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Research Article

Effectiveness of Novel 5-(5-amino-1-aryl-1*H*-pyrazol-4-yl)-1*H*-tetrazole Derivatives Against Promastigotes and Amastigotes of *Leishmania amazonensis*

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In this research, a series of substituted 5-(5-amino-1-aryl-1*H*-pyrazol-4-yl)-1*H*-tetrazoles were synthesized and evaluated for *in vitro* antileishmanial activity. Among the derivatives, examined compounds 3b and 3l exhibited promising activity against promastigotes and amastigotes forms of Leishmania amazonensis. The cytotoxicity of these compounds was evaluated on murine cells, giving access to the corresponding selectivity index (SI).

Key words: antileishmanial activity, *Leishmania amazonensis*, tetrazole derivatives

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Leishmaniasis, caused by protozoa of the genus *Leishmania,* remains as a significant health issue in large part due to the lack of effective and affordable drugs and to the increasing resistance against existing drugs. More than 2 million new cases of leishmaniasis occur each year, with approximately 350 million persons at risk of infection (1,2). All parasites of the genus *Leishmania* are obligate intracellular parasites that infect cells of the mononuclear phagocyte lineage of their vertebrate hosts, in which they exist as non-motile intracellular amastigotes (3). Within the insect vector, the female sandfly of the genus *Lutzomyia* in the New World and *Phlebotomus* in the Old World, they exist as extracellular motile promastigotes. In Brazil, *Leishmania amazonensis* is responsible for several clinical manifestations reported, which include cutaneous, mucosal, diffuse cutaneous, and visceral leishmaniasis (4,5) and is considered a species of epidemiologic importance. However, the chemotherapy for leishmaniasis is generally ineffective mainly due to the emergence of drug-resistant strains and toxicity of the therapeutic agents. The pentavalent antimonials compounds are widely used as primary therapy whereas other drugs such as amphotericin B, pentamidine, paromomycin, and azoles derivatives have been also employed (6). The azole antifungals ketoconazole, miconazole, and itraconazole have been used to treat cutaneous leishmaniasis with variable success rates (7). Allopurinol, a pyrazolopyrimidine, was described as a leishmanicidal drug in the late 1970s (8) and is considered an alternative to antimonials in some cases. In this context, our research group has been devoted to the synthesis and evaluation of antileishmanial activity of cyclic systems containing nitrogen (9–11), including the compound 5-amino-1-(3,5-dichlorophenyl)-4-(4,5-dihydro-1*H*-imidazol-2-yl)-1*H*-pyrazole, which was tested *in vivo* and exhibited significant inhibition relative to an untreated control (12). In addition, it has been reported that anti-inflammatory and related biological activities have been improved by the substitution of a 5-tetrazole group in place of carboxyl function (13–15). Recently, 5-substituted tetrazoles have been mentioned more and more frequently as non-classical isosteres of the carboxyl group. It has been also shown that ionized tetrazoles are ten times more lipophilic than the corresponding carboxylic acids, which in some cases enables these compounds to penetrate the cell membrane with greater ease (15). In this way, we have designed pyrazole–tetrazole hybrids by applying rational drug design approach. Twelve 5-(5-amino-1-aryl-1*H*-pyrazol-4-yl)-1*H*-tetrazoles (**3a**–**l**) were synthesized and evaluated against *Leishmania amazonensis*.

Methods and Materials

Chemistry

Unless otherwise noted, all the reagents and solvents were obtained from the market and used without further

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purification. Thin-layer chromatography (TLC) was performed on silica gel plates (60 F254). Melting points were determined with a Fisatom 430 apparatus and were not corrected. ^{1}H NMR spectra were recorded at room temperature on a Varian Unity plus 300 MHz employing tetramethylsilane as the internal reference. The chemical shifts (δ) are reported in ppm and the coupling constants (J) in hertz. Infrared (IR) spectra were recorded in a Perkin–Elmer Model 100 FT-IR spectrophotometer using attenuated total reflection (ATR) accessory. Mass spectral data were obtained using a Finingan MAT 711A.

