme values (*YI* and *YS*) in the data set for activity were IC = 580 nM and IC_{50} = 34,400 nM, respectively. For toxicity, these values were CC_{50} = 2500 nM and CC_{50} = 402,600 nM. The *s* parameter was adjusted for each endpoint to assign a value *d* = 0.5 to those molecules with IC_{50} = 5000 nM and CC_{50} = 10,000 nM (the top threshold to consider a compound as active and the minimum one to deem it non-cytotoxic, respectively). That resulted in *s* values of 4.95 and 0.17 for IC_{50} and CC_{50}, respectively.

Based on the above data, the resulting desirability functions were:

$$\text{Activity}: dIC_{50} = \left(\frac{IC_{50i} - 34,400}{580 - 34,400} \right)^{4.95}$$

$$\text{Cytotoxicity}: dCC_{50} = \left(\frac{CC_{50i} - 2500}{402,600 - 2500} \right)^{0.17}$$

Having calculated the desirability scores for each property, and giving the same importance to both responses in the overall desirability (selectivity), the overall desirability score (GD) for the *i*th compound was estimated as follows:

$$GDi = \sqrt[2]{dIC_{50i} * dCC_{50i}} \quad 0 \le GDi \le 1$$

Proper selection of *s* values allows a logical visualization of the data set when using the SARANEA

software. An effective and non-cytotoxic chemical will show desirability scores for both responses (IC_{50} and CC_{50}) over 0.5. Consequently, the overall desirability will also be larger than 0.5.

The input file for SARANEA was a zip file containing a subfolder including the above-mentioned information. An additional subfolder including the files with settings used for each applicable similarity threshold was also provided to the software.

Absorption, distribution, metabolism, excretion, and toxicology predictions. Absorption, distribution, metabolism, excretion, and toxicology (ADMET) properties of the most active compounds against amastigotes were predicted with the ADMETboost web server.[24]

Results

In vitro studies

A total of 13 out of 20 test compounds showed CC_{50} values above 5 μM and 8 of them had CC_{50} values above 10 μM (Table 2).

The MPC of the compounds studied shows that this group was not characterized by its parasiticidal activity, since most of them had IC_{50} values lower than 2 μM (Table 2). However, compounds displaying significant reduction of parasite density are also deemed active and merit further SAR studies, especially when considering that rapid parasite multiplication is the main factor determining the severity of the disease.

A comparative activity/cytotoxicity analysis evidenced that eight compounds were not only active ($IC_{50} < 2$ μM) against the promastigote stage, but also had low cytotoxicity for the host cell, showing selectivity indices over 10.

The compounds that showed selectivity indices over 10, were further tested in the intracellular amastigotes system (Table 2) and evidenced that 4 of them were also selectively active against the amastigote stage. The most outstanding results were obtained for compound VATR131 ($IC_{50} = 0.46$ μM against amastigotes) with comparable activity to that of the reference drug AmB ($IC_{50} = 0.036$ μM), but significantly higher selectivity [VATR131: Selectivity index (SI) = 875; AmB: SI = 161].

Structure–activity relationship

The goal of the SAR analysis was to graphically detect how small structural changes resulted in significant modifications on the selectivity profile. To this end, SARANEA software was used. It is a complementary tool to manual SAR that allows for an integral and more intuitive analysis of all structures by means of molecular network representations, SAR trees and so on. Figure 1 shows the NSG of twenty 2-bencyl-5-nitroindazolin-3-one derivatives with their IC_{50} and CC_{50} values reported. In this NSG, all compounds are linked forming one cluster and sharing at least 85% of structural similarity. Regions of small yellow/green nodes (continuous SAR) coexist with large red and green nodes (discontinuous SAR). Consequently, a heterogeneous SAR characterized the molecules in this dataset. In other words, their selectivity landscapes contain both gently sloped regions and selectivity cliffs. Similar and low continuity/discontinuity scores (0.062)/(0.049) determine a SARI (Structure–Activity Relationship Index) metric[25] of 0.502, which supports the hypothesis of a heterogeneous SAR. This implies a SAR space moderately useful to extract structural determinants of selectivity. For instance, compounds VATR81 and VATR80 form a dominant selectivity cliff as can be seen in the selectivity NSG marked with blue borders. Both molecules share 98% of structural similarity, however the GD (selectivity) values are very different. The GD value for VATR80 is 0.91 while the corresponding value for VATR81 is significantly lower (0.04). That revealed a huge increase in selectivity when the (2-piperidin-1-yl) ethyl fragment (VATR81) at position 1 of the 2-bencyl-5-nitroindazolin-3-one nucleus is replaced by the (2-pyrrolidin-1-yl)ethyl fragment (VATR80). Further analyses were conducted with more advanced structure data functions implemented on SARANEA to mine the information contained in the NSG: Structure−Selectivity Relationship (SSR) tree.

