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Optimization of Topical Therapy for Leishmania major Localized Cutaneous Leishmaniasis Using a Reliable C57BL/6 Model

Hervé Lecoeur1, Pierre Buffet2, Gloria Morizot2, Sophie Goyard1, Ghislaine Guigon3, Geneviève Milon1, Thierry Lang1*  

1 Unite´ d’Immunophysiologie et Parasitisme Intracellulaire, Institut Pasteur, Paris, France, 2 Unite´ de Recherche Clinique, Institut Pasteur, Paris, France, 3 Plate-forme Santé Pubblic, Institut Pasteur, Paris, France

Abstract

Background: Because topical therapy is easy and usually painless, it is an attractive first-line option for the treatment of localized cutaneous leishmaniasis (LCL). Promising ointments are in the final stages of development. One main objective was to help optimize the treatment modalities of human LCL with WR279396, a topical formulation of aminoglycosides that was recently proven to be efficient and safe for use in humans.

Methodology/Principal Findings: C57BL/6 mice were inoculated in the ear with luciferase transgenic L. major and then treated with WR279396. The treatment period spanned lesion onset, and the evolution of clinical signs and bioluminescent parasite loads could be followed for several months without killing the mice. As judged by clinical healing and a 1.5-3 log parasite load decrease in less than 2 weeks, the 94% efficacy of 10 daily applications of WR279396 in mice was very similar to what had been previously observed in clinical trials. When WR279396 was applied with an occlusive dressing, parasitological and clinical efficacy was significantly increased and no rebound of parasite load was observed. In addition, 5 applications under occlusion were more efficient when done every other day for 10 days than daily for 5 days, showing that length of therapy is a more important determinant of treatment efficacy than the total dose topically applied.

Conclusions/Significance: Occlusion has a significant adjuvant effect on aminoglycoside ointment therapy of experimental cutaneous leishmaniasis (CL), a concept that might apply to other antileishmanial or antimicrobial ointments. Generated in a laboratory mouse-based model that closely mimics the course of LCL in humans, our results support a schedule based on discontinuous applications for a few weeks rather than several daily applications for a few days.

Introduction

Of the 350 million people exposed to the risk of Leishmania parasite inoculation and further development, 2 million each year experience the discomfort and potential complications of cutaneous leishmaniasis (CL). Many active lesions are disfiguring, and remain so when healing as inesthetic scars that expose patients to social stigma, sometimes for life [1,2]. The demand for improved CL therapy has been fueled for decades by the lack of an efficient, social stigma, sometimes for life [1,2]. The demand for improved CL therapy has been fueled for decades by the lack of an efficient, non-irritative. This new formulation was recently shown to be discontinuous applications for a few weeks rather than several daily applications for a few days.

chloride, “Leshcutan”) [10,11] or well-tolerated but not efficient enough when first tested in humans [second generation paromomycin-urea “WHO formulation”) [12,13]. WR279396, a third-generation aminoglycoside ointment that contains 15% paromomycin formulated in a hydrophilic vehicle as well as a second aminoglycoside, 0.5% gentamicin, was designed to be effective but non-irritative. This new formulation was recently shown to be efficient and safe for the treatment of L. major localized cutaneous leishmaniasis (LCL) (Ben Salah, Buffet et al.,submitted and [14]). Although very encouraging, this result is only one step toward a simple and easily applicable therapy for this neglected disease. Various parameters such as frequency and duration of application or application in the presence or absence of an occlusive dressing may markedly influence the efficacy or safety of topically applied formulations [12,13,15,16]. For example, once-a-day applications of Leshcutan are associated with less frequent and less severe local
Author Summary

