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Full Length Research Paper

Searching for new antileishmanial lead drug candidates: Synthesis, biological and theoretical evaluations of promising thieno[2,3-*b*]pyridine derivatives

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Cutaneous leishmaniasis is a parasitic disease associated with high morbidity and mortality rates. This work reports the synthesis, biological and theoretical evaluations of a new antileishmanial series of 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-*b*]pyridine derivatives - 3 (H), 3a (*m*-CH_3), 3b (*m*-OCH_3), 3c (*m*-NO_2), 3d (*m*-F), 3e (*m*-Br), 3f (*p*-CH_3), 3g (*p*-OCH_3), 3h (*p*-NO_2), 3i (*p*-F), 3j (*p*-Br). Interestingly 3f and 3g showed a better profile against *Leishmania amazonensis* (EC_{50}=29.49 and 32.23 µM, respectively) than glucantime, the current drug on the market (EC_{50}=163.7 µM). The theoretical analysis pointed a correlation among the antileishmanial profile and the conformational and electrostatic features of these new molecules. ADMET and "Lipinski´s rule of 5" revealed higher theoretical biodisponibility, druglikeness and drugscore values for these derivatives compared to known antileishmanial drugs. Our results pointed these thieno[2,3-*b*]pyridine derivatives as lead compounds for designing new agents for treatment of cutaneous leishmaniasis.

Key words: Cutaneous leishmaniasis, antileishmanial, thieno[2,3-*b*]pyridine derivatives, structure-activity relationship (SAR).

INTRODUCTION

Leishmaniasis is a disease caused by protozoan that belongs to the genus *Leishmania,* a compulsory intracellular parasite of the mammalian host cell (Santos et al., 2008; Zanger et al., 2011). This disease is associated with high morbidity and mortality rates and currently affects about 12 million people worldwide in 88 countries, mainly in tropical and sub-tropical areas (Santos et al., 2008; Marinho et al., 2011; WHO, 2011).

Leishmania amazonensis causes the American tegumentary leishmaniasis (ATL) (Chakravarty and Sundar, 2010) that appears as simple or diffuse ulcerations on skin (mainly on the face) with patient mutilation and

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disfiguration (Delorenzi et al., 2001; Gontijo and Melo, 2004; Souza et al., 2010). This cutaneous form of leishmaniasis may spontaneously regress but usually evolves and requires treatment (Santos et al., 2008; Zanger et al., 2011).

Currently, different drugs used in leishmaniasis treatment are: a) pentostam allowed by Center for Disease Control and Prevention- (CDC) – Atlanta - USA , b) glucantime that is used in Brazil and some Latin America countries and c) antimonial that is not approved by Food and Drugs Administration – (FDA) - USA, (Santos et al., 2008; Cunha et al., 2010).

According to World Health Organization, glucantime therapeutic efficiency varies in different countries. Thus, the treatment protocols should be established based on the geographical area (Santos et al., 2008; WHO, 2011). Significant differences on clinical response are also observed when using pentavalent antimonials. Several collateral effects such as myalgy, pancreatitis, cardiac arrhythmia, hepatitis, and drug accumulation in different organs (such as spleen and liver) lead to treatment withdrawal or even resistance against these compounds (Yardley et al., 2002; Santos et al., 2008).

Several problems of using the current antileishmanial drugs (for example, high toxicity, several collateral effects, emergence of resistant strains and patients withdraw) together with the annual incidence of about two million new cases and 350 million people living in the endemic areas (Weniger et al, 2001; Yardley et al., 2002, Gontijo and Melo, 2004; WHO, 2011) reinforce the importance of finding new options for treating leishmaniasis (Yardley et al., 2002, WHO, 2011). The high cost of the current drugs that have gradually increased throughout the years (WHO, 2011) also increases the urgent need for searching for new antileishmanial agents with low toxicity, cost, and high efficacy against drug resistant strains (Ferreira et al., 2010). Recently, the literature pointed host defense peptides (HDPs) and new synthetic molecules as new anti-parasitic therapies alternatives. One example is the cathelicidin bovine myeloid antimicrobial peptide 28 (BMAP-28) that showed broad antimicrobial activities and protected animals models against bacterial infection or sepsis (Lynn et al., 2011).

