P-glycoprotein (Pgp) is a 170-kDa membrane transporter that mediates drug efflux and is an effector of multidrug resistance. SDZ PSC 833 (PSC), a nonimmunosuppressive cyclosporine that potently modulates Pgp, is currently under clinical evaluation in patients with cancer. We have developed a reversed-phase HPLC assay for determining PSC blood concentrations that utilizes a step gradient with linear segments to resolve PSC into two distinct peaks (likely to be keto and enol isomers). To clinically validate the assay, PSC concentrations were obtained by HPLC from nine patients receiving oral doses of 5 mg/kg every 6 h. Values ranged from 0.91 to 5.4 mg/L during the dosing period, comparable with concentrations of PSC that modulate Pgp in vitro. In addition, we investigated the immunoreactivity of the Abbott TDx cyclosporin A (CsA) monoclonal whole-blood assay for PSC. The TDx CsA assay cross-reacts ~17% with PSC as determined by adding known amounts of PSC to whole blood. When PSC concentrations obtained by the TDx CsA assay were divided by 0.17, we found agreement between the TDx CsA assay and the HPLC PSC assay for samples from nine patients.

INDEXING TERMS: P-glycoprotein • multidrug resistance • cyclosporine

A frequent limitation of successful chemotherapy in the treatment of cancer is the development of resistance to antineoplastic agents. Often these cells are resistant to multiple, unrelated chemotherapeutic agents. This phenomenon, referred to as multidrug resistance (MDR), may be caused by overexpression of the mdr1 gene, the product of which is a 170-kDa transmembrane glycosylated protein, P-glycoprotein (Pgp) [1–4]. Pgp is an ATP-dependent transporter that “pumps” hydrophobic molecules, including many chemotherapeutic agents, out of cells. The physiological role of Pgp is unclear, but it is related to the ATP-binding family of transporters that includes the cystic fibrosis transmembrane regulator [5].

The role of Pgp in MDR has led to efforts to modulate Pgp activity. A number of drugs modulate Pgp-mediated efflux, including calcium channel blockers, local anesthetics, calmodulin antagonists, and cyclosporins [6, 7]. Of these agents, cyclosporin A (CsA) has clinical potential, as the concentration required to modulate Pgp-mediated efflux in vitro is achievable in patients [8–10]. Because CsA is immunosuppressive and exhibits known toxicities during long-term therapy [11, 12], other nonimmunosuppressive analogs of CsA have been examined for their Pgp modulating activity. SDZ PSC 833 (PSC) is a nonimmunosuppressive analog that is ~10-fold more potent than CsA in reversing MDR in vitro [13–16]. One–two mg/L of PSC fully reverses Pgp in vitro, and these concentrations are achievable in the blood of healthy volunteers without significant side effects [13–17].

PSC is a cyclic undecapeptide differing from CsA at amino acids 1 and 2 [13]. At position 1, PSC has a 3-keto group modification (3-keto Bmt) of the unusual amino acid N-methyl-4-butanyl-4-methyl threonine (MeBmt) present in CsA. At position 2, PSC contains valine, like CsD, rather than α-aminobutyric acid, which is present in CsA. The other nine amino acids in PSC are identical to those in CsA. Because of their similarity in structure, it is postulated that PSC may cross-react with some CsA-specific immunoassays and, thus, the use of CsA immunoassays may ultimately be acceptable for monitoring PSC. Nevertheless, it is important to initially develop a PSC-specific method such as HPLC to determine the extent of any cross-reactivity and then to confirm the...
feasibility of CsA immunoassays to monitor PSC [18, 19]. Indeed, because of its complex metabolism and the cross-reactivity of CsA metabolites with CsA-specific immunoassays, HPLC has remained the established “gold standard” for monitoring blood concentrations of CsA [18, 19]. We report the development of a simple robust HPLC method for quantification of blood concentrations of PSC and examine the cross-reactivity of PSC in the Abbott TDx CsA monoclonal immunoassay.

**Materials and Methods**

**Reagents.** PSC (batch number 90902) was kindly provided by Sandoz Pharmaceuticals Corp., East Hanover, NJ. The HPLC internal standard, CsD, was also obtained from Sandoz by the Barnes Hospital Clinical Laboratories. Burdick and Jackson HPLC-grade acetonitrile (ACN), methanol (MeOH), and hexane were obtained from Baxter Healthcare Corp., McGaw Park, IL. Deionized H2O in all procedures was obtained from a MilliQ® system (Millipore Corp., Bedford, MA). 

