Discovery of Less Nephrotoxic FK506 Analogs and Determining Immunophilin Dependence of Immunosuppressant Nephrotoxicity with a Novel Single-Dose Rat Cisplatin Potentiation Assay

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ABSTRACT
Comparing nephrotoxicity of numerous drug analogs is impractical with chronic in vivo models. We devised a new cisplatin potentiation assay (CISPA) that sensitively detects renal injury as a serum creatinine increase when only one dose of test compound is followed by cisplatin. Reference nephrotoxins known to act on various sites in kidney tubules, glomeruli or renal papilla were all detected by the CISPA at single doses that without cisplatin gave little change, which showed that this simple, sensitive assay has broad potential utility for mechanistic studies of nephrotoxicity. We used the CISPA both to probe the nephrotoxic mode of action of immunosuppressants and to search for safer compounds. Although several non-nephrotoxic immunosuppressants were inactive, cyclosporine, FK506, ascomycin (C21-ethyl-FK506) and rapamycin were nephrotoxic in the CISPA at single doses equal to the daily amounts required to reduce creatinine clearance with 14 days of treatment. Similar therapeutic indices were derived comparing toxicity by either method to prevention of rat ear-heart allograft rejection. C18-OH-ascomycin, an FK506-binding protein (FKBP) antagonist, reversed in vivo immunosuppression by FK506 and ascomycin in the rat, and pretreatment in the CISPA blocked FK506 and ascomycin nephrotoxicity, which showed a common immunophilin dependence. Rapamycin nephrotoxicity was unaffected (as with cyclosporine), which indicated that binding to FKBP was not required. Rapamycin nephrotoxicity thus appears mechanistically unrelated to its immunosuppressive mode of action. Screening with the CISPA enabled discovery of A-119435, a less nephrotoxic ascomycin analog having a 10-fold higher therapeutic index.

The serine/threonine phosphatase, calcineurin, has been implicated as the common therapeutic target of the immunosuppressants FK506 (tacrolimus) and CsA. Although these drugs are structurally unrelated and bind to distinct cytoplasmic immunophilin proteins, FKBP and cyclophilin, respectively, the drug-protein complexes can interact with and inhibit the activity of calcineurin (Friedman and Weissman, 1991; Liu et al., 1991). The fact that CsA and FK506 both cause not only therapeutic immunosuppression by blocking calcineurin-dependent T-cell activation pathways, but also both interfere with brain, pancreas and kidney functions, makes a strong circumstantial case for inhibition of this enzyme as a common link between beneficial as well as side effects of both drugs (Dumont et al., 1992; Ho et al., 1996; Morris, 1992; Schreier et al., 1993; Sigal et al., 1991). Nonetheless, the detailed biochemical events linking calcineurin inhibition to the pathophysiological derangement resulting in loss of kidney function remain elusive.

Nephrotoxicity is a common side effect of pharmacological agents, but determining the precise mechanism of action for the toxicity, or exploring structure-activity relationships that may lead to separating this undesirable property from the therapeutic pharmacophore can be a daunting prospect. This is particularly true for compounds such as FK506, which requires chronic dosing to manifest this side effect (Kumano et al., 1991; Stephen et al., 1989). We were faced with this dilemma in attempting to design safer analogs of ASCO, a C21-ethyl analog of FK506 which was discovered previously (Arai et al., 1962) but not exploited for its immunosuppressive activity. Selective toxicity reduction of this class was achieved for ASCO analogs determined to be nontoxic to mouse pancreatic β cells by testing them in culture (Mollison

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ABBREVIATIONS: 2-BEA-HBr, 2-bromoethanamine hydrobromide; ASCO, ascomycin; CISPA, cisplatin nephrotoxicity potentiation assay; cisplatin, cis-platinum (II) diammine dihydrochloride; CsA, cyclosporine; FKBP, FK506 binding protein; GFR, glomerular filtration rate; HCBD, hexachlorobutadiene; mpk, mg/kg body weight; PLN, popliteal lymph node; RAPA, rapamycin; BUN, blood urea nitrogen.
et al., 1996). However, the mammalian kidney is anatomically and physiologically heterogeneous as a result of its complex functions, having 15 to 20 different cell types in the nephron alone (Kriz and Bankir, 1988). Unable to establish a suitable in vitro assay system in the absence of detailed knowledge concerning which cell type within the kidney is the most important proximal target of FK506 nephrotoxicity, development of a practical in vivo model represented the most reliable way to evaluate nephrotoxic potential of compounds from this class as a key element of the drug discovery strategy.

Cisplatin is one of the most widely used agents for treatment of solid tumors, but nephrotoxicity is a major side effect of its use (Jones et al., 1991). We exploited this property to devise a novel CISPA wherein a single dose of ASCO analogs was followed by a dose of cisplatin to further damage the kidneys in a consistent way and overcome their tremendous reserve capacity (Mollison et al., 1995). Serum creatinine is a stringent but normally insensitive marker of nephrotoxicity because considerable damage is required to induce an elevation. By adjusting the dose of cisplatin to raise the base-line to several-fold, levels increased sharply when there was potentiation from an injury induced by prior treatment. The CISPA was validated by showing its sensitivity to a wide range of reference nephrotoxins that target selectively differing cell types within the kidney.

Mechanistic studies were done with the CISPA to examine the immunophenolin dependence of immunosuppressant nephrotoxicity in vivo with the aid of C18-OH-ASCO, a specific antagonist of drug binding to the immunophilin target protein, FKBP (Dumont et al., 1992). This strategy was used to show for the first time that nephrotoxicity of RAPA (sirolimus), another natural product with promise for preventing allograft rejection (Sehgal et al., 1995), is not mediated through its interaction with FKBP, an observation with therapeutic implications for using RAPA combined with other immunosuppressants. We also report the discovery of an ASCO analog with reduced nephrotoxicity by use of the novel CISPA as an in vivo screening tool.

