Biomedicine & Preventive Nutrition 1 (2011) 168–171

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Original article

In vitro antileishmanial properties of new flavonoids against *Leishmania donovani*

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ARTICLE INFO

Article history:
Received 23 February 2011
Accepted 20 June 2011

Keywords:
Flavonoids
Leishmania donovani
Antileishmanial drugs
Luciferase assay

ABSTRACT

This study focuses on the *in vitro* antileishmanial evaluation of twenty original flavones which differ in substituent of the A-ring, mainly at C-7, and in substitution pattern of the lateral B-ring against *Leishmania donovani*. Flavonoids were evaluated *in vitro* against *L. donovani* promastigotes wild-type and drug-resistant lines using the MTT test and against intramacrophage amastigotes using a reporter gene luciferase assay. Cytotoxicity was evaluated on KB cell line. The most active compounds **7**, **11** and **14**, showed IC_{50} values on the intramacrophage amastigotes in a range from 1.7 to 3.6 μM with a selective index 29.9 for compound **14**. Compound **14** being about 6-fold more active than miltefosine on this *L. donovani* intramacrophage amastigote model, it is proposed for a further *in vivo* evaluation.

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1. Introduction

Leishmaniases are parasitic diseases that affect about 12 million people in tropical and subtropical areas provoking three clinical expressions: visceral leishmaniasis that is fatal in the absence of treatment, muco-cutaneous leishmaniasis and cutaneous leishmaniasis, this last one often self-curing. Classical drugs such as antimonials (Pentostam® and Glucantime®) are toxic and drug resistance dangerously increases in the field. A liposomal amphotericin B formulation (AmBisome®), less toxic than amphotericin B deoxycholate, progressively becomes the first line therapy mainly in immunocompromised patients but AmBisome® is administered by parenteral route. Thus, an orally safe and active drug is needed and miltefosine (Impavido®) is the first drug registered against visceral leishmaniasis in the last decade. However, its toxicity and the appearance of drug resistance justify the search for new chemical series. Flavonoids are a group of polyphenolic compounds having a basic flavan nucleus with two aromatic rings interconnected by a three-carbon-atom heterocyclic ring. These compounds are naturally present in fruits and vegetables and are known as antioxidants and preventive agents against cancer [1]. Moreover, flavonoids have shown *in vitro* activity against *Leishmania donovani* [2]. Since we had synthezised 3′,5′-di-tert.-butyl-4′-hydroxy flavones as potential inhibitors of low density lipoproteins (LDL) oxidation [3] and as multidrug resistance modulators [4,5], we decided to evaluate this series for antileishmanial activity.

2. Materials and Methods

2.1. *In vitro* evaluation on promastigote forms of *L. donovani*

Promastigote forms of wild-type *L. donovani* (MHOM/ET/67/HU3) clone called *L. donovani* LV9 WT and *L. donovani* (MHOM/IN/80/Dd8) called *L. donovani* Dd8 WT were grown in M-199 medium supplemented with 40 mM HEPES, 100 μM adenosine, 0.5 mg/L hemin, 10% heat-inactivated foetal bovine serum (FBS) and 50 μg/mL gentamycin at 26 °C in a dark environment under an atmosphere of 5% CO_2. The miltefosine and sitamaquine-resistant lines, named *L. donovani* LV9 HePC-R and *L. donovani* LV9 Sita-R, respectively, were obtained by *in vitro* continuous step-wise drug pressure from the *L. donovani* LV9 WT clone whereas the amphotericin B-resistant line, named *L. donovani* AmB-R, was obtained from the *L. donovani* Dd8 clone. The promastigote assay was carried out as previously described [6].

2.2. *In vitro* evaluation on intramacrophage amastigote forms of *L. donovani*

The antileishmanial evaluation of these compounds was then performed on *L. donovani* amastigotes by using the luciferase transfected *L. donovani* (strain MHOM/IN/80/Dd8) promastigotes maintained in the laboratory of Division of Parasitology, CDRI, Lucknow since 2005 as detailed by Sunduru et al. [7]. For assessing the activity of compounds against the amastigote stage of the parasite, mouse macrophage cell line (J-774A.1) infected with promastigotes expressing luciferase firefly reporter gene was used. Cells were seeded in a 96-well plate at the density of 4×10^4 cells per mL

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doi:10.1016/j.bionut.2011.06.012

under a final volume of 100 μL in RPMI-1640 containing 10% foetal calf serum and the plates were incubated at 37 °C in a CO_2 incubator. After 24 hours, the medium was replaced with fresh medium containing stationary phase promastigotes (4×10^5/100 μL/well). Promastigotes were engulfed by the macrophage and transformed there into amastigotes. The test compounds were added at two fold dilutions up to 7 points in complete medium starting from 100 μM concentration after replacing the previous medium and the plates were incubated at 37 °C in a CO_2 incubator for 72 hours. After incubation, the drug containing medium was decanted and 50 μL PBS was added in each well and mixed with an equal volume of Steady-Glo® Luciferase Assay Substrate dissolved in Steady-Glo® Luciferase Assay buffer. After gentle shaking for 1–2 minutes, the readings were recorded in a luminometer [8]. The values were expressed as relative luminescence units (RLU). Data were transformed into a graphic program (Excel). IC_{50} of antileishmanial activity was calculated by non-linear regression analysis of the concentration–response curve using the four parameter Hill equations. The *in vitro L. donovani* intramacrophage amastigote system used to evaluate the antileishmanial activity of the compounds was the most relevant one since it takes into account the pharmacokinetics barriers that a compound should overcome before entering the parasite.