The SAR tree utility implemented in SARANEA collects all pathways that contain a compound of interest and organizes these pathways in a SSR tree data structure. The SSR tree allows exploring overall trends encoding different structural transitions from poor to potent and selective antileishmanial compounds.

Figure 2 shows the SSR tree comprising fourteen out of the twenty 2-bencyl-5-nitroindazolin-3-one

Table 2. *In vitro* activity of 2-benzyl-5-nitroindazolin-3-one derivatives.

Products	Macrophages CC_{50} + SD (μM)	MPC (μM)	Promastigotes IC_{50} + SD (μM)	SI[a]	Amastigotes IC_{50} + SD (μM)	SI[b]	Control	
							BII %	Amas/ Macro
VA5-13L	4.0 ± 0.9	>120	2.0 ± 0.3	2	ND	–	–	–
VATR1	4.7 ± 1.7	>120	0.8 ± 0.2	6	ND	–	–	–
VATR3	5.1 ± 1.6	>120	0.6 ± 0.1	9	ND	–	–	–
VATR69	171.0 ± 22.1	>120	0.6 ± 0.3	285	>10	<17	89.3	8
VATR75	15.9 ± 9.2	>120	1.3 ± 0.5	12	9.1 ± 1.2	2	88.8	8
VATR79	177.6 ± 13.9	>120	34.4 ± 1.2	5	ND	–	–	–
VATR80	169.9 ± 21.3	>120	0.87 ± 0.6	195	6.3 ± 0.3	27	87.2	8
VATR81	12.1 ± 5.9	>120	24.2 ± 6.9	0.5	ND	–	–	–
VATR82	7.2 ± 5.4	>120	0.6 ± 0.3	12	0.9 ± 0.4	8	84	7
VATR83	8.3 ± 3.6	>120	0.8 ± 0.4	10	4.2 ± 2.4	2	83	7
VATR87	3.8 ± 1.7	>120	1.2 ± 0.5	3	ND	–	–	–
VATR89	5.1 ± 0.2	>120	1.0 ± 0.2	5	ND	–	–	–
VATR92	4.2 ± 0.1	>120	2.5 ± 2.2	2	ND	–	–	–
VATR93	4.3 ± 0.4	>120	0.9 ± 0.4	5	ND	–	–	–
VATR99	117.3 ± 5.6	>120	1.3 ± 0.2	90	8.0 ± 2.9	15	83.7	7
VATR100	2.5 ± 0.4	120–48	2.3 ± 0.3	1	ND	–	–	–
VATR101	5.0 ± 3.0	120–48	12.8 ± 0,7	0.4	ND	–	–	–
VATR120	139.5 ± 16.2	>120	0.9 ± 0.2	155	8.5 ± 2.1	16	84.5	7
VATR131	402.6 ± 13.4	>120	2.1 ± 0,9	191	0.46 ± 0.01	875	87.2	8
VATR136	4.8 ± 1.0	>120	1.7 ± 1.0	3	ND	–	–	–
Amphotericin B	5.8 ± 0.5	0.2	0.03 ± 0.014	196	0.036 ± 0.006	161	84.2	7