Materials and Methods

Animals and reagents. The male rats used were Brown Norway and Sprague-Dawley, ~200 g, from Charles River, Wilmington, MA. Fischer 344 rats, ~225 g, and Lewis rats, ~180 g, were from Harlan-Sprague-Dawley, Indianapolis, IN. All in vivo studies were conducted by protocols approved by an Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

CsA was purified from Sandimmune (Sandoz, Basel, Switzerland). FK506, RAPA and ASCO were obtained from fermentations and purified to homogeneity by HPLC. A-119435 and C18-OH-ASCO, the latter previously described as L-685,818 (Dumont et al., 1992) or A-82787 (Mollison et al., 1996), were synthesized from ASCO and their structures were confirmed by magnetic resonance imaging and mass spectrometric analysis. Sterile isotonic solutions for preparing vehicles were from Abbott Laboratories, North Chicago, IL. Dulbecco’s phosphate-buffered saline was from Gibco, Grand Island, NY. HCBD, sodium chromate and 2-BEA-HBr were from Aldrich, Milwaukee, WI. Mycophenolic acid was obtained from Ajino Moto, Tokyo, Japan, and azathioprine was from Burroughs-Wellcome, Tuckahoe, NY. All other reagents were from Sigma Chemical Co., St. Louis, MO.

Unless otherwise stated, all compounds were given in EPC vehicle, prepared by adding them to the following ingredients in order, at the indicated percent of total volume: 10% ethanol, 40% propylene glycol, with the balance dextrose (5%) in water containing cremophor EL to give a final concentration of 2%. CsA, mercuric chloride and amphotericin B were diluted in 0.2% hydroxypropyl methylcellulose. Sodium chromate and adriamycin were injected in saline and HCBD was given in corn oil.

Statistical analysis. Group means were compared for statistically significant differences by Student’s t-test.

Fourteen-day dosing studies. Fischer 344 rats were dosed intraperitoneally once a day for 14 consecutive days with vehicle alone or compound, 2 ml/kg b.wt. Rats were allowed to acclimate to individual metabolism cages for 24 h before the final dose, and urine was collected for 24 h after the final dose. Serum from cardiome blood was collected for BUN and creatinine determinations, and urine creatinine was determined from the supernatant of centrifuged (400 x g) urine. Weight normalized creatinine clearance was derived by the following equation: urine creatinine (mg/dl) x urine volume (ml)/[serum creatinine (mg/dl) x body weight (kg)].

CISPA. Fischer 344 rats were dosed intraperitoneally on day −1 with vehicle alone or compound, 2 ml/kg b.wt., followed 24 h later on day 0 by an intraperitoneal injection of cisplatin, 3 mg/kg, prepared fresh in dextrose (5%) in water and administered within 1 h. On day 6, rats were sacrificed by CO2 inhalation, and serum from centrifuged (1000 x g) cardiac blood was analyzed to determine blood urea nitrogen and serum creatinine with the VP analyzer (Abbott Laboratories, North Chicago, IL).

PLN hyperplasia. The PLN hyperplasia assay, was adapted from (Mollison et al., 1993), by suspending 5 x 10^7/ml of red blood cell-lysed, washed and irradiated (2000 rad) male Brown-Norway rat splenocytes in Dulbeco’s phosphate-buffered saline, and injecting 0.1 ml sc. under the plantar surface of the foot of male Lewis rats on day 0. Rats were dosed i.p. with EPC vehicle or compounds in EPC, 2 ml/kg, once on daily on day 0 through day 3. On day 4, the rats were sacrificed by CO2 inhalation and the PLNs from the injected limb of drug-treated animals, or from both limbs for the vehicle control, were excised and weighed on an M3P microbalance (Sartorius, Waukegan, IL). The difference between the average lymph node weights from the splenocyte-injected and contralateral limbs for the EPC control established the maximum hyperplasia response, and the percent inhibition of the PLN response for drug-treated animals was derived by subtracting the mean contralateral (noninjected) node weight of the EPC controls from the node of each splenocyte-challenged limb, dividing this difference by the maximum hyperplasia response, and multiplying by 100. ED50 values were determined from a least squares regression analysis of the day 14 dose-response curve.

Ear-heart allografts. The nonvascularized rat ear-heart method was adapted from the mouse method of (Mollison et al., 1993) by implanting longitudinally bisected neonatal (<60 h old), unsexed Brown-Norway hearts subcutaneously into the pinna of male Lewis rats. Compounds or vehicle alone were given i.p. once daily for 14 consecutive days starting on the day of implantation. Graft viability was assessed by visual observation of beating under stereoscopic 10 x magnification without anesthesia, with negative visual results confirmed by electrocardiography under anesthesia with 50 mg/kg ketamine HCl (Fort Dodge Labs, Fort Dodge, IA) and 3 mg/kg xylazine (Miles, Shawnee Mission, KS). Grafts on EPC-treated controls were uniformly nonviable on day 14, and ED50 values were determined from a least squares regression analysis of the day 14 dose-response curve.

