Quercetin interferes with iron metabolism in *Leishmania donovani* and targets ribonucleotide reductase to exert leishmanicidal activity

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**Objectives:** The possibility of developing antileishmanial drugs was evaluated by intervention in the parasite’s iron metabolism, utilizing quercetin (Qr) under *in vivo* conditions, and identifying the target of this lipophilic metal chelator against *Leishmania donovani*.

**Methods:** Interaction between Qr and serum albumin (SA) was studied by using the intrinsic fluorescence of Qr as a probe. The effect of treatment with Qr and SA on the proliferation of amastigotes was determined by evaluating splenic parasite load. Disintegration of parasites in response to combination treatment was assessed from ultrastructural analysis using a transmission electron microscope. Quenching of the tyrosyl radical of ribonucleotide reductase (RR) in treated amastigotes was detected by an electron paramagnetic resonance study.

**Results:** Treatment with a combination of Qr and SA increased bioavailability of the flavonoid and proved to be of major advantage in promoting the effectiveness of Qr towards the repression of splenic parasite load from 75%, *P* < 0.01 to 95%, *P* < 0.002. Qr-mediated down-regulation of RR (*P* < 0.05), catalysing the rate-limiting step of DNA synthesis in the pathogens, could be related to the deprivation of the enzyme of iron which in turn destabilized the critical tyrosyl radical required for its catalysing activity.

**Conclusions:** Results have implications for improved leishmanicidal action of Qr in combination with SA targeting RR and suggest future drug design based on interference with the parasite’s iron metabolism under *in vivo* conditions.

Keywords: visceral leishmaniasis, amastigotes, antileishmanial drugs, molecular mechanisms, metal chelators

**Introduction**

All living organisms including *Leishmania* sp. require iron (Fe) for their growth and survival.¹,² *Leishmania* is an intracellular parasite, which multiplies within phagosomes of macrophages.³,⁴ While residing within phagosomes, pathogens gain access to adequate Fe for their growth from endogenous macrophage sources.³ ³⁵ Recently, a *Leishmania* membrane protein has been identified that functions intracellularly as an Fe²⁺ transporter (LIT1) and appears to be essential for the replication of the parasite within macrophage phagolysosomes. LIT1 helps in the adaptation of the pathogen in the harsh intracellular environment and, to a large extent, contributes to the development of microbial virulence.⁵ Fe deprivation has been suggested to be an effective approach in the treatment of parasitic infections.⁵,⁷ In *vitro* studies have shown growth attenuation of promastigotes using metal chelators such as deferoxamine (DFO).⁸ This chelator being poorly lipid soluble suffers from limited membrane permeability and is therefore unsuitable for clinical use. In order to overcome this difficulty, we concentrated on a lipophilic metal chelator, quercetin (Qr) (3,3',4',5,7-pentahydroxyflavone). Qr, an abundant naturally occurring flavonoid, has generated considerable interest as a pharmaceutical compound with a wide range of therapeutic activities.⁹,¹⁰ In *vitro* studies indicated the possible domains in this flavonoid, which can interact with metal ions by hydrogen bonding.¹¹,¹² These are between (i) 3'- and 4'-hydroxy groups localized on the B ring and (ii) 3-hydroxy and 4-oxo in the C ring (Figure 1a). Protection of 3,6,7,3',4'-hydroxy moieties by methyl functions in 5-hydroxy-3,6,7,3',4'-pentamethoxyflavone (Figure 1b) is expected to reduce the interaction of this flavone with metal ions. Qr possesses strong affinity towards Fe, which promotes the formation
Quercetin targets ribonucleotide reductase in leishmaniasis

![Chemical structures](image)

Figure 1. Structures of flavonoids: (a) Qr and (b) 5-hydroxy-3,6,7,3’,4’-pentamethoxyflavone.

of free radicals. Thus, the antilipoperoxidative activity of Qr can be described as being concomitant with its free radical scavenging and iron-chelating activities.11,13

Qr also serves as an effectual antileishmanial agent of high potency and low systemic toxicity.12,14 To date, it is known to inhibit the growth of *Leishmania donovani* by inducing cell cycle arrest through topoisomerase II-mediated DNA cleavage.14 Recent reports, with implications for antiparasitic drug development by creating interventions in parasite Fe metabolism,6 made us interested to assess the contribution of Qr as a chelator, with an emphasis on the inhibition of Fe-mediated proliferation of the parasites in leishmanial infection.

