Introduction

All species of the genus *Leishmania* are obligate intracellular parasites of the monocyte-macrophage system. Leishmaniasis is a prevalent zoonotic disease in most developing countries, with clinical manifestations that depend not only on the protozoan species initiating infection but also on the general health as well as genetic potential and acute predisposition of the host defense system. In recent years, human populations of the developed world were also notably affected, probably compounded by facilitated international travel, carelessness of people, and improved climatic conditions for both the insect vector (sand-flies) and the parasites due to global warming [1]. Despite the major adverse impact of *Leishmania* parasites on human populations throughout the world and the increasingly observed partial resistance to antimonials, the first choice of drugs, only limited advances in the therapy of leishmaniasis have been achieved since the introduction of antimonials in the chemotherapy of these parasitic infections. Given the limitations of the current treatments for leishmaniasis reflected by severely toxic side effects and the annual incidence of about 2 million new cases [2], there is clearly an urgent need for the development of new drugs.

In addition to a range of beneficial pharmacological activities, resveratrol is recently reported to have potential antileishmanial activities *in vitro*. The present study was conducted to evaluate the effect of resveratrol on promastigotes and amastigotes of transgenic *Leishmania major* expressing green fluorescent protein in comparison with its direct cytotoxic effects on host cells (bone marrow-derived and J774-G8 macrophages, respectively). As assessed by FACS analysis, resveratrol showed moderate antipromastigote activity at < 35 µg/mL (153.2 µM) and promising effects at higher sample concentrations. In contrast, the green fluorescent protein signal as a measure of the intracellular parasites’ viability was reduced in a concentration-dependent manner. Resveratrol strongly inhibited NO production, but did not display direct NO-scavenging activity in sodium nitroprusside solution. Western blotting indicated that resveratrol reduced recombinant interferon-γ/LPS-induced expression of iNOS protein. Microscopic studies, MTT evaluation, and FACS analysis showed significant cytotoxic effects on host cells in a concentration-dependent manner. This finding suggests that the *in vitro* antileishmanial activity of resveratrol is due to cytotoxic effects on host cells rather than attributable to a specific antiparasitic potential.

Abbreviations

- BMMΦ: bone marrow-derived macrophages
- PI: propidium iodide
- GFP: green fluorescent protein
- FCS: fetal calf serum
- SNP: sodium nitroprusside
- rIFN: recombinant interferon

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica
most of their populations still rely on the use of plants as low-cost medication to treat infectious conditions. Advances in the research of natural plant products for the treatment of leishmaniasis have repeatedly been reviewed [3–7]. Many natural products did not progress through preclinical studies because of, e.g., low bioavailability. Another important consideration concerns the economics. The identification of an effectively antileishmanial herbal or even dietary source or natural product well tolerated in therapeutic doses that is readily available in endemic areas would therefore considerably contribute to the health care of people living at risk of leishmaniasis.

The stilbene resveratrol occurs in low amounts in various dietary and herbal sources including grapes, cranberries, blueberries, peanuts, **Veratrum grandiflorum**, Rheum rhabitcicum, and **Polygonum cuspidatum** [8]. Much interest in this secondary metabolite has been heightened by reports of a broad spectrum of health-promoting properties [8], ranging from putative cancer chemoprevention [9], beneficial effects on cardiovascular disease [10] and ischemic injury, enhanced stress resistance, extension of the lifespan of various organisms, a promising role in diabetes to anti-inflammatory effects [11]. In contrast, little is hitherto known about antimicrobial or antiparasitic activities of resveratrol. Following some documented clues against dermatophytes, parasitic fungi and viruses, the antileishmanial potential of resveratrol has been recently evaluated for the first time using **Leishmania major** as a test organism [12]. Based on microscopic examinations, resveratrol was found to exhibit pronounced activities against extracellular promastigotes and intracellular amastigotes, while revealing no adverse effects on murine macrophage J774 host cells. The mode of action, however, remains to be investigated. This and the limited number of stilbenes tested to date prompted the present study in the continuation of our work focusing on polyphenols as potential antileishmanial agents. Here, we report on the antileishmanial activity of resveratrol against promastigotes and amastigotes of transgenic **L. major** GFP in comparison with their direct cytotoxic effects on two murine macrophage cell cultures, BMMΦ and J774-G8, as their host cells. The experimental FACS-based protocol allowed the quantification of intracellular parasite survival and, in parallel, the cell viability of individual host cells [13]. In addition, cytotoxic effects on macrophage cell lines were measured by the MTT assay and concurrent microscopic examinations for comparison.

