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LC determination of Indinavir in biological matrices with electrochemical detection

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Abstract

A high performance liquid chromatographic (HPLC) method with electrochemical detection for the quantification of Indinavir in cell culture is described. The sample pre-treatment involved a protein precipitation procedure using acetonitrile. Chromatography was carried out on a base-deactivated reversed-phase column with an isocratic mobile phase. The method was validated with regard to specificity, linearity, limits of detection and quantitation, precision and accuracy, recovery and ruggedness. The proposed HPLC assay was utilised to directly evaluate the capability of P-glycoprotein expressing multidrug resistant cells in mediating the transport and efflux of protease inhibitor (PI) Indinavir, a basic compound in AIDS care. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The emergence of HIV-1 mutants in the *pol* gene and the corresponding amino acid substitutions on the reverse trascriptase (RT) protein is responsible for the selection of virus variants less susceptible to the current anti-retroviral therapy [1,2]. However, in vitro studies conducted in this and other laboratories have demonstrated that different cellular mechanisms, definitely resulting in the decrease of nucleoside analogous biodisponibility, might be also involved in the emergence of the drug resistance [3–7]. Unfortunately, this phenomenon seems to include the recently adopted chemotherapy based upon the administration to the AIDS patients of a single or combinations of the different commercially available protease inhibitors (PIs) [8]. In fact, the MDR1-P-glycoprotein that mediates PIs efflux and transport seems to be directly involved in the emergence of this form of drug-resistance [9,10]. In these studies, the PIs efflux P-glycoprotein mediated has been indirectly evaluated by measuring the transport inhibition of the well known P-glycoprotein fluorescence substrates daunorobicin or the FITC-labelled antitumoral drugs [11].

By developing a novel high performance liquid chromatographic (HPLC) method capable of directly determining very low Indinavir amounts in

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the cell culture supernatant, we intended to furnish a new and very effective tool for studying the cellular mechanisms involved in PIs transport and resistance.

Several HPLC methods to determine the Indinavir concentration in biological fluids with the use of UV detection at 210 nm have been reported [12–15]; however these methods have shown their limits in both sensitivity and specificity, bearing the risk of interferences from other compounds potentially present in the biological fluid. The Indinavir chemical structure with some oxidation sites [16] suggested that a very effective assay could be developed by coupling HPLC with electrochemical detection.

In this paper, a new and original HPLC system is described and its suitability for the determination of very low amounts (3–5 ng ml^{-1}) of Indinavir in cell culture is assessed. The sensitivity and specificity of this technique allow the study of Indinavir metabolism and transport also in those cells, such as infected lymphocytes and macrophages, where the reduced level of the P-glycoprotein expression and function may seriously limit the effectiveness on an indirect evaluation of the drug transport or metabolism.

2. Experimental

2.1. Chemicals

Indinavir was obtained from Merck & Co. Inc. (Whitehouse Station, NY, USA). Verapamil (Isoptin) was supplied by Knoll Farmaceutici (Milano, Italy). Acetonitrile was of HPLC grade (Lab-Scan Analytical Science, Dublin, Ireland). Water was bidistilled and all the other chemicals were of analytical reagent grade.

2.2. Chromatographic system

The chromatographic system consisted of a Series 410 LC Pump (Perkin Elmer, Norwalk, CT, USA), a Rheodyne Model 9125 injection valve (Rheodyne, Berkeley, CA, USA), and a Coulochem II electrochemical detector (ESA, Belford, MA, USA) equipped with a 5011 analytical cell. For the electrochemical detector (ED) the working parameters were $+400$ mV for the first electrode and $+750$ mV for the second; these settings were found to provide optimal detection conditions. The signals generated by the second electrode were used for the quantitation. For ED data collection and calculations a software Turbochrom 4 (Perkin Elmer) was used.