Visceral leishmaniasis (VL) is associated with depletion of serum albumin (SA).12 Since SA functions as the transport protein for Qr,15 the situation is likely to compromise the proficiency of Qr treatment in VL. In *in vitro* studies on the interaction of Qr with albumin provide direct spectroscopic evidence for protein–flavonoid interaction from both qualitative and quantitative perspectives.15,16

In this study, we have subjected animals to a combination treatment with Qr and SA. Here, we have shown the down-regulation of ribonucleotide reductase (RR), a Fe-dependent enzyme catalysing the rate-limiting step in DNA synthesis in the parasites as a possible mechanism behind the antileishmanial activity of the Fe chelator, Qr. Further, combination with SA increases the efficiency of Qr treatment by enhancing its bioavailability in VL.

Materials and methods

**Materials**

Unless indicated, all chemicals were obtained from Sigma (St Louis, MO, USA). 4-2-Amino ethyl benzene sulphoxyl fluoride hydrochloride (AEBSF) was from Calbiochem (San Diego, CA, USA). Trypanothione disulphide and glutathionylspermidine disulphide were from Bachem, Switzerland. DEAE-Sepharose FF, DE 52 and Sephadex G-25 were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Media 199 and RPMI 1640 were from Invitrogen-GIBCO-Cell Culture Services. Radioactive materials were purchased from Amersham Biosciences, UK.

**Animals**

Golden hamsters (*Mesocricetus auratus*) were maintained in the animal house of IICB, Kolkata, India, under controlled food, light/darkness cycles and temperature. All animal studies were performed following mandates approved by Animal Ethics Committee 147/1999/CPCSEA (committee for the purpose of control and supervision of experiments on animals, Government of India).

**Extraction of 5-hydroxy-3,6,7,3’,4’-pentamethoxyflavone**

The air-dried powdered leaves of *Vitex negundo* linn were subjected to extraction in a Soxhlet apparatus with petroleum ether (60–80°C) and methanol.12 The methanol extract was then fractionated with chloroform and *n*-butanol. Chromatography of the extract on silica gel yielded 5-hydroxy-3,6,7,3’,4’-pentamethoxyflavone. The structure of the compound was ascertained by spectroscopic analysis and superimposable infrared spectra with authentic samples.

**Isolation of parasites**

WHO reference strain *L. Donovanii* (MHOM/IN/1983/AG83) was maintained in hamsters by intracardial serial passages. Amastigotes were isolated from the spleens of *Leishmania*-infected hamsters.17,18 The spleens were rinsed and then lightly homogenized in ice-cold PBS/glucose (55 mM) containing ethylene diamine tetraacetic acid (EDTA) (2 mM). The macroscopic particles were allowed to settle and the upper layer containing a turbid suspension was decanted. This turbid suspension was centrifuged at 100 g for 10 min at 4°C. The amastigote-enriched suspension was centrifuged at 800 g for 10 min. The pellet was suspended in 45% percoll (8.0 mL). Finally, 25% percoll (4.0 mL) was layered over the amastigote suspension and centrifuged at 5000 g for 60 min. The band containing amastigotes was taken and washed three times with isotonic PBS (pH 7.4). Promastigotes were obtained by transforming amastigotes and were maintained under *in vitro* conditions in Media 199, supplemented initially with 20% fetal calf serum (FCS) and later with 8% FCS.

**Assessment of infection**

Hamsters at 4–5 weeks of age were inoculated with 10⁷ *L. Donovanii* amastigotes. Infection was monitored every fortnight until the end of the experiments.18,19 Immediately after the animals were killed, the spleen from each animal was removed and weighed. Multiple spleen impression smears from infected animals were prepared and stained with Giemsa stain. Spleen parasite burden was reported as total number of amastigotes per cell nucleus × (2 × 10⁴).

