

# Activity of Benzimidazole Derivatives and their *N*-Heterocyclic Carbene Silver Complexes Against *Leishmania major* Promastigotes and Amastigotes

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**Abstract:** Little progress was conducted concerning discovering new efficient antileishmanial drugs for many years. Hence, the disease has become a global health problem meanwhile. Benzimidazole derivatives and heavy metal complexes have shown potent antiparasitic activities. The present work is intended to evaluate fourteen synthetic benzimidazolium salts and *N*-heterocyclic silver carbene complexes against *Leishmania major*. Promastigotes and amastigotes of *L. major* were cultured *in vitro* to evaluate compound-induced inhibitory effects, and isolated mouse macrophages were used for cytotoxicity evaluation. Reactive oxygen species (ROS) formation was detected for all compounds as a possible mode of action. The silver complexes **3d** and **3e** revealed significant activity against *L. major* promastigotes with IC<sub>50</sub> values of 6.4 and 5.5 µg mL<sup>-1</sup>, and SI of 1.77 and 2.02, respectively. Both complexes showed higher ROS production in promastigotes than in macrophages. Further *in vivo* and enzyme inhibition studies are recommended to evaluate the potential of these compounds as new antileishmanial.

**Keywords:** *Leishmania major*; *N*-heterocyclic carbene; silver complexes; ROS production.

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## 1. Introduction

Leishmaniasis is a serious parasitic protozoal insect-borne disease with severe morbidity and mortality and commonly occurs in tropical and subtropical countries [1]. The causative agents are flagellated protozoans from the genus *Leishmania* comprising more than twenty species [2]. Two clinical forms dominate leishmaniasis diseases. The one form is cutaneous leishmaniasis (CL), the most common form. It has appeared as a serious international health problem based on its devastating skin effects, which affect the daily life of many patients.

Furthermore, a distinct morbidity increase becomes evident because of malnutrition, infections, and chronic stress. CL has globally reached endemic proportions in about 90

countries of five continents, with more than 700,000 reported cases annually [3, 4]. Skin ulcers are major disease lesions, which often lead to marked disfigurement. More than 90% of all CL cases are reported from eight countries: Saudi Arabia, Afghanistan, Pakistan, Syria, Peru, Iran, Algeria, and Sudan [5]. The absence of a vaccine aggravated the situation [6, 7]. Visceral leishmaniasis (VL) is the second form of leishmaniasis dominant in rural areas of India, Bangladesh, Brazil, Sudan, and Nepal [8].

*L. major* and *L. tropica* are the species that are responsible for the most CL cases worldwide. The majority of cases based on the infection with *L. major* in the arid regions of Saudi Arabia and other Arabian countries can be attributed to the presence of the sand fly *Phlebotomus papatasi* in these regions [9-11]. The estimated annual incidence in Saudi Arabia is higher than 4,000, and the zoonotic form of CL has emerged due to the spread of leishmaniasis during the 20<sup>th</sup> century [10, 11]. Because of the wide distribution of desert rodents (reservoir animals) and sand flies (vector of leishmaniasis), CL is endemic in many provinces of Saudi Arabia [12-14]. A recent evaluation of the prevalence of leishmaniasis in the Qassim province in Central Saudi Arabia showed that 50% of the cases were based on infection with *L. major*, 29% with *L. tropica*, and only 4% with the minor species *L. infantum/donovani* [2].

Since the 1960s, pentavalent antimonials have been the basic treatment regime for all forms of leishmaniasis. Meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®) are commonly applied drugs. These drugs are injected intravenously or via the intramuscular route. Still, many severe side effects were observed, such as accumulation of antimony in the pancreas, serum aminotransferase elevations, and electrocardiographic disorders [15]. However, the treatment with antimonials is not sufficient anymore because of the emergence of antimony resistance [16, 17]. Amphotericin B (AmB), its desoxycholate (Fungizone®), and its liposomal formulation (AmBisome®) can be applied as second-line therapy for VL patients [18]. Amphotericin B application is recommended for patients who do not respond to antimonial therapy anymore. Still, there are considerable side effects such as pain in the bones, fever, and renal toxicity. Moreover, the high cost of amphotericin B therapy limited its uses. Therefore, there is an urgent demand to discover and introduce new drugs for the safe and efficient treatment of leishmaniasis.

