

Determination of Immunostimulatory Efficacy of Benzimidazole Derivatives against *Leishmania infantum*

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ABSTRACT

Leishmaniasis is a parasitic disease affecting more than 12 million people worldwide. Although the use of chemotherapeutic drugs is among the current treatment methods, it is emphasized that the limitations of the drugs, their disadvantages such as insufficient safety and efficacy, the disease being endemic, and the need to develop drugs for this disease. In this study, *in vitro* anti-leishmanial activities of two benzimidazole derivatives (B1 and B2) were tested against *L. infantum* promastigote and amastigote for the first time. In our study, the anti-leishmanial effects of B1 and B2 on both amastigote and promastigote forms of *Leishmania infantum* were investigated using J774 macrophage cells. It was found that the tested compounds were not toxic to the host cell. In addition, when compared to the drug miltefosine, which is used worldwide in the treatment of leishmaniasis, it has been determined that it has a serious inhibitory effect by reducing the proliferation of promastigotes

and the metabolic activities of amastigotes, and it acts at lower concentrations than miltefosine. We found that benzimidazolium derivatives (B1 and B2) used for the first time in this study were more effective on both forms of *L. infantum*. The obtained results showed that benzimidazolium derivatives have high anti-leishmanial potential against *L. infantum*, which is the cause of Visceral Leishmaniasis (VL), which is known to be the deadly form of leishmaniasis. It has been shown that the obtained results will help the development of safe, stable, and effective anti-leishmanial drug formulations against VL.

Keywords: Benzimidazolium, *Leishmania infantum*, Visceral leishmaniasis, Treatment, Anti-leishmanial, Macrophage cells

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INTRODUCTION

Leishmaniasis, a parasitic disease caused by protozoa of the genus *Leishmania*, still affects more than 12 million people in 98 endemic countries, according to World Health Organization (WHO) data. Among its different clinical forms, VL causes fatal systemic diseases if left untreated (Abongomera C, et al., 2020). Symptoms of VL include anemia, weight loss, hepatomegaly, fever, and splenomegaly. An estimated 300,000 new cases and more than 20,000 deaths occur each year worldwide. Most of the cases are in developing countries, weak immune systems, wars, climate changes, immigration, and most importantly, resistance to drugs are the factors that increase the incidence of the disease (Desjeux P, 2001). VL is caused by *Leishmania infantum* in Central Asia, the Middle East, the Mediterranean Basin, and *Leishmania donovani* in Asia and Africa (Tabrez S, et al., 2021).

Although there is no official vaccine for the prevention of leishmaniasis, treatment methods are generally based on the use of antineoplastic drugs. Besides the use of antineoplastic drugs, liposomal amphotericin B, pentavalent antimonials, a combination of paromomycin, pentamidine, and miltefosine, and orally administered "azoles" (ketoconazole, itraconazole, fluconazole) are the main strategies in the treatment of leishmaniasis. Current treatments limit the use of drugs because of their disadvantages such as poor and variable efficacy, high toxicity, high cost, and the emergence of drug-resistant parasites. These reasons encourage the urgent investigation and optimization of new drug alternatives with new mechanisms of action.

When the literature is examined, benzimidazolium is a heterocyclic organic compound consisting of benzene and imidazole, which can interact with a wide variety of biological targets due to an electron-rich heterocyclic system and the presence of two hetero-nitrogen atoms (Tahlan S, et al., 2019). Benzimidazole and its

derivatives are antimicrobial (Marinescu M, 2021; Aragón-Muriel A, et al., 2021; Marinescu M, 2019) antifungal (Karaburun AÇ, et al., 2019), antihistamine (Hadole DC, et al., 2018), antineoplastic (Cheong JE, et al., 2018). It is known to show different biological and pharmacological properties such as anti-inflammatory (Gaba M, et al., 2014), anti-Alzheimer's (Chaves S, et al., 2018), anti-diabetic (Naim MJ, et al., 2018; Aboul-Enein and El Rashedy, 2015).

