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Quantitative Analysis of the Intracellular Efficacy of Conventional Antileishmanial Drugs Against *Leishmania tropica*: A Translational Approach Using the THP-1 Macrophage-Amastigote Model

Konvansiyonel Antileishmanial İlaçların Leishmania tropica'ya Karşı Hücre İçi Etkinliğinin Kantitatif Analizi: THP-1 Makrofaj-Amastigot Modelinde Translasyonel Bir Yaklaşım

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ABSTRACT

Objective: This study aimed to quantitatively assess the intracellular susceptibility of *Leishmania tropica (L. tropica)* to conventional antileishmanial drugs using the parasite rescue and transformation assay (PRTA), which involves controlled lysis of infected THP-1 macrophages, release of viable amastigotes, their transformation into promastigotes under defined *in vitro* conditions, and subsequent luminometric analysis.

Methods: *L. tropica* (MHOM/TR/2007/CBU001) promastigotes were cultured in Novy-MacNeal-Nicolle and Schneider's insect medium; THP-1 cells were cultured in RPMI-1640. PMA-differentiated THP-1 macrophages were infected to establish the macrophage-amastigote model. Intracellular susceptibility to five conventional drugs (amphotericin B, miltefosine, pentamidine, sodium stibogluconate, and meglumine antimoniate) was evaluated by PRTA through luminometric quantification (CellTiter-Glo®) of transformed promastigotes, with microscopic enumeration of amastigotes in Giemsa-stained slides used for validation. Inhibitory concentration 50 (IC50) values were calculated from dose-response curves and compared using Student's t-test and Pearson's correlation coefficient.

Results: Amphotericin B and pentamidine showed the highest efficacy; miltefosine demonstrated moderate activity; antimonials exhibited relatively low efficacy. IC50 values obtained by luminometric and microscopic methods were highly concordant: the paired t-test revealed no significant difference (t=0.2172, p=0.8387), and Pearson's correlation confirmed a strong positive relationship (r=0.9944, p=0.005).

Conclusion: Luminometric analysis of transformed promastigotes provides accuracy comparable to that of microscopic enumeration of intracellular amastigotes, while being more efficient in time, labor, and resources. By enabling functional assessment of parasite metabolism, supporting high-throughput and automation with low operator dependency, this standardized method represents a reliable, scalable, and translationally relevant platform with strong clinical predictive value, particularly for species-specific models such as *L. tropica*

Keywords: *Leishmania tropica*, intracellular drug susceptibility, THP-1, parasite rescue and transformation assay, translational medicine

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ÖZ

Amaç: Enfekte THP-1 makrofajlarının kontrollü lizisiyle serbest bırakılan canlı amastigotların uygun *in vitro* koşullarda promastigot forma dönüşmesine dayanan parazit kurtarma ve dönüştürme testi (PRTA) uygulanarak, dönüşen promastigotların luminometrik analizi ile konvansiyonel antileishmanial ilaçların *Leishmania tropica'ya (L. tropica)* karşı hücre içi duyarlılığının kantitatif olarak değerlendirilmesi amaçlanmıştır.

Yöntemler: *L. tropica* (MHOM/TR/2007/CBU001) promastigotları Novy-MacNeal-Nicolle ve Schneider's insect medium'da, THP-1 hücreleri ise RPMI-1640'ta kültüre edildi. PMA ile farklılaştırılan THP-1 makrofajları promastigotlarla enfekte edilerek makrofaj-amastigot modeli oluşturuldu. Hücre içi ilaç duyarlılığı, beş konvansiyonel ajan (amfoterisin B, miltefosin, pentamidin, sodyum stiboglukonat ve meglumin antimonat) için, dönüşen promastigotların (PRTA) luminometrik analizi (CellTiter-Glo®) ile kantitatif olarak belirlendi. Giemsa boyalı preparatlarda hücre içi amastigotların mikroskobik sayımı ise doğrulama yöntemi olarak kullanıldı. İnhibitör konsantrasyonu 50 (IC50) değerleri doz-yanıt eğrilerinden hesaplandı; yöntemler t-testi ve Pearson korelasyon analiziyle karşılaştırıldı.

