Cyclosporin G and Metabolite Binding to Cyclophilin and a 50-kDa Binding Protein Related to In Vitro Immunosuppression

James G. Donnelly, 1–3 Yajun Chen, 3 Randall W. Yatscoff, 4 Kenneth R. Copeland, 5 Edmund W. Palaszynski, 6 and Steven J. Soldin 2, 3, 7, 9

Seven purified metabolites of cyclosporin G (CsG) were studied for binding to cyclophilin and a 50-kDa binding protein (50-kDa BP). The ratios of the metabolite dissociation constants with respect to CsG were compared with in vitro immunosuppression by using the primary mixed lymphocyte suppression assay. The immunosuppressive potency ratio of the parent compounds, both cyclosporin A (CsA) and CsG, compared favorably with the drug dissociation constants for cyclophilin and the 50-kDa BP. Three of the seven metabolites had comparable binding and potency ratios for the 50-kDa BP. In contrast, none of the seven metabolites appreciably bound to cyclophilin in the concentration range tested.

Additional Keyphrases: cyclosporin A · drug metabolites · mixed lymphocyte culture assay · immunosuppression · ligand-binding studies

Cyclosporin G (CsG), a natural analog of cyclosporin A (CsA), has a norvaline residue substituted for α-aminobutyric acid at the amino acid 2 position. 10 Although CsA (combined with steroid therapy) is efficacious in preventing allograft rejection, its use is limited by both reversible acute and irreversible chronic nephrotoxicity. Thus, numerous natural and synthetic analogs of CsA have been screened for similar immunosuppressive action in hopes that one or more analogs will be immunosuppressive but less toxic than CsA. Only a few of the analogs screened have shown promise as candidates to replace CsA (1). CsG has immunosuppressive potency similar to that of CsA (2–4), although in initial animal studies some conflicting toxicity data, which may be species dependent, were reported (5–8).

CsG is now in clinical trials in humans. Phase-one studies for metabolism and pharmacokinetic studies have been carried out (9), and the metabolite pattern for CsG encountered in humans is similar to that encountered for CsA (9). CsA metabolites have limited immunosuppressive action with respect to the parent drug; however, the major metabolite, AMI, does demonstrate minor immunosuppressive action (10).

In a previous study, we (10) determined the immunosuppressive potency ratios of CsA metabolites with respect to the parent compound and used these data to correlate immunosuppression with binding to two intracellular drug-binding proteins. To extend our study, we examined the binding of CsG and its metabolites to cyclophilin and a 50-kDa protein that binds CsA, FK-506, and rapamycin (11, 12).

Materials and Methods

Mixed lymphocyte culture (MLC) suppression assay. The MLC assay was previously described (9). Potency ratios for the seven metabolites were determined relative to CsG by dividing the MLC 50% inhibitory concentration (IC50) for each metabolite by the IC50 of CsG.

Cell culture. The Jurkat cell line was maintained in complete medium containing RPMI 1640 supplemented with L-glutamine, penicillin–streptomycin antibiotics (Life Technologies, Gaithersburg, MD), and 100 mL/L fetal calf serum (MA Bioproducts, Walkersville, MD) at 37°C in a CO2-enriched atmosphere (CO2, 50 mL/L).

Protein purification. Jurkat T cells were separated from complete medium by centrifugation at 600 × g, washed with phosphate-buffered saline (pH 6.8), and then stored as a pellet at −70°C until ready for use. The protein purification procedure for cyclophilin was as previously described (11). The 50-kDa binding protein (50-kDa BP) was obtained from calf thymus (Pel-Freeze, Rogers, AR). Two calf thymus glands (200 g) were thawed and then homogenized in 500 mL of homogenization buffer: per liter, 5 mmol of potassium phosphate, pH 6.8, 10 mmol of phenylmethylsulfonyl fluoride, 5 mmol of EDTA, and 5 mmol of ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid. The crude homogenate was centrifuged at 100 000 × g for 1 h at 4°C to obtain the S100 fraction. This fraction was then electrofocused (Rotoflex; Bio-Rad, Richmond, CA) and the tubes between pH 6.2 and 6.4 were collected and concentrated by using a Centricon 30-kDa cutoff filter (Amicon, Danvers, MA). The electrofocusing procedure removes all of the high-abundance binding protein, cyclophilin (isoelectric points 7.4 and 9.0), permitting binding studies to be performed on the 50-kDa BP, which has an apparent isoelectric point of 6.2–6.4 (12).

Radiolabeled cyclosporin A. [Mebmt-β-3H]cyclospoor-
raine (\(^{3}H\)CsA) was obtained from Amersham (Arlington Heights, IL) at a specific activity of 13 Ci/mol.

**Isolation and identification of CsG metabolites.** Seven CsG metabolites were obtained from the urine of normal healthy individuals who were receiving CsG. The extraction, purification, and characterization procedures were as previously published (9). The metabolites were >98% pure.

