Intracellular concentration of the HIV protease inhibitors indinavir and saquinavir in human endothelial cells

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Human vascular endothelial cells may serve as targets and a reservoir for human immunodeficiency virus type 1 (HIV-1). The antiviral activity of HIV protease inhibitors is reported to be related directly to the intracellular amount of the drug. To assess intracellular concentrations of two HIV protease inhibitors, human umbilical venous endothelial cells (HUVECs) were exposed for 3 h and 24 h to 100, 10 and 1 mg/L indinavir and saquinavir. Intracellular drug concentrations and the total drug amount in the supernatant were measured by means of high-performance liquid chromatography (HPLC). Exposure of HUVECs to 10 and 1 mg/L indinavir and saquinavir resulted in undetectable intracellular drug levels in \(6 \times 10^5\) cells/well. Incubation of cells with solutions of 100 mg/L indinavir and saquinavir led to mean intracellular concentrations of indinavir (132 ± 56 mg/L after 3 h and 150 ± 29 mg/L after 24 h, respectively) and of saquinavir (96 ± 10 mg/L after 3 h and 100 ± 5 mg/L after 24 h) that were comparable to the levels determined for the substances in the supernatant over time (\(P > 0.001\)). These data indicate that intracellular concentrations of indinavir and saquinavir correlate well with the extracellular levels. Consequently, measurements of drug concentrations in patient’s plasma by HPLC are assumed to be a good means of monitoring the intracellular drug concentration.

Introduction

HIV protease inhibitors (PIs) are widely used to reduce viral replication in order to reverse or stop the pathogenic process of HIV infection and to improve survival and decrease mortality in HIV-infected patients. Human vascular endothelial cells have been implicated in the dissemination of human immunodeficiency virus type 1 (HIV-1) by being activated by HIV-1 to induce the expression of various adhesion molecules that may play a role in the extravasation of HIV-infected cells.\textsuperscript{1} Additionally, it has been shown that HIV infection alters the interaction of monocytes with endothelial cells (ECs).\textsuperscript{2} This effect is mediated through the interaction of HIV-tat, a viral gene product essential for HIV replication.\textsuperscript{2} Furthermore, results of an \textit{in vitro} study confirmed that ECs serve as targets and reservoirs for HIV.\textsuperscript{3}

Billelo and co-workers\textsuperscript{4} demonstrated that in HIV-1-infected peripheral blood mononuclear cells and cell lines the antiviral activity of PIs is highly correlated with the intracellular amount of these drugs.

Therefore we performed a series of experiments to examine intracellular concentrations of two HIV PIs, indinavir and saquinavir, using an \textit{in vitro} system of human umbilical venous endothelial cells (HUVECs). Furthermore, we evaluated the influence of these agents on the expression of adhesion molecules on HUVECs.

Materials and methods

Endothelial cells were prepared using human umbilical veins. Cells were isolated and cultured according to a modified standard procedure.\textsuperscript{5} The confluent primary monolayers consisted of c. 8 000 000 cells/flask. Only cells from these first subcultures were used for the experiments. Since no differences are reported in the kinetics of HIV PI uptake by uninfected and HIV-infected cells we carried out the experiments with uninfected cells.\textsuperscript{5}

Indinavir sulphate (Merck Research Laboratories, Rahway, NJ, USA) and saquinavir mesylate (Hoffmann-La Roche, Inc., Nutley, NJ, USA) were each dissolved in

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distilled water and diluted in cell culture medium containing 10% fetal calf serum (FCS) to final concentrations of 100, 10 and 1 mg/L.

For the experiments the culture medium was removed and the cell layers were washed with Dulbecco’s phosphate-buffered saline (PBS; Gibco, Paisley, Scotland, UK). Indinavir and saquinavir were added to the endothelial cells at final concentrations of 100, 10 and 1 mg/L, and incubated for 3 h and 24 h. All incubations were carried out in a humidified incubator at 37°C under 5% carbon dioxide.