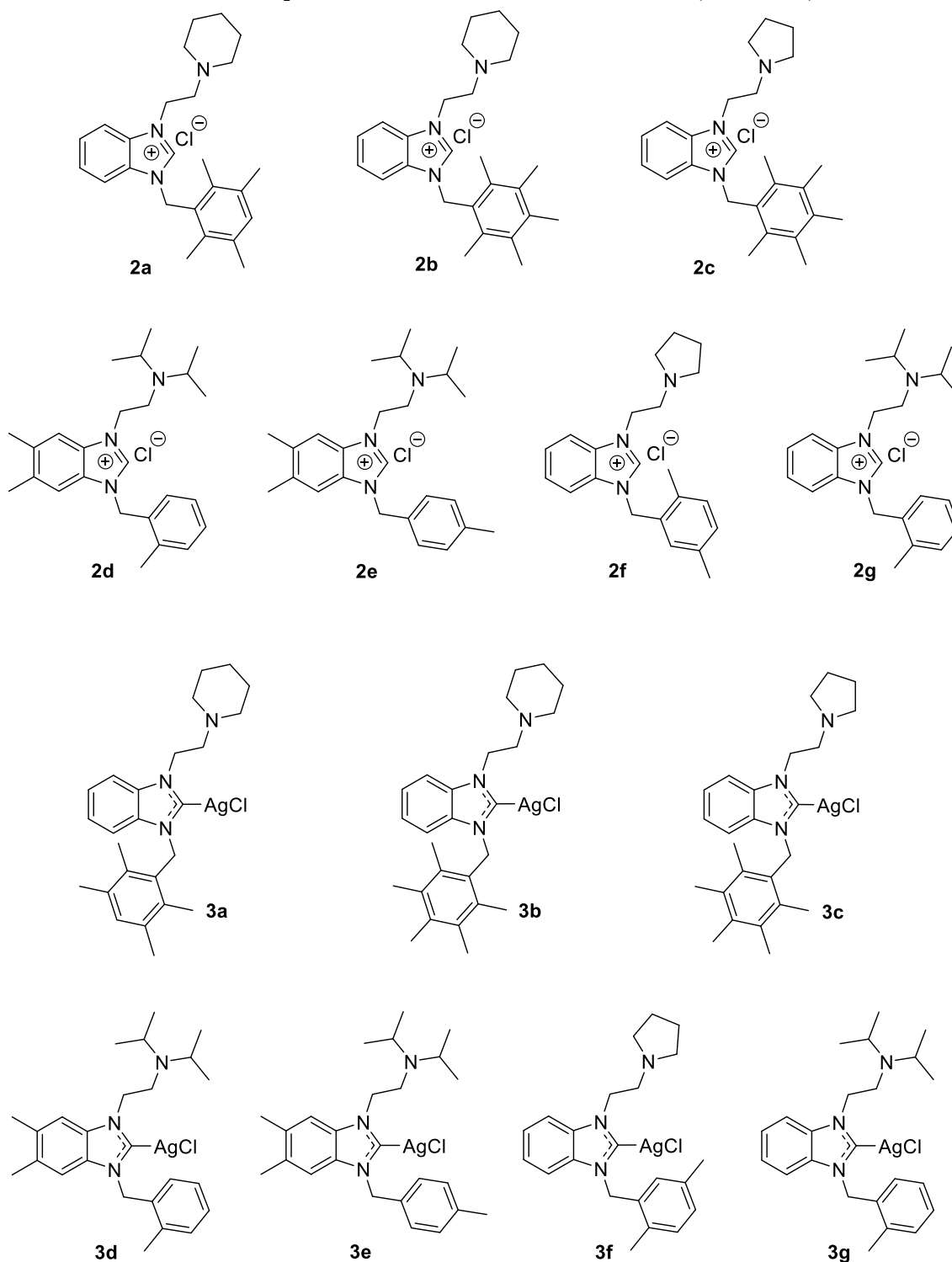
Metal-based drugs are very efficient in treating many human clinical disorders, including cancer and infectious diseases [19]. Among the transition metal NHC (*N*-heterocyclic carbene) complexes, NHC-silver complexes have been widely studied for various medicinal applications due to their simple synthesis, high air and moisture stability, and biological properties. Their properties comprise antibacterial, anticancer, anti-inflammatory, and antiseptic activities [20]. NHC-silver complexes with significant antimicrobial and anticancer properties were disclosed, which were more effective than compounds of other transition metals while showing only low toxicity for humans. Silver-NHCs were also able to overcome drug resistance and tackle antibiotic-resistant bacteria, fungi, and parasites [21, 22]. Sporadic studies of the cellular toxicity mechanisms of silver(I) compounds suggest that Ag<sup>+</sup> ions kill organisms by various mechanisms [23-25].

In the present work, 14 benzimidazolium salts and their NHC-silver complexes were investigated for their *in vitro* antileishmanial activities against *L. major* amastigotes and promastigotes. They were also evaluated for their toxicity against mice macrophages *in vitro* as well as for their ability to induce reactive oxygen species (ROS) production in *L. major* promastigotes.

## 2. Materials and Methods

### 2.1. Preparation of chemical compounds.

The synthesis of the test compounds **2a–g** and **3a–g** was published before, and the enumeration of these compounds was taken from this reference (Scheme 1) [26].