PSC and CsD solutions (1 g/L) were prepared by adding 5 mg of PSC or CsD to 5 mL of MeOH. Stock solutions (100 mg/L) of PSC and CsD were prepared by making 1:10 dilutions of the 1 g/L solutions in MeOH. These solutions were aliquoted and stored at −70 °C. Whole-blood (K+EDTA anticoagulant) pools used for preparation of calibrators and quality-control (QC) material were made with excess sample received by the Barnes Hospital Clinical Laboratories before discarding. Samples were restricted to those from private physician office outpatients that did not have requests for therapeutic drug monitoring. PSC calibrators containing 10, 7.5, 5, 2.5, and 1 mg/L were prepared before each run by adding 100, 75, 50, 25, and 10 μL of the 100 mg/L stock PSC to aliquots of the whole-blood pool to a final volume of 1 mL. Fifty microliters of the 100 mg/L CsD stock was added to 1 mL of all samples (calibrators, QC material, and unknowns) to provide a 5 mg/L internal standard. Control samples containing 7.5 and 2.5 mg/L PSC were prepared in whole blood and 1-mL aliquots frozen at −70 °C.

The CsA-specific immunoassay is a fluorescence polarization immunoassay performed on the Abbott TDx analyzer (Abbott Labs., Abbott Park, IL) [20]. To establish the cross-reactivity of PSC in the TDx CsA assay, CsA-free whole-blood samples were prepared to contain 2.5 and 5.0 mg/L PSC from the 100 mg/L PSC stock solution.

**Patient material.** Whole blood was obtained from patients with advanced cancers enrolled in a phase I trial for paclitaxel and PSC at Washington University School of Medicine. This trial received approval of the Washington University School of Medicine Human Studies Committee, and patients enrolled in the trial provided informed consent. All patients were given a total of 10 or 12 oral doses of 5 mg/kg of PSC every 6 h. Patients were given 86–210 mg of paclitaxel (40.5–105 mg/m2) by continuous intravenous infusion over a 3-h period beginning 2 h after the fifth dose of PSC.

**Chromatography.** Chromatography was performed on a gradient HPLC system consisting of two Waters 510 pumps, a Waters 717 plus autosampler, a column heater, and a Waters 486 tunable absorbance detector set at 203 nm (Waters Corp., Milford, MA). Proprietary Millennium software from the Waters Corp. was used to control the system and to perform integration of peak areas. Reversed-phase chromatography was performed with a 25 cm x 4.6 mm C18 silica column containing 5-μm particles with 30-nm pore size (Vydac Separations Group, Hesperia, CA). The mobile phase for the isocratic C18 chromatography was prepared by combining 500 mL of ACN, 300 mL of MeOH, and 200 mL of H2O (50:30:20). Isocratic chromatography was performed at 2 mL/min with an injection volume of 35 μL as previously described for CsA [18, 21].

Various gradient conditions were examined, and the optimal condition for PSC reported here was a binary step gradient from ACN:H2O (57:43) to ACN:H2O (68:32) over 11 min, followed by a gradient to ACN:H2O (90:10) over the next 9 min. The flow rate was 1.5 mL/min, the injection volume was 250 μL, and the column was maintained at 75 °C during chromatography. Column regeneration was performed by holding the mobile phase at ACN:H2O (90:10) for 7 min, returning to ACN:H2O (57:43) over the next 2 min, and holding an additional 13 min before the next injection.

**Extraction procedures.** Several extraction methods were examined to determine optimal recovery of PSC from whole-blood lysates. Solid-phase extraction used in these studies was performed by using a 1-mL Sep-Pak® Plus C18 cartridge (Waters Corp.). Briefly, 1 mL of a whole-blood lysate sample (patient, QC, or calibrator) was added to 2 mL of ACN:MeOH (90:10), vortex-mixed, and centrifuged at 1000g for 5 min to remove precipitated protein. H2O (500 μL) was added to the supernatant, mixed, and applied to a cartridge preconditioned with 3 mL of MeOH. The cartridge was then washed with 1 mL of 700 mL/L MeOH followed by 1 mL of hexane. Internal standard (CsD) and PSC were eluted from the column with 2 mL of ACN:H2O (80:20). The eluted material was evaporated to dryness and reconstituted in 400 μL of mobile phase A (ACN:H2O, 57:43) before injection.