Synthetic routes and structural analysis of thienopyridine derived molecules have also been described (Kaigorodova et al., 2000; El-Kashef et al., 2010; Testa et al., 2010) together with antiviral (Bernardino et al., 2004), anti-inflammatory (Moloney, 2001), antibacterial (Leal et al., 2008; Pinheiro et al., 2008; Panchamukhi et al., 2011) and antiparasitic reports (Bernardino et al., 2006). Interestingly, thienopyridine derivatives are analogous to amodiaquine, and pyrazolopyridine derivatives (Mello et al., 2004; Dias et al., 2007), which have been described as antiprotozoa agents, acting against resistant *Trypanosome* and *Leishmania* strains (Silva et al., 2007).

Due to our expertise on synthesizing thienopyridine derivatives (Bernardino et al., 2006; Pinheiro et al., 2008), in this work, we explored the addition of imidazol group to thieno[2,3-*b*]pyridine derivatives and compared their antileishmanial profile with glucantime, an antileishmanial drug. In addition, we theoretically evaluated the structure-activity relationship of these 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-*b*]pyridine derivatives as well as their *in vitro* and *in silico* toxicity, to identify them as feasible antileishmanial prototypes for cutaneous leishmaniasis treatment.

MATERIALS AND METHODS

Chemistry

The 4-chlorothieno[2,3-*b*]pyridine-5-carbonitrile (1) were prepared as described in literature (Bernardino et al., 2006; Pinheiro et al., 2008), underwent a nucleophilic substitution with aniline derivatives to afford the 4-(arylamino)thieno[2,3-*b*]pyridine-5-carbonitrile derivatives (2, 2a-j). The compounds - 3 (H), 3a (*m*-CH_3), 3b (*m*-OCH_3), 3c (*m*-NO_2), 3d (*m*-F), 3e (*m*-Br), 3f (*p*-CH_3), 3g (*p*-OCH_3), 3h (*p*-NO_2), 3i (*p*-F), 3j (*p*-Br) - were obtained in good yields (60 to 85%) from heating of 2, 2a-j with ethylenediamine and carbon disulfide at 100°C for 24 h (Scheme 1). The structures of the compounds were elucidated by using IR, ^{1}H, ^{13}C NMR spectroscopy and mass spectrometry, and all parameters were in the expected ranges (Supplementary material).

Biological evaluations

Drugs

The stock solutions of the 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-*b*]pyridine derivatives (50 µM) were prepared in dimethyl sulfoxide (DMSO). DMSO has no effect on the proliferation or morphology of parasites when its concentration does not exceed 1% v/v in cell culture.

Leishmania culture

Promastigote infective forms of *L. amazonensis* were maintained by weekly transfers in brain heart infusion medium (BHI) supplemented with 10% fetal bovine serum (FBS) at 26°C. The infectivity of the parasites was maintained by performing a periodic inoculation into hamster footpads.

Antileishmanial evaluation

Promastigote forms of *L. amazonensis* [3 × 10^6 cell/ml] were incubated (24 h) in BHI supplemented with 10% FBS in the absence or presence of the 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-*b*]pyridine derivatives (6.25 to 50 µM). Glucantime was used as the positive control and in previous pilot, 200 µM led to 73.9% inhibitory effect. Untreated controls were performed with or without DMSO. The growth inhibitory effect was quantitatively monitored by direct counting of parasites in a Neubauer chamber using optical microscopy (Olympus B × 41). Each experiment was performed in triplicate, and control groups (non-treated parasites) were performed in presence of DMSO 1% as it showed no effect on the assays. Serial dilutions of 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-*b*]pyridine derivatives was performed promastigotes forms of *L. amazonensis*

[3 × 10^6 cell/ml] were incubated with the derivative (6.25, 12.5, 25 and 50 µM) for 24 h. Cell density was determined by direct counting in a Neubauer chamber. For the most active compounds, we determined the effective concentration that is able to inhibit 50% of the *L. amazonensis* growth after 24 h (EC_{50}). EC_{50} and graphs were determined using the Microcal Origin program. The results were analyzed using Student's t test, and significant differences were determined at $P < 0.05$.