**Scheme 1.** Synthesis of benzimidazoles salts **2a–g** and Ag(I)-NHC complexes **3a–g**.

### 2.2. *L. major* parasite maintenance and bioassays.

*L. major* samples were collected from a male patient (February 2016) and cryopreserved in liquid nitrogen. The study was conducted according to the National

Committee of Bioethics guidelines and approved by the Committee of Research Ethics, Deanship of Scientific Research, Qassim University, Saudi Arabia (20-03-02/30, September 2020). The method described by Osorio *et al.* [27] was used to maintain the parasite virulence by injection of  $1 \times 10^6$  promastigotes of the stationary-phase to the hind footpads of BALB/c mice (passing maintenance). Eight weeks after inoculation, *L. major* amastigotes were collected. For the transformation of amastigotes to promastigotes, Schneider's medium supplemented with fetal bovine serum (FBS) 10% and antibiotics were used to incubate the parasites in culture flasks at 26°C. Amastigote-derived promastigotes of less than three passages were used for infection [28].

### 2.3. Activity of compounds against *L. major* promastigotes.

Logarithmic-phase promastigotes were cultured in a complete RPMI 1640 medium (Invitrogene, USA) supplied with FBS of a concentration of 10%. Parasites after dispatching were placed at concentrations of  $10^6$  cells  $\text{mL}^{-1}$  into 96-wells plates (the final volume was completed to 200  $\mu\text{L}$ /well). The test compounds were tested at different concentrations (25, 8.3, 2.9, and 0.93  $\mu\text{g mL}^{-1}$ ). Amphotericin B (reference compound) at concentrations of 25, 8.3, 2.9, and 0.93  $\mu\text{g mL}^{-1}$  was used as a positive control. Plates were incubated for 3 days at 26°C for the evaluation of the antiproliferative effects. Spectrophotometric techniques by applying the MTT (tetrazolium salt of (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay were used for assessing the number of viable promastigotes. Result data was generated with an ELISA reader (spectrophotometer) at 570 nm. Three data sets were obtained from three independent experiments. The results were expressed as  $\text{IC}_{50}$  values (inhibitory concentration killing 50% of the parasites) [28].

### 2.4. Activity of compounds against *L. major* intramacrophage amastigotes.

Drug evaluation against *L. major* intramacrophage amastigotes was carried out according to the method described previously by Calvo-Álvarez *et al.* [29]. Briefly, peritoneal macrophages were obtained as previously mentioned by Dos Santos *et al.* [30]. Then, 96-wells ELISA plates were used for harvesting  $5 \times 10^4$  cells/well with phenol red-free RPMI 1640 medium supplied by 10% FBS, and kept for 4 h at 37°C and 4%  $\text{CO}_2$ . Thereupon, a pipette was used for media discarding and washing with PBS 150  $\mu\text{L}$ . Then, *L. major* promastigotes were added to each well (at a ratio of 1 macrophage in phenol red-free RPMI 1640 medium with 10% FBS per 10 promastigotes). The plates were incubated for 24 h at 37°C in humidified 5%  $\text{CO}_2$  atmosphere to increase the rate of amastigote infection and differentiation, followed by washing with PBS three times in order to remove free promastigotes. The cells were overlaid with free phenol red RPMI 1640 medium with or without test compound, which was added at concentrations of 25, 8.3, 2.9, and 0.93  $\mu\text{g mL}^{-1}$ , and the cells were incubated at 37°C in humidified 5%  $\text{CO}_2$  atmosphere for 72 h. Amphotericin B was used as a positive control at concentrations of 25, 8.3, 2.9, and 0.93  $\mu\text{g mL}^{-1}$ . In order to evaluate the infected macrophage percentage, microscopy was used after removing the medium and washing, fixation, and staining of the cells with Giemsa dye. The assay was performed three times. The results were expressed as  $\text{IC}_{50}$  values [31].

## 2.5. Toxicological evaluation of compounds by MTT assay.

Macrophages were collected peritoneally from mice, as previously reported by Dos Santos *et al.* [30]. Complete phenol red-free RPMI 1640 medium supplied with 10% FBS was used for the cultures incubated at 37°C in 5% humidified CO<sub>2</sub>. The test compounds were added at different concentrations (25, 8.3, 2.9, and 0.93 µg mL<sup>-1</sup>) to 96-well plates containing viable macrophages at a concentration of 5×10<sup>3</sup> cells/well. After 72 h incubation, the cultures were washed with PBS, then 100 µL MTT was added to each well at a concentration of 1 mg mL<sup>-1</sup>. The cells were incubated for 4 h whereupon the supernatant was removed, and 150 µL DMSO was added to each well. A spectrophotometer was used for the colorimetric evaluation of the cells at 540 nm. DMSO (1%) without compounds was applied as a negative control. The results were visualized as the concentration of the test compound that caused 50% cell growth inhibition (CC<sub>50</sub>) [31].

