Supporting Information

Table of contents

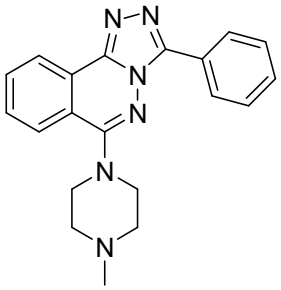
I. Chemistry Experimental Information

All reagents and starting materials were purchased from commercial sources and used without further purification; solvents were anhydrous HPLC grade. Melting points are recorded with a micro melting point apparatus und uncorrected. ^{1}H NMR spectra are recorded at JEOL Eclipse Plus 400. Multiplicity is indicated as follows: s (singlet), d (doublet), t (triple), m (multiplet), dd (doublet of doublets), br s (broad singlet); chemical shifts were measured in parts per million (δ) and coupling constant (*J*) are given in Hz. Proton chemical shifts were given in relative to tetramethylsilane (δ 0.00 ppm) in $CDCl_3$ or DMSO-d_6 solvents. IR spectra were recorded on NICOLET Magna IR 760 using KBr tablets. Elemental analyses of the synthesized compounds were performed using a Perkin Elmer 2400 CHN analyser: results fell in the range of 0.4% of the required theoretical values.

II. Synthesis procedures and spectroscopic data

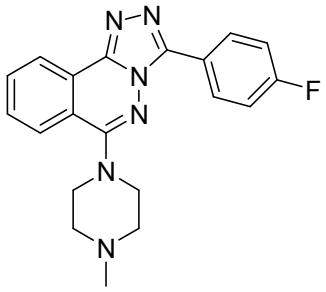
II.1. General procedure for the preparation of 3-aryl-6-(*N*-methylpiperazin)-1,2,4-triazolo[3,4-α]phthalazines 2a-e using silver (I) nitrate: The 4-chloro-1-phthalazinyl-aryl/heteroarylhydrazones **1a-e** were prepared following a similar procedure reported in literature [1]. Then, 4-chloro-1-phthalazinyl)-arylhydrazones **1a-e** (1 mmol, 1 equiv.) was dissolved in DMF (5 mL) and subsequently, *N*-methylpiperazine (5 mmol, 5 equiv.), silver nitrate (2.5 mmol, 2.5 equiv.) and K_3PO_4 (3 mmol, 3 equiv.) were added. The mixture was heated at 100 ºC with stirrring for 48 hours and, monitored by TLC using solvent mixture Hex/AcOEt (7:3). The reation mixture was then cooled to room temperature and, the solvent was evaporated under reduced pressure to give a dark solid. This solid was purified by flash columm chromatography using hexane-ethyl acetate (9:1), giving the corresponding 3-aryl-6-piperazin-1,2,4-triazolo[3,4-α]phthalazines **2a-e**. All synthesized compounds have been previously reported and, herein the ^{1}H-NMR spectra, IR data, elemental analysis and melting point were collected for each compound in order to compare with reported data. Spectroscopy data and melting points for the synthesized compounds are in concordance with previous reported data. All spectra of synthesized compounds **2a-e** can be found in our previous report [2].

II.1.1. 3-Phenyl-6-(4-methylpiperazin-1-yl)-1,2,4-triazolo[3,4-α]phthalazines 2a. Yellow solid, yield 83%, m.p. 160-162ºC (Lit.[3] 163-164). FT-IR (KBr, ν cm^{-1}): 2953 (st. C-H), 1611-1577-1512 (st. C=C, Ar). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.69 (d, 1H, *J* = 8.3); 8.47 (d, 2H, *J* = 6.8); 8.02 (d, 1H, *J* = 8.3); 7.87 (dd, 1H, *J* = 8.3; *J* = 7.0); 7.74 (dd, 1H, *J* = 8.3; *J* = 7.0); 7.51 (m, 3H); 3.56 (s, 4H, *N*-CH_2); 2.83 (s, 4H, CH_2-*N*); 2.49 (s, 3H, *N*-CH_3). Anal. Calc. for $C_{20}H_{20}N_6$: C, 69.75; H, 5.85; N, 24.40. Found: C, 69.52; H, 5.71; N, 24.29.



