Short Communication

Effects of Macrolide Antibiotics on CYP3A Expression in Human and Rat Hepatocytes: Interspecies Differences in Response to Troleandomycin

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ABSTRACT:

The effects of various macrolide antibiotics [triacetyloleandomycin (TAO), clarithromycin, azithromycin, roxithromycin, erythromycin base] and the new ketolide HMR3004 on CYP3A expression were evaluated in human and rat hepatocytes. Cells were treated for 3 days with nontoxic concentrations of the drugs, and CYP3A induction was assessed through midazolam hydroxylase activity and Western and Northern blot analyses. In rat hepatocytes, no induction of CYP3A1 expression was observed following exposure to macrolides, even to erythromycin base and TAO (well known in vivo CYP3A1 inducers), whereas dexamethasone and phenobarbital were confirmed to induce this enzyme. In contrast, treatment of fresh and thawed human hepatocytes with TAO, produced an increase of midazolam hydroxylation (4-fold over control). This result was in agreement with the high amount of CYP3A4 protein and mRNA revealed by Western and Northern blot analyses. Other tested macrolides had no induction effect on CYP3A expression. These results confirmed the interspecies variability of CYP3A regulation in hepatocytes and raised the question of its mechanism of induction by macrolides in human liver.

Macrolide antibiotics have been widely used for treatment of a large number of human and animal infections. Some of these compounds have been proved to undergo specific metabolic interactions with cytochrome P-450s, and more particularly with the CYP3A subfamily (Tinel et al., 1989). To prevent potential drug interactions, many efforts have been devoted to the evaluation of the effects of macrolide antibiotics on the CYP3A protein itself (Von Rosensteil and Adam, 1995).

However, even if a large number of studies has reported the effect of macrolide antibiotics on cytochrome P-450s in vivo and in hepatic microsomes, no comparative study has been published yet on the action of these compounds in human and rat hepatocytes primary cultures. Controversial effects have already been demonstrated for TAO, whether it was examined in vivo or in hepatocytes. Indeed, studies performed in vivo in rat have described a strong inducing effect of TAO on CYP3A1 (Wrighton et al., 1985), whereas results obtained in primary cultures of rat hepatocytes have not shown any increased expression of this isoenzyme. The complete induction mechanism therefore probably requires extrahepatic events (Watkins et al., 1986).

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1 Abbreviations used are: TAO, triacyloleandomycin; ERY, erythromycin base; CLA, clarithromycin; ROX, roxithromycin; AZI, azithromycin; CYP, cytochrome P-450; DEX, dexamethasone; MDZ, midazolam; 3-MC, 3-methylcholanthrene; PB, phenobarbital; RIF, rifampicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction.

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The objective of this study was primarily to investigate the effects of five macrolide antibiotics including TAO, erythromycin base (ERY), clarithromycin (CLA), roxithromycin (ROX), and azithromycin (AZI) and the new ketolide HMR3004 on the expression of one of the major CYP families involved in drug metabolism and toxicity, namely, CYP3A subfamily. Experiments were performed in primary cultures of fresh and thawed human hepatocytes and on rat hepatocytes.

Materials and Methods

Chemicals. Williams’ E medium, fetal bovine serum, and penicillin/streptomycin solutions were obtained from Life Technologies (Cergy Pontoise, France). β-NADP reduced form, DMSO, and TAO were from Sigma (St. Quentin Fallavier, France). Other macrolide antibiotics and the ketolide HMR3004 were provided by Hoechst Marion Roussel (Romainville, France). Oligonucleotides were obtained from Eurogentec (Angers, France). All other products were of analytical grade. Radiolabeled midazolam was from Hoffmann-La Roche (Basel, CH).

Primary Cultures of Human Hepatocytes. Hepatocytes from human surgical liver biopsies were isolated by a reverse two-step collagenase perfusion (Nicolas et al., 1995) and seeded at the density of 8 × 10**2** cells/cm**2** on 60-mm plates coated with rat type I collagen. Cells were seeded in medium I, consisting of Williams’ E medium with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and insulin (0.1 U/ml). After 24 h, the medium was replaced with serum-free medium (medium II) containing hydrocortisone hemisuccinate (1 μM), and BSA (240 μg/ml) with or without the test compounds. Cryopreserved human hepatocytes were briefly thawed, recovered by centrifugation (50g, 3 min) and seeded as described previously (Dou et al., 1992).

