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Isolation of a taxol-resistant *Leishmania donovani* promastigote mutant that exhibits a multidrug-resistant phenotype

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Abstract

We raised a strain of *Leishmania donovani* in the laboratory that was resistant to 500 nM taxol. The IC_{50} of the wild-type strain for taxol was 35 nM and that of the taxol-resistant strain (T-500) was 1 μM. The T-500 strain exhibited a Mdr phenotype; it was also resistant to other unrelated drugs like vinblastine, adriamycin and the commonly used antimonial drugs pentostam and glucantime. Verapamil (20 nM), a calcium channel blocker, was found to reverse the resistance of T-500 to taxol. Acquired resistance to taxol has been reported to be mediated by alterations involving tubulin in cancer cells. Thus polymerisation assays with tubulin fractions in wild-type versus taxol-resistant cells (T-500) were performed in vitro. The tubulin fraction from T-500 was more resistant to in vitro polymerisation than the tubulin isolated from the wild-type, suggesting that this is one means by which the parasite may acquire resistance to taxol.

Keywords: *Leishmania donovani*; Drug resistance; Taxol

1. Introduction

Drug resistance is a common obstacle to chemotherapy and cure of diseases. Parasites like *Leishmania* are rapidly becoming refractory to commonly used antimonial drugs, leading to an urgent need for novel chemotherapeutic strategies against this disease. But in addition to developing novel chemotherapeutics, there is also a need to explore possible mechanisms of resistance occurring in *Leishmania* and means to circumvent it.

Taxol is a plant alkaloid that has antimitotic activity and appears to stabilise microtubules [1,2]. Earlier reports have shown that taxol is effective in blocking the replication of *Trypanosoma cruzi* parasites and is also effective against drug-resistant *Trypanosoma* strains [3]. We have reported earlier that taxol at nanomolar concentrations selectively inhibits proliferation of *Leishmania donovani* promastigotes as well as amastigote multiplication within J774A.1 macrophages in vitro. In this study, variants of this parasite resistant to taxol have been characterised.

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2. Materials and methods

2.1. Chemicals and parasite

Powdered medium M-199 was purchased from Sigma Chemical Co., St. Louis, MO, USA. Minimal essential medium with alpha modification (α-MEM) was procured from Gibco, USA. Foetal calf serum (FCS) was obtained from Biological Industries, Israel. The drugs taxol, adriamycin (doxorubicin), vinblastine and verapamil were also obtained from Sigma Chemical Co. Pentostam and glucantime were from Wellcome, UK and Specia, France respectively.

Freshly transformed promastigotes of *L. donovani* (strain AG83) (MHOM/IN/1983/AG83) [4] were used for raising the resistant strain. Parasites were suspended in minimal essential medium (α-MEM) prior to their use in the drug studies.

2.2. Selection of Leishmania variants resistant to taxol

Promastigotes of *L. donovani* (strain AG83) were made resistant to taxol by gradual increase in the concentration of the drug as described by Coderre et al., 1983 [5]. Wild-type cells were gradually acclimatised to growth in increasing concentrations of the inhibitor. The doses used for the selection procedure were 35 nM, 50 nM, 100 nM, 150 nM, 250 nM, 350 nM, 450 nM and 500 nM. The cells were maintained in each concentration of taxol until their growth rate reached that of the control group. The entire selection procedure took about 3 months. All the studies in this paper were done using the T-500 strain that was resistant to 500 nM taxol.

2.3. Drug studies

The effective concentration of the drug which inhibited the growth of the cells by 50% (IC_{50}) was determined for the wild-type and taxol-resistant cells (T-500) at the late exponential growth phase of the cells [6]. Verapamil was used to check if it could render the T-500 cell line sensitive to taxol [7]. The wild-type and T-500 cells were simultaneously incubated with 20 nM verapamil and varying concentrations of taxol. Verapamil (20 nM) by itself had a marginal inhibitory effect on promastigote growth.