General procedure for the preparation of 5-(5-amino-1-aryl-1H-pyrazol-4-yl)-1H-tetrazoles (3a–l)

The key intermediates 5-amino-1-aryl-1*H*-pyrazole-4-carbonitriles (**2a**–**l**) were obtained from the reaction between arylhydrazine hydrochlorides (**1a**–**l**) and ethoxymethylenemalononitrile (0.01 mol), using sodium acetate (0.02 mol) in ethanol (40 mL), under reflux, during 1 h. After, the mixture was poured in cold water, and the precipitate formed was filtered out and recrystallized from ethanol/water. The reactions were accomplished by means of TLC using silica gel plate with fluorescent indicator and hexane/ethyl acetate (1:1) as eluent (16).The 5-amino-1-aryl-1*H*-pyrazole-4-carbonitriles (**2a**–**l**) (0.002 mol) were mixed with sodium azide (0.004 mol), ammonium chloride (0.004 mol), and DMF (15 mL). The reaction mixture was heated at 130–135 °C, during 14 h. After, the mixture was poured in cold water, and the precipitate formed was filtered out and recrystallized from ethanol/water. The targets (**3a**–**l**) were characterized by Fourier transform infrared (FT-IR), Nuclear magnetic resonance (NMR), and mass spectrometry (MS).

Antileishmanial activity assay

Promastigotes in late log phase in Schneider's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) were incubated at an average density of 10^7 parasites/mL in sterile 96-well plates with various concentrations of compounds dissolved in DMSO (final concentration <0.5% v/v). The assay was carried out in triplicate. Appropriate controls containing DMSO, pentamidine (reference drug) were added to each set of experiments. Parasite viability was assessed by a dye-reduction assay employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (17), and the absorbance measured in a spectrophotometer at 570 nm. Inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of *Leishmania* promastigotes compared with the control. IC_{50} /24 h was calculated by nonlinear regression analysis processed on dose–response curves, using GRAPHPAD PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA). IC_{50} values represent the mean value calculated from three independent experiments.

Measurement of antileishmanial activity in intracellular amastigote

Murine macrophages were seeded in Lab-Tek chamber slide (2.0×10^6 cells/mL) and allowed to adhere (about 1 h) at 37 °C, 5% CO_2. After removal of non-adherent macrophages with RPMI medium, cells were infected by 4 hours in the same environment with stationary phase promastigotes at a parasite: macrophage ratio of 3:1. Subsequently, non-phagocytized parasites were removed by gentle washing, and infected macrophages were incubated with tetrazole derivatives for an additional 72 h. The slides were stained using an Instant Prov hematological dye system (Newprov, Curitiba, Brazil) for microscopic evaluation of amastigote burden. At least 100 macrophages/well were analyzed, and IC_{50} was established, considering equal proportion of DMSO treated cells as control.

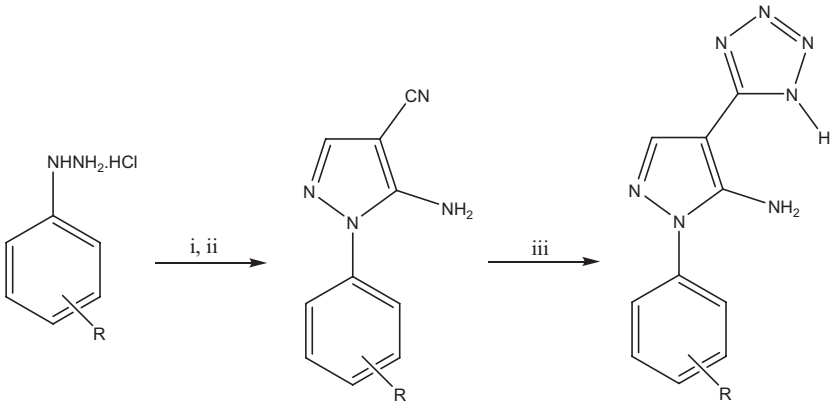
Cytotoxic assays on murine peritoneal macrophages