When initiating the cutaneous disease named cutaneous leishmaniasis (CL), Leishmania parasites develop within the parasitophorous vacuoles of phagocytes residing in and/or recruited to the dermis, a process leading to more or less chronic dermis and epidermis-damaging inflammatory processes. Topical treatment of CL could be a mainstay in its management. Any improvements of topicals, such as new vehicles and shorter optimal contact regimes, could facilitate their use as an ambulatory treatment. Recently, WR279396, a third-generation aminoglycoside ointment, was designed with the aim to provide stability and optimal bioavailability for the molecules expected to target intracellular Leishmania. Two endpoints were expected to be reached: i) accelerated clearance of the maximal number of parasites, and ii) accelerated and stable repair processes without scars. A mouse model of CL was designed: it relies on the intradermal inoculation of luciferase-expressing Leishmania, allowing for in vivo bioluminescence imaging of the parasite load fluctuation, which can then be quantified simultaneously with the onset and resolution of clinical signs. These quantitative readout assays, deployed in real time, provide robust methods to rapidly assess efficacy of drugs/compounds i) to screen treatment modalities and ii) allow standardized comparison of different therapeutic agents.

In vivo bioluminescence imaging of the luciferase-transgenic Leishmania population size fluctuations in the ear dermis

Luciferin (D-Luciferin potassium salt, Xenogen, California), the luciferase substrate, was intra-peritonealy inoculated into mice at a concentration of 150 mg/kg 25 minutes before bioluminescence analysis. Mice were anaesthetised in a 2.5% isoflurane atmosphere (Aerane, Baxter SA, Maurepas, France) for 5 minutes and maintained in the imaging chamber for analysis. Emitted photons were collected by 1 minute acquisition with a charge couple device (CCD) camera (IVIS Imaging System 100 Series) using the high resolution (small bining) mode. Analysis was performed after defining a region of interest (ROI) that delimited the surface of the entire ear. The same ROI was applied to every animal at every time point. Total photon emission from the ventral image of each mouse ear was quantified with Living Image software (Xenogen Corporation, Alameda, California), and results are expressed in number of photons/second/ROI. The photon signal from the ear is presented as a pseudocolor image representing light intensity (red = most intense and blue = least intense) and superimposed on the grey scale reference image. Of note, the lower threshold concentration of a mixture of Ketamine (120 mg/kg) and Xylazine (4 mg kg⁻¹; Rompun 2%, Bayer, Leverkusen, Germany). Ten thousand metacyclic promastigotes per 10 μl of Dulbecco’s phosphate buffered saline (PBS) were injected in the right ear dermis. Images of ketamine-xylasine anaesthetised mice were captured each day bioluminescence analyses were performed. The clinical features of parasite-loaded ear were examined based upon three phases: 1) early, leucocyte infiltrate-positive inflammatory, processes, 2) leucocyte infiltrate-positive inflammatory processes and 3) late repair processes could be distinguished. Only one name, “lesion”, was used to designate these different processes. The “lesion” size measurement (mm²) was approximated from the picture by fit within a rectangle.

Materials and Methods

Mice

Female C57BL/6 (5 week old) and Swiss nu/nu mice were purchased from Charles River (Saint Germain-sur-l’Arbresle, France) and were housed under institutional guidelines of the A3 Animal facility at Institut Pasteur (Paris, France).

Generation of bioluminescent Leishmania major

A 1.66 kbp finitl luciferase coding region was cut from pGL3 basic (Promega, Madison WI) using NcoI/EagI and subsequently cloned into the Leishmania expression vector pF4XL.HYG (Jenabioscience, Jena, Germany) with a marker gene for selection with hygromycin B (Casyla, Toulouse, France) which was previously cut with Ncol/NotI. In this construct, 18s rRNA flanked the luciferase and HYG genes. Following linearization with SwaI, luciferase and HYG genes were integrated into the 18s rRNA locus of the nuclear DNA of Leishmania. Transfections were realized by electroporation with the following conditions: 25 μF, 1500 V, in 4 mm cuvette; 3.75 kV/cm [21]. Following electroporation, cells were incubated 24 h in media without drug and plated on semisolid media containing 100 μg/ml of hygromycin B [20].

