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In Vitro Metabolism of Dexamethasone (DEX) in Human Liver and Kidney: The Involvement of CYP3A4 and CYP17 (17,20 LYASE) and Molecular Modelling Studies

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ABSTRACT. Dexamethasone (DEX) has previously been shown to be extensively metabolised to 6-hydroxylated and side-chain cleaved metabolites in human liver *in vitro*. CYP3A4 is responsible for 6α- and 6β-hydroxylation of DEX and CYP17 is thought to mediate side-chain cleavage to generate 9αfluoro-androsta-1,4-diene-11β-hydroxy-16α-methyl-3,17-dione (9αF-A). Although 9αF-A has not previously been isolated as a metabolite in its unhydroxylated form in human liver incubations, it is formed as an intermediate metabolite, which is subsequently rapidly hydroxylated to OH-9αF-A. A main part of this study has been to conclusively show that DEX undergoes extensive side-chain cleavage to form 9αF-A in human kidney fractions, which is in contrast to profiles obtained for DEX metabolism in parallel human liver microsomal incubations where 6-hydroxylation is the predominant pathway. Furthermore, molecular models of CYP3A4 and CYP17 (17,20 lyase) have been used to model the enzyme fits of DEX. From these modelling studies it has been shown that DEX complements both putative enzyme active sites in orientations likely to lead to the formation of the metabolites identified *in vitro*. We have also been able to rationalise the preferential formation of the 6βOH-DEX isomer. BIOCHEM PHARMACOL **54**;5:605–611, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. dexamethasone, metabolism, CYP3A4, CYP17 (17,20 lyase), enzyme modelling

Dexamethasone (DEX)[‖] metabolism has been rigorously studied *in vitro* (human liver) and *in vivo* [1–3]. Based on these studies a metabolite profile for DEX has been determined and is outlined in Fig. 1. Hydroxylation at both 6α and 6β positions is cytochrome P450 3A4 (CYP3A4) mediated and 6-hydroxylation forms the predominant metabolic pathway both *in vitro* and *in vivo* [1, 3]. The synthesis of both hydroxylated metabolites combined with NMR and mass spectral analysis has meant that the identification of each isomer has been possible; the ratio of epimer formation is 1:3 for 6αOH-DEX and 6βOH-DEX, respectively. Side-chain cleaved DEX, thought to be a product of CYP17 (17,20 lyase) metabolism [4], has also been identified *in vitro*, although only as the monohydroxylated derivative of 9α-fluoro-androsta-1,4-diene-11β-hydroxy-16α-methyl-3,17-dione (9αF-A). The involvement of CYP17-mediated side-chain cleavage in steroidogenesis [4] suggests that it is likely to be predominant in extrahepatic tissues. In this study we have examined the metabolism of [^{3}H]DEX in subcellular human kidney fractions as well as human liver microsomes to investigate the relative importance of CYP17 (17,20 lyase) and CYP3A4 activity. We have also examined the likely interaction of DEX with the putative active sites of homology models for both CYP3A4 and CYP17 and linked the findings to the data obtained from *in vitro* metabolic studies with DEX.

The structural model for CYP3A4 has been constructed on the basis of amino acid sequence alignment between CYP3A4, as determined by Aoyama *et al.* [5], and CYP102, a bacterial P450 of known crystal structure [6]. This model has been used to rationalise the known positions of metabolism for both substrates and inhibitors of CYP3A4 [7]. With the compounds examined, specific residues in the putative active site of CYP3A4 appear to be commonly involved in hydrogen bonding with CYP3A4-specific compounds. Steroidal substrates such as progesterone, testosterone, cortisol, and gestodene were tested in the model; and in each case, hydrogen bond interactions in the CYP3A4 active site were shown to hold the steroid in an orientation

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‖ *Abbreviations:* DEX, dexamethasone; 9α-FA, 9αfluoro-androsta-1,4-diene-11β-hydroxy-16α-methyl-3,17-dione; 6αOH-DEX, 6α-hydroxy-dexamethasone; and 6βOH-DEX, 6β-hydroxydexamethasone.