Cytotoxicity was assessed on murine peritoneal macrophages by a colorimetric assay based on the mitochondrial reduction in MTT as described previously (17). The cells were isolated from peritoneal cavity of BALB/c mice with cold RPMI 1640 medium, supplemented with 10% FCS. The 2×10^6 cells/well were cultivated on microplate and incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 2 h of incubation, no adherent cells were then removed and the adhered macrophages were washed with RPMI. Compounds were solubilized in DMSO at concentrations ranging from 500 to 12.5 μM and added to the cell culture for 72 h incubation at 37 °C and 5% CO_2. After that, culture supernatant was removed. The macrophage viability was measured by adding MTT (0.5 mg/mL in PBS, 200 μL/well), incubating plates for 2 h at 37 °C, and the colored product formazan was solubilized with DMSO. The results were read in spectrophotometer with wavelength of 570 nm (17).

Results and Discussion

Chemistry

The synthesis of the compounds 5-amino-1-aryl-1*H*-pyrazole-4-carbonitriles (**2a**–**l**) and 5-(5-amino-1-aryl-1*H*-pyrazol-4-yl)-1*H*-tetrazole (**3a**–**l**) are summarized in Scheme 1. The key intermediates 5-amino-1-aryl-1*H*-pyrazole-4-carbonitriles (**2a**–**l**) were obtained from the reaction between arylhydrazine hydrochlorides (**1a**–**l**) and ethoxymethylenemalononitrile, in ethanol. Previously, sodium acetate was added to provide an acid–basic reaction with hydrochloride. The reaction occurs *via* a similar Michael addition mechanism followed by cycloaddition. In the second step, the compounds 5-(5-amino-1-aryl-1H-pyrazol-4-yl)-1H-tetrazole (**3a**–**l**) were obtained via [3 + 2] cycloaddition by the reaction of the compounds **2a**–**l** with sodium azide and ammonium chloride, in *N*, *N*-dimethylformamide (DMF), according to similar methodology published by our



1a-l 2a-l 3a-l

R= H, 2-Cl, 3-Cl, 4-Cl, 2,4-diCl, 2,6-diCl, 3,4-diCl, 3,5-diCl, 2-F, 3-F, 4-F, 3-Br

Scheme 1: Reagents and conditions: (i) sodium acetate, ethanol, 0.5 h, reflux; (ii) ethoxymethylenemalononitrile, ethanol, 1 h, reflux; (iii) NaN_3, NH_4Cl, DMF, 14–18 h, 120–130 °C.

research group recently (15). All these final compounds **3a**–**l** are new, except 5-(5-amino-1-phenyl-1*H*-pyrazol-4-yl)-1*H*-tetrazole (**3a**) which was obtained by Walter and Becker (18).

The structure of compounds 5-(5-amino-1-aryl-1*H*-pyrazol-4-yl)-1*H*-tetrazoles (**3a**–**l**) were confirmed by their FT-IR, ^{1}H NMR, ^{13}C NMR, and mass spectrometry analysis. The FT-IR spectrum showed the absence of the cyano group (2209–2234 cm^{-1}), absorption characteristic of the key intermediates 5-amino-1-aryl-1*H*-pyrazole-4-carbonitriles (**2a**–**l**), and the presence of a strong band at 1638–1613 cm^{-1} characteristic of the C=N bond of the tetrazole ring. In the ^{1}H NMR spectrum of each of these tetrazoles (**3a**–**l**), the NH/NH_2 appeared at 3.40-4.95 and 6.34–6.68 ppm. The hydrogen atom at C-3 position in the pyrazole ring gave characteristic singlets at 8.00–8.06 ppm. The hydrogens present in benzene ring were assigned according to ortho and meta couplings. The ^{13}C NMR spectrum showed the C = N bond of the tetrazole ring at 149.5–149.7 ppm, the C-3 at 138.1–138.4 ppm, and the C-5 at 146.3–147.9 ppm. 2D NMR techniques COSY and HSQC were used to assign hydrogen and carbon atoms.