Cytotoxicity assay in mammalian cell

The cytotoxic effects of all compounds were evaluated in monocytes-derived human macrophages isolated from peripheral blood mononuclear cells. Briefly, human monocytes were purified from human blood using Ficoll-Hypaque gradient as previously described (Meddeb-Garnaoui et al., 2009). For adhesion and complete differentiation to macrophage, the human monocytes (10^6 cell/ml) cells were kept at 37°C, in an atmosphere of 5% CO_2, in Dulbecco's modified minimum essential medium (Sigma Chemical Co., St. Louis, MO), supplemented with 10% fetal bovine serum (Highclone) and Hepes buffer (Sigma) in 8-well LabTech™ tissue cultures slides (Life technologies) during 7 days. After that, the compounds diluted in DMSO (50 µM) were added to the macrophages in duplicate assays. After 24 h-incubation, treated and untreated cells were washed twice with PBS and fixed with methanol for 15 min. Cells were stained with Giemsa and the quantification was performed by counting 100 randomic fields in an optical microscopy (Olympus B x 41). The results were analyzed using Student's t test, and significant differences were determined at $P < 0.05$. As macrophages are adherent cells, the number of the stained cells represents viable macrophages. Our results were expressed as: % of cytotoxicity = [(compounds treated cells-control) ÷ control] x 100.

Theoretical evaluation

Molecular modeling and SAR studies

Molecular modeling was performed using SPARTAN'08 (Wavefunction Inc. Irvine, CA, 2000). Structures were minimized and the equilibrium geometry was obtained in vacuum using a semi-empirical AM1 module. In order to evaluate the electronic properties of the AM1 minimal energy conformations, they were submitted to a single-point calculation using the density functional theory (DFT) method, B3LYP functional and a 6-31G* basis set of SPARTAN'08. The electronic properties, HOMO energy and coefficient distribution, LUMO density, and dipole moment vector were calculated for all compounds. Theoretical logP (clogP) was calculated at the AM1 semi-empirical level using the Villar method, included in SPARTAN. Molecular electrostatic potential isoenergy surface maps were also generated with a range of energies from -25 (deepest red) to 30 (deepest blue) Kcal/mol and superimposed on a surface having a constant electron density of 0.002e/au3.

In silico ADMET screening

In the effort to study the hydrophobic pattern, we analyzed different descriptors of the compounds, including calculated octanol/water partition coefficient (cLogP), molecular weight (MW), molecular volume (MV), and number of hydrogen bond donor (HBD) and acceptor (HBA) as determined by Lipinski's "rule-of-five" (cLogP ≤ 5, MW ≤500, HBD ≤5 and HBA ≤10) (Lipinski et al., 2007), which evaluates theoretical oral biodisponibility. We also submitted the most potent compounds to an *in silico* ADMET screening using the program OSIRIS available at http://www.organic-chemistry.org/ to analyze their overall drug-score and drug-likeness potential and toxicity risks (mutagenic, irritant, tumorigenic, and reproductive effects) (Sander et al., 2009). In addition, we compared them to the available drugs currently in use on Leishmaniasis treatment.

RESULTS AND DISCUSSION

In this study, we evaluated the antileishmanial profile of these thieno[2,3-*b*]pyridine derivatives against *L. amazonensis* promastigote form at a screening concentration of 50 µM. Interestingly, most of the compounds presented a significant antileishmanial activity (>50%) after 24 h incubation, including the compound with no substituent at the phenyl ring. This suggested that the addition of the imidazol group in thieno[2,3-*b*]pyridine derivatives may lead to antileishmanial active compounds. Compared with the non substituted compound, the substitution at phenyl ring *para* or *meta* positions seems to affect positively the biological activity with *para*-position leading to the highest antileishmanial profile (that is, *p*-OCH_3) (Table 1 and Figure 2).