**Materials and Methods**

**Chemicals**

Amphotericin B, resveratrol, and DMSO were purchased from Sigma-Aldrich. All chemicals were free of LPS.

**Cell culture**

The murine macrophage J774-G8 cell line, obtained from Bernhard Nocht-Institut, was cultured in RPMI 1640 medium (Gibco Invitrogen) supplemented with HEPES (10 mM; Biochrom AG), Na-pyruvat (1 mM; Sigma-Aldrich Chemie), penicillin (100 U/mL; PAA Laboratories), streptomycin (100 µg/mL; PAA Laboratories), and 5 or 10% heat-inactivated FCS at 37°C in a humidified atmosphere with 5% CO₂. The culture medium is herein designated R5 or R10.

**Parasites**

**GFP transfected L. major** parasites (**L. major** GFP, strain LT 252, clone CC-1pXG-GFP), stable under the influence of geneticin [14], were kindly provided by S.M. Beverley (Department of Molecular Biology, Washington University School of Medicine, St. Louis, MO, USA). The parasites were cultured in **Leishmania** growth medium consisting of R5 and 10% MΦ-conditioned medium at 25°C in a humidified atmosphere with 5% CO₂. MΦ-conditioned medium consisted of the cell-free supernatant of 4- to 6-day-old murine MΦ cultured in RPMI 1640 supplemented with 10% FCS and hemin (0.25% v/v solution B of Hosmen II medium) [15].

**Mice**

Six- to 12-week-old female C57 BL/6 mice were supplied by Zentraler Versuchsstierzucht, Federal Institute for Risk Assessment (BfR), Berlin, Germany. Animal care and the experimental procedures were in accordance with the institutional guidelines and German law. The animal experiments were approved by the Landesamt für Gesundheit und Soziales, Berlin on April 27, 2004 under the number 01707/04.

**BMMΦ**

Mice were killed by cervical dislocation and bone marrow was obtained by flushing the femur and tibia with ice-cold Ca²⁺ and Mg²⁺-free PBS. BMMΦ were produced as described previously [12, 16]. For harvesting, the medium was replaced by cold PBS to remove most nonadherent cells and debris. BMMΦ were chilled to 4°C for 1 h, rinsed off the plastic and washed by centrifugation (200 × g, 10 min, 4°C). The viable cells (trypsin-blue exclusion) were counted using a Neubauer Chamber and kept in the dark at 4°C until use.

**In vitro infection**

BMMΦ and J774-G8 cells, respectively, were infected with parasites as described previously [12]. Briefly, macrophage cultures (1 × 10⁶ cells/mL R10) and **L. major** GFP promastigotes (8 × 10⁷ parasites/mL R10) were combined, giving an approximate macrophage/parasite ratio of 1:8. The suspension was centrifuged (250 × g, 5 min, 4°C) to facilitate cell contact and incubated at 37°C for 2–3 h. Subsequently, the cells were centrifuged (200 × g, 10 min, 4°C), and the supernatant discarded and replaced by ice-cold PBS. After washing to remove remaining extracellular parasites, the pellet was resuspended in R10 at 1 × 10⁶ BMMΦ/mL medium, transferred into 5-mL sterile polystyrene round-bottom tubes (Falcon) and incubated for 24 h at 37°C to allow internalization **L. major** parasites to transform into amastigotes. Aliquots of infected macrophages were treated with different concentrations of resveratrol for 48 h, washed carefully, centrifuged (200 × g, 10 min, 4°C), resuspended in 300 µL ice-cold PBS and stored on ice until use.

