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Use of human organ slices to evaluate the biotransformation and drug-induced side-effects of pharmaceuticals

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Abstract

Human liver and kidney organ slices were used to investigate the biotransformation competence of the slices in combination with several markers of cell viability and function. The immunosuppressant cyclosporin A (CSA) is extensively metabolized in liver slices to the three known primary metabolites and many secondary metabolites. In kidney cortex slices the biotransformation of CSA is far more pronounced in humans than in rats. In human liver slices, levels of CYP3A, the proteins metabolizing CSA, are depressed about 25% by 1 and 10 μmol/L CSA within 24 h, indicating that high blood or tissue concentrations will inhibit CSA clearance.

A clinical marker for liver damage is the release of cellular α-glutathione-*S*-transferases (αGST). In this study the αGST levels were used to assess donor organ quality, organ slice incubation conditions, and compound exposure. A marker for cell death in human cells is the solubilization and release of nuclear matrix proteins (Numa). Increases were apparent only after 48 h of culture.

A side-effect of CSA is that it induces hypertension and perturbs the lipid profile of transplant recipients. A potential marker for lipid disturbances is levels of serum lipoprotein (a) (Lp(a)), which is synthesized in the liver and found only in humans, apes, and nonhuman primates. CSA increases Lp(a) levels in the human liver slice cultures about 2-fold.

This study has demonstrated that the biotransformation capability of the organ slices contributes to the optimization of the *in vitro* system and to the evaluation of markers for drug induced side-effects or toxicity. Assays were identified that could be used clinically to monitor CSA-induced organ damage or rejection (αGST), hypertension (Lp(a)), and toxicity (Numa).

Abbreviations: αGST, α-glutathione-*S*-transferases; CSA, cyclosporin A; CYP3A, cytochrome P4503A; CSX, undefined cyclosporin; Lp(a), lipoprotein (a); IMM, SDZ IMM 125; Numa, nuclear matrix proteins

Introduction

In many regards, organ slice cultures are an attractive *in vitro* system for investigating the biotransformation and drug-induced side-effects of pharmaceuticals. The slices are prepared in a similar manner regardless of the species or organ, yielding a flexibility and ease in comparing species and organ differences. Critical to performing predictive bio- transformation and toxicity studies *in vitro* is the need to have all the cell types representative of the organ in the culture, since many cell types can participate in the metabolism and contribute to the toxicity of the drug under investigation. The technique is also an efficient use of tissue, in that any remaining tissue can be used to prepare microsomes or other subcellular fractions.

In this study the biotransformation of the immunosuppressant cyclosporin A (CSA) was used to evaluate the metabolic competence of the organ slices in combination with several markers of cell viability and function. An elevation of serum α-glutathione-*S*-transferase (αGST) levels is becoming a marker for liver or kidney rejection and toxicity, because αGST increases occur prior to increases in the aminotransferases, ALT or AST, and the αGSTs are more uniformly distributed throughout the liver (periportally and pericentrally), representing a greater proportion of the total soluble cellular protein (4–5% versus <1% for ALT) (Beckett and Hayes, 1993). The αGST levels of human liver and kidney slices were used to evaluate two culture methods and the effect of cyclosporins on slice viability.

CSA, at concentrations of 1–10 μmol/L, inhibits DNA and protein synthesis and disrupts transport processes in the absence of cell necrosis (Mason, 1990). A marker for cell death is the solubilization and release of nuclear matrix proteins (Numa) from human cells (Miller et al., 1993). This cellular program of self-destruction, apoptosis, is considered to be a very conserved process among cells, regardless of the mechanism by which it is induced, and may occur within a particular concentration range for a compound, while higher concentrations may cause necrosis.

A side-effect of CSA therapy is hypertension, and CSA may contribute to the increased incidence of coronary heart disease in transplant recipients (Sturrock et al., 1993). CSA binds and is transported by plasma lipoproteins and perturbs the lipid profile of transplant recipients, raising plasma cholesterol, LDL-cholesterol, triglycerides, VLDL-triglycerides and apolipoprotein B. A marker for an increased risk toward premature coronary heart disease and stroke is the elevation of serum lipoprotein (a) (Lp(a)) levels, which may prove to be a marker for lipid disturbances as well as liver recovery posttransplantation (Malmendier et al., 1992; Kostner, 1993; Scanu, 1993). Two recent studies demonstrate that CSA elevates serum Lp(a) levels in renal transplant recipients (Brown et al., 1993; Webb et al., 1993). The liver is the main site of lipoprotein synthesis as well as CSA metabolism. A possible link between disturbances in lipid metabolism and biotransformation pathways, including bile acid synthesis, may exist. Furthermore, Lp(a) is found in humans, apes, and nonhuman primates (Scanu, 1993).