## 2.6. ROS formation assay.

Reactive oxygen species (ROS) were evaluated spectrophotometrically using the nitroblue tetrazolium (NBT) dye. The slightly modified method previously described by Pramanik *et al.* was used for the test with the intracellular amastigotes [32]. After incubation of infected and non-infected macrophages with test compounds (concentrations of 8.3 and 2.9 µg mL<sup>-1</sup>) for 2 days in 96-well plates, the cells were washed with PBS. Then 100 µL of NBT (0.5 µg mL<sup>-1</sup>) was added to each well and incubated for 20 min in a CO<sub>2</sub> incubator. Thereupon, 2M KOH (120 µL) was added to each well, followed by incubation for 5 min. Then, DMSO (140 µL) was added to each well, and the plates were put in a shaker for 10 min. Finally, the optical density was read at 620 nm using a spectrophotometer. Lipopolysaccharide (LPS) of a concentration of 1 µg/mL was used as a positive control. For the evaluation of ROS in *L. major* promastigotes, the procedure of Tunc *et al.* was applied with slight modifications [33]. Promastigotes were cultured in a complete RPMI 1640 medium (Invitrogene, USA) supplied with 10% FBS. Dispatched parasites were used at a concentration of 10<sup>6</sup> cells mL<sup>-1</sup> in 96-wells plates. Test compounds were added at concentrations of 8.3 and 2.9 µg mL<sup>-1</sup>, while NBT was added at a concentration of 0.5 µg mL<sup>-1</sup>, followed by incubation at 26 °C for 24 h. 2M KOH (120 µL) was added to each well followed by incubation for 5 min. Then, DMSO (140 µL) was added to each well. After that, the plates were put in a shaker for 10 min. Finally, the optical density was read at 620 nm by a spectrophotometer. Lipopolysaccharide (LPS, 1 µg/mL) was used as a positive control.

## 2.7. Statistical analysis.

The average from three experiments was mentioned as mean ± SD (standard deviation). Mean differences were calculated with ANOVA, and significant difference levels between the groups were analyzed by LSD. Significant different level values were considered at  $p \leq 0.05$  and  $p \leq 0.001$ . SPSS 21.0 computer software was used to perform the above methods. IC<sub>50</sub> and CC<sub>50</sub> values were calculated by Boltzmann's dose-response analysis employing Sigmoidal Curve Fit, using Origin 8.1 computer software. SI (selectivity index) was calculated by dividing CC<sub>50</sub> over IC<sub>50</sub>.

### 3. Results and Discussion

#### 3.1. Antileishmanial activity.

Compounds **2a–g** and **3a–g** were initially tested for their activity against *L. major* promastigotes. Four compounds (**2d**, **2e**, **3d**, and **3e**) revealed activity at doses of 25 µg mL<sup>-1</sup> with EC<sub>50</sub> values of 25 (**2d**), 22 (**2e**), 6.4 (**3d**) and 5.5 µg mL<sup>-1</sup> (**3e**), respectively (Figure 1, Table 1). The other compounds were inactive against *L. major* promastigotes. The methyl groups of the benzimidazolium scaffold and the diisopropylamine side chain of compounds **2d** and **2e** seem to exert improved activities against promastigotes compared with the benzimidazolium systems of the other derivatives of the compound **2** series. In addition, a significant increase of the intrinsic activity of **2d** and **2e** against promastigotes was achieved by conversion of these benzimidazolium compounds into the corresponding chloride-Ag(I)-NHC complexes **3d** and **3e**.

Unlike the results from the experiments with *L. major* promastigotes, all test compounds **2a–g** and **3a–g** showed moderate activities against *L. major* amastigotes with EC<sub>50</sub> values in the range of 13.7 to 17.8 µg mL<sup>-1</sup> (Figure 2, Table 1). There was no significant activity difference between the benzimidazolium compounds **2** and the silver complexes **3**. Indeed, the benzimidazolium derivative **2a** was the most active compound against the *L. major* amastigotes. Except for the silver complexes **3d** and **3e**, all test compounds were more active against amastigotes than against promastigotes. This is an interesting discovery since effects on amastigotes are considered to be more relevant for antileishmanial drug design than effects on promastigotes [3].