2a

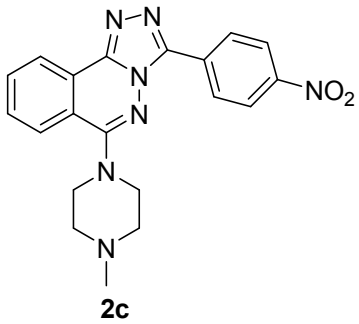
II.1.2. 3-(4-Fluorophenyl)-6-(4-methylpiperazin-1-yl)-1,2,4-triazolo[3,4-α]phthalazines 2b. Yellow solid, yield 79%, m.p. 218-220ºC (Lit.[2] 220-222 ºC). FT-IR (KBr, ν cm^{-1}): 2934 (st. C-H), 1609-1552-1511 (st. C=C, Ar). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.69 (d, 1H, *J* = 7.7); 8.50 (m, 2H); 8.04 (d, 1H, *J* = 8.1); 7.87 (ddd, 1H, *J* = 7.7; *J* = 7.7; *J* = 0.7); 7.74 (ddd, 1H, *J* = 7.8; *J* = 7.8; *J* = 1.0); 7.22 (dd, 2H, *J* = 8.8; *J* = 8.8); 3.49 (s, 4H, *N*-CH_2); 2.75 (s, 4H, CH_2-*N*); 2.44 (s, 3H, *N*-CH_3). Anal. Calc. for $C_{20}H_{19}FN_6$: C, 66.28; H, 5.28; N, 23.19. Found: C, 66.23; H, 5.24; N, 23.00.



2b

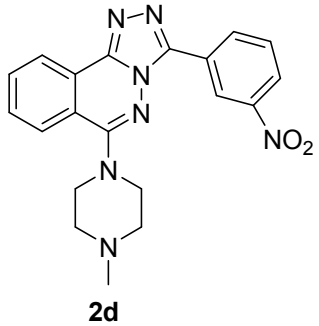
II.1.3. 3-(4-Nitrophenyl)-6-(4-methylpiperazin-1-yl)-1,2,4-triazolo[3,4-α]phthalazines 2c. Orange solid, yield 73%, m.p. >300ºC (Lit.[2] < 300ºC). FT-IR (KBr, ν cm^{-1}): 2952 (st. C-H), 1609-1547-1499 (st. C=C, Ar). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.70 (d, 2H, *J* = 7.0); 8.53 (dd, 1H, *J* = 5.8); 8.43 (d, 2H, *J* = 7.0); 8.14 (d, 1H, *J* = 6.2); 8.02 (m, 1H); 7.92 (m, 1H); 3.54 (s, 4H, *N*-CH_2); 2.71 (s, 4H, CH_2-*N*); 2.31 (s, 3H,

N-CH_3). Anal. Calc. for $C_{20}H_{19}N_7O_2$: C, 61.69; H, 4.92; N, 25.18. Found: C, 61.42; H, 4.81; N, 25.06.



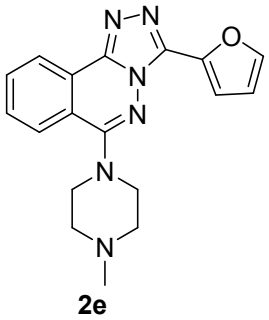
2c

II.1.4. 3-(3-Nitrophenyl)-6-(4-methylpiperazin-1-yl)-1,2,4-triazolo[3,4-α]phthalazines 2d. Orange solid, yield 76%, m.p. 228-229ºC (Lit.[2] 226-228 ºC). FT-IR (KBr, ν cm^{-1}): 2944 (st. C-H), 2847 (st. C-H), 1601-1536-1500 (st. C=C, Ar). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 9.55 (s, 1H); 8.81 (d, 1H, *J* = 8.1); 8.67 (d, 1H, *J* = 7.7); 8.32 (d, 1H, *J* = 9.1); 8.05 (d, 1H, *J* = 8.1); 7.90 (dd, 1H, *J* = 8.1; *J* = 7.7); 7.79 (dd, 1H, *J* = 7.7; *J* = 7.5); 7.71 (dd, 1H, *J* = 8.4, *J* = 8.1); 3.80 (s, 4H, *N*-CH_2); 3.00 (s, 4H, CH_2-*N*); 2.60 (s, 3H, *N*-CH_3). Anal. Calc. for $C_{20}H_{19}N_7O_2$: C, 61.69; H, 4.92; N, 25.18. Found: C, 61.50; H, 4.81; N, 25.03.



