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Synthesis and in vitro evaluation of Ca^{2+} channel blockers 1,4-dihydropyridines analogues against *Trypanosoma cruzi* and *Leishmania amazonensis*: SAR analysis.

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Abstract

Drugs containing the1,4-dihydropyridine (DHP) core have recently attracted attention concerning their antiparasitic effect against various species of *Leishmania* and *Trypanosoma*. This approach named drugs repositioning led to interesting results, which have prompted us to prepare 21 DHP's analogues. The 1,4-DHP scaffold was decorated with different function groups at tree points including the nitrogen atom (NH and N-phenyl), the aryl group attached to C-4 (various substituted aryl residues) and the carbon atoms 2 and 6 (bearing Ph or Me groups). Moreover, the products were evaluated for their cytotoxicity on three cancer and a non-tumoral cell lines. Only 6 of them were antiproliferative and their weak effect (CC_{50} comprised between 27-98 µM) suggested these DHPs as good candidates against the intracellular amastigote forms of *L. amazonensis* and *T. cruzi*. *L. amazonensis* was sensitive to DHPs **5**, **11** and **15** (IC_{50} values at 15.11, 45.70 and 53.13 µM, respectively) while 12 of them displayed significant to moderate trypanocidal activities against *T. cruzi*. The best trypanocidal activities were obtained with compounds **2**, **18** and **21** showing IC_{50} values at 4.95, 5.44, and 6.64 µM, respectively. A part of the *N*-phenylated DHPs showed a better selectivity than their NH analogues towards THP-1 cells. 4-Chlorophenyl, 4-nitrophenyl and 3-nitrophenyl residues attached to the carbon atom 4 turned to be important sub-structures for the antitrypanosomal activity.

Keywords

1,4-dihydropyridines, Leishmanicidal activity, Trypanocidal activity, SAR.

1. Introduction

Data reported in 2016 by WHO revealed that 6 to 7 million people worldwide are suffering from Chagas disease, which is endemic in 21 Latin America countries with an annual incidence of 28 thousand cases [1]. In the particular case of *Trypanosoma cruzi*, the transmission to humans mainly occurs through excreta deposition, after biting of contaminated triatomines, blood transfusion from infected donors, the consumption of infected food, organ transplantation and childbirth from infected mother [1]. Leishmaniasis, another worldwide vector-borne disease is caused by protozoan parasites of the genus *Leishmania* with an estimation of 1.5 to 2 million new cases annually. Human leishmaniasis ranges from self-healing skin ulcers to mucosal lesions up to a life-threatening progressive visceral disease [2]. Moreover, about 90% of reported human visceral leishmaniasis occurs in Brazil, Ethiopia, India, Somalia, South Sudan and Sudan [2]. Chagas disease and leishmaniasis represent a difficult healthcare problem in Brazil, especially because of the lack of vaccines and the existence of pernicious side effects associated with these diseases treatments [3, 4]. Moreover, the cure also requires a long-term therapy [4]. Therefore, search for unmet efficient and inexpensive chemotherapy remain imperative. DHPs acting as Ca^{2+} channel blockers (CCB) such as amlodipine, lacidipine, nimodipine, lercanidipine, nicardipine, bepridil, cilnidipine nefidipine, azelnidipine and nitrendipine have been largely studied for their antiparasitic effect against various species of *Leishmania* and *Trypanosoma* [5-8]. It turned out that several of these pyridine analogues are endowed with promising antiprotozoal activity either as a single compound or in combination with other drugs [6]. Moreover, synthetic N-aryl-4-glycosylDHPs derivatives showed interesting inhibitory effect against *L. donovani* [9]. The theory put forward is based on the ability of DHPs containing compounds to suppress the calcium channel action in cell membranes [10]. This mechanism has been considered for the search of new antiparasitic compounds since Ca^{2+} ions in eukaryotic organisms (trypanosomatids) act as a second messenger that regulates various cellular processes [10]. Moreover, previous studies demonstrated that during the invasion of the host cells, there is an increase in the cytolosic calcium concentration in *T. cruzi* trypomastigotes [11]. Likewise, the virulence during the invasion in *L. mexicana amazonensis* amastigotes is associated to their intracellular Ca^{2+} pool content [12]. Therefore, search for compounds that can compromise the Ca^{2+} channel action in these parasites represents one of the approaches to be explored. The use of Ca^{2+} channel blockers derivatives in this study to target parasitic diseases joins the drug discovery strategy named drugs repositioning or repurposing. Drug repositioning consists in giving an additional pharmacological potential to a drug by targeting illness other than that for which it was initially intended. Based on the results obtained from the repurposing assay, the drug itself can undergo some hemisynthesis or can inspire the preparation of analogues. A number of drugs repurposing has been successfully made in neglected tropical diseases (NTDs) and has also inspired the preparation of biological promising entities; for instance, eflornithine an anticancer compound, is being used for the treatment of the African sleeping sickness (caused by *T. brucei gambiense* and *T. brucei rhodesiense*) whereas pentanidine and amphotericin B initially antifungal drugs are prescribed today as leishmaniasis chemotherapies. Besides, miltefosine developed for breast cancer and other tumors, is actually employed as topical formulation against skin metastases and for the treatment of leishmaniasis [13, 14]. The hemisynthetic derivative of artemisinin, dihydroartemisinin used for the treatment of malaria is also endowed with promising activity against the human metastatic melanoma cells [15]. On the Basis of the aforementioned observation, this study aims to report the synthesis of 21 DHPs including 13 NH analogues and eight *N*-phenyl derivatives. These compounds have been evaluated for their cytotoxic activity against three cancer cells and a non-tumoral cell line as well as for their inhibitory effect against the intracellular amastigotes form of *L. amazonensis* and *T. cruzi*.

2. Experimental section

2.1. General methods

Vacuum liquid chromatography (VLC), column chromatography (CC) and thin layer chromatography (TLC) were performed on silica gel 60 (particle size 90 % <45 mm), 200–300 mesh silica gel, and silica gel GF254 (Merck), respectively. The spots were visualized using ultraviolet light at 254 and 366 nm. NMR spectra were recorded on a Bruker Avance operating at 300 MHz. The accurate mass spectra were recorded on a Xevo G2-S QTOF machine equipped with an electrospray probe. xlogP and PSA values were calculated by using SwissTargetPrediction website.

2.2. Preparation of DHPs **1**-**7**.

An amount of 0.2 g of an aldehyde (purchased from Sigma Aldrich) was dissolved in ethanol to give a 0.25 M solution, then 2.4 equivalents of ethyl acetoacetate (purchased from Sigma Aldrich) was added with 1.2 equivalents of NH_4OAc (purchased from Sigma Aldrich) in the reaction flask, the reaction mixture was stirred under reflux temperature between 16 to 68 hours. The reaction was stopped and the solvent was removed under reduced pressure, the crude reaction mixture was then subjected to a liquid/liquid extraction system with brine/ethyl acetate, the combined organics were dried with Na_2SO_4, concentrated under reduced pressure, and submitted to a chromatographic purification.