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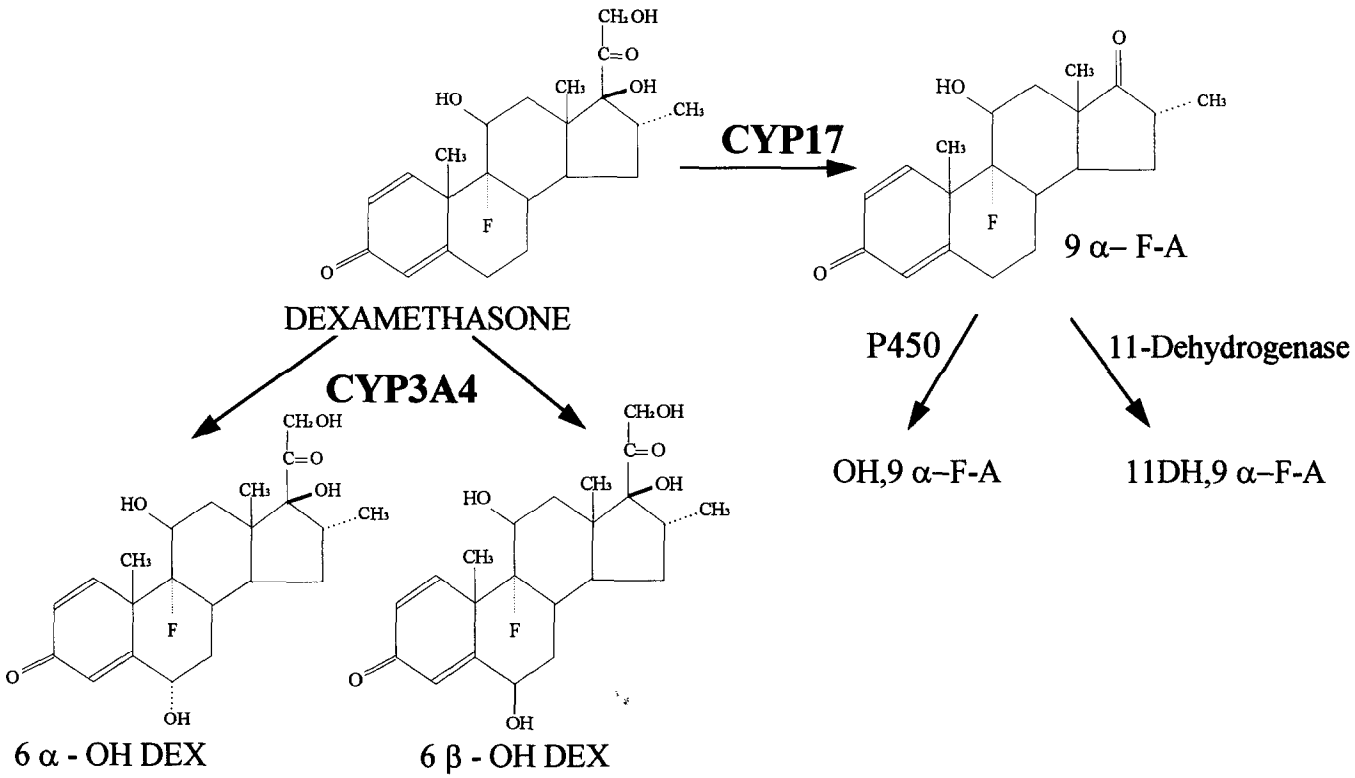


FIG. 1. Proposed metabolite profile for hepatic dexamethasone metabolism *in vitro*.

whereby the 6β-position might be susceptible to hydroxylation by interaction with the haem moiety [7]. In the case of DEX, we have to consider the possibility of an interaction with the haem proto-porphyrin, which may rationalise the formation of both 6α- and 6β-hydroxylated metabolites.