ASCO and A-119435 pharmacokinetics. ASCO or A-119435, 10 mg/kg i.p., in EPC vehicle were given to rats, either alone or followed immediately by 3 mg/kg cisplatin prepared in water with 5% dextrose. Blood samples were collected in sodium heparin (10 U/ml) blood from the retro-orbital plexus under 60% CO2/40% O2 anesthesia at 0.5, 1, 2, 4, 6, 24 and 48 h after dosing. Whole blood was hemolyzed by addition of 20% methanol in water (1:2.5 v/v) and extracted with ethyl acetate/hexane (1:1 v/v). Samples were centri-
fuged at 1200 × g for 10 min (4°C), and a constant volume of the organic layer was transferred to a conical centrifuge tube in a 35°C warming block and evaporated to dryness with dry air. Samples were reconstituted with 40% (v/v) acetonitrile in water and injected into a warming block and evaporated to dryness with dry air. Samples were organic layer was transferred to a conical centrifuge tube in a 35°C temperature and extracted with chloroform by vortexing for 5 min, steam bath at 85°C for 10 min. The mixtures were cooled to room temperature (45:5:50 by volume), at a flow rate of 1.0 ml/min and with UV detection at 205 nm.

**Cisplatin pharmacokinetics.** Rats were dosed as discussed above with ASCO (10 mg/kg, i.p.) in EPC, then 24 h later with cisplatin (3 mg/kg i.p.). Heparinized blood samples were collected by cardiac puncture after sacrificing by CO₂ inhalation at 0.25, 1, 2, 3, 4, 5 and 6 h after cisplatin dosing. Plasma concentrations of cisplatin were determined by an HPLC method as described (Borch et al., 1979). Plasma samples were deproteinized with acetonitrile and supernatants (0.3 ml) were derivatized with freshly made 10% sodium diethyldithiocarbamate water solution (10 μl) by heating in a steam bath at 85°C for 10 min. The mixtures were cooled to room temperature and extracted with chloroform by vortexing for 5 min, then 100 μl of 0.2 M NaSH was added and mixed. The compound of interest in the chloroform layer was separated from the co-extracted components by a YMC ODS-A, 10-μm, 4.6 × 150 mm column (YMC, Morris Plains, NJ) at room temperature with 70:30 acetonitrile/water (10 μl) by heating in a steam bath at 85°C for 10 min. The mixtures were cooled to room temperature and extracted with chloroform by vortexing for 5 min, then 100 μl of 0.2 M NaSH was added and mixed. The compound of interest in the chloroform layer was separated from the co-extracted components by a YMC ODS-A, 10-μm, 4.6 × 150 mm column (YMC, Morris Plains, NJ) at room temperature with 70:30 acetonitrile/water (by volume) at a flow rate of 1.0 ml/min. Detection of cisplatin was accomplished at a wavelength of 345 nm.

**Pharmacokinetic data analysis.** The concentration of each sample was calculated by a least squares linear regression analysis of the peak area compared with spiked rat blood standards. The following pharmacokinetic parameters were determined. Maximum whole-blood concentrations (Cmax) and the time of their occurrence (tmax) were compiled from the raw data. Terminal half-life (t½) was calculated from the elimination rate constant. Area under the blood drug concentration-time curve (AUC) up to the last measured blood concentration was also analyzed.

**Results**

**Nephrotoxicity of reference immunosuppressants in 14-day studies.** Comparative data in the 14-day dosing model for the reference immunosuppressants CsA, ASCO, FK506 and RAPA are shown in table 1. When given parenterally via i.p. route, all compounds showed significant nephrotoxicity as evidenced by reduced creatinine clearance. The changes were dose-related, and in each compound, the reduction in kidney function reached levels below approximately 50% of normal. These results provided a basis for calculating a therapeutic index in conjunction with immunosuppressive potencies and for comparing nephrotoxic potential in the CISPA (see below).

**Immunosuppressive efficacy of reference agents.** To provide data for comparison with nephrotoxic potential in the rat, the in vivo immunosuppressive potency of CsA, FK506, ASCO and RAPA was determined in an ear-heart allograft model. The Brown Norway (RT1b) to Lewis (RT1l) strain combination represents a major MHC mismatch, providing a rigorous therapeutic challenge for defining immunosuppressive potencies. Figure 1 shows results with daily i.p. dosing for inhibition of ear-heart allograft rejection. All compounds were able to block graft rejection effectively. Potency estimates are shown in table 2, where the calculated ED₅₀ values for preventing graft rejection through day 14 were used to derive a therapeutic index by dividing them into the respective nephrotoxic doses for each compound seen with 14-day administration in the rat. By this analysis, there was little difference in therapeutic index among ASCO, FK506 and CsA. RAPA appeared to be less toxic, showing a 3- to 11-fold greater therapeutic index than the other compounds.

**Cisplatin nephrotoxic injury assessment.** The dose response to cisplatin of Fischer 344 rats for causing an increase in serum creatinine is shown in figure 2A. Note that a statistically significant increase did not occur until a cisplatin dose of 3 mg/kg i.p. was reached, but then the serum creatinine level increased sharply at 4.5 mg/kg. These results suggest that the reserve capacity of the kidneys was gradually impaired as the cisplatin dose was increased, with a continued ability to regulate creatinine levels until the magnitude of injury was severe, resulting in serum creatinine elevation. In figure 2B, the percent decrease in creatinine clearance is depicted for individual rats treated with varying doses of cisplatin. This curve illustrates that significant nephrotoxicity occurred before effects on serum creatinine became apparent, conversely showing that because serum creatinine elevation is a less sensitive marker of impaired.

