The Cytotoxic Effect and the Multidrug Resistance Reversing Action of Lignans from Phyllanthus amarus

Abstract

Multidrug resistance (MDR) constitutes the major obstacle to the successful treatment of cancer. In several cancer cells, MDR is thought to be mediated by the super-expression of P-glycoprotein (Pgp). Pgp extrudes drugs from the cells, therefore reducing their cytotoxicity, and its activity inhibition may reverse the MDR phenotype. The present study evaluated the possible cytotoxic effect and MDR reversing properties of the extract and compounds isolated from Phyllanthus amarus. To this purpose, two human leukaemia cell lines were employed: K-562 and its vincristine-resistant counterpart Lucena-1, a Pgp-overexpressing subline. We report here that Lucena-1 was significantly more resistant to the cytotoxicity of P. amarus derivatives: the hexane extract (HE, 100 μg/mL), the lignans-rich fraction (LRF, 100 μg/mL) and the lignans nirtetralin (NIRT, 43.2 μg/mL), niranthin (NIRA, 43 μg/mL) or phyllanthin (PHYL, 43 μg/mL) exerted cytotoxic effects on K-562 cells with 40.3, 66.0, 62.0, 61.0 or 24.1% of cell death, respectively. The cellular toxicity observed on Lucena-1 was 16.3, 40.4, 29.4, 30.2, or 24.8 %, respectively. However, cell treatment with the lignan phylltetralin (PHYLT) up to 41.6 μg/mL had no cytotoxic action on either of the cell lines. P. amarus derivatives were also found to be effective in inhibiting Pgp activity as assessed by rhodamine accumulation in Lucena-1 cells, as were the classical Pgp inhibitors, cyclosporine A (160 nM), PSC-833 (2 μM) and verapamil (5 μM). The lignan NIRT produced the most potent inhibition (EC50 = 29.4 μg/mL) followed by NIRA (44.3 μg/mL), LRF (49.1 μg/mL), PHYT (99.4 μg/mL), PHYL and HE (>100 μg/mL). Lucena-1 cells were more resistant to daunorubinc-induced cell death (LC50 = 50 μM) than K562 cells (LC50 = 4.95 μM). Of note, the P. amarus derivatives significantly potentiated 5μM daunorubicin-induced cell death in Lucena-1 cells (P < 0.01) but not in K562 cells. After treatment only with P. amarus derivatives (100 μg/mL HE, 30 μg/mL LRF, 12.9 μg/mL NIRA, 43.2 μg/mL NIRT, 43 μg/mL PHYL or 41.6 μg/mL PHYT), the Lucena-1 cellular viability was 83.7, 85.3, 101, 69.7, 75.6 or 88.7 %, respectively, whereas the in the presence of daunorubincin, which was not cytotoxic per se, the cell viability decreased to 42.9, 42.2, 64.2, 35.4, 30.4 or 52.6 %, respectively. Together, these results suggest a potential action of P. amarus derivatives as MDR reversing agents, mainly due to their ability to synergize with the action of conventional chemotherapeutics.

Key words
Phyllanthus amarus · Euphorbiaceae · lignans · multidrug resistance · P-glycoprotein · human leukaemia cells · cancer

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Bibliography
Introduction

The multidrug resistance (MDR) phenomenon was first described in 1970 by Biedler and Riehm [1]. It is characterized by a cross-resistance of tumour cells to a variety of structurally and functionally unrelated anticancer drugs and is still one of the major obstacles in cancer treatment. In 1976, Juliano and Ling identified a plasma membrane protein named P-glycoprotein (Pgp) which was highly expressed in drug-resistant cells, but not in their non-resistant counterparts [2]. Known as ABCB1, Pgp belongs to the ABC family of transporter proteins. Many other members of this family are also involved in the MDR phenomenon. P-glycoprotein acts as an energy-dependent efflux pump, performing the extrusion of several chemotherapeutic drugs, decreasing their intracellular concentration, and thereby reducing their cytotoxicity [3].

It has been reported that Pgp-mediated MDR both in vitro and in vivo can be reversed by a variety of hydrophobic agents such as verapamil [4], trifluoperazine [5] or cyclosporin A [6] and by a non-immunosuppressive cyclosporin derivative, PSC 833 [7]. The majority of these drugs are structurally and functionally distinct, but their common action is the inhibition of Pgp activity. However, the association of a Pgp inhibitory agent with a chemotherapeutic drug, which produces a satisfactory inhibition of Pgp function without any damage to normal tissues, is not yet well established. Much effort has been focused in recent years on finding new pharmacological inhibitors of Pgp. In this regard, naturally-occurring active substances of plant origin might constitute an important source for the study of Pgp inhibitors with applications in chemoresistance of tumour cells [8], [9].

Phytochemical and biological studies have identified potential antitumoural substances including some lignans [10]. These substances may display a variety of pharmacological properties including immunosuppressive [11], vasoactive [12], antioxidant [13], antiviral [14], anti-inflammatory [15] and antitumoural [16] actions. Lignans are an important class of secondary metabolites found in many plant species belonging to more than seventy families [17]. They are the major active components encountered in the plants of the *Phyllanthus* genus, from the botanical family Euphorbiaceae [17].

*Phyllanthus* sp. are widely distributed in most tropical and subtropical countries and have long been used in folk medicine [17]. Recently, Rajeshkumar and Kuttan have demonstrated that the aqueous extract of *Phyllanthus amarus* increases the life span of hepatocellular carcinoma-harbouring animals by decreasing the proliferation of tumour cells [18]. The same authors have shown that this extract exhibits potent anticarcinogenic activity against different kinds of cancer such as sarcomas, carcinomas and lymphomas [19].

Although *P. amarus* may have anti-tumoural properties and some lignans inhibit Pgp activity, it was not clear whether the lignans from *P. amarus* encompassed anti-tumoural and/or anti-MDR properties. Thus, in the present study we assessed the cytotoxic effect of the *P. amarus* hexane extract, its lignans-rich fraction, and the following isolated lignans: nirtretalin, niranthin, phyltetralin and phyllanthin, on two human cancer cell lines, K-562 and its MDR counterpart Lucena-1. In addition, we also investigated the ability of these compounds to inhibit Pgp activity and, therefore, their multidrug resistance reversing action.

Materials and Methods

Reagents

Foetal calf serum (FCS) and RPMI 1640 medium were obtained from Gibco (New York, NY, USA); l-glutamine, HEPES, rhodamine 123, cyclosporin A, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and verapamil were purchased from Sigma-Aldrich (Saint Louis, MO, USA). PSC 833 (kindly donated by Novartis, Basel, Switzerland) was first dissolved in 50% ethanol and kept as a stock solution of 1 mM. Culture medium was composed of RPMI 1640 medium supplemented with 1 mM l-glutamine and 25 mM Hepes. Lignans were freshly prepared in dimethyl sulfoxide (DMSO) as a stock solution of 100 mM.

Plant material and phytochemical studies

*P. amarus* was obtained from the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas – CPQBA/UNICAMP, Brazil, and was authenticated by Prof. Grady L. Webster (University of California; Davis, CA, USA). A voucher specimen (number UEC 127.411) of *Phyllanthus amarus* Schumach & Thonn was deposited at the herbarium of the Institute of Biology, UNICAMP, Brazil. The detailed procedures for preparing the hexane extract, lignans-rich fraction and purified lignans from *P. amarus* have been previously described [20].

Cell lines

The human leukaemic cell line K-562 (ATCC; Rockville, MD, USA) and its vincristine-resistant derivative Lucena-1 were used. Lucena-1 cells were developed in our laboratory by continuous exposure of K-562 cells to increasing concentrations of vincristine [21]. Both cell lines were maintained in RPMI medium at 37°C in a humidified atmosphere of 5% CO₂. Cultured Lucena-1 cells were maintained in the presence of vincristine 60 nM until the time of the experiments.

Cytotoxicity assay

Cell survival studies were performed for each cell line using an MTT assay as previously described by Mosmann [22]. Briefly, cells were incubated with drug-free medium or medium containing four different concentrations of the *P. amarus* hexane extract (HE) and its lignans-rich fraction (LRF) (10 – 300 μg/mL) or purified lignans nirtretalin (NIRT), niranthin (NIRA), phyllanthin (PHYLLA) and phyltetralin (PHYLIT) (10 – 300 μM), or vehicle only (0.1% DMSO) (see Supporting Information for detailed description).

Rhodamine 123 extrusion assay

The Pgp activity was analysed as previously described by Neyfakh [23]. The efflux of the Pgp fluorescent substrate rhodamine was studied on the K-562 and Lucena-1 cells. Briefly, cell suspensions at 10⁶ cells/mL were incubated with 200 ng/mL rhodamine, in the presence or absence of the Pgp inhibitors cyclosporin A (CSA, 160 nM), verapamil (VP, 5 μM) or PSC 833 (2 μM) for 30 min at 37°C. The experiment was performed as detailed in Supporting Information. Drug concentrations were chosen based on the published literature data [24].
Daunorubicin resistance
For both cell lines seeded at $10^6$ cells/mL in 96-well plates, we observed the cytotoxic effect of another Pgp substrate, the chemotherapeutic drug daunorubicin (DAUNO, 0.5 – 50 μM), after 72 h incubation using the MTT assay. In order to detect any cytotoxic drug synergism, Lucena-1 cells were treated with the Pgp inhibitors VP (5 μM), CSA (160 nM) or PSC 833 (2 μM) or P. amarus derivatives in the absence or presence of daunorubicin (5 μM), and the MTT reaction was performed.

Statistical analysis
The percentage of cell viability was calculated using the absorbance values obtained after the MTT assay. Percentage results are relative to each control experiment, consisting of cells cultured in the presence of the vehicle only.

All values are expressed as mean ± SEM of 3 – 7 experiments performed in triplicate, except the median lethal concentration (LC), which is reported as the geometric mean accompanied by its respective 95% confidence limits. The LC values were obtained from the concentration of extract and lignans (LC50) or daunorubicin (LC50) necessary to reduce cell viability to 25 or 50% of the control values, respectively. The EC50 values were obtained from concentrations of the P. amarus derivatives necessary to increase rhodamine fluorescence to 50% of the PSC 833-induced response. The effect was calculated by linear regression analyses for individual concentration-response curves ($n = 3 – 7$) using the GraphPad Prism software.

Statistical significance between different groups of treatment was performed using Student's t test for unpaired samples and one- or by two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Differences were considered statistically significant when $P < 0.05$.

Supporting information
Complementary information for Materials and Methods (cytotoxicity assay and rhodamine 123 extrusion assay) and the rhodamine accumulation cytogram profile of K-562 and Lucena-1 cells are available as Supporting Information.

Results and Discussion
In order to analyse the potential anti-tumoural effect of P. amarus derivatives, our study was performed using two cancer cell lines exhibiting different phenotypes regarding chemotherapy resistance. The erythroleukaemia cell line K-562 is vincristine-sensitive and was established from the pleural effusion of a patient with chronic myelogenous leukaemia in terminal blast crisis [25]. The vincristine-resistant Lucena-1 cells are a Pgp-over-expressing K-562 subline which was obtained after progressive adaptation of cultured cells to vincristine [21].

Firstly, both tumour cell lines were incubated with three different concentrations of the P. amarus derivatives, and their effect on cell survival was measured by a tetrazolium-based assay. As shown in Fig. 1A – E, the HE (100 μg/mL), the LRF (100 μg/mL) and the lignans NIRT, NIRA and PHYLLA (100 μM) exerted a cytotoxic effect on K-562 cells with a relative cell death of 40.3 (± 6.3)%, 66.0 (± 2.5)%, 62.0 (± 5.6)%, 61.0 (± 4.3)%, and 24.1 (± 7.6)%, respectively. At the same concentrations of HE, LRF, NIRT, NIRA or PHYLLA, the cellular toxicity observed on vincristine-resistant Lucena-1 cells was 16.3 (± 6.4)%, 40.4 (± 11.5)%, 29.4 (± 10.7)%, 30.2 (± 4.9)%, and 24.8 (± 6.2)%, respectively. Therefore, these results reveal that Lucena-1 cells are significantly more resistant than K-562 cells to the cytotoxic effect of 100 μg/mL HE (2.5-fold, $P < 0.001$) or LRF (1.5-fold, $P < 0.05$), and 100 μM NIRT (2.1-fold, $P = 0.01$) or NIRA (2-fold, $P < 0.001$) (Figs. 1A, B, C and D, respectively). However, cell treatment with PHYLT (Fig. 1F) up to 100 μM had no cytotoxic action on either of the cell lines.

Some cytotoxic lignans have been described [16], [26], but the mechanisms involved in the cytotoxicity of P. amarus-derived...
lignans remains unknown. Nevertheless, other works have failed to demonstrate this cytotoxic effect [10, 13], suggesting that the cytotoxic activity is not a common characteristic of all lignans. In fact, our results showed that the lignan PHYLT had no significant effect on either K-562 or Lucena-1 tumour cells (Fig. 1). Moreover, the weak effect of HE, LFR, NIRT, NIRA and PHYLLA on the vincristine-resistant Lucena-1 cell line suggests that these compounds might not be useful as single chemotherapy in myeloid leukaemia treatment.

On the other hand, there are many studies, including phytochemicals, focused on identifying new substances to overcome MDR, seeking new inhibitors or drugs capable of destroying MDR cells [8, 9, 27]. The low cytotoxicity of the lignans observed on Lucena-1 cells does not discard a possible MDR reversing action of these products. Interestingly, Somananbandhu and collaborators [10] have demonstrated that the lignans PHYLLA and hypophyllanthin are not cytotoxic per se, but that they enhance the cytotoxic response mediated by vinblastine in multidrug resistant cells. Of note, those lignans are able to displace the binding of vinblastine on membrane vesicles derived from these cells, suggesting a relevant interaction of PHYLT and hypophyllanthin with the multidrug resistance transporter Pgp [10].

Our data also show that Lucena-1 cells were more resistant than K-562 cells to the cytotoxic effect of some P. amarus derivatives, indicating that these cells are phenotypically different and that those compounds might be Pgp substrates. The most prominent feature of Lucena-1 cells seems to be the Pgp over-expression [21], and our experiments confirm this feature (Fig. 1S in the Supporting Information).

Since the Pgp activity was demonstrated in Lucena-1 cells, we next investigated the effect of P. amarus derivatives on the functionality of this transporter protein. The results in Fig. 2 show that HE, LFR, and lignans from P. amarus were effective in modulating the rhodamine accumulation, as did the classical Pgp inhibitors, CSA, PSC 833 and VP. Intracellular rhodamine accumulation in K-562 cells was significantly higher than that of untreated Lucena-1 cells (P < 0.001, Fig. 2A). The effect of CSA or PSC 833 treatment on Lucena-1 cells was similar to that obtained by LRF and NIRT (100 μM), increasing the rhodamine intracellular accumulation by about 12–15-fold. The Pgp inhibition obtained by the 2 μM PSC 833 treatment (Fig. 2A) was assumed to be the maximal effect on rhodamine accumulation in Lucena-1 cells. Thus, the following EC50 values were found after the cell treatment with some lignans: 49.1 ± 8.5, 29.4 ± 5.3 and 44.3 ± 2.7 μg/mL for LRF, NIRT and NIRA, respectively. The hexane extract as well as PHYLLA and PHYLT had the EC50 values extrapolated from their concentration-response curves of rhodamine accumulation (259.3 ± 76, 159 ± 27 and 99.4 ± 36.2 μg/mL, respectively). Our data show that the P. amarus derivatives were able to reverse Pgp activity in the following rank order of potency: NIRT > NIRA > LRF > PHYLT > PHYLHA > HE. These data support the ability of these lignans to reverse Pgp-generated MDR.

The increased expression of Pgp is one of the causes of drug resistance in myeloid leukaemia [28]. The anthracycline daunorubicin, used for the treatment of leukaemias, is a Pgp substrate and may be exported from the cell by this transporter [29]. As previously described, Lucena-1 cells are characterised by increased Pgp expression [21] and consequently they are resistant to daunorubicin-induced cell death [30]. Our results confirmed this phenomenon in Lucena-1 cells by demonstrating that the LC50 of daunorubicin on these cells was 49.99 (48.08–51.97) μM, while the K-562 cells were markedly less resistant to this drug [LC50 = 4.95 (3.93–6.23) μM] (not shown). Based on these data, the concentration of daunorubicin was established for the next experiments.