2.3. Chromatographic conditions

The column was a 5 μm Hypersil 120-5 BDS C18, 250×3 mm I.D. (Macherey-Nagel, Düren, Germany). The elution was carried out under isocratic conditions at room temperature and at a flow rate of 0.6 ml min^{-1}. The mobile phase consisted of sodium dihydrogen phosphate (pH 6.3; 10 mM) acetonitrile (65:35 v/v). The injection volume was 20 μl. The mobile phase, filtrated through a 66 nylon membrane (0.45 μm) before use, was continuously recycled to the solvent reservoir and freshly prepared weekly.

Cleaning of the electrode system was performed weekly with a mixture of methanol–water (70:30 v/v).

2.4. Standard solutions

Stock solutions of Indinavir were prepared by dissolving the appropriate amount of Indinavir, accurately weighed, in acetonitrile:water (50:50, v/v) to yield a final drug concentration of 200 μg ml^{-1}. All stock solutions were stored at -20°C and no significant degradation occurred over a period of 30 days.

Working solutions of 6, 25, 100, 300, 500, 1000 ng ml^{-1} were prepared by adding the appropriate amount of Indinavir stock solution to blank cell medium and treating and processing them as samples.

2.5. Cells and media

For this study, the parental drug-sensitive human T-lymphoblastoid CEM cells and their derivative MDR variant CEM-VBL100 were used. The cells growth was sustained by basic medium (BM) consisting of RPMI-1640 (Euro-

clone Ltd., UK) enriched with 10% foetal bovine serum (FBS, HyClone Laboratories Inc., Logan, Utan, USA) and antibiotics using standard conditions for mammalian cells growing in suspension. The MDR phenotype was routinely tested by determining the binding level to cells of the Mab MM4.17 specifically reacting with an external P-glycoprotein epitope. Flow-cytometry procedures and cells staining techniques were previously described [17]. The P-glycoprotein drug transporter activity was evaluated according to the detailed procedures elsewhere described [18].

2.6. Incubation conditions

Extensively washed drug sensitive/MDR CEM cell pairs were pelleted and resuspended with 500 μl of RPMI-1640 containing 100 μg of Indinavir. As control, parallel incubations of cells with Indinavir and Verapamil, an MDR reversing modulating agent (RMA), were also performed to control the PI retention in MDR cells. After 1 h at 37°C the cells were washed with cold phosphate buffer solution (PBS), pelletted by centrifugation (1500 rpm × 5 min) and again resuspended in 500 μl of RPMI-1640 for the scheduled times. After centrifugation 200 μl of each supernatant sample were recovered and treated as further described.

2.7. Sample preparation

To the cell medium sample (200 μl, in a centrifugation tube) 1 ml of acetonitrile was added. The tube was shaken with a vortex mixer for 2 min and then centrifuged at 1800 rpm for 8 min. The supernatant was evaporated under vacuum at 25°C and the dried residue was reconstituted with 100 μl of distilled water. Aliquots (20 μl) of the resulting solution were analysed directly by HPLC to assess the amounts of released Indinavir. All the samples were stored at −20°C until analysis.

3. Results and discussion

Detector settings reported under Section 2 were the result of an optimisation process aimed at obtaining maximum sensitivity with sufficient specificity. The two electrodes were used in the oxidation screen mode. For the determination of the optimum working parameters for the electrochemical detection of Indinavir, the current–voltage curve was investigated. The curve was obtained by varying the downstream electrode potential from +400 to +900 mV. Fig. 1 shows the hydrodynamic voltammogram of Indinavir standard solution. The curve showed a voltage dependent increase starting from +500 mV before reaching the final plateau from +750 mV. Consequently, the first electrode potential was set at +400 mV, near the low end of the oxidation–voltage curve, to remove compounds with lower oxidation potential than the analyte. For the detection, +750 mV was used because the response of the compound reached a plateau at this value. Under these conditions, the detection of Indinavir showed to be twice more sensitive than that obtained with an UV detector set at 210 nm [12–15].

