Hepatic Cytochrome P450s Attenuate the Cytotoxicity Induced by Leflunomide and Its Active Metabolite A77 1726 in Primary Cultured Rat Hepatocytes

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The Black Box Warning section of the U.S. drug label for leflunomide was recently updated to include stronger warnings about potential hepatotoxicity from this novel anti-arthritis drug. Because metabolic activation is a key mechanism for drug-induced hepatotoxicity, we examined whether leflunomide and its major metabolite, A77 1726, are cytotoxic to primary rat hepatocytes and whether their toxicity is modulated by hepatic cytochrome P450s (CYPs). As measured by lactate dehydrogenase leakage, time-dependent cytotoxicity was observed at 250–500 μM for leflunomide and 330–500 μM for A77 1726 within 20 h. Unexpectedly, three nonisoenzyme–specific CYP inhibitors, including SKF-525A, metyrapone, and 1-aminobenzotriazole, did not reduce but remarkably enhanced the cytotoxicity of leflunomide or A77 1726. SKF-525A pretreatment notably rendered hepatocytes susceptible to as low as 15 μM leflunomide or A77 1726. Three isoenzyme-specific CYP inhibitors including alpha-naphthoflavone, ticlopidine, and ketoconazole that mainly target CYP1A, CYP2B/2C, and CYP3A, respectively, also enhanced the cytotoxicity. A strong synergistic effect, similar to SKF-525A alone, was noted using a combination of all three of the isoenzyme-specific inhibitors. Hepatocytes pretreated with the CYP inducer dexamethasone for 24 h exhibited decreased cytotoxicity to leflunomide and A77 1726. At the concentrations tested, the CYP inhibitors and inducer showed no cytotoxicity. These data demonstrate that the parent forms of leflunomide and A77 1726 are more toxic to hepatocytes than their poorly characterized metabolites, indicating that the metabolic process of leflunomide is a detoxification step rather than an initiating event leading to toxicity.

Key Words: rat; hepatocytes; hepatotoxicity; leflunomide; A77 1726; cytochrome P450s.

Leflunomide is a noncompetitive inhibitor of mitochondrial dihydroorotate dehydrogenase, the rate-limiting enzyme in the fourth step of de novo biosynthesis of pyrimidine (Breedveld and Dayer, 2000). It has immunomodulatory, anti-inflammatory, and antipyretic activities and was approved by the Food and Drug Administration (FDA) as an oral drug to treat rheumatoid arthritis in 1998 (Alcorn et al., 2009). Leflunomide was also approved by the regulatory agencies in many other countries such as Canada and countries in the European Union (Bertele et al., 2007). Shortly after its approval, cases of severe liver injury, including fatal hepatitits and acute liver failure, were noted during postmarketing surveillance of adverse drug event reports. Due to the hepatotoxicity concern, the drug label (package insert) was revised several times so as to increase the prescribers’ awareness of such potential side effects (Breedveld, 2001). Some even suggested the withdrawal of this drug from the market due to the risk of liver injury, but the FDA concluded that the overall benefits of leflunomide significantly outweighed the hepatic risks (Moynihan, 2003). However, very recently, due to increased concerns about clinical cases of hepatotoxicity, the FDA required that severe liver injury information must be put in the Black Box Warning (BBW) section of the prescription drug label for leflunomide (http://www.fda.gov/Drugs/DrugSafety/Postmarket-DrugSafetyInformationforPatientsandProviders/ucm218679.htm). Despite these growing concerns, the clinical use of leflunomide will likely remain unchanged due to the lack of therapeutically equivalent drug products. Therefore, identifying the risk factors and understanding the mechanisms of leflunomide-induced liver injury are of critical importance to protect patients from suffering life-threatening side effects.