This paper presents the use of human slices for the evaluation of biotransformation and compound-induced side-effects and toxicity.

Materials and methods

Organ slices (liver and kidney, 200 ± 25 μm) were prepared from 8-mm-diameter cores in tissue culture medium using a Krumdieck tissue slicer. The human organs had been perfused with Belzer's University of Wisconsin solution but could not be suitably used for transplantation. Kidney slices of human and rat (male Wistar, 200 g) were prepared from the cortex region of the kidney, which is the site of the cytochrome P450s, and the site of CSA-induced toxicity. Organ quality was checked

visually within 15 min by the slice reduction of the tetrazolium salt MTT (150 μg/ml EBSS) to MTTF at 37°C. Controlling slice thickness (200 ± 25 μm) will affect both slice viability and its biotransformation capacity.

The decline in cytochrome P450 levels can be in part prevented by the supplementation of the culture medium with hormones and ligands, such as a cold oxygenated EBSS-based media without phenol red containing the recommended amounts of MEM-essential and nonessential amino acids, BME-vitamins, glutamax, and Mito+™ serum extender, antibiotic/antimycotic solution, and supplemented with 25 mmol/L Hepes, 1 μmol/L dexamethasone, 25 mmol/L nicotinamide, 10 nmol/L glucagon, 25 mmol/L glucose, 0.1 mmol/L δ-aminolevulinic acid, 0.2 mmol/L adenosine, 0.33 mmol/L L-cysteine, 5 mmol/L glutathione, 0.3 μmol/L aprotinin, and 0.1 mmol/L AEBSF (pH 7.4). Two slices were loaded onto each roller insert and maintained in 2 ml of medium containing 10% NuSerum plus 1 or 10 μmol/L CSA (3.2 μCi [^{3}H]CSA/ml medium in the biotransformation cultures) at 37°C (95:5, air:CO_2) for up to 48 h, after a 90-min preincubation period. The DMSO vehicle had a final concentration of 0.1%. At various times the slice and medium were separated and stored at –80°C. Recovery of the radioactivity was achieved by rinsing the insert and roller culture with 1.5 ml of methanol. Supernatants of the biotransformation samples were prepared for HPLC analysis (Kronbach, 1988) by homogenizing the slice in methanol, adding the medium, and pelleting the cellular protein. Slice protein was determined with bovine immunoglobulin as the standard (Bradford, 1976). Drug stability was checked under the experimental conditions in the absence of a slice.

CYP3A Western Blot: CYP3A protein levels were determined in the human liver slice cultures following 24 h of CSA exposure (1 and 10 μmol/L). The liver slice S9 fraction (10 μg) was prepared as described for microsomes (Vickers et al., 1992), mixed with sample loading buffer and separated for 1 h on a 12% SDS-PAGE gel by electrophoresis at 200 V (Mini-PROTEAN II, Bio-Rad, Glattbrugg, Switzerland). The protein bands were transferred to Hybond-C nitrocellulose membrane (Amersham), incubated for 3 h with a polyclonal rabbit anti-human CYP3A primary antibody diluted 1:2000 (Oxygene Int., Dallas, TX, USA), followed by a 1-h incubation with a secondary anti-rabbit antibody (Amersham) at a 1:1000 dilution. The membranes were exposed to Hyperfilm ECL film (Amersham) and band intensity was quantified by densitometry (Bio-Rad).

Viability: αGST levels were determined in the human slice homogenates and medium using the Hepkit™ enzyme immunoassay (Biotrin International, Dublin, Ireland). Aliquots (200 μl) of slice homogenate (0.75–2.5 μg protein/ml) and medium (diluted 400×) were incubated for 1 h in microtiter plates precoated with a monospecific antibody to human αGST on a plate rocker at room temperature. The secondary biotinylated antibody, a rabbit anti-human-αGST, was added for 1 h followed by the enzyme conjugate solution, streptavidin peroxidase complex, for 1 h. Following color development the optical density was read at 490 nm with unknown values extrapolated from the standard curve.