Concentrations of indinavir and saquinavir were measured by means of modified high-performance liquid chromatography (HPLC). Compounds were separated by injecting 100 μL of the neutralized supernatant on to a C18 reversed-phase column (Chemcon, Vienna, Austria) using a gradient (buffer A, 0.15 mol/L KH2PO4, pH 3.45; buffer B, 0.15 mol/L KH2PO4:acetonitrile 50:50, pH 3.45). A linear gradient rising from 0 to 100% buffer B in 40 min was used with a total running time of 60 min and a retention time of 3.5 min (indinavir) and 8 min (saquinavir).

The flow rate was 1.2 mL/min and the detection was made at a wavelength of 214 nm. Linearity of the method was obtained in the concentration range 10–10 000 ng/mL for the two substances. The average recovery of each of the two PIs was determined in three analytical runs (10–1000 ng/mL, 10–10 000 ng/mL and 10–100 000 ng/mL) by calculating the ratio of the slope of a calibration curve in HPLC buffer without protein and the slope of a calibration curve in extracted cell culture medium. A concentration of 10 ng/mL defined the lower limit of quantification. The stability of indinavir and saquinavir under various conditions has already been investigated.

In order to evaluate intracellular drug concentrations, the supernatant was aspirated after 3 h and 24 h exposure of the HUVECs to the drugs and the cells were washed three times with 2 mL PBS/well. Subsequently 1 mL lysis buffer (0.5 mol/L perchloric acid) was added to each well and the six-well plates were put into a −30°C freezer for 30 min. Afterwards the well content was homogenized. The content of each well was transferred to an Eppendorf tube with 0.6 mL buffer (1 mol/L Na2HPO4/1 mol/L NaH2PO4, pH 7.4). After centrifugation (3 min, 15 000 rpm) the drug concentration in the supernatant was analysed by means of modified HPLC as described above.

The intracellular volume was calculated after determination of the cell pellet volume (after centrifugation, 10 min, 600g). Experiments performed in our laboratory showed that a pellet volume of $2 \times 10^7$ cells corresponds to 100 μL and that this volume is comparable to the mean cell density ($1.76 \times 10^8$ cells/cm$^3$) in human liver.

The detection of cell surface protein expression was performed by means of an enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis, MN, USA).

Results

In this report, we examined intracellular concentrations of two HIV PIs using an in vitro system within HUVECs. All experiments were performed in duplicate consecutively in order to prove reproducibility of the results. The recovery of the compounds in cell culture medium 199 containing 10% FCS is presented in the Table.

The intracellular concentrations of indinavir and saquinavir after exposure of HUVECs to 100 mg/L of these drugs for 3 h and 24 h were comparable to the extracellular quantities ($P = 0.22$, $P = 0.26$ and $P = 0.025$, $P = 0.02$ for indinavir and saquinavir after 3 h and 24 h exposure, respectively) (Table). Exposure of HUVECs to 10 mg/L resulted in concentrations close to the detection limit (data not shown). Incubation with 1 mg/L led to undetectable intracellular concentrations of these drugs.

Additional investigations were performed to assess the influence of these PIs on the expression of cell surface proteins. In contrast to interleukin 1 (IL-1) the drugs did not induce the cell surface expression of endothelial leucocyte adhesion molecule 1 (ELAM-1), vascular cell adhesion molecule 1 (VCAM-1) and of intercellular adhesion molecule 1 (ICAM-1, Figure).
Intracellular concentration of protease inhibitors

Discussion

The use of highly active antiretroviral therapy to control HIV-1 infection is in many ways a success story, but high concentrations of PIs are required to suppress HIV replication in chronically infected cells, such as monocytes/macrophages and lymphocytes.9

Based on the results reported by Corbeil et al.3 that ECs serve as targets and reservoirs for HIV-1 we employed an in vitro system of HUVECs to determine extracellular and intracellular concentrations of indinavir and saquinavir. The recovery rates of these drugs in the cell culture medium were 83–89%, and the intracellular amounts of these PIs correlated well with those in the supernatant, indicating a passive uptake of the drugs as reported by Bilello et al.4

Indinavir and especially saquinavir are highly bound to plasma proteins, predominantly to α1-acid glycoprotein.10 The amount of PIs entering cells is inversely proportional to the concentration of α1-acid glycoprotein and directly proportional to the amount of extracellular non-protein-bound drug.4 The specific binding to α1-acid glycoprotein of these PIs was not analysed, but the binding to the total protein in the supernatant can be calculated from the Table.