2d

II.1.5. 3-(Furyl)-6-(4-methylpiperazin-1-yl)-1,2,4-triazolo[3,4-α]phthalazines 2e. Yellow solid, yield 81%, m.p. 196-198ºC (Lit.[2] 196-198 ºC). FT-IR (KBr, ν cm^{-1}): 2937 (st. C-H), 1612-1549-1515 (st. C=C, Ar). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.69 (m, 1H); 8.04 (d, 1H, *J* = 8.1); 7.85 (dd, 1H, *J* = 7.0; *J* = 7.0); 7.75 (dd, 1H, *J* = 7.7; *J* = 7.7); 7.66 (m, 1H); 7.42 (d, 1H, *J* = 3.0); 6.62 (d, 1H, *J* = 1.4); 3.50 (s, 4H, *N*-CH_2); 2.75 (s, 4H, CH_2-*N*); 2.43 (s, 3H, *N*-CH_3). Anal. Calc. for $C_{18}H_{18}N_6O$: C, 64.66; H, 5.43; N, 25.13. Found: C, 64.52; H, 5.34; N, 25.01.



2e

III. Biological Experimental Information

Promastigotes and amastigote of *Leishmania (V) braziliensis* (MHOM/BR/75/M2903) (provided by Instituto de Biomedicina "Dr. Jacinto Convit," Hospital Vargas, Caracas, Venezuela) were isolated from footpad lesions in BALB/c mice previously infected. For differentiation and maintenance of promastigote culture was used medium LIT (tryptose 15 g/L, yeast extract 5 g/L, liver extract 2 g/L, hemin-NaOH 0.02 g/L, glucose 4 g/L, NaCl 9 g/L, KCl 0.4 g/L, Na_2HPO_4 7.5 g/L, pH 7.4) supplemented with 10% fetal bovine serum and maintained at 29°C. Extracelullar clinical amastigote were isolated from footpad lesions in BALB/C mice previously infected with human strain called MHOM/VE/2017/M2903/MR. The mentioned strain was initially isolated from a bone narrow aspirate taken from Venezuelan CL patient treated in the Instituto de Biomedicina "Dr. Jacinto Convit" of Hospital Vargas (Caracas, Venezuela). Peritoneal macrophages were obtained from mouse peritoneal cavity and differentiated in a conditioned medium (medium RMPI) as described in previous methodologies [4-5].

IV. Biological procedures

IV.1. *In vitro* antileishmania evaluation against promastigote form of parasite. In order to evaluate the effect of five synthesized compounds on the promastigotes viability of *L. braziliensis* strain, colometric MTT (3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide) method was used according to reported protocol [6]. The screening was performed in 96-well microliter plates maintained at 25°C. Briefly, $1x10^6$ parasites/mL were seeded in a 96-well plate and exposed to increasing concentrations between 1.0 and 50.0 μM (1.0, 5.0, 10.0, 20.0, and 50.0 μM) of each compound by 72 h at 25°C. Three independent experiments were independently performed for each

concentration of the corresponding compounds. Controls contained 1% of DMSO and medium. Glucantime was used as reference drug. Parasite proliferation was evaluated by using ELISA plate reader (800 TS Absorbance Reader) at 72 h. Untreated control parasites were used to calculate the relative proliferation. The IC_{50} value (50% growth inhibitory concentration) was determined by using five concentrations of compounds. The percentage of cell growth was assayed by measuring the absorbance (A) of the formazan in solution at 570 nm and calculated as follows: % parasite growth = $(A_p - A_{0p})/(A_c - A_{0c})x100$, where A_p is absorbance (at 570 nm) of the culture containing the compound at 72 h; A_{0p} is the absorbance (at 570 nm) of the culture containing the compound at hour 0; A_c is the absorbance (at 570 nm) of the culture in the absence of any drug (control) at 72 h; and A_{0c} is the absorbance (at 570 nm) in the absence of any drug at hour 0. Dose-response curves were recorded and the IC_{50} values were determined using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA). The results are presented as averages SD with SD below 10 %.