When the literature is examined, it is seen that benzimidazole derivatives are used as an antiparasitic drug against protozoan infections by inhibiting the polymerization of tubulin, which is known to play a key role in the growth and differentiation (Valdez J, et al., 2002) of protozoa (Croft SL, 1997; Werbovetz K, et al., 1999; Jayanarayan KG and Dey CS, 2004). It is known that α , β - and γ -tubulins are essential for microtubule formation (Katiyar SK, et al., 1994; Wang YT, et al., 2019). Benzimidazoles prevent tubulin formation by binding to microtubules and disrupting their polymerization (Wang YT et al., 2019; Wu K, et al., 2022). Disruptions in the tubulin structure prevent the division of parasite cells by blocking the cell cycle in the G2/M phase, and may disrupt the cellular microtubule network by inducing apoptosis (Son DS, et al., 2020). It is seen that there are a limited number of studies in the literature investigating the antileishmanial effects of benzimidazoles against all these anti-parasitic effects. The most common parasite in research is *L. major*, and it is known to be active against *L. donovani*, *L. infantum*, and *L. mexicana*, respectively.

When the literature was examined, we could not find any study examining the anti-leishmanial activities of the benzimidazolium compounds (B1 and B2), which are the subject of our study, against *L. infantum*, which was a VL agent before. Therefore, the anti-leishmanial effects of benzimidazolium compounds B1 and B2 investigated in this study were evaluated by us *in vitro* against amastigote and promastigotes of *L. infantum*, which is a visceral

leishmaniasis agent. The antileishmanial effect and cytotoxicity of B1 and B2 components against the drug miltefosine (Dorlo TP, *et al.*, 2012), which is known to be widely used in the treatment of Leishmaniasis, were investigated comparatively.

MATERIALS AND METHODS

RPMI-1640, Fetal Bovine Serum (FBS), Dimethylsulfoxide (DMSO), Trypsin-EDTA, gentamicin, filter paper (Hoffmann), 0.45 and 0.22 μm filters were all purchased from Sigma-Aldrich. All aqueous solutions were prepared with milli-Q water. An inverted microscope Olympus CKX 41 was used to observe morphological changes.

Preparation of benzimidazolium derivatives

The benzimidazolium derivatives (B1 and B2) that were the subject of the study were commercially available in powder form with 98% purity. Benzimidazolium components were dissolved in 200 μl of methanol and benzimidazolium solutions of various concentrations were prepared using RPMI-1640 culture medium and stored at 4°C for later use (Figures 1 and 2).

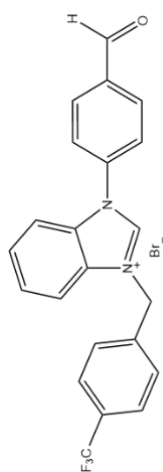


Figure 1: B1: 1-(4-Bromofenil)-4,4,4-trifloro-3-[(4-metilfenil)-imino]-1-okso2 (piridinyum-1-il) bütan-2-id

Note: ($\text{C}_{22}\text{H}_{16}\text{BrF}_3\text{N}_2\text{O}$) and molecular weight: 461.28 g/mol

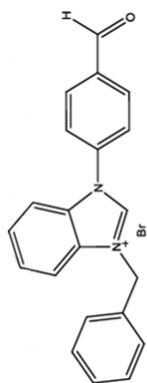


Figure 2: B2: 3-[5-(4-Bromophenyl)-2,3-dihydro-1H-pyrrolizin-6-yl]-1,3-dihydro-2H-indol-2-one

Note: ($\text{C}_{22}\text{H}_{16}\text{BrF}_3\text{N}_2\text{O}$) and molecular weight: 393.28 g/mol

Leishmania parasite and J774 macrophage cell culture

L. infantum promastigotes were cultured in flasks in RPMI-1640 medium supplemented with L-glutamine, antibiotic (Gentamycin), and 10% fetal bovine serum at 27°C. The culture was passaged after 3-4 days of incubation

by monitoring the proliferation of promastigotes daily using an inverted microscope.

