Interaction of rocuronium with human liver cytochromes P450

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Rocuronium is a neuromuscular blocking agent acting as a competitive antagonist of acetylcholine. Results of an inhibition of eight individual liver microsomal cytochromes P450 (CYP) are presented. As the patients are routinely premedicated with diazepam, possible interaction of diazepam with rocuronium has been also studied.

Results indicated that rocuronium interacts with human liver microsomal CYPs by binding to the substrate site. Next, concentration dependent inhibition of liver microsomal CYP3A4 down to 42% (at rocuronium concentration 189 μM) was found. This effect has been confirmed with two CYP3A4 substrates, testosterone (formation of 6β-hydroxytestosterone) and diazepam (temazepam formation). CYP2C9 and CYP2C19 activities were inhibited down to 75–80% (at the same rocuronium concentration).

Activities of other microsomal CYPs have not been inhibited by rocuronium.

To prove the possibility of rocuronium interaction with other drugs (diazepam), the effect of rocuronium on formation of main diazepam metabolites, temazepam (by CYP3A4) and desmethyldiazepam, (also known as nordiazepam; formed by CYP2C19) in primary culture of human hepatocytes has been examined. Rocuronium has caused inhibition of both reactions by 20 and 15%, respectively.

The results open a possibility that interactions of rocuronium with drugs metabolized by CYP3A4 (and possibly also CYP2C19) may be observed.

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1. Introduction

Rocuronium is a neuromuscular blocking agent (NMBA) acting as a competitive antagonist of acetylcholine on the postsynaptic nicotinic receptor of neuromuscular junction (1). Its advantage is relatively quick onset of the neuromuscular block (the most quick among currently used non-depolarizing NMBA, 60 s after 0.6 mg/kg dose) and an intermediate duration of its action (35–50 min after the same dose) (1,2). Rocuronium is predominantly used to facilitate endotracheal intubation, to provide skeletal muscle relaxation during surgery, or to facilitate mechanical ventilation in intubated, critically ill patients (1–3).

Rocuronium is according to its structure (Fig. 1) an aminosteroid compound similar to vecuronium or pancuronium (1,2). It is in principle a basic derivative of androstane with acetylated hydroxyl group and with a substituent containing a quaternary nitrogen atom; this is why the bromide salt is used in drug formulation. Steroid nature of the molecule is an advantage as it is not a substrate of pseudocholinesterase whose genetically determined deficiency is a serious complicating factor causing prolonged muscle relaxation e.g. after suxamethonium (succinylcholine) (1–4). Primary route of rocuronium elimination is the liver re-uptake and biliary excretion (5). Rocuronium is excreted from human organism in the urine (26% in 48 h), in the bile (7% in 48 h) and in faeces (up to 31% during 7 days). Two metabolites of rocuronium were found (5); 17-desacetylrocuronium is formed in relatively small amount, this metabolite was found in the plasma, urine, bile and faeces and in amount less than 10%. It was suggested that a
cytochrome P450 enzyme, presumably CYP3A4, takes part in this reaction (6,7). An N-desallylrocuronium metabolite was found to be even less formed than the previous compound as it was detected in the bile only, however, a study on formation of this metabolite is lacking. As the amount of totally excreted rocuronium was below 75% of the doses, a formation of other (more polar) metabolites as glucuronides cannot be ruled out (5).

Rocuronium, however, exhibits a great variability in the effect and in the course of neuromuscular block as well in the time of recovery. Variability of response to rocuronium is according to the literature primarily caused by pharmacokinetics of this drug. In the literature there are reports indicating that an interaction of rocuronium with liver monoxygenase system of cytochromes P450 (CYP) (8) may contribute to these differences (9). Also, in patients with liver dysfunction or after application of inhalational anesthetics, a decrease of the rocuronium clearance and increase of elimination half-time have been observed (2); these effects may be due to CYP-mediated formation of reactive species known to be formed from inhalation anesthetics (10).

An increase in effect of rocuronium described after the use of anesthetics (11) is not only the single example of interaction of other drugs with rocuronium. Also, antibiotics as aminoglycosides (e.g. gentamicin) and lincosamides (as clindamycin) potentiate neuromuscular blockade and should be used with caution (12,13). Inter- gentamicin) and lincosamides (as clindamycin) poten- tiate neuro- rhythms and should be used with caution (12,13). In the literature (12), clindamycin, metabolized by cytochrome P450 form 3A4 (CYP3A4), was found also to be an inhibitor of this form (14). On the contrary, drugs known as inducers of CYP enzymes, e.g. carbamazepine or phenytoin, appear to cause a decrease of the effect of rocuronium (15). In general, the key for understanding drug interactions may often be a dependence of drug (e.g. rocuronium) levels on the activity of liver drug metabolizing enzymes (mostly CYPs). In vitro studies on drug interactions are commonly used to get an insight into the nature of these unwanted effects (16).