2.4. Effect of taxol on leishmanial tubulin assembly in vitro in wild-type versus taxol-resistant cells

AG83 and T-500 promastigotes were harvested from 200-ml cultures each, washed with PBS and re-suspended in phosphate glutamate buffer (PG: 20 mM $NaHPO_4$, 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 [8]. The pellet was thawed on ice and resuspended in 1 ml MEMED buffer (100 mM MES pH 6.7, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM EDTA and 1 mM DTT). GTP (2 mM), EGTA (1 mM) and $MgCl_2$ (1 mM) were added to the above solution and it was sonicated (7×20 s) 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 [9]. Microtubule polymerisation was measured in vitro in both strains. The effect of different concentrations of taxol on tubulin assembly in both the wild-type and T-500 strains was measured in vitro. The tubulin samples were incubated with different concentrations of taxol and the absorbance was measured in a temperature-controlled spectrophotometer at 37°C [2,3,10]. The absorbance was measured at 30-s intervals for a period of 1 min. Since the maximum assembly was observed within 30 s, the absorbance value at the 30-s time point was used for making the graph.

3. Results

Taxol-resistant *Leishmania* were raised by stepwise selection of the wild-type cells in gradually increasing concentrations of taxol until the cells were resistant to 500 nM taxol. This selection procedure took about 3 months. As shown in Fig. 1, the T-500 cell line was approximately 30-fold more resistant to taxol than the wild-type strain.

We tested T-500 for resistance to adriamycin and

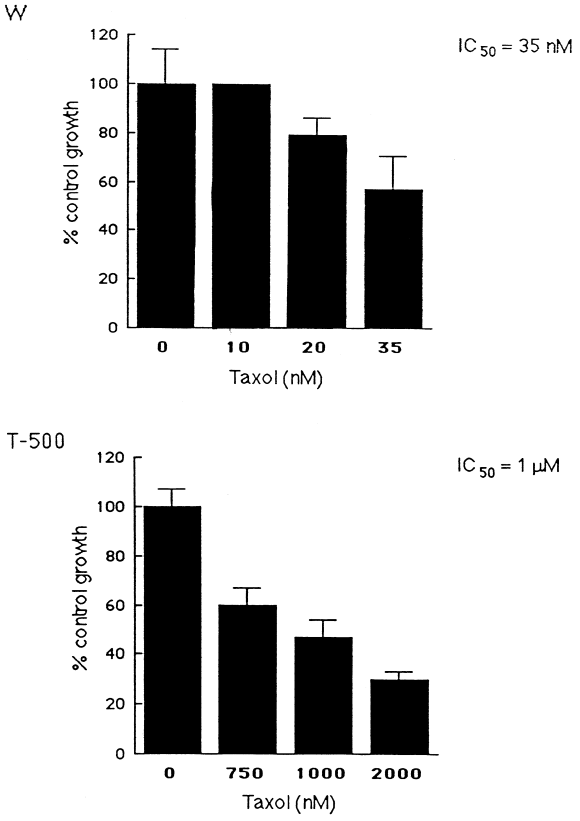

Fig. 1. Effect of different concentrations of the anti-microtubule agent taxol on the growth of wild-type (W) and taxol-resistant *L. donovani* promastigotes (T-500). These results are representative of triplicate samples.

found it to be 2-fold more resistant (IC_{50} 5.0 μM) than the wild-type parental strain (IC_{50} 2.5 μM). Moreover, T-500 cells were also 5-fold more resistant to vinblastine (IC_{50} 10.0 μM) than the wild-type (IC_{50} 2.0 μM). These cells were also found to be 2-fold and 5-fold more resistant respectively to pentostam (IC_{50} 0.05 mg ml^{-1}) and glucantime (IC_{50} 2.5 mg ml^{-1}), the drugs commonly used for treating leishmaniasis. The IC_{50} values for the wild-type cells to pentostam and glucantime were 0.025 mg ml^{-1} and 0.5 mg ml^{-1} respectively. The resistance exhibited by T-500 to unrelated drugs suggested that the T-500 cell line exhibited a multidrug-resistant (MDR) phenotype.