2.3. Cytotoxicity evaluation

KB cells were used to evaluate the cytotoxicity of the compounds, then allowing determining an *in vitro* selectivity index. The cell viability was determined using the MTT assay. Exponentially growing KB cells at the density of 1×10^5 cells per mL under a final volume of 100 μL were incubated in a 96-well plate with test drugs for 72 hours. The test compounds were added at three fold dilutions up to 7 points in complete medium starting from 400 μM concentration, and were incubated at 37 °C in a humidified mixture of CO_2 and 95% air in an incubator. Podophyllotoxin was used as reference drug and control wells containing DMSO without drugs were also included in the experiment. Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. After incubation, 25 μL of MTT reagent (5 mg/mL) in PBS medium, followed by syringe filtration were added to each well and incubated at 37 °C for 2 hours. At the end of the incubation period, the supernatant were removed by tilting plate completely without disturbing cell layer and 150 μL of pure DMSO are added to each well. After 15 minutes of shaking the readings were recorded as absorbance at 544 nM on a micro-plate reader. The cytotoxic effect were expressed as 50% lethal dose, i.e., as the concentration of a compound which provoked a 50% reduction in cell viability compared to cell in culture medium alone. CC_{50} values were estimated as previously described [9]. The selective Index (SI) for compound was calculated as the ratio between cytotoxicity (CC_{50}) and the activity (IC_{50}) against *Leishmania* amastigotes. This cytotoxicity assay was performed on compounds exhibiting an IC_{50} value on *L. donovani* intramacrophage amastigotes less than 20 μM.

3. Results and discussion

Flavonoid appears as a promising series which still requires improvements and here we report the *in vitro* antileishmanial evaluation of twenty original flavones which differ in substituent of the A-ring (mainly at C-7), and in substitution pattern of the lateral B-ring. The synthesis of flavonoids has been previously described [3–5].

The preliminary antileishmanial *in vitro* screening was performed against promastigotes of two *L. donovani* wild-type (WT) lines, one from Ethiopia (LV9) and the second from India (Dd8). The results are gathered in Table 1. Both the lines exhibited similar sensitivity to flavonoids except compound **8** less active on the Dd8 line. Six compounds, **10**, **11**, **18**, **19**, **20** and **21** had IC_{50} values less than 10 μM against the LV9 WT line and seven (the same plus **7**) against the Dd8 line whereas about ten compounds were inactive ($IC_{50} > 100$ μM). Compound **15**, the scaffold of this series, was completely inactive on both the strains whereas a significant activity emerged against resistant parasites. It is noteworthy that compounds active on WT lines also appeared to be active against lines resistant to miltefosine, amphotericin B and sitamaquine, except compound **8**, inactive on the Sita-R line. This last compound displayed also some discrepancies within WT lines, since it was active on the LV9 line and inactive on the Dd8 line demonstrating the susceptibility variations of the *Leishmania* parasites as a function of their geographical origin. From the results on promastigote forms, importance of the C-7 substitution was very clear, that all the most active flavones on the five lines (average $IC_{50} < 10$ μM) bring at C-7 a substituent with an amine function. The best response of **11** *vs* **16**, both substituted at C-7 by the same dimethylaminopropyloxy chain, indicates that substitution pattern of the B-ring has an influence on the activity. We think that the best activity of **11** can be related to the strong lipophilic character of the *o,o′*-di-*t*-butylphenol group since the predicted logP values for **11** and **16** were 5.97 and 3.05, respectively.

The *in vitro L. donovani* intramacrophage amastigote system used to evaluate the antileishmanial activity of the compounds is the most relevant one since it takes into account the pharmacokinetics barriers that a compound should overcome before entering the parasite. Seven compounds exhibited IC_{50} values less than 10 μM. The IC_{50} values obtained from the promastigote and the intramacrophage amastigote screening systems were positively correlated for most of the 21 tested compounds with the three following notable exceptions: the 7-hydroxyethoxyflavone **14**, inactive on promastigotes, was strongly active on amastigote form, while aminoflavones **18** and **20** appeared active only on promastigote forms. Compounds **7**, **11** and **14** were the most promising with IC_{50} values less than 5 μM on intramacrophage amastigotes (selectivity index ≈ 29.9 for compound **14**). The fact that compound **15**, the basis scaffold, was totally inactive on intramacrophage amastigotes and WT promastigotes but very active on resistant-parasites suggests specific mechanims to be explored further. So, the present study allowed to establish evident structure–activity relationships for this flavone series with promastigote tests, but not with amastigote forms as previously described for other derivatives in the flavonoid series [10].

Among flavonoids, quercetin, had been shown to inhibit *L. donovani* amastigote growth *in vitro* with an IC_{50} value at 45.5 μM [11]. Despite this low *in vitro* activity, the action of quercetin in *L. donovani* was further investigated *in vivo*. Thus, quercetin and some of its glycoside derivatives have shown to be active *in vivo* after oral administration on the *L. amazonensis in vivo* mice model [12]. Moreover, an albumin-quercetin combination had the advantage to prevent reduced survival of erythrocytes in visceral leishmaniasis [13]. Our present results showed that quercetin was totally inactive *in vitro* both on promastigote and intramacrophage amastigote models. The poor *in vitro* activity previously described, the absence of activity on our *in vitro* models but the *in vivo* activity demonstrated in a previous study suggest that quercetin could need to be bioactivated to be active against *Leishmania* parasites. In a further study, quercetin metabolites could be identifid and evaluated *in vivo*.

In conclusion, this study pointed out interesting compounds, highly active *in vitro* against intramacrophage amastigote forms of *L. donovani* in comparison to quercetin. Despite the toxicity of these flavonoids, an *in vivo* evaluation on the *L. donovani*/Balb/c

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