The present work is aimed to investigate the influence of rocuronium on the activities of the eight principal drug-metabolizing enzymes, CYPs, of human liver microsomes (CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4). As diazepam (substrate of CYP3A4 and CYP2C19 (17)) is often used in premedication of the patients, in addition, micro- somes and human hepatocytes were also used for studying of possible drug interaction of rocuronium and diazepam.

2. Material and methods

2.1. Materials

Rocuronium was obtained from N.V. Organon, Oss, Netherlands (10 mg/ml). The structure of tested compound is shown in Fig. 1. 7-Ethoxy-4-(trifluoromethyl)coumarin was supplied by Fluka (Buchs, Switzerland). Acetonitril, methanol and HPLC column were from Merck (Darmstadt, Germany). All other chemicals were purchased from Sigma Aldrich CZ (Prague, Czech Republic). Pooled human liver microsomes were obtained from Biopredic International (Rennes, France). Microsomes were from 36 donors (25 males and 11 females) with a total cytochrome P450 content 582 pmol/mg of protein.

2.2. Spectral study of interaction of rocuronium with hepatic microsomal CYP

Binding difference spectra of interactions of rocuronium with microsomal CYP enzymes were followed according to established procedure (18). The cuvette contained microsomes diluted to final concentration of CYP 1 µM in 50 mM potassium phosphate buffer (pH 7.4). The tested compound was dissolved in 50 mM phosphate buffer (pH 7.4) and the concentration range in experiments were 0–210 µM. Taking into account that rocuronium level in patients reach routinely from 33 µM in plasma to 160 µM in the bile (after an i.v. application of 0.3–0.9 mg kg⁻¹ (5), the concentration range used here can be considered as adequate.

Spectra were recorded at room temperature by repetitive scanning between 300 and 700 nm, at a scan speed of 150 nm/s. Baseline of reference cuvette contained microsomes diluted to final concentration of CYP 1 µM in 50 mM KPO₄ buffer (pH 7.4). Spectra were recorded on a Varian 4000 UV VIS spectrophotometer (Varian, Palo Alto, USA). Absorbance change at about 418 nm was plotted against concentration of compound tested. Data were analyzed using a Sigma Plot v. 8.0 graphing software (Jandel Scientific, Chicago, IL, USA).

2.3. Enzyme assays

The microsomal CYP activities were measured according to established protocols. The following enzyme assays were performed to determine activities of specific CYP enzymes: CYP1A2, 7-ethoxyresorufin 0-deethylolation (19); CYP2B6, 7-ethoxy-4-(tri- fluoromethyl)coumarin 0-deethylolation (20); CYP2C9, diclofenac 4’-hydroxylation (21), CYP2D6, bufuralol 1’-hydroxylation (22); CYP2E1, chlorozoxazone 6-hydroxylation (23); CYP3A4, testosterone 6β-hydroxylation (24) and diazepam 3-hydroxylation (see Method for CYP2C19); CYP2A6, coumarin 7-hydroxylation (25), CYP2C19, diazepam N-demethylation (http://www.cypex.co.uk/2c19info.htm). Incubation mixtures contained 100 mM potassium phosphate buffer (pH 7.4) except for determination of CYP3A4 activity (with substrate testosterone), where 50 mM Tris/KCl buffer, pH 7.4 was used, NADPH-generating system (0.8 mM NADP⁺, 5.8 mM isocitrate, 0.3 unit/ml of isocitrate dehydrogenase and 8 mM MgCl₂), human liver microsomes and individual probe substrate. Time of incubation was 20 min.

For determination of metabolites formed from specific substrates, HPLC system Shimadzu Class VP (Kyoto, Japan) with UV detection or fluorescence detection was used. As a rule, a reversed phase chromatography was applied with Merck (Darmstadt, Germany) LiChroCART 250 × 4 mm (i.d.) cartridges packed with a LiChrospher RP-18 silica gel (5 µm particle size) with C-18 reversed phase properties; a 4 × 4 mm (i.d.) guard column was also used. For composition of mobile phase in the respective analyses, see the original literature, further details of the analyses incl. The concentrations of the respective substrates and experimental conditions are presented in Table 1.