J774 Murine macrophage cells to be used for cytotoxicity experiments and amastigote-macrophage culture, 1 M HEPES, 10% Fetal Bovine Serum (FBS), RPMI medium containing antibiotics (Gentamycin), 5% CO_2 in 37 °C ambient conditions were cultured in T25 culture flasks. Macrophage cells were passaged when they reached 80%-90% confluency and cell proliferation was monitored daily with an inverted microscope.

Determination of cytotoxic concentrations

To determine non-toxic concentrations of benzimidazolium components in J774 macrophage cells, 5×10^4 J774 macrophage cells and *L. infantum* parasites as hosts were seeded into each well of a 96-well microplate and incubated at 37°C for 24 hours. After adhesion of macrophages, B1 and B2 compounds, which were homogenized by dissolving in DMSO/ H_2O (10%) and sterilized by a 0.20- μm membrane filter, were obtained in 8 different concentrations (0.8 $\mu\text{g}/\text{ml}$, 1.6 $\mu\text{g}/\text{ml}$, 3.2 $\mu\text{g}/\text{ml}$, 6.4 $\mu\text{g}/\text{ml}$, 12.8 $\mu\text{g}/\text{ml}$, 25.6 $\mu\text{g}/\text{ml}$, 50 $\mu\text{g}/\text{ml}$, 100 $\mu\text{g}/\text{ml}$) were diluted and added to the wells and incubated for another 24 hours. After 48 hours of incubation, an MTT assay was performed on macrophage cells. Miltefosine was used as the reference drug in the study. 10 μl of MTT solution with a final concentration of 10 mg/ml in PBS was transferred to each well of the microplate and cells were incubated at 37°C for 4 hours. Then, 100 μl of DMSO was added to each well. Absorbance was measured at 570 nm using an ELISA reader. All studies were performed in 3 replicates. Half maximum cytotoxicity concentration (CC_{50}) was determined.

Determination of the antileishmanial effects of B1 and B2 on *L. infantum* promastigotes

To determine the antileishmanial effects of benzimidazolium derivatives (B1 and B2) on *L. infantum* promastigotes, $1 \times 10^6/\text{ml}$ *L. infantum* promastigotes in log phase were transferred to 2 ml Eppendorf tubes and incubated overnight at 27°C. *L. infantum* promastigotes taken simultaneously into a different eppendorf tube were exposed to non-toxic concentrations of B1 and B2 for 3 days. To determine the antileishmanial activity, promastigote counts were determined with the Thoma slide at certain hours (24, 48 and 96 hrs) during the incubation period of all samples tested. Thus, the antileishmanial activities of benzimidazolium derivatives on the proliferation of parasites were determined and compared.

In vitro infection of J774 macrophage cells with *L. infantum* promastigotes

To detect the antileishmanial effects of benzimidazolium components on *L. infantum* amastigotes, J774 murine macrophage cells were infected with *L. infantum* promastigotes. 5×10^4 macrophages and promastigotes were seeded into microplates at a 1:10 ratio of 200 $\mu\text{L}/\text{well}$ and incubated for 24 hours at 33°C, 5% CO_2 . The incubation temperature was increased to 37°C for 24 hours. Cells were washed 3 times with PBS to remove non-infective free promastigotes. Benzimidazole compounds ranging from 100 to 0.8 $\mu\text{g}/\text{mL}$ were diluted to form 8 different concentrations and 200 $\mu\text{L}/\text{well}$ was added to the microplates. The culture medium was incubated for 4 hours at 37°C to enable the transformation of promastigotes into intracellular amastigotes. All assays were performed in triplicate. In these assays, miltefosine, which is used in the treatment of *L. infantum*, was used as the reference drug.