**Isolation of SA**

Plasma was separated from the blood collected from hamsters.20 Residual haemoglobin and γ-globulin were removed from the plasma (pH 7.2) by adding a mixture of 19% cold ethanol and 0.6% chloroform at 4°C and then keeping it overnight at −5°C. α,β-Globulin was then precipitated from the supernatant I by keeping it at −5°C for 6 h at pH 6.0. Ethanol and chloroform were
then removed and supernatant II was concentrated at pH 6.6. Fatty acids, haemagglutinin and residual globulin were then removed by thermocagulation at 70°C. Finally, SA was isolated from supernatant III at pH 5.4 and purified by using the ion-exchange gel DEAE-Sepharose FF. SA thus obtained had 99% purity. N-Acetyl-DL-tryptophan and sodium caprylate were used as stabilizers. Viral inactivation was performed by incubation for 10 h at 60°C.

**Isolation of peritoneal macrophages (PM)**

Macrophages were collected by peritoneal lavage from hamsters given an ip injection of 0.5 mL of 4% thioglycolate 5 days before harvest.21 Thioglycollate-elicited peritoneal exudates (1.5 × 10^7 macrophages) were allowed to adhere to glass coverslips (20 mm × 25 mm) in RPMI 1640 supplemented with 10% FCS and cultured for 5–7 days at 37°C in 5% CO₂ before in vitro infection with *L. donovani*.

**Fe acquisition by *L. donovani* from an endogenous source**

To study the effect of Qr on Fe acquisition by *L. donovani* from intracellular pools, PM were pre-loaded with 59Fe transferrin to ensure Fe incorporation. Internal Fe (endogenous source) was available to the parasites for Fe acquisition. Since no extracellular Fe was present during incubation, only internal Fe (endogenous source) was available to the parasites for acquisition.

**Analysis of metal-chelation properties of Qr and 5-hydroxy-3,6,7,3’,4’-pentamethoxyflavone**

The metal chelation ability of Qr and 5-hydroxy-3,6,7,3’,4’-pentamethoxyflavone was directly assessed from the spectral characterization of each flavonoid in terms of shifts in band I (300–550 nm) and band II (240–285 nm), which relate to B and A ring absorption, respectively.11 Solutions of both flavonoids (25 μM) were added to an equimolar concentration of Fe³⁺ in 10 mM PBS, pH 7.4, at room temperature. Shifts in the absorption spectra of the flavonoids after complex formation with metal were analysed with a UV-VIS spectrophotometer22 (Specord 200, Analytikjena).

**Qr analysis**

After 60 min of feeding Qr, animals were anaesthetized and livers were perfused in situ with chilled 0.15 M KCl. Organs were then removed and homogenized. Qr extract was transferred from the homogenized samples by shaking in 60% methanol for 60 min at 60°C in a water bath. The Qr extract was centrifuged at 2300 × g for 10 min and filtered through a 0.45 μm filter. An aliquot of the filtered sample (10 μL) was injected into an HPLC system (Waters, Vienna, Austria) having a 150 m × 4.6 mm C18 column.23 The mobile phase consisted of 44% methanol in 0.1 M ammonium acetate (pH 5.15) containing 0.27 mM EDTA. The HPLC analysis was carried out at a flow rate of 0.7 mL/min with UV detection at 360 nm. This study was carried out at 27°C. An external standard was used and the calibration curve showed good linearity over a range of 6–100 mg/L.

**Drug treatment**

Hamsters were grouped according to drug treatment with five animals in each group. In the monotherapy study, animals received Qr, 5-hydroxy-3,6,7,3’,4’-pentamethoxyflavone orally and DFO intravenously, three times a week in doses ranging from 5 to 50 mg/kg. SA was injected intravenously three times a week at a dose of 20 mg/kg. For the combination therapy study, Qr was given orally and SA was administered intravenously three times a week both at a dose of 20 mg/kg. One group of animals independently received Qr at 20 mg/kg alone. Hamsters in the control and untreated infected groups received isotonic PBS, pH 7.4, by oral feeding. Treatment was started after 30 days of infection and continued for the next 4 weeks of the post-infection period. Animals were sacrificed on day 60. 5-Hydroxy-3,6,7,3’,4’-pentamethoxyflavone was extracted from *V. negundo* leaf following the procedure described previously.12

**Spectrophotometric measurement of SA**

SA was determined by a dye-binding technique using Bromocresol Blue.24 The results obtained by this method agreed well with those obtained by electrophoresis, and the absorbance-concentration maintained a linear relationship under the experimental conditions. Determination of the amount of protein in all samples was carried out with a commercial source of human SA as the standard.