2.4. Enzyme inhibition studies

Initially, for each enzyme assay, a preliminary experiment was done to determine Kᵣ and Vₘₜₜ to obtain the appropriate concentra- tion of the specific substrates for the inhibition experiments (substrate concentration was chosen in the range corresponding to the value of the Kᵣ for each particular reaction). Data were analyzed
1.4 were seeded on collagen-coated culture dishes at density 105 cells/cm2. Culture medium, made as described previously (26), was enriched for seeding with 5% bovine serum (v/v). The preparative isolation of cytochrome P450 CYP enzymes was described (27), was enriched for seeding with 5% bovine serum (v/v). The preparative isolation of cytochrome P450 CYP enzymes was investigated. Results of the inhibition experiments are summarized in Fig. 3. Rocuronium concentrations ranging from 2.6 to 100 µM were used. In both cases rocuronium inhibited activity of CYP3A4 and secondly diazepam with its speciﬁc metabolite temazepam was used.”

Table 1
Experimental conditions for individual CYP enzyme activity assays.

<table>
<thead>
<tr>
<th>CYP</th>
<th>Substrate</th>
<th>Conc. (µM)</th>
<th>Incubation Volume (µL)</th>
<th>CYP pmol Conc.</th>
<th>Quench agent</th>
<th>Incubation Time (min)</th>
<th>Detection</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>7-Ethoxyresorufin</td>
<td>2.6</td>
<td>100</td>
<td>35</td>
<td>0.2 ml MetOH</td>
<td>15</td>
<td>Flu</td>
<td>UV</td>
</tr>
<tr>
<td>2A6</td>
<td>Coumarin</td>
<td>6.2</td>
<td>100</td>
<td>35</td>
<td>0.2 ml MetOH</td>
<td>15</td>
<td>Flu</td>
<td>UV</td>
</tr>
<tr>
<td>2B6</td>
<td>7-Ethoxy-4-(trifluoromethyl) coumarin</td>
<td>4</td>
<td>100</td>
<td>35</td>
<td>0.2 ml MetOH</td>
<td>15</td>
<td>Flu</td>
<td>UV</td>
</tr>
<tr>
<td>2C9</td>
<td>Diclofenac</td>
<td>16</td>
<td>200</td>
<td>35</td>
<td>0.05 ml 94%ACN/4% acetic acid</td>
<td>25</td>
<td>UV</td>
<td></td>
</tr>
<tr>
<td>2D6</td>
<td>Bufuralol</td>
<td>13.2</td>
<td>200</td>
<td>70</td>
<td>0.02 ml 70% perchloric acid, 0.05 ml 42% phosphoric acid</td>
<td>15</td>
<td>Flu</td>
<td>UV</td>
</tr>
<tr>
<td>2E1</td>
<td>Chloroxazone</td>
<td>26</td>
<td>1000</td>
<td>160</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3A4</td>
<td>Testosterone</td>
<td>100</td>
<td>500</td>
<td>100</td>
<td>2 ml dichloromethane</td>
<td>20</td>
<td>UV</td>
<td></td>
</tr>
<tr>
<td>2C19 and 3A4</td>
<td>Diazepam</td>
<td>100</td>
<td>200</td>
<td>70</td>
<td>0.1 ml ACN</td>
<td>20</td>
<td>UV</td>
<td></td>
</tr>
</tbody>
</table>

using the Sigma plot software as mentioned above with spectral studies. Inhibition experiments were performed with five concentration levels of rocuronium (1.5, 5.9, 23.6, 94.4, 188.8 µM). As rocuronium level in patients reach routinely from 33 µM in plasma to 160 µM in the bile, the concentration range used here is adequate (5). Experimental conditions were the same as for determination of individual CYP activities; the assays were performed in three separate experiments in duplicates with individual values differing less than 10%. Statistical analyses were done as described in 2.6. Preincubation of reaction mixtures with inhibitor (rocuronium) for 30 min at 37 °C was kept in all assays. Inhibition of individual CYP activities was in all cases evaluated by plotting respective remaining activity against the inhibitor concentration.

