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Identification of enzymes involved in Phase I metabolism of ciclesonide by human liver microsomes

C. F. PEET[1,4], T. ENOS[2], R. NAVE[3], K. ZECH[3] and M. HALL[1]

Departments of [1] In Vitro Metabolism and [2] Experimental Biology, Huntingdon Life Sciences Ltd, Huntingdon, Cambridgeshire, UK [3]ALTANA Pharma AG, Konstanz, Germany [4] Present address: GlaxoSmithKline, Harlow, Essex, UK

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SUMMARY

Ciclesonide, a novel inhaled corticosteroid, is currently being developed for the treatment of asthma. Here, the enzymes catalysing the human hepatic metabolism of ciclesonide were investigated. When incubated with human liver microsomes (HLM), [^{14}C]ciclesonide was first metabolised to the active metabolite M1 (des-isobutyryl-ciclesonide, des-CIC) and to at least two additional metabolites, M2 and M3. M3 comprises a 'family' of structurally similar metabolites that are inactive. 16-Hydroxyprednisolone was also formed in microsomal incubations of [^{14}C]des-CIC, but at approximately one-tenth the amount of both M2 and M3. bis-*p*-Nitrophenylphosphate and SKF 525-A respectively inhibited des-CIC formation from [^{14}C]ciclesonide by 82% and 49% and M2/M3 formation by 82-84% and 87-89%. Regression analysis showed significant negative correlations (r = -0.96, -0.79 and -0.71, respectively) of M2 formation with CYP3A4/5, CYP2B6 and CYP2C8 activities; M3 formation significantly correlated with CYP4A9/11 (r = 0.47). Troleandomycin and diethyldithiocarbamate inhibited M2 and M3 formation by 85% and 45%, respectively. Sulphaphenazole and quinidine had no inhibitory effects. CYP3A4 Supersomes® catalysed notable formation of both M2 and M3 from [^{14}C]des-CIC; CYP2C8 and CYP2D6, but not CYP4A11 formed smaller amounts. It is concluded that the human hepatic metabolism of ciclesonide is primarily catalysed by one or more esterases and, subsequently, by CYP3A4.

INTRODUCTION

Medicines are often concurrently administered to patients, thereby leading to the possibility that drug-drug interactions may occur. Although only a small proportion of such interactions prove to be clinically relevant, the potential exists for serious, even fatal adverse reactions to arise, particularly with drugs having a narrow therapeutic range. One important type of interaction is when one drug inhibits the metabolism of another drug (1-3). The majority of drugs presently prescribed are metabolised by the cytochrome P450-dependent monooxygenases (CYP), a superfamily of enzymes that are found in most organs of the body, but especially in the liver (4). Due to the routine availability of human-derived biological material, together with an increased knowledge of the enzymes themselves, it is now common practice during the development of a drug candidate to use *in vitro* techniques to investigate the potential for drug interactions to occur at the level of CYP-catalysed metabolism (5, 6).

Ciclesonide (Fig. 1) is a synthetic inhaled corticosteroid that is currently being developed as a glucocorticoid receptor agonist for asthma therapy. Ciclesonide is converted to the major pharmacologically active

Please send reprint requests to: Dr Michael Hall, Department of In Vitro Metabolism, Huntingdon Life Sciences Ltd, Woolley Road, Alconbury, Huntingdon, Cambridgeshire, PE28 4HS, UK.

metabolite, M1 (des-isobutyryl-ciclesonide, des-CIC) by ester cleavage. Early experiments have indicated that ciclesonide is metabolised by both human liver microsomes (HLM) *in vitro* and *in vivo* according to the scheme shown in Fig. 1. The present study was conducted to investigate the human enzymes involved in the metabolism of this compound *in vitro*, in order to predict any contraindications with other compounds that may be co-administered during the course of treatment.