**Flow cytometric measurements of infected macrophages**

Information on the rate of infection was obtained by comparing the GFP signal with that of noninfected cells. After treatment with resveratrol, cells were resuspended in 300 µL of PBS and stored on ice to facilitate the detachment of the remaining adherent cells. To discriminate dead from living cells, PI (Sigma-Aldrich) solution was added ca. 30 s before the FACS measurement, giving a final concentration of 0.33 µg PI/tube. A total amount of
20000 events were counted using a FACSCalibur cytometer (Becton Dickinson) and FlowJo software (FlowJo v8.8.4) with the following instrument settings: forward scatter (E-1, linear mode; threshold 52), side scatter (344V, linear mode), fluorescence 1 (540 V, log mode), and fluorescence 3 (580 V, log mode).

Microscopic examination of infected macrophages
Macrophase suspensions (5 × 10^4 cells/100 µL R5) were plated on sterile 13-mm cover slips and the cells were left to rest for 2 h to allow to adhere. Medium was carefully removed, and 500 µL of L. major GFP cultures added (ratio of macrophages/parasites 1:8) and incubated for 2 h at 37°C, 5% CO2. Culture medium (R5) was aspirated, and cover slips were carefully rinsed two times with warm medium to remove extracellular parasites [17]. After 24 h, the medium was replaced by different concentrations of resveratrol and incubated for 48 h in a humidified atmosphere with 5% CO2. Finally, the incubations were washed twice in warm PBS. Infected cells as well as uninfected and untreated cells were included as controls in each experiment. Following Diff-Quik® staining, cover slips were placed on glass slides and sealed with a solution of polymer in xylene (Entellan®; Merck). Cell morphology was examined microscopically (conventional microscope: Axioskop 2; Carl Zeiss).

Assay for cytotoxicity of resveratrol against macrophages (MTT assay)
The direct effect of resveratrol on the viability of macrophages was assessed by monitoring the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] metabolism after a 48-h culture period. Noninfected macrophages (1 × 10^5 BMMΦ/well; 2.5 × 10^4 J774-G8/well) were seeded into 96-well plates and exposed to different concentrations of resveratrol. Following incubation at 37°C, MTT (20 µL/well of a 5 mg/mL stock solution) was added to each well. After 6 h, the MTT processing was stopped, cells lysed and formazan crystals solubilized by adding acidified SDS (20%; 50 µL/well) [16,18]. The relative amount of formazan/well produced by viable cells was determined spectrophotometrically at 570 nm by blanking against an appropriate control. Absorbance was measured using a Tecan Infinite M 200 Pro plate reader. The experiments were performed at least in triplicate and data are expressed as mean ± standard deviation.

Griess assay for NO production by activated J774-G8 macrophages
Cells were seeded at 5 × 10^4/mL R5 medium in 96-well microtiter plates. The medium was replaced by fresh medium containing different concentrations of resveratrol (4.4–43.8 µM). After incubation at 37°C for 48 h, the supernatants were collected as a source of secreted NO which was quantitated by determining the nitrite concentration using the Griess assay [equal volumes of A) 1% sulfanilamide, and B) 0.1% naphthylethyldiamine dihydrochloride/3% H3PO4] [19]. For this, aliquots of 100 µL of the supernatants were mixed with 100 µL of Griess reagent. After 5 min at room temperature, the absorbance was measured at 550 nm using an ELISA reader. Cells activated with rIFN-γ (100 U/mL) (Genentech) plus LPS (10 ng/mL) (Salmonella friedenau, kindly provided by O. Holst, Forschungsinstitut Borstel, Germany) at 37°C for 12 h served as a positive control, and non-treated cells were used as a negative control. The experiments were performed in duplicate and repeated three times in independent experiments. Results are given relative to positive control values, set to 100%.

In a modified procedure, cells were incubated with resveratrol for 48 h and post-treated with rIFN-γ (100 U/mL) plus LPS (10 ng/mL). After incubating for 12 h, NO levels were measured as described above. In parallel, cells were first activated with rIFN-γ (100 U/mL) plus LPS (10 ng/mL) for 5 h followed by adding resveratrol. After 24 h incubation, the NO levels relative to that of the positive control were determined (vide supra).