The nuclear matrix protein (Numa) levels in the slice and medium were measured using the Apo-Pak (Advanced Magnetics, Inc., and Maritech Inc., Cambridge, MA, USA). Aliquots (100 μl) of slice homogenate (60–200 μg protein/ml) and medium were exposed to microtiter plates coated with murine monoclonal antibodies for NMP 41/7 for 2 h at room temperature on a plate rocker. The optical density following color development was read at 490 nm and the concentration of NMP 41/7 in the sample was extrapolated from a reference dose–response curve.

Liver slice homogenate Lp(a) levels were

determined using the Coaliza Lp(a) Assay (Kabi Diagnostica, Moelndal, Sweden). Aliquots (100 µl) of liver slice homogenate (300 µg – 1 mg of protein) were incubated with mouse anti-human Lp(a) monoclonal antibody coated onto microtiter plates for 3 h at 37°C with gentle shaking, followed by a 1-h incubation with an enzyme-labeled anti-human apo B polyclonal antibody (conjugate). The optical density was read following color development at 450 nm and the amount of Lp(a) was determined from a standard curve.

Results and discussion

Biotransformation

A method of validation that the liver slice preparation is metabolically competent is the routine inclusion of an internal standard for which the biotransformation is well defined both *in vivo* and *in vitro*, and ideally a compound that is metabolized by several cytochrome P450 families. Such a validation procedure will lead to an optimization of the culture conditions, aid in the judgment as to the quality of the tissue, and increase the power of the prediction for an unknown compound. In this study the biotransformation capability of the liver slices was demonstrated with the metabolism of CSA (1 µmol/L) to the primary hydroxylated metabolites M1 and M17 and the *N*-demethylated metabolite M21 and many secondary metabolites (Figure 1). An example of human liver that had poor biotransformation capability and was not used for further investigation is shown with HL-2. Microsomes derived from this liver formed the three primary metabolites, indicating that the CYP3A was functional, but not adequate for performance of predictive studies (Figure 1). The monitoring of cyclosporin blood levels or CYP3A levels initially after transplantation could give some insight as to the quality of the liver transplanted. Lucey et al. (1990) reported a liver transplant