A model of human CYP17, which is consistent with available evidence from site-directed mutagenesis experiments [8], has also been used to examine the binding of DEX in an orientation within its active site, which could result in side-chain cleavage.

MATERIALS AND METHODS

Chemicals

[1,2,4,6,7-^{3}H]Dexamethasone (89 Ci/mmol) was obtained from Amersham Int. (Bucks, UK). Dexamethasone and sodium bismuthate were obtained from Sigma Chemical Co. (Poole, UK). HPLC-grade solvents were supplied by Fisher Scientific (Loughborough, UK). ECI Western blotting kits were purchased from Amersham International. All other chemicals were from BDH (Poole, UK).

Synthesis of 6β-Hydroxydexamethasone and 9α-Fluoro-androsta-1,4-Diene-11β-hydroxy-16α-methyl-3,17-dione (9αF-A)

6β-Hydroxydexamethasone (6βOH-DEX) was synthesised as described by Gentile *et al.* [1]. A method for the oxidation of corticosteroids using sodium bismuthate was used for the synthesis of 9αF-A [9–11], also described in Gentile *et al.* [1].

Human Liver Samples

Histologically normal livers were obtained from transplant donors. Consent for their removal was obtained from the donors' relatives, and Ethics Committee consent was granted for their use in this study. Livers were stored as 10 to 20 g portions at −80°C until use. The four human livers used in this study were from patients between 19 to 60 years old.

Human Kidney Samples

Histologically normal kidney tissue was obtained from patients undergoing surgery (diagnosis, kidney tumour, or hydronephrosis). Tissue was stored at −80°C until use. The 10 kidneys were from male and female patients aged 49 to 82 years. Ethics committee consent was granted for use of kidney tissue in this study.

Microsomal and Cytosolic Incubations With [^{3}H]DEX

Washed human liver microsomes were prepared by the classical differential sedimentation method [12]. Cytosol from the first 105,000 × *g* spin was retained.

Kidney microsomes were prepared by the centrifugation of homogenised kidney portions in buffered sucrose solution

(pH 7.4). The homogenate portions were centrifuged at 13,000 × g for 20 min at 4°C in a Beckman TLA100 ultracentrifuge. The resulting supernatant was then centrifuged at 105,000 × g for 60 min at 4°C to obtain microsomal pellets. Supernatant from the first 60 min spin (i.e. cytosol) was decanted and stored at −80°C until used. The pellets were resuspended in sucrose buffer (pH 7.4) and centrifuged at 105,000 × g for a further 60 min at 4°C to wash the microsomal material. Microsomal and cytosolic protein was determined by the method of Lowry *et al.* [13].

Microsomal or cytosolic protein (1.5 mg) was incubated at 37°C for 20 min with 1 μM [^{3}H]DEX, $MgCl_2$ (10 mM), NADPH (5 mM), and 0.067 M phosphate buffer (pH 7.4) in a final volume of 0.5 mL. Incubations were terminated and DEX and metabolites were extracted with ethyl acetate (4 mL × 2). The organic extracts were evaporated to dryness and the residues reconstituted into methanol (120 μL) before analysis by on-line radiometric HPLC.

HPLC Analysis

[^{3}H]DEX and its metabolites formed from incubation with human kidney fractions were resolved by HPLC on a Nucleosil 5C8 column (5 μm, 25 cm × 4.6 mm i.d. Phenomenex, Macclesfield, Cheshire, UK) with aqueous acetic acid, pH 3.0 (A) and acetonitrile, (B) using the following gradient profile: t = 0 min, 10% B; t = 10 min, 50% B; t = 12.5 min, 60% B; t = 17.5 min, 65% B; t = 20 min, 10% B. The gradient was formed using a Spectra Physics 500 high pressure mixer. The flow rate was 1.0 mL min^{-1}. The mobile phase was delivered by an SP8800 ternary pump. The column was linked directly to a Radiomatic A250 Flo One/β radioactivity detector (Canberra Packard). Extracts from human liver incubations were also resolved by HPLC as described above, and elution was carried out using an isocratic mixture of acetonitrile and aqueous acetic acid, pH 3.0 (75:25, v/v).