[a]Selectivity indexes for promastigotes (SI = CC_{50} macrophages/IC_{50} promastigotes).
[b]Selectivity indexes for amastigotes (SI = CC_{50} macrophages/IC_{50} amastigotes).
BII, Baseline Infectious Index; CC_{50}, 50% cytotoxic concentration; IC_{50}, 50% inhibitory concentration; MPC, minimum parasiticidal concentration

derivatives. At the top, green nodes represent compounds with low or null selectivity, for example, VATR79, 81 and 100. At the bottom, red nodes (VATR69, 80, 99, 120 and 131) depict significantly selective molecules. The observed pattern in the SSR tree evidences the positive effect of hydrophilic fragments substituted at the 1 position of the 2-bencyl-5-nitroindazolin-3-one scaffold on selectivity. For example, replacing propyl and butyl fragments in chemicals VATR1 and VATR3 by 2-aminoethyl or 3-aminopropyl moieties in chemicals VATR120 or VATR99 radically

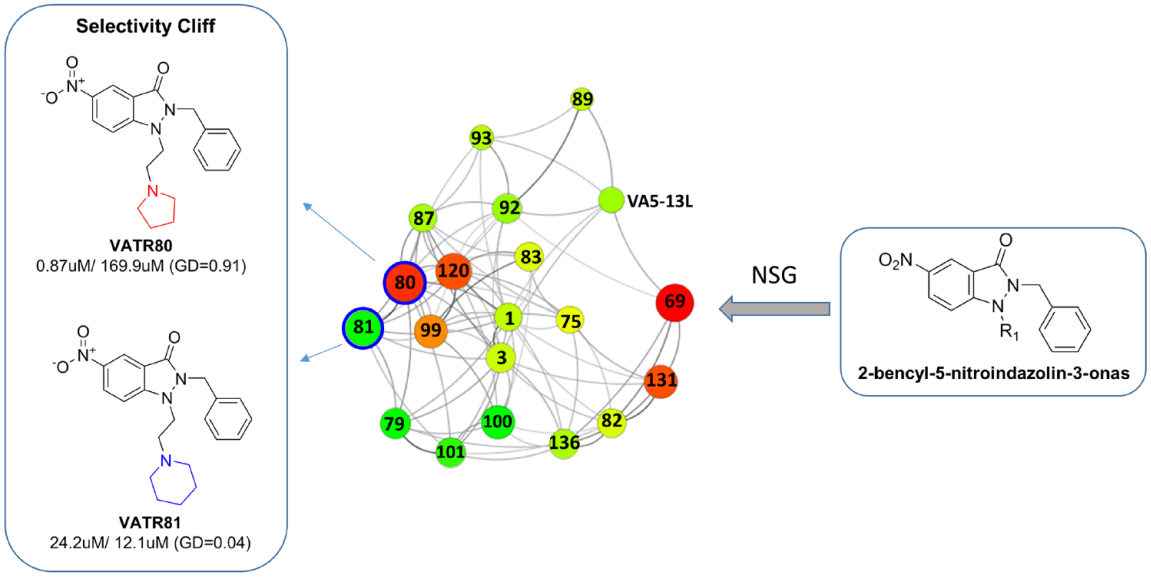


Figure 1. Selectivity NSG of the twenty 2-bencyl-5-nitroindazolin-3-one derivatives.
Circles represent compounds which are connected by edges since they share 2D similarity above a predefined Tanimoto coefficient (Tc) of 0.85. The colour and size of every node reflect the overall desirability and the contribution to the global discontinuity (GD) score (SARI) of each compound, respectively. For each compound, the IC_{50} and CC_{50} values are shown separated by a slash, while the GD scores are enclosed in parenthesis.
NSG, network similarity graphs; SARI, Structure–Activity Relationship Index.

increased the selectivity of the latter. In the same way, the introduction of ester and alcohol groups (VATR82, 75, 131 and 69) increased selectivity, resulting in the most selective compounds. Among them, VATR69 displayed the highest GD score of 0.93. All fragments positively contributing to GD present in VATR75, 99, 120, 131 and 69 can also be associated with the existence of hydrogen acceptor or donor sites, which could increase the antileishmanial activity and decrease compounds' cytotoxicity.

