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Identification of the human P450 enzymes involved in the *in vitro* metabolism of the synthetic steroidal hormones Org 4060 and Org 30659

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1. The type of human P450 enzymes involved in the *in vitro* metabolism of Org 4060 and Org 30659, two synthetic steroidal hormones currently under clinical development by NV Organon for use in oral contraceptive and hormone replacement therapy, was investigated.

2. Both steroids were mainly hydroxylated at the 6β-position in incubations with human liver microsomes.

3. The results from experiments with supersomes, correlation studies as well as inhibition studies with ketoconazole, a selective inhibitor of CYP3A, strongly suggest that the CYP3A family plays a significant role in the 6β-hydroxylation of both steroids.

4. Measurements of kinetic parameters of P450 enzymes that could metabolize both steroids, combined with the fact that CYP3A4 is known to be the most abundant P450 enzyme in the human liver, indicate that CYP3A4 will be of major importance for the *in vivo* human metabolism of Org 4060 and Org 30659.

Introduction

Knowledge on the P450 enzymes involved in the metabolism of new chemical entities is relevant in predicting drug interactions and explaining interindividual variation of drug metabolism. Large interindividual variability in the expression of the P450 enzymes and the related differences in metabolic clearance may be caused by genetic differences (genetic polymorphism) or differences in exposure to xenobiotics, which may induce or inhibit P450s. Disease states, diet and hormonal influences may also change the levels of P450 expression (Wrighton *et al.* 1996). The P450 enzymes are known to catalyse a large number of Phase I reactions in steroid metabolism. CYP3A4, for example, is the major P450 enzyme involved in the 6β-hydroxylation of testosterone, androstenedione and progesterone in human liver microsomes (Waxman *et al.* 1988), whereas the CYP2C family seems to have an important role in the 3-hydroxylation of desogestrel (Gentile *et al.* 1998). A

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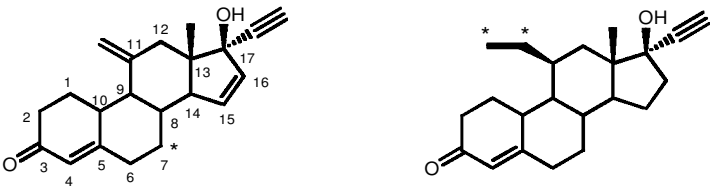

Figure 1. Structures of Org 30659 (left) and Org 4060 (right).

major contribution of CYP2C9 is suggested for the transformation of the prodrug mestranol to ethinylestradiol (Schmider *et al.* 1997). This enzyme also has progesterone 21-hydroxylation activity, although to a lower extent than CYP2C19. In addition, the oxidation of testosterone at the 17-position to form androstenedione is catalysed by the CYP2C19 enzyme (Yamazaki and Shimada 1997).

Org 30659 ((17α)-17-hydroxy-11-methylene-19-norpregna-4,15-dien-20-yn-3-one) and Org 4060 ((11β, 17α)-11-ethyl-17-hydroxy-19-norpregn-4-en-20-yn-3-one) (figure 1) are both synthetic steroidal hormones currently under clinical development by NV Organon for use in oral contraceptive and hormone replacement therapy. Org 30659 shows a very high progestagenic activity in pharmacological studies in rat and rabbit. In addition, a lack of androgenic activity (according to the Hershberger test) distinguishes Org 30659 from other frequently used synthetic progestagens. Except for some weak oestrogenic activity in rat, Org 30659 is devoid of other hormonal activities, including glucocorticoid and anti-glucocorticoid activity (Deckers *et al.* 1992). Org 4060 is strongly oestrogenic and progestagenic in rat and rabbit. In rat, Org 4060 displays minimal androgenic activity and only at a high dose, very weak glucocorticoid activity.

The present investigation was performed to identify the specific human P450 enzymes involved in the Phase I metabolism of both steroids. In addition, kinetic parameters of P450 enzymes capable of the conversion of Org 4060 and Org 30659 were determined.