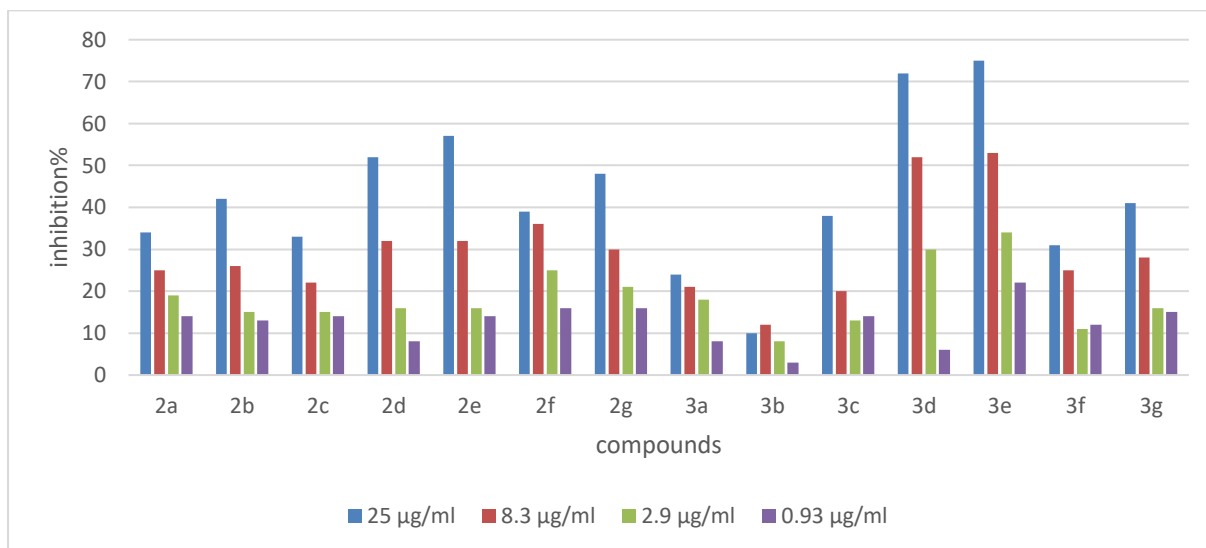
**Table 1.** *In vitro* activity of compounds **2a–g** and **3a–g** against *L. major* promastigotes and amastigotes, against macrophages, and the resulting SI values.

| Compound   | EC <sub>50</sub> against <i>L. major</i> promastigotes | EC <sub>50</sub> against <i>L. major</i> amastigotes | CC <sub>50</sub> against macrophages | SI for promastigotes | SI for amastigotes |
|------------|--------------------------------------------------------|------------------------------------------------------|--------------------------------------|----------------------|--------------------|
| <b>2a</b>  | >25                                                    | 13.7 ± 3.4                                           | 12.4 ± 2.1                           | -                    | 0.91               |
| <b>2b</b>  | >25                                                    | 14.9 ± 3.1                                           | 14.2 ± 2.4                           | -                    | 0.95               |
| <b>2c</b>  | >25                                                    | 16.1 ± 2.9                                           | 13.3 ± 2.5                           | -                    | 0.83               |
| <b>2d</b>  | 25 ± 4.2*                                              | 14.4 ± 2.7                                           | 11.1 ± 1.9                           | 0.44                 | 0.77               |
| <b>2e</b>  | 22 ± 3.9*                                              | 17.8 ± 3.5                                           | 14.1 ± 2.6                           | 0.64                 | 0.79               |
| <b>2f</b>  | >25                                                    | 16.4 ± 2.8                                           | 14.3 ± 2.4                           | -                    | 0.87               |
| <b>2g</b>  | >25                                                    | 16.2 ± 3.3                                           | 10.0 ± 1.8                           | -                    | 0.62               |
| <b>3a</b>  | >25                                                    | 15.3 ± 3.4                                           | 7.5 ± 1.8                            | -                    | 0.49               |
| <b>3b</b>  | >25                                                    | 16.8 ± 3.0                                           | 14.9 ± 2.3                           | -                    | 0.89               |
| <b>3c</b>  | >25                                                    | 17.4 ± 3.3                                           | 12.3 ± 2.0                           | -                    | 0.71               |
| <b>3d</b>  | 6.4 ± 1.1**                                            | 16.8 ± 3.6                                           | 11.3 ± 2.2                           | 1.77                 | 0.67               |
| <b>3e</b>  | 5.5 ± 0.9**                                            | 14.7 ± 2.8                                           | 11.1 ± 1.8                           | 2.02                 | 0.76               |
| <b>3f</b>  | >25                                                    | 17.2 ± 3.4                                           | 10.9 ± 1.9                           | -                    | 0.63               |
| <b>3g</b>  | >25                                                    | 14.2 ± 2.9                                           | 12.3 ± 2.2                           | -                    | 0.87               |
| <b>AmB</b> | 1.6 ± 0.3                                              | 0.93 ± 0.2                                           | 16.5 ± 2.8                           | 10.3                 | 17.7               |