As represented in the SAR tree, VATR120 and 131 are two compounds that have the same GD index (GD = 0.89), they are not the two best molecules, but they have a significant selectivity (Table 2). All fragments that contribute positively to GD present in VATR120 and 131 can also be associated with the existence of hydrogen acceptor or donor sites, which could increase antileishmanial activity and decrease the cytotoxicity of the compounds. Both molecules were selected to analyze the effect of different concentrations of the compounds on parasite culture growth. The growth curves as a function of time for these compounds were evaluated in a range of 12–0.02 μM.

Figure 3 plots the promastigotes count for the different concentrations of VATR120 and VATR131. To do this, the Neubauer hemocytometry chamber method was used. This study was conducted to only evaluate the effect of different concentrations of the compound on the culture of promastigotes over time. However, the IC_{50} values of both products were determined with the fluorometric method of transformation of resazurin (Table 2). The fluorometric method is the most recommended one since it allows for faster, objective and reproducible tests.[26] Therefore, it is not possible to establish comparisons between inhibitory concentrations obtained with different methods. This means that the values in Table 2 are not statistically comparable with the values in Figure 3 during the 72 h time interval.

Compound VATR120 was much more active in the promastigote stage. The inhibitory concentrations 12 and 4.8 μM show significant differences ($p < 0.05$) at 24, 48 and 72 h of treatment compared to the untreated control. Although there are no significant differences for the rest of the test concentrations, a reduction in the density