**Fluorescence studies**

Fluorescence quantum yields of SA were determined at an excitation wavelength of 290 nm and emission spectra were recorded from 300 to 450 nm. SA has a very high conformational adaptability to a great variety of ligands.25 This property assists in its role as a carrier for numerous endogenous and exogenous compounds including Qr.15,16 We studied the interaction of Qr with SA, using the intrinsic fluorescence of Qr as a probe. The fluorescence spectra of Qr in the presence of SA were determined with excitation at 370 nm and emission from 400 to 650 nm. Recordings were made using a Fluoromax 3, Horiba spectrofluorometer.15

**Fe incorporation studies**

Fe acquisition by *L. donovani* was measured under *in vivo* conditions by using a tracer dose of 59Fe-citrate (370 kBq of 59Fe suspended in 1.5 mL of 0.9% NaCl). Infected animals were injected with 2 μCi of 59Fe intracardially. Amastigotes were isolated from the splenic aspirates collected on day 3 and day 7 after the transfusion of 59Fe and were used for the measurement of Fe incorporation.

**Ultrastructure of amastigotes**

*L. donovani* amastigote ultrastructure was studied by examining thin sections of spleen from infected animals using transmission electron microscopy.26 Spleen cells from infected animals were fixed with 6% glutaraldehyde in 0.125 M phosphate buffer (pH 7.2) for 16 h and then 1% osmium tetroxide for 20 h at room temperature. The fixed cells were washed for 2 h in 0.5% uranyl acetate, dehydrated with increasing concentrations of ethanol and embedded in spurr medium at 70°C for 48 h. Sections were cut with a Dupont diamond knife in an LKB ultra microtome and examined using a JEOL 1000 X transmission electron microscope.

**Isolation and purification of RR**

Frozen amastigotes were disintegrated by sonicication and suspended in a buffer containing 50 mM Tris, pH 7.6, 20% glycerol, 10 mM MgCl₂, 2 mM dithiothreitol (DTT) and 1 mM PMSF. Nucleic acids...
in the crude extracts were precipitated by addition of neutralized streptomycin sulphate to a final concentration of 2%. Proteins were then subjected to ammonium sulphate precipitation (40% to 60% saturation). The pellet of ammonium sulphate precipitated protein was dissolved in 60% of buffer A [0.75 M (NH₄)₂SO₄ and 5 mM DTT] and 40% of buffer B (50 mM Tris–HCl, pH 7.6, and 5 mM DTT) and loaded onto a 20 mL bed volume HiLoad’s Phenyl Sepharose’s high-performance column, equilibrated with 0.75 M (NH₄)₂SO₄. The column was washed with 60 mL of buffer A followed by a 100 mL gradient from 0% to 100% of buffer B. The 100% buffer B was then washed for another 140 mL.

The R1 protein eluted when the gradient reached 100% of buffer B. R2 protein was purified on a DE52 column. A gradient from 0 to 200 mM KCl (buffered with 10 mM potassium phosphate, pH 7.0) was used. R2 protein, eluted between 100 and 130 mM KCl, was desalted on a Sephadex G-25 column equilibrated with 50 mM Tris–HCl (pH 7.6). Protein-containing fractions were analysed by SDS–PAGE. R1 and R2 fractions were concentrated and then stored at -80°C.

**Assay of RR**

RR activity was determined from the rate of conversion of guanosine 5'-diphosphate (GDP) into 2'-deoxyguanosine 5'-diphosphate (dGDP) as described for cytidine 5'-diphosphate (CDP). The consumption of nicotinamide adenine dinucleotide phosphate, reduced (NADPH) was monitored spectrophotometrically for 5–10 min. RR activity is defined as the amount of protein R1 that converts 1 nmol of substrate per minute in the presence of an excess of R2 protein at 25°C. The specific activity is expressed as nmol/mg of protein.²⁷,²⁸

**Electron paramagnetic resonance (EPR) measurement**

Immediately after isolation, the amastigotes (2 × 10⁸) were directly transferred to standard quartz EPR tubes, frozen in liquid nitrogen and scanned for the typical EPR spectrum using a Bruker X-band EMX spectrometer. EPR spectra were recorded at 77 K.²⁹ Instrumental parameters were: microwave frequency, 9.84 GHz; microwave power, 0.4 mW; modulation amplitude, 3 gauss; and modulation frequency, 100 kHz.