2.2.1. Diethyl 4-(3,4-dimethoxyphenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate (**1**).
The reaction ran for 48 h and the compound was purified by flash column chromatography (FCC) using a gradient from 100% CH_2Cl_2 to 10% MeOH in CH_2Cl_2, yielding a pale green solid (0.2000 g, 43%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.88 (d, J = 1.85 Hz, 1H), 6.80 (dd, J = 1.85, 8.30 Hz, 1H), 6.73 (d, J = 8.30 Hz, 1H), 5.63 (s, 1H), 4.94 (s, 1H), 4.10 (dq, J = 2.48, 7.13 Hz, 4H), 3.84 (s, 3H), 3,82 (s, 3H), 2.34 (s, 6H), 1.24 (t, J = 7.13 Hz 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.7, 148.1, 147.3, 146.6, 119.8, 111.7, 110.8, 104.3, 59.8, 55.8, 39.1, 19.7, 14.4; HRESIMS m/z 412.1728 ($[C_{21}H_{27}NO_6+Na]^+$ calcd for m/z 412.1731).

2.2.2. Diethyl 2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**2**).
The reaction last 68 h and the compound was purified by FCC, in a gradient from 100% CH_2Cl_2 to 10% MeOH in CH_2Cl_2, yielding a pale green solid (0.0655 g) 13 %. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (d, J = 8.36 Hz, 2H), 7.45 (d, J = 8.36 Hz, 2H), 5.78 (s, 1H), 5.10 (s, 1H), 4.10 (q, J = 7.12 Hz, 4H), 2.36 (s, 6H), 1.21 (t, J = 7.12 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.1, 155.1, 144.6, 128.9, 123.3, 103.2, 92.1, 60.0, 40.1, 19.7, 14.3; HRESIMS m/z 397.1367 ($[C_{19}H_{22}N_2O_6+Na]^+$ calcd for m/z 397.1370).

2.2.3. Diethyl 2,6-dimethyl-4-(4-hydroxyphenyl)-1,4-dihydropyridine-3,5-dicarboxylate (**3**).
The reaction ran for 48 h and the compound was purified by FCC, in a gradient from 100% CH_2Cl_2 to 10% MeOH in CH_2Cl_2, yielding a pale yellow solid (0.3217 g, 57%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.10 (s, 1H), 8.72 (s, 1H), 6.93 (d, J = 8.43 Hz, 2H), 6.58 (d, J = 8.43, 2H), 4.74 (s, 1H), 3.98 (m, 4H), 2.23 (s, 6H), 1.13 (t, J = 7.06 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 167.6, 155.9, 145.2, 139.4, 128.8, 115.0, 102.7, 59.4, 38.3, 18.7, 14.7; HRESIMS m/z 368.1419 ($[C_{19}H_{23}NO_5+Na]^+$ calcd for m/z 368.1468).

2.2.4. Diethyl 4-(4-hydroxy-3-methoxyphenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5- dicarboxylate (**4**)
The reaction ran for 44 h and the compound was purified by FCC, in a gradient from 100% CH_2Cl_2 to 2% MeOH in CH_2Cl_2, yielding a pale green solid (0.2093 g, 43 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09 (d, J = 8.36 Hz, 2H), 7.45 (d, J = 8.36 Hz, 2H), 5.78 (s, 1H), 5.10 (s, 1H), 4.10 (q, J = 7.12 Hz, 4H), 2.36 (s, 6H), 1.21 (t, J = 7.12 Hz 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.8, 145.8, 143.9, 143.6, 140.1, 120.5, 113.8, 110.9, 104.3, 59.8, 55.8, 39.1, 19.7, 14.4; HRESIMS m/z 398.1525 ($[C_{20}H_{25}NO_6+Na]^+$ calcd for 398.1574).

2.2.5. (*E*)-diethyl 2,6-dimethyl-4-styryl-1,4-dihydropyridine-3,5-dicarboxylate (**5**)
The reaction ran for 29 h and the compound was purified by flash column chromatography, in a gradient from 100% CH_2Cl_2 to 2% MeOH in CH_2Cl_2 yielding a pale yellow solid (0.5088 g, 63 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25 (m, 5H), 6.25 (d, J = 15.9 Hz 1H), 6.14 (dd, J = 15.9, 6.15 Hz, 1H), 5.89 (s, 1H),

4.64 (d, J = 6.14 Hz, 1H), 4.2 (m, 4H), 2.33 (s, 6H), 1.30 (t, J = 7.10 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.7, 145.0, 137.8, 131.8, 128.4, 128.1, 126.9, 126.2, 101.4, 59.8, 36.5, 19.5, 14.4; HRESIMS m/z 378.1633 ($[C_{21}H_{24}NO_4+Na]^+$ calcd for 378.1676).

2.2.6. Diethyl 2,6-dimethyl-4-(3,4,5-trimethoxyphenyl)-1,4-dihydropyridine-3,5- dicarboxylate (**6**).
The reaction ran for 16 h and the compound was purified by FCC, in a gradient from 100% CH_2Cl_2 to 2% MeOH in CH_2Cl_2 yielding a bright yellow solid (0.3967 g, 93 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.52 (s, 2H), 5.68 (s, 1H), 4.98 (s, 1H), 4.14 (m, 4H), 3.80 (s, 9H), 2.36 (s, 6H), 1.26 (t, J = 7.1 Hz 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.7, 152.6, 143.8, 143.4, 136.4, 104.9, 104.0, 60.8, 59.8, 55.9, 39.7, 19.7, 14.4; HRESIMS m/z 442.1837 ($[C_{22}H_{29}NO_7+Na]^+$ calcd for m/z 442.1836).

2.2.7. Diethyl 4-(4-hydroxy-3,5-dimethoxyphenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5- dicarboxylate (**7**).
The reaction ran for 20 h and the compound was purified by FCC, in a gradient from 100% CH_2Cl_2 to 2% MeOH in CH_2Cl_2 yielding a bright yellow solid (0.1991 g, 45 %). ^{1}H NMR (300 MHz, $CDCl_3$) data: δ 6.54 (s, 2H), 5.65 (s, 1H), 5.38 (s,1H), 4.96 (s, 1H), 4.13 (m, 4H), 3.84 (s, 6H), 2.35 (s, 6H), 1.26 (t, J = 7.12 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) data: δ 167.7, 146.5, 143.6, 139.1, 133.2, 104.8, 104.2, 59.8, 56.2, 39.5, 19.7, 14.4; HRESIMS m/z 428.1654 ($[C_{21}H_{27}NO_7+Na]^+$ calcd for m/z 428.1680).

2.3. Preparation of N-phenyl DHPs (**8**-**15**).
An amount of 0.2 g of the aldehyde was dissolved in ethanol to reach a 0.25 M solution, then 2.4 equivalents of ethyl acetoacetate were added with 1.2 equivalents of aniline in the reaction flask, the reaction mixture was stirred under reflux temperature between 16 to 30 hours. The reaction was stopped and the solvent was dried under reduced pressure, the crude reaction mixture was then subjected to a liquid/liquid extraction system with brine/ethyl acetate, the combined organics were dried with anhydrous Na_2SO_4, concentrated under reduced pressure, and purified by a chromatographic column.
2.3.1. Diethyl-4-(4-chlorophenyl)-2,6-dimethyl-1-phenyl-1,4-dihydropyridine-3,5-dicarboxylate (**8**).
The reaction ran for 20 h and the compound was purified by FCC, in a gradient from 70% to 100% CH_2Cl_2 in hexane yielding a yellow solid (0.2770 g, 44 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46 (m, 3H), 7.35 (d, J 8.32 Hz 2H), 7.28 (d, J = 8.32 Hz, 2H), 7.14 (m, 2H), 5.12 (s, 1H), 4.16 (q, J = 7.08 Hz 4H), 2.06 (s, 6H), 1.26 (t, J = 7.08 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.0, 147.5, 145.7, 140.3, 131.8, 130.2, 129.5, 128.9, 128.8, 128.2, 105.5, 60.1, 38.4, 18.7, 14.3; HRESIMS m/z 440.1642 ($C_{25}H_{26}ClNO_4+H]^+$ calcd for m/z 440.1623).

