Effect of the microtubule stabilising agent taxol on leishmanial protozoan parasites in vitro

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Abstract

Taxol, a mitotic spindle toxin, was found to selectively inhibit the proliferation of Leishmania donovani in vitro at nanomolar concentrations with an IC₅₀ of 35 nM. Concentrations of taxol as high as 50 nM, however, did not affect J774A.1 murine macrophages. Taxol (30 nM) also inhibited amastigote multiplication within a J774A.1 macrophage cell line when used in a 10-day experiment. It resulted in the in vitro assembly of L. donovani microtubules in a dose-dependent manner. When promastigotes were exposed to different concentrations of taxol for 24 h, cells were largely blocked in the G₂-M phase of the cell cycle and there was a marked reduction in the percentage of cells in the S phase. The selective nature of taxol action against the parasite and its effectiveness in controlling amastigote multiplication emphasise its use as a promising chemotherapeutic against kala-azar. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Leishmania donovani; Antileishmanial; Taxol

1. Introduction

Leishmania donovani is a protozoan parasite that causes visceral leishmaniasis. Globally, millions of people are infected with this parasite and options for drug treatment are limited primarily to antimonial drugs. The high toxicity of these drugs makes alternative approaches attractive. In addition, the development of resistance is a grave concern as it is further accentuated by the small range of antileishmanial drugs. There is an urgent need for new molecular targets against which we can design new and more effective drugs. Since tubulin is the most abundant leishmanial protein, perhaps the microtubule inhibitory agents can serve as antileishmanial compounds.

Taxol is a mitotic spindle toxin isolated from the bark of the Pacific yew Taxus brevifolia and is a naturally occurring chemotherapeutic against cancer [1]. While the exact mechanism of taxol cytotoxicity is unclear, there is evidence that its antitumor effect results from its binding tightly to microtubules and preventing their disaggregation [2,3]. Taxol blocks the transition of cycling cells from the late G₂ to M phase of the cell cycle [4].

Earlier reports have shown that taxol is effective in blocking the replication of Trypanosoma cruzi para-
sites [5] and is also effective against drug-resistant Trypanosoma strains [6]. Taxol is also known to have an effect on the cell cycle and cytoskeleton of L. donovani promastigotes [7]. In this paper we explore the effect of taxol on L. donovani promastigotes and amastigotes in vitro and its mechanism of action. Taxol, at nanomolar concentrations, was found to selectively inhibit the proliferation of L. donovani promastigotes as well as the multiplication of amastigotes within J774A.1 macrophages in vitro. The effects of taxol on host macrophages, on the cell cycle of Leishmania promastigotes and on tubulin assembly in these promastigotes in vitro are also reported.

2. Materials and methods

2.1. Chemicals and parasites

Powdered media RPMI 1640 and M-199 were purchased from Sigma Chemical Co., St. Louis, MO, USA. Minimal essential medium with alpha modification (α-MEM) was procured from Biological Industries, Israel. Taxol, propidium iodide, MES (2[N- morpholino] ethane sulfonic acid) and β-mercaptoethanol were also obtained from Sigma Chemical Co. All other chemicals were purchased locally and were of analytical grade.

Freshly transformed promastigotes of L. donovani (strain AG83) (MHOM/IN/1983/AG83) were used in this study [8]. They were suspended in minimal essential medium (α-MEM) prior to use in the drug studies and in RPMI 1640 medium containing 10% FCS (RPMI) for use in the amastigote multiplication assay.

2.2. Effect of taxol on promastigote growth

Taxol was dissolved in methanol and filter-sterilised before addition to the medium containing parasites. The toxicity of the drug was determined by adding a range of drug concentrations (0–100 nM) to a suspension of promastigotes (1×10^6 cells ml⁻¹) and incubating them at 22°C for 48 h. The minimum concentration of the drug that caused inhibition of approximately 50% of the parasites at 48 h of drug treatment was designated IC₅₀ [8]. The time-dependent effect of the inhibitor was determined by adding the inhibitor to the promastigote suspension at 24 h after plating in α-MEM medium and determining the cell density after every 24 h until 120 h. Triplicate incubations in the absence of drug were maintained in parallel as controls. The protozoal counts were taken using a Neubauer haemocytometer.