Some calcium channel blockers, such as verapamil [7,11], can reverse multidrug resistance mediated by P-glycoprotein. The effect of verapamil was therefore tested in the wild-type and T-500 cell lines. Verapamil (20 nM) by itself resulted in marginal growth inhibition in both wild-type and T-500 strains. After plating 1×10^6 promastigotes ml^{-1} for 24 h in α-MEM media, verapamil (20 nM) and different concentrations of taxol were added to the media containing promastigotes. The cells were harvested 48 h later and counted using a Neubauer haemocytometer. Different concentrations of taxol (10–30 nM) had no effect on the growth of T-500 promastigotes in the absence of verapamil (Fig. 2). In the presence

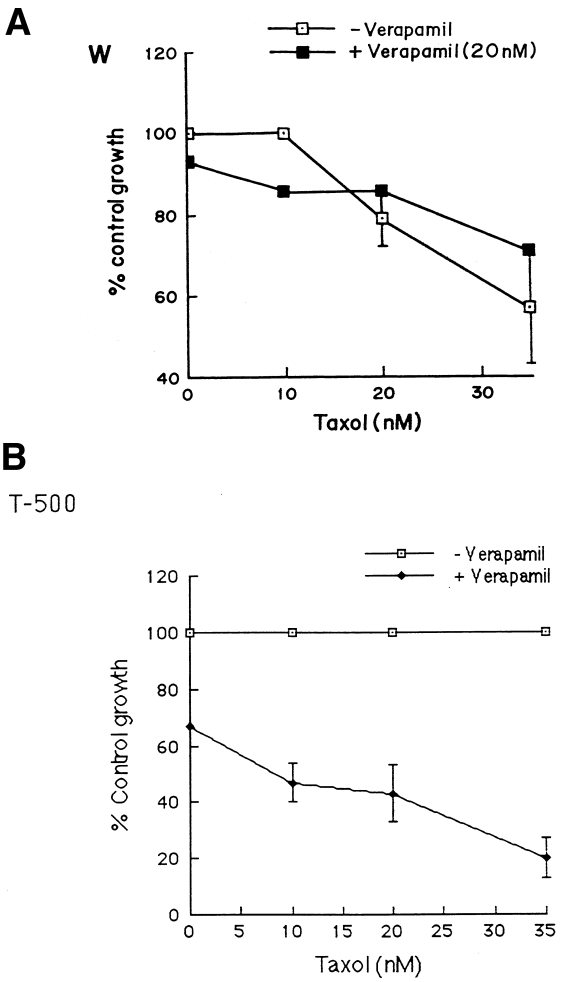

Fig. 2. Combined growth inhibitory effect of verapamil (20 nM) and different concentrations of taxol on wild-type (W) (A) and taxol-resistant promastigotes (T-500) (B). These results are representative of triplicate samples.

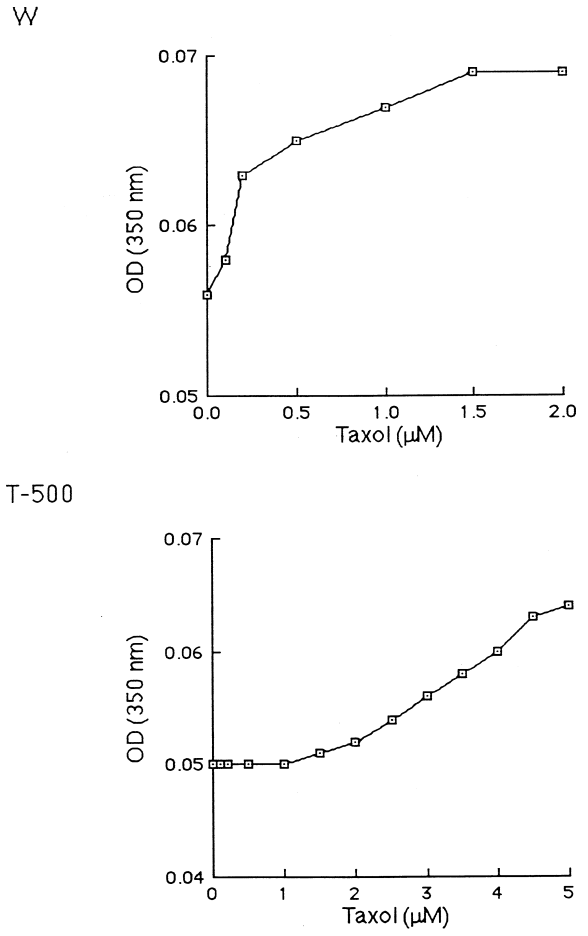

Fig. 3. Effect of taxol on microtubule assembly in vitro in wild-type (W) and taxol0resistant (T-500) cells. *L. donovani* tubulin was prepared as described and analysed in Section 2.

of verapamil, however, there was a highly significant inhibitory effect of these concentrations of taxol on the growth of the T-500 strain (Fig. 2B). In the case of the wild-type AG83 strain, taxol resulted in a dose-dependent inhibition of promastigotes in the absence of verapamil. Addition of 20 nM verapamil did not alter the inhibitory effect of taxol on promastigote growth (Fig. 2A).