2.3.2. Diethyl-(4-nitrophenyl)-2,6-dimethyl-1-phenyl-1,4-dihydropyridine-3,5-dicarboxylate (**9**).
The reaction ran for 28 h and the compound was purified by FCC, in a gradient 100% CH_2Cl_2 to 2% MeOH in CH_2Cl_2 and further separated by reverse phase chromatography with 20% water in MeOH, yielding a yellow solid (0.0459 g, 7%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 (m, 2H), 7.57 (m, 2H), 7.47 (m, 3H), 7.13 (m, 2H), 5.24 (s, 1H), 4.15 (q, J = 7.10 Hz, 4H), 2.07 (s, 6H), 1.25 (t, J = 7.10 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.6, 154.6, 148.2, 146.4, 140.0, 130.1, 129.7, 129.0, 123.5, 104.7, 60.3, 39.2, 18.8, 14.3; HRESIMS m/z 451.1853 ($[C_{25}H_{26}N_2O_6+H]^+$ calcd m/z 451.1864).

2.3.3. Diethyl 4-(4-bromophenyl)-2,6-dimethyl-1-phenyl-1,4-dihydropyridine-3,5- dicarboxylate (**10**).
The reaction ran for 24 h, the crude mixture was left to crystallize over 48 h by slow solvent evaporation, the solid were filtered and washed with ethanol, yielding a white solid (0.1561 g, 30%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45 (m, 5H), 7.28 (m, 2H), 7.13 (m, 2H), 5.10 (s, 1H), 4.15 (q, J = 7.08 Hz, 4H), 2.06 (s, 6H), 1.26 (t, J = 7.08 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.9, 147.5, 131.1, 130.2, 129.5, 129.3, 128.8, 105.4, 60.1, 38.5, 18.7, 14.2; HRESIMS m/z 484.1116 ($[C_{25}H_{26}BrNO_4+H]^+$ calcd for m/z 484.1118).

2.3.4. Diethyl 4-(4-hydroxyphenyl)-2,6-dimethyl-1-phenyl-1,4-dihydropyridine-3,5- dicarboxylate (**11**).
The reaction ran for 16 h, and the compound was purified byFCC, in a gradient from 100% CH_2Cl_2 to 2% MeOH in CH_2Cl_2 yielding a yellow solid (0.1203 g, 17%). ^{1}H NMR (300 MHz, $CDCl_3$) data: δ 7.43 (m, 3H), 7.23 (d, J 8.45 Hz 2H), 7.14 (m, 2H), 6.74 (d, J = 8.45 Hz, 2H), 5.08 (s, 1H), 4.15 (q, J = 7.26 Hz, 4H), 2.05 (s, 6H), 1.25 (s, J = 7.12, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) data: δ 168.8, 154.7, 147.2, 140.4, 138.9, 130.2, 129.5, 128.7, 128.6, 115.0, 106.1, 60.3, 38.0, 31.0, 18.8, 14.3; HRESIMS m/z 422.1924 ($[C_{25}H_{27}NO_5+H]^+$ calcd for m/z 422.1962), m/z 376.1530 $[M\text{-}EtOH]^+$.

2.3.5. Diethyl 2,6-dimethyl-1-phenyl-4-(3,4,5-trimethoxyphenyl)-1,4-dihydropyridine-3,5- dicarboxylate (**12**).
The reaction last 30 h, and the compound was purified by flash column chromatography, in a gradient from 50% CH_2Cl_2 in hexane to 100% CH_2Cl_2 and to 2% MeOH in CH_2Cl_2 yielding a yellow solid (0.1824 g, 36%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39 (m, 3H), 7.08 (m, 2H), 6.61 (s, 2H), 5.11 (s, 1H), 4.14 (q, J = 7.10 Hz 4H), 3.81 (s, 6H), 3.79 (s, 3H), 2.03 (s, 6H), 1.24 (t, J = 7.10 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.1, 152.8, 147.2, 142.7, 140.3, 136.3, 130.1, 129.5, 128.7, 119.9, 105.6, 104.3, 60.7, 60.0, 55.9, 38.8, 18.7, 14.4; HRESIMS m/z 496.2360 ($[C_{28}H_{32}NO_7+H]^+$ calcd for m/z 496.2330), m/z 518.2161 $[M+Na]^+$.

2.3.6. Diethyl 4-(3,4-dimethoxyphenyl)-2,6-dimethyl-1-phenyl-1,4-dihydropyridine-3,5- dicarboxylate (**13**).
The reaction ran for 30 h, and the compound was purified by flash column chromatography, in a gradient from 70% CH_2Cl_2 in hexane to 100% CH_2Cl_2, yielding a yellow solid (0.2275 g, 41%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37 (m, 3H), 7.07 (m, 2H), 6.96 (d, J = 1.62 Hz, 1H), 6.88 (dd, J = 1.62, 8.12 Hz 1H), 6.78 (d, J 8.12 Hz 1H), 5.06 (s, 1H), 4.11 (q, J = 7.10 Hz, 4H), 3.83 (s, 3H), 3.80 (s, 3H), 2.01 (s, 6H), 1.21 (t, J = 7.10 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.2, 148.3, 147.3, 147.1, 140.4, 140.0, 130.2, 129.4, 128.7, 119.1, 111.2, 110.9, 105.9, 60.0, 55.8, 55.7, 38.2, 30.8, 18.6, 14.3; HRESIMS m/z 466.2261 ($[C_{27}H_{32}NO_6+H]^+$ calcd for m/z 466.2224), m/z 488.2027 $[M+Na]^+$.
2.3.7. Diethyl 4-(4-hydroxy-3-methoxyphenyl)-2,6-dimethyl-1-phenyl-1,4-dihydropyridine-3,5-dicarboxylate (**14**).
The reaction ran for 16 h, and the compound was purified by flash column chromatography, in a gradient from 70% CH_2Cl_2 in hexanes to 100% CH_2Cl_2, yielding a brown solid (0.1375 g, 23%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (m, 3H), 7.12 (m, 2H), 6.97 (s, 1H), 6.84 (s, 2H), 5.08 (s, 1H), 4.14 (q, J = 7.12 Hz, 4H), 3.84 (s, 3H), 2.04 (s, 6H), 1.25 (t, J = 7.12 Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.3, 147.1, 146.2, 144.0, 140.4, 139.2, 130.2, 129.5, 128.7, 119.7, 114.1, 110.6, 105.9, 60.0, 55.7, 38.3, 18.7, 14.3; HRESIMS m/z 452.2027 ($[C_{26}H_{30}NO_6+H]^+$ calcd for m/z 452.2068), m/z 474.1886 $[M+Na]^+$.