Materials and methods

Chemicals

[^{3}H]-Org 4060 and [^{3}H]-Org 30659 labelled at the C11 ethyl and the C7, respectively, with radiochemical purities $\geq$ 98% were prepared by the Organic Isotope Section of the Department of Process Chemistry, NV Organon. The specific activities of Org 4060 and Org 30659 were 560 and 888 GBq mmol^{-1}, respectively. Unlabeled Org 4060, Org 30659, the 6β-OH metabolite of Org 4060, the 6β-OH metabolite of Org 30659 and ketoconazole (chemical purity $\geq$ 95%) were synthesized by the Department of Process Chemistry, NV Organon. All other chemicals were obtained from commercial sources and were of analytical grade.

In vitro *incubations*

Metabolites of Org 4060 and Org 30659 in human liver microsomal incubations. Human liver microsomes were prepared from surgical waste liver tissue, after consent from legal authorities and patients was obtained, in cooperation with the Human Liver Group Groningen (Dr G. M. M. Groothuis). Liver samples were homogenized using a Potter-Elvehjem homogenizer at 0°C in a 50 mm Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose. Microsomes were prepared by centrifugation (20 min, 100 000*g*; supernatant 2 $\times$ 75 min, 1.000 000*g*). The microsomal pellet was resuspended in 100 mm potassium phosphate (pH 7.4) containing 20% (v/v) glycerol. The microsomal suspensions were stored at –80 °C, until used. The microsomal suspensions were characterized by the determination of P450 and protein contents. The concentration of P450 was calculated from the CO-reduced difference spectrum according to Omura and Sato (1964). The concentration of protein was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Incubations were performed with a pool of four human liver microsomes. Human liver microsomes were incubated (final volume of 0.2 ml) at 37 °C in 100 mM potassium phosphate (pH 7.4), containing

(final concentration) 3 mM NADPH (the pre-incubation mixture) and an appropriate concentration of test compound. [^{3}H]-Org 4060 was incubated with human liver microsomes (final protein concentration range: 0.03–1 mg ml^{-1}) at a final concentration of 1.3 or 10 μM (170 kBq ml^{-1}). [^{3}H]-Org 30659 was incubated with human liver microsomes (final protein range 0.1–1 mg ml^{-1}) at a concentration of 1 or 10 μM (90 kBq ml^{-1}). After a pre-incubation of 2 min, incubations were started by the addition of the pre-incubation mixture to the test compound. Incubations were carried out in a shaking Eppendorf Thermomixer 5436 and were stopped after 5, 10 and 15 min by the addition of 20% ice-cold acetonitrile. After centrifugation, supernatants were subjected to HPLC analysis. All incubations were performed in duplicate. In addition, an incubation (final protein concentration 1 mg ml^{-1}) without NADPH was performed to determine the non-NADPH-dependent reactions.

In vitro *incubation studies with supersomes.* Incubations were performed using commercially available supersomes (CYP1A2, CYP2A6, CYP2D6, CYP2E1, CYP2C9, CYP2C19, CYP3A4 or CYP3A5) (Gentest Corporation). Supersomes were derived from baculovirus-insect cells with a specific human P450 enzyme expressed, co-expressed with human NADPH-cytochrome P450 reductase. The insect host cell line has been demonstrated not to contain a detectable P450 activity (in accordance with the Catalogue of Gentest Corporation). The conversion of suitable substrates by these P450 enzymes has been done by the supplier and is specified in their catalogue (Catalogue of Gentest Corporation).