<p>| TABLE 1 |
| Renal toxicity of immunosuppressants with 14-day administration |</p>
<table>
<thead>
<tr>
<th>Treatment</th>
<th>I.P. Dose</th>
<th>Creatinine Clearance</th>
<th>% reduction</th>
<th>Serum BUN</th>
<th>mg/dl</th>
</tr>
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<tr>
<td>Control</td>
<td>–</td>
<td>7355 ± 700</td>
<td>–</td>
<td>15.4 ± 0.6</td>
<td>0.60 ± 0.04</td>
</tr>
<tr>
<td>ASCO</td>
<td>0.3</td>
<td>8083 ± 668</td>
<td>–10</td>
<td>21.5 ± 0.7***</td>
<td>0.5 ± 0.03</td>
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<tr>
<td>ASCO</td>
<td>1</td>
<td>5850 ± 635</td>
<td>20</td>
<td>25.7 ± 0.3***</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>ASCO</td>
<td>3</td>
<td>3383 ± 531</td>
<td>54***</td>
<td>34.1 ± 4.1**</td>
<td>0.61 ± 0.03</td>
</tr>
<tr>
<td>ASCO</td>
<td>10</td>
<td>2665 ± 317</td>
<td>64***</td>
<td>65.5 ± 16.1***</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>FK506</td>
<td>0.1</td>
<td>8689 ± 1352</td>
<td>–18</td>
<td>20.9 ± 0.7***</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>FK506</td>
<td>0.3</td>
<td>5413 ± 690</td>
<td>26</td>
<td>22.7 ± 0.6***</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>FK506</td>
<td>1</td>
<td>3244 ± 631</td>
<td>56***</td>
<td>36.8 ± 4.9**</td>
<td>0.87 ± 0.03***</td>
</tr>
<tr>
<td>FK506</td>
<td>3</td>
<td>3115 ± 700</td>
<td>58***</td>
<td>62.6 ± 11.2***</td>
<td>0.69 ± 0.08</td>
</tr>
<tr>
<td>RAPA</td>
<td>0.3</td>
<td>6512 ± 807</td>
<td>11</td>
<td>18.9 ± 0.8*</td>
<td>0.64 ± 0.02</td>
</tr>
<tr>
<td>RAPA</td>
<td>1</td>
<td>7072 ± 1327</td>
<td>4</td>
<td>21.2 ± 0.8***</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td>RAPA</td>
<td>3</td>
<td>5268 ± 265</td>
<td>28**</td>
<td>19.2 ± 0.7***</td>
<td>0.77 ± 0.06***</td>
</tr>
<tr>
<td>RAPA</td>
<td>10</td>
<td>3776 ± 432</td>
<td>49***</td>
<td>21.2 ± 0.5***</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>CsA</td>
<td>1.5</td>
<td>6006 ± 730</td>
<td>18</td>
<td>20.1 ± 0.5**</td>
<td>0.63 ± 0.02</td>
</tr>
<tr>
<td>CsA</td>
<td>5</td>
<td>5776 ± 882</td>
<td>21</td>
<td>20.7 ± 0.5***</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td>CsA</td>
<td>15</td>
<td>3616 ± 454</td>
<td>51***</td>
<td>34.5 ± 3.6***</td>
<td>0.73 ± 0.03**</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.; n = 10; **P < .01; ***P < .001 vs. vehicle control.
glomerular filtration, significant increases reflect substantial loss of kidney function.

Based on these relationships, a cisplatin dose of 3 mg/kg i.p. was chosen as a standard condition for drug potentiation studies in the CISPA. This dose caused the normal level of serum creatinine in the Fischer 344 rat of 0.3 to 0.9 mg/dl (Charles River, Wilmington, MA) to increase significantly to a concentration of approximately 1 to 2 mg/dl, a level corresponding to a 60 to 70% loss of creatinine clearance (fig. 2B). BUN levels increased 2- to 4-fold from an average of approximately 20 mg/dl. These conditions were considered ideal for revealing any additional injury by prior treatment with test compounds because the combined injuries should result in an exponential increase in serum creatinine.

**Drug interactions with use of cisplatin.** To address the potential pitfall of possible pharmacokinetic drug interactions between the administered test compound and cisplatin, studies were done with ASCO as a representative of the FK506 class of drugs to rule out an effect on cisplatin pharmacokinetics. As shown in figure 3, cisplatin was very long-lived in the rat as described previously (Appenroth et al., 1988). Peak blood levels and the time course for clearance of cisplatin appeared unchanged by ASCO given either simultaneously or 1 day before cisplatin.

In contrast, there was a significant effect of cisplatin on ASCO pharmacokinetics when cisplatin and ASCO were given together (fig. 4). The whole-blood levels were increased and the rate of elimination was reduced. However, with interposition of the 1-day pretreatment interval for ASCO, its levels were undetectable at the time of cisplatin administration, so that any effect by cisplatin on ASCO elimination at that stage was unlikely to have had a measurable influence on kidney function. Accordingly, all subsequent studies were
done by administering test substances and imposing a 1-day pretreatment interval before dosing with cisplatin to minimize artifacts from possible drug interactions.

Another consideration in the timing of events was that concomitant stressful procedures were found to potentiate cisplatin nephrotoxicity, which resulted in a higher serum creatinine (data not shown). This effect was noted in preliminary studies when subjecting rats to the added stress of tail vein injection at the same time as cisplatin administration, but was avoided by carrying out such procedures 1 day before cisplatin.