The analysis of the effective concentration able to inhibit 50% of the *L. amazonensis* growth after 24 h (EC_{50}) revealed 3f and 3g (EC_{50} = 29.49 and 32.23 µM, respectively) with a better antileishmanial profile than glucantime (EC_{50} = 163.7 µM) (Figure 2). This infers that the methyl group in 3f and 3g is important for interacting with the target in the parasite. The level of activity of these compounds was better than andrographolide, a diterpenoid lactone isolated from the leaves of *Andrographis paniculata* (160 µM), than 1,4-diamino-2-butanone, a putrescine analogue (144 µM) and similar to quercetin (31.4 µM), the most common polyphenolic flavonoids present in plants such as onions, ginko biloba and tea (Fonseca-Silva et al., 2011; Vannier-Santos et al., 2008, Roy et al., 2010).

Analogously to our work, other derivatives containing imidazol group with antileishmanial profile have been recently described (Monzote et al., 2011; Marrapu et al., 2011). The literature described that, in general, the azole drugs get into the *Leishmania* cells and bound to the cytochrome P-450 14α-demethylase. Therefore, these molecules effectively block 14α-methyl sterol demethylation, affecting the parasite metabolism. These data may help to identify a feasible target for our molecules to be further explored (Mishra et al., 2007).

Herein, to determine the relation between the structure and the cytotoxic profile of these *p*-substituted compounds, we tested them at the screening concentration (50 µM) using human macrophages-derived peripheral blood mononuclear cells. Importantly, the substituents of 3f and 3g did not lead to cytotoxic effects (Figure 3), pointing these derivatives as promising for continuous work.

We used molecular modeling tools to identify some features of the structure-activity relationship of this series. Thus, we performed the calculation of some molecular electronic properties (dipole moment, cLogP and energy

Table 1. Comparison of the antileishmanial profile (%) of thieno[2,3-b]pyridine derivatives (50 M) with their molecular electronic properties (Energy of HOMO and LUMO, Dipole moment and LogP) and the Lipinski Rule of 5 values (cLogP, Molecular Weight - MW, Hydrogen Bond Acceptor and Donor Groups –HBA and HBD).

Compound	R	Antileishmanial profile (%)	Energy(Ev)		Dipolo debye	Lipinski Rule of 5			
			HOMO	LUMO		CLogP	HBA	HBD	MW
3	H	59.6	-5.40	-1.28	2.98	2.57	4	1	294.38
3a	*m*-CH_3	71.7	-5.35	-1.26	3.12	3.05	4	1	308.41
3b	*m*-OCH_3	66.8	-5.29	-1.28	3.09	2.44	5	1	324.41
3c	*m*-NO_2	69.8	-5.94	-2.37	4.77	2.60	7	1	339.38
3d	*m*-F	88.10	-5.57	-1.39	2.63	2.73	4	1	312.37
3e	*m*-Br	51.9	-5.66	-1.45	2.74	3.40	4	1	373.28
3f	*p*-CH_3	88.30	-5.26	1.25	3.14	3.05	4	1	308.41
3g	*p*-OCH_3	95.45	-5.02	-1.20	3.18	2.44	5	1	324.41
3h	*p*-NO_2	80.7	-6.06	-2.18	5.48	2.60	7	1	339.38
3i	*p*-F	69.4	-5.40	-1.35	2.63	2.73	4	1	312.37
3j	*p*-Br	36.4	-5.54	-1.45	2.79	3.40	4	1	373.28

(%) of thieno[2,3-*b*]pyridine derivatives (50 µM) with their molecular electronic properties (energy of HOMO and LUMO, dipole moment and LogP) and the Lipinski Rule of 5 values (cLogP, molecular weight - MW, hydrogen bond acceptor and donor groups –HBA and HBD).