Supersomes (final protein concentration 250 μg ml^{-1}) were incubated at 37 °C in 200 μl potassium phosphate (pH 7.4) containing 3 mM NADPH (final concentration) and the test compound in a final incubation volume of 0.2 ml. The final concentrations of [^{3}H]-Org 4060 and [^{3}H]-Org 30659 were 10 μM (170 kBq ml^{-1}) and 10 μM (90 kBq ml^{-1}), respectively. Incubations with CYP2A6 and CYP2C9 were performed using Tris buffer (100 mM, pH 7.4). After a pre-incubation of 2 min, incubations were started by the addition of the pre-incubation mixture to the test compound. Incubations were carried out in a shaking Eppendorf Thermomixer 5436 (Eppendorf, Germany) and were stopped after 15 min by the addition of 20% ice-cold acetonitrile. After centrifugation, supernatants were subjected to HPLC analysis. All incubations were performed in duplicate.

Kinetic studies with CYP3A4, CYP3A5 and CYP2C19 supersomes. Studies with CYP3A4, CYP3A5 and CYP2C19 (only Org 30659) supersomes were performed to determine apparent K_m and V_{max} for the 6β-hydroxylation of Org 4060 and Org 30659. Supersomes (final protein concentration 100–250 μg ml^{-1}) were incubated at 37 °C for 15 min in 100 mM potassium phosphate (pH 7.4) containing (final concentration) 3 mM NADPH and the test compound at various concentrations (0.3–100 μM) in a total volume of 200 μl. All incubations were performed in duplicate. Apparent K_m and V_{max} were determined by fitting the data to the standard Michaelis–Menten equation: $v = V_{max} * [S]/(K_m + [S])$, where $[S]$ is the substrate concentration, using the program of EZ-FIT (v.1.1, Medical Products Department, E. I. Dupont de Nemours & Co., 1988).

Correlation studies. A set of ten human liver microsomes with specified activities for selected P450 substrates (testosterone for CYP3A activity and *S*-mephenytoin for CYP2C19 activity) was obtained from the International Institute for the Advancement of Medicine (IIAM). Tissues were obtained by IIAM as a result of a medical/surgical procedure, recovery for transplantation (of non-transplantable organs or tissues) or *post mortem* recovery of tissues for research. All tissues were procured with the appropriate legal consent of the donor or the donor's next of kin. Human liver microsomes (final protein concentrations 100 μg ml^{-1} (Org 4060) and 250 μg ml^{-1} (Org 30659)) were incubated at 37 °C in 100 mm potassium phosphate (pH 7.4), containing 3 mM NADPH (final concentration) in a total volume of 200 μl. After a pre-incubation of 2 min, incubations were started by the addition of the pre-incubation mixture to the test compound (two different concentrations). The final concentrations of [^{3}H]-Org 4060 were 1.3 and 10 μM (170 kBq ml^{-1}) and the final concentrations of [^{3}H]-Org 30659 were 1 and 10 μM (90 kBq ml^{-1}). Incubations were carried out in a shaking Eppendorf Thermomixer 5436 (Eppendorf) and were stopped at 10 min (Org 4060) or 15 min (Org 30659) by the addition of 20% ice-cold acetonitrile. After centrifugation, supernatants were subjected to HPLC analysis.

Inhibitor studies with human liver microsomes. Inhibitor studies were performed with the human liver microsomes as described above. A pool of four different preparations of human liver microsomes was used. Microsomal incubations with [^{3}H]-Org 4060 and [^{3}H]-Org 30659 were performed in the presence of ketoconazole, a selective inhibitor for CYP3A (Baldwin *et al.* 1995). Incubations were performed at four inhibitor concentrations (0.01, 0.1, 1, 10 μM). Incubations containing ketoconazole were pre-incubated for 5 min before addition of the substrate. The incubation conditions were as described for the microsomal incubations in the correlation studies. All incubations were performed in duplicate. An additional incubation was performed under the conditions described above in the absence of inhibitor.

Sample analysis

Determination of radioactivity. The concentration of radioactivity in incubation samples was determined by liquid scintillation counting (LSC) (Tri-Carb 2500 TR/2, Canberra Packard, Germany).