LC-MS

Samples for LC-electroscopy MS were obtained from incubations containing 10 μM DEX to ensure sufficient metabolite mass. They were eluted from a Nucleosil 5C8 column (5 μm, 25 cm × 4.6 mm i.d.) with aqueous acetic acid (pH 3.0) and acetonitrile (75:25, v/v). The flow rate was 1.0 mL min^{-1}. The mobile phase was delivered by two Jasco PU-975 pump units. Eluate was directed via a Valco T-piece stream splitter at ca 40 μL min^{-1} to the electrospray probe and interface of a Quattro II tandem quadrupole mass spectrometer (Micromass Ltd., Manchester, UK). The stream splitter and probe were connected by 95 cm of fused silica capillary. Nebulizing and drying gas (nitrogen) was delivered at 13 1 hr^{-1} and 300 1 hr^{-1}, respectively. The interface temperature was 60°C, and the capillary voltage, 4 × 10^3 V.

The cone voltage was 30 V. Compressed centroid spectra were acquired between either m/z 100 to 650 or m/z 100 to 850, over a scan duration of 4.91 sec, at a photomultiplier voltage of 540 V. Ion current chromatograms were processed by a single smoothing. Spectra (3–5) within a chromatographic peak were averaged, and averaged background scans were subtracted from them.

Molecular Modelling

The three-dimensional models of CYP3A4 and CYP17 were constructed from the P450BM3 (CYP102) crystallographic template as described by Lewis *et al.* [7]. The sequence alignment between P450BM3 (CYP102) and CYP3A4 has been published [7]. CYP17 was modelled in a similar manner to CYP3A4, utilizing an alignment between CYP102 and CYP17 family proteins [8]. The molecular geometries of DEX and 9αF-A were energy minimised prior to interactive docking with the enzyme models. As these structures are steroidal, they have only minimal conformational flexibility so there was close agreement between the optimised geometries and those obtained from the crystal structures of analogous compounds [7]. All molecular modelling was carried out using the Sybyl Bipolymer module (Tripos Associates, St Louis, MO) running on an Evans and Sutherland ESV30 Unix work station.

RESULTS

Metabolism of [^{3}H]DEX by Human Liver Microsomes in vitro

[^{3}H]DEX metabolism in human liver microsomes was consistent with previous studies described in Gentile *et al.* [1]. 6β-HydroxDEX was identified as the major metabolite by mass spectral comparison and cochromatography with the chemical standard. This was formed with 6α-hydroxDEX at a ratio of 3:1 (Fig. 2a). Both isomers gave a pseudomolecular ion at m/z 409. A monohydroxylated derivative of 9αF-A was detected by LCMS at m/z 349, and a metabolite with a pseudomolecular ion at m/z 331 was also seen, which would correspond to a side-chain cleaved 11-dehydrogenated derivative of DEX. The MS data are summarised in table 1.

Metabolism of [^{3}H]DEX by Human Kidney in vitro

[^{3}H]DEX was metabolised in both human kidney microsomes and cytosol to one major metabolite (Fig. 2b), which yielded a pseudomolecular ion at m/z 333 (Table 1). This was identified as 9αF-A by cochromatography and mass spectral comparison with the chemical standard. 6βOH-DEX and OH-9αF-A formation were not detected in kidney incubations.