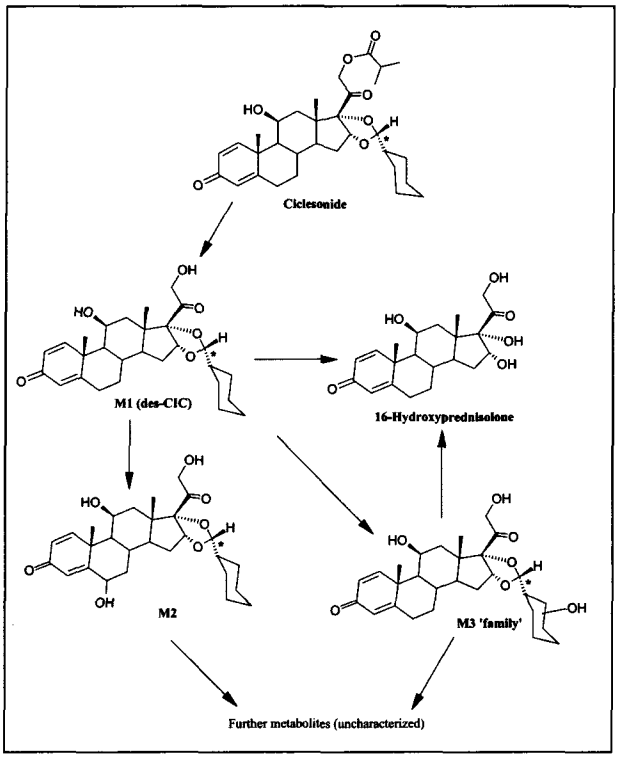

Fig. 1: Proposed scheme for human metabolism of ciclesonide.
* Denotes the position of the radiolabel

MATERIALS AND METHODS

Chemicals

Authentic non-radiolabelled ciclesonide ([11β,16α(R)]-16,17-[(Cyclohexylmethylene)bis(oxy)]-11-hydroxy-21-(2-methyl-1-oxopropoxy)pregna-1,4-diene-3,20-dione), des-CIC, M2, M3 and 16-hydroxyprednisolone (Fig. 1), [^{14}C]ciclesonide (radioactive concentration 96.4 μCi/mg; >98% radiochemically pure) and [^{14}C]des-CIC (radioactive concentration 107.11 μCi/mg; >97% radiochemically pure) were synthesised by ALTANA Pharma AG. [^{14}C]M2 and [^{14}C]M3 'family' metabolites were isolated from rat and guinea pig in vitro microsomal incubations of [^{14}C]ciclesonide and identified by liquid chromatography with mass spectrometric detection (LC-MS) and nuclear magnetic resonance (NMR) technology. β-NADPH (tetrasodium salt), sodium dithionite, SKF 525-A (proadifen), troleandomycin (TAO), bis-*p*-nitrophenyl-phosphate (BpNP), sulphaphenazole, tolbutamide, quinidine, diethyldithiocarbamate (DEDC), lauric acid and testosterone were obtained from Sigma Chemical Company Ltd (Poole, Dorset, UK). High-performance liquid chromatography (HPLC) grade methanol and pentane were purchased from Fisher Laboratory Supplies (Loughborough, Leicestershire, UK). Dichloromethane and ethyl acetate were obtained from Rathburn Chemicals Ltd (Walkerburn, Scotland). Debrisoquine sulphate was obtained from ICN Chemicals (Thame, Oxfordshire, UK). Dexamethasone was procured from Acros, Fisher Scientific (Loughborough, Leicestershire, UK). Nitrogen gas was obtained from BOC Ltd (London, UK). [4-^{14}C]Testosterone (specific activity 56 μCi/μmol), [*ring*-U-^{14}C]tolbutamide (specific activity 60 μCi/μmol), [*guanidine*-^{14}C]debrisoquine (specific activity 55 μCi/μmol) and [1-^{14}C]lauric acid (specific activity 58 μCi/μmol) were all supplied by Amersham International (Little Chalfont, Buckinghamshire, UK). MI-31 liquid scintillant was purchased from Canberra Packard (Groningen, The Netherlands).