HPLC analysis of the metabolite profiles of Org 4060 and Org 30659. Supernatants of the incubation samples, obtained after precipitation of microsomal protein with acetonitrile, were analysed by HPLC. HPLC analysis was performed using a μ-Bondapak C18 (internal length 300 mm, internal diameter 7.8 mm) column and a gradient of 0.1 M ammonium acetate buffer (pH 4.2) (solvent A) and methanol (solvent B). Elution was performed with a linear gradient from 10–90% (v/v) solvent B in 35 min at 500 °C. The flow rate was 2.5 ml min^{-1}. HPLC analysis was performed with a HP1090 liquid chromatograph equipped with a HP1040 diode array detector (Hewlett Packard, Germany) and a Flo-one/Beta model A525 on-line radioactivity detector (Canberra Packard). Samples were spiked with unlabeled Org 4060 and the 6β-OH metabolite of Org 4060 or Org 30659 and the 6β-OH metabolite of Org 30659 as references for retention times (UV signal at 254 nm).

Spearman Rank correlation test. The correlation between the rates of formation of the metabolites of Org 4060 and Org 30659 and the activity of microsomes with selective P450 substrates (testosterone for CYP3A activity and *S*-mephenytoin for CYP2C19 activity) as specified by the supplier (IIAM) was calculated using the Spearman-Rank correlation test.

Results

In vitro *incubations*

In the present study, the specific P450 enzymes involved in the Phase I metabolism of Org 4060 and Org 30659 were investigated. Three different *in vitro* experiments, namely incubations with supersomes, correlation analysis and incubations with ketoconazole (a selective inhibitor of CYP3A), were done to identify the P450 enzymes involved in the Phase I metabolism of the two synthetic steroidal hormones. It has been shown that the involvement of a particular enzyme can only be reliably established if multiple approaches are used (Guengerich 1996).

Metabolites of Org 4060 and Org 30659 in human liver microsomal incubations. The HPLC metabolite profile of the incubation of Org 4060 with pooled human liver microsomes in the presence of NADPH, shows the formation of one major metabolite (figure 2) under the conditions used. By co-chromatographic analysis with the 6β-OH Org 4060 reference compound, the presence of the 6β-OH metabolite of Org 4060 was confirmed. The initial rate of the formation of the 6β-OH metabolite of Org 4060 was linear with the microsomal protein concentrations between 0.03 and 0.1 mg ml^{-1} over a 10-min incubation (data not shown). To avoid the possibility of secondary metabolism, the final concentration of microsomal protein in further incubations was set at 0.1 mg ml^{-1} and incubation was set at 10 min. HPLC analysis of incubations with Org 30659 in human liver microsomes also shows the presence of one major metabolite (figure 3) under the conditions used. The formation of the 6β-OH metabolite was also confirmed for Org 30659 on the basis of co-chromatography with the authentic reference compound. For Org 30659, the initial rate of the formation of the 6β-OH metabolite was linear with the microsomal protein concentrations between 0.1 and 1 mg ml^{-1} over a 15 min incubation (data not shown). Based on these results, the final concentration of microsomal protein in further incubations with Org 30659 was set at 0.25 mg ml^{-1} and the incubation at 15 min to avoid possible secondary metabolism. For both test compounds, no metabolites were formed in incubations in the absence of NADPH (data not shown).

