dilute specimen. Usually, the intense contribution from polyclonal free light chains is sufficient to enable the correct interpretation in the absence of visible α2-microglobulins; however, in the present case this was not true because of a lack of polyclonal immunoglobulin synthesis. This case illustrates the importance of comparing serum and urine results whenever possible.

A second misinterpretation that may emerge from inadequate concentration of urine is failure to identify a BJP. In the case described here, although nothing was observed in the γ region of the 20-fold concentrate with UPE, a diffuse region of staining was seen in the 100-fold concentrate. After IFE, this diffuse staining clearly appears as a band of restricted migration, which stained as a κ-BJP (Fig. 1B). The problem of screening for BJPs with UPE alone is further accentuated by the patterns shown in Fig. 1C, from a second patient, where nothing is seen after UPE of a 100-fold concentrate, but a κ-BJP is seen clearly after IFE of the same sample. In the case shown in Fig. 1C, the total protein was found to be 150 mg/24 h, an amount that is within the reference interval. Most laboratories quantify urine protein with a precipitation method, such as trichloroacetic or sulfosalicylic acid, or with a protein binding dye such as Coomassie blue or Ponceau S. The degree to which these methods measure free light chains is unclear. Very few laboratories use the biuret method, which reacts with the peptide bonds in proteins, measuring all proteins more equally. As a result, except for biuret method, these methods cannot always be relied on to provide useful information regarding quantification of free light chains. Furthermore, the detection limit of all of these methods, including the biuret, are higher than that of IFE (5). Although disagreement remains as to which combination of UPE and IFE provides the most efficient and cost-effective screening approach to detecting BMP (6), the cases presented here show that even with a 100-fold concentration, UPE in the absence of IFE is an inadequate screen for low concentrations of BMP in samples.

Some commercial manufacturers of kits indicate that the urine sample should be concentrated to a defined protein concentration, which in our experience often turns out to be only a 10- to 20-fold concentration for UPE or IFE. The importance of greater mechanical concentration is emphasized in this study. In our opinion, the best approach may reduce the more tedious IFE by ~30%.

Identification of a Pharmacologically Active Metabolite of Mycophenolic Acid in Plasma of Transplant Recipients Treated with Mycophenolate Mofetil, Ekkehard Schütz, Maria Shipkova, Victor W. Armstrong, Eberhard Wieland, and Michael Oellerich (Abteilung Klinische Chemie, Georg-August-University, D-37075 Göttingen, Germany; * address correspondence to this author at: Abteilung Klinische Chemie, Zentrum Innere Medizin, Georg-August-Universität, Robert Koch Strasse 40, D-37075 Göttingen, Germany; fax 49 (0)391-398551, e-mail eschuetz@med.uni-goettingen.de)

Mycophenolic acid (MPA), the active moiety of mycophenolate mofetil (MMF), is an antiproliferative agent that acts by inhibition of inosine monophosphate dehydrogenase type II (IMPDH-II), a key enzyme in the de novo purine biosynthetic pathway (1,2). Several studies have documented that MMF is effective in the treatment of refractory rejection in renal, heart, and liver transplant recipients (2). The major pathway for elimination of MPA involves glucuronidation (3) at the phenolic hydroxyl group to form mycophenolate 7-O-glucuronide (7-O-MPAG). Modification of this phenolic hydroxyl residue leads to a loss of pharmacological activity toward IMPDH-II (4,5).

Most studies on the pharmacokinetics of MPA have utilized HPLC procedures (6,7) to measure both MPA and MPAG. Recently, the first immunoassay became available for the quantification of MPA (Emit-MPA, Dade Behring). 7-O-MPAG does not cross-react with this assay. Comparison of plasma MPA concentrations from clinical samples determined with HPLC showed an overestimation in relation to those obtained with the Emit of up to 100%, with an average of 35% in a group of 37 kidney recipients, which accounts for a mean overestimation of 20% for the calculated areas under the concentration-time curve (8). Through a modification of our HPLC procedure (7), we were able to identify two putative MPA metabolites, M-1 and M-2, from the plasma of transplant recipients, of which M-2 was found to cross-react in the immunoassay (8). Recently, we showed that in clinical samples from heart, kidney, and liver recipients, the relative amounts of M-2 correlate with the bias between MPA values determined with HPLC and the immunoassay (9). In pharmacokinetic studies, it was shown that the areas under the concentration-time curve for both M-1