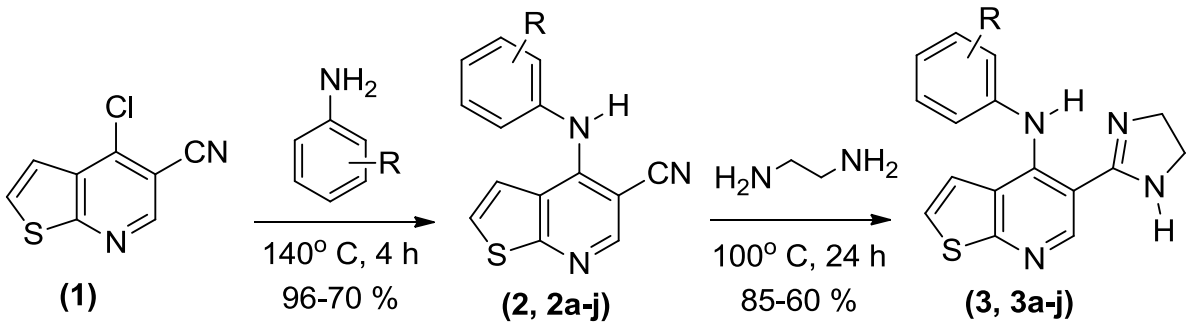
of HOMO-highest occupied molecular orbital and LUMO-lowest unoccupied molecular orbital energy) and compared them with the antileishmanial activity (Table 1). Overall, no clear or direct correlation was observed for most of the parameters evaluated except for the orbitals HOMO and LUMO that presented the lowest values for 3f and 3g. Since these orbitals may be directly involved in the interaction with the parasite target and the low values pointed these molecules reactivity as important for the highest antileishmanial activity (Table 1).

Interestingly, the analysis of the derivatives 3-D structure pointed the occupation of *para*-position and the increase of the electrostatic potential at the phenylamine ring as important for improving their antileishmanial activity (Figure 1). In fact, the individual analyses of biological activity together with the compounds conformation infer that the occupation of their *meta*-position may lead to a steric effect and the lowest antileishmanial profile (Figure 1 and Table 1).

In the effort to study the hydrophobic pattern related to the oral bioavailability, we also calculated some theoretical parameters according to Lipinski rule of 5 (Table 1). Our results revealed that the thienopyridine lipophilicity is not greater than 5 (2.44>cLogP<3.40); that, according to Lipinski, is an important feature for good drug absorption and permeation (Table 1) (Lipinski et al., 2007). The molecular volume and weight of derivatives (289.85Å^3>MV<316.96Å^3 and 294.38Da>MW<373.28Da) are similar to more than 90% of all Fluka traded drugs (MW<450Da) as well as the number of hydrogen bond acceptors (HBA= 4-7) and donors (HBD= 1), which are in accord to Lipinski "Rule of 5" (LogP ≤ 5, molecular weight ≤500Da, number of hydrogen bond acceptors ≤ 10, and donors ≤ 5) (Table 1).

Currently, the initial treatment against leishmaniasis includes pentavalent antimonials (that is, sodium stibogluconate and N-methylglucamine antimoniate forms) used since 1940 decade (Santos et al., 2008; WHO, 2011). However, other drugs are selected as a second option for treating against resistant strains despite their great toxicity to the host (such as, pentamidine, and amphotericin B). Recently, pentamidine resistance was described by the literature as well as problems on treating immunosupressed patients (HIV and organ transplanted patients) (Santos et al., 2008; Chakravarty and Sundar, 2010; WHO, 2011). In this work, we performed an *in silico* ADMET screening that calculates the theoretical pharmacokinetic profile of the derivatives compared to a database of molecules available on the market (Sander et al., 2009). In this evaluation, we compared the most active compounds of this new thieno[2,3-*b*]pyridine series with glucantime, pentamidine, miltefosine, and amphotericin B. Interestingly, the thienopyridine derivatives presented a druglikeness and drugscore close to or better than some of the antileishmanials tested, which reinforced their promising profile (Figure 4).

The comparison of some theoretical toxicity risks (tumorigenic, irritant and reproductive effects) calculated for the most active compounds using Osiris program (Sander et al., 2009) revealed a low theoretical toxicity profile in agreement to the experimental cytotoxicity results (Figure 3). It is important to notice that the toxicity predicted herein is neither a fully reliable toxicity prediction, nor guarantee that these compounds are completely free of any toxic effect. However it reinforced the promising profile of these compounds, also detected *in vitro,* for further experimental investigation.