2.3.8. Diethyl 4-(4-hydroxy-3,5-dimethoxyphenyl)-2,6-dimethyl-1-phenyl-1,4-dihydro pyridine-3,5-dicarboxylate (**15**).
The reaction ran for 20 h, and the compound was purified by flash column chromatography, in a gradient from 70% CH_2Cl_2 in hexane to 100% CH_2Cl_2, yielding a dark brown solid (0.0740 g, 15%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (m, 3H), 7.11 (m, 2H), 6.64 (s, 2H), 5.09 (s, 1H), 4.16 (q, J = 7.12 Hz 4H), 3.85 (s, 6H), 2.05 (s, 6H), 1.26 (t, J = 7.12 Hz 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.2, 147.1, 146.7, 140.4, 138.3, 133.2, 130.2, 129.5, 128.7, 105.8, 104.2, 60.0, 56.2, 38.6, 30.9, 18.7, 14.4; HRESIMS m/z 482.2185 ($[C_{27}H_{32}NO_7+H]^+$ calcd for m/z 482.2185), m/z 518.1970 $[M+Na]^+$.

2.4. Cytotoxicity assay.
Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1640 medium, penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and resazurin were acquired from Sigma-Aldrich (Missouri, EUA). Fetal bovine serum was acquired by Gibco® (Waltham, MA USA) and Dimethylsulfoxide (DMSO) was purchased from Vetec (Rio de Janeiro, Brazil). All compounds were dissolved in DMSO for the study of the viability in cancer cells, stock solution of 30 μM, and stored at -20°C.

2.4.1. Cell Culture

Cell lines were obtained from Rio de Janeiro Cell Bank (BCRJ, Brazil). The human breast cancer MCF-7 and mouse breast cancer 4-T1 cell lines were cultured in RPMI and human melanoma SK-MEL-28, human breast cancer MDA-MD-231 and normal mouse fibroblast NIH-3T3 cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 10 mM HEPES. Cells were maintained at 37 °C in a CO_2 5% humidified atmosphere.

2.4.2. Evaluation of cell viability in cancer cell lines and selectivity index

The cell viability was evaluated by Alamar Blue (resazurin) assay. Cells (1×10^4/well) were plated in 96-well culture microplate and incubated for 24 h with increasing concentrations of the compounds, ranging from 5 µM to 100 µM. The same final concentration of DMSO (1%) without compounds was used as negative control. Resazurin (150 µM/mL) was incubated by 4 h and the plate was read in fluorescence spectrometer – LS55 PerkinElmer (530 nm excitation, 590 nm emission). The concentration required to reduce the cell number by 50% (CC_{50}) was determined by nonlinear regression analysis of the logarithm of concentration as a function of the normalized response using the software Prism 5.0 (GraphPad Software). To obtain the selectivity index (SI), the CC_{50} of non-tumoral cell was divided by CC_{50} of tumoral cell.

2.5. Antiparasitic assay

2.5.1. Trypanocidal and Leishmanicidal screening against intracellular amastigotes.

T. cruzi trypomastigotes expressing β-galactosidase were raised from infected L929 cell line maintained at 37°C, CO_2 5% in RPMI-1640 medium supplemented with FBS 5%. *L. amazonensis* MHOM/BR/77/LTB0016 promastigotes, expressing β-galactosidase, were grown at 26°C, in Schneider's insect medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with heat inactivated FBS 5% and human urine 2%.

THP-1 cell line (ATCC TIB202) was grown at 37°C, 5% CO_2, in RPMI-1640 without phenol red (Sigma-Aldrich, CO. St. Louis, MO, USA) supplemented with FBS 10%, HEPES 12.5 mM, penicillin (100 U/mL), streptomycin (100 µg/mL) and Glutamax (2 mM).

For assays, cells were cultivated in 96 well microplates (4.0×10^4 cells/well) in supplemented RPMI-1640 and treated with phorbol 12-myristate 13-acetate (PMA) 100 ng/mL for 72 h (37°C, CO_2 5%) to allow cell differentiation into non-dividing macrophages.

Four days old culture promastigotes of *L. amazonensis* (4.0×10^6 parasites/mL) was washed with phosphate buffered saline (PBS, pH 7.4) and incubated in RPMI-1640 supplemented with 10% of heat-inactivated human B+ serum for 1 h (34°C) to parasite opsonization. THP-1 cells were then incubated with a parasite/cell ratio of 10:1 for 3 h (34°C, CO_2 5%) for *L. amazonensis* and with a parasite/cell ratio of 2:1 overnight (37 °C, CO_2 5%) for *T. cruzi*. Thereafter, non-adherent parasites were removed by two washes with PBS and infected cells incubated with 180 µL of full supplemented RPMI-1640 medium for another 24h (34°C or 37°C, CO_2 5%) to allow the transformation of promastigotes/trypomastigotes into intracellular amastigotes.

The infected cells were treated with 20 µL of each compound serially diluted in DMSO 50 µM to 1.56 µM in triplicate, followed by incubation for 48h (34°C or 37°C, CO_2 5%). Subsequently, cells were carefully washed with PBS and incubated for 16h (34°C, 5% CO_2) with 250 µL of chlorophenol red-ß-d-galactopyranoside (Sigma–Aldrich Co., St. Louis, MO, USA) (CPRG) at 100 µM and Nonidet P-40 (Amresco Inc, Solon, Ohio, USA) (NP-40) 0.1%. Optical density was read at 570/630 nm in an Infinite M200 TECAN, Austria. Amphotericin B (Bristol-Myers, Squibb) and benznidazol (Sigma Aldrich) were used as positive control and DMSO 1% as negative control. The concentration of each

sample that reduced parasite viability by 50% when compared to untreated control (IC50) was estimated by non-linear regression of concentration-response curves using the GraphPad Prism program.

2.5.2. Cytotoxicity assay

THP-1 cells were cultivated in 96 well microplates (4.0×10^6 cells/well), and treated with the compounds serially diluted in concentrations ranging from 500 µM to 15.6 µM and incubated for 72h (37°C, 5% CO_2). Cell viability was assessed by the MTT assay. DMSO 1% (Merck), Benznidazol (Sigma Aldrich) and Amphotericin B (Squibb) were used as negative and positive controls, respectively. The optical density was read at 540 nm in an Infinite M200 TECAN microplate reader immediately after the dissolution of formazan crystals with DMSO [16, 17]. DMSO 1% (v/v) and DMSO 50% (v/v) were used as negative and positive controls, respectively. The IC_{50} values were calculated by a non-linear regression using the GraphPad Prism program.

3. Results and discussion

3.1. Chemistry

DHPs were prepared on a one-pot manner based on the catalyst-free Hantzsch reaction [18]. So, 2.4 equivalents of ethyl acetoacetate and 1.2 equivalents of ammonium acetate (compounds **1**-**7**) or aniline (compounds **8**-**15**) were added to an ethanolic solution of the aromatic aldehyde. The reaction mixture was stirred under reflux conditions and monitored by thin layer chromatography (TLC) (Fig. 1). The desired products were purified by silica gel chromatographic column. Their structures were established by the mean of NMR spectroscopy and HR-ESI-QTOFMS data. The yields of DHPs containing a NH group ranged 14 to 93% while the *N*-phenyl products were obtained with quantities comprised between 14 and 44%.

The synthesis of compounds **16**-**21** was performed as previously reported by Sandjo et al. [19] and the preparation process is illustrated in figure 2. The cytotoxic [19] and antiparasitic effects of DHPs **16**-**21** (tables 1 and 2) guided the structural design used for the preparation of compounds **1**-**15**.