**Statistical analysis**

Results are expressed as means ± SEM. Two-tailed Student’s t-tests were used to determine P values.

**Results**

**Phenolic hydroxyl group in Qr empowers it with metal-chelating property**

Interaction of Fe³⁺ with Qr at a 1:1 metal to flavonoid ratio produced a bathochromic shift in band I of 25 nm. This was accompanied by a small 7 nm red shift at the position of band II (Figure 2a). Under similar conditions, no changes were observed in the positions of both the bands in the 5-hydroxy-3,6,7,3',4'-pentamethoxyflavone spectrum at a 1:1 metal to flavonoid ratio (Figure 2b).

**Lipophilic metal chelator, Qr, shows high antiproliferative activity against L. donovani**

To assess the efficacy of metal chelation on the proliferation of the parasites, we began by testing the antileishmanial function of Qr, 5-hydroxy-3,6,7,3',4'-pentamethoxyflavone and DFO under an in vivo treatment schedule (Figure 2c). Among the compounds studied, the antiproliferative activity of Qr was significantly more effective than the other two. Treatment for 30 days decreased the splenic parasite burden of the infected hamsters in a dose-dependent fashion. However, under similar...
experimental conditions, 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone failed to show any significant effect on the parasite load, thereby confirming the importance of the Fe-binding domain in the flavonoids for their antileishmanial function. Treatment with Qr at a dose of 20 mg/kg decreased the proliferation of the parasites in the infected animals by \( \sim 80 \pm 9.7\% \) \((P < 0.001, n = 6)\) compared with the untreated group. Negligible decreases in parasite load with further dose increases made us utilize Qr at this dose for the subsequent experiments.

**Qr inhibits Fe acquisition by L. donovani from an endogenous source**

Figure 3(a) shows the total Fe content in PM pre-loaded with \(^{59}\text{Fe}\). Decrease in this Fe content was accompanied by a corresponding increase in parasite Fe content after 48 h of incubation of PM with *L. donovani* (Figure 3b). This observation demonstrates Fe acquisition by intraphagosomal parasites from endogenous sources. A drastic fall in Fe acquisition by the parasites (Figure 3c; \( P < 0.01 \)) could be related to the significant decrease in Fe content in the PM (Figure 3c; \( P < 0.02 \)), after incubation in the presence of 30 \( \mu \text{M} \) Qr. This is a reflection of Qr-mediated change in the Fe distribution in PM (Figure 3d; \( P < 0.01 \)), which resulted in the decreased availability of Fe required for use by the pathogens.

**Interaction between Qr and SA increases the bioavailability of the flavonoid in the *L. donovani*-infected hamsters**

In order to replenish the paucity of SA in VL, we subjected the infected animals to SA treatment. Figure 4(a) compares the fluorescence emission spectra of SA from control animals with the samples collected from the serum of infected animals before and after SA treatment. The figure shows successful repletion of infection-induced depletion of SA from the control level after SA treatment and was further confirmed by spectrophotometric measurements (Figure 4b). With a view to facilitate the transport of Qr, infected animals were subjected to combination treatment with Qr and SA. Figure 4(c) shows a typical dual fluorescence behaviour with marked alteration in the fluorescent emission intensity of Qr depending upon the depletion and repletion of SA levels in the infected animal compared with the control. The importance of combination treatment with Qr and SA, in comparison with treatment with Qr alone, towards increased bioavailability of the flavonoid was obvious from the consequential enhancement in the Qr level in the target organ liver of the infected hamsters during the treatment period (Figure 4d).