5-[5-amino-1-(2′-chlorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3b)

yield 57%; mp 134–135 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3437, 3331, 3230, 3164, 3112, 1633, 1610; ^{1}H-NMR (300 MHz, DMSO) δ 3.46 (br NH), 6.34 (br, NH), 7.65–7.73 (3H, m), 7.83 (1H, d, J = 8.7 Hz), 8.00 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 86.7, 128.3, 130.3, 130.4, 131.7, 131.9, 135.0, 138.1, 147.8, 149.6; TOF-MS (ES^+): ($C_{10}H_8ClN_7$) calcd $[M + H]^+$ 262.0608; found 262.0628.

5-[5-amino-1-(3′-chlorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3c)

yield 74%; mp 164–165 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3473, 3425, 3296, 3111, 1635, 1595; ^{1}H-NMR (300 MHz, DMSO) δ 3.45 (br, NH), 6.57 (br, NH), 7.61–7.64 (1H, m), 7.67–7.72 (2H, m), 7.81 (1H, t, J = 1.8 Hz), 8.05 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 87.8, 122.3, 126.0, 129.2, 131.6, 135.6, 138.3, 139.5, 146.5, 149.6; TOF-MS (ES^+): ($C_{10}H_8ClN_7$) calcd $[M + H]^+$ 262.0608; found 262.0629.

5-[5-amino-1-(4′-chlorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3d)

yield 72%; mp 139–140 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3417, 3361, 3250, 3189, 1634, 1602; ^{1}H-NMR (300 MHz, DMSO) δ 3.47 (br, NH), 6.53 (br, NH), 7.72 (2H, d, J = 9.0 Hz), 7.79 (2H, d, J = 9.0 Hz), 8.06 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 88.2, 120.3, 126.3, 133.4, 136.0, 138.4, 146.3, 149.7. TOF-MS (ES^+): ($C_{10}H_8ClN_7$) calcd $[M + H]^+$ 262.0608; found 262.0625.

5-[5-amino-1-(2′,4′-dichlorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3e)

yield 64%; mp 155–157 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3447, 3359, 3245, 3108, 1630, 1610; ^{1}H-NMR (300 MHz, DMSO) δ 3.45 (br, NH), 6.56 (br, NH), 7.67 (1H, dd, J = 9.0;2.4 Hz), 7.72 (1H, d, J = 9.0 Hz), 7.85 (1H, d, J = 2.4 Hz), 8.03 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 87.6, 128.2, 130.4, 131.5, 131.9, 132.4, 135.0, 138.1, 147.4, 149.6; TOF-MS (ES^+): ($C_{10}H_7Cl_2N_7$) calcd $[M + H]^+$ 296.0218; found 296.0254.

5-[5-amino-1-(2′,6′-dichlorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3f)

yield 62%; mp 233–234 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3410, 3283, 3154, 3113, 1628, 1609; ^{1}H-NMR (300 MHz, DMSO) δ 3.48 (br, NH), 6.45 (br, NH), 7.62–7.67 (3H, m); 8.04 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 87.6, 127.8, 128.8, 133.5, 136.8, 138.2, 147.9, 149.5; TOF-MS (ES^+): ($C_{10}H_7Cl_2N_7$) calcd $[M + H]^+$ 296.0218; found 296.0252.

5-[5-amino-1-(3′,4′-dichlorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3g)

yield 67%; mp 160–162 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3415, 3277, 3128, 3110, 1629, 1605; ^{1}H-NMR (300 MHz, DMSO) δ 3.46 (br, NH), 6.53 (br, NH), 7.53 (1H, dd, J = 8.7;2.4 Hz), 7.68 (1H, d, J = 8.7 Hz), 7.77 (1H, d, J = 2.4 Hz), 8.06 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 87.9, 128.5, 130.4, 131.2, 131.9, 132.8, 135.3, 138.2, 146.3, 149.5; TOF-MS (ES^+): ($C_{10}H_7Cl_2N_7$) calcd $[M + H]^+$ 296.0218; found 296.0258.