**Results**

**Optimization of chromatographic conditions.** Initial studies of chromatographic conditions examined resolution and separation of PSC and CsD at 10 and 5 mg/L dissolved in the initial mobile phase without extraction. None of the
isocratic methods examined with either biphenyl or C<sub>18</sub> columns, including the isocratic mobile phase of ACN: MeOH:H<sub>2</sub>O commonly used for CsA [18, 21], resolved PSC into a distinct peak (not shown). Therefore, a variety of gradient conditions were examined, with the C<sub>18</sub> gradient conditions described in Materials and Methods providing optimal resolution. Fig. 1a depicts a typical chromatogram in which, under these conditions, CsD elutes at 14.3 min and PSC elutes as two distinct peaks at 15.5 and 16.5 min.

**PSC extraction from whole blood and quantification by chromatography.** Solid-phase extraction was chosen for convenience and resulted in similar recoveries as a common double (acid/base) liquid extraction procedure used for CsA [18] (data not shown). Recovery of PSC from whole blood with the Sep-Pak Plus C<sub>18</sub> cartridges was 44% ± 4.1% and 39% ± 3.2% (n = 3) at PSC concentrations of 20 and 5 mg/L, respectively, whereas recovery of CsD was 48% ± 6.4% at 5 mg/L (n = 3). This was determined by comparing peak areas after extraction from whole blood with the peak areas of the same concentration of drug in the initial mobile phase but not extracted. The ratio of peak areas of PSC to CsD was used to develop a calibration curve. Linear regression was performed on each calibration curve to quantify PSC in QC and patient samples. Fig. 1b depicts a typical chromatogram of a whole-blood calibrator containing 5 mg/L PSC and 5 mg/L CsD. Fig. 2 depicts a chromatogram from a whole-blood sample from a patient receiving PSC to which 5 mg/L CsD, the internal standard, was added. The ratio of peak 2 (16.5 min) area to peak 1 (15 min) area was remarkably consistent regardless of PSC concentration or whether PSC was from calibrators or patient samples. The peak 2:peak 1 ratio was 1.36 ± 0.12 determined from three each of the 1.0, 2.5, 5.0, and 7.5 mg/L calibrators, and 1.46 ± 0.13 for 12 patient samples from 7 patients.

**HPLC assay performance.** Intrarun precision was determined by running whole-blood QC samples five times each in a single run and was 3.7% at a mean concentration of 2.6 mg/L, 4.1% at a mean concentration of 6.2 mg/L, and 9.1% at a mean concentration of 7.7 mg/L. Interrun precision from 18 runs was 6.5% at a mean of 7.66 mg/L of PSC, 8.2% at a mean of 5.24 mg/L (n = 5), and 8.5% at a mean of 2.58 mg/L. The HPLC method was sensitive to 800 μg/L as determined by performing parallel dilutions on a sample containing 2.5 mg/L PSC and noting deviations of >20% from expected values. This was consistent with a mean value of 1.02 ± 0.16 (n = 18) for the 1.0 mg/L calibrator when this calibrator was calculated as an unknown against the calibration curve.

To examine potential interferences, the following drugs were added to a whole-blood sample containing 5 mg/L PSC and CsD: acetaminophen (164 mg/L), amikacin (29 mg/L), amitryptiline (470 μg/L), chloramphenicol (55 mg/L), CsA (360 μg/L), digoxin (2.3 μg/L), disopyramide (5.2 mg/L), ethosuximide (108 mg/L), gentamicin (6 mg/L), lidocaine (6.5 mg/L), methotrexate (8 μmol/L), phenobarbital (35 mg/L), phenytoin (22 mg/L), primidone (10.1 mg/L), procainamide (8.9 mg/L), N-acetylpromiactamid (10.5 mg/L), propanolol (128 μg/L), quinidine (4.7 mg/L), salicylate (330 mg/L), theophylline (24.07 mg/L), tobramycin (7.3 mg/L), valproic acid (105 μg/L), and warfarin (10 mg/L).

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Fig. 1. Chromatogram of CsD internal standard at 5 mg/L and PSC calibrator (a) at 5 mg/L in mobile phase A and (b) at 7.5 mg/L after extraction from drug-free whole blood.

Full scale is 0.09 and 0.075 absorbance units in (a) and (b), respectively.

Fig. 2. Typical chromatograms of extracts from whole blood of a patient receiving PSC, with a whole-blood PSC concentration of 3.3 mg/L. Full scale is 0.11 absorbance units.
mg/L), and vancomycin (36 mg/L). At least seven additional peaks were observed, compared with the sample without these drugs, but the retention times and recovery of CsD and PSC were not affected (Fig. 3).

PSC was stable in whole blood for at least 14 days at 4 °C, as three samples with initial values between 2.53 and 7.42 mg/L produced values that averaged 2.6% higher after 14 days at 4 °C. Similarly, five patient samples with values between 2.7 and 5.3 mg/L produced values that averaged 2.0% higher after 18 months at −70 °C.