2.3. Effect of taxol on J774A.1 macrophages

J774A.1 macrophages were plated onto petri dishes at a density of 1×10⁶ cells per plate and incubated at 37°C in a CO₂ incubator (5% CO₂) for 24 h. Taxol was added at this stage and the macrophages were incubated for another 48 h at 37°C. The cells were then harvested by trypsinisation and the number of live cells in each set was determined using the trypan blue dye exclusion test. The results were expressed as percentage of cells in control cultures of triplicate samples.

2.4. Effect of taxol on intracellular amastigote multiplication

J774A.1 macrophages were plated onto adherent petri dishes at a cell density of 1×10⁶ cells ml⁻¹ and incubated overnight at 37°C in a CO₂ incubator (5% CO₂). Parasites were added onto the macrophages at a ratio of 10:1 and the plates were re-incubated at 37°C for 3 h. After 3 h, the excess promastigotes were washed off and the plates were incubated at 37°C for another 12 h to enable transformation of promastigotes to amastigotes. At this stage, the drug was added to the plates and they were kept at 37°C. The drug was replenished on days 3, 5 and 7. On day 10, the plates were harvested for microscopic examination after staining with Giemsa. The data were expressed as the percentage of infected macrophages ± S.D. and the mean number of amastigotes per 100 infected macrophages ± S.D. Each data point represents mean ± S.D. of triplicate samples [9].

2.5. Effect of taxol on the cell cycle of leishmanial promastigotes

Exponential phase AG83 promastigotes were treated with different concentrations of taxol at
22°C for 24 h. 2×10⁶ cells were harvested after 24 h, fixed in 70% ethanol and stored at 4°C. Before the assay, the ethanol was removed by centrifugation. The cells were stained with 1 ml propidium iodide staining solution (50 μg ml⁻¹ PI, 100 Kunitz units RNase A and 1 mg ml⁻¹ glucose in PBS without Ca²⁺ or Mg²⁺) and incubated for 30 min at room temperature with constant shaking. The cells were examined within 12 h using EPICS® XL-Software (Coulter, Miami, FL, USA) and then analysed using MULTICYCLE Software (Phoenix Flow Systems, K-OS Un Ltd. Inc., USA) [10]. These experiments were performed twice.

2.6. Effect of taxol on tubulin assembly in vitro

AG83 promastigotes were harvested from 200-ml cultures, washed with PBS and re-suspended in phosphate glutamate buffer (PG: 20 mM NaHPO₄, 100 mM glutamic acid, 1 mM β-mercaptoethanol, adjusted to pH 7.0 with 1 mM NaOH), PMSF (10 μg ml⁻¹) and Triton X-100 (0.5% w/v) were added to the PG buffer and the cells were homogenised. Detergent-insoluble cytoskeleton was collected, washed in PG-PMSF (3×), frozen in liquid nitrogen and stored at −70°C according to the method of Chan and Fong [11]. The pellet was thawed on ice and re-suspended in 1 ml MEMED buffer (100 mM MES pH 6.7, 1 mM EGTA, 1 mM MgSO₄, 1 mM EDTA and 1 mM DTT). GTP (2 mM) and MgCl₂ (1 mM) were added to the above solution and it was sonicated for 20 s (7×) to release the cytoskeletal proteins with 1 min cooling on ice after each sonication and a liquid nitrogen freeze-thaw after the third sonication. The solution was centrifuged and the supernatant was collected. It was assayed for protein using the micro Lowry method [12]. In vitro microtubule polymerisation was measured by incubating tubulin samples (0.8 mg ml⁻¹) with different concentrations of taxol and measuring the absorbance in a temperature-controlled spectrophotometer at 37°C [1,2,5]. The absorbance was measured at 30-s intervals for a period of 1 min. Since the maximum assembly was observed within 30 s, the maximum absorbance value at this time point was used for making the graph.