**Binding studies.** This procedure was previously described for determining cyclophilin-CsA binding (13) and for determining CsA and metabolite binding to cyclophilin and 50-kDa BP (10, 11). In brief, the binding proteins cyclophilin and 50-kDa BP are incubated for 15 min at 22 °C with a fixed quantity of \(^{3}H\)CsA and various concentrations of CsA, CsG, or metabolites. The LH-20 assay (13) was used to separate \(^{3}H\)CsA-cyclophilin or \(^{3}H\)CsA-50-kDa BP from unbound \(^{3}H\)CsA. Nonspecific binding was determined by adding a 200-fold molar excess of unlabeled drug to the mixture. The drug or radiolabeled compound that is bound to the protein elutes from the LH-20 column in the void fraction, whereas unbound radiolabeled compound is adsorbed to the hydrophobic matrix. The dissociation constants for the 50-kDa BP were determined by using the ligand-binding analysis program (14). The observed dissociation constants for CsA and CsG were used to derive binding ratios for the seven CsG metabolites.

**Results and Discussion**

Cyclophilin and the 50-kDa BP dissociation constants for CsA, CsG, and the seven metabolites are presented in Table 1 along with the MLC IC_{50} results. Both CsA and CsG bound to cyclophilin almost equally, with 50% bound values of 200 and 210 nmol/L. None of the seven metabolites displaced \(^{3}H\)CsA from cyclophilin in the concentration range used. The 50-kDa BP also did not distinguish between CsA and CsG in these binding studies (Table 1 and Figure 1). This indicates that the loss of a methyl group at amino acid 2 is not important for drug binding to the 50-kDa BP or cyclophilin (Table 2). The hydroxylation of amino acid 1 (GM1) also does not affect the ability of the 50-kDa BP to bind; however, cyclization at amino acid 1 to form an intramolecular lactone ring significantly affected protein binding. This observation was confirmed by using GM1c9, which also demonstrated no significant binding in the concentration range analyzed. The N-demethylation at amino acid 4 (GM4N) also had a significant effect on binding to the 50-kDa BP. The lack of binding to 50-kDa BP by the N-demethylated species at amino acid 4 may be opposed by hydroxylation at amino acid 9, as observed in

**Table 1. CsA, CsG, and CsG Metabolite Binding to Cyclophilin and 50-kDa BP Compared with the Mixed Lymphocyte Culture Suppression Assay**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Bound_{50} (nmol/L)</th>
<th>CsA ratio</th>
<th>CsG ratio</th>
<th>K_{D, nmol/L}</th>
<th>CsA ratio</th>
<th>CsG ratio</th>
<th>IC_{50}, nmol/L</th>
<th>IC_{50} Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA</td>
<td>200</td>
<td>1</td>
<td>1.1</td>
<td>39 ± 48</td>
<td>1</td>
<td>1</td>
<td>15 ± 3</td>
<td>2.2</td>
</tr>
<tr>
<td>CsG</td>
<td>210</td>
<td>1</td>
<td>1</td>
<td>37 ± 27</td>
<td>1</td>
<td>1</td>
<td>33 ± 6</td>
<td>1</td>
</tr>
<tr>
<td>GM1</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>23 ± 11</td>
<td>1</td>
<td>1.6</td>
<td>329 ± 99</td>
<td>0.1</td>
</tr>
<tr>
<td>GM1c</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>&gt;830</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>3300 ± 220</td>
<td>0.01</td>
</tr>
<tr>
<td>GM1c9</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>&gt;830</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&gt;18 000</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>GM4N</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>&gt;830</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>425 ± 103</td>
<td>0.08</td>
</tr>
<tr>
<td>GM4N9</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>225 ± 220</td>
<td>0.2</td>
<td>0.2</td>
<td>3550 ± 120</td>
<td>0.01</td>
</tr>
<tr>
<td>GM9</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>6 ± 1</td>
<td>7.1</td>
<td>6.7</td>
<td>403 ± 78</td>
<td>0.08</td>
</tr>
<tr>
<td>GM19</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>24 ± 11</td>
<td>1.6</td>
<td>1.5</td>
<td>6550 ± 250</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Bound 50% (nmol/L) of CsG or metabolite/bound 50% (nmol/L) of CsA.

*Bound 50% (nmol/L) of CsA or metabolite/bound 50% (nmol/L) of CsG.

*Potency ratio (IC_{50} CsG/IC_{50} CsA).

ND, binding not detectable.
GM4N9. Supporting this observation is the low dissociation constant of GM9 from the protein, possibly attributable to hydrogen binding between the hydroxy group at amino acid 9 and an amino acid in the protein’s binding site.