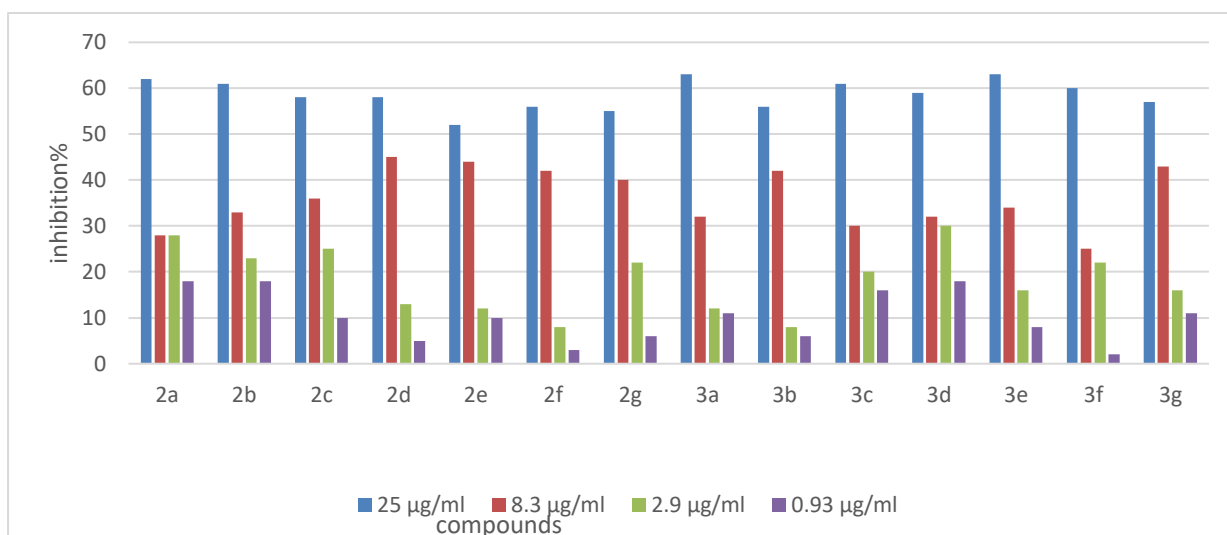
\* P < 0.05, \*\* P < 0.01, amphotericin B (AmB) was used as positive control.

#### 3.2. Cytotoxic activity against macrophages.

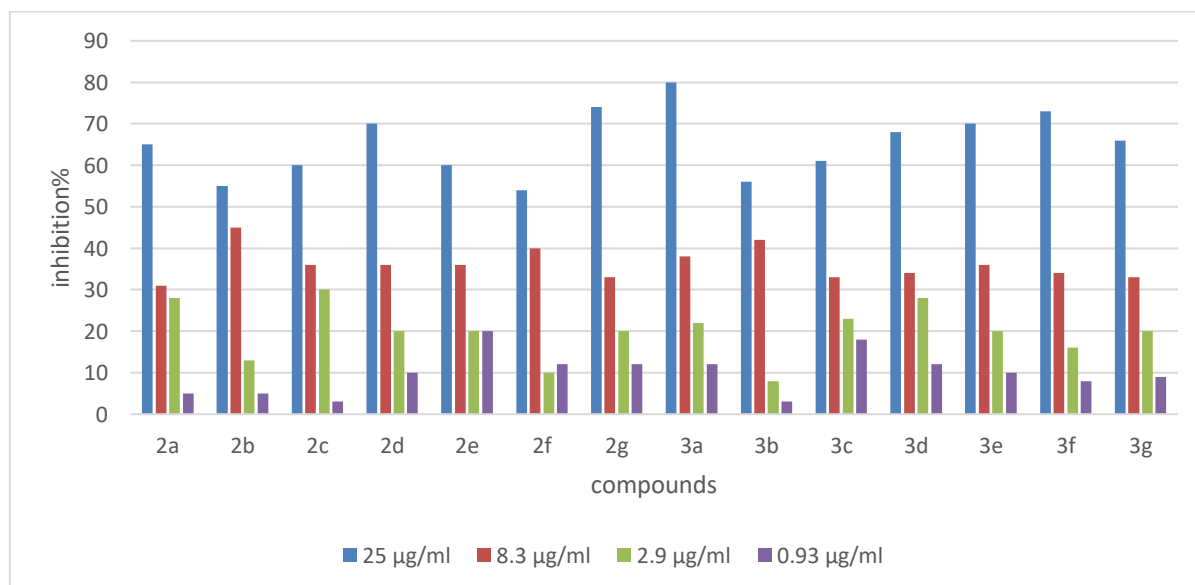
Next, all test compounds were investigated for their antiproliferative activity against macrophages to determine whether there is a reasonable selectivity of the test compounds for the parasite cells. All compounds possess a dose-dependent cytotoxic activity against the isolated macrophages (Table 1).