Parasite preparation, inoculation in the ear dermis and parasite-loaded ear feature monitoring

Transgenic luciferase L. major strain NIH173 (MHOM/IR/173) amastigotes were isolated from infected Swiss nude mice. Briefly, the promastigote developmental stage was grown at 26°C in M199 media supplemented with 10% FBS, 25 μM HEPES pH 6.9, 12 mM NaHCO3, 1 mM glutamine, 1xRPMI 1640 vitamin mix, 10 μM folic acid, 100 μM adenosine, 7.6 mM hemin, 50 U/ml of penicillin and 50 μg/ml of streptomycin [21]. Infective-stage metacyclic promastigotes were isolated from stationary phase cultures (6 day old) using density gradient centrifugation, as previously described [22].

C57BL/6 mice were anaesthetised by intraperitoneal administration of a mixture of Ketamine (120 mg/kg) and Xylazine (4 mg kg⁻¹; Rompun 2%, Bayer, Leverkusen, Germany). Ten thousand metacyclic promastigotes per 10 μl of Dulbecco’s phosphate buffered saline (PBS) were injected in the right ear dermis. Images of ketamine-xylasine anaesthetised mice were captured each day bioluminescence analyses were performed. The clinical features of parasite-loaded ear were examined based upon three phases: 1) early, leucocyte infiltrate-positive inflammatory, processes, 2) leucocyte infiltrate-positive inflammatory processes and 3) late repair processes could be distinguished. Only one name, “lesion”, was used to designate these different processes. The “lesion” size measurement (mm²) was approximated from the picture by fit within a rectangle.

Topical Therapy of L. major Cutaneous Lesions
Distribution of mice within groups on the basis of median bioluminescence values

Forty to 60 animals per experiment were inoculated with transfected *L. major* and the total photon emission of each ear was quantified 11 days later. Mice were monitored and distributed in groups according to an equal median bioluminescence value (1 × 10⁶–5 × 10⁹ photons/sec/ROI) and standard deviation. Each experimental group contained 7 to 10 mice, each individually ear-tagged (the contralateral ear with respect to the inoculation site).

Topical formulation

Topical formulations were prepared at the Walter Reed Army Institute of Research (Washington DC). WR279396 consists of paromomycin sulphate (15%) plus gentamicin (0.5%) in a vehicle as previously described [23].

Topical ointment regimen design

From day eleven post-*L. major* inoculation, topical ointments were applied to parasite-loaded ears once every two days for 10 days or once everyday for 5 days. Each formulation was applied using a sterile tip directly onto the ears to form a thin layer. Control groups were treated with the vehicle used in the medication without any of the active ingredients, i.e., the paromomycin and gentamicin. The ointment was either left open without dressing or covered with an occlusive dressing. The occlusive dressing was an adhesive polyurethane membrane (Tegaderm; 3M Health Care, St Paul, USA) that keeps water but is permeable to both water vapour and oxygen. Then two independent leaflets of 3M Micropore Surgical Tape (3M Health Care) were directly applied to the Tegaderm. This tape permitted maintenance of Tegaderm and formulation in contact with the ear during the two days.

Quantification of parasites

We estimated the number of parasites present in parasite-loaded ears as previously described [24]. Ears were cut off. The dorsal ear half was separated from the cartilage-containing ventral ear half, cut into small pieces and ground in HOSMEM-II culture medium and then dispensed into 96-well plates containing semi-solid agar (Becton-Difco, Detroit, MI) supplemented with 10% sterile rabbit serum. Tissue/organ homogenates were serially diluted in HOSMEM-II culture medium and then inoculated into 96-well plates containing semi-solid agar (Becton-Difco, Detroit, MI) supplemented with 10% sterile rabbit serum and blood collected on heparin. Plates were incubated for ten days and each well was then examined and classified as positive or negative according to whether or not viable promastigotes were present. Limiting dilution analysis was then applied to the data to estimate the number of viable parasites, expressed in limiting dilution assay units (LDAU) [25]. Statistical analysis of the results was based on the maximal likelihood method [26,27].