As a prodrug, leflunomide undergoes rapid and extensive metabolism in vivo in both humans and experimental animals such as rats, mice, and beagle dogs (http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/20905_ARAVA_PHARMR_P2.PDF), but detailed information on its metabolic pathways and the relationship to hepatotoxicity is unknown (Rozman, 2002). It is generally accepted that leflunomide is mainly converted into a predominant metabolite, A77 1726, to exert its pharmacological effects in vivo, and A77 1726 is further biotransformed into an oxanilic acid derivative that is
eliminated by the kidney in the urine (Rozman, 2002). The above information was mainly obtained by the sponsor of leflunomide during the early drug development process. In the literature report, original studies on leflunomide metabolism were scarcely found. In human liver microsomes, hepatic cytochrome P450 1A (CYP1A) has been implicated to play a role in the biotransformation of leflunomide to A77 1726, but CYP2C and CYP3A may also be involved as determined by the purified recombinant human CYPs (Kalgutkar et al., 2003). In animal-based studies, the liver microsomes of both rats (Kalgutkar et al., 2003) and mice (Chan and New, 2007) were shown to be capable of catalyzing this reaction, but the responsible CYP isoenzymes have not been identified. The enzymes involved in the downstream metabolism of A77 1726 remain unknown, though the possible role of CYPs has been suggested (Rozman, 2002). In addition to hepatic CYP-mediated biotransformation, the plasma and gut wall also play a contributory role (Kalgutkar et al., 2003; Rozman, 2002).

Some drugs associated with liver injury have been shown to be metabolized by CYPs to reactive metabolites that react with cellular macromolecules and subsequently disrupt hepatocellular homeostasis (Lammert et al., 2010; Walgren et al., 2005). We therefore tested the hypothesis that the cytotoxicity of leflunomide and A77 1726 in primary rat hepatocytes is associated with reactive metabolite formation. We unexpectedly found that the cytotoxicity induced by leflunomide and A77 1726 was enhanced rather than decreased when the activities of CYPs were inhibited, and the cytotoxicity was reduced rather than increased when CYPs were induced, indicating that their metabolism is a detoxification step rather than an initiating event leading to toxicity. Data presented here will provide novel hypotheses into why some subjects are more susceptible than others to leflunomide-induced liver injury and hopefully will help form a theoretical basis for the development of new biomarkers of sensitivity to leflunomide hepatotoxicity.

MATERIALS AND METHODS

Chemicals and reagents. Leflunomide with a purity of ≥99% and A77 1726 with a purity of ≥98% were obtained from Enzo Life Sciences International (Plymouth Meeting, PA). PureCol (bovine collagen I) was from Advanced BioMatrix (San Diego, CA). Fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA). Dexamethasone, proadifen (also known as SKF-525A, SKF), metyrapone (10 μM), ANF (10 μM), KET (25 μM), or TIC (20 μM) for 30 min followed by treatment with the inhibitor plus leflunomide or A77 1726 for 20 h. For CYP induction experiments, 10 μM of dexamethasone was added to the media of 4 h attached cells and incubated for an additional 24 h followed by leflunomide or A77 1726 treatment. All chemicals were dissolved in dimethyl sulfoxide (DMSO) with the final DMSO concentration in the medium being <0.5%. For a given experiment, the DMSO concentration was the same for all treatments, including controls. All the experiments were repeated at least three times using cells isolated from at least three different rats.

Cell viability measured by lactate dehydrogenase leakage. Cell viability was determined by the modified lactate dehydrogenase (LDH) leakage assay (Chao et al., 1988) using an uQuant Microplate Reader (BioTek, Winooski, VT). Briefly, 10 μl of medium was taken out of each well in the six-well plates at the specified time point and Triton-X-100 was added to the cells to achieve a final concentration of 1% in order to lyse the cells. After 1 h, another 10 μl of medium was taken from the lysed cells. These 10-μl samples were placed in parallel into a 96-well plate and 240 μl buffer containing 81 mM Tris, 204 mM NaCl, 0.2 mM NADH, and 1.7 mM monosodium pyruvate, pH 7.2, were added to each well. The absorbance at 340 nm (A340) was immediately measured at 60-s intervals for 5 min using the kinetic mode. The software KCjunior, provided by the microplate reader manufacturer, was used to determine the rate of decrease at A340 due to the conversion of NADH to NAD+ by LDH. The rate obtained before cell lysis was divided by those after cell lysis to calculate the percentage of LDH leakage. Cell viability thus obtained correlated very well with the trypan blue exclusion method in selected experiments.