References

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and M-2 can account for ~10% of those of MPA, whereas M-2 can be present in predose trough samples at concentrations up to those of MPA (9). Mass spectrometric analyses are consistent with M-1 being the 7-O-glucoside conjugate and M-2 being an acyl glucuronide of MPA (10). In addition, both metabolites could be produced by incubation of MPA with human liver microsomes and the respective cosubstrates (10). In a recent review Bullingham et al. (11) pointed out that an acyl glucuronide conjugate of MPA was observed in the urine of patients in amounts similar to those of MPA. Knowledge of the pharmacological potential of drug metabolites is essential for judging their role in drug therapy. We, therefore, investigated the ability of the newly discovered metabolites to inhibit human IMPDH-II.

MPA, 7-O-MPAG, M-1, and M-2 were isolated from the plasma of transplant recipients under treatment with MMF, using HPLC as described elsewhere (10). After collection, the fractions containing metabolites were desalted by column chromatography, concentrated by evaporation, and reconstituted in water. The purity of the isolated metabolites was confirmed by rechromatography on HPLC.

Human IMPDH-II was expressed in *Escherichia coli* (DH10B; Life Technologies), using the pProEX-HTb plasmid (Life Technologies), which was modified to express the unmodified recombinant human IMPDH-II. Briefly, an 85-bp fragment of pProEX-HTb containing the ribosome binding site was enzymatically amplified with a restriction site for RsaI at the 5′ end. The 3′ end was constructed to include the translation initiation codon and a 22-bp fragment of the human IMPDH-II mRNA sequence. ssDNA of this construct was used as the forward primer with a human lymphocyte cDNA library, whereas the specific reverse primer was equipped with a NotI restriction site. After enzymatic amplification, the resulting construct was ligated into the plasmid after RsaI and NotI digestion of both insert and plasmid with a T4 DNA ligase (Life Technologies). The IMPDH-II insert was confirmed by complete sequencing using a LI-COR Model 4200 infrared dye sequencer (MWG-Biotec). Cells were harvested 10–20 h after induction of expression in transformed *E. coli* with isopropyl-β-d-thiogalactoside, and recombinant human IMPDH-II (rh-IMPDH-II) was extracted with 4 mol/L urea and purified by chromatography over phosphocellulose followed by blue-Sepharose as described elsewhere (12). The purity after these steps was >99% as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). The integrity of the protein was confirmed by N-terminal amino acid sequencing. The specific activity of the rh-IMPDH-II was determined to be 800 U/g protein. The conditions for determination of IMPDH-II activity were as follows (final concentrations in reaction): 100 mmol/L Tris-HCl, 200 μmol/L IMP, 300 μmol/L NAD⁺, 3 mmol/L EDTA, 1 mmol/L dithiothreitol, and 1.5 U/L rh-IMPDH-II. Inhibition of the enzymatic activity of the rh-IMPDH-II was used to assess the pharmacological activity of the metabolites as follows: 2.0–12 μL of sample containing either metabolite, MPA, or H₂O as control was mixed with 250 μL of reaction mixture without NAD⁺, and after incubation for 5 min, the reaction was started by the addition of 30 μL of NAD⁺ solution; the enzymatic activity was followed by measurement of absorbance at 340 nm for 5 min. The enzyme reactions were carried out at 37 °C on a Cobas Mira Plus analyzer (Hoffmann-La Roche).