Statistics

Lesion size or log transformed parasite loads were analyzed with a two-way analysis of variance (ANOVA). The two factors examined were the treatment (untreated, vehicle, drug, drug with occlusion) and the period of observation (treatment, post-treatment and final) in the statistical environment R. The assumption of homoscedasticity and normality were tested with the Bartlett and Kolmogorov-Smirnov test, respectively. If the interaction term was significant, pair wise comparisons using t tests were realized for each combination of factors. A probability level of p≤0.05 was accepted for the purpose of declaring statistically significant treatment effects.

Results

Design of a reliable mouse model of LCL

The first objective of this study was to design and validate standardized readout assays for assessing different drug regimens using C57BL/6 mice inoculated with *Leishmania major*. To carry out these experiments, 10⁶ luciferase-expressing *L. major* metacyclic promastigotes were inoculated intradermally into the mouse ear. Parasites produced a significant bioluminescent signal in situ allowing parasite load expansion and reduction to be monitored non-invasively. The development and outcome of parasite burden and parasite-loaded ear features were examined simultaneously over a period of 3 months. The relationship between bioluminescence and the clinical features of the ears were respectively assessed by quantifying the number of photons per second per ear and measuring the “lesion” area.

Figures 1A and 1B illustrate the real-time bioluminescent images and clinical signs displayed by the *L. major*-inoculated ear from a representative C57BL/6 mouse (untreated group). The first post-inoculation phase (days 0–11) was characterized by a sharp increase of the bioluminescent signal at the inoculation site (from 7 × 10⁶ to 4.4 × 10⁹ photons/sec/ear at day 11; Figure 1B, C). By day 7, mouse ears displayed no clinically detectable sign (Figure 1A). However, a leukocyte infiltrate-free tiny red spot (5 mm²) was observed at day 11 (Figure 1A, C). Thus, during the first stage of parasite development no significant correlation was found between the bioluminescence value at the inoculation site and the clinically detectable features. By day 22, the parasite load peaked (Figure 1B and 1C) with a median value of 1.5 × 10⁶ photons/sec/ear which was associated with the first bona fide cutaneous clinical signs (Figure 1A and 1C). The next phase of *L. major*-driven processes was characterized by a relatively sharp decrease in bioluminescence followed by healing of the ear lesion (Figure 1A, 1B and 1C). Following the complete and stable healing of this dermal lesion, no more bioluminescent signal was detected in the ear tissue (Figure 1B and 1C). We are aware that any persisting parasite load with a population size value ≤5000 per ear is not detectable using bioluminescence: thus, between days 80–96 post inoculation at the time of mouse sacrifice, mouse ears were recovered in the control and treated group. Using the LDA readout assay, these ears were monitored for the presence of persisting parasites. 40% of the ears were positive in all groups (≥500 parasites per ear) and these percentages were obtained from two independent experiments (data not shown).

In contrast, the persistent presence of a low number of parasites as measured by LDAU was noted in the inoculation site (3 positive mice out of 7) for up to 90 days post-inoculation. These measurements helped us to define the onset of the first topical ointment application (WR279396 vehicle or WR279396 ointment with or without dressing). We decided to initiate treatment at day 11 post-inoculation for three reasons. First, at this time point, a high parasite load (bioluminescence value; >1 × 10⁶ photons/sec/ear) was reproducibly measured. Secondly, these values were observed in the median part of acute-phase load, allowing for monitoring of either an increase in parasite load in the absence of any topical application or a decrease in treated groups. Thirdly, the last topical ointment application in the group of mice treated with WR279396 was coincident with the highest parasite load measured in the control group.