Before the HPLC analyses a protein precipitation was performed. This procedure resulted in no loss of PIs when the drugs were diluted in cell culture medium without FCS or in cell culture medium with 10% FCS as shown in the Table. Since antiviral activity of PIs is related directly to the intracellular concentration, protein binding influences the inhibition of HIV protease.4

The efflux of the drugs was not determined in this study. However, it has been demonstrated previously that the efflux of the PI A-80987 is rapid and dependent on the concentration–time profile of the drug at the site of action.4 Very recently the interaction of PIs with the multidrug transporter P-glycoprotein (P-gp) was the subject of one study.11 Data from this investigation suggest that saquinavir is an inhibitor and possibly a substrate of P-gp, in contrast to indinavir.11 Since P-gp is expressed in the intestinal mucosa and in epithelial cells at the blood–brain barrier, the limiting oral absorption and CNS exposure to saquinavir may be explained by the interaction of saquinavir with P-gp.11,12

Our study addressed a second issue: do PIs induce HUVECs to express adhesion molecules? In contrast to IL-1, indinavir and saquinavir did not influence the cell surface expression of ELAM-1, VCAM-1 or ICAM-1, which play a role in the extravasation of HIV-infected cells.1

The data presented herein demonstrate clearly that the intracellular and extracellular amounts of PIs correlate well. Consequently, we assume that measurements of drug

<table>
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<tr>
<th>Concentration of PIs</th>
<th>Concentration of PIs in mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>indinavir</td>
<td>0.825</td>
</tr>
<tr>
<td>saquinavir</td>
<td>0.837</td>
</tr>
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</table>

Table. Recovery of PIs in medium 199 containing 10% FCS

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>µg/10⁶ cells after 3 h</th>
<th>mg/L cell volume after 3 h</th>
<th>Sample no.</th>
<th>µg/10⁶ cells after 24 h</th>
<th>mg/L cell volume after 24 h</th>
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</thead>
<tbody>
<tr>
<td>Indinavir</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.440</td>
<td>88</td>
<td>5</td>
<td>0.651</td>
<td>130</td>
</tr>
<tr>
<td>2</td>
<td>0.403</td>
<td>81</td>
<td>6</td>
<td>0.595</td>
<td>119</td>
</tr>
<tr>
<td>3</td>
<td>0.835</td>
<td>167</td>
<td>7</td>
<td>0.859</td>
<td>172</td>
</tr>
<tr>
<td>4</td>
<td>0.960</td>
<td>192</td>
<td>8</td>
<td>0.883</td>
<td>177</td>
</tr>
<tr>
<td>mean</td>
<td>0.660 ± 0.28</td>
<td>132 ± 56</td>
<td>mean</td>
<td>0.747 ± 0.15</td>
<td>150 ± 29</td>
</tr>
<tr>
<td>Saquinavir</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.533</td>
<td>107</td>
<td>13</td>
<td>0.480</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>0.467</td>
<td>93</td>
<td>14</td>
<td>0.533</td>
<td>107</td>
</tr>
<tr>
<td>11</td>
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<td>83</td>
<td>15</td>
<td>0.507</td>
<td>101</td>
</tr>
<tr>
<td>12</td>
<td>0.493</td>
<td>99</td>
<td>16</td>
<td>0.483</td>
<td>97</td>
</tr>
<tr>
<td>mean</td>
<td>0.477 ± 0.05</td>
<td>96 ± 10</td>
<td>mean</td>
<td>0.501 ± 0.025</td>
<td>100 ± 5</td>
</tr>
</tbody>
</table>
concentrations in patient’s plasma by HPLC are a good means to monitor the intracellular levels of these two PIs. Nevertheless, investigations are necessary using drug concentrations equivalent to those in patient’s plasma. Furthermore, patients’ plasma concentrations have to be compared with the intracellular amounts of the same compounds in patients’ lymphocyte and macrophage cultures in order to be able to ascertain the clinical relevance of the data from our study. Similar effort should be devoted to carefully evaluating the intracellular pharmacokinetics of existing and future PIs.

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References


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