The assay was selective for Indinavir in cell culture medium and chromatograms of blank cell culture medium did not show any peak at the retention time of Indinavir (Fig. 2).

3.1. Specificity

The specificity of the assay was determined by the analysis of a blank medium incubated with cells as described. There were no interfering peaks at the retention time for Indinavir peak. The RMA Verapamil used in the studies did not show interference with the analytical method.

3.2. Linearity

The linearity was calculated by linear regression in the range from 6 to 1000 ng ml^{-1} giving a correlation coefficient of 0.998. The equation for the calibration curve was $y = 11\,500x + 91\,793$. The coefficients of variation of the slope and the intercept were 2.9 and 2.4, respectively.

3.3. Precision and accuracy

In Table 1 precision and accuracy are reported for cell culture medium and supernatant. The intra-day precision calculated as the coefficient of

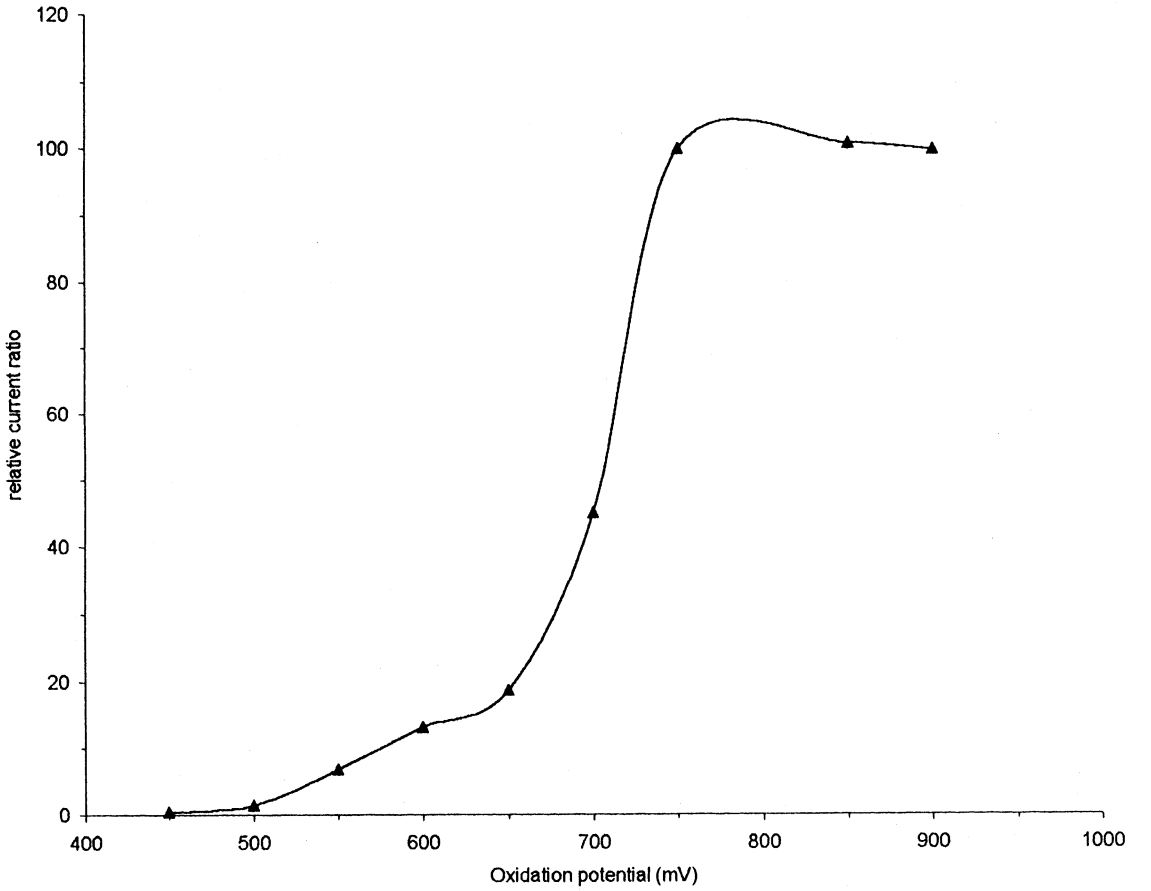

Fig. 1. Hydrodynamic voltammogram of Indinavir standard solution.