Efficiency of WR279396 ointment under an occlusive dressing

By day 11 post-parasite inoculation, C57BL/6 mice were distributed in different groups on the basis of equal median
bioluminescence values. WR279396 was applied topically to the \textit{L. major}-inoculated ear every two days for 10 days. Occlusive dressing was performed by covering the \textit{L. major}-loaded ears with adhesive polyurethane dressing (Tegaderm) and a surgical tape to maintain the formulation for 2 days (Figure 2). An evaluation of the effect of WR279396 with an occlusive dressing was monitored by measuring the bioluminescence and ear "lesion" area (Figures 3B and 3C). Three periods of observation have been defined i) the 10-day treatment period ii) the post-treatment period, which ends with the absence of any bioluminescence signals in the control group and iii) the late period. As controls, three groups were analysed. In the first group, ears were left untreated. In the second group, the paromomycin- and gentamicin-free vehicle was applied to \textit{L. major}-inoculated ears that remained uncovered after application. In the third group, the WR279396 vehicle was applied and ears were immediately covered with an occlusive dressing. In all control groups, parasite load as well as lesion onset development and healing were simultaneously assessed. No statistical differences in parasite loads and lesion area were observed in any period between untreated and ointment vehicle-treated groups regardless of the period under study with respect to the measurement (not shown).

Monitoring of bioluminescence values showed that topical treatment with WR279396 (without a dressing) accelerated the decrease of both the parasite load (Figure 3A) and "lesion" area (Figure 3B and 3C). Two-way ANOVA analysis indicated a significant effect of treatment \((P\text{-value}<9.2\times10^{-6})\) and period \((P\text{-value}<2.2\times10^{-5})\) on parasite load for the whole experimental group, and there was a significant interaction between treatment and period effect \((P\text{-value}<0.008)\). The parasite load (grey line; Figure 3A) decreased rapidly after the fourth application (day 18; Figure 3A). Median values of bioluminescence indicated that parasite loads in the group of mice left without dressing were significantly lower than the control group during the treatment period (Figure 3A and 3D; grey line vs blue line and box plot; \(p\text{-value}=0.0033\)). Of note, during the post-treatment period, a rebound pattern of parasite load was observed in mice treated with ointment without occlusive dressing (grey line;
Two independent leaflets of surgical tape were applied directly on the occlusive dressing. This surgical tape permitted maintenance of the dressing and kept the formulation in contact with the parasite-loaded site for two days.

The optimal application regimen

We also monitored application regimens of WR279396 to determine which one might have a superior therapeutic index against the parasite. The experimental protocol shown in figure 5 was as previously described except for a different schedule of drug ointment application. At day 11, the topical ointment was applied on parasite-loaded ears either daily for 5 days or once every two days for 10 days. A control group (no medication) was used in parallel for determining comparability and efficacy of the different topical therapy regimens. As previously described, all parasite-loaded ears exposed to five applications for 10 days were healed by day 21 (Figure 5A) without relapse by day 64. All lesions (7/7) treated with WR279396 daily for 5 days had healed at day 21 (end of the topical therapy), but 71% (5/7) and 14% (1/7) of mice relapsed at day 50 and day 60, respectively. The clinical aspect of the lesions at day 36 post-inoculation (Figure 5B) confirmed the greater efficiency of the 5 every two days application over a 10 day schedule. By two-way ANOVA, it was shown that the difference between treatments depends on the observation periods considered (significant interaction term, p-value<0.05 ; Figure 5C). Pair wise comparisons using t-tests for each combination of factors are shown in figure 5D.

The integrative analysis of parasite load evolution in 4 experiments, involving mice receiving 5 applications for 10 days either with (n = 31) or without (n = 23) an occlusive dressing, shows that 74% of mice treated without a dressing controlled parasite loads (17/23) without relapse. Of the 6 remaining mice, 2 were unresponsive, as shown by parasite load values similar to untreated mice. The other mice initially controlled parasite loads and lesion size during the treatment period, but relapsed by day 30 as shown by parasite load values similar to or higher than measured from the ears of untreated mice. In contrast, 94% of mice treated with an occlusive dressing healed (29/31) by day 30. Only 6% (2/31) of mice treated with WR279396 daily for 5 days had healed at day 21 (end of the topical therapy). The results allow us to establish the greater parasitological efficacy of the schedule using an occlusive dressing, with a trend toward a prophylactic effect on relapse after a successful course of WR279396.

