Cytotoxicity of macrolide antibiotics in a cultured human liver cell line

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Cytotoxicity of erythromycin base, erythromycin estolate, erythromycin-11,12-cyclic carbonate, roxithromycin, clarithromycin and azithromycin was compared in cultured human non-malignant Chang liver cells using reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and cellular protein concentration as end points of toxicity. Erythromycin estolate was the most toxic macrolide in all tests differing clearly from all the other macrolides studied. Erythromycin-11,12-cyclic carbonate was also more toxic than the other macrolides. Roxithromycin and clarithromycin were the next toxic derivatives, while erythromycin base and azithromycin were least toxic. Thus, cytotoxicity of the new semisynthetic macrolides, roxithromycin, clarithromycin and azithromycin, is not substantially different from that of erythromycin base. In view of the low level of hepatotoxicity of macrolides hitherto reported in humans, the results do not suggest any substantial risk for hepatic disorders related to the use of azithromycin, clarithromycin and roxithromycin.

Introduction

Erythromycin base (EB) and some of its chemical derivatives have been widely used since 1952 for treatment of a variety of human infections (Periti et al., 1993). Their usefulness, however, has been limited by their inactivation by gastric acid, resulting in poor oral bioavailability. Although the incidence of significant toxic effects caused by macrolides is remarkably low, erythromycin derivatives, especially erythromycin estolate (2'-propionyl erythromycin lauryl sulphate, EE) have been reported to cause hepatic side-effects, including elevated serum transaminases and, uncommonly, potentially serious cholestatic hepatitis (Anderson et al., 1959; Tolman, Sannella & Freston, 1974; Ginsburg, 1980; Keller & Bircher, 1980; Inman & Rawson, 1983).

To overcome these problems and to improve the antibacterial spectrum and potency as well as patient compliance, new macrolides have been developed. However, no comparative studies on the hepatotoxic potential of these new derivatives have been published yet. Cultured cells of hepatic origin, including Chang liver cells, rat hepatoma cells and isolated rat hepatocytes, have been shown to be selectively responsive to potentially hepatotoxic macrolides (Dujovne et al., 1970; Dujovne, 1975, 1978; Dujovne
& Salhab, 1980; Richelmi et al., 1984; Villa, Begue & Guillouzo, 1984, 1985; Villa et al., 1988). Studies with these in-vitro models have clearly demonstrated the toxicity of EE compared with EB.

The Chang liver cell line is a well-differentiated non-malignant liver epithelial cell line of human origin (Chang, 1954; Hung et al., 1993). In addition to its established sensitivity to EE (Dujovne, 1975, 1978; Dujovne & Salhab, 1980), this cell line has been shown highly susceptible to mebendazole, a benzimidazole derivative with ability to cause hepatic damage in man (Hung et al., 1993). In this study we have utilized Chang liver cells to compare the cytotoxicity of three new semisynthetic macrolide antibiotics, roxithromycin, clarithromycin and azithromycin with that of three older erythromycins, EB, EE and erythromycin-11,12-cyclic carbonate (EC). The chemical structures of these macrolides are shown in Figure 1.

![Chemical structures of the study agents](image)

<table>
<thead>
<tr>
<th>Macrolide</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>H</td>
<td>O</td>
<td>H</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>CH₃</td>
<td>O</td>
<td>H</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>H</td>
<td>NOCH₂O(CH₂)₂OCH₃</td>
<td>H</td>
</tr>
<tr>
<td>Propionylerthromycin</td>
<td>H</td>
<td>O</td>
<td>COCH₃</td>
</tr>
</tbody>
</table>

*aErythromycin estolate is a lauryl sulphate salt of propionylerthromycin.*

**Figure 1.** Chemical structure of the study agents.
Toxicity of macrolides in human liver cells

Materials and methods

Chemicals

The macrolide antibiotics were provided by the following sources: azithromycin dihydrate (Pfizer, Groton, CT, USA), clarithromycin (Abbot, Queenborough, England), roxithromycin (Roussel-Uclaf, Paris, France), erythromycin estolate (Pierrel S.p.A., Milan, Italy), erythromycin-11,12-cyclic carbonate (Polfa, Poland) and erythromycin base (Orion Pharmaceutica, Espoo, Finland). For stock solutions the drugs were dissolved in ethanol and further diluted with the cell culture medium.