**Kinetics of nephrotoxic injury potentiation with cisplatin.** A time course of cisplatin nephrotoxicity and its potentiation by ASCO in the Fischer 344 rat are shown in figure 5. Serum creatinine peaked on day 5 after cisplatin administration (3 mg/kg i.p.) on day 0. In animals treated with ASCO, 10 mpk i.p. on day –1, before cisplatin, a significant further elevation of creatinine occurred at all time points, consistent with potentiation of an ASCO-induced injury of the kidneys by the subsequent cisplatin dose. Figure 5 also illustrates a consistent feature of the cisplatin response, because once creatinine levels of vehicle-treated control animals reached a peak, they began to decline toward normal on day 6. Creatinine levels in rats with a drug-induced preinjury typically showed a slower decline. Therefore, day 6 was chosen as an endpoint for potentiation studies with combined treatments in the CISPA, because the percent increase over controls of the serum creatinine levels was typically greatest on day 6.

**CISPA sensitivity to reference nephrotoxins.** Substances known to be nephrotoxic were tested by CISPA to determine the ability of this novel assay to detect injury produced by diverse nephrotoxic mechanisms and at a variety of target sites within the kidney. Table 3 shows effects on serum creatinine levels which result from single doses of nephrotoxins known to injure various sites within the kidney, tested either alone or followed by cisplatin. For every agent, the doses tested produced little or no effect on creatinine levels compared with vehicle-treated control rats when they were administered alone. However, when followed by cisplatin treatment, in every case there was a significant potentiation of the elevation of serum markers compared with controls given only cisplatin. BUN was elevated in parallel (not shown).

These results were consistent with the expectation that a drug-induced injury to the kidney that was insufficient in magnitude by itself to manifest a change in serum creatinine, would be potentiated by a subsequent cisplatin dose, resulting in a measurable increase in serum creatinine levels. These results further demonstrate sensitivity of the model for detecting agents with a wide range of nephrotoxic mechanisms.

In contrast, reference immunosuppressants not known to cause nephrotoxicity, including azathioprine, prednisolone and cyclophosphamide, as well as the FKBP antagonist, C18-OH-ASCO, were inactive in the CISPA when tested at high doses. None of these agents altered the serum BUN or creatinine elevation induced by cisplatin alone, which indicates that pretreating the kidney with potent immunosuppressants conferred no protection from cisplatin-induced injury. Mycophenolic acid has not been reported to be nephrotoxic, so its creatinine elevation was unexpected. The reason for this apparent toxicity is not clear.

**Comparison of CISPA to 14-day dosing.** The dose-response relationship for nephrotoxicity of FK506, ASCO, RAPA and CsA in the CISPA are shown in figure 6. The elevation of both creatinine and BUN in concert resulted in a fairly constant BUN/creatinine ratio, indicative of a renal, as opposed to prerenal or postrenal injury (Kassirer, 1971). Single immunosuppressant doses in the CISPA capable of causing an increase in serum creatinine to approximately double the base-line response to cisplatin alone were very close to doses required to show a 50% reduction in creatinine clearance with 14-day chronic administration, as summarized in table 2. In a practical sense, the CISPA is thus a more simple and efficient assay for comparing nephrotoxicity of many compounds. By combining the effect of the cisplatin dose, which itself was shown to reduce creatinine clearance by 60 to 70% (fig. 2B), the significant but otherwise undetectable preinjury caused by the single dose of immunosuppressant was made readily apparent.
Immunophilin dependence of nephrotoxicity in CISPA. The simplicity of this assay was exploited to probe the requirement for immunosuppressant binding to FK506 binding protein (FKBP) in causing nephrotoxicity. For this purpose, a known FKBP antagonist was used, C18-OH-ASCO, which binds to FKBP without inhibiting calcineurin (Dumont et al., 1992). To first validate the in vivo use of this reagent in the rat and establish dose requirements for antagonism, its effect on therapeutic immunosuppression was investigated. Rats challenged with allogeneic splenocytes to induce PLN hyperplasia were treated for 4 days with a range of daily doses of C18-OH-ASCO given simultaneously with a 1 mg/kg i.p. immunosuppressive dose of ASCO (fig. 7A). There was a dose-related blockade of ASCO immunosuppression, with a statistically significant effect using as little as 1 mg/kg i.p. of C18-OH-ASCO. When given by itself at 10 mg/kg i.p., C18-OH-ASCO had no immunosuppressive activity in the PLN assay.

Based on these results, doses of FK506 and CsA chosen to give immunosuppression in the range seen with 1 mg/kg ASCO were similarly studied to ascertain whether their immunosuppressive activity would be blocked by 10 mg/kg i.p. of C18-OH-ASCO antagonist. As shown in figure 7B, although immunosuppression of both ASCO and FK506 was blocked, there was no effect on activity of CsA, consistent with its known dependence on binding to cyclophilin rather than FKBP (Handsclumacher et al., 1984).

A dose-response study to determine the effect of C18-OH-ASCO pretreatment on ASCO nephrotoxicity in the CISPA is shown in figure 8A. The nephrotoxicity of ASCO was essentially abrogated with doses of 10 mg/kg i.p. and higher. When C18-OH-ASCO was tested by itself in the CISPA, it did not potentiate or inhibit the cisplatin response (table 3 and fig. 8B), which showed that it was devoid of nephrotoxicity. In a follow-up study with a 100 mg/kg i.p. dose of C18-OH-ASCO, the statistically significant nephrotoxic potentiation by both FK506 and ASCO was blocked by pretreatment with C18-
OH-ASCO, consistent with their toxicity depending on FKBP binding in the kidney (fig. 8B). In contrast, CsA and RAPA nephrotoxicities were unaffected, which suggests that their nephrotoxicity is mediated by different immunophilins or via a nonimmunophilin-dependent mechanism.