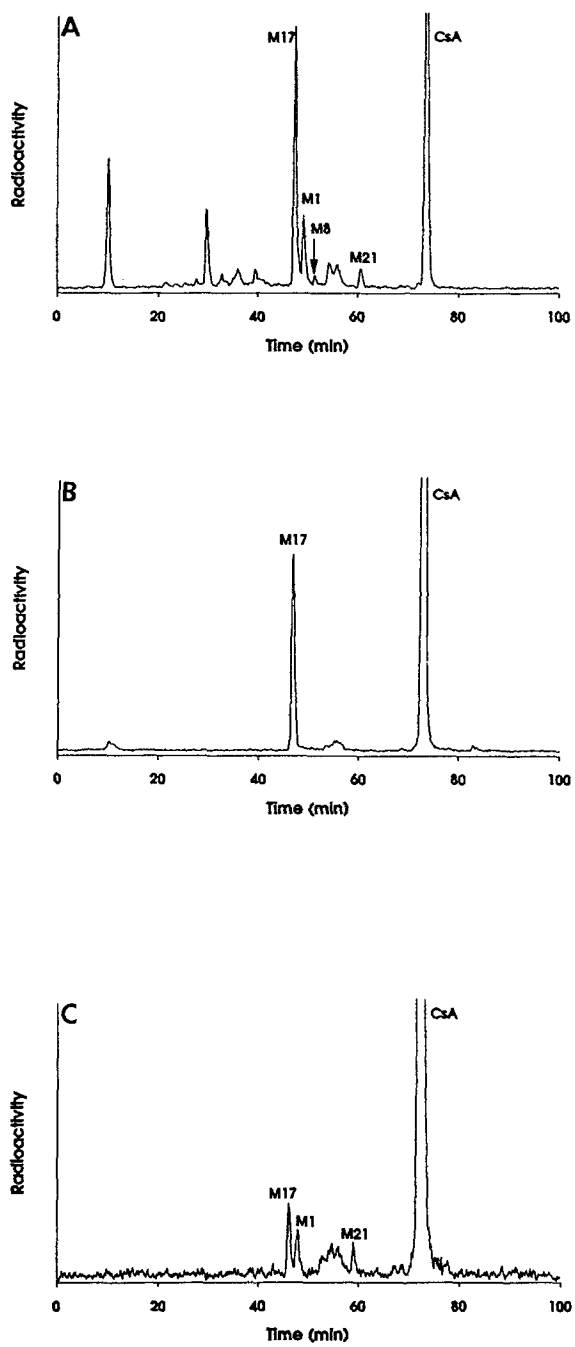

Figure 1. HPLC radiochromatograms of CSA and metabolites from human liver slice cultures exposed to CSA (1 µmol/L) for 24 h: (A) HL-1, (B) HL-2, and (C) HL-2 microsomes incubated with 1 µmol/L CSA for 10 min

recipient with low CYP3A activity and renal failure that could be rechallenged with double the dose of CSA following rifampicin induction. Another study also suggests that a low level of CYP3A in the donor liver contributes to the CSA-associated toxicity and could be deleterious to the recipient (Lemoine et al., 1993).

Liver slice CYP3A levels decrease with 1 µmol/L CSA (20%) and 10 µmol/L CSA (24%) exposure for 24 h as compared to DMSO-exposed slices, indicating that high blood or tissue concentrations will inhibit the clearance of CSA (Figure 2). In liver transplant recipients the median whole blood CSA concentration is 1025 ng/ml, 0.9 µmol/L, (range 18–1925 ng/ml, 0.15–1.6 µmol/L (Kahn et al., 1992). CSA (1 µmol/L) biotransformation plateaued in the human liver slices, with the 24-h slice being equivalent to the 48-h slices (290 pmol/mg slice protein); however, for the cyclosporin derivative IMM (1 µmol/L) biotransformation increased another 36%. Slice viability decreases with culture time, compounded by an inhibition of CYP3A and cyclosporin biotransformation. Either the parent compound directly inactivates the CYP3A proteins or the immunomodulating activities of CSA affect the CYP3A levels.

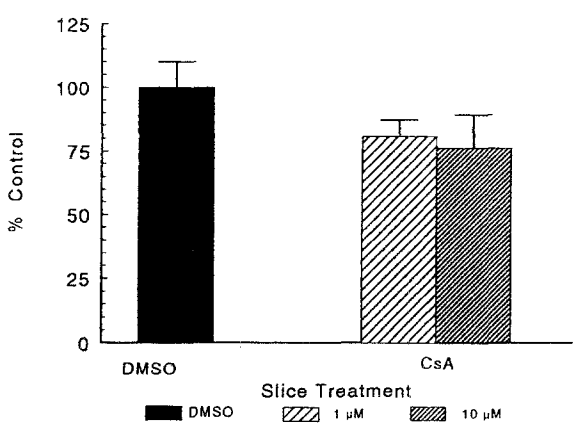

Figure 2. Human liver slice CYP3A levels are decreased upon exposure to 1 and 10 µmol/L CSA compared to 0.1% DMSO-exposed slices. Results of three independent experiments.

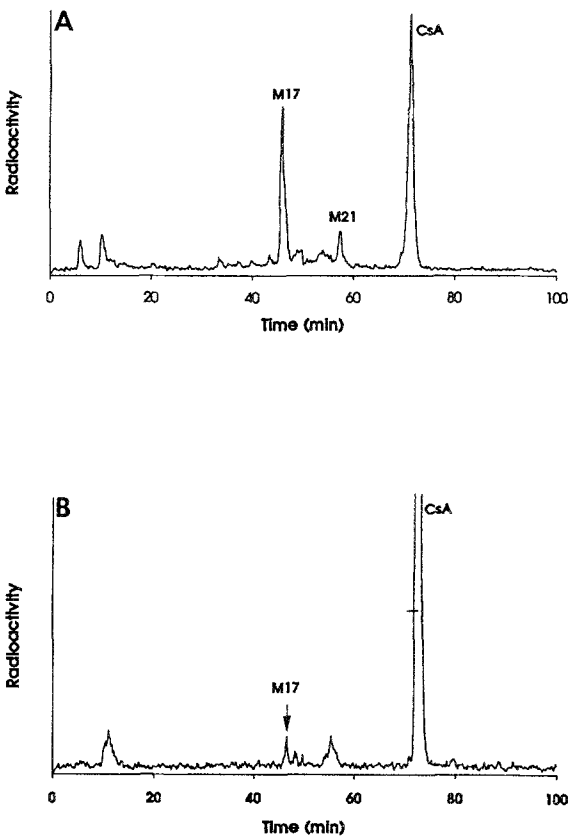

Figure 3. HPLC radiochromatograms of CSA (1 µmol/L) biotransformation in (A) human kidney slices after 24 h and (B) rat kidney slices after 48 h.