Statistical analysis

The data obtained in the study were calculated as mean \pm standard deviation. For statistical analysis, parametric tests (unpaired sample t-test, analysis of variance, and Mann-Whitney U test) were used using the "SPSS 16.0 for Windows" program. The significance level was accepted as $p < 0.05$

RESULTS AND DISCUSSION

Firstly, the cytotoxic effects of benzimidazolium salts (B1 and B2), on the J774 macrophage cell line were investigated. In our study, it was determined that B1 and B2 did not show significant cytotoxicity at low concentrations (0.8 µg/mL). It has been observed that B1 and B2 have an inhibitory effect in direct proportion to increasing concentrations. However, B2 was less toxic than B1 at all tested concentrations ($p < 0.05$).

To investigate the survival percentages of *L. infantum* promastigotes, the MTT cellular viability assay was performed after 24 hours of incubation. It shows that benzimidazolium compounds are not very toxic to macrophage cells compared to leishmania parasites. The activity of compounds B1 and B2 was evaluated against *L. infantum* promastigotes. The results are shown in Table 1. It has been determined that B1 and B2 compounds are more active than Miltefosine used as a reference drug. Benzimidazolium derivatives have been found to have superior inhibitory effects on the growth of parasites. For both compounds B1 and B2, cell viability decreased significantly due to the increase in concentration 24 hours after inhibition. In addition, it has been determined that the tested benzimidazolium derivatives are less toxic than miltefosine, which is used as an antileishmanial drug. It was found to be much more effective than miltefosine (IC₅₀ value 30.12 µM+(-1.36), with an IC₅₀ value of 3.42 µM+(-1.2) for B1 and 2.23 µM+(-1.34) for B2. In the light of these data, we can say that the B2 component is less toxic than B1. B1 and B2 benzimidazolium derivatives have been found to have an inhibitory effect on the metabolic activity and proliferation of *L. infantum* promastigotes. According to the results of our study, it can be said that miltefosine (30.12 µM) exhibits very high values against *L. infantum* promastigotes compared to the IC50 values detected in

the literature (15.0 µM; 16.7 µM; 19.6 µM) (Plano D, et al., 2011; Wulsten IF, et al., 2017). Our results show us that B2 is much more effective than B1 and miltefosine on *L. infantum* amastigotes. Compared to miltefosine, B2 appears to be approximately 13.5 times more effective against *L. infantum*, while B1 is approximately 9 times more effective. The selectivity indexes (SI) of B1 and B2 are between 82 and 239. It can be said that the tested compounds showed promising antileishmanial activity against *L. infantum*.

The cytotoxic effects of B1 and B2 were tested using J774 cells. The data obtained are presented in Table 1. It has been shown to exhibit less toxicity for J774 macrophages than drugs currently used in the treatment of leishmaniasis, such as miltefosine. The CC₅₀ value for miltefosine, which was used as the reference drug, was found to be 270.12 µM. B1(280.97 µM) and B2 (534.91 µM) appeared to respond more strongly to the reference drug miltefosine. It is known that compounds B1 and B2 show a much higher Selectivity Index (SI) than miltefosine used as a reference drug. The selectivity index is obtained by the ratio of the CC₅₀ value to the IC50 value. Compounds B1 and B2 were found to have higher SI values against *L. infantum* promastigotes compared to miltefosine. It was determined that B2 was 26.77 times more selective than miltefosine, and B1 was 9.17 times more selective.

When Table 2 is examined, B1 and B2 IC₅₀ values on *L. infantum* amastigotes were observed to be 0.37 µM and 0.18 µM, respectively. Compared to miltefosine (1.04 µM), these data appear to be quite valuable. B1 and B2 were found to have SI values of 759.37 and 2971.72 against *L. infantum* amastigote. B2 was found to be 11.44 times more selective than miltefosine.