2.5. Primary cultures of human hepatocytes

Primary cultures of human hepatocytes were prepared using liver tissue obtained from multiorgan donors, for whom suitable recipient was not available. Tissue acquisition protocol was in accordance with requirements issued by local ethics committee of the University Hospital, Olomouc, Czech Republic. Human liver samples used in the experiments were from two donors; from each liver sample, two experiments were performed. Hepatocytes were isolated as previously described (26). Following isolation, the cells were seeded on collagen-coated culture dishes at density 1.4 × 105 cells/cm2. Culture medium, made as described previously (27), was enriched for seeding with 5% bovine serum (v/v). The medium was exchanged for serum free medium (1.5 ml) the day after and the culture was allowed to stabilize for an additional 48 h prior to the treatment. Cultures were maintained at 37 °C and 5% CO2 in humidified incubator. Then rocuronium (final concentration 100 µM), diazepam (final concentration 50 µM) and both rocuronium and diazepam (final concentration 100 µM and 50 µM) were applied to the medium and hepatocytes were incubated for 12 h. Levels of both compounds corresponded to the values which may be reached in patients (rocuronium, see above, diazepam, levels up to 20–30 µM may be found (28)). Diazepam was added in methanol solution in concentrations for which the final solvent concentration did not exceed 0.5% (v/v) when added to incubation medium. Substrates were absent in the control sample. Subsequently, 200 µl sample was deproteinated by adding 100 µl of acetonitril. After centrifugation at 9000 g for 10 min, supernatants (200 µl) were subjected to HPLC analysis to detect diazepam metabolites.

2.6. Statistical analysis

All calculations were done using Microsoft Excel 2010 and Statistica 12 (Systat Software, San Jose, CA). One-way ANOVA followed by t-test was used for the statistical evaluation of differences between treated groups and control. The differences were regarded as significant when P < 0.05.

3. Results

3.1. Spectral studies of the rocuronium interaction with human liver microsomal CYP enzymes

Spectral studies in the Soret region (Fig. 2) were used to detect the substrate-induced difference spectra of cytochromes P450. From the inspection of the course of the difference spectra for tested compound it follows, that the rocuronium binds to human liver microsomal CYP enzymes as substrate. The spectral change corresponds to the formation of a minimum at about 420 nm with a maximum formed at about 380 nm (Fig. 2). The value of the corresponding spectroscopic binding constant (Kd) for tested compound was 30.31 ± 9.31 µM. This value indicates the relatively specific binding of rocuronium on microsomal cytochromes P450.

3.2. Inhibition of specific CYP activities in human liver microsomes

In the present study, inhibitory effect of rocuronium on the activities of eight microsomal CYP enzymes was investigated. Results of the inhibition experiments are summarized in Fig. 3. Rocuronium displayed no or weak inhibition of activities of CYP1A2, 2A6, 2B6, 2C9, 2D6 and 2E1. A moderate inhibition was observed for CYP2C19 (diazepam N-demethylation). Only the CYP3A4 activity was inhibited significantly. For measurement of the activity of CYP3A4, metabolism of two different substrates was studied. Firstly, testosterone with its specific metabolite 6β-hydroxytestosterone and secondly diazepam with its specific metabolite temazepam were used. In both cases rocuronium inhibited activity of CYP3A4 similarly. IC50 values for inhibition of testosterone hydroxylation were found to be 115.8 ± 108.6 µM for the second reaction, i.e. for inhibition of temazepam formation.

3.3. Effect of rocuronium on metabolism of diazepam in human hepatocytes

In hepatocytes, similarly to microsomes, diazepam is metabolized to N-desmethyl/diazepam by CYP2C19 (with contribution of CYP3A4) and by CYP3A4 to temazepam, which are further metabolized and conjugated (17). Cytochrome P450 enzymes CYP2C19 and CYP3A4 are hence the main enzymes involved in the diazepam metabolism. In this work, effect of rocuronium presence on formation of both two main metabolites of diazepam formed in hepatocytes was followed. Here, an advantage was taken from the fact that one substrate (diazepam) can be taken as a probe of activity of...
two CYP enzymes forming two metabolites, as desmethyldiazepam is a product of CYP2C19 and CYP3A4 and hydroxylated diazepam or temazepam is formed by CYP3A4. Rocuronium, applied simultaneously with diazepam into primary culture of human hepatocytes, slightly inhibited metabolism of diazepam. The concentration of both diazepam metabolites was lower in this case by 11% for N-desmethyldiazepam and by 15% for temazepam. Results are displayed in Fig. 4.