In addition to leishmania preparations, microtubules from a mammalian source, rodent brain, were used as control. Freshly collected brains from rats were homogenised in MES-EGTA-guanosine 5’-triphosphate buffer (0.1 M MES, 1 mM EGTA, 1 mM GTP, 0.5 mM MgCl₂ (pH 6.4) at 4°C [13]. The minced tissue was washed twice with this buffer and homogenised in 1 ml of buffer per g of tissue. The homogenate was centrifuged at 100000 ×g for 1 h at 4°C and the tubulin was prepared by two cycles of assembly-disassembly and stored at −20°C in MES buffer (0.1 M MES, 1 mM EGTA, 0.5 mM MgCl₂, adjusted to pH 6.4 with NaOH) containing 1 mM GTP and 4 M glycerol [13]. Before the experiment, tubulin was dialysed for 3 h at 4°C against 100 volumes of MES buffer and centrifuged at 120000 ×g for 20 min at 4°C. The fraction was quantitated for protein and the assembly was monitored using 0.8 mg ml⁻¹ of tubulin and different concentrations of taxol as described above for the leishmanial tubulin. The absorbance was measured at 50-s interval for a period of 10 min. Since the maximum assembly was observed at 5 min in this case, the maximum absorbance value at this time point was used for making the graph.

All results are means ± S.D. of triplicate samples at each time point. Student’s t-test was performed to determine the level of significance and P < 0.05 was regarded as significant.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean % infected cells ± S.D. (nM)</th>
<th>Mean number of amastigotes per 100 infected macrophages ± S.D. (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43 ± 1</td>
<td>149 ± 11</td>
</tr>
<tr>
<td>Taxol (20 nM)</td>
<td>15 ± 1 (65)*</td>
<td>136 ± 8 (9)**</td>
</tr>
<tr>
<td>Taxol (30 nM)</td>
<td>10 ± 1 (77)*</td>
<td>127 ± 3 (15)*</td>
</tr>
</tbody>
</table>

*For details of treatment see Section 2.
**Significant decrease from control (P < 0.01–0.001). ***Not significantly different from control values.
3. Results

Taxol was found to inhibit leishmanial growth with an IC<sub>50</sub> of 35 nM. A concentration of 100 nM inhibited promastigote growth completely (Fig. 1). The drug kinetics of taxol on AG83 promastigotes over a period of 48 h is shown in Fig. 2. Maximum inhibition was observed 24 h after drug addition (Fig. 2). Taxol also resulted in morphological changes like cell rounding of the parasite as observed under the phase contrast microscope. The organisms continued to exhibit motility.

Table 1 shows the effect of taxol on intracellular amastigote multiplication within J774A.1 murine macrophages. A concentration of 20 nM reduced the percentage infectivity significantly (P<0.001) but it had no significant effect on amastigote numbers. A 30 nM concentration, however, reduced both parameters in a significant manner (P<0.01–0.001).

Taxol was also tested on host macrophages in order to determine the selectivity of its action on promastigotes. Fig. 3 shows the effect of different concentrations of taxol on J774A.1 host macrophages at 48 h of macrophage treatment. A 50 nM concentration of taxol, which reduces promastigote number to 30% of control values, resulted in a 77% survival of J774A.1 macrophages. Higher concentrations of taxol (100 nM, 500 nM and 1000 nM) resulted in 57%, 44% and 28% survival respectively of J774A.1 macrophages.

The differential effect of taxol against the parasite and the host macrophages led us to examine the mechanism of action of this compound. Since taxol has been found to arrest cancer cells in the G<sub>2</sub>-M phase of the cell cycle, we studied the effect of taxol on the cell cycle of AG83 promastigotes. A range of taxol concentrations from 1 nM to 10 nM was tried and cells were harvested at 24-, 48- and 72-h intervals after drug treatment. PI staining followed by flow cytometry showed that there was a significant blockage of cells in the G<sub>2</sub>-M phase of the cell cycle and the percentage of cells in S phase decreased significantly at 24 h of treatment in taxol-treated cells. Higher concentrations of taxol, 5 nM and 10 nM, did not further alter the cell cycle pattern when compared to 1 nM (Table 2).