Molecular Modelling

CYP3A4 Model. The overall geometry of the CYP3A4 enzyme model, with DEX docked interactively in the active site, is shown as an overview in Fig. 3(a). The active site

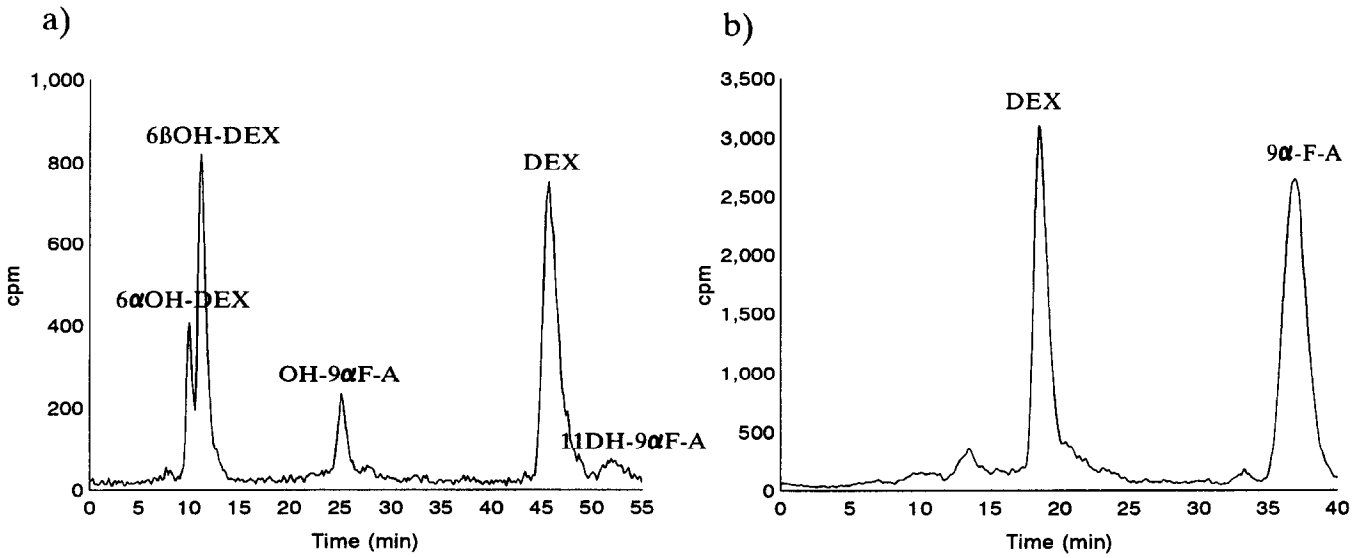


FIG. 2. High-performance liquid chromatograms indicating the metabolites formed during incubation of [^{3}H]DEX (1 μM) with (a) human liver microsomes and (b) human kidney microsomes. Note: Different retention time in (a) and (b) due to different HPLC conditions (see Materials and Methods).

region has been focused upon for putative substrate binding, and DEX has been shown in orientations likely to occur for two energetically feasible interactions, which could result in either 6α- or 6β-hydroxylation, respectively (Fig. 3B and C). No single orientation could be found in which the interaction would allow both 6α- and 6β-positions to be available to interact simultaneously with the haem iron-oxygen moiety (maximum distance of approximately 3 Å is required).

Hydrogen bonding between amino acid residues lining the pocket of the CYP3A4 active site and DEX are represented by the yellow dashed lines and may be seen in Fig. 3B and C. For a binding interaction to result in 6β-hydroxyDEX formation the Ser271 residue would form a hydrogen bond with the 3-keto group of the A-ring. In addition, DEX may be firmly held in this position by hydrogen bonding of the amine nitrogen of Asn74 with the 17β-OH group, and of the carbonyl oxygen of the amide group of Asn74 with the C21-hydroxyl group of DEX.