![Fig. 2. Spectral determination of interaction between rocuronium and cytochromes P450 in human liver microsomes. Insert, plot of the spectral difference (ΔA) at about 417 nm vs. rocuronium concentration for determination of Ks (see text). Concentration of CYP in microsomal preparation, 1 μM in 50 mM K/phosphate buffer (pH 7.4).](image)

![Fig. 3. Effects of rocuronium on catalytic activities of CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (3A4 I, substrate testosterone, 3A4 II, substrate diazepam, for other substrates, see Methods, 2.3 and 2.4) enzymes in human liver microsomes. Inhibition of catalytic activities is expressed as the activity remaining relative to control (100%, without rocuronium) in percent. Concentration of rocuronium in the reaction mixture was 0, 1.47, 5.90, 23.60, 94.4, and 188.8 μM. For experimental details, see Methods (2.4 and 2.6). Activities represent the mean from three independent experiments in duplicates and values significantly different from control are labeled (N = 6, *P < 0.05, **P < 0.01).](image)

![Fig. 4. Inhibition of diazepam metabolism (formation of desmethyldiazepam and temazepam) in the presence of rocuronium in primary human hepatocytes. Concentration of diazepam, 50 μM; rocuronium, 100 μM; results shown as means ± S.E.M.(bars); bars marked with an asterisk represent statistically significant results compared to control (N = 4, P < 0.01, see Methods, 2.6 Statistical analysis).](image)
4. Discussion

Results obtained in our study indicate that rocuronium interacts with two forms of human liver microsomal CYPs. First, the difference spectra were employed to confirm the presence of an interaction of rocuronium with (unspecified) microsomal CYPs. This experiment is based on the fact, that in the liver microsomes, CYPs are known to exist in an equilibrium between two forms with the heme central ferric iron atom either in the high spin (approx. 390 nm absorption maximum in the absolute spectra) or low spin (417 nm absorption maximum in the absolute spectrum) state. A simplified explanation of the function of CYP enzymes begins with binding of a substrate resulting in a conformational and structural change in favor of the high spin form (8,10,18). Hence, in case a substrate is bound, relative content of the high spin form increases, which is reflected in formation of a maximum in the difference spectrum corresponding to the high spin form (at about 390 nm); concomitantly, a minimum in the difference spectrum is observed at about 420 nm corresponding to a decrease of the low spin content (18).

Spectroscopic binding constant \( K_i \) (found in half saturation in the plot of the absorbance change at selected wavelength vs. substrate concentration, in analogy to the \( K_m \) in enzyme kinetics) expresses the relative affinity of a substrate to microsomal cytochromes P450. The results presented here (Fig. 2) (the value of the spectroscopic binding constant \( K_i \) for rocuronium 30.31 ± 9.31 μM) indicated that there is a possibility of an interaction of rocuronium with human liver microsomal cytochrome P450 enzymes.

In the next step, rocuronium was added to human microsomes with the aim to find the particular cytochrome P450 form with which the tested compound interacts. Inhibition of specific activities of individual CYP enzymes by rocuronium was followed. The results show (Fig. 3) that rocuronium was able to inhibit enzyme activities of two forms of P450, namely, of the CYP3A4 and CYP2C19. The activities of these two enzymes were measured using their prototypic substrates: For CYP2C19, diazepam N-demethylation leading to formation of desmethyldiazepam metabolite was used; to follow the activity of CYP3A4, inhibition of two activities (testosterone 6β-hydroxylation and diazepam hydroxylation to temazepam) was analyzed. The results confirm the interaction of rocuronium with both these activities. In fact, as the CYP3A4 form is known to metabolize steroids and related compounds (as rocuronium (6,7)), there is a possibility of binding of rocuronium to this enzyme. In other words, concomitantly administered drugs metabolized, or more generally, bound or interacting with CYP3A4 may contribute to a prolongation of rocuronium effect when they are more strongly interacting with CYP3A4 than rocuronium itself. This effect may in part contribute to increased sensitivity to rocuronium during therapy with clindamycin which has been already mentioned in the Introduction (13). As clindamycin has been shown to be a substrate and inhibitor of the CYP3A4 enzyme (14).

The interactions of rocuronium described in this work may also contribute to the shift of dose–response curve of rocuronium to higher doses due to administration of diazepam in experimental model (29) as well to prolonged effect of structurally very similar vecuronium (30). It may be speculated that binding of diazepam to CYP enzymes may also contribute to this effect as diazepam is a substrate of both the CYP3A4 and CYP2C19 enzymes (17).

In summary, the results of spectral analysis of interaction (of the sum of CYP enzymes present in the microsomal fraction of human liver homogenate) with compound tested as well as studies on inhibition of individual microsomal CYP activities by rocuronium revealed an interaction indicating the binding of rocuronium to the CYP enzymes. A detailed study on the possible influence of this interaction to enzyme activities of eight individual CYP forms known to be important for drug metabolism revealed significant interactions with CYP3A4 and CYP2C19. On the basis of these results with microsomal fraction, the attention was focused on possible interactions of rocuronium and diazepam (substrate of CYP3A4 as well as of CYP2C19). The experiments using human hepatocytes indicated a possibility of drug–drug interaction when rocuronium and diazepam are applied concurrently.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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