Bulgular: Amfoterisin B ve pentamidin en yüksek etkinliği, miltefosin orta düzeyde, antimon bileşikleri ise görece düşük etkinlik göstermiştir. Luminometrik ve mikroskobik yöntemlerle elde edilen IC50 değerleri arasında yüksek düzeyde uyum saptandı; eşleştirilmiş t-testi fark olmadığını (t=0,2172, p=0,8387), Pearson analizi ise güçlü pozitif korelasyonu (r=0,9944, p=0,005) ortaya koydu.

Sonuç: Dönüşen promastigotların luminometrik analizi, hücre içi amastigotların mikroskobik sayımı ile karşılaştırılabilir doğruluk sunarken; zaman, iş gücü ve kaynak açısından daha verimli bir yöntemdir. Canlı parazitlerin metabolik aktivitesine dayalı fonksiyonel ölçüm yapabilmesi, yüksek örnekleme kapasitesi, otomasyona uyumluluğu ve düşük operatör bağımlılığı başlıca avantajlarıdır. Bu yönleriyle yöntem, standardize yapısı sayesinde *L. tropica* gibi türe özgü modellerde klinik öngörü açısından güvenilir, translasyonel araştırmalara uygun ve ölçeklenebilir bir analiz platformu olarak önerilmektedir.

Anahtar Kelimeler: *Leishmania tropica,* hücre içi ilaç duyarlılığı, THP-1, parazit kurtarma ve dönüştürme testi, translasyonel tıp

INTRODUCTION

Leishmaniasis, a zoonotic parasitic disease caused by protozoan parasites of the genus *Leishmania*, is a significant public health problem widely distributed in tropical and subtropical regions. The parasite exhibits a heteroxenous life cycle; the aflagellated amastigote form resides intracellularly within the macrophages of mammalian hosts, whereas the flagellated promastigote form develops in the digestive tract of sand fly vectors belonging to the genera *Phlebotomus* and *Lutzomyia* (1). The World Health Organization estimates that about 350 million people worldwide are at risk of infection. The clinical spectrum ranges from self-healing cutaneous lesions to the visceral form, which is fatal if left untreated (2).

Currently, there is no effective or approved vaccine against leishmaniasis; therefore, disease control relies primarily on chemotherapeutic agents. The most commonly used drugs are pentavalent antimonials, amphotericin B, miltefosine (MLT), and paromomycin (PMD) (3). However, these agents present significant limitations in clinical use due to severe systemic side effects, drug resistance, high treatment costs, and pharmacokinetic constraints. Invasive forms, such as mucocutaneous and visceral leishmaniasis, remain particularly challenging, with treatment success difficult to sustain. Thus, identifying safer, more effective, and affordable compounds, or improving current formulations, is now a major research priority (4).

The success of studies aimed at discovering new antileishmanial compounds depends on the use of highly sensitive, reliable, and translationally relevant screening methods. In this context, the promastigote-based models commonly employed in *in vitro* drug screening have limited physiological validity compared to amastigotes, the natural biological form of the parasite in mammalian hosts (5). Accordingly, intracellular amastigote-based *in vitro* models have gained prominence (5,6). However, classical methods used to apply these models, such as microscopic counting, are time-consuming, operator-dependent, and poorly reproducible. Although modern techniques using fluorescent markers, monoclonal antibodies, transgenic parasites, or colorimetric reagents have been developed, they cannot distinguish viable parasites from dead ones. Moreover, they fail to differentiate between leishmanicidal and leishmaniostatic effects. Furthermore, methods based on radioactive isotopes measure host cell activation rather than direct parasite viability and present significant operational challenges (7). In light of these limitations, there is a need for quantitative approaches capable of directly assessing viable intracellular parasite burden with improved analytical robustness. Parasite rescue and transformation assay (PRTA) has been introduced as one such strategy, based on controlled host-cell lysis, recovery of intracellular amastigotes, and subsequent transformation into promastigotes under defined *in vitro* conditions.

Because only promastigotes derived from viable amastigotes are quantified, PRTA enables selective evaluation of viable parasites. Adenosine triphosphate (ATP)-based luminometric quantification has emerged as a promising complementary approach for measuring metabolically active cells in this context (8).

The study aimed to quantitatively assess the intracellular susceptibility of *Leishmania tropica (L. tropica)*, the causative agent of cutaneous leishmaniasis (CL), to conventional antileishmanial drugs [amphotericin B (AmB), miltefosine, pentamidine (PEN), sodium stibogluconate (SSG), and meglumine antimoniate (MA)] using the PRTA, which involves transforming viable amastigotes released by controlled lysis of infected THP-1 macrophages into promastigotes under appropriate *in vitro* conditions, followed by luminometric analysis of the transformed promastigotes.