R - **2,3** = H; **2a,3a**=*m*-CH_3; **2b,3b** = *m*-OCH_3; **2c, 3c** = *m*-NO_2; **2d, 3d** = *m*-F; **2e,3e** = *m*-Br;

2f, 3f = *p*-CH_3; **2g, 3g** = *p*-OCH_3; **2h, 3h** = *p*-NO_2, **2i,3i** = *p*-F; **2j, 3j** = *p*-Br.

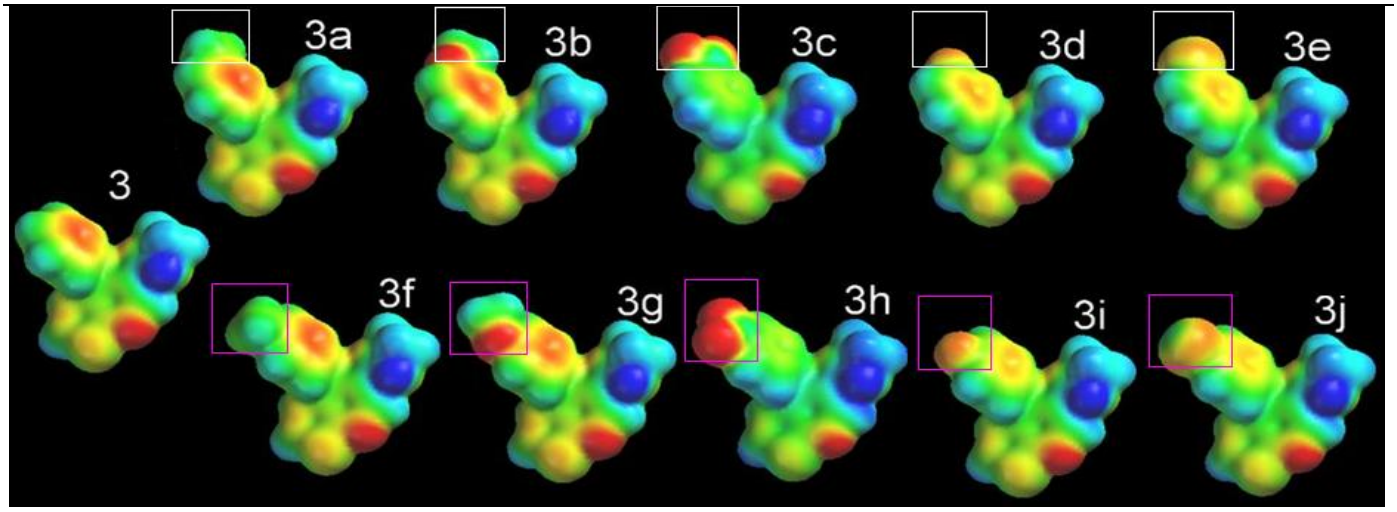


Figure 1. The 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-*b*]pyridine derivatives. Synthetic route (up) and Best conformational 3D structure according to the molecular modeling evaluation (down), which reveals the molecular electrostatic potential energy isosurface (MEP) (down) superimposed onto total electron density of 0.002 e/au3 The color code is in the range of - 25 (deepest red) to +30 (deepest blue) kcal/mol calculated as described in the material and methods. The light gray and pink squares mark the substituted *meta* and *para*-positions, respectively.

Conclusion

Overall our results pointed 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-b]pyridines as an interesting series to be further explored to find new antileishmanial compounds. 3f and 3g showed suitable antileishmanial activity against *L. amazonensis* and *in vitro* toxicity profile. Our theoretical analysis suggested that the antileishmanial activity detected in this series is correlated with the molecular electrostatic potential properties and the *para*-position on the phenyl ring of these molecules. These derivatives fulfilled the Lipinski ule of 5 and presented a drug-like profile similar or better than antileishmanial drugs. In addition, they presented a low theoretical risk profile, which suggested their potentiality for pursuing *in vivo* tests.