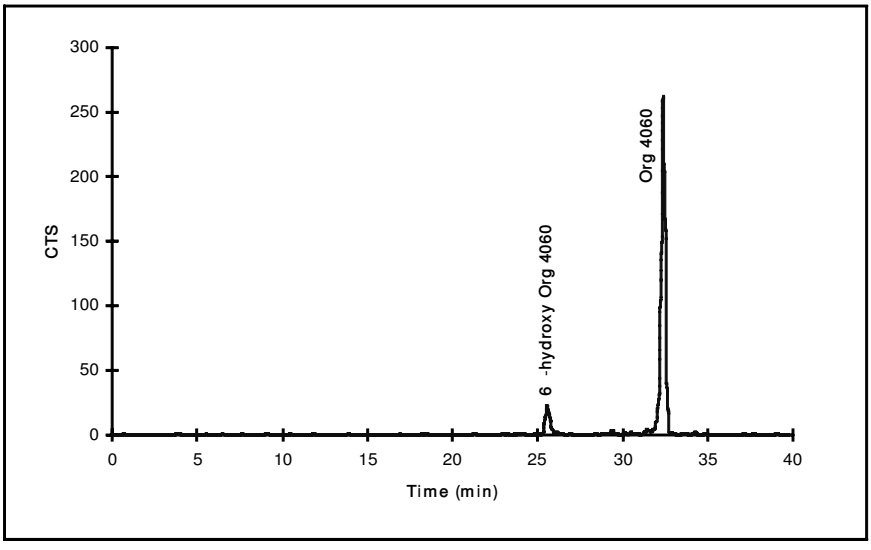

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Figure 2. Typical chromatogram derived from incubations of Org 4060 for 10 min with human liver microsomes.

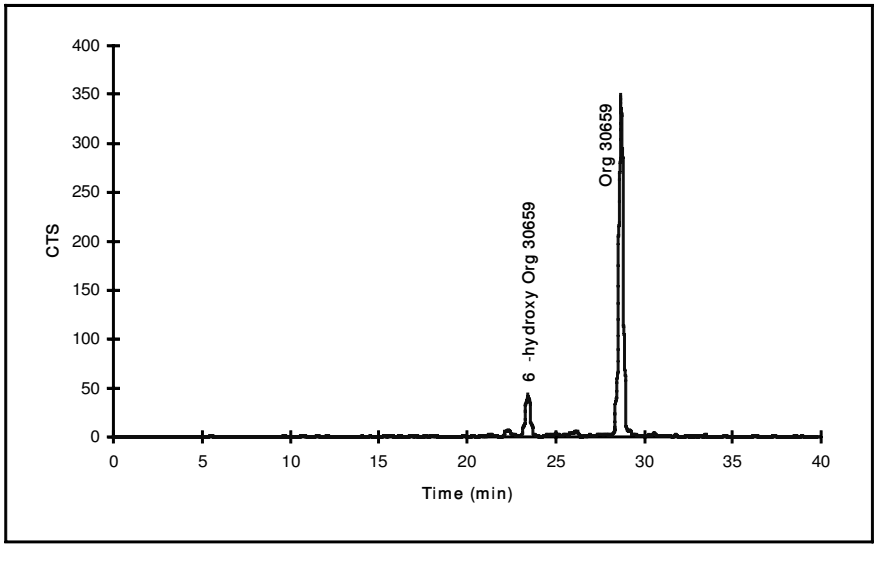

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Figure 3. Typical chromatogram derived from incubations of Org 30659 for 15 min with human liver microsomes.

In vitro *incubation studies with supersomes*. In a first approach to identify the human P450 enzymes involved in the 6β-hydroxylation of Org 4060 and Org 30659, the formation of the 6β-OH metabolite of Org 4060 and Org 30659 was determined in incubations of Org 4060 and Org 30659 with supersomes (CYP1A2,

CYP2A6, CYP2D6, CYP2E1, CYP2C9, CYP2C19, CYP3A4 or CYP3A5). The formation of the 6β-OH metabolite of Org 4060 and Org 30659 by the individual supersomes, expressed as min^{-1}, is given in table 1. Formation of the 6β-OH metabolite of Org 4060 was only observed in incubations with CYP3A4 and CYP3A5, with CYP3A4 being the most active. The other enzymes showed no activity in the formation of the 6β-OH metabolite of Org 4060. The CYP3A4 and CYP3A5 supersomes also metabolized Org 30659 to its 6β-OH metabolite. However, the conversion rate for Org 30659 with CYP3A5 supersomes was ~10-fold lower than with CYP3A4 supersomes. In addition, the CYP2C19 microsomes could form the 6β-OH metabolite of Org 30659, although at a rate that was ~8-fold lower than observed with CYP3A4 supersomes. All the other microsomes failed to metabolize Org 30659 to a detectable level.