Statistical analysis. The software GraphPad Prism 5 was used to perform one-way and two-way ANOVA followed by Dunnett’s test. A p value less than 0.05 was considered as statistically significant. Data are means and SDs from at least three batches of cells isolated from at least three different rats.
RESULTS

**Leflunomide and A77 1726 Caused Time- and Concentration-Dependent Cytotoxicity in Primary Rat Hepatocytes**

The average human steady-state plasma concentration of A77 1726 is about 250 μM after repeated therapeutic dosing of leflunomide (Rozman, 2002). This concentration was therefore chosen as a median to tentatively design the drug exposure levels in primary rat hepatocytes. The same concentration range was applied to leflunomide so as to facilitate a head-to-head comparison. Preliminary studies showed that neither leflunomide nor A77 1726 interfered with the LDH assay (data not shown), excluding the possibility of false interpretation of cell viability. In selected cases, trypan blue exclusion was carried out in parallel plates to validate the LDH data and the two methods gave comparable results (data not shown). As shown in Figures 1A and 1B, no cytotoxicity was observed at 170 μM for both drugs as determined by LDH leakage. Statistically significant cytotoxicity began to appear at 250 μM leflunomide (Fig. 1A) and 330 μM A77 1726 (Fig. 1B), suggesting that leflunomide is more cytotoxic than A77 1726. The toxicity of both drugs increased with the exposure time (Figs. 1A and 1B). To minimize hepatocyte dedifferentiation, that is, time-dependent loss of normal phenotype such as CYP activity, drug treatment was started at 4 h and ended at 24 h after cell isolation.

**Isoenzyme Nonspecific CYP Inhibitors Remarkably Enhanced the Cytotoxicity of Leflunomide or A77 1726 in Primary Rat Hepatocytes**

SKF, ABT, and MET are three widely used CYP inhibitors that are not isoenzyme selective and have commonly been used in primary cultured hepatocytes to investigate the involvement of metabolic enzymes in chemically induced hepatotoxicity (Ishihara et al., 2006; Kostrubsky et al., 2006). The concentrations used for them, that is, 20 μM for SKF, 1 mM for ABT, and 2 mM for MET, were based on both previous reports (Ishihara et al., 2006; Kostrubsky et al., 2006) and preliminary experiments using fluorescent probes to measure the CYP isoenzyme activity in intact cells, which showed that CYP activity was decreased (data not shown). As shown in Figures 2A and 2B, when cells were treated without (i.e., 0 μM) leflunomide (Fig. 2A) or A77 1726 (Fig. 2B), no significant cytotoxicity was induced by these CYP inhibitors alone. However, pretreatment with either of the three CYP inhibitors caused remarkably enhanced cytotoxicity of both leflunomide and A77 1726 (Figs. 2A and 2B). The most striking enhancement was observed with SKF, which caused a statistically significant increase in cytotoxicity at as low as 15 μM for both leflunomide and A77 1726 (Figs. 2A and 2B). Less remarkable enhancement was found with ABT and MET, both of which caused a significant

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**FIG. 1.** (A–B) Cytotoxicity of leflunomide and A77 1726 in primary cultured rat hepatocytes. Freshly isolated rat hepatocytes were seeded in six-well plates for 4 h. Then, 3-ml serum-free medium containing 0, 170, 250, 330, 420, and 500 μM leflunomide (Fig. 1A) or A77 1726 (Fig. 1B) was added. Cytotoxicity was determined by LDH leakage at 1, 3, 6, and 20 h. Data are means and SDs from three batches of cells isolated from three different rats. *Statistically significant compared with the control, p < 0.05.

**FIG. 2.** (A–B) Effects of three isoenzyme–specific CYP inhibitors on the cytotoxicity induced by leflunomide or A77 1726 in primary rat hepatocytes. Primary rat hepatocytes were treated with 1 mM ABT, 2 mM MET, or 20 μM SKF for 30 min after seeding in six-well plates for 4 h. Leflunomide (Fig. 2A) or A77 1726 (Fig. 2B) was then added to achieve final concentrations of 0, 15, 31, 63, 125, or 250 μM. After 20 h, cytotoxicity was measured by LDH leakage. Data are means and SDs from three batches of cells isolated from three different rats. *Statistically significant compared with cells not treated with CYP inhibitors, p < 0.05. #Statistically significant compared with cells treated with ABT or MET, p < 0.05.
increase in cytotoxicity for leflunomide and A77 1726 at 125 and 250\(\mu\)M (Figs. 2A and 2B). Of note, in parallel experiments, when the CYP inhibitors were added together with Kava extract, a dietary supplement associated with hepatotoxicity, no significant changes in cytotoxicity were observed (data not shown), indicating that the enhanced cytotoxicity of leflunomide and A77 1726 is not due to nonspecific stress.