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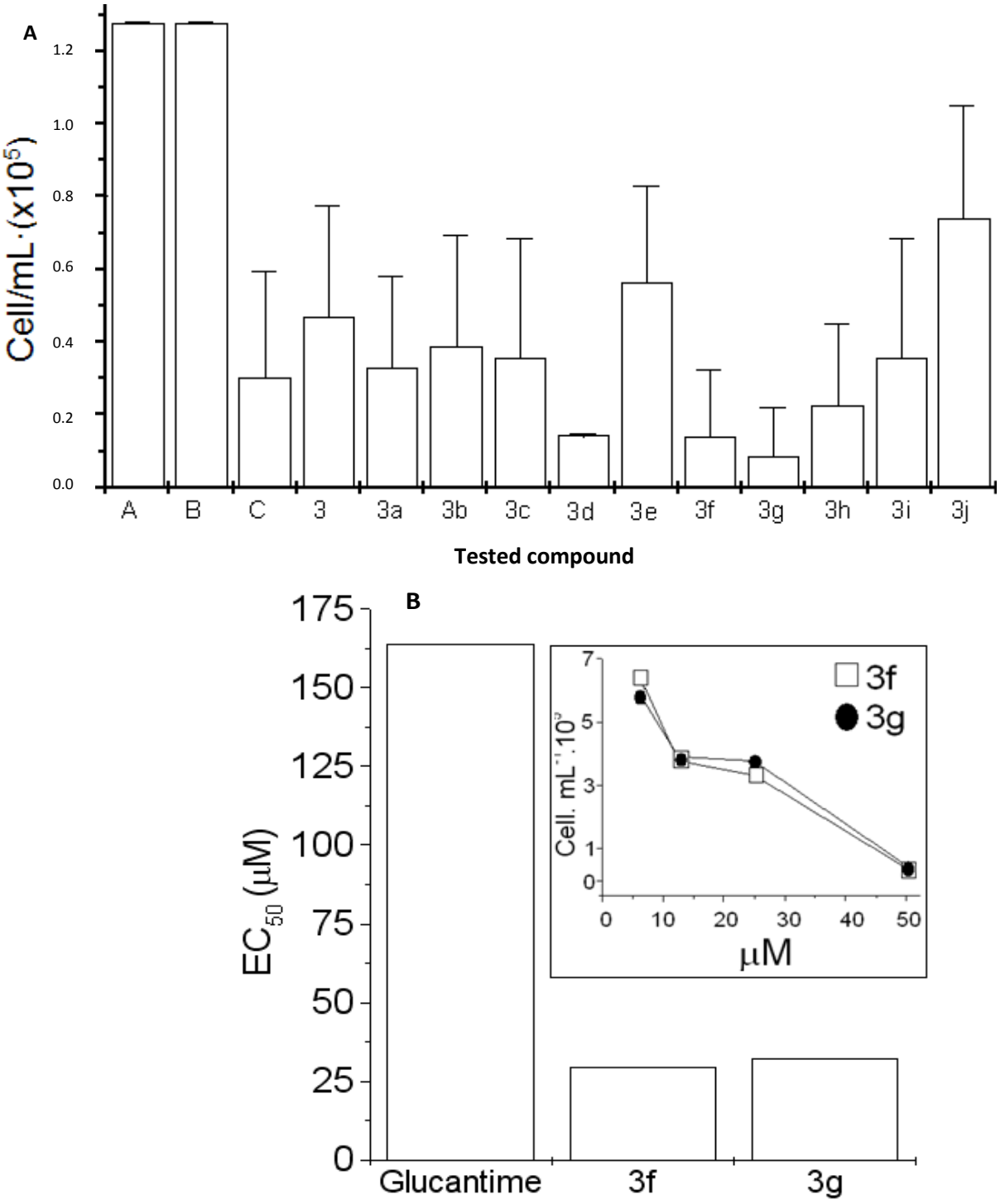

Figure 2. Comparison of the antileishmanial effects of the 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-*b*]pyridine derivatives against *Leishmania amazonensis* proliferation after 24 h of incubation. A) Antileishmanial profile at screening concentration (50 µM), A = control; B = DMSO; C = glucantime (200 µM); B) effective concentration (EC_{50}) of the most active derivatives (3f and 3g) and glucantime able to inhibit 50% of the *L. amazonensis* growth after 24 h. The inset shows the serial dilutions (6.25 to 50 µM) curves of 3f (*p*-CH3) and 3g (*p*-OCH3).