METHODS

Ethical Approval

This study was approved by the Health Sciences Ethics Committee of the Faculty of Medicine, Manisa Celal Bayar University (decision no: 20.478.486/3131, date: 07/05/2025). Patient consent was not required, as the study was performed using *L. tropica* strains and THP-1 cells obtained from a parasite bank, without direct use of human clinical specimens.

Preparation of Culture Media

Novy-MacNeal-Nicolle (NNN) medium was prepared with agar, peptone, sodium chloride, and defibrinated rabbit blood; it was supplemented with antibiotics and stabilized at 4 °C. Roswell Park Memorial Institute 1640 (RPMI-1640) (pH 7.2) was enriched with 10% fetal bovine serum (FCS) and antibiotics, stored at 4 °C, and equilibrated before use. Schneider's insect medium (SIM)

(pH 5.5) was supplemented with 20% FCS, L-glutamine, and antibiotics, sterilized by 0.22 µm filtration, and stored at 4 °C for use in experiments.

Preparation of Drug Solutions

To assess antileishmanial drug susceptibility, the reference compounds SSG (Pentostam®), MA (Glucantime®), PMD (Chemcruz®), MLT (Calbiochem®), and AmB (Boc Sciences®) were employed. Stock solutions of SSG (100 mg/mL, 100 mL) and MA (1.5 g/5 mL) were prepared in their respective formulations. PMD, MTF, and AmB were solubilized in 100% dimethyl sulfoxide (DMSO) to obtain 10 mM stock solutions. For experimental use, the required working concentrations were generated by diluting the stock solutions in RPMI-1640 medium, while ensuring that the final DMSO concentration did not exceed 0.2%.

Parasite Culture and Maintenance

The *L. tropica* strain (MHOM/TR/2007/CBU001) was isolated from a CL case in Manisa, Türkiye. Parasites were first cultured in NNN medium, then adapted to RPMI-1640 with 10% FCS, 1% gentamicin, and 1% penicillin-streptomycin, and routinely maintained in SIM (pH 5.5) supplemented with 20% FCS and antibiotics at 26 °C. Experiments used promastigotes at ~1×10^8/mL, and long-term stocks were cryopreserved in RPMI-1640 with 10% DMSO in liquid nitrogen.

Culture and Differentiation of the Human Monocytic Cell Line (THP-1)

The THP-1 monocytic cell line (Parasite Bank, Manisa Celal Bayar University, Türkiye) was maintained in RPMI-1640 medium supplemented with 10% FCS at 37 °C in 5% carbon dioxide (CO_2). Cells were subcultured every three days to keep densities below 1×10^6 cells/mL. For differentiation, THP-1 cells (2.5×10^5 cells/mL) were exposed to 100 ng/mL PMA for 24 h, then seeded into 96-well plates, and incubated under standard conditions. After PMA removal and medium replacement, cultures were maintained for 96 h to obtain differentiated macrophages, which were then prepared for cytotoxicity and intracellular drug susceptibility assays (9).

Macrophage-amastigote Infection Model

Differentiated THP-1 macrophages were detached with a sterile scraper, pelleted by centrifugation, and adjusted to 5×10^5 cells/mL. Cells were seeded into flasks, incubated for 24 h at 37 °C with 5% CO_2, and then infected with *L. tropica* promastigotes at a 1:10 ratio. After washing with sterile 1x phosphate-buffered saline (PBS) to remove free parasites, fresh RPMI-1640 was added, and cultures were maintained under the same conditions for 48 h (10).

Intracellular Efficacy of Conventional Antileishmanial Drugs

This study tested MA (500-3.9 µM), SSG (500-3.9 µM), MLT (125-0.98 µM), PEN (31.25-0.24 µM), and AmB (7.8-0.06 µM) at eight concentrations. Stock solutions in RPMI-1640 (10% FCS) were serially diluted (1:2) and dispensed into 96-well plates. *L. tropica*- infected THP-1 macrophages were washed with PBS and then treated with 100 µL medium plus 100 µL drug dilution (final volume 200 µL). Infected, untreated controls were included, and plates were incubated at 37 °C with 5% CO_2 for 48 h. The intracellular susceptibility of standard antileishmanial drugs was evaluated in an *L. tropica*- infected THP-1 macrophage model using both microscopic and luminometric methods (11,12).