In order to check if acquired resistance to taxol can be mediated by alterations involving tubulin, tubulin was isolated from wild-type and T-500 cells and its polymerisation pattern in these two cell types was studied in the presence of different concentrations of taxol. Fig. 3 shows the comparative assembly patterns of tubulin from wild-type and T-500 leishmanial promastigotes in the presence of different concentrations of taxol. The concentration of tubulin used for the polymerisation experiment with the wild-type strain was 1 mg ml^{-1} and for the T-500 strain it was 2 mg ml^{-1}. The above two tubulin concentrations were chosen after preliminary polymerisation experiments were performed with tubulin from wild-type and T-500 cells in the absence of taxol. These preliminary results showed that there was an approximately comparable polymerisation at these two concentrations of tubulin.

The comparative assembly patterns of tubulin from wild-type and T-500 leishmanial cells using 1 mg ml^{-1} and 2 mg ml^{-1} of tubulin respectively showed that T-500 tubulin requires a much higher concentration of taxol for its polymerisation as compared to the wild-type strain. These results show that acquired resistance to taxol in the T-500 strain is probably due to resistance to polymerisation by taxol in this strain, which may be due to alterations in the tubulin of the T-500 strain.

4. Discussion

Drug resistance is a common obstacle to leishmanial therapy today. The inability of currently used drugs to control *Leishmania*, on account of the widely increasing incidence of drug-resistant organisms, makes it necessary not only to devise alternative chemotherapeutic strategies but also to explore the mechanisms by which the parasite becomes resistant to different drugs.

The mechanism by which *L. donovani* could become resistant to taxol was explored. A taxol-resistant *L. donovani* cell line (T-500) exhibited cross-resistance to several unrelated drugs like adriamycin, vinblastine and the antimonial compounds pentostam and glucantime indicating that T-500 had an MDR phenotype. Gueiros-Filho et al. [7] have shown that the MDR phenotype can be reversed by the calcium channel antagonist verapamil, a feature that was also found in the T-500 cell line. Moreover, in the case of the wild-type strain, it was observed that there was absence of enhancement of inhibition with verapamil, indicating the absence of any synergistic inhibition effect of verapamil in com-

bination with taxol. However, hybridisation studies with a mammalian *mdr* probe showed that taxol-resistant cells did not contain amplified *MDR* DNA (data not shown), suggesting that resistance to taxol may be mediated by some mechanism other than amplification of the gene encoding the mammalian MDR phenotype.

Multidrug resistance does not necessarily require gene amplification. Increased expression of the multidrug gene without *MDR* gene amplification is commonly observed in different types of cancers and appears to be a significant marker of clinical drug resistance [12]. It is possible that this mechanism may be involved in resistance to taxol in *L. donovani*.

There are other mechanisms involved in resistance to taxol. Acquired resistance to paclitaxel can be mediated by P- glycoprotein or by alterations involving tubulin. The latter is observed in taxol-resistant cell lines derived from 1A9 human ovarian carcinoma cells [13]. No *MDR-1* mRNA can be detected in these cells. The total tubulin content in taxol-sensitive and -resistant cells remains the same but the polymerised fraction increases in wild-type cells as compared to resistant cells following taxol addition.

The present in vitro tubulin polymerisation studies of wild-type and taxol-resistant leishmanial cells with taxol showed that T-500 cells are indeed more resistant to polymerisation with taxol. The taxol concentrations needed to cause the same degree of polymerisation in T-500 cells were much higher as compared to the wild-type cells. However, whether the exact mechanism involved is mutation or change in the tubulin isotype still remains to be explored.

Acknowledgements

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