Primary Cultures of Rat Hepatocytes. Fresh rat hepatocytes were obtained from male Sprague-Dawley rats (220–240 g) by a two-step collagenase perfusion method (Berry and Friend, 1969). Cells were seeded as for human hepatocytes. Medium I was replaced 4 h later with medium II supplemented or not with the test compounds.
Cytotoxicity Test. The cytotoxic effects of macrolide antibiotics on rat hepatocytes were assessed after 24 h of exposure, using Neutral Red assay and MTT test (Nicolas et al., 1995).

Cells Treatments. Hepatocytes were exposed to 50 μM macrolide antibiotics (except 25 μM ROX and 10 μM HMR3004). Prototypical CYP inducers were used: 50 μM dexamethasone, 25 μM rifampicin, and 2 mM phenobarbital. All compounds were dissolved in DMSO (final concentration ≤0.25%). After treatment, medium was discarded and plates were stored at −80°C. After thawing, cells were harvested into 100 mM potassium buffer, pH 7.4. Protein content was determined by BCA assay (Pierce, Rockford, IL).

Midazolam Hydroxylase Activity. MDZ metabolism was measured by incubating 60 μl of cellular extract for 30 min at 37°C with 60 μl of reactive solution (100 mM KH2PO4, pH 7.4, 2 mM NADPH, 20 μM [3H]midazolam; 0.104 μCi/ml) (Fahre et al., 1988; Dou et al., 1992). Reactions were stopped by adding 120 μl of cold acetonitrile. The tubes were then centrifuged for 10 min at 13,000 g and analyzed by HPLC.

Northern Blot Analysis. Total RNA were isolated by acidic phenol extraction (Chomczynski and Sacchi, 1987). Twenty micrograms of RNA were size-fractionated on a 0.9% agarose gel containing 10% formaldehyde, and transferred to nitrocellulose membrane. Hybridization was performed in the presence of the appropriate 32P-labeled probes. CYP3A4 and CYP3A1 cDNA sequences (position +1096 to +1517) were obtained by PCR using the following oligonucleotides: 5′-CCCCACCTATGATACTGTG-3′ as first primer for both human and rat CYP3A, and 5′-GGTTGAGAAGCTGTGCTGC-3′ or 5′-GGTTGAGAAGCTGTGCTCCTAAGC-3′ as reverse primers for CYP3A4 and CYP3A1 cDNA probes, respectively.

Western Blot Analysis. Ten micrograms of protein extract were analyzed on SDS-10% polyacrylamide gel electrophoresis. Corresponding blots were then incubated with anti-CYP3A4 antibodies (Oxford Biomedical Research, Oxford, MI). Specific signals were detected by ECL (Amersham Life Science, Orsay, France).

Statistical Analysis. Statistical significance was evaluated using the non-parametric Mann-Whitney U test, and the levels of probability were noted (*P < .05; **P < .01). Data were expressed as means ± S.D. from at least three experiments.

Results and Discussion

A large number of studies have demonstrated the effects of macrolide antibiotics on CYP3A activity, by using human and animal microsomes. The use of primary cultured hepatocytes has already demonstrated its capability to provide relevant information on the metabolism and hepatotoxicity of various chemicals (Nicolas et al., 1995; Viluksela et al., 1996; Reinach et al., 1999). However, the effects of macrolides on CYP expression in this cellular model have been less considered as yet. The purpose of this study was to compare the activities of macrolides on CYP3A4 and CYP3A1 cDNA probes, respectively.

Comparative cytotoxicity was assessed on rat hepatocytes by using two different endpoint assays: the reduction of MTT and the Neutral Red assay. As shown in Table 1, significant differences were observed between the different compounds. HMR3004 appeared to be the most toxic chemical with an IC50 value of 25 to 30 μM. The rank order of toxicity was as follows: HMR3004 > AZI > ROX > CLA > TAO > ERY. In addition, an important accumulation of light-reflecting intracellular bodies was observed both in human and rat hepatocytes exposed to 25 μM AZI and to a lesser extent with 50 μM CLA. These results were in agreement with the lysosomal alteration de-

### Table 1

Comparison of IC50 values (μM) of different macrolide antibiotics measured using MTT and Neutral Red tests

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MTT (μM)</th>
<th>Neutral Red (μM)</th>
</tr>
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<tbody>
<tr>
<td>ERY</td>
<td>794.3 ± 61.7</td>
<td>464.6 ± 79.0</td>
</tr>
<tr>
<td>TAO</td>
<td>409.1 ± 57.3</td>
<td>369.8 ± 1.7</td>
</tr>
<tr>
<td>CLA</td>
<td>444.7 ± 32.9</td>
<td>246.1 ± 30.1</td>
</tr>
<tr>
<td>ROX</td>
<td>193.5 ± 11.8</td>
<td>164.9 ± 38.9</td>
</tr>
<tr>
<td>AZI</td>
<td>208.9 ± 53.8</td>
<td>143.0 ± 47.1</td>
</tr>
<tr>
<td>HMR3004</td>
<td>33.9 ± 3.5</td>
<td>24.5 ± 0.9</td>
</tr>
</tbody>
</table>