Discussion

WR279396, a third-generation aminoglycoside-based ointment, was efficient on L. major-induced localized cutaneous lesions (LCL) in C57BL/6 mice. Five applications for 10 days under occlusion induced a 94% healing rate by day 30, without re-expansion of parasite loads. This high cure rate, as well as the general evolution profile in both treated and control mice, is strongly reminiscent of...
what has been observed in clinical trials (Ben Salah, Buffet et al.-submitted and [14]), providing a strong validation of this new model for drug-testing purposes.

The adjuvant use of an occlusive dressing significantly enhanced control of parasite loads. Several non-mutually exclusive mechanisms may account for these effects. First, the dressing prevented removal of the ointment from the lesion by protecting the skin from scratching, rubbing and scraping. These latter observations have been made in patients treated without occlusion, an important proportion of the ointment being wiped off by clothes during the day, sheets during the night or even attracted to a "protective" gauze put on the top of ulcerated lesions. Second, occlusion on burns or wounds favours epidermal regeneration (ie, ulceration closure). Finally, water retention by semi-permeable occlusive dressing (like the polyurethane film used here) results in hydration of the ointment application zone [28] and likely improves the penetration and diffusion of hydrophilic antiparasitic compounds into the dermis [15], where intracellular amastigotes multiply. The aminoglycosides paromomycin and gentamicin, the active ingredients in the WR279396 ointment, are OH-rich hydrophilic compounds. Whether the dominant mechanism of the adjuvant effect is merely mechanical (enough ointment maintained on the lesion) or linked to dermal diffusion issues, the occlusive dressing enhanced the healing process induced by active...
Figure 4. Individual follow up of bioluminescence (parasite load) and “lesion” area in mice treated with WR279396 without any dressing. $10^4$ luciferase-expressing *L. major* metacyclic promastigotes were inoculated into the dermis of the right ear of C57BL/6 mice ($n=10$; day 0) treated with WR279396 (5 applications ($q$) for 10 days) at day 11 post-inoculation. The parasite load (photons/sec/ear) in individual mice (A, B) and the area (mm)$^2$ (C) of the lesion displayed by the same mice were followed for 3 months. (A, B, C) Green colour assesses the profile in mice that controlled their ear parasite load, i.e. exhibiting a bioluminescence value $<1 \times 10^6$ photons/sec/ear at day 33 (green points in panel A and green lines in panels B and C). In contrast, red colour corresponds to mouse ears that display a high bioluminescence value at day 33 ($>1 \times 10^6$ photons/sec/ear; red points in panel A and red lines in panels B and C). Note that lesion area values (C) did not assess any clinical failure except in 1 mouse (arrow). Values obtained for control mice are shown in the grey areas indicated in each graph.

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ingredients, and prevented persisting parasite loads to re-expand. Now that this adjuvant effect is established, future studies should be set up for dissecting its fine mechanisms.

Apart from a mild difference in thickness, LCL lesions in mice resemble human lesions both clinically and histologically. As opposed to systemic drug testing, topical drug testing in mice will be relevant since potential pharmacokinetic differences between mouse and human skin are expected to be minor and easily tractable to further analyses. These observations, along with the careful validation of the model, support the assumption that our results are likely to apply to human therapy. To our knowledge, occlusion has never been fully validated as an adjuvant for the topical therapy of human cutaneous lesions driven by invasive microorganisms, but several case reports have proposed this approach both in CL [29,30] and non-infectious dermatologic conditions [15]. We provide here a strong validation of a concept that might apply to other antileishmanial or antimicrobial ointments. Ointments are usually painless, their application requires no sophisticated expensive device or local anaesthesia and they can be applied easily to both children and adults by a primary care health provider with minimal training. Excluding those L. braziliensis foci where the incidence of mucosal extension can be high, ointment therapy of cutaneous lesions otherwise declared as neglected diseases should be favoured since the potential adjuvant effect of occlusion might help some ointment formulations to reach the required efficacy for development.