Other chemicals and solvents were supplied by Sigma Chemical Company Ltd, Fisher Laboratory Supplies, Rathburn Chemicals Ltd or BDH Ltd (Poole, Dorset, UK). Super pure water was prepared in the laboratory using an Elgastat Option 4 plant (High Wycombe, Buckinghamshire, UK).

Microsomes

Pooled HLM used for incubations with [^{14}C]ciclesonide and [^{14}C]des-CIC were prepared from 6 individual donors (1 female and 5 male subjects) supplied by the International Institute for the Advancement of Medicine (Scranton, PA, USA). These microsomes had a protein concentration of 25.4 mg/ml and a CYP concentration of 376 pmoles/mg protein (mean of 2 separate determinations). The phenotyped bank of 16 individual HLM was obtained from XenoTech LLC (Kansas, KS, USA) as their Reaction Phenotyping Kit. Each batch of microsomes had a protein concentration of 20 mg/ml and a CYP concentration ranging from 276 to 733 pmoles/mg protein (data provided by XenoTech LLC).

Gentest Corporation (Woburn, MA, USA) supplied microsomes prepared from baculovirus-infected insect cells expressing individual human CYP (Supersomes®). The CYP concentration (pmoles/mg protein) of each preparation was determined to be 423 (CYP2C8), 97 (CYP2D6), 65 (CYP3A4) and 111 (CYP4A11),

respectively. NADPH-P450 reductase was co-expressed in all Supersomes®, while cytochrome b_5 was co-expressed with CYP3A4. Microsomes containing baculovirus vector only were used as controls in experiments with Supersomes®.

The protein concentration of pooled HLM and Supersomes® was determined by the method of Lowry et al. (7). Diluted microsomal suspension (pooled HLM were diluted 1 in 300 and Supersomes® were diluted 1 in 75 to 200, in water) was successively mixed with alkaline copper tartrate reagent and Folin-Ciocalteu's phenol reagent before measuring the UV absorbance at 750 nm. The total CYP concentration of pooled HLM was assayed by the method of Rutten et al. (8). Aliquots of microsomal suspension were mixed with 100 mM phosphate buffer, pH 7.4 containing 20% (v/v) glycerol and 4.6 mM sodium dithionite and the carbon monoxide absorption difference spectrum measured between 400–500 nm.

Incubations of [^{14}C]ciclesonide and [^{14}C]des-CIC with human liver microsomes

Incubations were initially conducted to determine optimal conditions under which the human hepatic metabolism of ciclesonide should be studied. All reaction mixtures contained Na/K phosphate buffer (100 mM, pH 7.4), microsomal protein (0.5 mg/ml) and NADPH (2 mM) in a final total volume of 1 ml. Following a pre-incubation of 3 minutes at 37°C, the reactions were started by addition of a solution of [^{14}C]ciclesonide dissolved in acetone (final volume of acetone: 1% of incubation, v/v) to give final concentrations of [^{14}C]ciclesonide of either 1 µM or 10 µM. Na/K phosphate buffer (100 mM, pH 7.4) replaced the microsomal protein in control incubations, which were performed simultaneously. Incubations were open to the atmosphere and were performed in an oscillating water bath at 37°C. After 15, 30 or 60 minutes, each reaction was terminated by transferring the mixture from its incubation vial to an Eppendorf® tube, which was capped and dropped into liquid nitrogen.

Subsequent incubations were conducted under the conditions described above, each in duplicate. Reactions were initiated by addition of either [^{14}C]ciclesonide or [^{14}C]des-CIC (final concentration of both of 10 µM) dissolved in acetone. The final concentration of organic solvent in the incubation mixture, including any required to dissolve other incubation components, was restricted to 1% (v/v). Control incubations with [^{14}C]ciclesonide and [^{14}C]des-CIC contained the human liver microsomal protein (0.5 mg/ml), in place of native HLM, that had been previously denatured by heating to ≥80°C for 10 minutes.