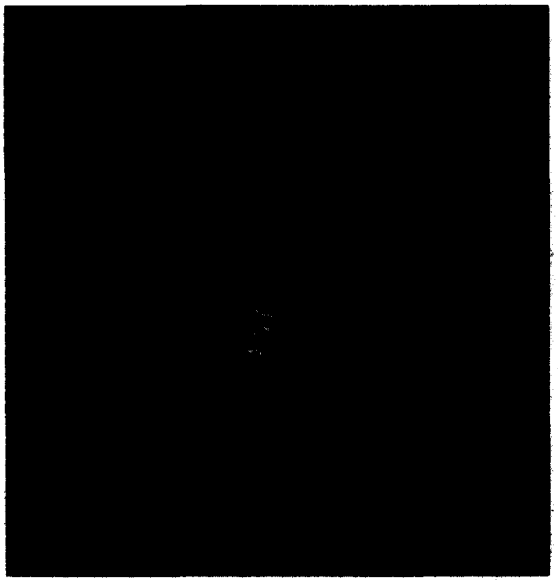
The orientation of DEX that would be required for 6α-hydroxylation in the active site model involves hydrogen bonding interactions with the side chain only. These exist between both the C20 keto and the 17β-OH groups of DEX and Ser271. There may also be hydrogen bonding of the C21-hydroxyl group with Ser436. In this orientation, the 6α-position is only 1.6 Å away from the haem iron oxene. There is no possible amino acid interaction with the A-ring of DEX in this configuration, suggesting that it is rigidly fixed at only one end of the molecule.

The hydrophobic residues Phe181 and Phe78, which line the top of the active site, could also be involved in hydrophobic interactions with DEX. Additionally, Asn74 has been shown to interact with almost all CYP3A4 substrates [7] and is locally present within the binding region for all of the orientations described.

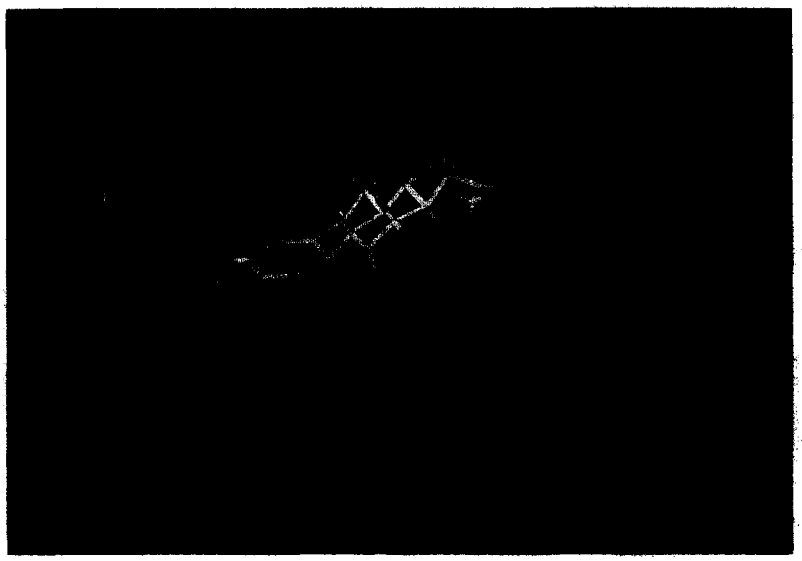
CYP17 Model. An overview of the three-dimensional structure of CYP17 (17,20-lyase) with DEX in the putative active site is illustrated in Fig. 4A. Again, we have focused on the active site region to study possible interactions with DEX that might lead to oxidative side-chain cleavage. The orientation shown in Fig. 4B is the most likely to obtain a favourable interaction with the enzyme when DEX is positioned so that the C17-C20 bond is

TABLE 1. Pseudomolecular Ions and Major Fragment ions of Dexamethasone Metabolites Isolated From Renal and Hepatic *In Vitro* Studies