Microscopic Quantification of Intracellular Amastigotes

To microscopically assess the susceptibility of intracellular *L. tropica* amastigotes to reference drugs, Giemsa staining-based infection analysis was performed. Infected cells were treated with reference drugs [MA (500-3.9 µM), SSG (500-3.9 µM), MLT (125-0.98 µM), PEN (31.25-0.24 µM), and AmB (7.8-0.06 µM)] for 48 h, fixed with chilled methanol, and stained with Giemsa. Infection was evaluated by counting 100 cells in triplicate to determine the proportion of infected macrophages, the mean number of intracellular amastigotes per cell, and the reduction in infection rate. Inhibitory concentration 50 (IC50) values were subsequently derived through linear regression analysis (11).

Luminometric Assessment of Transformed Promastigotes

To assess the intracellular susceptibility of reference antileishmanial drugs, the PRTA was applied, in which parasite viability was quantified through luminescence signals (RLU) generated by the ATP-based CellTiter-Glo® assay. Infected THP-1 macrophages were treated with reference drugs: MA (500-3.9 µM), SSG (500-3.9 µM), MLT (125-0.98 µM), PEN(31.25-0.24 µM), and AmB (7.8-0.06 µM) for 48 h; they were then washed, lysed with 0.05% SDS, and cultured in SIM (20% FCS) at 26 °C for 72 h to enable amastigote-to-promastigote transformation. Viability of transformed promastigotes was quantified using the ATP-based CellTiter-Glo® assay, with RLU reflecting metabolic activity, and IC50 values calculated from dose–response curves in GraphPad Prism (9,12).

Statistical Analysis

All experiments were performed in triplicate, and data are expressed as mean ± standard deviation. IC50 values were calculated by nonlinear regression analysis of dose-response curves using GraphPad Prism 9 (San Diego, USA). Agreement between microscopy- and luminometry-derived IC50 values was evaluated using a paired t-test, while correlation between the two methods was assessed by Pearson correlation analysis. A $p<0.05$ was considered statistically significant.

RESULTS

Inverted microscopy demonstrated the presence of *L. tropica* amastigotes within differentiated THP-1 macrophages (Figure 1A). Parasites were clearly localized within the cytoplasm of host cells, confirming their intracellular persistence and reflecting their differentiation from promastigotes to amastigotes. Upon further *in vitro* cultivation, these intracellular amastigotes transformed into motile promastigote forms, which appeared as extracellular, elongated parasites (Figure 1B). The observed transition from amastigotes to promastigotes not only confirmed parasite viability but also validated the robustness of the experimental infection model employed in this study.

The intracellular susceptibility of *L. tropica* amastigotes to five conventional antileishmanial drugs—MA, SSG, MLT, PMD, and AmB—was subsequently evaluated. Two complementary and independent quantitative approaches were employed to ensure methodological reliability. First, microscopic examination following Giemsa staining enabled direct visualization and

enumeration of intracellular amastigotes, thereby providing morphological confirmation of drug-induced parasite clearance. Second, luminometric quantification using the PRTA measured parasite viability indirectly via ATP-dependent RLU of transformed promastigotes. The use of dual methodologies enabled a more robust and reproducible assessment of intracellular drug susceptibility.

Microscopic evaluation involved direct counting of intracellular amastigotes in Giemsa-stained slides. Both microscopic and luminometric assays produced IC50 values that fell within comparable ranges for all tested compounds (Table 1 and Figure 2). Among the evaluated drugs, AmB (microscopy: 0.48±0.05 µM; luminometry: 0.42±0.03 µM) and PMD (1.62±0.17 µM; 1.58±0.15 µM) exhibited the highest potency, yielding nearly identical IC50 values across both quantification methods. MLT displayed intermediate activity (11.33±1.07 µM; 12.15±1.12 µM), while the antimonial compounds MA (56.12±4.31 µM; 52.78±3.45 µM) and SSG (31.55±2.17 µM; 35.42±2.45 µM) showed comparatively lower efficacy.

Statistical analyses further reinforced these findings. A paired t-test confirmed that the IC50 values obtained by the two quantification methods did not differ significantly (t=0.2172, p=0.8387). Additionally, Pearson correlation analysis revealed a strong positive association between microscopy and luminometry (r=0.9944, p=0.0005), underscoring the high concordance between the two approaches. Taken together, these results demonstrate that luminometric analysis via PRTA is not only consistent with conventional microscopic evaluation but also a reliable, quantitative, and high-throughput alternative for assessing the intracellular drug susceptibility of *L. tropica* amastigotes.