NO-scavenging activity
The scavenging effects of resveratrol on NO were measured using SNP as an NO donor. SNP (4.5 µM) was incubated for 3 h alone or in combination with different concentrations of resveratrol. Incubations were performed in daylight at room temperature, and nitrite levels were determined after 3 h by Griess reaction.

Western blotting
J774-G8 cells were exposed to vehicle control or resveratrol for 48 h. Supernatants were removed and cells stimulated with 10 ng/mL LPS (Salmonella friedenau) and 100 U/mL murine rIFN-γ for an additional 12 h. Untreated cells in fresh medium (R5) served as a negative control.

Cell lysates (lysis buffer: 10 mM Tris (pH 7.2; Roth), 150 mM NaCl (Merck), 1% Triton® X-100 (Merck), 1% Na-desoxycholat (Sigma); protease inhibitor mix, complete EDTA-free (Roche)) were ultrasonicated, and the homogenate was centrifuged at 9600 × g for 10 min at 4°C to remove insoluble material. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagent (Pierce) using BSA as a standard. Equal amounts of macrophase-lysate proteins (20 µg) for iNOS were separated on 10% SDS-polyacrylamide gels (precast gels,peqLab). A PageRuler Plus Prestained Protein Ladder (Fermentas, Thermo Scientific) was used as a size reference. Proteins were transferred to pure nitrocellulose membranes (Whatman Protran®). The membranes were incubated in blocking buffer, 5% milk powder (Sigma-Aldrich) in PBS (phosphate buffer saline), at room temperature for 1 h with agitation. The membranes were then incubated with rabbit iNOS polyclonal antibody (rabbit polyclonal to iNOS; Abcam, 1:200) overnight. Membranes were washed twice with PBS-T (0.05%) and incubated sequentially with blocking buffer and HRP-conjugated goat anti-rabbit IgG (H&L) at a dilution of 1:3000 (5% milk powder in PBS). Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham ECL Prime Western Blotting Detection System; GE). Expression levels of the β-actin protein were used for standardization.

Statistical analysis
Data are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed by Student’s t-test; p values < 0.05 were considered to be statistically significant.

Supporting information
The following are available as Supporting Information: figures of the antileishmanial activity of resveratrol against promastigotes (Fig. 15); cytotoxic effects of resveratrol on noninfected BMMΦ using FACS analysis (Fig. 25); changes in cell morphology of BMMΦ upon treatment with resveratrol (Diff-Quick® stain).
Results and Discussion

Leishmania protozoa require an insect vector and a vertebrate host to complete their biological cycle. Within the former, the parasites exist as extracellular, motile flagellates in the gut, while phagocytosed promastigotes transform into nonmotile amastigotes and reside in compartments of macrophages and monocytes. Massive amastigote multiplication leads to host cell disruption and release of the intracellular forms to infect newly recruited host cells. Since promastigotes are encountered in the mammalian host only for a very short time after transmission of the insect vector, it is the intracellular amastigote that is of clinical and pharmacological importance. Attention was therefore given to the antiamastigote effects of resveratrol. Having in mind that a difference in antileishmanial activity against the two stages of the parasite may well exist, promastigotes were included in this study.

Using transgenic L. major GFP, resveratrol exhibited pronounced antileishmanial effects on promastigotes but at therapeutically unacceptably high sample ranges. At 45 µg/mL (196.9 µM), the GFP signal as a measure of the parasites’ viability was significantly reduced by ca. 97% as assessed by FACS analysis (Fig. 15). Conspicuously, moderate killing rates (ca. 10%) were observed at sample concentrations ≤35 µg/mL (≤153.3 µM). This finding may be indicative of a limited tolerance of L. major GFP to resveratrol, which appears crucial when establishing a relevant dose effective against the extracellular form. Parallel staining with PI to discriminate viable from dead parasites confirmed the antileishmanial activity profile of resveratrol. Although previous work showed similar antipromastigote activities, the issue of a required minimal concentration has not been documented. The fairly high rationale dose of at least ca. 40 µg/mL (175 µM) calls for reliable data about the safety of resveratrol, which is claimed to be generally well tolerated.