5-[5-amino-1-(3′,5′-dichlorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3h)

yield 66%; mp 210–211 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3466, 3427, 3336, 3297, 3113, 1634, 1608; ^{1}H-NMR (300 MHz, DMSO) δ 3.46 (br, NH), 6.68 (br, NH), 7.79 (1H, t, J = 1.8 Hz), 7.82 (2H, d, J = 1.8 Hz), 8.06 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 87.9, 128.0, 130.5, 133.7, 135.7, 138.2, 146.3, 149.5; TOF-MS (ES^+): ($C_{10}H_7Cl_2N_7$) calcd $[M + H]^+$ 296.0218; found 296.0254.

5-[5-amino-1-(2′-fluorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3i)

yield 47%; mp 120–121 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3436, 3321, 3164, 3117, 1638, 1614; ^{1}H-NMR (300 MHz, DMSO) δ 4.95 (br, NH), 6.43 (br, NH), 7.47–7.62 (2H, m), 7.66-7.73 (2H, m), 8.04 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 87.0, 117.0, 125.5, 125.7, 129.6, 131.7, 138.5, 147.9, 149.7, 155.8; TOF-MS (ES^+): ($C_{10}H_8FN_7$) calcd $[M + H]^+$ 246.0903; found 246.0942.

5-[5-amino-1-(3′-fluorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3j)

yield 74%; mp 179–180 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3361, 3260, 3174, 3085, 1629, 1599; ^{1}H-NMR (300 MHz, DMSO) δ 4.40 (br, NH), 6.56 (br, NH), 7.35–7.41 (1H, m), 7.58-7.63 (2H, m), 7.67–7.75 (1H, m), 8.05 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 88.2, 110.5, 114.3, 119.6, 131.6, 138.3, 139.8, 146.8, 149.7, 160.1; TOF-MS (ES^+): ($C_{10}H_8FN_7$) calcd $[M + H]^+$ 246.0903; found 246.0948.

5-[5-amino-1-(4′-fluorophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3k)

yield 62%; mp 172–173 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3468, 3320, 3215, 3106, 1613, 1603; ^{1}H-NMR (300 MHz, DMSO) δ 3.40 (br, NH), 6.43 (br, NH), 7.51 (2H, t, J = 9.0 Hz), 7.75 (2H, dd, J = 9.0;4.8 Hz), 8.01 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 88,0, 116.3, 126.3, 134.2, 138.1, 146.8, 149.6, 159.8; TOF-MS (ES^+): ($C_{10}H_8FN_7$) calcd $[M + H]^+$ 246.0903; found 246.0944.

5-[5-amino-1-(3′-bromophenyl)-1H-pyrazole-4-yl]-1H-tetrazole (3l)

yield 68%; mp 175–176 °C; FT-IR (ATR-diamond/ZnSe) cm^{-1}: 3468, 3422, 3301, 3200, 3108, 1631, 1610; ^{1}H-NMR (300 MHz, DMSO) δ 3.45 (br, NH), 6.58 (br, NH), 7.63 (1H, t, J = 8.1 Hz), 7.76 (2H, t, J = 8.4 Hz), 7.93 (1H, t, J = 1.8 Hz), 8.05 (1H, s); ^{13}C-NMR (75 MHz, DMSO) δ 88.2, 122.0, 122.2, 126.0, 130.2, 133.7, 138.4, 139.6, 146.3, 149.5; TOF-MS (ES^+): ($C_{10}H_8BrN_7$) calcd $[M + H]^+$ 306.0103; found 306.0135.