**Isoenzyme-Specific CYP Inhibitors Significantly Increased the Cytotoxicity of Leflunomide or A77 1726 in Primary Rat Hepatocytes**

ANF, KET, and TIC are three FDA-recommended isoenzyme-specific CYP inhibitors for drug development and drug interaction studies (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm081177.htm#4). Their principle targets are CYP1A, CYP3A, and CYP2B/2C, respectively. ANF and KET have been used in rat hepatocytes to probe the CYP contributions to chemically induced liver injury (Ishihara et al., 2006; Jurima-Romet et al., 1991), and the reported concentrations, that is, 10\(\mu\)M for ANF and 25\(\mu\)M for KET, were adopted in the present study. For TIC, 20\(\mu\)M was adopted based on preliminary experiments showing that it was a nontoxic concentration and CYP2C activity was ~90\% inhibited (data not shown). To clarify which CYP isoenzymes play a contributory role in the cytotoxicity induced by leflunomide and A77 1726, hepatocytes were pretreated with either one of these three CYP inhibitors, or a combination of all three, and their effects examined. As shown in Figures 3A and 3B, all three inhibitors consistently led to enhanced toxicity of leflunomide and A77 1726, with the CYP3A inhibitor, KET, being the most potent, which caused significant cytotoxicity at 63\(\mu\)M leflunomide and 125\(\mu\)M A77 1726 (Figs. 3A and 3B). For leflunomide, but not A77 1726, the enhanced cytotoxicity by KET appeared even stronger than the nonisoenzyme–specific inhibitors ABT and MET (Figs. 2A and 3A). Interestingly, cells pretreated with a mixture of these three inhibitors (10\(\mu\)M ANF, 12\(\mu\)M KET, and 20\(\mu\)M TIC) displayed remarkable cytotoxicity even at 31\(\mu\)M leflunomide or 63\(\mu\)M A77 1726, which were nontoxic concentrations when the three CYP inhibitors were used alone with leflunomide or A77 1726 (Figs. 3A and 3B). None of the inhibitors by themselves were significantly toxic to the cells at the concentrations tested. Based on preliminary experiments, the KET concentration was reduced from 25 to 12\(\mu\)M in the mixture of the three CYP inhibitors in order to prevent cytotoxicity triggered by these inhibitors themselves.

**CYP Inducer Dexamethasone Caused a Reduction in the Cytotoxicity Induced by Leflunomide or A77 1726 in Primary Rat Hepatocytes**

To further establish the roles of CYPs in the cytotoxicity induced by leflunomide or A77 1726, dexamethasone was used to induce CYP activity. Treatment of the cells with dexamethasone lasted for 24 h before addition of leflunomide or A77 1726 to provide sufficient time for the induction of CYP enzymes (Nishimura et al., 2007). As illustrated in Figures 4A and 4B, the cytotoxicity of both leflunomide and A77 1726 was significantly decreased by dexamethasone. In the control groups, that is, cells not treated with dexamethasone, the cells appeared more sensitive to leflunomide and A77 1726 than those in Figures 1A and 1B, possibly due to decreased CYP activity in cells attached for 28 h in the CYP induction study versus 4 h for the CYP inhibition study.

**DISCUSSIONS**

Although leflunomide-induced hepatotoxicity has been recognized in clinical practice for over a decade, no well-designed studies are available for the cytotoxicity induced by leflunomide or A77 1726 in primary hepatocytes. Despite some known limitations, this remains an essential model to explore the mechanisms of drug-induced hepatotoxicity (Hewitt et al., 2007). Indeed, some authors have touched on the issue of...
leflunomide cytotoxicity for other purposes. For example, one paper showed that A77 1726 was not toxic to commercial human primary hepatocytes at 10–50 μM when the production of serum amyloid A protein was inhibited (Migita et al., 2005). Another report showed that A77 1726 did not induce necrotic cell death in rat primary hepatocytes at 1–50 μM when bile acid-induced apoptotic cell death was prevented (Vrenken et al., 2008). Our data are in line with these publications in that we did not see increased LDH leakage even when leflunomide or A77 1726 concentrations were increased to 170 μM. However, we did observe obvious cytotoxicity at 250 μM for leflunomide and 330 μM for A77 1726 and we clearly showed that leflunomide was more toxic than A77 1726 to normal hepatocytes. This observation is reminiscent of a previous report showing that leflunomide appeared more toxic than A77 1726 at 300 μM in the immortalized human hepatic cell line HC-04 (Seah et al., 2008). However, the comparisons between our results and published data may not be straightforward due to the species difference between humans and rat, particularly their CYP profiles (Martignoni et al., 2006). For example, CYP1A2 is highly expressed in human livers and is very sensitive to inhibition by furafylline, but its expression is very low in rats and it is resistant to inhibition by furafylline (Martignoni et al., 2006). Species-dependent responses should be taken into consideration for further mechanistic studies using cells of human origin.