2.2. Biological assays.

2.2.1. Cytotoxicity

Compounds **1**-**21** were evaluated for their cytotoxicity on three cancer cell lines including the human melanoma SK-MEL-28, the breast cancer MCF-7 and the mouse breast tumor 4-T1. Toxicity of the compounds was also assayed on the murine fibroblast cells NIH-3T3, a non-tumoral cell line, to calculate the selectivity index (Table 1). Among the tested compounds, only DHPs **2**, **11**, **15**, **18**, **19**, and **21** showed antiproliferative activity with CC_{50} values ranged between 27-84 µM. Compound **18** was the most potent against SK-MEL-28 with CC_{50} of 27 µM. Furthermore, MCF-7 and 4-T1 were more sensitive to compound **19** with CC_{50} of 28 µM and 30 µM, respectively. Compound **14** showed cytotoxicity exclusively on 4-T1 and was 2.4-fold less toxic for NIH-3T3. In general, the active 1,4-dihypyridines did not present the expected selectivity for the non-tumoral cells lines. While **11**, **18**, **19**, and **21** displayed SI values greater than 1.3, compounds **2** and **15** strongly inhibited the growth of non-tumoral cells than cancer cells. The weak antiproliferative activity of the prepared DHPs against tumoral cells made them good candidates for the evaluation against the parasites. This strategy was opted because the antiparasitic bioassay was carried out on intracellular amastigote forms. Thus, the cytotoxic activity and the selectivity values obtained were used as an initial insight into the THP-1 (host cells) sensitivity while they are exposed to **1**-**21**.

3.2.2. Antiparasitic activity.

3.2.2.1. Trypanocidal activity

Compounds were further evaluated for antiprotozoal activity against the amastigote form of two trypanosomatids namely *T. cruzi* and *L. amazonensis*. The inhibitory concentration of 50% of the parasite growth (IC_{50}) was calculated for each compound. Besides, their toxicity to THP-1 (human monocytic cell line) used as host cells for both parasites were performed in order to evaluate the selectivity index (SI = $CC_{50THP-1}/IC_{50parasite}$) for each compound.

The first antiparasitic assays performed with compounds **16**-**21** against the intracellular amastigotes of *T. cruzi* revealed interesting inhibitory effect with IC_{50} (SI) values at 5.44 µM (20.91) and 6.64 µM (17.03) for compounds **18** and **21**, respectively (Table 2). Compounds **18** and **21** had the Me group attached to C-2 and C-6 while those bearing phenyl group at the position was devoid of trypanocidal effect. Assuming that bulky groups attached to C-2 and C-6 of the DHP core disadvantage the potency of **16**, **17**, **19**, and **20**, Me groups were used to replace to phenyl groups for the next series of compounds to be prepared. Among the products (**1**-**7**), **1**-**5** showed interesting trypanocidal activity with IC_{50} (SI), respectively at 15.74 µM (8.19), 4.95 µM (9.30), 42.54 µM (>11.75), 35.78 µM (1.87), and 20.30 µM (17.1). Less bulky *N*-phenylDHP analogues **8**-**15** were prepared and assayed on the abovementioned parasites. Except for **10**, **12** and **14**, DHPs **8**, **9**, **11**, **13**, and **15** showed trypanocidal activity with IC_{50} (SI) at 10.91 µM (38.17), 30.23 µM (>16.54), 16.79 µM (5.32), 24.39 µM (11.14), and 26.14 µM (5.43), respectively.

3.2.2.2. Leishmanicidal activity

All prepared DHPs were tested on *L. amazonensis* and among the 21 compounds, only **5**, **11**, and **15** showed leishmanicidal activity with IC_{50} (SI) values at 15.11 µM (22.98), 45.7 µM (1.95), and 53.13 µM (2.67), respectively. Compound **5** was highly selective towards the TPH-1 cell line compared to other DHPs and was the most potent chemical entity.

3.2.3. SAR study

3.2.3.1. Trypanocidal effect and DHP structures

A diagnostic analysis of the tested DHP structures regarding their trypanocidal activity revealed that 4-(para-nitrophenyl)-1,4-dihydropyridine (**2**) was more potent than 4-(meta-nitrophenyl)-1,4-dihydropyridine (**21**). However, the position of NO_2 in *meta* reduces the toxicity against THP-1 cell by almost 2-fold if comparing their selectivity indexes. Replacing the NO_2 group in **2** by a Cl group reduces the toxicity of compound **18** by 2.25-fold even though its anti-*T. cruzi* effect is slightly inferior (1.12-fold) to that of **2**. A *para* H-donor group strongly decreases the anti-*T. cruzi* activity as observed for the activity of **3** and by comparing to those of **2** and **18**. Moreover, 4-(para-bromophenyl) DHP (**19**) inhibited *T. cruzi* with IC_{50} higher than 55 µM. This loss of the activity might be caused by the size of the bromine atom instead of its electronic property, which is similar to that of chlorine.

In general, polyoxygenated compounds were moderately to weakly trypanocidal agents or not active. However, the presence of a OMe group at the ortho position of the phenol in DHP **3** structure increase the anti-*T. cruzi* effect as noticed with 4 but increase also the toxicity of this eventually (Fig. 3). An additional OMe group to compound **4** to afford **7** abolishes the antiprotozoal activity meanwhile, the methoxylated form of **4** (compound **1**) was more potent than **4** by 2.23-fold. Compound **1** showed also a good tolerance to THP-1 cell compared to **4** whereas an additional MeO group in **1** to form DHP **6** completely abolishes the activity.

The presence of a phenyl moiety in compound **9** strongly decreases the antiparasitic effect by 6.12-fold compared to its free NH-containing derivative **2**. Nevertheless, this *N*-phenyl residue (DHP **9**) also contributes to reduce the toxicity. Similar biological feature was observed with compound **8** (the *N*-phenyl derivative of **18**), which showed a better selectivity than **18** even though its anti-*T. cruzi* effect was diminished by almost 2-fold (Fig. 4). In contrast, the *N*-Ph moiety in **11** (the *N*-phenyl derivative of **3**) rather improved the trypanocidal activity and increased the toxicity comparing to the activity of **3**. 1-Ph-4-(4-chlorophenyl) DHP (**8**) was more active than its congeners 1-Ph-4-(4-nitrophenyl) DHP (**9**) and 1-Ph-4-(4-hydroxyphenyl) DHP (**11**) with a better selectivity towards the THP-1 cell line. Additional MeO group in compounds **14** and **15** led to respectively, a loss and a decrease of the trypanocidal activity when compared their data to those of **11** (Fig. 4). DHP **14** initially inactive on *T. cruzi*, recovered the antitrypanosomal activity in its methoxylated form (**13**). Compounds bearing OH and MeO groups were

moderately to weakly trypanocidal agents suggested the substituents unfavorable for the activity. Moreover, DHPs bearing these functional groups did no show good selectivity.