**Figure 1.** Activity of selected compounds against *L. major* promastigotes by using four different concentrations.



**Figure 2.** Activity of selected compounds against *L. major* amastigotes by using four different concentrations

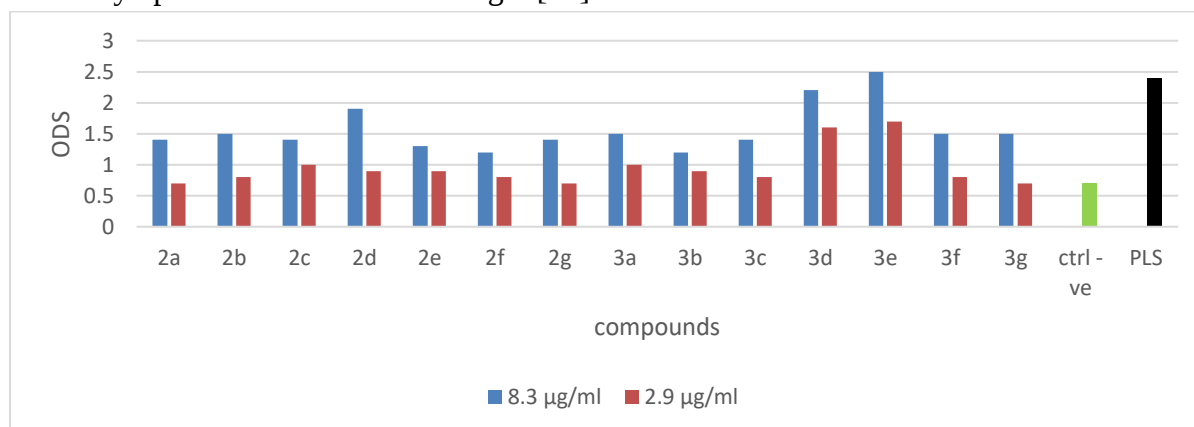


**Figure 3.** The activity of selected compounds against isolated macrophages by using four different concentrations.

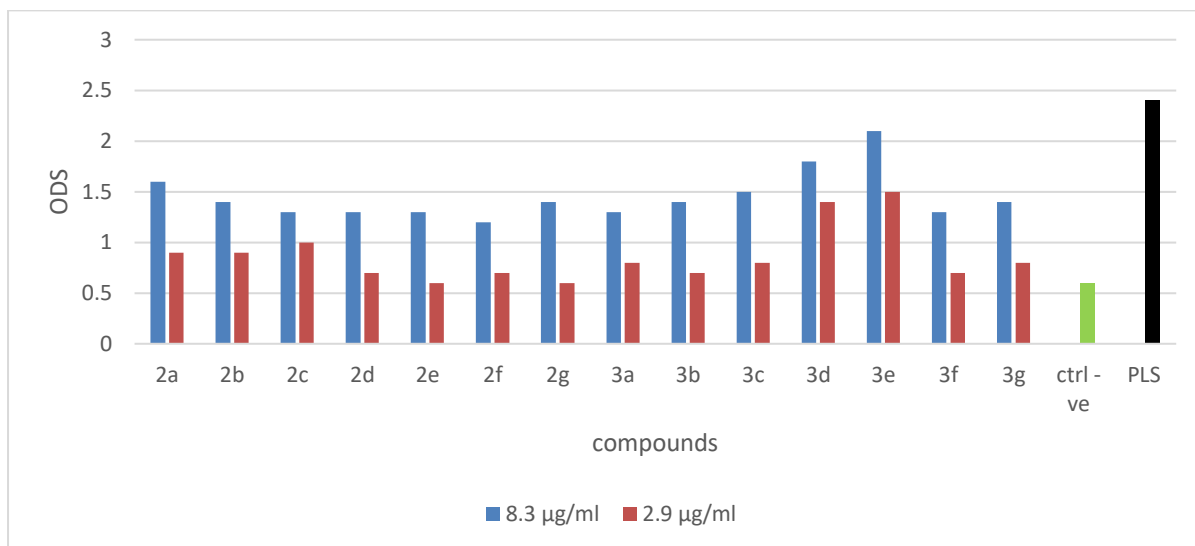
The  $CC_{50}$  values of all test compounds are in the range of 7.5 to 14.9  $\mu\text{g mL}^{-1}$ , which are in the activity range of the reference drug AmB (Figure 3, Table 1). Silver complex **3a** was the most toxic compound ( $CC_{50} = 7.5 \mu\text{g mL}^{-1}$ ), while its close silver analog **3b** was the least toxic one ( $CC_{50} = 14.9 \mu\text{g mL}^{-1}$ ). Both compounds differ only by one methyl substituent at the *N*-benzyl moiety. Thus, already slight modifications of this moiety have the potential to modulate the toxicity to macrophages distinctly. The SI values of all test compounds for the amastigotes were less than 1, indicating no selectivity here. In the case of the promastigotes, silver complexes **3d** and **3e** displayed a slight selectivity with SI values of 1.8 and 2.0, respectively (Table 1).