Kinetic studies with CYP3A4, CYP3A5 and CYP2C19 supersomes. Kinetic data for the 6β-hydroxylation of Org 4060 and Org 30659 by the three types of supersomes which could metabolize Org 4060 and Org 30659 are shown in table 2. The apparent K_m for the 6β-hydroxylation of Org 4060 in CYP3A4 was 2.0 µM. For Org 30659 an ~20 times higher apparent K_m = 41µM was observed in CYP3A4 supersomes, whereas the V_{max} = Org 4060 was three times lower as compared with the V_{max} determined for Org 30659 converted by CYP3A4. Both steroids were converted by CYP3A5 with relatively high K_m of 141 µM (Org 4060) and 390µM (Org 30659) and also high V_{max}. The apparent K_m of CYP2C19 for Org 30659 (17 µM) was approximately two times lower than the apparent K_m for CYP3A4 conversion of Org 30659. The V_{max} determined for the conversion of Org 30659 by CYP2C19 was relatively low; 0.26 min^{-1}. Org 4060 was not converted by CYP2C19 to a detectable extent.

Correlation studies. To investigate further the enzyme involvement in the 6β-hydroxylation of both steroids, a correlation study between the formation of the 6β-OH metabolite of Org 4060 or Org 30659 (expressed as pmol min^{-1} mg $protein^{-1}$) and the 4-hydroxylation of *S*-mephenytoin (marker substrate for

Table 1. Formation of the 6β-OH metabolite of Org 4060 and Org 30659 by supersomes at a substrate concentration of 10µM.

Supersomes	6β-OH Org 4060 (min^{-1})	6β-OH Org 30659 (min^{-1})
CYP1A2	n.a.	n.a.
CYP2A6	n.a.	n.a.
CYP2D6	n.a.	n.a.
CYP2C9	n.a.	n.a.
CYP2C19	n.a.	0.17
CYP2E1	n.a.	n.a.
CYP3A4	1.44	1.34
CYP3A5	0.50	0.13

Radioactive peaks in the HPLC metabolite profiles were characterized as a metabolite if the peak area was least the average area of five times 1−min integrated background noise (cpm) + 3*SD (standard deviation), the peak height was at least three times the average peak height (cpm) of five times 1−min integrated background noise and the peak duration was at least 0.5 min.

n.a. No detectable activity.

Table 2. Kinetic parameters for the formation of the 6β-OH metabolite by CYP3A4, CYP3A5 and CYP2C19 supersomes.

	CYP3A4		CYP3A5		CYP2C19	
	Org 4060	Org 30659	Org 4060	Org 30659	Org 4060	Org 30659
V_{max} [a]	1.74 ± 0.16	5.27 ± 0.29	8.39 ± 2.11	4.70 ± 0.64	–	0.26 ± 0.02
K_m [a]	2.0 ± 0.9	41 ± 5	141 ± 46	390 ± 64	–	17 ± 3
V_{max}/K_m	0.87	0.13	0.06	0.01	–	0.02

[a] Values are the mean ± SD of the fit, where individual data points are the mean of two replicates. V_{max} (min^{-1}); K_m (μM); V_{max}/K_m (min^{-1} μM^{-1}).

CYP2C19, expressed as pmol min^{-1} mg protein^{-1}) and the 6β-hydroxylation of testosterone (marker substrate for CYP3A, expressed as pmol min^{-1} mg protein^{-1}) was performed. The Spearman-Rank correlation data obtained from these experiments are given in table 3. The activities of the marker substrates were used as specified by the supplier. When Org 4060 and Org 30659 were incubated with a set of ten human liver microsomes, a significant correlation was observed between the formation of the 6β-OH metabolite of Org 4060 and Org 30659 and the activity of these microsomes in the selective assay for CYP3A activity, testosterone 6β-hydroxylation. No significant correlation was observed between the formation of the 6β-OH metabolite of Org 4060 and Org 30659 and the metabolism of *S*-mephenytoin, a substrate selective for CYP2C19.