**Qr and SA combination (Qr–SA) increases antileishmanial activity of Qr**

The antiproliferative activity of Qr–SA \((P < 0.002, n = 6)\) was more pronounced than that of Qr \((P < 0.01)\) as revealed in Figure 5(a). The decrease in splenic parasite load in the infected hamsters was much higher after 30 days of treatment with the Qr–SA combination in comparison with that with Qr alone \((P < 0.05)\). Increased incorporation of \(^{59}\text{Fe}\) in the amastigotes from the third to the seventh day \((P < 0.02, n = 6)\) after radioactive Fe injection \((2 \mu \text{Ci})\) during the post-infection period (Figure 5b) indicated the utilization of the labile Fe pool by the parasites required for their survival. The figure shows a preventive role of Qr–SA towards Fe utilization by the parasites. It is evident from the significant decrease in \(^{59}\text{Fe}\) incorporation in Qr–SA-treated infected animals both on day 3 \((P < 0.05)\) and day 7 \((P < 0.01)\) after \(^{59}\text{Fe}\) injection. Antileishmanial activity of Qr–SA was further supported by the non-significant increase in \(^{59}\text{Fe}\) incorporation in the treated group with the progress of infection.

**Transmission electron micrograph of phagolysosome showing antiproliferative function of Qr–SA against Leishmania amastigotes**

Figure 6(a) shows the transmission electron micrograph of amastigotes within a phagolysosome compartment of spleen after 60 days of infection. Ultrastructures of the parasites illustrate disintegration of the amastigotes (Figure 6b) in response to Qr–SA treatment during the latter half of the infection period. The combination treatment caused a significant decrease (5-fold) in splenic weight in the infected hamsters when expressed as percentage of body weight \((0.1 \pm 0.016\%, n = 10)\) in comparison with the untreated animals \((0.5 \pm 0.084\%; n = 15)\). The average
length of the spleen in the treated animals (1.5 ± 0.22 cm) was significantly \( (P < 0.01, \ n = 8) \) smaller than in the untreated infected animals (5.5 ± 1.55 cm) (Figure 6c).

**Quercetin targets ribonucleotide reductase in leishmaniasis**

In an attempt to define the mechanism behind the inhibition of parasite proliferation by iron chelators, we have measured the effect of Qr–SA on the activity of RR, an iron-containing enzyme that catalyses the reduction of ribonucleotide to deoxyribonucleotide, the rate-limiting step of DNA synthesis,\(^{30}\) in an in vivo model. A significant decrease \( (P < 0.05, \ n = 5) \) was noted in the activity of RR in the amastigotes isolated from the spleen of Qr–SA-treated infected hamsters compared with that from the untreated animals (Figure 7a). The down-regulation of RR could be related to a Qr-mediated decline in the Fe acquisition by the amastigotes (Figure 3), which is likely to destabilize the critical tyrosyl radical in the R2 subunit of the enzyme, required for its catalytic activity.\(^{31}\) EPR detects the absorption of electromagnetic radiation of unpaired electrons such as the tyrosyl radical.\(^{32}\) To determine the effect of Qr–SA on RR, we analysed the EPR signal of the tyrosyl radical in the enzyme of *Leishmania* amastigotes. The amount of tyrosyl radical, which is directly proportional to the RR activity, was determined from the peak to peak amplitude of the tyrosyl EPR signal \((Y)\). Figure 7(b) shows 65.5 ± 9.8% \( (P < 0.05, \ n = 4) \) quenching of the \( g = 2.009 \) feature of the signal after 30 days of treatment in comparison with that exhibited by the amastigotes from the untreated infected animals. This is consistent with the decreased activity of amastigote RR in response to flavonoid–protein combination treatment (Figure 7a). Results indicate iron depletion to be an effective mechanism against *L. donovani* infection under in vivo conditions.
Discussion

Limiting the microorganism’s access to Fe is an evolutionary strategy of host defence.3 Leishmania sp. are defective in the haem biosynthetic pathway and acquire this metalloporphyrin from exogenous sources, in order to satisfy their demand for Fe, required for the growth and multiplication of the parasites.53–35 This forms the basis for targeting Fe in antileishmanial therapeutics—an area that has not been adequately investigated. Fe chelation has been proposed as a useful tool to control VL under in vitro conditions.8 The most commonly used Fe chelator is DFO, the potency of which under in vivo conditions is largely restrained by a range of disadvantages, like a short plasma half-life, weak membrane permeability and the requirement for a long subcutaneous infusion.36 Lipophilicity of a compound is a crucial factor in determining its permeability across the cell membrane.37 Thus, the greater effectiveness of Qr may be partly due to its lipophilicity, allowing the flavonoid to permeate the cell membrane and reach the Fe pools more readily than the less lipid soluble chelator, DFO.