The duration of treatment is another important determinant of reaching a stable cure. A very short 5-day daily application

**Figure 5. Determination of the most suitable regimen for WR279396 application under an occlusive dressing.** Eleven days post-inoculation of $10^4$ luciferase-transfected L. major parasites, three groups of 7 mice were constituted: In the control group, mouse ears were left without any ointment (blue plain line), and in the other group WR279396 was applied on the mouse ear under an occlusive dressing with different application frequencies; either 5 applications/10 days (brown plain line) or 5 applications/5 days (brown dotted line). Bioluminescence evaluation of parasite load was performed over the course of 64 days post-inoculation (A). Note that the rebound of the parasite load in the every day-application group can be associated to a more severe lesion as illustrated by a representative mouse at day 36 post-inoculation (B/5 days). White squares delimit the "lesion" area. (C, D) Parasites loads were analysed with ANOVA whose two factors were the treatment (WR279396 10 days or 5 days) and the period of observation. Pair wise comparisons using t-tests were realized for each combination of factors.

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schedule under occlusion led to a “rebound” pattern similar to that displayed in mice treated for 10 days without occlusion. In other words, too short of an application period may lead to parasite load rebound, this latter risk being partially controlled by an occlusive dressing. The ability to perform individual mouse follow-up revealed a dichotomic pattern of parasite load evolution (‘‘sustained control’’ versus ‘‘unstable control with parasite rebound’’), pervasive over many weeks post transient topical ointment application. Interestingly, these patterns were displayed over several weeks, i.e., well beyond the treatment application period. Parasite load level at the end of applications was not a good predictor of further evolution (Figure 4). So, not only parasite killing but also some modification of parasite environment determined the long-term outcome of tissue damage and repair processes. It is then very likely that, during the treatment application period, some integrated programs are triggered that will be the dominant determinant of evolution. Those results fit well with observations in human CL, such as the low prognosis value of parasitological tests at the end of therapy or the efficacy of therapeutic schedules stopped before lesion healing [4,31]. Taken together, these experiments show that parasites must be exposed to the drug for >5 days to drive evolution toward long term sustained control of parasite loads and clinical healing. Duration of drug exposure was a stronger determinant of outcome than the total amount of drug used. Intracellular pharmacokinetics of aminoglycosides helps understand the mechanism leading to this observation. In eukaryotic cells exposed to aminoglycosides in vitro, a slow (2–4 days) lysosomal accumulation is observed, followed, when aminoglycosides are removed from the extracellular medium, by an even slower (2–5 days) release [32]. Interestingly, the lysosome is the only subcellular compartment in which aminoglycosides accumulate, an important feature of their antileishmanial efficacy [32,33]. So, provided that appropriate concentrations of aminoglycosides are reached in the dermal intercellular space, relatively discontinuous applications would probably suffice to allow intracellular killing of replicating amastigotes and long term sustained control of parasite loads. Taken together, our observations will help select the most efficient ointment application schedules for implementation, in the context of the therapy of this neglected disease, by health care providers with little resources and heavy duty. Even if relatively discontinuous applications for a few weeks should be preferred to many daily applications for a few days.

Our model offers relevant preclinical readout assays i) of the efficacy of a topical ointment delivered under occlusion or not ii) for establishing the proper regimen/schedule that allows sustained parasite load reduction and lesion healing during post-treatment period features. This luciferase-based imaging study might be useful for pre-clinical evaluation of novel formulations containing molecules that target parasite-loaded cells residing in the dermis as well as molecules that contribute to damaged skin-repair processes. Next challenges will be to screen molecules expected to act on the amastigote population that persist in the dermis or in distant sites [34] and to investigate the acquisition in real time of long-term protective immunity.

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Author Contributions

Conceived and designed the experiments: GM PB TL HL. Performed the experiments: PB TL HL SG. Analyzed the data: PB TL HL. Contributed reagents/materials/analysis tools: TL HL GM SG. Wrote the paper: GM GG PB TL HL. Statistical analysis: GG.

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