Cell culture

The Chang liver cell line (CCL 13) was obtained from the American Type Culture Collection (ATCC). The cells were cultured at 37°C in a 5% CO₂-95% air humidified atmosphere. The culture medium was Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum, penicillin (100 IU/mL), streptomycin (100 mg/L) and nystatin (8.2 mg/L). For testing, the cells were plated on 96-well microtitre plates, 5000 or 10,000 cells in 100 μL per well (MTT-test) and 10,000 or 20,000 cells in 100 or 200 μL per well (cellular protein assay). Twenty four hours later the medium was replaced with the test medium containing the macrolides at concentrations 1-5000 μM in DMEM supplemented with 0.2% fetal calf serum and no other antibiotics. The selection of the fetal calf serum concentration (0.2%) was based on preliminary experiments to show the greatest sensitivity of the cells to EE without adverse effect on cell viability.

MTT-test

The MTT-test for cytotoxicity was performed as described by Supino (1990) using incubation times of 4, 48 and 96 h. In brief, at the end of the test period 10 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma), dissolved in Ca- and Mg-free phosphate buffered saline (PBS, Gibco, USA) 5 g/L was added to each well of the 96-well plate and incubated for 2 h at 37°C. The incubation medium was aspirated and replaced with 100 μL of dimethylsulphoxide (DMSO, Merck, Darmstadt, Germany), shaken for 5 min and the absorbances measured at 550 nm (reference at 620 nm) with Labsystem Multiscan MCC eight-channel microtitre plate reader (Labsystem, Helsinki, Finland).

Cellular protein assay

The cellular protein concentration was determined by the Bradford method (Bradford, 1976) applied for cell cultures on microtitre plates as described by Otoguro et al. (1991), using incubation times of 48 and 96 h. In brief, at the end of the test period the medium was aspirated, washed twice with 200 μL of PBS, and lysed with 100 μL of 0.1 N NaOH. The plates were incubated for 1 h at 37°C, 60 μL of the contents of each well was transferred to a new 96-well plate, 120 μL of PBS and 60 μL of Coomassie protein assay reagent (Pierce, Rockford, IL, USA) were added. The plates were shaken for 5 min and the absorbances measured at 620 nm (reference at 405 nm).
Calculations

Dose-response curves were generated by plotting the cytotoxicity responses relative to the mean control value of the same microtitre plate against logarithms of the nominal macrolide concentration. The EC₅₀ values for different macrolides were interpolated from the dose-response curves.

Results

Dose-responses of the macrolides measured in MTT-tests and cellular protein assays are shown in Figures 2 and 3, respectively, and the EC₅₀ values estimated from the dose-response curves of these experiments are given in the Table. All the macrolides showed a concentration-dependent increase in cytotoxicity within a range of concentrations up to the limits of solubility. The results obtained using the two different end points of cytotoxicity were largely uniform. Similar to earlier findings in rat primary hepatocytes (Otoguro et al., 1991) the cellular protein assay was, to some extent, more sensitive than the MTT-test, resulting in slightly lower EC₅₀ values. On the other hand, the MTT-test, as a more specific end point of cytotoxicity, was able to accurately demonstrate the response to the macrolides after only 4 h of treatment. Also the sensitivity of the cells to all macrolides increased with the incubation time, whereas in the protein assay the EC₅₀ values were nearly identical at both incubation times (48 and 96 h).