Interaction with metal ions can result in the formation of chelates and the reduction of metal ion, both depending on the flavonoid structure.38 The Fe$^{3+}$-reducing capacity of the flavonoids has been assigned to the simultaneous presence of catechol groups in the B ring and the 3-hydroxy group in the C ring.22,38 Qr satisfies these structural features, which is reflected from the 25 nm bathochromic shift in the band I spectrum in the presence of Fe$^{3+}$. 5-Hydroxy-3,6,7,3’,4’-pentamethoxyflavone lacks these structural advantages essential for the metal-chelating ability,22 thus exhibiting no spectral shift under similar experimental conditions. Methylation prevented 5-hydroxy-3,6,7,3’,4’-pentamethoxyflavone from binding with Fe$^{3+}$ and inducing antileishmanial activity.

The presence of Leishmania membrane protein LIT1 plays an essential role in the acquisition of Fe by the parasites. This was further strengthened by the deletion of LIT1, which curtails Leishmania’s virulence and renders it incapable of replication.3 Here, we have demonstrated that intraphagosomal L. donovani readily acquire $^{59}$Fe from PM pre-loaded with $^{59}$Fe prior to their

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Discussion

Limiting the microorganism’s access to Fe is an evolutionary strategy of host defence.3 Leishmania sp. are defective in the haem biosynthetic pathway and acquire this metalloporphyrin from exogenous sources, in order to satisfy their demand for Fe, required for the growth and multiplication of the parasites.53–35 This forms the basis for targeting Fe in antileishmanial therapeutics—an area that has not been adequately investigated. Fe chelation has been proposed as a useful tool to control VL under in vitro conditions.8 The most commonly used Fe chelator is DFO, the potency of which under in vivo conditions is largely restrained by a range of disadvantages, like a short plasma half-life, weak membrane permeability and the requirement for a long subcutaneous infusion.36 Lipophilicity of a compound is a crucial factor in determining its permeability across the cell membrane.37 Thus, the greater effectiveness of Qr may be partly due to its lipophilicity, allowing the flavonoid to permeate the cell membrane and reach the Fe pools more readily than the less lipid soluble chelator, DFO.

Interaction with metal ions can result in the formation of chelates and the reduction of metal ion, both depending on the flavonoid structure.38 The Fe$^{3+}$-reducing capacity of the flavonoids has been assigned to the simultaneous presence of catechol groups in the B ring and the 3-hydroxy group in the C ring.22,38 Qr satisfies these structural features, which is reflected from the 25 nm bathochromic shift in the band I spectrum in the presence of Fe$^{3+}$. 5-Hydroxy-3,6,7,3’,4’-pentamethoxyflavone lacks these structural advantages essential for the metal-chelating ability,22 thus exhibiting no spectral shift under similar experimental conditions. Methylation prevented 5-hydroxy-3,6,7,3’,4’-pentamethoxyflavone from binding with Fe$^{3+}$ and inducing antileishmanial activity.

The presence of Leishmania membrane protein LIT1 plays an essential role in the acquisition of Fe by the parasites. This was further strengthened by the deletion of LIT1, which curtails Leishmania’s virulence and renders it incapable of replication.3 Here, we have demonstrated that intraphagosomal L. donovani readily acquire $^{59}$Fe from PM pre-loaded with $^{59}$Fe prior to their
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Figure 7. Effect of in vivo treatment of infected (Inf) hamsters with Qr–SA on RR in *L. donovani* amastigotes. (a) RR activity in the amastigotes of Inf hamsters after 30 days of treatment with Qr–SA. RR activity was determined from the rate of conversion of GDP into dGDP. RR activity in the Inf + Qr–SA group is significantly different (*P < 0.05) from the Inf group. Values are the mean ± SEM, *n* = 8–10. (b) EPR signal of tyrosyl radical in RR of *L. donovani* amastigotes. EPR spectra of the g = 2.009 region, before and after the treatment of Inf hamsters with Qr–SA. EPR analysis was performed at 77 K. Spectra shown were corrected for small frequency differences in the background signals. The peak-to-peak amplitude of tyrosyl EPR signal Y of amastigotes from Inf + Qr–SA animals is reported as the percentage of the g = 2.009 feature of the signal exhibited by amastigotes from Inf animals. The tyrosyl EPR signal in the Inf + Qr–SA group is significantly different (*P < 0.05; *n* = 4) from the Inf group. Results are representative of a total of six experiments.