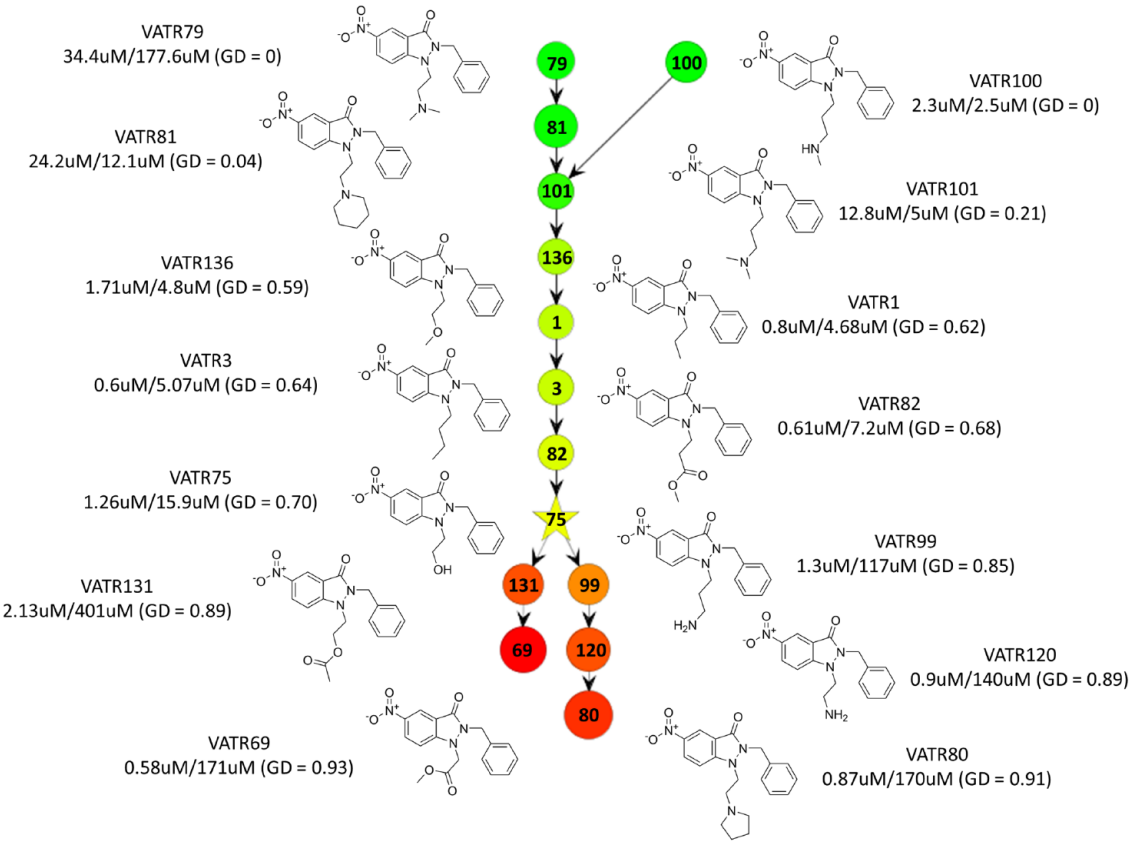


Figure 2. SSR tree generated for the twenty 2-bencyl-5-nitroindazolin-3-one derivatives.
Green and red nodes represent compounds with low and high desirability, respectively. The molecular structures, IC_{50}/CC_{50} (GD) values of 2-bencyl-5-nitroindazolin-3-ones represented by single nodes are also shown in the figure.
CC_{50}, 50% cytotoxic concentration; IC_{50}, 50% inhibitory concentration.

of the culture at a concentration of 0.77 μM was microscopically appreciable after 72 h of treatment. Meanwhile, VATR131 only showed significant differences ($p < 0.05$) on the promastigote stage with respect to the untreated control at the maximum concentration (12 μM) and a reduction in the density of the culture was not observed.

ADMET predictions

ADMET properties of the most active compounds against amastigotes were predicted with the ADMETboost web server. The results of these predictions are presented in Table 3 and show that the newly reported compounds have physicochemical properties more favorable for oral bioavailability than the reference drug AmB. Likewise, nitroindazole derivatives are predicted with slightly better cell permeability and intestinal absorption than the reference compound, although their water solubility in some cases is lower. In terms of distribution, AmB has a lower blood-brain barrier permeability than the compounds reported herein. Nitroindazoles also show high probability of interacting with different CYP450 isoforms and are excreted at higher rates than AmB. For toxicity, predictions show a similar profile for the newly reported compounds and the reference drug.

Discussion

Derivatives of 5-nitroindazole have shown various biological and pharmacological activities such as antiprotozoal activity.[9] The study of the activity of indazole against trypanosomatids began with *T. cruzi* due to its similarity to the reference drugs Nifurtimox and Benznidazole. This led to the investigation of this family of compounds against