In both tests and with all incubation times EE was the most toxic macrolide differing clearly from all the other macrolides studied. EC was the next most toxic derivative. Depending on the test, clarithromycin or roxithromycin was the next most toxic derivative, while EB or azithromycin, again depending on the test, were the least cytotoxic macrolides. Thus, based on the MTT-test with incubation times of 4 and 48 h, the cytotoxicity ranking order of the macrolides was EE > EC > clarithromycin > roxithromycin > EB > azithromycin, and based on the MTT-test (96 h) and the cellular protein concentration assay (48 h and 96 h), EE > EC > roxithromycin > clarithromycin > azithromycin > EB. Taken together these observations suggest that the cytotoxicity of the new semisynthetic macrolides (roxithromycin, clarithromycin and azithromycin) is not substantially different from the cytotoxicity of EB.

Discussion

The mechanism of hepatotoxicity of macrolide antibiotics is incompletely understood, but has been suggested to involve cytotoxicity combined with a hypersensitivity reaction (Pessayre et al., 1985; Periti et al., 1993). A problem for mechanistic and prognostic hepatotoxicity studies has been the difficulty in finding a responsive animal model. Dog biliary epithelium, however, has been shown to be sensitive to EE (Viluksela et al., 1988), the macrolide most frequently related to hepatic disorders in clinical practice (Keller & Bircher, 1980; Inman & Rawson, 1983). On the other hand, in-vitro studies with cultured cell lines or primary hepatocytes have proved to be promising methods both for predicting hepatotoxicity and for clarifying mechanisms of toxicity (Dujovne et al., 1970; Dujovne, 1975, 1978; Richelmi et al., 1984; Villa et al., 1984, 1985, 1988).
Toxicity of macrolides in human liver cells

Figure 2. The effect of macrolide antibiotics on the reduction of MTT by Chang liver cells. The cells were exposed to the macrolides for (a) 4h, (b) 48h and (c) 96h before the assay. Each value is a mean of a total of 12 wells analysed in two separate experiments. (○, Erythromycin base (EB); △, azithromycin; ▽, clarithromycin; ■, roxithromycin; □, erythromycin-11,12-cyclic carbonate (EC); ●, erythromycin estolate (EE)).

The outcome of these studies emphasize the essential role of cytotoxicity in inducing the toxic effects of macrolides in liver.

In the present study, the hepatotoxic potential of the new generation of macrolides (roxithromycin, clarithromycin, azithromycin) was estimated by comparing their cytotoxicity with that of EB, EE and EC. The concentrations used were clinically
Figure 3. The effect of macrolide antibiotics on the total protein concentration of the cultures of Chang liver cells. The cells were exposed to the macrolides for (a) 48h and (b) 96h before the assay. Each value is a mean of a total of 12 wells analysed in two separate experiments. Symbols legend as in Figure 2.

relevant, since serum peak concentrations of slightly less than 10 µmol/L have been reported for EB and roxithromycin (Nilsen, 1987), and higher concentrations have been measured in bile. Cytotoxicity was assessed as the ability of the cells to reduce MTT, a measure of mitochondrial function also reflecting the viability of the cells (Supino, 1990), and as the total protein concentration, which demonstrates the growth and survival of the cells. In cultured Chang liver cells EE was clearly and EC slightly more toxic than the other macrolides. Roxithromycin and clarithromycin were slightly more

Table I. EC₅₀ values (µM) measured for different macrolides in the MTT-test and cellular protein concentration analysis with Chang liver cells

<table>
<thead>
<tr>
<th>Macrolide</th>
<th>4h</th>
<th>MTT-test 48h</th>
<th>MTT-test 96h</th>
<th>Protein concentration analysis 48h</th>
<th>Protein concentration analysis 96h</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE</td>
<td>69.8</td>
<td>35.5</td>
<td>34.6</td>
<td>23.6</td>
<td>26.2</td>
</tr>
<tr>
<td>EC</td>
<td>&gt;503</td>
<td>148</td>
<td>85.6</td>
<td>59.1</td>
<td>78.3</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>817</td>
<td>259</td>
<td>253</td>
<td>228</td>
<td>186</td>
</tr>
<tr>
<td>Roxithromycin</td>
<td>1002</td>
<td>516</td>
<td>173</td>
<td>148</td>
<td>164</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>2574</td>
<td>1168</td>
<td>295</td>
<td>326</td>
<td>273</td>
</tr>
<tr>
<td>EB</td>
<td>1669</td>
<td>719</td>
<td>503</td>
<td>343</td>
<td>530</td>
</tr>
</tbody>
</table>