Table 1
Determination of Indinavir in cell culture — precision and accuracy

Nominal standard concentration (ng ml^{-1})	Calculated concentration (ng ml^{-1}) mean ± S.D.	Accuracy (%)	CV
Intra-day determination[a]			
100	100.15 ± 1.04	100.1	1.04
300	283.36 ± 17.25	94.4	6.09
500	494.71 ± 14.27	98.9	2.88
Inter-day determination[b]			
100	96.76 ± 3.20	96.8	3.31
300	283.59 ± 2.62	94.5	0.92
500	472.12 ± 16.81	94.4	3.61

[a] Results are the mean of five experiments.
[b] Results are the mean of two experiments a day over a period of 5 days.

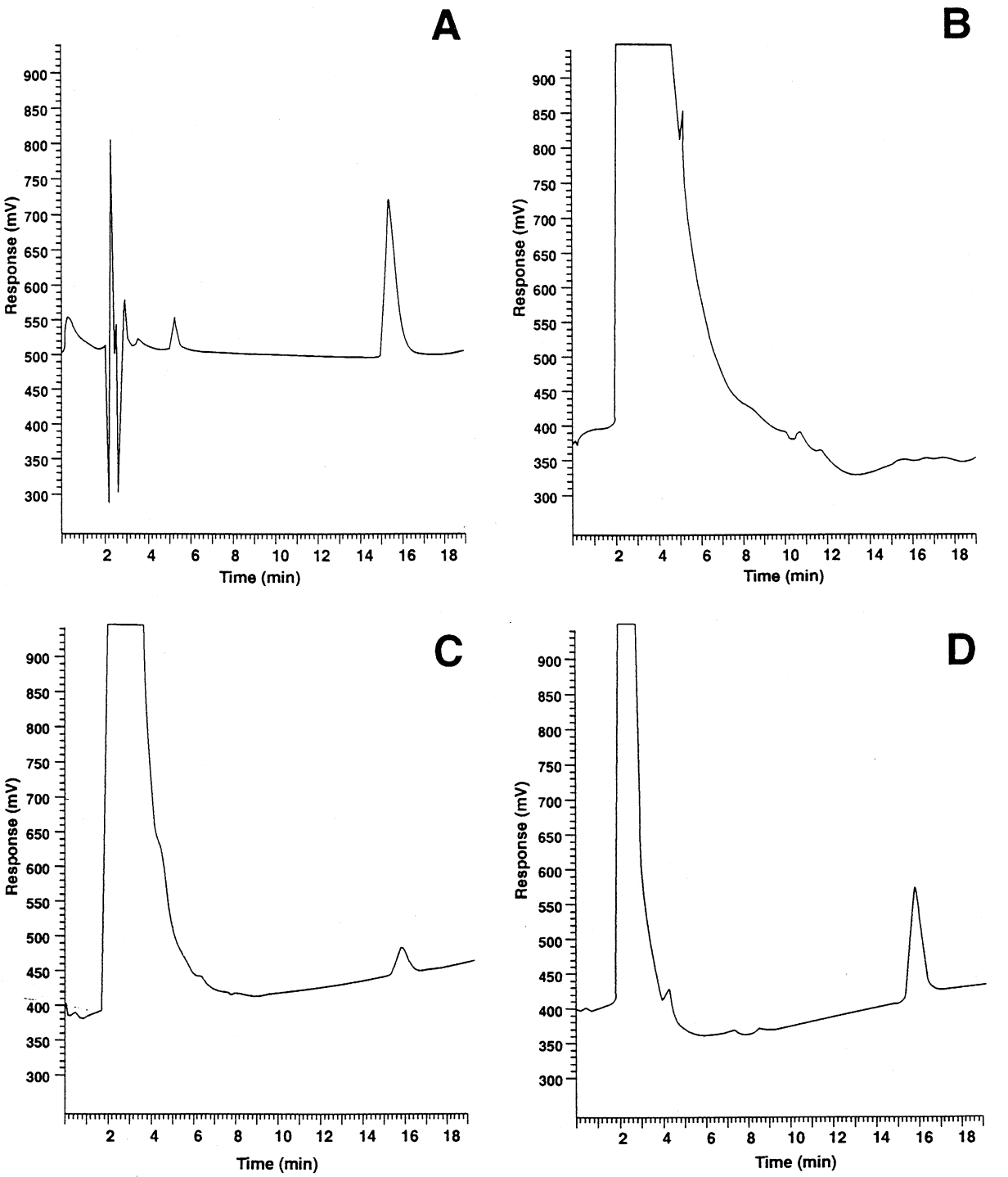

Fig. 2. Typical chromatogram of (A) standard solution containing 1 μg ml^{-1} of Indinavir; (B) extract of a blank cell medium; (C) extract of a cell medium spiked with a 200 ng ml^{-1} Indinavir solution; (D) extract of a cell culture incubated with Indinavir for 30 min as described in the text.

Table 2
Recovery of Indinavir from cell culture

Concentration (ng ml^{-1})	Recovery (%)[a]	CV
6	97.2	0.1
25	99.0	0.1
100	100.0	0.2
300	99.9	0.9
500	98.1	4.0
1000	95.4	3.7

[a] Results are the mean of three experiments.

variation (CV) was always below 7% and ranged from 1.04 and 6.09%. The mean accuracy for calculated Indinavir concentrations ranged from 94.4 to 100.1%. The inter-day CV ranged form 0.92 to 3.61 and the accuracy was between 94.4 and 96.8%.

3.4. Limits of detection and quantitation

The limit of detection was defined by the injected amount giving a peak with a height at least three times as high the baseline noise level and it resulted to be 2 ng ml-1. The limit of quantitation was determined using the EURACHEM approach [19]. A number of samples with decreasing amounts of Indinavir were injected three times. The calculated precision was plotted against the Indinavir amount. The amount that corresponds to the definite precision of 12% is the limit of quantitation and it resulted to be 4 ng ml^{-1}.