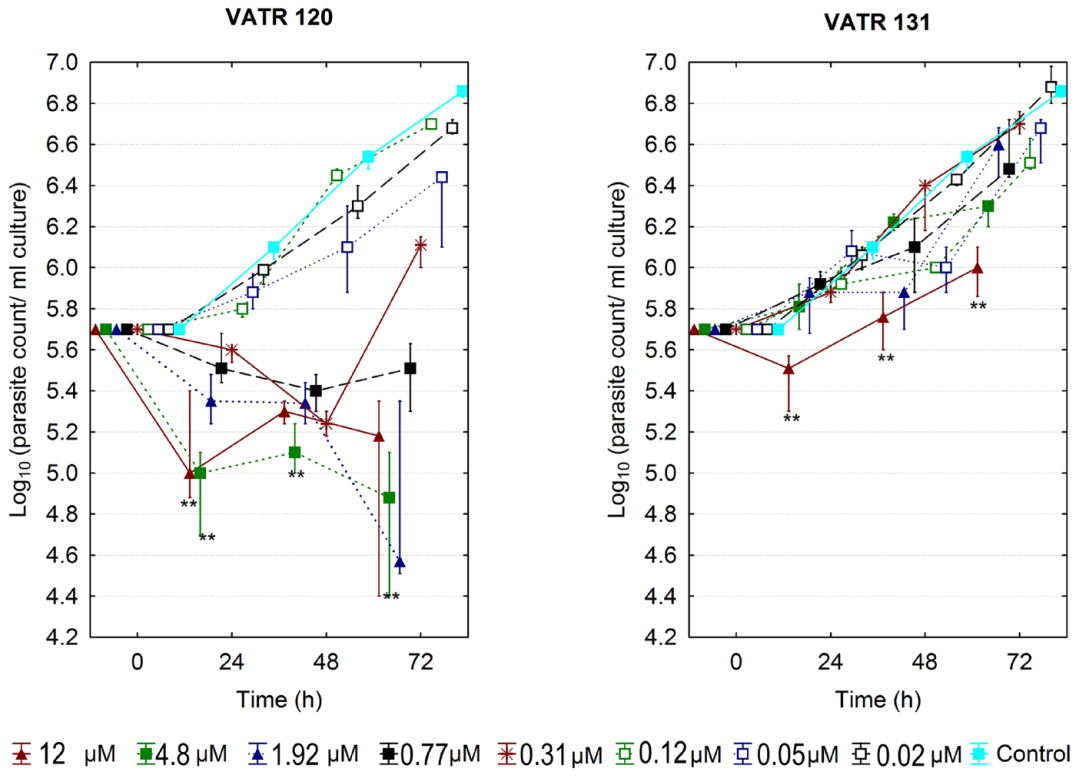


Figure 3. Parasite growth curves. The median (whisker: 25–75%) of parasite counts per mL of culture (*y*-axis) are plotted against time (VATR120 and VATR131).
The groups marked with asterisks (*) show significant differences with the control group ($p < 0.05$), according to Kruskal–Wallis and post hoc multiple comparison tests.

other parasitic diseases such as leishmaniaisis, considering the degree of kinship between *T. cruzi* and *Leishmania*.

During the *in vitro* screening of these compounds, the analysis of their cytotoxicity is of special interest, since the adverse effects of conventional drugs currently used in antileishmanial chemotherapy are severe. In this study, the effect of indazoles on peritoneal macrophages showed lower cytotoxicity values than AmB, but only six of them with CC_{50} values greater than 100 µM. This group of indazoles has been previously evaluated against another murine fibroblasts cell line (L929) with CC_{50} values greater than 100 µM.[12] Differences in toxicity in different cell lines may be due to continuous cell lines being constantly growing and a product may have an effect on the cell, but not inhibit culture growth. In peritoneal macrophages, as they do not have the capacity to divide, not only viability is affected but their number is too.

The *in vitro* activity against promastigotes of *L. amazonensis* of 2-benzyl-5-nitroindazolin-3-ones showed that, although they are not characterized by their parasiticidal activity, these compounds could be classified as potent growth inhibitors. Values of $IC_{50} < 1$ µM were obtained for eight of the studied compounds, with four of them having a similar SI that for VATR69 was higher than for the reference drug AmB (Table 2).

Compounds designed by other authors with the same chemical scaffold have not shown a potent antileishmanial activity, so these results highlight the potential of the derivatives reported herein. For example, other derivatives of 5-nitroindazole showed IC_{50} values higher than the indazoles studied, with IC_{50} values between 11 and 52 µM, against *L. infantum* and *L. braziliensis*.[15]

The preliminary studies in promastigotes of *L. amazonensis* provided information on the

Table 3. ADMET predictions for the most active compounds and the reference Amphotericin B.