Table 1: IC_{50} [a] (μM) values of 5-(5-amino-1-aryl-1*H*-pyrazol-4-yl)-1*H*-tetrazoles (**3a**–**l**) on promastigotes and amastigotes of *Leishmania spp*. and the cytoxicity in murine macrophages

Compound	Promastigote ($IC_{50}/\mu M$) *Leishmania amazonensis*	Amastigote intracellular ($IC_{50}/\mu M$) *Leishmania amazonensis*	Mø peritoneal murine ($LD_{50}/\mu M$)	Selectivity index (SI) (LD_{50}/ IC_{50} in amastigote intracellular)
3a (H)	388 ± 6.0	130.5 ± 0.0	>500	ND
3b (2-Cl)	75.8 ± 7.2	46.5 ± 4.8	185.2 ± 5.7	3.98
3c (3-Cl)	379 ± 3.1	ND	>500	ND
3d (4-Cl)	>800	ND	ND	ND
3e (2,4-diCl)	>800	ND	ND	ND
3f (2,6-diCl)	>800	ND	ND	ND
3g (3,4-diCl)	>800	ND	ND	ND
3h (3,5-diCl)	195.8 ± 18.8	ND	192.2 ± 13.0	ND
3i (2-F)	91.4 ± 0.1	>100	93.9 ± 4.7	ND
3j (3-F)	144.8 ± 0.0	ND	>500	ND
3k (4-F)	102.6 ± 13.1	97.0 ± 2.8	>250	ND
3l (3-Br)	78.5 ± 3.4	106.6 ± 7.45	500 ± 0.0	6.41
Pentamidine	13.0 ± 0.1	1.9 ± 0.121	8.49 ± 1.25	4.46

[a]IC_{50} values were obtained from the drug concentration-response curve, and the results were expressed as the mean ± standard deviation determined from three independent experiments.

Biological activity

In this work, we evaluated the synthesis, cytotoxicity and *in vitro* leishmanicidal activity of twelve 5-(5-amino-1-aryl-1*H*-pyrazol-4-yl)-1*H*-tetrazole (**3a**–**l**) derivatives. Table 1 displays IC_{50} values of these derivatives against *L. amazonensis* promastigotes and intracellular amastigotes forms. Cytotoxic activity (LC_{50}) was related to anti-amastigote activity(IC_{50}) for determining their corresponding selectivity index (SI = LC_{50}/IC_{50}). Among the compounds tested **3a**–**l**, four compounds **3b** (R = 2-Cl), **3i** (R = 2-F), **3k** (R = 4-F), and **3l** (R = 3-Br) presented interesting anti-promastigote activity (IC_{50} 75.83, 91.41, 102.58, and 78.53 μM, respectively), with greater selectivity index for bromine derivative **3l** (SI = 6.41). We carried out amastigote assays with these four active compounds to evaluate its ability for reducing the parasite load in infected host cells. The results showed that derivative **3b** is able to reduce significantly the number of intracellular parasites with IC_{50} = 46 μM (Table 1), while compounds **3a**, **3k**, and **3l** displayed an average activity (IC_{50} of 130.5, 97.0, and 106.6 μM, respectively). Concerning compound **3i** exhibited significant cytotoxicity on macrophages cells (LC_{50} = 93.92 μM), the presence of the 2-fluoro in the aryl group seems to play a more important role for the cytotoxicity profile. In a previous report (9), we synthesized and evaluated the *in vitro* leishmanicidal activity of 5-amino-1-aryl-4-(4,5-dihydro-1*H*-imidazol-2-yl)-1H-pyrazoles. Thus, considering the similarity of imidazole and tetrazole rings, this study could compare the leishmanicidal activity of new tetrazole derivatives. However, a close examination of the similar compounds imidazole and tetrazole groups revealed that their leishmanicidal activity is strongly bound to the nature and position of the substituent on the phenyl group (Scheme 1). Compound with no structural modification (R = H) compared with tetrazole (**3a**), IC_{50} = 388 ± 6.0 μM (Table 1), or imidazole rings, IC_{50} = 365±3.5 μM showed similar activity (11).

Conclusion

This study achieved the synthesis of a novel series of tetrazole ring bearing arylpyrazol derivatives. This study confirmed this new class of compounds as innovative antileishmanial agents. However, through a preliminary study of the structure activity, we find that there is no significant difference in the effect antileishmanial between tetrazole and imidazole groups. Future studies toward this objective, based on animal models, may resolve these issues.

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