incubation with the parasites. This indicates that the parasites are able to acquire Fe from endogenous PM sites in addition to the acquisition from extracellular TF sources. Qr decreased Fe acquisition by PM from TF resulting in an altered internal PM Fe pool. Thus, the antileishmanial effect of Qr can be partly ascribed to the depletion of Fe from the intracellular sites within the PM, which is critical for the replication of intraphagosomal *L. donovani*.

SA is often termed a ‘transport protein’ and is a target for therapeutically active flavonoids of both natural and synthetic origin. The role of SA in increasing the solubility of Qr is evident from fluorescence spectroscopic studies indicating high-affinity interaction between the protein and the flavonoid. Binding has been proposed for fluorescence energy transfer, which takes place in the interdomain cleft region of SA proximal to the tryptophan-214 residue. However, no direct evidence has been demonstrated of this interaction under in vivo conditions. Considering the development of hypoalbuminemia during leishmanial infection, which is likely to reduce the therapeutic efficacy of Qr in the infected animals, we decided to exploit this high-affinity interaction between Qr and SA to increase the bioavailability of the flavonoid in VL. A combination treatment with Qr and SA proved to be highly promising as evident from the conspicuous changes both in the emission intensity and emission profile of Qr. To our knowledge, this is the first direct spectroscopic evidence showing the interaction between Qr and SA in a natural medium under in vivo conditions. Moreover, the combination was quite effective in enhancing the level of Qr in the infected animals, which was a major advantage in favour of the antileishmanial activity of the flavonoid. As a result of this observation, we utilized the combination of Qr–SA in our subsequent experiments.

That Fe was essential for the survival of intracellular pathogens was obvious from the enhanced 59Fe incorporation in the amastigotes as the infection progressed, thus demonstrating a predictable liaison between the parasites and increased acquisition of Fe in VL. This received further confirmation from the decreased incorporation of 59Fe in the amastigotes collected from animals challenged with Qr–SA, which also reflected the contribution of chelatable Fe in the process.

Ultrastructure studies present visual evidence for the leishmanicidal effect of Qr–SA treatment. A declining trend in amastigote viability could be explained by the observed disintegration of the *Leishmania* pathogen in response to the combination treatment. This may be related to the pronounced decrease in splenic weight in Qr–SA-treated hamsters, supporting the antileishmanial efficacy of the combination treatment. RR catalyses the rate-limiting step in the *de novo* synthesis of DNA precursors and is a key enzyme for the replication of an organism. The enzyme is a tetrameric protein composed of R1 and R2 subunits. The large R1 protein lodges in the active site as well as the regulatory site and the small R2 protein contains a di iron site that produces a stable tyrosyl free radical essential for the enzyme activity. Interestingly, RR is the only known enzyme that carries a stable free radical in its resting state. Although differential cells such as PM have negligible RR activity, the enzyme is critical for the DNA synthesis in the parasites. Our results displayed a marked diminution of the EPR spectroscopy signal of the tyrosyl radical in RR after Qr–SA treatment. Considering this finding, we suggest that RR is an important intracellular target for Qr, which upon binding with Fe forms the cytotoxic Fe complex that leads to the inhibition of the growth of rapidly proliferating amastigotes.

In conclusion, this study elucidates improved efficacy of Qr–SA over Qr in antileishmanial therapeutics, exploiting high-affinity protein–flavonoid interaction in a natural medium. We have also identified RR as a target for Qr action with an implication for antileishmanial drug development based on intervention with the parasites iron metabolism under in vivo conditions.
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Transparency declarations

None to declare.

References

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36. Lovejoy DB, Richardson DR. Novel ‘hybrid’ iron chelators derived from aroylhydrazones and thiosemicarbazones demonstrate high anti-proliferative activity that is selective for tumor cells. Blood 2002; 100: 666–76.