Molecule property	**Unit**	**VATR69**	**VATR80**	**VATR99**	**VATR120**	**VATR131**	**Amphotericin B**	**Values desirable**
Molecular weight	g/mol	341.1	366.17	326.14	312.12	355.12	923.49	100–600
Number of heteroatoms		8	7	7	7	8	18	1–15
Number of rotatable bonds		5	6	6	5	6	3	0–11
Number of rings		3	4	3	3	3	3	0–6
Number of HA		7	6	6	6	7	17	0–12
Number of HD		0	0	1	1	0	12	0–7
log KOW	log (mol/L)	1.93	2.86	2.11	1.72	2.32	0.71	0–3
Absorption	**Unit**	**VATR69**	**VATR80**	**VATR99**	**VATR120**	**VATR131**	**Amphotericin B**	**Values desirable**
Caco-2 permeability	log (cm/s)	−4.96	−5.22	−5.15	−5.1	−5.16	−5.49	>−5.15
HIA	%	74.89	75.98	74.83	76.17	74.44	65.92	⩾80 E 30–80 M
Pgp inhibition	%	45.66	50.69	51.45	50.26	46.9	45.35	<30 E 30–70 M
log D7.4	log (mol/L)	1.83	1.8	1.5	1.53	1.89	1.56	1–3
Aqeuous solubility	log (mol/L)	−4.17	−4.26	−3.9	−3.88	−4.17	−3.9	−4–0.5
Oral bioavailability	%	46.01	42.27	45.24	45.36	43.59	37.84	
Distribution	**Unit**	**VATR69**	**VATR80**	**VATR99**	**VATR120**	**VATR131**	**Amphotericin B**	**Values desirable**
BBB	%	34.74	41.31	42.29	42.39	39.1	23.16	⩽30 E 30–70 M
PPBR	%	48.53	51.93	49.55	50.06	49.21	35.31	⩽90
VDss	L/kg	3.6	3.53	3.57	3.5	3.43	4.13	0.04–20
Metabolism	**Unit**	**VATR69**	**VATR80**	**VATR99**	**VATR120**	**VATR131**	**Amphotericin B**	**Values desirable**
CYP2C9 inhibition	%	61.57	62.56	65.25	64.83	54.61	51.38	
CYP2D6 inhibition	%	92.96	91.8	93.33	89.99	95.98	100.7	
CYP3A4 inhibition	%	35.62	32.12	33.82	33.44	38.82	28.68	
CYP2C9 substrate	%	31.22	30.57	32	31.57	31.98	31.81	
CYP2D6 substrate	%	61.42	57.09	60.41	62.36	61.17	59.35	
CYP3A4 substrate	%	34.75	38.32	34.82	35.56	38	26.88	

(Continued)

Table 3. (Continued)

Excretion	Unit	VATR69	VATR80	VATR99	VATR120	VATR131	Amphotericin B	Values desirable
Half life	h	76.61	71.23	71.35	72.47	76.23	119.76	
CL-Hepa	μL min^{-1} $(106\ cells)^{-1}$	41.38	40.51	41.25	38.58	45.54	38.72	>15 high 5–15 moderate
CL-Micro	mL min^{-1} g^{-1}	40.85	38.72	35.4	35.81	41.31	43.63	>15 high 5–15 moderate
Toxicity	**Unit**	**VATR69**	**VATR80**	**VATR99**	**VATR120**	**VATR131**	**Amphotericin B**	**Values desirable**
hERG blockers	%	42.45	46.77	43.33	42.07	42.15	41.73	⩽30 E 30–70 M
Ames	%	48.46	49.43	51.87	49.27	50.57	42.63	⩽30 E 30–70 M
DILI	%	48.48	50.11	51.47	53.1	52.67	42.38	⩽30 E 30–70 M
LD50	–log (mol/kg)	2.28	2.6	2.17	2.28	2.22	2.48	

ADMET, Absorption, distribution, metabolism, excretion, and toxicology; Ames, Probability of being mutagenic; BBB, Blood-brain barrier permeation; CL-Hepa, Clearance hepatocyte; CL-Micro, Clearance microsome; DILI, Probability to produce drug-induced liver injury (DILI); E, Excellent; hERG Blockers, Probability of being hERG (Human ether-a-go-go-related gene) blocker; HIA, Human intestinal absorption; LD50, Acute toxicity; log D7.4, Lipophilicity; log KOW, Log *n*-octanol-water partition coefficient; M, Medium; Number of HA, Number of hydrogen bond acceptors; Number of HD, Number of hydrogen bond donors; Pgp Inhibition, P-glycoprotein inhibition; PPBR, Plasma protein binding rate; VDss, Volume of distribution at steady state.

possible SAR of the compounds evaluated and constitute a first screening to rule out those that have no activity. However, the assays that provide the most valuable information are those using intracellular amastigotes, which are the infective form in the mammalian host.[27]

In the intracellular amastigotes system, five compounds showed SI higher than 10, but only one compound showed activity comparable to AmB. VATR131 was found to be the most active compound against the amastigote stage, with SI five times higher than AmB. In another research, this compound presented similar activity against epimastigotes and intracellular amastigotes of *T. cruzi*, significantly improving the activity of Benznidazol ($p < 0.05$).[13] In a similar study, four compounds derived from benzimidazole and azabenzoxazole were evaluated against macrophages infected with *L. amazonensis*, with only one of them resulting active with an IC_{50} value of 4 μM.[28] In the amastigote stage (Table 2), compound VATR131 showed the best values of IC_{50}.