Calculated physical properties including the lipophilicity (xlogP) and the polar surface area (PSA) revealed that compounds with the best trypanocidal IC_{50} have xlogP values ranging 3.81 to 4.03 and PSA values of 110.45 $Å^2$. Nevertheless, no real correlations were found between the activity (IC_{50}) and these two parameters. It can be noted in table 2 that some compounds have the same xlogP or PSA values and revealed different antiparasitic activity. Moreover, compounds **2**, **5**, **18**, and **21**, the most potent active 1,4-dihydropyridines corroborate the Lipinski rule of 5 (H-bond donors <5, H-bond acceptors <10, the molecular weight < 500, and ClogP < 5) [20]. Thus, 4-(3-nitrophenyl)DHP, 4-(3-nitrophenyl)DHP and 4-(Cl-nitrophenyl)DHP cores seem to be the pharmacophoric unit that can inspire preparation of hit analogues. The 4-(3-nitrophenyl)DHP and 4-(Cl-nitrophenyl)DHP scaffolds can easily be framed within the chemical structure of NO_2 containing Ca^{2+} channel blockers (azelnidipine, cilnidipine, lercanidipine, nicardipine, nifendipine, nimodipine, nitrendipine, and isradipine) and the chlorinated DHP amlodipine previously reported as antiparasitic entities against the trypomastigote form of *T. cruzi* [5] with interesting inhibitory values. However, compounds **2**, **18** and **21** can still be decorated with different function groups in order to improve to antichagasic activity and still meet the rule of 5.

3.2.3.2. Leishmanicidal effect and DHP structures

The styryl group in compound **5** turned to be favourable for the leishmanicidal activity and less aggressive towards THP-1 cells. In contrast, **11** and **15** the weakest active compounds contain hydroxylated and methoxylated aryl groups. No concrete structure-activity relationships were found between the active compounds except for **11** and **15** that are N-phenylated and contain OH and/or MeO groups. The lack of leishmanicidal effect from the remaining DHPs might be explained by the lack of any correlations between the Ca^{2+} channel action and the survival of *L. amazonensis* in its host. Otherwise, the inhibition of the Ca^{2+} channel action perhaps does occur, but does not affect the proliferation of the parasite in its host.

A comparison of various former studies on antiparasitic effect of DHPs used as CCB drugs revealed that nifedipine and verapamil inhibited the macrophage growth but did not show any antiparasitic effect on *L. donovani*. Despite the observation, the report claimed a critical contribution of Ca^{2+} in the interaction macrophage-*Leishmania* [21]. In contrast, another study reported the proliferation of *L. donovani* in macrophage despite the presence of nifedipine [22]. Because of these controversial results, Tempone and co-workers studied a different CCB (nimodipine) on *L. chagasi*. During the *in vitro* investigation, no decrease in *L. chagasi* growth was observed. Despite their results, the authors correlated the leishmanicidal effect of the compound with its ability to induce apoptosis by affecting the mitochondrial function [7]. Moreover, another interesting result revealed that nifedipine and verapamil inhibited the sphingosine-activated plasma membrane Ca^{2+} in *L. mexicana* promastigotes [23]. On the basis of these previous data, we concluded that the leishmanicidal effect of DHPs **11** and **15** might occurred by a similar mechanism pathway namely the inhibition of the sphingosine-activated plasma membrane Ca^{2+}. This deactivation of the Ca^{2+} channel might also affect the host cell supporting the low selectivity of **11** and **15** in assays reported in tables 1 and 2.

4. Conclusion

The study aimed to prepare 1,4-dihydropyridines related to Ca^{2+} channel blockers drugs and to evaluate their cytotoxic and antiparasitic effects on *L. amazonensis* and *T. cruzi*. The results showed that some of the compounds had a significant trypanocidal activity with a good selectivity on THP-1 cells. Their selectivity was increased when a phenyl group was attached to the nitrogen atom although a slight decrease of the antiparasitic effect was observed. Moreover, Br atom, MeO and OH groups seemed not to be suitable substituents for the trypanocidal activity because of the weak effect of the compound or the low selectivity. The study also showed that except for compound **5**, the leishmanicidal effect of some DHPs might be associated to their capability of inhibiting the Ca^{2+} channel.

Supplementary Material

^{1}H and ^{13}C NMR data of compounds **1-15** are associated with this work can be found online.

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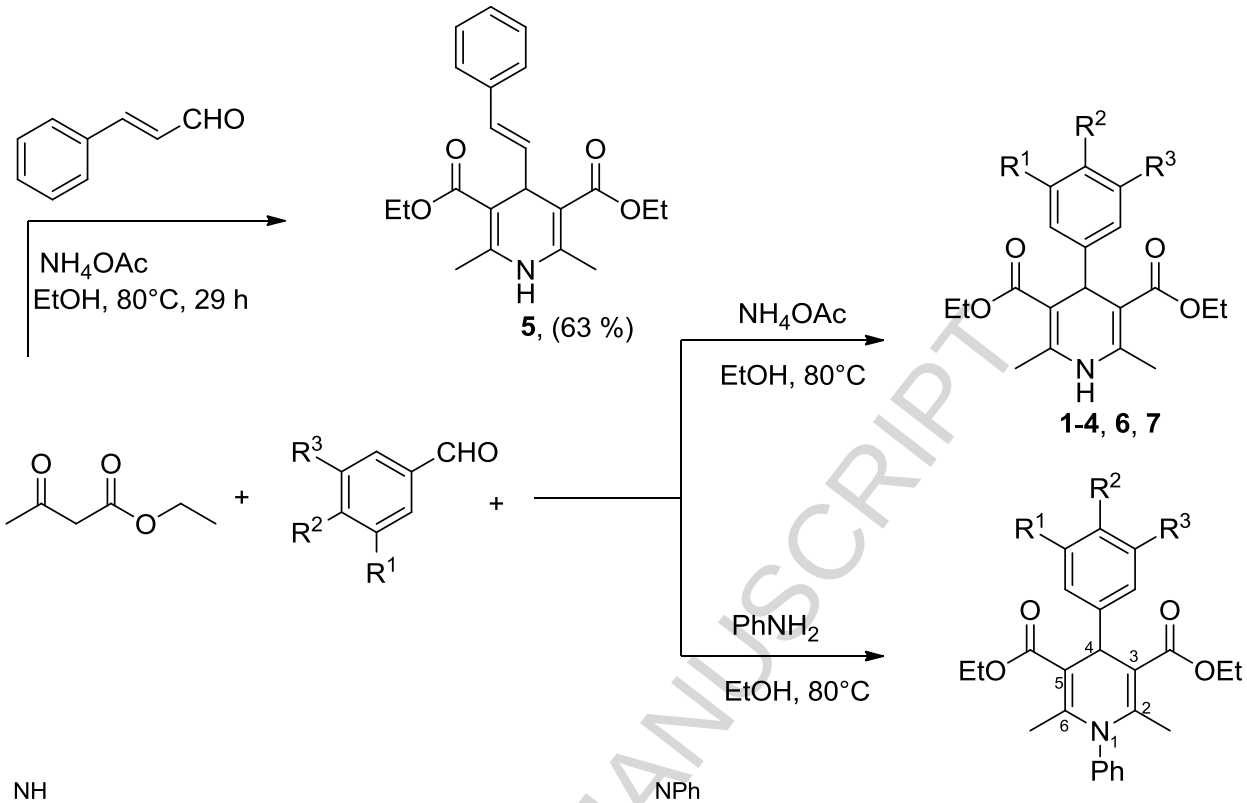