Table 1: In vitro data on antileishmanial activity and cytotoxicity of benzimidazole derivatives (B1 and B2) against *L. infantum* promastigotes on the J774 macrophage cell line

Benzimidazolium derivatives	IC ₅₀ (µM)+SD	J774 CC ₅₀ (µM)+SD	SI
B1	3.42+(-1.2)	280.97+(-0.17)	82.155
B2	2.23+(-1.34)	534.91+(-0.13)	239.86
Miltefosin	30.12+(-11.3)	270.12	8.96

Note: *IC₅₀: Concentration that provides a 50% reduction in *L. infantum* parasites; CC₅₀: Concentration with 50% reduction in J774 macrophage cells; SD: Standard Deviation from three replicates; SI: Selectivity Index, (CC₅₀/IC₅₀)

Table 2: In vitro data on antileishmanial activities of benzimidazole derivatives (B1 and B2) against *L. infantum* amastigotes

Benzimidazolium derivatives	IC ₅₀ (µM)+(-SD)	SI
B1	0,37+(-0.01)	759.37
B2	0,18+(-0.07)	2971.72
Miltefosin	1,04+(-0.06)	259.73

Note: *IC₅₀: Concentration that provides a 50% reduction in *L. infantum* parasites; CC₅₀: Concentration with 50% reduction in J774 macrophage cells; SD: Standard Deviation from three replicates; SI: Selectivity Index, (CC₅₀/IC₅₀)

It should be noted that B2 showed the highest selectivity against *L. infantum* promastigotes (239.86) and amastigotes (2971.72). Among the formulations investigated, B2 was found to be more effective against both forms of *Leishmania* parasites (amastigote-promastigote), as they significantly abolished the parasites' important biological properties, proliferation, and metabolic activities compared to B1 and miltefosine. It is possible to say that the data obtained from this study is supported by the literature. Besides, as mentioned earlier, there are very limited studies investigating the biological activities of benzimidazolium derivatives in the literature, and cytotoxic analysis of the benzimidazolium derivatives used in our study in the J774 macrophage cell line has not been performed before. In line with this information, we can clearly state that this study is the first to examine the cytotoxic behavior of benzimidazolium derivatives B1 and B2. Compound B1 at all concentrations tested showed important inhibitory effects on the metabolic activity and growth of *L. infantum* amastigotes and promastigotes compared to B2 and miltefosine. Since miltefosine shows much higher toxicity to macrophage cells than B1 and B2, B2, in particular, turned out to be quite safe for eradicating *L. infantum* parasites. Of course, when compared with the literature, it is possible to say that possible differences in IC_{50} and CC_{50} values are related to different factors such as parasite strain, growth medium, and the method used for IC_{50} and CC_{50} determination.

Although leishmaniasis is an endemic disease, affecting more than 12 million people, it is a parasitic disease that is not well known and there is no effective vaccine and treatment method.

In this study, the activities of two different benzimidazolium derivatives against *L. infantum*, which is the causative agent of visceral leishmaniasis, were tested. The results of the study revealed that benzimidazolium derivatives have a high potential to be used in the treatment of leishmaniasis and opened a new avenue for further research. It has been determined that benzimidazolium derivatives that we have examined within the scope of this study, especially B2, have significant antileishmanial activity against *L. infantum* when compared with miltefosine used in the treatment of leishmaniasis. Our results also prove the interaction between benzimidazoles and tubulin, as mentioned before.

CONCLUSION

As a result of these findings, more detailed studies are needed for potential antileishmanial therapy using benzimidazole derivatives for the treatment of visceral leishmaniasis. To examine the *in vivo* antileishmanial performance of B2, which is a benzimidazolium derivative, we suggest that experimental studies be carried out on animals as soon as possible. On the other hand, we think that B2 should be investigated in combination with traditional antileishmanial drugs and antileishmanial activities can be increased by increasing synergistic effects with combination therapy. We also hope that the promising results obtained in this study will motivate researchers to synthesize new benzimidazolium compounds. Thus, we believe that new drug formulations that can eliminate leishmaniasis-related infections in endemic areas will be developed.

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