Metabolite	Metabolite formed in Liver (L) or Kidney (K) microsomes	Pseudomolecular (M + 1) and fragment ions (relative intensity)
6α-HydroxyDEX	L	409(M+1:100)
6β-HydroxyDEX	L	409(M + 1:100), 345(56), 227(30)
Hydroxy-9α-F-A	L	349(M + 1:100), 142(21)
11-Dehydro-9α-F-A	L	331(M + 1:100)
9α-F-A	K	333(M + 1:100)
Dexamethasone		393(100), 373(25)



A



B

C

FIG. 3. (A) An overview of dexamethasone within the active site of the CYP3A4 model. (B) Dexamethasone within the active site of the CYP3A4 model and potential hydrogen bonding and haem interactions likely to exist for 6α-hydroxylation. (C) Dexamethasone within the active site of the CYP3A4 model and potential hydrogen bonding and haem interactions likely to exist for 6β-hydroxylation.

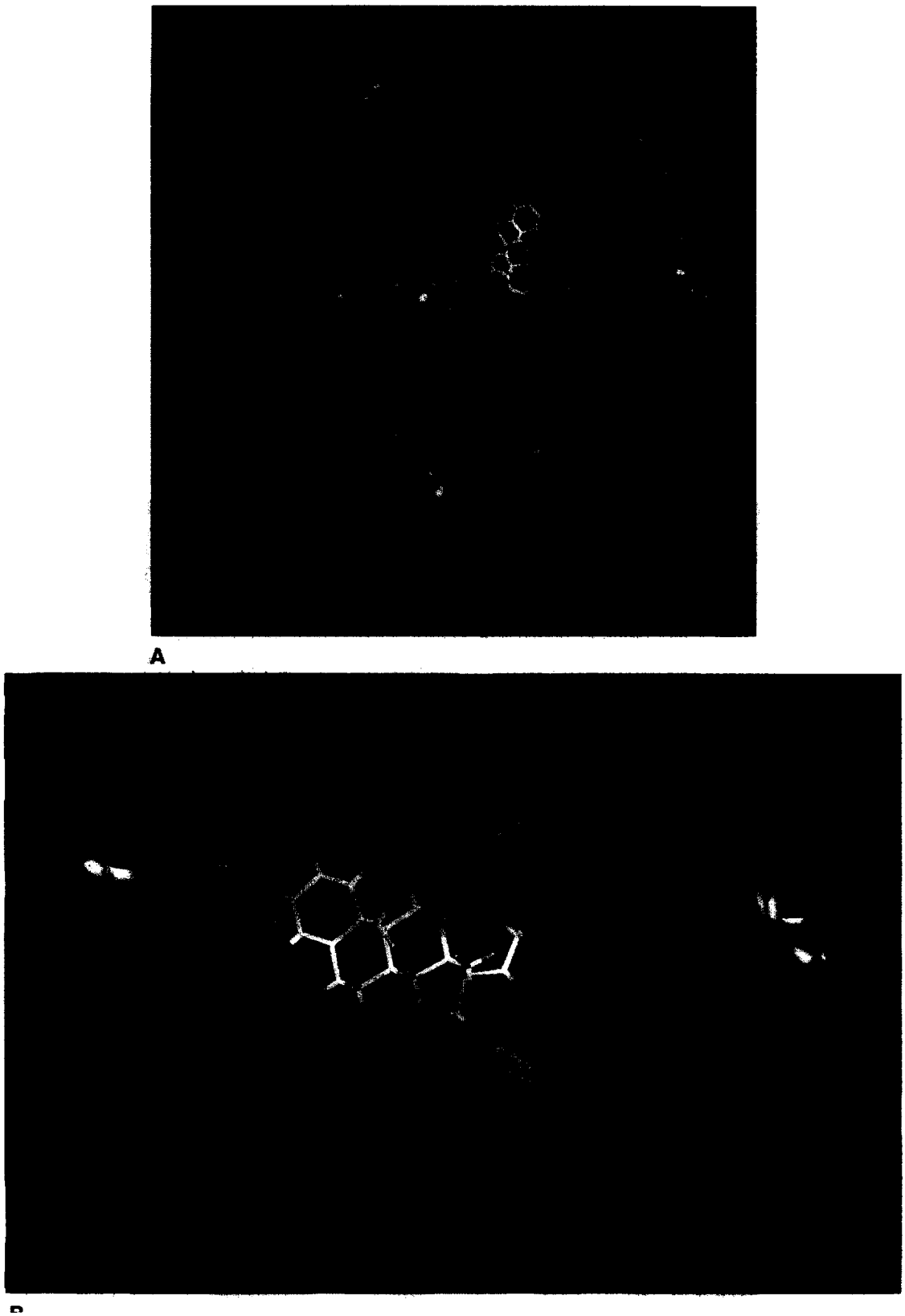


FIG. 4. (A) An overview of dexamethasone within the active site of the CYP17 (17,20-lyase) model. (B) Dexamethasone within the active site of the CYP17 (17,20-lyase) model and potential hydrogen bonding and haem interactions likely to exist for side-chain cleavage.

directly above the haem iron. Hydrogen bonding interactions are represented by the dashed yellow lines. As shown in Fig. 4(B), the 3-keto group of DEX is likely to interact with Thr77 and the 21-hydroxyl group with Tyr177. Hydrophobic interactions with Ile181 and Leu78 make a contribution to the binding of the steroid in this configuration, in agreement with site-directed mutagenesis studies on CYP17.

DISCUSSION

Contrasting metabolic profiles were obtained for DEX metabolism in human liver and kidney fractions (Fig. 2). There was extensive CYP3A4 dependent 6-hydroxylation in hepatic microsomes and evidence of side-chain cleavage, although 9αF-A was only isolated as the hydroxylated metabolite. In human kidney microsomes and cytosol there was extensive side-chain cleavage and 9αF-A was the major metabolite formed. This is thought to be extensively formed in the kidney because CYP17 (17,20 lyase) is predominantly localised in certain extrahepatic tissues [4]. However, 9αF-A has not been detected as a urinary excreted metabolite of DEX [3], and this suggests that although CYP17 (17,20 lyase) is involved in DEX metabolism *in vitro* it is of limited significance *in vivo*. The absence of DEX 6-hydroxylation in the kidney confirmed the low CYP3A activity in this organ [14].