IV.2. *In vitro* evaluation on intracellular amastigotes. Intracellular amastigote were directly extracted from footpad lesions in BALB/c mice previously infected with *L. braziliensis* (MHOM/BZ/82/M2903). The isolate contained amastigote and small portions of infected macrophages and macrophages. These two last portions were removed from cellular mixture by controlled centrifugation (at 3000 rpm by 2-3 min) to obtain a culture enough pure in amastigote. Amastigote culture was maintained at 37°C and pH 5.5 in M199 medium (Invitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g/L *L*-alanine, 100 mg/L *L*-asparagine, 200 mg/L sucrose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L α-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L 2-(*N*-morpholino) ethanesulfonic acid (MES), 0.4 mg/L hemin, and 10 mg/L gentamicin. The screening was performed in 96-well microtiter plates maintained at 37°C. Briefly, $2x10^6$ parasites/mL were exposed to increasing concentrations between 1.0 and 50.0 μM (1.0, 5.0, 10.0, 20.0, and 50.0 μM) of each compound **(2b, 2c** and **2d)**. Controls contained 1% of DMSO and medium. Glucantime was used as reference drug. The analysis was performed by triplicate. The effect of the most active compounds **2b, 2c** and **2d** against amastigote forms was tested at 48 h using conventional counting parasite in Neubauer chamber (optical microscopy, 1000x magnification) [5]. Untreated control parasites were used to calculate the relative

proliferation. Dose-response curves were recorded and the LD_{50} values were determined using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA). The results are presented as averages SD with SD below 10 %.

IV.3. *In vitro* evaluation using the axenic amastigote form. Axenic amastigote forms of *L. braziliensis* (MHOM/BR/75/M2903) were cultured following the methodology described previously [7]. Promastigote transformation to amastigotes was achieved after 3 days of culture in M199 medium (Invitrogen) supplemented with 10% heat-inactivated FCS, 1 g/L *L*-alanine, 100 mg/L *L*-asparagine, 200 mg/L sucrose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200mg/L α-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.4 mg/L hemin, and 10 mg/L gentamicin, pH 5.5 at 35°C. Briefly, $2x10^6$ parasites/mL were exposed to increasing concentrations between 1.0 and 50.0 μM (1.0, 5.0, 10.0, 20.0, and 50.0 μM) of each compound **(2b, 2c** and **2d)**. Controls contained 1% of DMSO and medium. Glucantime was used as reference drug. Untreated control parasites were used to calculate the relative proliferation. The analysis was performed by triplicate. The effect of the compounds **2b, 2c** and **2d** against axenic amastigote forms was tested at 48 h by MTT protocol using ELISA plate reader (800 TS Absorbance Reader). The analysis was performed by triplicate. The LD_{50} value was determined by using five concentrations of compounds as was described in point V.1. Dose-response curves were recorded and the LD_{50} values were determined using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA). The results are presented as averages SD with SD below 10 %.

IV.4. *In vitro* evaluation on a clinical amastigote strain. Extracelullar clinical amastigote were isolated from footpad lesions in BALB/c mice previously infected with human strain called MHOM/VE/2017/M2903/MR. The mentioned strain was previously isolated from a bone narrow aspirate taken from Venezuelan CL patient. Similarly to section IV.2, the infected macrophages and macrophages were removed from cellular mixture by controlled centrifugation (at 3000 rpm by 2-3 min) to obtain a neat amastigote culture. Amastigote culture was maintained at 37°C and pH 5.5 in M199 medium (Invitrogen) supplemented with 10% heat-inactivated FCS, 1 g/L *L*-alanine, 100 mg/L *L*-asparagine, 200 mg/L sucrose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200 mg/L α-ketoglutaric

acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.4 mg/L hemin, and 10 mg/L gentamicin, at 37°C. Briefly, $2x10^6$ parasites/mL were exposed to increasing concentrations between 1.0 and 50.0 μM (1.0, 5.0, 10.0, 20.0, and 50.0 μM) of each compound **(2b, 2c** and **2d)**. Controls contained 1% of DMSO and medium. The analysis was performed by triplicate. The effect of the most active derivatives **2b, 2c** and **2d** against clinical amastigote isolates was tested at 48 h using a Neubauer hemocytometer. The analysis was performed by triplicate. Untreated control parasites were used to calculate the relative proliferation. Dose-response curves were recorded and the LD_{50} values were determined using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA). The results are presented as averages SD with SD below 10 %.