**Discovery of less nephrotoxic ASCO analogs with CISPA.** To support an ongoing medicinal chemistry effort to make improved and less toxic ASCO analogs, numerous compounds were tested in CISPA. This screening was done at a specific dose ratio for each compound based on its in vivo immunosuppressive potency in the rat PLN hyperplasia assay described above. Dose-response curves from the CISPA are shown in figure 9 for ASCO and the ASCO analog A-119435. (Structures are shown in figure 10.) Serum creatinine levels are expressed as the percent increase over controls given cisplatin alone, and there is clearly a shift to the right in the A-119435 curve, which indicates a lower nephrotoxic potency than ASCO. This was not attributable to a
difference in pharmacokinetic behavior between ASCO and A-119435. A pharmacokinetic analysis (table 4) showed that the maximal blood level and AUC for A-119435 were slightly higher than for ASCO when given i.p. in the rat.

Table 4 also shows that the in vivo immunosuppressive potency of A-119435 was similar to ASCO in the rat PLN, consistent with comparable FKBP binding and calcineurin inhibition because of their similar pharmacokinetic behavior. A-119435 was equivalent to FK506 in potency for inhibiting FKBP binding and human T cell proliferation in the mixed leukocyte reaction in vitro (data not shown). However, as described above, A-119435 had less-than-expected nephrotoxicity. The estimated dose of A-119435 required to double the increase in serum creatinine produced by cisplatin alone in the CISPA was significantly higher than with ASCO. Although A-119435 is not free of nephrotoxicity, it has a significantly improved safety margin compared with ASCO, with a therapeutic index approximately 10-fold better.

**Discussion**

Successful drug discovery depends on having robust, reproducible assays to support an intelligent iterative process of evaluating synthetic drug analogs for their pharmacologic, and sometimes toxicologic, effects. Therefore, it was critically important to have both an adequately sensitive and mechanistically appropriate readout to probe ASCO analog nephrotoxic potential to select against this side effect.

The genesis of the cisplatin potentiation approach was an appreciation of the fact that profound loss of GFR must occur before the serum creatinine increases above normal, but once this degree of impairment is reached, creatinine increases exponentially (Thurau et al., 1985). We sought a way to exploit the use of this simple marker in the rat by chemically injuring the kidney so that a modest immunosuppressant-induced preinjury would immediately be revealed as a serum creatinine elevation. Our data with cisplatin injury in the rat showed that once substantial loss of creatinine clearance occurred, relatively small additional changes markedly increased serum markers of toxicity (fig. 2). Cisplatin was chosen to develop the model because of its ability to cause severe renal impairment with a single dose (Choie et al., 1981), and because it damaged the proximal tubule (Jones et al., 1985), the site of injury caused by CsA (Rosen et al., 1990; Ryffel et al., 1985) and FK506 (Andoh et al., 1994; Kumano et al., 1991; Yamada et al., 1991).

In addition to providing a highly sensitive model for detecting immunosuppressant nephrotoxicity, the CISPA was sensitive to nephrotoxins known to act on a variety of other sites in the kidney. This was not surprising in terms of the need for the various renal tissues to work in concert to maintain normal glomerular filtration, and represented an advantage in terms of the model having broad sensitivity for detecting many types of mechanisms of renal injury. As seen with a single high dose of CsA, doses of reference nephrotoxins that showed little discernible effect when they were given alone resulted in marked potentiation with cisplatin, which caused significant further elevation of serum creatinine above the increase in base line produced by cisplatin (table 3). These reference agents were typically active at one third to one half the doses required to show acute biochemical changes in the urine, e.g., enzymuria (Gartland et al., 1988).

In use of the CISPA, attention was given to some methodological details to minimize artifacts. Significantly higher serum creatinine elevations were seen in animals exposed to transport stress in moving them to an adjacent building to carry out the cisplatin injections, and in rats concomitantly given painful tail vein injections (data not shown). Although the mechanism for this presumed stress potentiation is unknown, it could be a significant source of variability in studies with this nephrotoxin and will require further study to

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**Table 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PLN ED50</th>
<th>CISPA Toxic Dose*</th>
<th>Therapeutic Index</th>
<th>Cmax</th>
<th>AUCb</th>
<th>t½</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mpk/day</td>
<td>mg/kg</td>
<td></td>
<td>µg/ml</td>
<td>µg·mL·day·h</td>
<td></td>
</tr>
<tr>
<td>ASCO</td>
<td>0.31c</td>
<td>3c</td>
<td>9.7</td>
<td>1.04</td>
<td>3.24</td>
<td>4.7</td>
</tr>
<tr>
<td>A-119435</td>
<td>0.27sa</td>
<td>27sa</td>
<td>100</td>
<td>1.64</td>
<td>3.99</td>
<td>7.6</td>
</tr>
</tbody>
</table>

* Approximate 2-fold serum creatinine increase.

* 0–6 h.

* n = 48.

* n = 112.

* n = 16.

* n = 40 pooled from multiple experiments.
elucidate. Accordingly, for the present work, a consistent technique and controls carefully matched in terms of comparable handling and vehicle injections were used. Administering test substances 1 day before giving cisplatin minimized this problem in our studies. However, agents causing persistent severe irritation might conceivably show false-positive nephrotoxic potentiation when followed by cisplatin.

Drug interactions are also possible, e.g., the decreased ASCO clearance observed when it was given at the same time as cisplatin. Although the latter interaction might make the model more sensitive to ASCO nephrotoxicity by causing a more prolonged persistence of the drug in the blood, this possible variable, as well as the effects of stress attendant to the additional manipulation of the animal for drug administration, were minimized by giving the test agents 1 day before cisplatin. This protocol also had the advantage that whatever the properties of the drug under investigation, it was allowed to exert its effects on the normal kidney before the addition of cisplatin-induced alterations. Although not the case for the macrolactam immunosuppressants, other test substances that are long-lived may continue to act concomitantly with cisplatin, and this persistent presence may be required to induce injury. For careful investigations of a given drug class, the possibility of pharmacokinetic interactions would need to be ruled out, particularly an effect on cisplatin blood levels, as we did with ASCO (fig. 3).