### 3.3. ROS production.

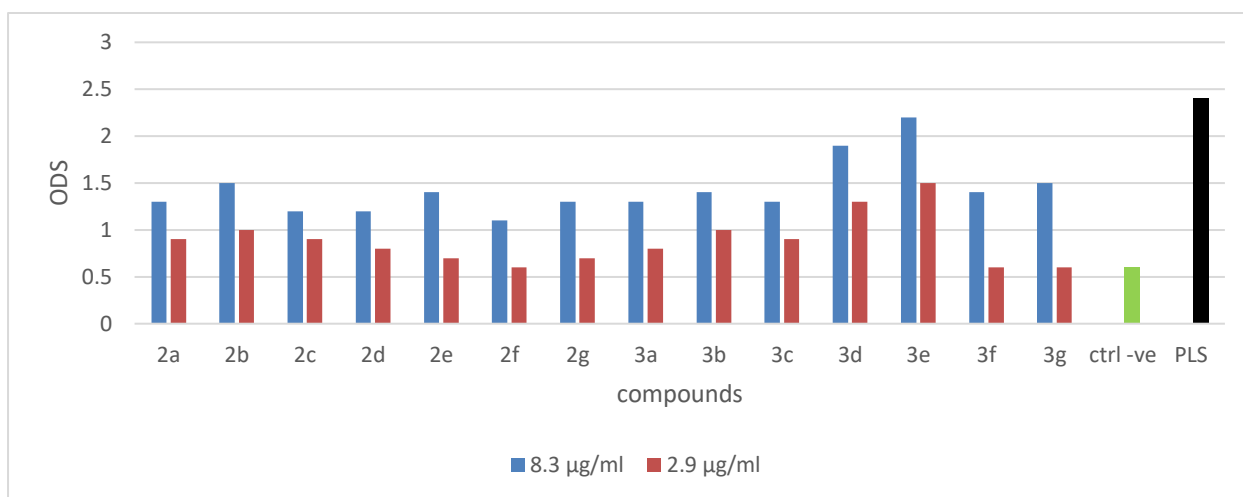
*Leishmania* parasites causing cutaneous leishmaniasis can be especially sensitive for ROS, and, thus, the design of new compounds, which induce ROS formation in parasite cells and infected macrophages, is a promising strategy to obtain new antileishmanial drug candidates [35]. Hence, compounds **2a–g** and **3a–g** were evaluated for their ability to induce ROS formation in *L. major* promastigotes and macrophages. All test compounds showed dose-dependent ROS formation. The compounds **2d**, **3d**, and **3e** exhibited significantly increased ROS production ( $p < 0.01$ ) in promastigotes at a concentration of 8.3  $\mu\text{g mL}^{-1}$ , which is in line with their inhibitory effects on promastigotes shown in Table 1 (Figure 4a). ROS formation by **3d** and **3e** reached or exceeded the ROS induction by the positive control LPS. Hence, **3d** and **3e** can be considered as potent ROS inducers in *L. major* promastigotes. In infected macrophages, only the silver complexes **3d** and **3e** showed significant ROS increase at a concentration of 8.3  $\mu\text{g mL}^{-1}$  (Figure 4b). Similar results were observed for non-infected macrophages (Figure 4c). Thus, there is no difference between infected and non-infected macrophages in terms of compound-induced ROS formation. However, ROS formation by **3d** and **3e** in macrophages was lower than in promastigotes and lower than ROS formation by LPS in macrophages. The differing ROS-inducing activities of **3d** and **3e** depending on the cell line can explain the higher inhibitory activities of both complexes against promastigotes when compared to their activities against macrophages (Table 1). A number of antileishmanial drugs showed ROS-inducing properties in promastigotes correlated with parasite cell death, and the 8-aminoquinoline derivative sitamaquine caused oxidative stress in *L. donovani* promastigotes by targeting succinate dehydrogenase [36]. In terms of silver compounds, silver nanoparticles induced ROS formation in *L. tropica* promastigotes and amastigotes, which was increased distinctly upon irradiation with UV light [37].



**Figure 4a.** ROS production in *L. major* promastigotes treated with **2a–g** and **3a–g**.



**Figure 4b.** ROS production in *L. major*-infected macrophages treated with **2a–g** and **3a–g**.



**Figure 4c.** ROS production in non-infected macrophages treated with **2a–g** and **3a–g**.

#### 4. Conclusions

The antileishmanial activities of the compounds presented in this study deserve further research efforts based on two reasons. On the one hand, the high activity of the silver carbene complexes **3d** and **3e** against promastigotes depends strictly on the silver atom and the benzimidazolylidene ligands. The distinct increase of ROS levels in promastigotes treated with **3d** and **3e** provides evidence for an efficient parasite-eliminating mechanism of these complexes. Further *in vivo* studies with compounds **3d** and **3e** as prophylaxis for *L. major* infection seem to be promising and studies concerning structure-activity relations and inhibition of parasite enzymes.

On the other hand, the general activities of the test compounds **2a–g** and **3a–g** against *L. major* amastigotes, which are independent of the presence of silver and from benzimidazolium modifications, suggest a different mode of action in these intramacrophage cells. Except for **3d** and **3e**, all compounds of the tested series showed higher activity against amastigotes than against promastigotes. Hence, based on the lead structures of the described compounds, the design of further new drug candidates with improved and selective activity against intracellular amastigotes appears to be possible in the future.

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## Conflicts of Interest

We declare no conflict of interest.

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