Taxol has been shown to bind to the microtubules...
in cancer cells. Thus, we used this compound in binding assays with leishmanial tubulin. Tubulin was extracted from AG83 promastigotes \[6,14\]. A range of taxol concentrations was added and tubulin assembly was monitored at 30-s intervals for a period of 1 min per sample \[1\]. Taxol caused an increase in the absorbance and hence tubulin assembly within 30 s of addition; after this there was no further increase in the absorbance. Since there was no further increase in the turbidity 30 s after taxol addition, the dose-dependent effect of taxol on tubulin assembly depicted here is at the 30-s time point for all concentrations used. Concentrations ranging from 0.1 to 0.5 \(\mu\)M showed a dose-dependent increase in tubulin assembly. Higher concentrations did not lead to any further increase in tubulin assembly (Fig. 4A). These data confirm that taxol mediates its effect on AG83 promastigotes by binding to the microtubules.

In the case of rat brain, a range of taxol concentrations was added and tubulin assembly was monitored at 50-s intervals for a period of 10 min per sample \[1\]. The dose-dependent effect depicted here is at the 5-min time point for all the concentrations used. Concentrations ranging from 0.1 to 0.5 \(\mu\)M showed a dose-dependent inhibition of tubulin assembly (Fig. 4B).

4. Discussion

The parasite \textit{L. donovani} exists in two forms – a
flagellated, motile, extracellular promastigote form that survives within the salivary glands of the Phlebotomine sandfly vector and a nonmotile, intracellular amastigote form that exists within the macrophages of the vertebrate host. Antimonials are currently the only viable options for drug treatment. However, the high toxicity of these drugs and the emergence of resistance render chemotherapy difficult. Hence there is an urgent need for novel chemotherapeutic agents against kala-azar. Since tubulin is one of the most abundant leishmanial proteins, microtubule inhibitory agents may provide effective chemotherapeutics. Previous reports have shown the antileishmanial effects of two such agents – trifluralin [11] and oryzalin [14]. Both these drugs prevent microtubule assembly. They have also been found to act selectively on the parasite and do not affect the host cells.

Taxol is a cancer chemotherapeutic which binds to tubulin and prevents disassembly of microtubules [2,3], thereby blocking cells in the G2-M phase of the cell cycle [4]. Taxol has been previously against T. cruzi and L. donovani promastigotes and it has been found to block replication of the parasite at micromolar concentrations [5,7]. Drug-treated parasites were found to have multiple cell organelles but they could not divide fully, confirming that taxol causes inhibition of microtubule disassembly in these cells [5].

In the present study taxol inhibited leishmanial growth at nanomolar concentrations. It also inhibited infectivity and amastigote multiplication within a J774A.1 murine macrophage cell line. When taxol was used against J774A.1 cells, it was found that it had a selective action against the parasite. A 100 nM concentration of taxol resulted in complete inhibition of parasite growth whereas concentrations of taxol up to 500 nM resulted in only 56% inhibition of the macrophage growth (a concentration 14 times the IC50 value for the parasite). Treatment of promastigotes with taxol at different time intervals was found to arrest cells in the G2-M phase of the cell cycle and inhibited the percentage of cells in the S phase significantly. Earlier work by Moulay et al. [7] also showed that taxol interferes with G2-M progression in L. donovani promastigotes.

In order to confirm the mechanism by which taxol exerts its effect, in vitro tubulin binding assays with taxol were performed. Taxol increased leishmanial tubulin assembly at 37°C in a cell-free system in a dose-dependent manner. In contrast, taxol in the rat brain acted as an inhibitor of tubulin assembly in vitro. Thus, it appears that in vitro, the mechanism of action of taxol on leishmanial tubulin is different from that on the mammalian tubulin. These results show that taxol binds to leishmanial tubulin in vitro and that it may be important for cytoskeleton-targeted chemotherapy against visceral leishmaniasis.

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**References**