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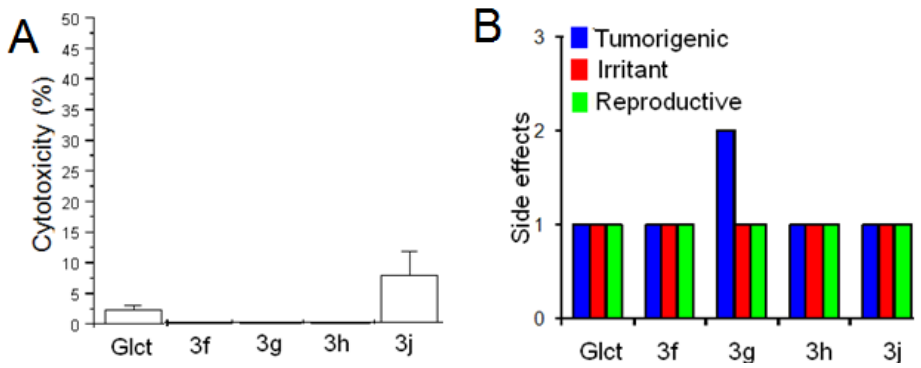

Figure 3. *In vitro* (A) and *in silico* (B) toxicity profile of the antileishmanial 5-(4,5-dihydro-1*H*-imidazol-2-yl)-4-(arylamino)thieno[2,3-*b*]pyridine derivatives. A) experimental cytotoxicity effect of the derivatives (50 µM) against monocytes derived human macrophages after 24 h of incubation; B) theoretical toxicity risks (tumorigenic, irritant and reproductive effects) calculated using Osiris program. The scale of side effects is low (0 to 1), medium (1 to 2), and high (2 to 3) toxicity profile. (Glct = glucantime).

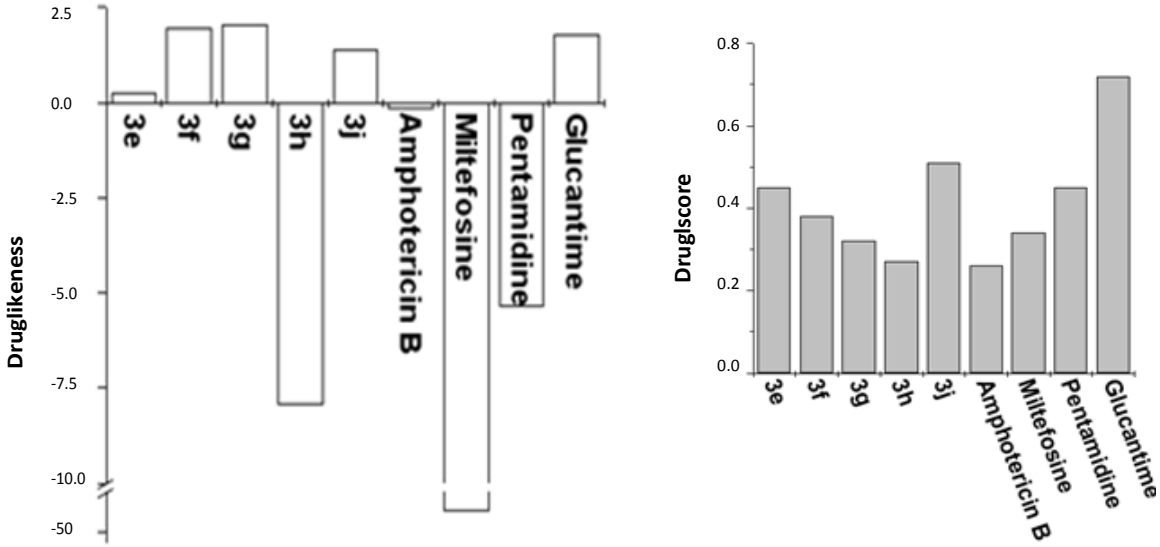

Figure 4. Comparison of the drug-like profile (druglikeness and drugscore values) of the most potent compounds (3, 3e, 3f, 3g, 3h and 3j) and of the antileishmanial agents currently in use. These parameters were calculated using Osiris program as described on the experimental section.

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