EE, Erythromycin estolate; EC, erythromycin-11,12-cyclic carbonate; EB, erythromycin base.
Toxicity of macrolides in human liver cells

Toxic than EB, and depending on the test, azithromycin or EB was the least toxic macrolide. Previous studies in various cell lines of hepatic origin (Dujovne, 1975, 1978; Dujovne & Salhab, 1980) and in rodent primary hepatocytes (Dujovne et al., 1970; Richelmi et al., 1984; Villa et al., 1984, 1985, 1988), have also shown that, compared with EB, EE is more toxic. In those studies the leakage of cytoplasmic enzymes or morphological and ultrastructural changes were used as endpoints of toxicity. Cytotoxicity of EE and EC in Chang liver cells also correlates well with liver damage observed in EE and EC-treated dogs (Viluksela et al., 1988).

There are no published data available about the cytotoxicity of clarithromycin and azithromycin. Pharmacokinetic factors seem to explain at least some of the relatively small differences in cytotoxicity between derivatives. The higher cytotoxicity of roxithromycin in rat primary hepatocytes compared with EB is likely to result from 2–3 times better accumulation of roxithromycin into the cells (Villa et al., 1988). However, it seems evident that the drug metabolizing capacity of the cell is not an important factor in determining the sensitivity to macrolides (Villa et al., 1984, 1985).

EE is a lauryl sulphate salt of the 2'-propionyl ester of erythromycin. It is a prodrug, which is hydrolyzed to form the active drug EB. Toxicity of EE is not related to EB, but rather to the combined effect of 2'-propionyl erythromycin and lauryl sulphate (Dujovne, 1975, 1978). Both EE and lauryl sulphate, but not EB, were also shown to effectively disrupt the intracellular calcium homeostasis in isolated rat primary hepatocytes at a concentration of 100 μM (Richelmi et al., 1984). At least two hypotheses have been presented to explain the hepatotoxicity of EE. Firstly, the ability of the macrolide to decrease the surface activity is related to hepatocellular damage by causing damage to cell membranes (Dujovne, 1978). Correspondingly, 2'-propionyl erythromycin, a compound with high surface activity, was shown to significantly increase the adsorption of lauryl sulphate on Chang liver cells. Secondly, hydrophobic macrolides containing an unhindered, readily accessible, N-dimethylamino group (such as EE) can be metabolized to form nitrosoalkanes, which are able to covalently bind to sulphhydryl groups of proteins, thereby inducing liver damage (Delaforge, Jaouen & Mansuy, 1983; Periti et al., 1992, 1993). Furthermore, formation of metabolic nitrosoalkanes from macrolides seems to be correlated with their hepatotoxic potential (Pessayre et al., 1985; Franklin, 1991; Periti et al., 1993).

Structural modification of erythromycins can result in altered production of nitrosoalkanes. Among the macrolides with semisynthetic modifications of the aglycone ring, roxithromycin and clarithromycin have a decreased potential, and azithromycin is completely unable, to form nitrosoalkanes (Periti et al., 1992). Accordingly, compared with erythromycins, roxithromycin and clarithromycin are generally considered to have a low hepatotoxic potential in humans and that of azithromycin is considered to be negligible or lacking (Periti et al., 1993). Results of the present study are in full agreement with these observations.

In conclusion, EE was significantly and EC slightly more cytotoxic in Chang liver cells than the other macrolides studied. The new macrolides clarithromycin and roxithromycin were only slightly more toxic than EB, while azithromycin was, depending on the test, equally or even less toxic than EB. The results do not suggest any substantial risk for hepatic disorders related to the use of azithromycin, clarithromycin or roxithromycin.
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References


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