Immunosuppressant nephrotoxicity in the CISPA was validated by several approaches. First, non-nephrotoxic immunosuppressants were tested at high doses as negative controls. Because they showed no evidence of cisplatin potentiation, exposure to immunosuppressive activity per se had no ability to prevent the injury induced subsequently by cisplatin administration, nor to potentiate it. Thus, in general, neither false-positive nor false-negative responses should result from prior administration of a single dose of immunosuppressants. The reason for the significant increase in creatinine with mycophenolic acid is unknown and may have been the result of an idiosyncratic effect in the rat because the nephrotoxicity of this agent has not been reported.

RAPA was initially tested as a member of the non-nephrotoxic immunosuppressant group, based on early reports of its safety with chronic dosing in the Sprague-Dawley rat at 1.5 mpk i.p. (Whiting et al., 1991) or 10 mpk p.o. (Dijoseph et al., 1992). RAPA is particularly relevant to a dissection of FK506 nephrotoxicity because the two are structurally related and depend on FKBP binding for their immunosuppression (fig. 10). Surprisingly, it was nephrotoxic in the CISPA. When we compared it with CsA and FK506 with conventional chronic administration and creatinine clearance measurements in 14-day studies in the Fischer 344 rat, RAPA nephrotoxicity was confirmed at the higher doses of 3 and especially 10 mpk i.p. (table 1). The reported lack of activity with oral administration at 10 mpk is likely because of its poor oral bioavailability of 3.5% in rat (data not shown). A recent study of RAPA in the spontaneously hypertensive rat with i.v. administration showed nephrotoxicity in a range 10-fold higher than therapeutic doses (Dijoseph et al., 1994). The mechanism of RAPA toxicity has not been established, and although RAPA has not yet been found to be nephrotoxic in humans (Sehgal et al., 1995), our results suggest there is potential for this side effect.

Calculation of a therapeutic index with either 14-day dosing data or CISPA results showed that RAPA has a significantly wider therapeutic window than ASCO, CsA or FK506 when comparing their toxicities with immunosuppressive efficacy in the rat ear-hair transplant model. The latter compound, although differing markedly in immunosuppressive potency, showed similar therapeutic indices, consistent with the clinical observations that when used at their effective doses, FK506 has no advantage over CsA in the prevalence of nephrotoxicity in patients receiving transplants (Vincenzi et al., 1996). Thus, the lack of clinical nephrotoxicity seen with RAPA may reflect its relatively greater safety margin or that the side effect in rats is a species-specific pathology.

The toxic dose levels and therapeutic indices of FK506, ASCO and CsA were also similar whether derived from 14-day dosing data or CISPA results, which suggests that the nephrotoxic effects detected in the CISPA are mechanistically similar to those seen with chronic dosing. Of practical significance, the threshold of the CISPA with a single dose of immunosuppressants was as sensitive as 2-week chronic daily administration for revealing nephrotoxicity (table 2).

The effects of CsA and FK506 on the kidney have been studied intensively, and a number of pathophysiological changes have been implicated in the gradual loss of function. First and foremost, these agents both cause a profound effect on renal blood flow (Engelsh et al., 1987; Youngelman et al., 1991), which is expected to be deleterious to an organ with such high oxygen demand (Thurau et al., 1985). This property is also observed with cisplatin (Offerman et al., 1985; Winston and Safirstein, 1985) and represents another mechanistic similarity, along with the shared site of injury, which supports the rationale for its use in establishing the present potentiation model. The gradual loss of kidney function with long-term chronic administration is believed to be the result of a cumulative effect of repeated insults that occur with each dose, effects that should therefore be present and likely contribute to a positive response in the CISPA. Although a detailed discussion of the numerous reports of CsA and FK506 observations is beyond the scope of this paper, these have been reviewed extensively (Bennett, 1995; de Mattos et al., 1996).

Probing immunosuppressant nephrotoxicity with the FKBP antagonist, C18-OH-ASCO, afforded both an opportunity for mechanistic exploration as well as additional validation of the nephrotoxic specificity detected by CISPA. This analog has been shown to bind with high affinity to FKBP12, a predominant immunophilin in T cells, and thereby block T-cell immunosuppression induced by both FK506 and ASCO, which depend on interaction with FKBP for their calcineurin inhibitory activity (Dumont et al., 1992). In contrast, the immunosuppressive activity of CsA is not blocked, because its calcineurin inhibition is mediated by binding to cyclophilin (Friedman and Weissman, 1991). RAPA is mechanistically distinct from both CsA and FK506 because it does not prevent the early events leading to cytokine transcription and production of the growth factor, interleukin-2, but rather blocks the T-cell response to interleukin-2 stimulation (Dumont et al., 1990b). Despite its different mode of action, RAPA also depends on FKBP binding for its immunosuppressive activity. Although this FKBP interaction does not lead to calcineurin inhibition, prevention of binding with an excess
of FK506 (Dumont et al., 1990a, 1994) or C18-OH-ASCO (Dumont et al., 1992) abrogates the effect of RAPA on T cells. C18-OH-ASCO was used in a pioneering study to examine the effects of FK506 given to mice along with a cytochrome P450 inhibitor to delay its metabolism (Dumont et al., 1992). C18-OH-ASCO treatment blocked the elevation of serum BUN induced by FK506, which indicated that this abnormality depended on immunophilin binding and therefore was mechanistically related. In our experience with this method, the fact that only BUN levels were affected by FK506 (data not shown) raised doubt about whether nephrotoxicity was indeed occurring in this model as opposed to some other toxic effect. For example, high doses of FK506 cause gastrointestinal side effects and inhibit food intake and weight gain (Kumano et al., 1991; Ohara et al., 1990; Ryffel et al., 1994). Reduction in food intake in rodents can be expected to result in dehydration and may cause BUN elevation. We therefore used C18-OH-ASCO both to help validate the CISPA for detecting ASCO nephrotoxicity and to probe in vivo the immunophilin dependence for nephrotoxicity of FK506 and other immunosuppressants not previously studied by this approach.