As shown in Table 1, M-2 isolated from the plasma of a liver transplant recipient under treatment with MMF displayed a concentration-dependent inhibition of rhIMPDH-II similar to that obtained with MPA. The ability of M-2 to inhibit rh-IMPDH-II was further investigated by use of a total of 21 separate HPLC preparations of this metabolite obtained from the plasma of six kidney transplant recipients, one heart transplant recipient, and one liver transplant recipient at different time points after transplantation. The concentration of M-2 in each preparation was estimated using the MPA Emit assay and compared with the MPA concentration that caused the same inhibition of rh-IMPDH-II within the same analytical run (inhibition/concentration ratio). The inhibitory effect of M-2 from different preparations was found to be

**Table 1. Concentration-dependent inhibition of rh-IMPDH-II by MPA and metabolites isolated from plasma of a liver transplant recipient under MMF therapy.**

<table>
<thead>
<tr>
<th>MPA/metabolite</th>
<th>Concentration, μg/L</th>
<th>Inhibition, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPA</td>
<td>5.6</td>
<td>22.4</td>
</tr>
<tr>
<td></td>
<td>11.1</td>
<td>49.6</td>
</tr>
<tr>
<td></td>
<td>22.0</td>
<td>69.5</td>
</tr>
<tr>
<td></td>
<td>32.5</td>
<td>87.2</td>
</tr>
<tr>
<td>M-2</td>
<td>5.6</td>
<td>24.0</td>
</tr>
<tr>
<td></td>
<td>11.1</td>
<td>52.5</td>
</tr>
<tr>
<td></td>
<td>22.0</td>
<td>75.2</td>
</tr>
<tr>
<td></td>
<td>32.5</td>
<td>89.1</td>
</tr>
</tbody>
</table>

*Final concentrations of MPA and M-2 in the enzyme assay are given. Percentage of inhibition of rh-IMPDH-II vs H₂O as control. Results are means of duplicates.*

![Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant IMPDH-II expressed in *E. coli* after purification by column chromatography and reconcentration.](image)
somewhat variable compared with that of MPA: the median inhibition/concentration ratio of M-2 to MPA, as a measure of the relative effectiveness compared to MPA, was 0.91 (range, 0.48–1.23; n = 21).

In contrast, 7-O-MPAG, also fractionated by HPLC, showed no measurable enzyme inhibition. When five fresh preparations of 7-O-MPAG from the plasma of kidney recipients with final concentrations of 8–23 mg/L were tested immediately, the enzyme activity in the presence of 7-O-MPAG was not significantly different from that of the respective control assay using H2O (ratio of enzyme activity with 7-O-MPAG to the control activity: median, 0.998; range, 0.94–1.03; P = 0.61). The purity of these preparations was confirmed by HPLC analysis.

The 7-O-glucoside metabolite M-1, which like 7-O-MPAG does not cross-react in the immunoassay, also did not inhibit rh-IMPDH-II at concentrations up 500 µg/L estimated by HPLC; this concentration estimation was based on the assumption that both 7-O-MPAG and M-1 have a similar ultraviolet absorption at 215 nm.

This is the first study to demonstrate the presence of a metabolite of MPA that possesses a pharmacological potency almost as high as that of the parent drug. Like MPA, this metabolite (M-2) cross-reacts with the antibody used in the Emit immunoassay. M-2 has been observed regularly in the plasma of liver, kidney, and heart transplant recipients undergoing treatment with MMF (8) and has been identified as the acyl glucuronide of MPA (10). This is in agreement with the observation that modifications at the acyl residue do not lead to a substantial loss of IMPDH-II inhibitory efficacy, in contrast to modifications at the phenolic hydroxyl group (13). Results from a recent study (3) into the glucuronidation of MPA with human UDP-glucuronosyltransferase (UGT1A10) showed that in addition to the 7-OH glucuronide, a second product was formed, which was proposed to be the acyl glucuronide of MPA. Some variability was observed in the ability of different preparations of M-2 to inhibit IMPDH-II. This may be related to the fact that acyl glucuronides undergo intramolecular rearrangement at physiological pH from position 1 to positions 2, 3, and 4 in the glucuronic acid moiety (14). The metabolite M-2 isolated from the plasma of patients is, therefore, likely to be a mixture of these different isomers. Whether these isomers differ in their ability to inhibit rh-IMPDH-II must await their separation and isolation.