### Table 2

MDZ hydroxylase activity in human and rat hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Human Hepatocytes</th>
<th>Rat Hepatocytes</th>
</tr>
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<tbody>
<tr>
<td>DMSO</td>
<td>3.50 ± 0.37</td>
<td>1.31 ± 0.16</td>
</tr>
<tr>
<td>PB</td>
<td>10.60 ± 2.90</td>
<td>3.62 ± 0.51**</td>
</tr>
<tr>
<td>RIF</td>
<td>15.91 ± 1.09**</td>
<td>7.35 ± 1.74*</td>
</tr>
<tr>
<td>DEX</td>
<td>N.D.</td>
<td>3.44 ± 0.36*</td>
</tr>
<tr>
<td>TAO</td>
<td>13.11 ± 3.21*</td>
<td>5.19 ± 0.36**</td>
</tr>
<tr>
<td>ERY</td>
<td>4.19 ± 1.03</td>
<td>1.59 ± 0.36</td>
</tr>
<tr>
<td>ROX</td>
<td>2.45 ± 1.30</td>
<td>1.49 ± 0.48</td>
</tr>
<tr>
<td>CLA</td>
<td>1.59 ± 0.28**</td>
<td>1.26 ± 0.09</td>
</tr>
<tr>
<td>AZI</td>
<td>N.D.</td>
<td>1.81 ± 0.06*</td>
</tr>
<tr>
<td>HMR3004</td>
<td>1.66 ± 1.21</td>
<td>0.89 ± 0.19</td>
</tr>
</tbody>
</table>

**N.D.** not determined.

Fig. 1. Western blot analysis of CYP3A4 protein levels in fresh human and rat hepatocytes (A); and effect of macrolides antibiotics on CYP3A mRNA levels in human and rat hepatocytes (B).
scribed by Van Bambeke et al. (1998) in rat fibroblasts treated with low concentrations of AZI.

Effects of macrolides on CYP3A expression were characterized using the MDZ hydroxylation activities, as well as by Western and Northern blot analyses. In rat hepatocytes, MDZ hydroxylase activity was increased only after treatment with DEX (4.2-fold) and to a lesser extent with PB (1.6-fold). No induction was observed when hepatocytes were treated with macrolides (Table 2). Conversely, TAO, which has been described as inactivating CYP3A4 in human microsomes, strongly induced MDZ metabolism in human hepatocytes. As shown in Table 2, treatments with TAO led to a 4-fold induction of MDZ hydroxylase activity, compared with a 5-fold induction with RIF and a 3-fold induction with PB. CLA and AZI seemed to have inhibitory and induction effects, respectively. However both effects were not confirmed by Western and Northern blots, a result that could be explained for CLA by an inhibition of the enzyme activity probably via the formation of an inactive metabolite-CYP3A complex.

Western blot analyses (Fig. 1A) confirmed that no rise in CYP3A protein levels was observed after treatments with macrolide antibiotics, except in human hepatocytes challenged with TAO. Figure 1B shows that TAO also was a strong inducer of CYP3A4 mRNA in human hepatocytes, but failed to induce CYP3A1 expression in rat cells. This result is completely in agreement with the activity data and suggests a transcriptional regulation of CYP3A4 gene by TAO. ERY increased the level of CYP3A4 mRNA in human hepatocytes, but not the corresponding MDZ hydroxylase activity. This controversial data may result from ERY ability to form a metabolite complex with CYP3A4 and therefore to provoke a decrease in enzyme activity.

In summary, this study has demonstrated the absence of any induction effect of ERY, ROX, CLA, AZI, and the new ketolide HMR3004 on CYP3A. Our data confirm that in human hepatocytes, TAO treatment provoked an accumulation of CYP3A4 protein (Watkins et al., 1986; Daujat et al., 1991; Schuetz et al., 1993), and reveal that this macrolide antibiotic also increased CYP3A4 mRNA levels. This finding suggests two distinct induction mechanisms: i) a decreased degradation rate of the enzyme by formation of a stable metabolite complex in vivo; and ii) an increased level of CYP3A mRNA probably via a transcriptional activation.

In conclusion, even if in vivo experiments are still needed in drug development, the use of human hepatocytes provides useful information on drug metabolism and effects on CYP regulation. Moreover the use of cryopreserved cells offers considerable advantages and allows a better extrapolation to human than rat cells.

**References**