Experimental infection of macrophages constitutes a particularly versatile model for assessing selective direct effects of an agent on intracellular pathogens as well as indirect antiparasitic effects via macrophage activation. In our initial work, BMMΦ experimentally infected with L. major GFP promastigotes were rested for 24 h at 37°C to allow internalized parasites to transform into amastigotes. When infected cells were exposed to resveratrol for an additional 48 h, the resulting GFP signal was reduced in a concentration-dependent manner compared to the non-treated population (IC50 = 9.9 µg/mL (43.6 µM)) (Fig. 1). Amphotericin B served as a positive control.

To gain insight into the underlying antileishmanial mode of action, the NO-inducing potential of resveratrol was evaluated. Activated macrophages are known to produce a variety of potentially cytotoxic effector molecules, of which nitric oxide and its congeners represent the most effective microbialicidal species against Leishmania parasites [20,21]. Aliquots of the culture supernatants were collected for measuring NO concentrations using the Griess assay. Consistent with previous findings, the combination of rIFN-γ plus LPS strongly induced NO production (ca. 28 µM) [13]. In contrast, the NO-inducing potential of resveratrol was relatively weak with similar levels noted for noninfected and infected macrophages (ca. 3 µM) (data not shown). This finding suggested that resveratrol did not stimulate the infected macrophages for NO release and that its antileishmanial activity was apparently mediated by a different mode of action. Before investigating alternative antiparasitic mechanisms, it appeared meaningful to focus on NO scavenging and iNOS inhibiting effects of resveratrol to rationalize the low NO levels. That resveratrol is capable of decreasing the NO level in cells has been demonstrated for a range of cell types, including macrophages [22,23], skeletal muscle [24], and cardiomyocytes [25]. However, resveratrol has also been reported to enhance NO production and to modify expression of NOS isoforms [26–28]. In a first set of experiments, NO was generated in a cell-free system using sodium nitroprusside as an NO donor and measured spectrophotometrically. Addition of resveratrol moderately decreased the produced NO level in a concentration-dependent manner to ca. 65% of the control value in test concentrations ≥30 µg/mL (data not shown), indicating that direct NO scavenging effects played a minor role in reducing NO production.

Next, J774-G8 cell cultures stimulated with rIFN-γ plus LPS were incubated with resveratrol. As shown in Fig. 2, NO production was significantly decreased by the presence of resveratrol in a concentration-dependent manner (IC50 = 5.2 µg/mL; 22.8 µM), indicating inhibition of iNOS mRNA/protein expression or enzyme activity. This finding was consistent with similar reports on a range of cell types [22–25].

In a modified procedure, J774-G8 cell cultures were pretreated with resveratrol for 48 h, followed by incubating with the known macrophage-stimulating agents rIFN-γ and LPS. Again, the release of NO was similarly reduced in a concentration-dependent manner (data not shown). Accordingly, the inhibition of NO production by resveratrol may largely be ascribed to inhibition of iNOS.

A Western blot-based protocol was subsequently employed to examine the expression of iNOS at the protein level. Results showed that J774-G8 cells treated with resveratrol exhibited a concentration-dependent decrease in the levels of iNOS when compared with the control levels (Fig. 3). This finding was consistent with...
observed reduced NO production upon resveratrol treatment (vide supra) and supported the conjecture that resveratrol inhibited NO production primarily by suppression of iNOS expression. Nonstimulated cells expressed very low levels of iNOS protein. Resveratrol is reported to be generally well tolerated, and only minor side effects have been observed in short-term studies. Based on animal experiments, toxicological effects were evident at high doses and from long-term administration [29]. Insufficient clinical studies and lacking data on the toxicity of long-term intake of resveratrol in humans reflect inadequate scientific evidence for the safety of resveratrol in medical practice. Adverse effects are one of the most critical pharmacological features. Accordingly, the clinical relevance of intracellularly persisting Leishmania amastigotes prompted special attention to the evaluation of possible cytotoxic effects of resveratrol on macrophages as host cells.

In a first set of experiments, the effect of resveratrol on the viability of BMMΦ host cells was tested using the MTT assay and related to untreated cells, defined as 100%. Cytotoxicity increased in a concentration-dependent manner, indicating an IC50 value of 16.3 µg/mL (71.6 µM) (Fig. 2). This finding contrasts with the reported absence of any toxic effects of resveratrol up to 45 µg/mL (196.9 µM) [12].