Table 1. *In vitro* intracellular susceptibility of *Leishmania tropica* amastigotes to reference antileishmanial drugs, expressed as IC50 values (µM ± SD). IC50 values were determined by two independent quantitative methods: luminometric analysis using the PRTA (PRTA; based on transformed promastigotes) and microscopic enumeration following Giemsa staining (intracellular amastigotes)

Reference drugs	IC50 (µM ± SD)	
	Luminometry	Microscopy
Meglumine antimoniate	52.78±3.45	56.12±4.31
Sodium stibogluconate	35.42±2.45	31.55±2.17
Miltefosine	12.15±1.12	11.33±1.07
Pentamidine	1.58±0.15	1.62±0.17
Amphotericin B	0.42±0.03	0.48±0.05

PRTA: Parasite rescue and transformation assay, SD: Standard deviation, IC50: Inhibitory concentration 50

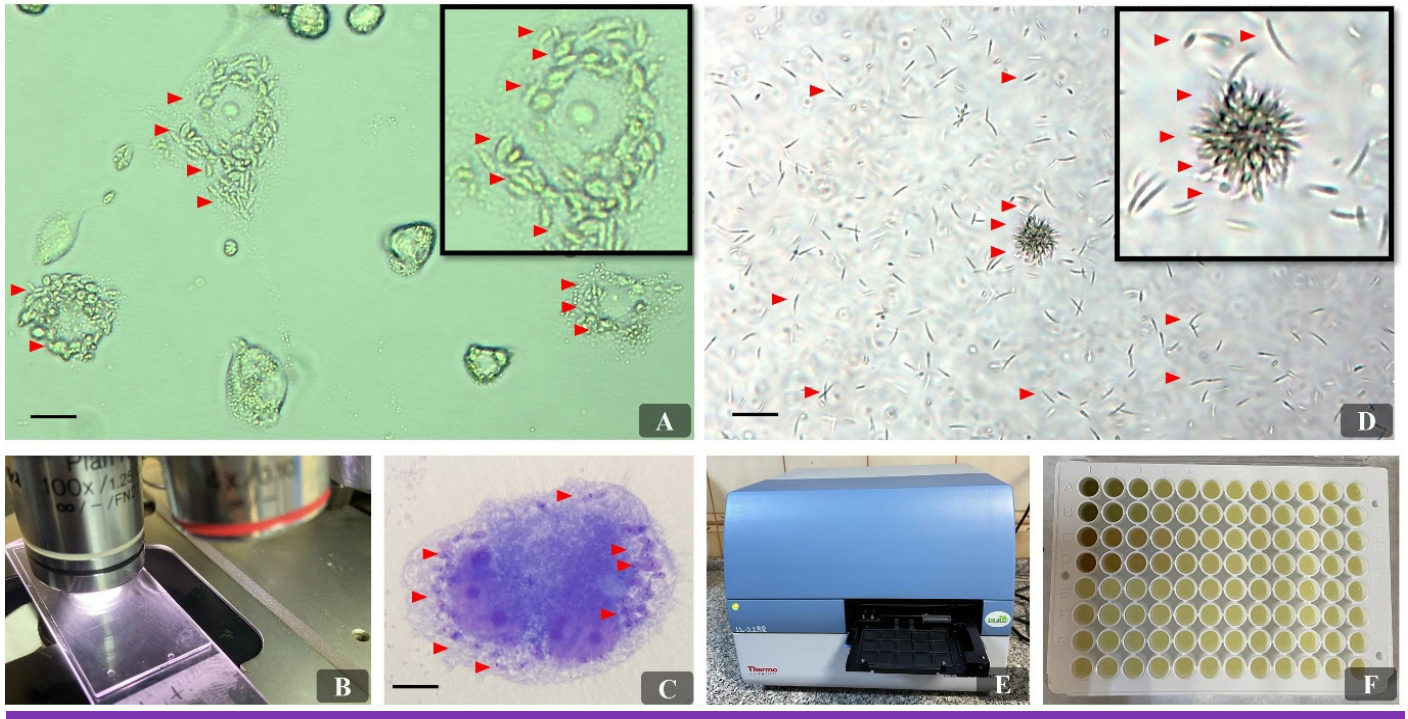

Figure 1. Representative images illustrating the *in vitro* experimental workflow of the PRTA-based intracellular susceptibility assay. (A) Intracellular *Leishmania tropica* amastigotes (red arrowheads) within differentiated THP-1 macrophages observed by inverted phase-contrast microscopy (400× magnification; scale bar=10 µm). A magnified inset is provided to enhance visualization of intracellular amastigotes under unstained live-cell conditions. (B) Slide-coverslip preparation used for microscopic examination. (C) Giemsa-stained intracellular amastigotes (red arrowheads) confirming parasite morphology at higher magnification (1000×; scale bar=5 µm). (D) Promastigotes, indicated by red arrowheads, transformed from viable intracellular amastigotes following host-cell lysis and incubation in SIM medium (200× magnification; scale bar=5 µm). A magnified inset is included to improve structural visualization despite the high density and motility of promastigotes. (E) A microplate reader used for ATP-based luminometric measurements. (F) Representative 96-well microplate layout applied in the PRTA

SIM: Schneider's insect medium, PRTA: Parasite rescue and transformation assay

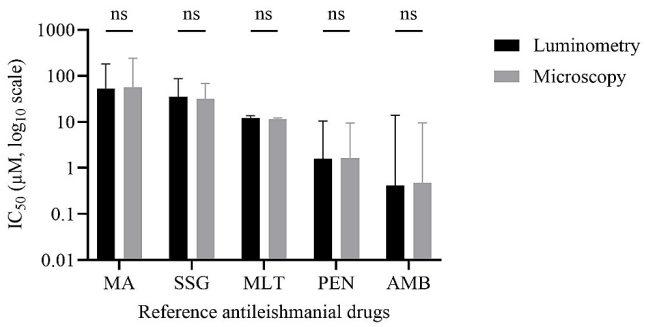

Figure 2. Comparative intracellular susceptibility of *Leishmania tropica* amastigotes to reference antileishmanial drugs, expressed as IC50 values (µM). Data were obtained using two independent methods: luminometric PRTA analysis and microscopic enumeration. Bars represent mean ± SD from independent experiments. The y-axis is presented on a logarithmic (log_{10}) scale to facilitate comparison across compounds with different activity ranges. Tested compounds included meglumine antimoniate, sodium stibogluconate, miltefosine, pentamidine, and amphotericin B. No statistically significant differences (ns) were observed between the two methods for any tested compound ($p>0.05$)