As *Leishmania* is a digenetic organism, there are specific metabolic steps for each stage of life, which is reflected in the different susceptibility of the parasite to the same compound depending on its life stage. Some active products in promastigote forms are inactive in amastigote forms. This result is expected considering that a bioactive product needs to pass different barriers to meet the parasite within the phagolysosome, thus tests in amastigotes are preferred because they are very similar to what happens in nature. The product VATR131 is of special interest, as it showed activity against promastigotes and amastigotes, combined with low cytotoxicity and

activity similar to the reference drug while having higher selectivity (SI = 875).

The study in SARANEA allows visualizing the high similarity (Tc ⩾ 0.85) of the compounds in the series under study, and provides details of the indazole derivatives that can be inferred as general rules. The presence of the 5-nitro group plus the 2-benzyl substituent in the indazole ring positively influences the activity against *Leishmania*. In fact, seventeen out of the twenty 2-bencyl-5-nitroindazolin-3-one derivatives tested had dIC_{50} values ⩾ 0.5 or IC_{50} ⩽ 5000 nM. Esters and alcohol groups, as well as primary alkylamines substituents in N1 improve the antileishmanial activity and the selectivity, as was shown above. The 20 indazoles studied were previously evaluated against *T. cruzi* and five compounds (VATR69, 75, 82, 99 and 120) were shown to be active for both *Leishmania* promastigotes and *T. cruzi* epimastigotes.[12] These structures could be studied as possible hits with anti-tripanosomatidae activity.

The mechanism of action for *Leishmania* remains unknown but there are some studies in *Trypanosoma* that suggest the importance of the 5-nitro group in indazoles that could induce oxidative stress in the parasite.[29] This radical may act through a previous intracellular reduction by nitroreductases yielding nitro-anion radicals, followed by redoxcycling with oxygen producing reactive oxygen species, able to damage the parasite's macromolecules. This compound merits further research as a possible drug candidate against leishmaniasis.

ADMET predictions were performed for the nitroindazoles that displayed the best activity against the amastigote form of the parasite. These predictions show a better profile for oral bioavailability for these compounds compared to the reference drug AmB. In addition, the calculations show that in future development steps, properties such as water solubility, permeability, CYP450 interaction and toxicity must be jointly optimized with potency to ensure not only potent derivatives but also safe drug candidates with better pharmacokinetic profiles.

Conclusion

Compound VATR131 [ethyl 2-(benzyl-2,3-dihydro-5-nitro-3-oxoindazol-1-yl) acetate] showed outstanding results against the amastigotes stage having an activity comparable to that of the reference drug AmB, but with significantly higher selectivity. The study of the SAR showed the positive effect on the selectivity of the hydrophilic fragments in position 1 of the structure 2-benzyl-5-nitroindazolin-3-one, which played a critical role in improving the selectivity profile of this series of compounds. Derivatives of 2-benzyl-5-nitroindazolin-3-one showed potent and selective *in vitro* activity, supporting further investigations on this family of compounds as potential antileishmanial agents.

Declarations

Ethical approval and consent to participate

Directive 2010/63/EU of the European Parliament (on the protection of animals used for scientific purposes, 22 September 2010) was followed to cause the least suffering. The protocol for the use and care of animals was previously approved by the Institutional Ethics Committee – Instituto de Medicina Tropical 'Pedro Kourí' (IEC-IPK), La Habana, Cuba and the Scientific Council of Centro de Bioactivos Quimicos from Universidad Central de las Villas (CC-CBQ-UCLV), Santa Clara, Cuba (Approval No: IEC-IPK-15/2021, date of approval 12/10/2021).

Consent to publication

Not applicable.

Author contributions

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Aliuska Morales-Helguera: Data curation; Validation.

Yunierkis Perez-Castillo: Data curation; Validation; Writing – review & editing.

Vicente Arán-Redó: Resources; Supervision.

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Competing interests

The authors declare that there is no conflict of interest.

Availability of data and materials

Data is available as Supplemental material. Materials are available upon request to the authors.

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Supplemental material

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