**PSC cross-reactivity in the TDx CsA assay.** To determine the immunologic cross-reactivity of PSC in the TDx monoclonal CsA assay, CsA-free whole-blood calibrators containing 2.5 mg/L and 5.0 mg/L PSC were assayed in multiple TDx runs. CsA values for these samples were 0.45 ± 0.047 mg/L and 0.86 ± 0.10 mg/L (n = 10), respectively, indicating that PSC exhibits ~17% cross-reactivity with CsA in the TDx CsA-specific assay and that these samples had a CV of 10–11% in this assay. Therefore, CsA values obtained from this TDx method were multiplied by 5.7 to obtain approximate PSC concentrations in whole-blood samples of patients receiving PSC. PSC values obtained from the TDx CsA assay compared well by linear regression with those obtained by the HPLC method for 86 samples from nine patients: TDx = 0.88HPLC + 0.44 mg/L, r = 0.93 (Fig. 4). Deming correction [22] of the least-squares analysis was TDx = 0.93HPLC + 0.32 mg/L. Analysis of the differences between these methods with the method of Bland and Altman [23] showed that PSC values averaged 0.14 ± 0.36 mg/L higher by the TDx method than by HPLC (Fig. 5).

Whole-blood concentrations of PSC during the dosing regimen ranged from 0.91 to 5.42 mg/L by HPLC and 0.92 to 5.41 mg/L by the Abbott TDx CsA assay for nine patients in this study. Whole-blood PSC concentrations for samples taken within 1 h before the next dose (“trough” concentrations) ranged from 0.96 to 3.72 mg/L by HPLC and 0.99 to 5.41 mg/L by the Abbott TDx CsA assay.

**Discussion**

Development of a precise HPLC assay for PSC was not as straightforward as initially anticipated. Isocratic conditions that work well for CsA [18, 21] resulted in very poor...
resolution of PSC even at temperatures of 70–75 °C, which are necessary to minimize peak broadening of CsA due to interconversion of its conformers [21]. Under these conditions, we consistently observed a very broad bilobed peak that spanned 2–2.5 min. The gradient conditions used greatly enhanced peak resolution but still consistently produced two peaks. The ratio of areas of the second peak to the first peak (~16 min) was consistent regardless of whether the sample was pure PSC in mobile phase or PSC in patient whole blood. A likely explanation for the consistent presence of two peaks is keto-enol isomerization at the 3' position of the amino acid, MeBmt, at position one of the undecapeptide.

When PSC values from the HPLC assay were compared with putative CsA concentrations of patient samples containing PSC from the TDx CsA immunoassay, we found that PSC cross-reacts ~17% with CsA in this assay. Both the good correlation and the general agreement in absolute values suggest that the HPLC and TDx methods are primarily detecting the parent molecule. The slightly higher values observed with the TDx assay may be due to detection of PSC metabolites that are similar in structure to CsA metabolites, which exhibit some cross-reactivity in the TDx CsA assay [20]. For instance, the CsA metabolite AM9, which shows the highest cross-reactivity (~13%) in the TDx [20], is a result of hydroxylation of amino acid 9 in CsA. It is likely that similar, simple metabolites will be present for PSC and cross-react to a similar extent in this immunoassay. Clearly, additional studies will be required to identify PSC metabolites, their elution patterns in chromatography, and their reactivity with anti-CsA antibodies.

The HPLC method described for quantifying PSC in whole blood is sufficiently precise and accurate for clinical use. However, the gradient conditions and subsequent column equilibration restrict sample throughput to one injection every 60 min. Thus, this HPLC method will likely be most useful for some limited pharmacokinetic studies during clinical trials and for determining PSC cross-reactivity in other CsA immunoassays rather than for routine monitoring of PSC. For instance, we found that PSC in whole blood from these nine patients had a reproducible immunologic cross-reactivity of ~17% in a commonly used immunoassay for CsA [20]. Whole-blood PSC concentrations determined by HPLC correlated well with those determined by the TDx monoclonal CsA immunoassay after accounting for this cross-reactivity. Other CsA immunoassays exhibit different patterns of cross-reactivity with CsA metabolites [19]. Thus, it will be important to determine the extent of PSC cross-reactivity in these assays to assess their potential utility to monitor this CsA analog. On the basis of our observations with the TDx CsA immunoassay, it is likely that at least some CsA immunoassays will be acceptable for routine monitoring of PSC, when and if this becomes clinically necessary.

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