In accordance with the results of previous investigations (5), purified 7-O-MPAG did not inhibit rh-IMPDH-II. The higher IMPDH-II inhibition of 7-O-MPAG seen in lymphoblasts (15) could not be confirmed with the recombinant enzyme. According to our experience, during 7-O-MPAG isolation and storage, hydrolysis to MPA can occur in trace amounts, which then inhibit IMPDH activity, when 7-O-MPAG is tested at high concentrations (data not shown). This phenomenon may also explain the apparent IMPDH inhibition by 7-O-MPAG obtained in a test system that used a cell lysate as the source of IMPDH-II (15). The second metabolite, M-1, has been shown to be the 7-O-glucoside of MPA (10). In accordance with the modification at the phenolic hydroxyl group, this metabolite, like the 7-O-MPAG, did not inhibit rh-IMPDH-II when tested at concentrations up to 40-fold higher than those that achieved a 50% inhibition with MPA or M-2. The 7-O-glucoside metabolite, like 7-O-MPAG, did not cross-react in the immunoassay. In conclusion, a pharmacologically active metabolite of MPA has been identified, which is present in the plasma of patients undergoing immunosuppressive therapy with MMF. The magnitude of the observed rh-IMPDH-II inhibition and the presence of M-2 in the plasma of transplant recipients (9) in concentrations up to those of MPA in predosetrough samples is of interest with regard to its contribu-tion to the immunosuppressive and toxic effects of MPA.

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A Gap Between Total Prostate-specific Antigen and the Sum of Free Prostate-specific Antigen Plus α1-Antichymotrypsin-Prostate-specific Antigen in Patients with Prostate Carcinoma but not in Those with Benign Prostate Hyperplasia, Klaus Jung,1* Brigitte Brux,2 Angela Knäbich,1 Michael Lein,1 Pranav Sinha,2 Dietmar Schnorr,2 and Stefan A. Loening1 (Departments of 1 Urology and 2 Laboratory Medicine, University Hospital Charité, Humboldt University Berlin, Berlin, Germany; * address correspondence to this author at: Department of Urology, University Hospital Charité, Humboldt University, Schumannstrasse 20/21, D-10098 Berlin, Germany; fax 49 30 28021402, e-mail klaus.jung@charite.de)

Approximately 70–90% of the total serum prostate-specific antigen (t-PSA) in serum is complexed to α1-antichymotrypsin (ACT), whereas 10–30% of t-PSA is not bound to serum proteins and is called free PSA (f-PSA). The determination of f-PSA and the calculation of the ratio of free to total PSA has proven to be a promising tool for differentiating between prostate cancer (PCa) and benign prostate hyperplasia (BPH), because the ratio is lower in PCa than in BPH (1, 2). Although the determination of ACT-PSA would have the analytical advantage of measuring the major and not the minimal portion and the clinical advantage of measuring the portion of serum PSA apparently directly related to PCa (3–5), overrecovery problems related to interferences with the ACT-cathepsin G complex for ACT-PSA assays are obstructive to accurate ACT-PSA measurement (6). Thus, it is generally assumed that the sum of f-PSA plus ACT-PSA yields t-PSA, whereas the small amounts of PSA being bound to other proteins such as α1-antitrypsin and protein C may be neglected. However, there is no information concerning whether the occurrence of minor forms of PSA is different between patients with PCa and those with BPH. We have now studied this problem by using a reliable ACT-PSA assay.

t-PSA, f-PSA, and ACT-PSA were measured on an ES immunoanalyzer (Boehringer Mannheim GmbH). The principle of the tests is based on the one-step (for t-PSA; cat. no. 1555332) and/or two-step (for f-PSA, cat. no. 1776444; for ACT-PSA, a research assay) sandwich technique. For the determination of t-PSA, samples (calibrator, calculated on the basis of the mean value + 2 SD) of 0.068 μg/L; intraassay imprecision (CV; n = 21) of 1.51–3.48% in five pooled human sera with ACT-PSA values between 1.05 and 22.0 μg/L; interassay CV (n = 8–17) of 2.08–6.32% in four pooled sera and one control material with ACT-PSA values between 0.32 and 32.2 μg/L. Measurement of 53 female sera (41 healthy females and 12 with pronounced inflammation indicated by highly increased C-reactive protein between 144 and 284 mg/L; reference value, <5 mg/L) showed a mean value...