The incubation of [^{14}C]des-CIC for the determination of levels of 16-hydroxyprednisolone formation used three different controls: (i) in the absence of substrate to determine whether the microsomes contained residual 16-hydroxyprednisolone, (ii) in the absence of NADPH to determine whether this cofactor was essential to 16-hydroxyprednisolone formation, and (iii) in the presence of heat-denatured HLM. The incubation periods varied from 10 to 60 minutes in these experiments as the extent of metabolism formation was optimised for different purposes.

After stopping the reaction, each sample containing [^{14}C]ciclesonide or [^{14}C]des-CIC was freeze-dried in a Modulo 4K Freeze Dryer (Edwards High Vacuum International, Surrey, UK). The dried residues from the initial incubation only were reconstituted in 200 µl of methanol and an aliquot (10 µl) was removed, mixed with MI-31 scintillation fluid and subject to liquid scintillation counting (LSC). Each sample was then concentrated to dryness under a gentle stream of nitrogen at 25°C and the residues reconstituted in 300 µl of methanol. The dried residues from all subsequent incubations were reconstituted in 300-500 µl of methanol, vortex mixed and centrifuged for 4-5 minutes at 14,000 rpm. The supernatants were transferred to clean Eppendorf® tubes from where 10-20 µl aliquots were removed for LSC to determine the achieved radioactive concentration prior to analysis by thin-layer chromatography (TLC).

Microsomal incubations of [^{14}C]ciclesonide in the presence of chemical inhibitors were performed using the following (final concentrations and solvents): BpNP (1 mM, in water), SKF 525-A (1 mM, in water), TAO (100 µM, in acetone), sulphaphenazole (20 µM, in ethanol), quinidine (10 µM, in acetone) and DEDC (300 µM, in water). Samples containing TAO and DEDC only, were pre-incubated for 15 minutes with microsomes and NADPH prior to initiating the reaction, as these are known to be mechanism-based inhibitors of CYP (9).

Incubations of [^{14}C]des-CIC with Supersomes® contained either 20 or 100 pmoles of CYP in place of human liver microsomal protein. Control incubations contained insect cell control Supersomes® at the protein concentration equivalent to either 20 or 100 pmoles CYP, respectively.

Thin-layer chromatography analysis

One aliquot (15-30 µl) of each reconstituted sample was individually applied to a silica gel 60 F254 (20 x 20 cm) TLC plate (Merck, Darmstadt, Germany) and left to dry at ambient temperature. Chromatography was performed using a mobile phase of *n*-pentane : dichloromethane : methanol : ammonium hydroxide (15 : 68 : 15 : 2, by

volume). Under these conditions, the reference standards had the following approximate R_f values: ciclesonide (0.83), des-CIC (0.67), M2 (0.49) and M3 (0.46). The TLC plates were allowed to dry before exposing them against a Fujix imaging plate (Fuji Photo Film Co., Ltd., Japan) for 4-24 hours. Each imaging plate was viewed and the bands of radioactivity that chromatographed with R_f values equivalent to ciclesonide, des-CIC, M2 and M3 were quantified using a Fujix BAS 2000 Autoradiographic Imaging System, utilising BAS reader 2.8 and Tina 2.09 (LabLogic, Sheffield, Yorkshire, UK) imaging/evaluation software.

A two-dimensional TLC analysis of the sample derived from incubation of [^{14}C]ciclesonide with liver microsomes from a single donor was performed to confirm the designation of radiolabelled components as M2 and M3. Aliquots of solutions of each of [^{14}C]M2 and [^{14}C]M3 'family' metabolites were applied to one corner of separate TLC plates and were overspotted with an aliquot of the reconstituted HLM incubation mixture. Each plate was developed in the first direction using ethyl acetate : ethanol : ammonium hydroxide (80 : 16 : 4, by volume) as the solvent system. After drying, the plates were rotated through 90° and chromatographed in the second direction using a solvent system of *n*-pentane : dichloromethane : methanol : ammonium hydroxide (15 : 68 : 15 : 2, by volume).