The interactive docking studies of both CYP17 and CYP3A4 with DEX complement the experimental findings. DEX is accommodated by the active site of CYP17 (17,20 lyase) and is held by hydrogen bonding residues at each end of the molecule in a position suitable for oxidative side-chain cleavage by the haem (Fig. 4B).

DEX exhibited complementary binding within the active site of the CYP3A4 model in the orientation designated for 6β-hydroxylation. The structural requirements deemed necessary for binding in this position are a 3-keto group on the A-ring and a 17β-hydroxyl or 17-keto group on the D-ring of the respective steroid. Hydrogen bonding of these functional groups with Ser271 and Asn74, or with Asn74 alone firmly hold the molecule in an orientation which allows the 6β-position to interact with the haem (Figs. 3B and C).

DEX could also bind within the CYP3A4 active site in an orientation where the 6α-position could be hydroxylated. Hydrogen bonds would exist between functional groups on the side chain of the substrate and serine residues Ser271 and Ser436 (Fig. 3C). DEX is not fixed rigidly in this conformation, as it is only held at one end of the molecule and has the potential to move within the active site to some extent. An interaction between the 6α-position and the haem iron might be hindered by this flexibility and inhibit the formation of 6αOH-DEX explaining the 3:1 ratio in the formation of 6β and 6αOH-DEX *in vitro*.

In summary, the use of molecular modelling in the rationalisation of drug metabolism by human cytochromes P450 is useful both in determining the configuration of metabolite enantiomers and the likelihood of metabolite formation.

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