IC50: Inhibitory concentration 50, PRTA: Parasite rescue and transformation assay, SD: Standard deviation, MA: Meglumine antimoniate, SSG: Sodium stibogluconate, MLT: Miltefosine, PEN: Pentamidine, AmB: Amphotericin B

DISCUSSION

CL, caused by various species of the genus *Leishmania*, remains a significant public health challenge in tropical and subtropical regions. Despite the long-standing use of conventional antileishmanial agents, increasing reports of therapeutic failure and drug resistance in recent decades have considerably limited the efficacy of current treatment strategies. Field studies conducted in endemic areas such as India, Sudan, and the Middle East have documented resistance rates to pentavalent antimonials as high as 30-60%, underscoring the growing clinical concern regarding the sustainability of these compounds as first-line therapies (13). This situation has raised substantial concerns regarding the safety and efficacy profiles of many drugs, particularly pentavalent antimonial compounds. Moreover, most of the currently available therapeutic agents present major drawbacks, including the requirement for parenteral administration, the risk of systemic toxicity, and the need for prolonged treatment regimens (14,15). It has been consistently emphasized in the literature that treatment failures cannot be solely attributed to parasite resistance; host immune responses, pharmacokinetic/pharmacodynamic (PK/PD) parameters, drug penetration into cutaneous lesions, and field-related implementation factors also play critical roles in determining therapeutic outcomes (15,16). The increasing prevalence of drug resistance has called into question the efficacy of current treatment regimens. Still, it has underscored the urgent need for novel antileishmanial agents that are more effective, safer, and more accessible. The successful advancement of novel therapeutic approaches against CL depends on the establishment of reliable intracellular drug screening assays. Such assays should accurately assess intracellular drug activity, reflect parasite viability and functional status, and provide clinically meaningful predictive value. Traditional methodologies are often limited in accuracy, fail to adequately reflect clinical reality, and exhibit low throughput, thereby restricting their utility in drug development pipelines. Here, the term "translational" refers to the capacity of *in vitro* IC50 measurements to demonstrate at least a partial correlation with clinical treatment responses. However, the strength of this bridge must also be supported by PK/PD parameters, host immune responses, and field-related considerations.