CsA, CsG, GM1c, GM4N, and GM4N9 binding to the 50-kDa BP correlated reasonably well with in vitro immunosuppression. In contrast, GM1, GM9, and GM19 binding did not correlate with in vitro immunosuppression. The binding of these metabolites may not be related to immunosuppressive activity. One possible reason for this is that metabolite diffusion across the cell membrane may be, in part, a limiting factor in the immunosuppressive potency of these metabolites. If this hypothesis is correct, then any immunosuppressive metabolite that does not displace radiolabeled CsA from either cyclophilin or 50-kDa BP suggests that the binding protein is not involved in immunosuppression. None of the seven CsG metabolites bound to cyclophilin in the concentration range tested; however, two of the metabolites did exhibit ~10% of the immunosuppressive activity of the parent compound. In our previous CsA metabolite study, we observed immunosuppressive activity with AM9, AM1, and AM4N (10). The 50-kDa BP did not bind AM9 and AM4N but bound AM1. Cyclophilin bound AM9, AM1, and AM4N, which in this study alone would make it a better target protein for CsA. However, with the observations from the CsA and CsG metabolite study, the results are more equivocal, because both proteins apparently do not bind to some of the immunosuppressive metabolites.

Cyclophilin is a rotamase enzyme (15), and this activity can be inhibited by CsA. The 50-kDa BP is likely not a rotamase enzyme, because it failed to catalyze the cis-trans folding of several different peptides (J. G. Donnelly, S. J. Soldin, manuscript in preparation). Several groups have observed that the inhibition of rotamase activity is not required for immunosuppression (16, 17). However, it also was observed that the CsA–cyclophilin (18, 19) and FK-506–FK BP (20) complexes are capable of associating with and inhibiting calcineurin (calmodulin-binding protein) phosphatase in vitro. If immunosuppressive activity is dependent on the complexation of the drug–immunophilin complex to other proteins in the cytosol, then the dissociation constant of drug with regard to immunophilin may not be as important as the dissociation constant of the drug–immunophilin complex with regard to the ultimate target protein. These observations increase the importance of identifying the minor (low-abundance) immunophilins present in the cytosol of T cells and determining whether their in vivo function is similar to that of the major immunophilins.

This paper is part of a doctoral dissertation in pharmacology at George Washington University by J.G.D.

References
Discordant Results for Determinations of Triglycerides in Pig Sera

Tom Tuten, Keith A. Robinson, and Demetrios S. Sgoutas

We recently determined triglyceride concentrations in pig sera by three fully enzymatic methods (Kodak Ektachem 700, Hitachi 707, and Abbott EPx) and obtained significantly lower values than those obtained with chemical or enzymatic methods based on chemical hydrolysis. All methods used involve microbial lipases for liberating glycerol from triglycerides and glycerol phosphate dehydrogenases or oxidases for subsequent oxidation. The methods were validated against reference methods by using fresh human sera and survey materials. The discordant results were not from matrix sample–method interaction but from incomplete hydrolysis of pig serum triglycerides by the lipolytic enzymes. When serum triglycerides from 10 pigs showing the highest biases were hydrolyzed by microbial lipases and the reaction mixture was subjected to thin-layer and gas–liquid chromatography, the predominant end products were palmitoyl monoglyceride and a mixture of free fatty acids with the following composition (fatty acid as percent of total ± SD): 16:0, 7.8 ± 2; 18:0, 5.4 ± 2.2; 18:1, 53 ± 12; 18:2, 31 ± 4.6; and 18:3, 2.5 ± 1.

Additional Keyphrases: enzymatic methods • lipase • variation, source of • fatty acids

Methods for analyzing triglycerides are based on the determination of the glycerol portion of the triglyceride molecule after hydrolysis to remove fatty acids and liberate glycerol (1). Colorimetric and fluorometric methods depend on chemical hydrolysis followed by an oxidation of glycerol to formaldehyde with periodate and the subsequent reaction of formaldehyde with chromotropic acid or with acetylacetone and ammonium acetate to yield colored or fluorescent products (2, 3). Enzymatic methods are based on the formation of glycerol phosphate from glycerol, with the ultimate conversion of NADH to NAD⁺ through coupled reactions and the monitoring of the decrease in NADH absorption at 340 nm (4). In other enzymatic schemes, glycerophosphate is oxidized by glycerophosphate oxidase (EC 1.1.3.21) to dihydroxyacetone phosphate and hydrogen peroxide (1, 5), which in the presence of peroxidase (EC 1.11.1.7) oxidizes a chromogen to produce a colored dye, and the color intensity is monitored.

Lipases (e.g., triacylglycerol acylhydrolase; EC 3.1.1.3) of animal or microbial origin, which catalyze the hydrolysis of triglycerides, have been substituted for chemical saponification, making possible the development of methods that are direct, rapid, and specific (5, 6). Paramount, however, for an accurate determination of serum triglycerides is the complete hydrolysis of all triglycerides. Because serum triglycerides are esterified with different fatty acids, efforts were made to develop lipolytic systems that completely hydrolyze all triglycerides in human serum and are stable, specific, and simple (7, 8).