Inhibitor study with human liver microsomes. Finally, the effect of the selective P4503A inhibitor ketoconazole (Baldwin *et al*. 1995) was investigated to corroborate the conclusion that CYP3A4 is the P450 enzyme preferentially involved. Ketoconazole showed a potent inhibitory effect on the formation of 6β-OH metabolite of Org 4060 and Org 30659 in a concentration-dependent manner (figure 4a and b respectively). From these results, it follows that the IC_{50} for the inhibition of the conversion of both steroids by ketoconazole is < 0.1 μM.

Discussion

In the present study, the type of human P450 enzymes involved in the *in vitro* metabolism of Org 30659 and Org 4060 was investigated. Both steroids were mainly hydroxylated at the 6β-position. The 6β-hydroxy metabolite was previously identified in human liver microsomes for Org 30659 (Verhoeven *et al*.

Table 3. Spearman-Rank correlations (r) between the formation of the 6β-OH metabolite of Org 4060 and Org 30659 and the metabolism of the marker substrates for CYP2C19 and CYP3A activity.

P450	Marker substrate	Org 4060		Org 30659	
		1.3 μM	10 μM	1 μM	10 μM
CYP2C19	*S*-mephenytoin	0.22	0.35	0.35	0.26
CYP3A	testosterone	0.87***	0.88***	0.92***	0.95***

Statistical significance: *** $p < 0.001$.

1998) and Org 4060 (unpublished data). The 6β-hydroxylation is also the major metabolic pathway for testosterone, progesterone and androstenedione in human liver microsomes (Waxman *et al.* 1988). The results from the experiments with supersomes, correlation studies as well as the inhibition studies with ketoconazole, a selective inhibitor of CYP3A, strongly suggest that the CYP3A family plays a significant role in the 6β-hydroxylation of both steroids. The human CYP3A family, accounting for ~50% of total P450 in human liver, is clinically very important because the members of this family have been shown to metabolize a large number of drugs (Wrighton *et al.* 1989, Shimada *et al.* 1994, Wrighton *et al.* 1996). The human CYP3A subfamily includes family members CYP3A4, CYP3A5 and CYP3A7 (Hunt *et al.* 1992, Li *et al.* 1995). CYP3A4 is the major

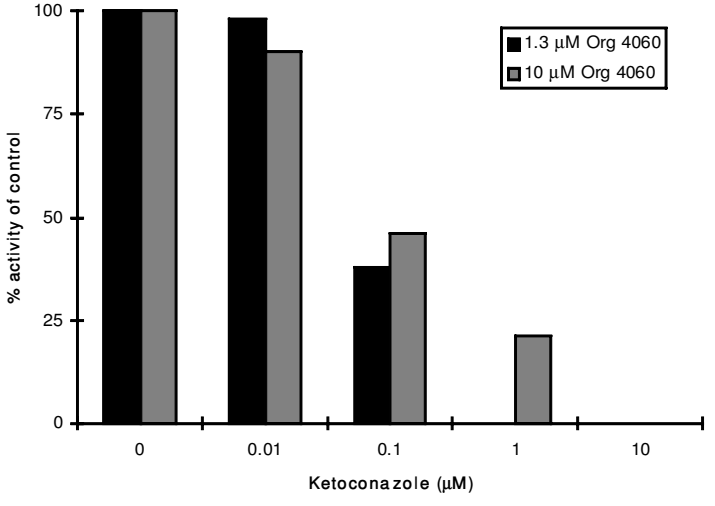

Figure 4a. Inhibition of the 6β-hydroxylation of Org 4060 by ketoconazole. (i) Absolute values of 100% control activities; (ii) data are the mean of duplicate incubations.