3.5. Recovery

The experimental recovery for Indinavir extracted from cell culture was determined in triplicate by comparing the areas under the peaks obtained from suitable dilutions of the standard stock solution with the peak areas from extracted cell culture to which known amounts of the analyte were added (working solutions). The recovery ranged from about 95 to 100% (Table 2).

3.6. Ruggedness

The determination of Indinavir in cell culture was not affected by slight variations either in the pH value of the phosphate buffer or in the electrode potential. On no occasion interferences from the compounds present in the cell medium were observed.

Slight variations in retention times were observed using mobile phases prepared on different days by different analysts and injecting the sample solution on three columns of the same manufacturer containing the same brand of packing material.

3.7. Applicability of the method

The present method for determining very low amounts of Indinavir in cell culture supernatant has been used to evaluate the ability of P-glycoprotein in mediating the PI transport. As it is shown in Fig. 3A, CEM–VBL 100 cells are characterised by the expression of the P-glycoprotein multidrug transporter system which, conferring the MDR phenotype, makes these cells capable of mediating the efflux of different cytotoxic compounds including the fluorescence substrate daunorobicin. In Fig. 3B it is shown that the efflux of this drug is inhibited by RMA or by an excess of Indinavir. These observations indirectly demonstrate that the PI Indinavir is mediated by P-glycoprotein expressing MDR cells.