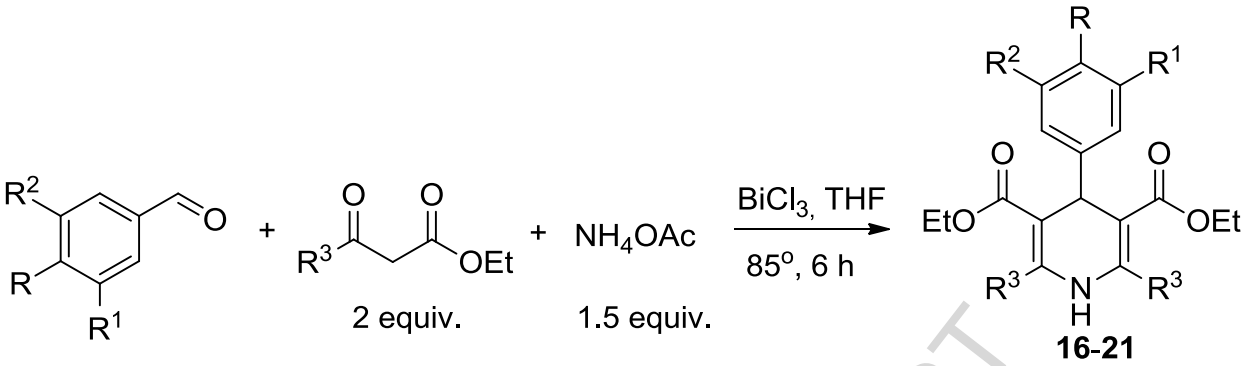
NH

Compounds	R^1	R^2	R^3	Times (h)	Yield (%)
1	OMe	OMe	H	48	43
2	H	NO_2	H	68	13
3	H	OH	H	48	57
4	OMe	OH	H	44	43
6	OMe	OMe	OMe	16	93
7	OMe	OH	OMe	20	45

NPh

Compounds	R^1	R^2	R^3	Times (h)	Yield (%)
8	H	Cl	H	20	44
9	H	NO_2	H	28	7
10	H	Br	H	24	30
11	H	OH	H	16	17
12	OMe	OMe	OMe	30	36
13	OMe	OMe	H	30	41
14	OMe	OH	H	16	23
15	OMe	OH	OMe	20	14

Figure 1: Synthesis route of compounds **1-15**



Compounds	R	R^1	R^2	R^3	Yield (%)
16	Br	H	H	Ph	90
17	Cl	H	H	Ph	91
18	Cl	H	H	Me	88
19	Br	H	H	Me	92
20	OMe	OMe	OMe	Ph	88
21	H	H	NO_2	Me	87

Figure 2: Synthesis route of compounds **16**-**21**[19].

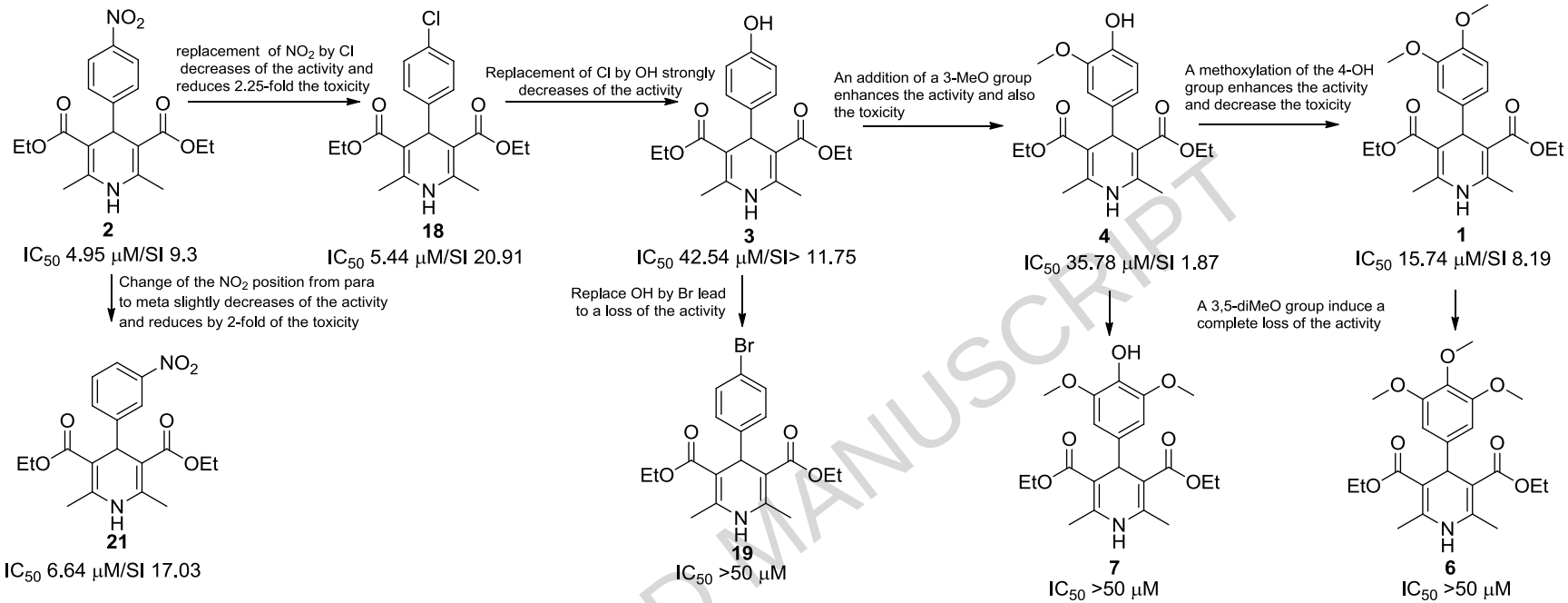


Figure 3Analysis of the structure-Activity relationship between DHPs bearing a di-substituted nitrogen atom