An additional set of FACS-based assays was therefore performed on BMMΦ in order to evaluate the relevance of the currently presented cytotoxicity of resveratrol. Again, cytotoxicity significantly enhanced with increasing concentrations, as evident from the number of PI-positive events (Fig. 3). The complementary assessment of cytotoxic effects carried out at single cell levels re-affirmed the toxic potential of resveratrol observed by means of the MTT assay. Importantly, cell cytotoxicity was negligible (≥ 0.5%) in all untreated incubations as assessed by PI staining. Since microscopic studies were exclusively used in previous work [12], we embarked on a similar protocol to further verify our results. Diff-Quik® stain revealed dramatic changes in the morphology of both non-infected and infected cells (Fig. 4), thus re-affirming a cytotoxic potential of resveratrol as assessed in the MTT assay. Cells clearly displayed highly condensed pyknotic nuclei, and showed more vacuoles and disintegration of cell membranes. These morphological changes were less evident in noninfected compared with infected cells. Infection per se may therefore have some impact on the cell integrity. Due to the absence of any image documentation in the previous paper [12], the conflicting results cannot satisfactorily be explained.

Finally, differences in the susceptibility of host cells to resveratrol represent a major parameter to provide a clue for the observed discrepancy in cell toxicity. BMMΦ were used in the current experiments, while J774 cultures have been included in the previous study. The panel of cell viability assays including the MTT assay (Fig. 5), FACS measurements (Fig. 6), and microscopic studies (Fig. 4) was therefore extended to J774-G8 macrophages. The pictures that emerged from examining the cytotoxicity and cytotoxic effects on host cells after PI staining (Fig. 6)
and changes in cell morphology (Fig 4S). This finding clearly excluded any dependency of cytotoxic effects on the kind of cells, at least for BMMΦ and J774-G8.

In conclusion, the claimed antileishmanial activity of resveratrol appears to be strongly associated with cytotoxic effects on host cells rather than with antiparasitic properties (SI = 1–1.6), taking into account the ineffective NO-inducing potential of resveratrol and induction of apoptosis in noninfected macrophages. MTT evaluation, FACS analysis, and microscopic examinations showed similar cytotoxic effects of resveratrol on noninfected J774-G8 and BMMΦ, respectively, thus rendering resveratrol less suitable as an antileishmanial agent. Having in mind that J774-G8 is a murine macrophage-like cancer cell line, the observed pronounced cytotoxic effects may be seen in line with the accumulating evidence for anticancer/chemopreventive activities of resveratrol [30]. Tolerability may well vary from tissue to tissue, and adverse effects occur somewhere in the human body. Clinical trials are therefore needed to evaluate the overall safety more faithfully than conventional in vitro experiments. Although this work demonstrates cytotoxic effects of resveratrol on macrophages under the experimental conditions, if and to what extent these immune cells are affected in vivo remains to be evaluated.

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Conflict of Interest

The authors declare none.

References


Fig. 5 Cytotoxic effects of resveratrol on noninfected J774-G8 cells as assessed by MTT. The results are shown relative to non-treated cells (negative control), defined as 100%. Data are expressed as mean ± SD (n = 3); * p < 0.05 and ** p < 0.001 compared to the control group (ctrl: cells + medium + 0.2% DMSO). The vehicle (medium and DMSO, respectively) did not affect cell viability (data not shown).

Fig. 6 Antileishmanial and cytotoxic effects of resveratrol on infected and noninfected J774-G8 cells as assessed by FACS analysis. Loss of the GFP signal and an increase in host cell cytotoxicity coincide in resveratrol-treated J774-G8 cells in a concentration-dependent manner. Non-treated cells, defined as 100%, were used as a negative control. Amphotericin B (1 µM) served as a positive control. Data are expressed as mean ± SD (n = 3); * p < 0.05; ** * p < 0.01; ** p < 0.001 compared to the control group (ctrl: cells + medium + 0.2% DMSO). The vehicle (medium and DMSO, respectively) did not affect antileishmanial and cytotoxic effects (data not shown).


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