It might be argued that the cytotoxicity presented here has limited clinical relevance because after a human therapeutic dose, the plasma concentration of leflunomide is barely detectable and the free circulating A77 1726 (i.e., nonprotein bound) is estimated to be 2.5 μM after taking into account that 99% of the 250 μM plasma A77 1726 is bound to albumin (Latchoumycandane et al., 2006; Tan et al., 2008). However, these findings are likely to be of clinical importance because (1) the exact exposure levels of leflunomide and A77 1727 to hepatocytes, that is, the local concentrations in the liver that is the most relevant, are not known, and there are many uncertainties in directly extrapolating plasma drug levels to hepatocyte exposure concentrations or simply assuming that only the free drug but not protein-bound drug exerts all the toxic effects and (2) remarkable interindividual variability in the pharmacokinetics of leflunomide have been reported (van Roon et al., 2005). Assuming a 10-fold difference in the maximal blood concentration of A77 1726 among individuals, it is possible that in rare cases, a subject could have as high as 25 μM free A77 1726 in the circulation. This concentration was shown to be toxic to hepatocytes when CYPs were inhibited by SKF (Figs. 2A and 2B), justifying the potential clinical significance of the present study. Of course, animal studies are needed to further enhance this speculation and such efforts are underway. It is worthwhile to point out that leflunomide was shown to cause liver necrosis in rats, mice, and dogs when administrated for a 1- to 2-year period at high doses (http://www.accessdata.fda.gov/drugsatfda_docs/nda/98/20905_ARAVA_PHARMR_P2.PDF).

The most important finding is that CYP inhibitors and an inducer significantly enhanced and decreased, respectively, the cytotoxicity of both leflunomide and A77 1726 in primary hepatocytes. The significance of this finding is twofold. First, it raises an interesting question if clinical cases of leflunomide-induced hepatotoxicity could be, at least in part, the result of drug-drug interactions or genetic or environmental alterations of CYPs. Because two of the CYP inhibitors we adopted here, KET and TIC, are prescription drugs in the United States, it is possible that subjects taking either of them or in combination will have a higher chance of suffering from liver problems when leflunomide is co-administered. Interestingly, fatal hepatitis was reported in a patient co-administrated with leflunomide and itraconazole, a structural analogue of KET, which is also an FDA-recommended CYP3A inhibitor for drug development and interaction studies (Legras et al., 2002). Similarly, our findings may suggest that genetic alterations in some CYP isoenzymes could predispose patients to this risk. It has been previously reported that the CYP1A2*1F allele, but not the CYP2C19*2, CYP2C19*17, CYP2C9*2, or CYP2C9*3
alleles, were associated with the overall toxicity of leflunomide in rheumatoid arthritis patients (Bohanec Grabar et al., 2008), and one case report showed that a patient with a CYP2C9 polymorphism developed acute hepatitis when treated with leflunomide (Sevilla-Mantilla et al., 2004). Our finding may provide an alternative interpretation of these clinical reports. Second, it implies that the parent forms of leflunomide and A77 1726 are more toxic than their downstream CYP-derived metabolites. This appears in sharp contrast with a previous report showing that leflunomide toxicity was attenuated by pretreatment with a CYP inhibitor ABT (Seah et al., 2008). A possible reason for this discrepancy is that we used primary cultured rat hepatocytes, but the previous study adopted an immortalized human cell line of liver origin (HC-04 cells) (Seah et al., 2008). Although some studies have characterized HC-04 cells in terms of normal hepatocyte function, including CYP activity (Lim et al., 2007), additional research is required to determine the relevance of this model for drug hepatotoxicity research versus primary rat hepatocytes. Based on the consistent results obtained in the present study with six different CYP inhibitors, all of which have been widely used in the study of drug-induced hepatotoxicity, and the finding that the CYP inducer dexamethasone notably decreased the cytotoxicity, it appears that leflunomide and A77 1726 are more toxic than their CYP-derived metabolites. However, the possibility that leflunomide and A77 1726 are converted to more toxic metabolites by enzymes that are not affected by the inhibitors we tested here could not be completely excluded.