The biotransformation of CSA by the adult human kidney to M17, and to M21 in some kidneys, could only be demonstrated clearly with slice cultures and not with microsomes (Figure 3). CSA biotransformation continues in human kidney cultures carried out to 48 h, with the formation of M17 often doubling. For CSA the human kidney slices were a better model for human kidney CSA metabolism than either the rat or dog kidney slices, in which metabolism is not clearly evident. Rat kidney slices maintained for 48 h formed small amounts of M17, less than 1% of the radioactivity in the culture (Figure 3). The human kidney cortex comprises about 72% of the kidney, indicating that the human kidney could produce considerable quantities of M17, substantiating the presence of

M17 as the main metabolite in human urine, while unchanged CSA represents only 0.1% of the administered dose (Maurer et al., 1984). M17 is less immunosuppressive, possessing 8–15% of the activity of CSA, and is less capable of eliciting a toxic response (Copeland et al., 1990).

Viability parameters

The leakage of αGST into the medium is a useful marker for judging the viability of both human liver or kidney slices. In this study, αGST leakage has also been used to compare two different culture methods: roller cultures versus plate cultures. Control liver or kidney slices generally exhibit medium αGST levels of 20–30% after 24 h in roller cultures, depending on the organ quality and total organ preservation time. The slices remain more viable in the roller cultures even up to 48 h of culture than slices that are constantly covered with medium (plate cultures) for 24 h (Figure 4). Exposure of human liver and kidney slices to CSA (1 μmol/L and 10 μmol/L) for 24 and 48 h did not affect αGST release, while the cyclosporin CSX caused a dose-dependent increase (1.5–2-fold) of αGST levels (Figure 4). Increases in serum αGST levels of liver transplant recipients precede increases of ALT, AST and bilirubin triggered by hepatocellular damage and allograft rejection, and fall more rapidly than ALT and AST following both liver transplantation and treatment of rejection, permitting a greater discrimination of subsequent changes in liver function (Beckett and Hayes, 1993).

The reduction of the tetrazolium salt MTT to MTTF is used as a marker for cell viability but is currently in question. The assay does not always correlate with other markers of cell death and the suspected mitochondrial involvement of this reaction is only about 40%, while extramitochondrial NADH- and NADPH-dependent reactions account for the rest (Berridge and Tan, 1993). For some compounds, like the cyclosporins, this assay is not indicative of just cell death. After a 24-h exposure the CSA-exposed slices displayed an equivalent activity for MTTF formation as control, while increases were seen with IMM (about 1.2-fold) and CSX (1.6-fold) (Figure 5). The increase induced by CSX was maintained even at 48 h.

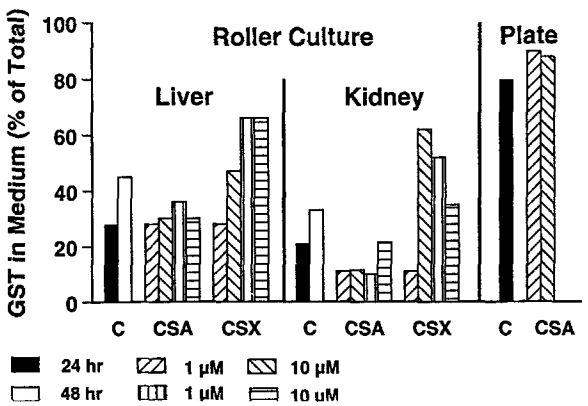

Figure 4. Human liver and kidney slice viability assessed by αGST leakage from slices exposed to 0.1% DMSO, 1 and 10 μmol/L CSA or CSX for 24 and 48 h in roller cultures, and after 24 h in the plate cultures