A variety of *in vitro assay* systems, based on the parasite's distinct life-cycle stages, namely promastigotes and amastigotes, have been developed to evaluate the susceptibility of *Leishmania* species to conventional therapeutic agents and to newly developed compounds. Assays employing the promastigote stage are frequently used because of their practical advantages, including simple culture conditions, rapid results, and the relative ease of measurement. Nevertheless, because the promastigote represents the extracellular vector stage rather than the intracellular mammalian stage, such assays exhibit limited capacity to predict clinical treatment responses (17). As highlighted by Croft (2001), only models based on the amastigote-macrophage interaction are capable of establishing a biologically meaningful correlation between *in vitro* susceptibility data and the clinical course of the disease (13). Subsequent studies have corroborated this observation, demonstrating that IC50 values derived exclusively from amastigote-based systems exhibit greater concordance with clinical efficacy (18,19). Within the mammalian host, the intracellular amastigote form represents the pathogenic stage of the parasite, directly interacting with the immune system. Consequently, amastigote-based, macrophage-centered assay systems provide higher translational accuracy and enhanced predictive power for PD evaluations and clinical outcomes (13).

Among the *in vitro* models developed for antileishmanial drug screening, one of the most prominent is the macrophage-amastigote infection system. Primary macrophages may be derived from several sources, including peritoneal exudate cells, peripheral blood mononuclear cells, and bone marrow-derived macrophages. Alternatively, continuously proliferating cell lines are widely used, including murine lines such as J774 and RAW264.7 and human-derived lines such as THP-1, U937, and HL-60 (17,20,21). In experiments employing proliferative host cells, it must be acknowledged that the tested compounds may exert simultaneous effects on host cells and parasites, necessitating cautious interpretation of the results in light of these potential interactions. Primary macrophages are generally non-dividing, terminally differentiated cells, a feature that minimizes artifacts arising from cell proliferation during drug efficacy assessments. Nevertheless, such cells are influenced by the biological variability of donor animals and are often heterogeneous, which can compromise experimental reproducibility and data reliability. By contrast, macrophages derived from monocytic cell lines display homogeneous phenotypic characteristics and provide a higher degree of standardization across experiments (22). In the present study, the THP-1 cell line was selected as the experimental model for *L. tropica*, the causative agent of CL in Türkiye. Its homogeneous phenotype and non-proliferative nature enable standardized and reproducible results.

Macrophage-amastigote-based drug screening systems can be implemented through a variety of methodological approaches.

The most widely employed classical strategy relies on the direct microscopic enumeration of infected host cells. However, this method is labor-intensive and time-consuming, and the lack of automation significantly limits its applicability for high-throughput analyses (23). In manual systems based on host-cell and parasite counting, reliable determination of parasite viability is challenging when staining techniques are employed, as their use may lead to inaccuracies in the calculation of PD parameters such as IC50. Consequently, results derived solely from microscopic enumeration are often criticized by reviewers for possessing "limited translational value." In flow cytometry-based systems, parasites can be detected using fluorescent dyes or monoclonal antibodies; however, these approaches also demonstrate limited sensitivity and generally allow the assessment of drug activity only over short-term exposures (e.g., 24 hours) (24). This limitation hinders monitoring of long-term treatment responses. As an alternative, reporter gene-based systems have been developed that enable the quantitative tracking of intracellular amastigote proliferation. These approaches offer significant advantages in high-throughput screening studies, particularly with respect to standardization, time efficiency, and compatibility with automation (25). Green fluorescent protein-based systems are limited by their inability to distinguish between viable and non-viable parasites. Luciferase-based systems, although highly sensitive, are constrained by high operational costs, which restrict their suitability for large-scale screening applications (5). Accordingly, ATP-based viability assays represent a highly robust and scalable platform for high-throughput applications, combining exceptional analytical sensitivity with operational simplicity. Their homogeneous, rapid, and automation-compatible format minimizes procedural variability while maximizing reproducibility across large compound libraries. These characteristics support ATP quantification as a gold-standard readout for cell viability in high-throughput screening workflows. This is particularly relevant when reliable discrimination of metabolically active cells is required (26).