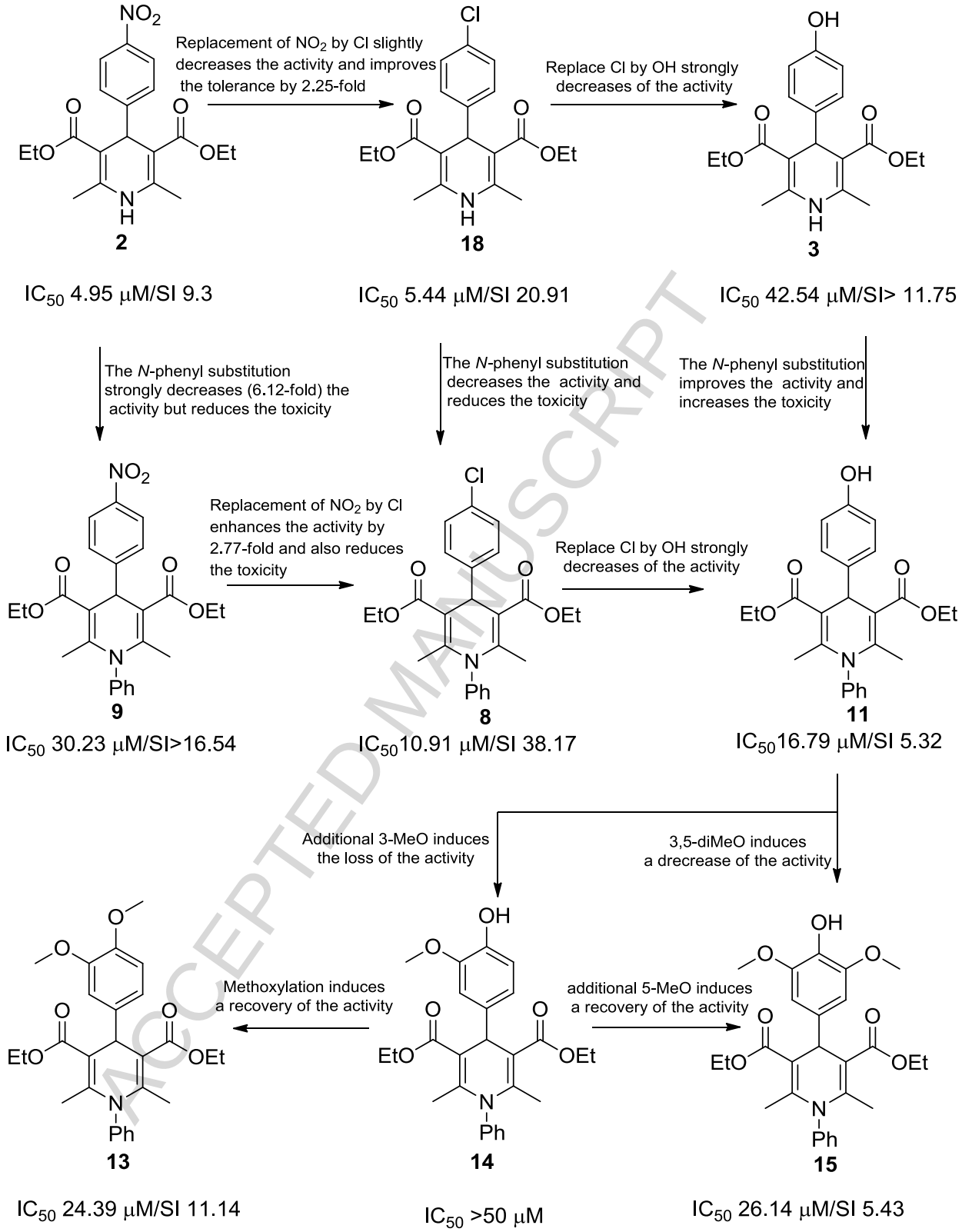


Figure 4. Analysis of the structure-Activity relationship between DHPs bearing NH and N-phenyl groups.

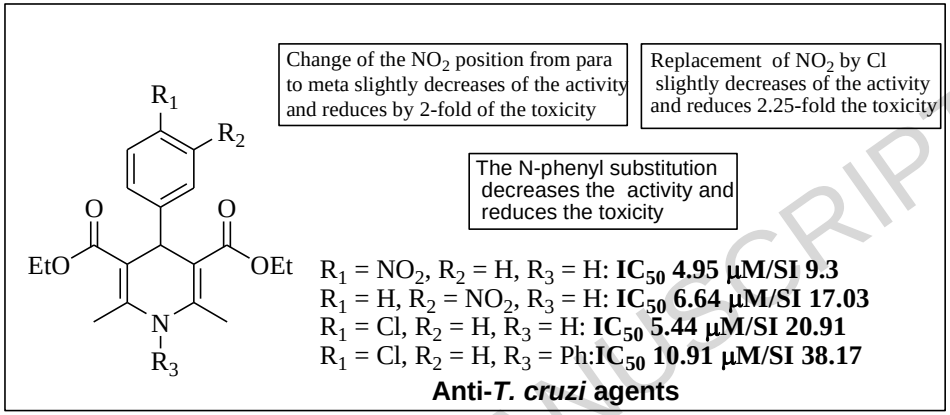
Table 1: CC_{50} and SI values of compounds against different cancer cell lines

Compound	CC_{50} (µM)					Selectivity Index (SI)*		
	SK-MEL-28	MCF-7	4-T1	NIH-3T3		NIH-3T3/ SK-MEL-28	NIH-3T3/ MCF-7	NIH-3T3/ 4-T1
1	>100	>100	>100	>100		-	-	-
2	41	68	57	59		1,5	0,9	1,0
3	>100	>100	>100	>100		-	-	-
4	>100	>100	>100	>100		-	-	-
5	>100	>100	>100	>100		-	-	-
6	>100	>100	>100	>100		-	-	-
7	>100	>100	>100	>100		-	-	-
8	>100	>100	>100	>100		-	-	-
9	>100	>100	>100	>100		-	-	-
10	>100	>100	>100	>100		-	-	-
11	41	67	49	44		1,1	0,6	0,9
12	>100	>100	>100	>100		-	-	-
13	>100	>100	>100	>100		-	-	-
14	>100	>100	84	200		-	-	2,4
15	71	43	81	44		0,6	1,0	0,6
16	>100	>100	>100	>100		-	-	-
17	>100	>100	>100	>100		-	-	-
18	27	37	31	58		2,2	1,6	1,9
19	32	28	30	41		1,3	1,5	1,4
20	>100	>100	>100	>100		-	-	-
21	56	55	64	98		1,7	1,8	1,5

Table 2 Antiparasitic effect of compounds **1-21** against the intracellular *T. cruzi* and *L. amazonensis* amastigotes as well as their selectivity towards the THP-1 cell line.

T. cruzi			**THP-1 cells**	***L. amazonensis***		xLogP	PSA ($Å^2$)
Compound	IC_{50} (µM)	SI	CC_{50} (µM)	IC_{50}	SI		
1	15.74 (±0.58)	8.19	128.90 (±19.10)	NA	-	3.39	83.09
2	4.95 (±0.40)	9.30	46.05 (±1.77)	NA		3.81	110.45
3	42.54 (±17.39)	>11.75	>500	NA	-	3.08	84.86
4	35.78 (±2.08)	1.87	91.66 (±18.01)	NA	-	3.09	94.09
5	20.30 (±2.16)	17.10	347.20 (±33.03)	15.11 (±3.67)	22.98	3.92	64.63
6	NA	-	NA	NA		3.40	92.32
7	NA	-	NA	NA	-	3.10	103.32
8	10.91 (±2.12)	38.17	416.50 (±92.01)	NA	-	5.68	55.84
9	30.23 (±5.49)	>16.54	>500	NA	-	5.46	101.66
10	NA	-	NA	NA		5.79	55.84
11	16.79 (±0.29)	5.32	89.34 (±4.79)	45.70 (±11.70)	1.95	4.74	76.07
12	NA	-	NA	NA	-	5.06	83.53
13	24.39 (±2.06)	11.14	271.70 (±41.50)	NA	-	5.05	74.30
14	NA	-	NA	NA	-	4.74	85.3
15	26.14 (±1.21)	5.43	142.00 (±10.00)	53.13 (±4.30)	2.67	4.75	94.53
16	NA	-	NA	NA	-	6.41	64.63
17	NA	-	NA	NA	-	6.30	64.63
18	5.44 (±0.13)	20.91	113.8 (±36.03)	NA	-	4.03	64.63
19	NA	-	~203.6	NA	-	4.14	64.63
20	NA	-	NA	NA	-	5.80	92.32
21	6.64 (±0.68)	17.03	113.1 (±13.10)	NA	-	3.81	110.45
ref1	10.18 (±0.3)	>49.11	>500	-	-		
ref2			27.86 (±0.98)	0.14 (±0.02)	199		

ref1: Benznidazol, ref2: Amphotericin B, NA: not active



Graphical abstract

Highlights

- Synthesis of 21 Ca^{2+} channel blocker 1,4-dihydropyridine analogues.
- Evaluation of their antiparasitic effects against *T. cruzi* and *L. amazonensis*.
- 4 of the 12 active compounds on *T. cruzi* revealed promising IC_{50} values.
- *L. amazonensis* was sensitive to three of the prepared 1,4-dihydropyridines.