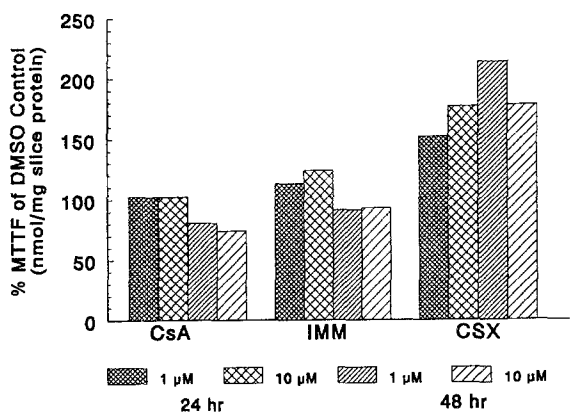

Figure 5. Reduction of MTT in human liver slices following exposure to 1 and 10 μmol/L CSA, IMM and CSX for 24 and 48 h compared to 0.1% DMSO-exposed slices

The release of nuclear matrix proteins by the human liver slices increased with culture time from 10% at 24 h to 25% by 48 h. An increase due to exposure to cyclosporin, CSA, IMM or

CSX, was only apparent at 48 h and 10 µmol/L concentrations (Figure 6). In a series of compounds tested, both this assay and the αGST assay flagged the same compounds, which could have a greater potential for toxicity than CSA.

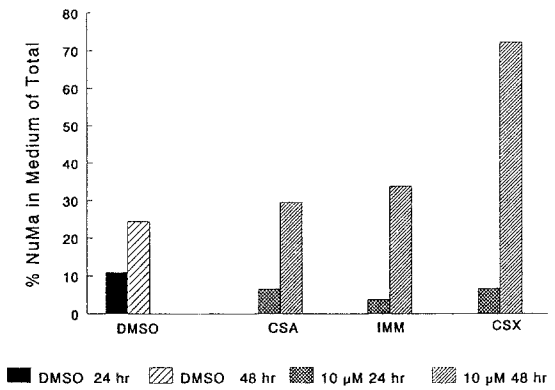

Figure 6. Human liver slice viability assessed by the leakage of nuclear matrix proteins into the medium following exposure to 10 µmol/L CSA, IMM or CSX for 24 and 48 h.

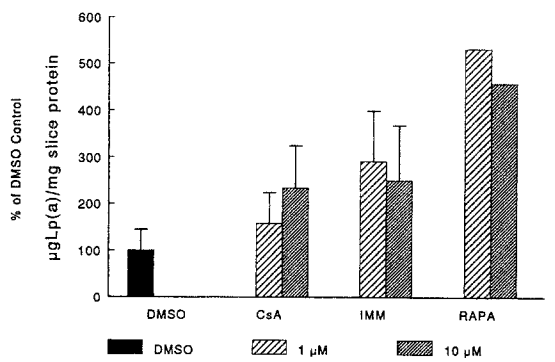

Figure 7. Human liver slice Lp(a) levels after 24 h of 1 and 10 µmol/L CSA and IMM (n=4 different livers), and rapamycin (RAPA, n=1).

Human liver slice Lp(a) levels are increased by the cyclosporins CSA and IMM (1 and 10 µmol/L) within 24 h, about 2-fold over DMSO control (Figure 7). This *in vitro* finding mimicked the increased serum Lp(a) levels (1.8- to 3.6-fold) induced by CSA in renal transplant recipients. Rapamycin increased Lp(a) levels 4- to 5-fold at 1 and 10 µmol/L, indicating that rapamycin derivatives will have a similar effect on liver function as the cyclosporins. One liver exhibited about 10-fold higher Lp(a) levels (35 µg Lp(a)/mg slice protein) such that differences due to CSA exposure could not be detected. The presence of Lp(a) in humans but not rodents suggests that this drug-induced side-effect will manifest itself better in a human *in vitro* system than in the rodent studies. It has also been suggested that a transplanted organ (liver, kidney) may be predisposed to genetic hypertension (Rettig et al., 1993).

This study has combined the investigation of the biotransformation of a compound with investigation of its potential to induce side-effects or toxicity. Markers that could be used clinically to monitor CSA-induced organ damage or rejection (αGST) and hypertension (Lp(a)) have been identified. Additionally, an assay indicative of organ damage (Numa) provided information about compounds potentially more toxic than CSA.

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