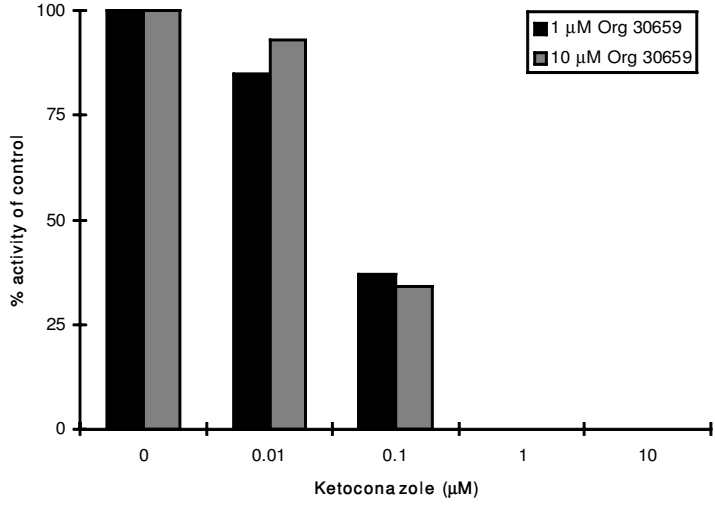

Figure 4b. Inhibition of the 6β-hydroxylation of Org 30659 by ketoconazole. (i) Absolute values of 100% control activities; (ii) data are the mean of duplicate incubations.

human liver CYP3A enzymes, whereas CYP3A5 is present in only ~10–30% of the human livers (Aoyama *et al*. 1989, Li *et al*. 1995, Wilkinson 1996). CYP3A7 is a major P450 enzyme present in foetal liver, but this enzyme appears not to be important in adults (Wilkinson 1996). Enzymes of the CYP3A superfamily are not only present in human liver, but also CYP3A-related P450 enzymes have been detected immunohistochemically in human kidneys, small intestine, pancreas, gall bladder, skin, testes and ovaries (Hunt *et al*. 1992).

Measurements of kinetic parameters of P450 enzymes able to metabolize Org 4060 and Org 30659, combined with the fact that CYP3A4 is known to be the most abundant P450 enzyme in human liver (Li *et al*. 1995), indicates CYP3A4 to be of major importance for the metabolism of both steroids *in vivo*. Kinetics of Org 4060 and Org 30659 metabolism revealed the relative differences in V_{max} and K_m for the conversion of Org 4060 and Org 30659 by CYP3A4, CYP3A5 and CYP2C19 (only Org 30659). The apparent K_m obtained for CYP2C19 for the 6β-hydroxylation of Org 30659 was ~2-fold lower than the K_m obtained for CYP3A4. The maximum human plasma concentration of Org 30659 after an oral administration of 250 µg/subject was ~7 nM (2.5 ng ml^{-1}) (unpublished data), which is several orders of magnitude below the K_m obtained. At very low substrate concentration, when $[S] < K_m$, the ratio of the enzymatic rate of CYP3A4 to CYP2C19 is given by the ratio V_{max}/K_m (enzyme efficiency) of both enzymes. This ratio of enzyme efficiency of both enzymes is 6.5, indicating that the formation of the 6β-OH metabolite by CYP3A4 is ~6–7 times higher than the formation by the CYP2C19 enzyme even at very low substrate concentrations. Furthermore, CYP3A4 appears to be the most abundant P450 enzyme in human liver (Li *et al*. 1995). Based on these findings, it may be predicted that the CYP3A4 enzyme is likely to play a major role in the 6β-hydroxylation of Org 30659 *in vivo*.

In conclusion, in this study, the involvement of the CYP3A family in the 6β-hydroxylation of the synthetic steroidal hormones Org 4060 and Org 30659 has been demonstrated by means of three different *in vitro* approaches.

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