Cytochrome P450 assays

Positive control incubations were performed using probe substrates for CYP2C8 ([^{14}C]tolbutamide, final concentration: 100 µM), CYP2D6 ([^{14}C]debrisoquine, final concentration: 500 µM), CYP3A4 ([^{14}C]testosterone, final concentration: 175 µM) and CYP4A11 ([^{14}C]lauric acid, final concentration: 100 µM). These were conducted in parallel to the incubations with either [^{14}C]ciclesonide or [^{14}C]des-CIC under identical conditions and were used to confirm the effectiveness of the chemical inhibitors and the activity of the Supersomes® of these CYP. At the end of the incubations, each positive control sample was freeze-dried in a Modulo 4K Freeze Dryer before the residues were reconstituted in 500 µl of methanol, vortex mixed and centrifuged for 4-5 minutes at 14,000 rpm. The supernatants were transferred to clean Eppendorf® tubes from where 10-20 µl aliquots were removed, mixed with 7 ml of MI-31 scintillation fluid and subject to LSC. The remaining volumes of positive control samples were concentrated to dryness under a gentle stream of nitrogen gas at 25°C, before reconstitution in the following solvents prior to analysis by HPLC with in-line radiodetection. [^{14}C]Tolbutamide: 100-200 µl of HPLC mobile phase (acetonitrile : water : phosphoric acid, 40 : 60 : 0.04, by volume), [^{14}C]debrisoquine: 30 µl of acetonitrile followed by 170 µl of trifluoroacetic acid (10 mM), [^{14}C]testosterone: 110-200 µl of methanol, [^{14}C]lauric acid: 80-100 µl of methanol containing 0.1% acetic acid (by volume) followed by 80-100 µl of water containing 0.1% acetic acid (by volume).

The same HPLC system components were used for the analysis of all positive control samples and comprised of the following instrumentation: P4000 quaternary pumps, AS3000 variable loop autosampler and UV2000 dual wavelength UV detector (Thermo Separation Products Ltd, Stone, Staffordshire, UK). Radioactivity was detected using an in-line Model 2B β-RAM radiodetector (LabLogic) and fluorescence was detected using an LS-4 Fluorescence Spectrometer (Perkin Elmer, Beaconsfield, Buckinghamshire, UK). The radio-chromatograms were evaluated using Laura system software (version 1.2D, LabLogic). All of the samples were maintained at 4°C in the autosampler until analysis.

[^{14}C]Tolbutamide incubation samples (60-140 µl aliquots) were analysed using a Hypersil C_{18} (3 µm), 150 x 4.6 mm i.d. column (Phenomenex, Macclesfield, Cheshire, UK) at ambient temperature and an isocratic mobile phase system consisting of acetonitrile : H_2O : phosphoric acid (40 : 60 : 0.4, by volume) (10). The flow rate was 2.0 ml/min. The eluate was monitored for UV absorbance at 230 nm. Using these conditions 1-butyl-3-(4-hydroxymethylphenyl) sulphonylurea and [^{14}C]tolbutamide eluted with retention times of 2 and 5 minutes, respectively. [^{14}C]Debrisoquine incubation samples (40-120 µl aliquots) were analysed using a Nucleosil 120-5C_{18} EXCEL (5 µm), 100 x 4.6 mm i.d. column and pre-column (Hichrom, Reading, Berkshire, UK) maintained at ambient temperature (11). Mobile phases A (10 mM trifluoroacetic acid) and B (acetonitrile) were pumped at a flow rate of 0.5 ml/min for the first 15 minutes and at 1.0 ml/min thereafter as a linear gradient: 90% mobile phase A for 0-10 minutes, 85% A from 15-20 minutes, 0% A at 25 minutes, 85% A at 30 minutes and initial conditions at 35 minutes. The eluate was monitored for UV absorbance at 211 nm. