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A Two-complementary Method Assay for Screening New Reversal Agents of Cancer Cell Multidrug Resistance

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Abstract

Multidrug resistance represents a major obstacle in the successful therapy of neoplastic diseases. P-Glycoprotein appears to play an important role in such cells by acting as an energy-dependent efflux pump which removes various natural drugs from the cell before they had any chance to exert their cytotoxic effects. In the present study, a two-method assay based on the direct interaction with a purified C-terminal recombinant cytosolic domain of P-glycoprotein and the increase of intracellular daunomycin in a multidrug resistant cell line has been adapted for the screening and detection of potential inhibitors of P-glycoprotein present in various plant extracts. This paper demonstrates that the two methods are simple and sensitive, and constitute quantitative tools to assess the capacity of complex samples such as plant extracts to inhibit P-glycoprotein transporters; they also are useful for screening or identifying new inhibitors of P-glycoprotein.

Keywords: Plants extracts, drug resistance, membrane P-glycoprotein, drug effects, cancer chemotherapy, screening.

Introduction

Clinical resistance to chemotherapeutic drugs is a major problem in the treatment of cancer. One form of drug resistance, termed multidrug resistance (MDR) is defined as the ability of cells exposed to a single drug to develop resistance to a broad range of structurally and functionally unrelated drugs (Lehnert, 1998).

Among the different mechanisms which confer MDR, one is often overexpressed in mammary tumours following drug treatment, namely the 170-kD plasma membrane-associated P-glycoprotein (Pgp) (Lehnert, 1994; Goldstein, 1995; Licht et al., 1996).

Pgp is an ATP-dependent multidrug pump, belonging to the ATP-binding cassette (ABC) superfamily of proteins (Hyde et al., 1990), which protects cells from cytotoxic compounds by transporting them out and reducing the intracellular levels below their effective concentrations. Physiologically, Pgp is widely expressed in the epithelial cells of intestine, liver and kidney, and in the endothelial cells of brain and placenta. The broad substrate specificity and differential expression locations suggest that Pgp may have a direct role in absorption and availability of drugs and xenobiotics. Consequently, the inhibition of this membrane transporter could result in far reaching implications, including drug bioavailability.

Since the early observation of Tsuruo et al. (1981) that non-cytotoxic doses of verapamil could restore the sensitivity to *Vinca* alkaloids in MDR cells, a large number of multidrug resistance modifiers have been identified. They include molecules with different activities, such as calcium-channel blockers, calmodulin inhibitors, anti-estrogen compounds and immunosuppressants (Gottesman & Pastan, 1993). Various problems are associated with the clinical use of these drugs, such as their inability to achieve plasma concentrations sufficiently high to inhibit Pgp activity, their short half-life and rapid clearance, and their unacceptable toxicities when administered at the concentration needed to sensitize cancer cells (Kellen, 1993). Several agents such as PSC 833

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(Twentyman & Bleehen, 1991) a non-immunosuppressive analog of cyclosporin A, and the cyclopeptolide SDZ 280–446 (Loor et al., 1992) have been recently described as more effective in sensitizing MDR cells *in vitro* than the initially examined MDR modulators. However, no definitive MDR inhibitor is still available in the clinic. Additional efforts have to be devoted to the development of more specific inhibitors of Pgp, lacking undesired side effects.

More recently, several reports have suggested that flavonoids, especially some isoprenylated derivatives, are efficient modulators of Pgp (Comte et al., 2001). These compounds are naturally present in some plant families, especially in the Fabaceae (Leguminosae), Rutaceae and Moraceae (Barron & Ibrahim, 1996). Their mechanism of Pgp inhibition is original since they do not behave as competitive substrates for Pgp, but rather are able to interact with the cytosolic ATP- and steroid-binding sites of the transporter (Conseil et al., 1998).

However, purification of individual compounds from plant extracts is a long-term process which is not compatible with fast selection of interesting plant sources. For this reason, the development of rapid assays performed on crude extracts is suitable. In this study, two complementary methods are developed to characterize potential Pgp inhibitors present in several plant extracts. The former is based on the direct interaction with the purified C-terminal cytosolic nucleotide-binding domain of Pgp. The latter is a drug-accumulation assay, that reveals the increase in the intracellular concentration of fluorescent compound inside resistant cells, as due to inhibition of Pgp activity. Both methods are simple and give complementary data about the interaction of new inhibitors with Pgp.

Materials and methods

Chemicals

Methyl 6-*O*-(*N*-heptylcarbamoyl)- α -D-glucopyranoside was purchased from Boehringer (Mannheim). Cyclosporin A came from Novartis (Basel, Switzerland), daunomycin was from Aventis (Vitry-Alforville, France). The latter compounds were used under their clinical formulations. All other reagents were purchased from Sigma Chemical Co. (Saint Quentin Fallavier, France).

Plant material

Stem bark of *Ficus nymphaeolia* Mill (Moraceae) was collected from the Mont Caraïbe-Vieux Fort area located in guadelupe in January 1999. The identity was verified by Dr. S. Rodin-Bercion, Laboratory COVACHIM, EA925, University of Antilles and Guyane. Roots of common licorice (*Glycyrrhiza glabra* Linn, Fabaceae) was purchased from Cooper, Melun, France. Roots of white-melilot (*Melilotus alba* Lam, Fabaceae) was collected from the Saint Priest flat country located in the Rhône department of France, in June

1998 and authenticated by Pr. J. Raynaud, Department of Botany and Pharmacognosy, Claude Bernard University, Lyon, France. Roots of sour orange (*Citrus aurantium* Linn, Rutaceae) and trifoliate orange (*Citrus trifoliata* Hort, Rutaceae) were collected in March 1998, in Corsica, France. The identity of plants was confirmed by Dr. R. Cottin from National Institut of Agronomic Research (INRA) of Corsica, France. Voucher specimens were deposited at the herbarium of the Department of Botany and Pharmacognosy, Claude Bernard University, Lyon, France.

Preparation of extracts

The powdered plant material was extracted with methanol at room temperature. The extract was filtered, concentrated under reduced pressure by rotary evaporation and taken in 300 ml of 50% aqueous methanol. It was finally extracted (3 $\times$ 150 ml each) with hexane, dichloromethane, ethyl acetate and butanol. Hexane and dichloromethane extracts of *Ficus nymphaeolia* were subjected to chromatography over a silica gel column, and eluted with hexane/dichloromethane (8:2; 6:4 and 3:7), dichloromethane, ethyl acetate and methanol. Fractions free of chlorophyll, as estimated by TLC, were combined and used for biological assays.

Fluorescence

The recombinant C-terminal cytosolic domain of Pgp, H₆-NBD2 was overexpressed and purified as described previously (Conseil et al., 1998). Fluorescence experiments were performed at 25.0 $\pm$ 0.1 °C using a SLM-Aminco 8000C spectrofluorimeter with spectral bandwidths of 2 and 4 nm, respectively, for excitation and emission. Fluorescence measurements were performed by diluting H₆-NBD2 in 1.2 ml of dialysis buffer at pH 6.8, containing 20 mM potassium phosphate, 0.5 M NaCl, 20% glycerol, and 0.01% methyl 6-*O*-(*N*-heptylcarbamoyl)- α -D-glucopyranoside, in the presence of increasing concentrations of dimethyl sulfoxide solutions of the plant extracts (0 to 14 μ g/ml). Tryptophan-intrinsic fluorescence of H₆-NBD2 was studied by scanning emission in the 310–360 nm range upon excitation at 295 nm. The binding of plant extracts was monitored by quenching of emission fluorescence produced by addition of increasing ligand concentrations. Corrections for inner-filter effect and for dimethyl sulfoxide were determined under the same conditions by using *N*-acetyltryptophanamide. Curve fitting of ligand binding related to fluorescence decrease was performed with the Graft program (Erithacus software) as described (Divita et al., 1993).

Cell culture

The human erythroleukemic cell line K562 was purchased from the American Type Culture collection. The K562/R7 Pgp-positive cell line was obtained by prolonged exposure of K562 cells to doxorubicin (Jeannesson et al., 1990).

Cells were cultured in RPMI 1640 supplemented with 10% newborn-calf serum, 2 mM glutamine, 200 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

Intracellular drug accumulation

For inhibition of Pgp-mediated drug efflux, 10⁶ human K562/R7 leukemic cells were incubated for 1 h at 37 °C in 1 ml of RPMI 1640 medium containing a final concentration of 10 µM daunomycin, in the presence or absence of plant extracts (2 µl of a 8.75 µg/ml dimethyl sulfoxide solution). Cells were then washed three times with ice-cold PBS and maintained on ice until analysis by flow cytometry on a FACS-II (Becton-Dickinson Corp., Mountain View, CA). Assays were performed in duplicate, in at least three separate experiments. Cyclosporin A, a potent modulator of Pgp, was used as a positive control at a 2 µM final concentration. The ability of plant extracts to inhibit Pgp-mediated drug efflux was quantified by comparing the shift in fluorescence

induced by these compounds to that obtained with cyclosporin A.

Results

Effect of plant extracts on the intrinsic fluorescence of Pgp cytosolic domain

A total of 20 plant extracts, from three families and five species, were assayed for direct binding to the purified C-terminal recombinant cytosolic domain H₆-NBD2 by quenching of its tryptophan intrinsic fluorescence. The results of the screening are summarized in Table 1. It appeared that all the dichloromethane extracts tested, except that of white-melilot, bound strongly to purified H₆-NBD2. Some activity was found in ethyl acetate extracts from sour orange, trifoliate orange and *Ficus nymphaeolia*. In contrast, most of the hexane extracts were inactive in our screening apart those from common licorice and white-melilot which bound to Pgp with an high affinity. Only the

Table 1. Parameters of binding to H₆-NBD2 of the different plant extracts.

Plant	Extract	Maximal quenching (%) ^a	Apparent K _D (µg/ml) ^a
Sour orange (<i>Citrus aurantium</i>)	Hexane extract	33.29 ± 5.90	3.90 ± 1.52
	Dichloromethane extract	76.25 ± 4.01	1.37 ± 0.32
	Ethyl acetate extract	73.74 ± 7.99	6.11 ± 1.61
	Butanol extract	18.38 ± 1.47	2.92 ± 0.77
Trifoliate orange (<i>Citrus trifoliata</i>)	Hexane extract	32.13 ± 2.05	5.04 ± 0.53
	Dichloromethane extract	80.36 ± 3.67	0.53 ± 0.16
	Ethyl acetate extract	45.17 ± 1.17	1.91 ± 0.16
	Butanol extract	33.55 ± 4.07	2.91 ± 1.17
Common licorice (<i>Glycyrrhiza glabra</i>)	Hexane extract	73.41 ± 5.68	2.84 ± 0.96
	Dichloromethane extract	51.41 ± 3.42	2.38 ± 1.23
	Ethyl acetate extract	33.46 ± 4.48	1.49 ± 0.32
	Butanol extract	25.48 ± 4.82	3.57 ± 2.00
White-melilot (<i>Melilotus alba</i>)	Hexane extract	61.18 ± 5.50	1.54 ± 0.40
	Dichloromethane extract	38.89 ± 5.04	7.51 ± 2.15
	Ethyl acetate extract	11.99 ± 1.63	2.12 ± 0.39
	Butanol extract	21.73 ± 1.35	2.56 ± 0.54
<i>Ficus nymphaeolia</i>	Hexane extract	18.12 ± 1.34	2.07 ± 0.60
	Dichloromethane extract	59.33 ± 5.46	22.4 ± 4.06
	Ethyl acetate extract	82.74 ± 2.93	1.19 ± 0.23
	Butanol extract	25.82 ± 2.11	6.62 ± 1.08

^a The H₆-NBD2 domain was incubated with increasing concentrations of each extract. The maximal quenching and apparent K_D values were determined by using the Graft program (Erithacus software).

active extracts were selected for further experiments with multidrug resistant cells.

Effect of plant extracts on intracellular drug accumulation

The effect of plant extracts on the intracellular accumulation of daunomycin were examined in Pgp-expressing K562/R7 leukemic cells. As shown in Figures 1a and 1b, efflux blocking values of 107.87 ± 3.60 and 96.92 ± 5.25

were observed, respectively, in the presence of the dichloromethane extracts of sour orange and trifoliate orange, at a $17.5 \mu\text{g/ml}$ concentration which is not toxic for these cells (data not shown). Efflux-blocking was at least as high as that obtained with $2 \mu\text{M}$ cyclosporin A (77.97 ± 5.32).

Similarly, the hexane extract of white-melilot (Fig. 1d) caused an increase in the cellular accumulation of daunomycin (141.46 ± 6.23) up to a value comparable to that observed with cyclosporin A (142.73 ± 3.77).

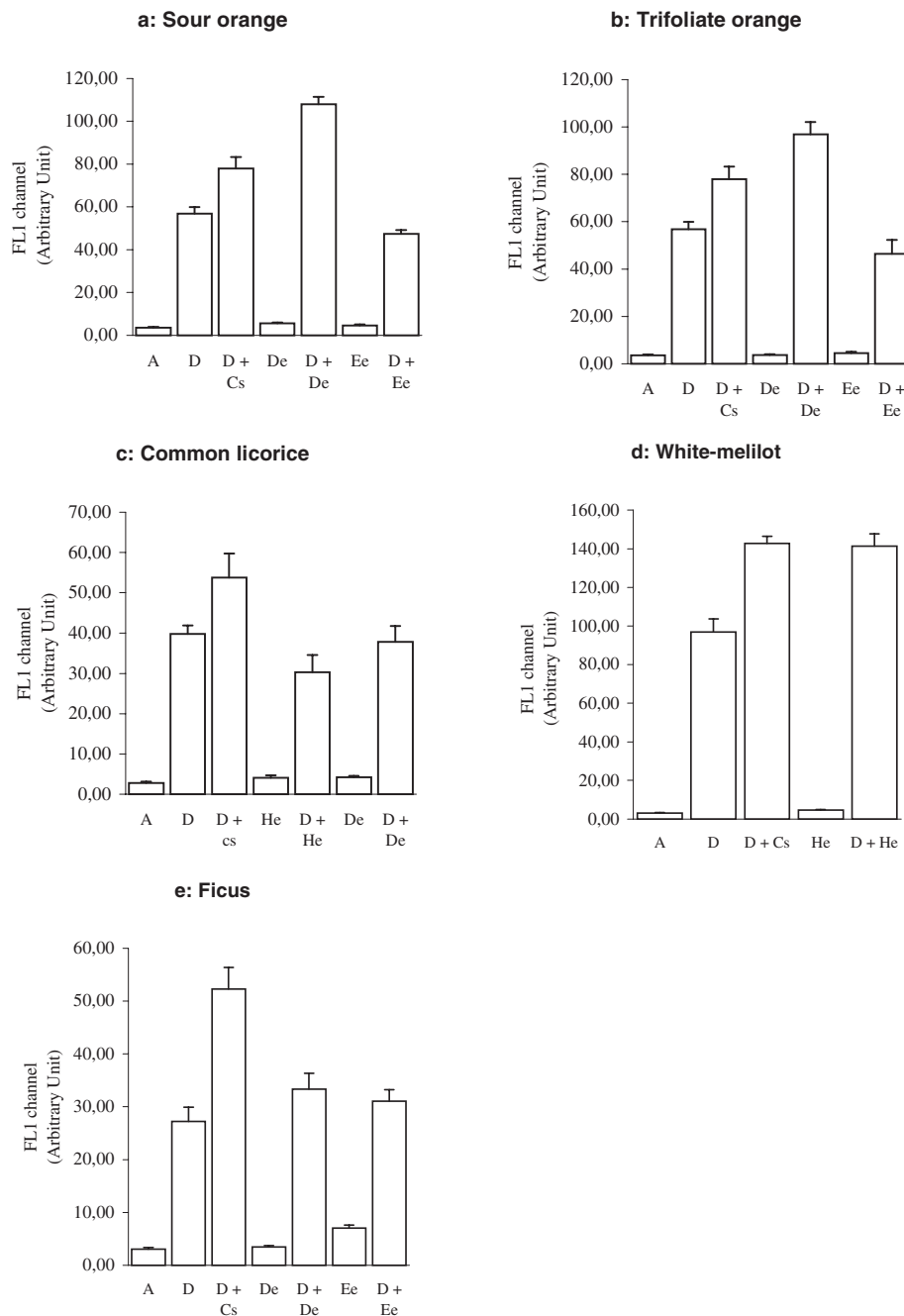


Figure 1. Effects of different plant extracts on the intracellular retention of daunomycin. Abbreviations: A, Autofluorescence; D, Daunomycin; Cs, Cyclosporin A; He, Hexane extract; Ee, Ethyl acetate extract; De, Dichloromethane extract.

In contrast, the ethyl acetate extracts of sour orange (Fig. 1a), trifoliate orange (Fig. 1b) and *Ficus nymphaeolia* (Fig. 1e), the dichloromethane extracts of common licorice (Fig. 1c) and *Ficus nymphaeolia* (Fig. 1e), as well as the hexane extract of common licorice (Fig. 1c), which are active on the purified C-terminal recombinant cytosolic domain H₆-NBD2, did not affect daunomycin accumulation inside K562/R7 cells.

Discussion

One of the important points of this study was to define a standardized extraction procedure, suitable for rapid biological screening of plant sources. The plant material is first treated with methanol, a solvent capable of extracting compounds of a wide range of polarity. After dilution to a final concentration of 50% aqueous methanol, the extract is partitioned against solvents of increasing polarity: hexane, dichloromethane, ethyl acetate and butanol (Fig. 2). Elimination of the latter pigments as chlorophyll is crucial since, due to their fluorescence properties, they may interfere strongly with one of our assay, itself based on fluorescence measurements. In the case of elevated chlorophyll content extracts like stem bark extracts (*Ficus nymphaeolia*), the pigments are spread within both the hexane and the dichloromethane extracts. In the latter case, a column of silica is necessary to completely get rid of the pigments before biological screening. Furthermore, following our extraction procedure, the constituents of the original methanolic extract are roughly separated according to their polarity. Since most known P-gp inhibitors share quite high hydrophobic properties, the less polar extracts like hexane, dichloromethane or ethyl acetate are expected to contain active compounds. On the contrary, the polar butanol and aqueous extracts are not.

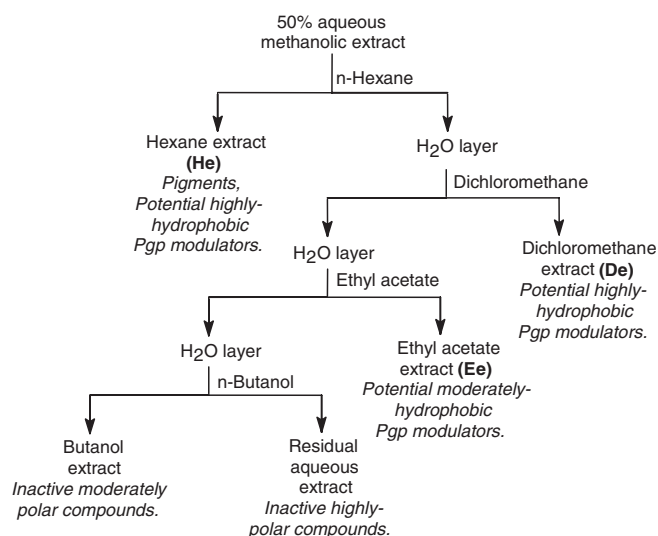


Figure 2. A standardized extraction procedure from plants, for a rapid screening of potential P-gp modulators.

Binding studies with a purified C-terminal recombinant cytosolic domain of P-gp ascertained that extracts were indeed able to target the multidrug transporter. A positive effect in the screening is detected by a marked quenching of intrinsic fluorescence, indicating modification of the tryptophan environment. Due to the high reproducibility of the method, the use of triplicates is sufficient for each sample. The results show that the dichloromethane extracts from trifoliate orange, sour orange and to a lesser extent common licorice, induced high quenching of fluorescence, suggesting that their constituents bind to a region close to the tryptophan residue of H₆-NBD2. Specific effects were displayed by the dichloromethane extract of *Ficus nymphaeolia*. In fact, a very bad apparent K_D value was recorded (22.4 µg/ml; Table 1), despite an appreciable quenching of fluorescence (59%; Table 1). The positive effect on quenching indicates that active compounds are probably present in the extract, however, at very low concentration, thus resulting in high apparent K_D value. The ethyl acetate extracts from *Ficus nymphaeolia*, sour orange and to a lesser extent trifoliate orange displayed marked quenching of fluorescence as well. Finally the hexane extracts from common licorice and white-melilot exhibited a high-affinity binding for H₆-NBD2. Thus, all these extracts contain potentially active components. It is noteworthy that none of the polar butanolic (Table 1) or residual water extracts (results not shown) displayed any activity. This is in accordance with our previous observation that polar compounds like flavonoid glycosides (Conseil et al., 1998) are ineffective in H₆-NBD2 binding.

Secondly, we investigated whether these extracts were able to enhance intracellular accumulation of P-gp substrates. Yoshimura et al. (1990) have described a method for the screening of agents able to overcome MDR which measures the increase of rhodamine 6 G accumulation in resistant cells, as caused by P-gp inhibition. We have modified this method by using daunomycin instead of rhodamine 6 G. The presence of dichloromethane extracts from sour orange, trifoliate orange or of hexane extract from white-melilot increased daunomycin accumulation in the resistant cells K562/R7 up to the levels obtained with cyclosporin A used as a positive control. It is noteworthy that the modulating effects of extracts were observed at low concentrations which were not toxic for the cells, suggesting that these extracts should be investigated *in vivo* for potential P-gp modulation in tumour cells. Such high-affinity inhibitors of P-gp might primarily bind to the cytosolic domains of the transporter, possibly inside, or close to, the ATP-binding sites. However, the dichloromethane extracts from common licorice and *Ficus nymphaeolia*, the ethyl acetate extracts from sour orange, trifoliate orange and *Ficus nymphaeolia* and the hexane extract from common licorice had no effects on the accumulation of daunomycin despite a high-affinity binding for H₆-NBD2. Constituents of these extracts might either not be able to properly cross the membrane, leading to limited accessibility to the transporter, or preferentially bind to other cellular, ATP-binding protein, targets.

In conclusion, binding to the purified C-terminal recombinant cytosolic domain of Pgp, on the one hand, and the daunomycin intracellular accumulation, on the other hand, as described here are complementary methods which constitute helpful tools for the screening and the detection of new potential inhibitors of Pgp in complex samples, such as plant extracts, that may contain very low concentrations of modulating agents. The major advantage of using a recombinant cytosolic domain of Pgp is that it allows a direct measurement of binding. An important advantage of the accumulation assay is the direct measurement of the inhibition of Pgp pumping activity. Both methods are highly sensitive and may be automated, allowing the handling of high numbers of samples, as required for a high-throughput screening. An extensive search for more potent Pgp inhibitors is indeed expected to lead to a significant improvement in anticancer chemotherapy.

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