The structures of FK506, C18-OH-ASCO and RAPA are shown in fig. 10. The binding domains involved in docking of the drugs to the FKBP protein are virtually identical in all three molecules. The right-hand portion of the macrocyclic ring constitutes the effector region, which differs for each compound. This illustrates why RAPA can bind to FKBP, but the complex interacts with a distinct protein unrelated to calcineurin, variously designated as FKBP12-rapamycin-associated protein or mammalian target of rapamycin; the mode of action of RAPA has been reviewed (Abraham and Wiederrecht, 1996; Sehgal et al., 1995).

The addition of the hydrophilic hydroxyl group to the C18 position of ASCO occupies the hydrophobic space generally required for docking to calcineurin, and therefore lacks the immunosuppressive effects of FK506 or ASCO. Likewise, C18-OH-ASCO would not be expected to interact with mTOR because it lacks the RAPA effector domain. However, by virtue of its ability to compete effectively with FK506, ASCO and RAPA for binding to FKBP, C18-OH-ASCO is able to inhibit the downstream immunosuppressive effects of all three drugs by preventing the formation of a drug/FKBP complex.

Because the in vivo effects of C18-OH-ASCO on immunosuppression had not been documented, we first determined its ability to block immunosuppression induced by ASCO and FK506 with the rat PLN model. These experiments showed that ASCO and FK506 inhibition of lymph node hyperplasia in response to alloantigen were blocked by pretreatment with the FKBP antagonist, without a similar effect on immunosuppression induced by CsA, which reflects the appropriate mechanistic dependence of these agents on FKBP (fig. 7, A and B). Correspondingly, at somewhat higher doses in the CISPA, to take into account the need to block toxic levels of the immunosuppressants, C18-OH-ASCO prevented the nephrotoxicity only of FK506 and ASCO, but not of CsA, consistent with their mechanistic dependence on FKBP/calcineurin inhibition for this side effect.

At a dose of 100 mg/kg i.p., which was greater than the C18-OH-ASCO dose needed to block FK506 nephrotoxicity, there was no effect in the CISPA on toxicity of RAPA (fig. 8B), despite its similar FKBP binding affinity (Dumont et al., 1992). Thus, whereas RAPA depends on FKBP binding for its immunosuppression (Dumont et al., 1990a), the nephrotoxicity of RAPA in the rat is not mediated by FKBP. Concomitant administration of RAPA at nontoxic doses has potentiated the nephrotoxicity of CsA in the rat (Andoh et al., 1996; Whiting et al., 1991). The safety of combining RAPA with CsA or FK506 in patients may ultimately depend on whether the nephrotoxic effects seen in the rat are additive, synergistic or simply not relevant to humans. The fact that C18-OH-ASCO fails to block the nephrotoxicity of RAPA in the CISPA provides strong evidence that its toxicity is not mechanism based. This perhaps increases the likelihood that these results reflect a species-specific toxicity unique to the rat, or that RAPA analogs can be designed to eliminate nephrotoxicity without affecting immunosuppressive activity.

Having validated the sensitivity and specificity of immunosuppressant nephrotoxicity in the CISPA, the assay was used to screen ASCO analogs to identify compounds with less nephrotoxic potential. One such compound was A-119435, which had in vivo immunosuppressive activity in the rat similar to ASCO but with a significantly decreased nephrotoxic potency in the CISPA. This resulted in a 10-fold improvement in therapeutic index relative to ASCO (table 4, fig. 9). The reasons for the shift in the dose-response curve for nephrotoxicity are not yet known.

Analogs of the FK506/ASCO class could differ in their nephrotoxic potential as a result of at least four possible mechanisms. First, there may be a difference in tissue distribution of the drug as a result of a lower affinity for accumulating in cells of the target organ of toxicity, in this case, the kidney. Second, there may be a difference in tissue distribution of FKBP or a predominance of different FKBP subtypes in renal cells to which A-119435 binds less avidly than lymphocyte immunophilins, thereby reducing its nephrotoxicity compared with its immunosuppressive potency. A third possibility is the presence in kidney of a unique calcineurin isoform that has a lower susceptibility to inhibition by the A-119435/FKBP complex than calcineurin in T cells, and which preserves normal function in the presence of the drug. A fourth possibility is that the substrate for calcineurin action whose dephosphorylation leads to toxicity in the kidney differs in some way from that in the T cell, which results in less susceptibility to inhibition by the A-119435/FKBP complex. Choosing among these hypotheses is difficult considering the present lack of knowledge of events on a molecular level leading to renal injury induced by nephrotoxic immunosuppressants. At a minimum, the discovery of A-119435 should provide a new pharmacological tool for further differentiating immunophilin-dependent interactions resulting in immunosuppressant efficacy vs. toxicity.

References