In the present study, the intracellular drug susceptibility of *L. tropica* amastigotes was assessed using two independent quantitative approaches: (i) direct microscopic enumeration of amastigotes within infected macrophages following Giemsa staining, and (ii) luminometric analysis based on the PRTA, which relies on the recovery of viable intracellular amastigotes and their subsequent differentiation into promastigotes. Microscopic enumeration remains a long-established standard method that provides direct visualization and quantification of the intracellular stage of *Leishmania* spp., thereby retaining its reliability for susceptibility assessments (27). The luminometric analysis of transformed promastigotes (PRTA) was first described in 2012 by Jain et al. (12), who employed the THP-1 cell line against *L. donovani* and demonstrated the reliability of this approach. Subsequent studies applied this method to other species, including *L. major* (28) and *L. martiniquensis* (29). The method has also contributed to investigations examining the impact of genetic modifications on parasite infectivity (30) In our study, luminometric analysis provided a reliable and reproducible model for evaluating intracellular drug efficacy. Strong concordance between IC50 values obtained by both methods indicates substantial clinical predictive value. Promastigote transformation was observed on approximately day 5. This finding parallels proliferation patterns previously reported for *L. major* and *L. braziliensis* and supports species- and culture-dependent variations described in the literature (31). While microscopic enumeration provides morphological validation, luminometric analysis offers a more objective and quantitative readout. Parasite subpopulations with reduced metabolic activity, such as stress-adapted, drug-exposed, or quiescent forms, may produce lower luminescent signals despite remaining viable. Metabolically attenuated yet persistent phenotypes have been described in kinetoplastid parasites and may contribute to variability in metabolic assays (32). Importantly, the strong concordance observed between luminometric PRTA measurements of transformed promastigotes and microscopic amastigote counts demonstrates that this theoretical limitation did not significantly affect quantitative outcomes under the experimental conditions employed. This methodological reliability, in turn, supports positioning luminometric analysis of transformed promastigotes as a robust alternative to classical microscopy, with clear potential to advance translational research in leishmaniasis.

CONCLUSION

In conclusion, luminometric analysis of transformed promastigotes (PRTA method) provides not only technical advantages such as quantitative accuracy and operator independence but also a human-relevant *in vitro* model with high translational validity because it uses the human monocytic THP-1 cell line. In this study, the application of the PRTA approach against *L. tropica,* the causative agent of CL endemic in Türkiye, enabled quantitative and objective monitoring of infection dynamics. The close concordance between IC50 values obtained through PRTA and those derived from conventional microscopic enumeration further substantiated the reliability of the method. Strengths of this work include the parallel application of two independent quantitative assays, the convergence of IC50 results across methods, the use of THP-1 cells, which enhance standardization, and the demonstration of PRTA's high-throughput and operator-independent measurement capacity. Together, these strengths support PRTA's predictive potential for infection dynamics. Nonetheless, several methodological limitations should be acknowledged: the study was restricted to a single species (*L. tropica*) and a limited panel of drugs; THP-1 cells, although advantageous for standardization, may not fully recapitulate the heterogeneity of primary macrophages; and microscopic enumeration is inherently labor-intensive. Taken together, luminometric analysis of transformed promastigotes offers a robust bridge between preclinical findings and clinical outcomes in therapeutic development for *L. tropica*, providing a practical, reproducible, and scalable alternative within *Leishmania*-infected macrophage models. While the method emerges as a reliable prescreening tool supported by consistent IC50 values and a standardized framework, further validation across diverse isolates, broader drug panels, primary macrophage systems, and time-dependent analyses will be essential to strengthen its clinical predictive capacity.

*Ethics

Ethics Committee Approval: This study was approved by the Health Sciences Ethics Committee of the Faculty of Medicine, Manisa Celal Bayar University (decision no: 20.478.486/3131, date: 07/05/2025).

Informed Consent: Patient consent was not required, as the study was performed using *Leishmania tropica* strains and THP-1 cells obtained from a parasite bank, without direct use of human clinical specimens.

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Footnotes

*Authorship Contributions

Surgical and Medical Practices: A.Ö., Concept: A.Y., T.A., Design: A.Y., T.A., Data Collection or Processing: A.Y., T.A., A.Ö., Analysis or Interpretation: A.Y., T.A., A.Ö., Literature Search: A.Y., T.A., Writing: A.Y., T.A.

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