Considering that some lignans are known to enhance the accumulation of rhodamine, which is also a Pgp substrate like the chemotherapeutic drug daunorubicin, we further assessed the possible reversing action of P. amarus derivatives on daunorubicin resistance. To do this, Lucena-1 cells were treated with P. amarus derivatives in the presence or absence of a non-toxic concentration of daunorubicin (5 μM). According to Fig. 3, HE, LRF, NIRT, NIRA, PHYLLA and PHYLT were able to synergise with daunorubicin to induce cell death. In addition, a similar synergistic effect was observed when Lucena-1 cells were incubated with daunorubicin in the presence of VP, CSA or PSC 833 (which were not cytotoxic per se) reducing cell viability to 68.2 ± 2.6 (P < 0.01), 61.3 ± 1.1 (P < 0.001) or 61.1 ± 2.7% (P < 0.01), respectively (data not shown). The minimal concentrations of P. amarus derivatives that significantly enhanced rhodamine accumulation (Fig. 2)
were also able to synergise with daunorubicin (Table 1). Table 1 also shows the LC_{25} mean values of HE, LRF, NIRT, NIRA, PHYLLA and PHYLT in the absence or in the presence of daunorubicin. However, the ratio between LC_{25} (− DAUNO/+ DAUNO) indicates that PHYLLA and LRF present the best synergistic actions, different from the potency rank order for Pgp function. This result suggests that the synergistic effect observed after co-treatment of Lucena-1 cells with *P. amarus* derivatives and daunorubicin is not only explained by the inhibition of Pgp. Further studies are required to explain this discrepancy. On the other hand, the single treatment

Table 1 Cytotoxic effects of the following compounds isolated from *P. amarus* on Lucena-1 cells: the hexane extract (HE), the lignans-rich fraction (LRF), and the purified lignans nirtretalin (NIRT), niranthin (NIRA), phytetralin (PHYLLA) and phyllanthin (PHYLT), in the presence or absence of daunorubicin (DAUNO, 5 μM).

<table>
<thead>
<tr>
<th>Compound</th>
<th>LC_{25} (μg/mL)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>− DAUNO</td>
<td>+ DAUNO</td>
</tr>
<tr>
<td>HE</td>
<td>115.0 ± 3.1</td>
<td>49.4 ± 9.4</td>
</tr>
<tr>
<td>LRF</td>
<td>98.4 ± 28.9</td>
<td>5.5 ± 2.7</td>
</tr>
<tr>
<td>NIRT</td>
<td>48.4 ± 8.9</td>
<td>8.7 ± 2.7</td>
</tr>
<tr>
<td>NIRA</td>
<td>47.9 ± 5.6</td>
<td>13.3 ± 1.6</td>
</tr>
<tr>
<td>PHYLLA</td>
<td>68.4 ± 19.5</td>
<td>3.9 ± 1.9</td>
</tr>
<tr>
<td>PHYLT</td>
<td>113 ± 26.9</td>
<td>20.2 ± 8.8</td>
</tr>
</tbody>
</table>

LC_{25} is the lethal concentration to 25% of each treatment and was calculated from Fig. 3 data.
The equivalent molar concentrations of the lignans are \textsuperscript{a} 30 μM and \textsuperscript{b} 100 μM.
Values are mean ± SEM of at least three independent experiments performed in triplicate. P values indicate statistical differences between both groups (Student Bonferroni’s multiple comparison test).
with daunorubicin had a cytotoxic effect (43.9 ± 3.2%) on K-562 cells; co-treatment with liganms resulted in an additive cytotoxicity. Thus, no synergistic effect could be observed (data not shown).

A previous study of our group had shown that some of the P. amarus derivatives such as HE, LRF, NIRA, NIRT and PHYLT had a marked anti-inflammatory action in vivo [20]. Moreover, 5-day treatments with HE and LRF did not display any toxic effect on mice [20]. This finding supports the traditional medical use of P. amarus’ therapeutic properties in hepatitis, diabetes and renal calculi and the absence of reported adverse effects [17]. Hence, the anti-tumoural properties of liganms isolated from P. amarus associated with its low potential toxicity in vivo could be advantageous against other MDR reversing agents, which are frequently responsible for side effects and/or toxicity [31]. However, much of these toxic effects may be related to the ubiquitous expression of Pgp which is responsible for the detoxification of many drugs [3]. In this way, Pgp inhibition could originate drug-drug interactions by altering the accumulation of co-administrated drugs. Thus, possible adverse interactions must be taken in account when performing in vivo studies with P. amarus derivatives.

Collectively, the present results demonstrate that the P. amarus hexane extract, the liganms-rich fraction and the purified liganms NIRT, NIRA, PHYLLA and PHYLT are effective in inhibiting Pgp function in vitro. Furthermore, these compounds exhibit remarkable synergism with daunorubicin in terms of antileukaemic activity, being potential candidates for combined treatment with conventional chemotherapies, acting as MDR reversing agents.

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