After 120 min incubation, 3.24 ($\pm$ 0.12) μg ml^{-1} of Indinavir were released by the cells in the culture supernatant. The amount of this drug determined in the various cell samples increased in parallel with incubation periods. In fact 3.70 ($\pm$ 0.08) and 4.52 ($\pm$ 0.40) μg ml^{-1} of Indinavir were assessed after additional 5 and 10 min of incubation. However, in more prolonged periods, the amount of Indinavir detected in the culture supernatant decreased, as a consequence of re-adsorption phenomena.

In contrast, the treatment of the cells with the RMA Verapamil, by inhibiting P-glycoprotein function, strongly reduces the efflux of Indinavir out of the cells. The drug amounts determined by HPLC after 125 and 135 min incubation were 1.79 ($\pm$ 0.13) and 2.17 ($\pm$ 0.12) μg ml^{-1}, respectively.

The differences in Indinavir concentration values obtained in presence or absence of the RMA Verapamil confirm the role of the multidrug transporter P-glycoprotein in mediating the efflux and transport of the PI Indinavir.

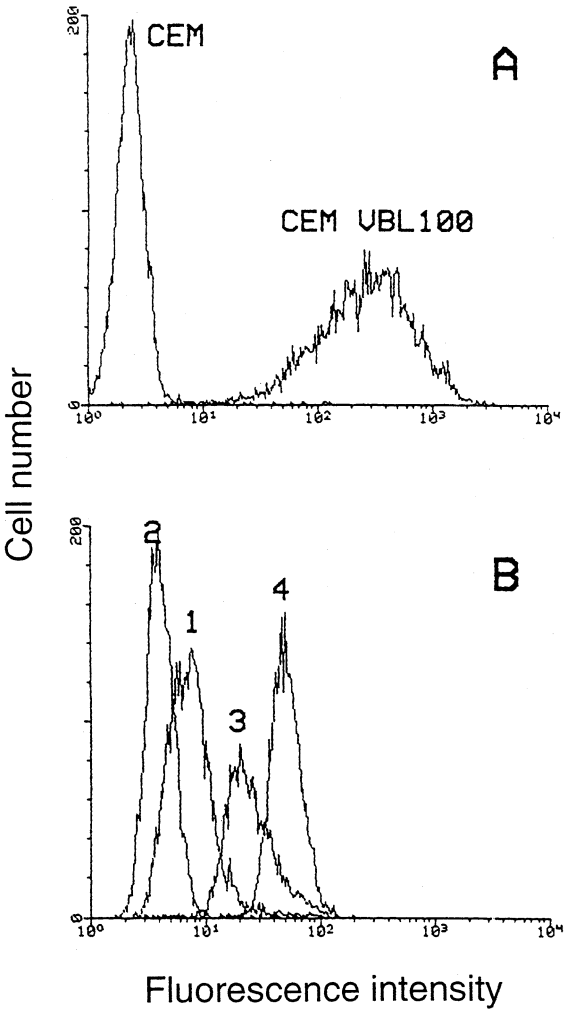

Fig. 3. Flow citometry determination of P-glycoprotein expression and function. Panel A, CEM cells react with the MAb MM4.17 to P-glycoprotein only in concomitance with the expression of the MDR phenotype (CEM–VBL 100). These MDR cells are capable to actively transport the fluorescence substrate daunorobicin. Panel B, the fluorescence profile of CEM–VBL 100 cells after 60 min incubation with daunorobicin is strongly reduced after an additional 60 min incubation at 37°C (profiles 1 and 2, respectively). The P-glycoprotein drug transport function is partially inhibited by treating the MDR cells with the RMA Verapamil or the PI Indinavir (profiles 4 and 3, respectively).

4. Conclusions

The HPLC assay here described represents an improved methodology to determine the PI Indinavir in organic solution such as FBS-enriched cell culture supernatant. In fact, this assay is precise and linear over the range studied and it implies a very easy sample preparation. The high specificity and sensitivity, compared with the current UV assay, of this original procedure in directly quantifying very low amounts of Indinavir allow its use for studying the role of PIs in cellular mechanisms such as the drug transport and efflux P-glycoprotein mediated.

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