Among the three nonisooenzyme–specific inhibitors, SKF was more potent than ABT and MET in enhancing the cytotoxicity. For ABT, one possible reason is that it is ineffective toward inactivating CYP 2C (Linder et al., 2009). In the case of MET, its strong induction of CYP3A may offset its inhibitory effects on other CYP isoenzymes (Wright et al., 1994). Regardless of the reasons, they all enhanced the cytotoxicity of leflunomide and A77 1726, clearly showing that CYP metabolism results in detoxification. Further supporting evidence is that primary rat hepatocytes exposed to either compound 4 h after isolation were less sensitive to the cytotoxicity when compared with hepatocytes exposed 28 h after isolation (Figs. 1A and 1B and Figs. 4A and 4B). CYP activity declines rapidly in isolated hepatocytes with overall CYP activity typically declining to < 50% of basal levels within 24 h of isolation (Vernia et al., 2001). The decreased CYP activity at 24 h would have resulted in less detoxification of both compounds, leading to the increased cytotoxicity as observed. Additional experiments with the CYP inducer dexamethasone (Figs. 4A and 4B) showed an obvious reduction of cytotoxicity, lending strong support that both compounds are detoxified in rat hepatocytes by CYP-mediated metabolism.

The finding that all three isoenzyme-specific inhibitors led to enhanced cytotoxicity of leflunomide and A77 1726 was quite unexpected. It suggests that all the CYP isoenzymes examined, including 1A, 2B/2C, and 3A, play a role in detoxifying leflunomide and A77 1726. However, as with the nonspecific inhibitors, differential sensitivity was observed with KET being the most potent. It is likely that leflunomide and A77 1726 are metabolized by several different CYPs with the rate and/or capacity of metabolism being CYP specific. This is not uncommon for drugs and is exemplified by acetaminophen, which is metabolized by at least three different CYPs (CYP1A, CYP3A, and CYP2E) at different capacities (Zaher et al., 1998). Therefore, it is likely that for leflunomide and A77 1726, inhibiting specific CYPs has a greater impact on cytotoxicity than others due to their rate and capacity for detoxifying both compounds. Most importantly, if any defects occur to the delicate balance of the CYP isoenzymes that metabolize leflunomide and A77 1726, the toxicity of both compounds

![Proposed pathways for the metabolism and detoxification of leflunomide and A77 1726 in primary cultured rat hepatocytes.](image-url)
will be increased in hepatocytes. Figure 5 presents an overview of the potential pathways for the metabolism and detoxification of both compounds based on data generated in this study.

Interestingly, at least 17 papers were published showing leflunomide and A77 1726 are protective against liver injury induced by various means such as acetaminophen overdose (Latchoumycandane et al., 2007) or ischemia-reperfusion (Karaman et al., 2006) in animal models. The rationale behind these studies was that leflunomide would attenuate the immune system response to the liver injury and prevent propagation of the injury such as through reactive oxygen species generation by Kupffer cells. Based on clinical cases of hepatotoxicity (Legras et al., 2002; Sevilla-Mantilla et al., 2004) and data from the present study, the mechanism of leflunomide inhibition of immune system function appears to be separate from that of hepatotoxicity. Therefore, our results are not necessarily contradictory to previous reports but do suggest that an intact functional CYP system is required for leflunomide and/or A77 1726 to be safely used against any forms of liver injury.

Our study has several limitations. First, only pure hepatocytes were used. The possible contribution of the non-parenchymal cells such as the Kupffer cells (Roberts et al., 2007) awaits further investigation. Second, metabolic profiles were not carried out. Third, data were generated from only rat hepatocytes and not cells of human origin. These limitations are expected to be addressed in future studies.

In summary, leflunomide and A77 1726 caused time- and concentration-dependent cytotoxicity in primary rat hepatocytes, with the latter being less cytotoxic. Inhibition of hepatic CYPs led to significantly enhanced rather than decreased cytotoxicity and CYP induction caused a decrease in cytotoxicity, indicating that the toxicity was mainly caused by the parent forms of both compound and not the downstream metabolites. These data provide novel insights into the mechanisms of leflunomide-induced hepatotoxicity and will contribute to the identification of new risk factors and biomarkers to prevent this severe and sometme fatal side effect.

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