IV.5. Toxicity assay. Peritoneal murine macrophages were grown in RPMI 1640 medium (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum, 1% of *L*-glutamine, 1% streptomicyn, 100 units/mL penicillin (all obtained from Sigma Aldrich USA) [8]. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for assay, which is based on the ability of viable cells to metabolically reduce a yellow tetrazolium salt (MTT; Sigma) to a purple formazan product. This reaction takes place when mitochondrial reductase enzymes are active. Cells were grown in 96-well plates ($5x10^4$ cells/well) for 24 h. Cultures were carried out at 37 °C in a humidified atmosphere with 5% CO_2 and incubated with the most active compounds **2b, 2c** and **2d** in 100 μL of complete culture medium containing 25.0, 50.0, 75, 100, 200.0 μM concentrations of each compounds for 48 h. After incubation, the medium was removed and the cells were treated with 100 μL 0.4 mg/mL MTT for 4 h at 37 °C. Subsequently, 100 μL DMSO was added to the mixture. The solubilized formazan product was quantified with the help of a microtiter plate reader TECAN-sunrise at 570 nm. In all cases, the compounds **2b, 2c** and **2d** were dissolved in DMSO, at the final concentration in the culture medium was lower than 1 %, a concentration that had neither cytotoxic effect nor caused any interference with the colorimetric detection method. All experiments were performed at least three times. Untreated control parasites were used to calculate the relative proliferation. Dose-response curves were recorded and the CC_{50} values were determined using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA). The results are presented as averages SD with SD below 10 %.

IV.6. Stadistical analysis. All experiments were performed at least three times. The results are expressed as mean ± SD. Anova test were performed. Only post hoc Dunnet test $p< 0.01$ was considered to be statistically significant. The dose-response curves as well as the 50% growth inhibitory concentrations (IC_{50}, LD_{50} or CC_{50}) of synthetics products or chemotherapeutic drugs were determined by a non-linear regression of individual experiments calculated through computation with GraphPad prism v.5.02 software [9] program (Intuitive Software for Science, San Diego, CA, USA).

V. References

[1] (a) Romero, A.H., Medina, R., Alcala, A.M., García-Marchan, Y., Nuñez-Duran, J., Leañez, J., Mihoba, Ali, Ciangherotti, C., Serrano-Martín, X., Lopez, S.E., *Eur. J. Med. Chem.*, **2017**, 127, 606-620. (b) Romero, A.H., Rodríguez, J., García-Marchan, J., Leañez, J., Serrano-Martín, X., López, S.E., Bioorg. Chem., 2017, 72, 51-56.

[2] A.H. Romero, F. Sojo, F. Arvelo, C. Calderón, A. Morales, S.E. López, *Bioorg. Chem.* 2020, 101: (DOI: 10.1016/j.bioorg.2020.104031).

[3] Tarzia G.; Occelli, E.; Toja, E.; Barone, D.; Corsico, N.; Gallico, L.; Luzzani, F.; J. Med. Chem. 1988, 31, 1115.

[4] (a) A.H. Romero, S.E. Lopez, N. Rodriguez, H. Oviedo, Arch. Pharm. (Weinheim), 2018, 351 (8): e1800094 (DOI: 10.1002/ardp.201800094). (b) A.H. Romero, N. Rodríguez, H. Oviedo, *Bioorg. Chem.* 2019, 83, 145-153.

[5] (a) A.H. Romero, N. Rodriguez, S.E. Lopez, H. Oviedo, *Archiv Pharm.* 2019, 352 (5): e1800281 (DOI: 10.1002/ardp.201800281). (b) A.H. Romero, N. Rodriguez, S.E. Lopez, H. Oviedo, *Archiv Pharm.* 2019, 352 (6): e1800299 (DOI: 10.1002/ardp.201800299**).**

[6] T. Mosmann, J. Immunol. Methods, 65 (1983) 55-63.

[7] P. S. Doyle, J. C. Engel, P. F. P. Pimenta, P. Pinto da Silva, D. M. Dwyer, Exp. Parasitol. 1991, 73, 326.

[8] A.E. Nugroho, A. Hermawan, D.D. Putri, A. Novika, E. Meiyanto, M. Kawaichi, Asian Pac. J. Trop. Biomed. 3 (2013) 297-302.

[9] Graph Pad Prism Software Inc. 4.02 for windows. May 17th 1992-2004.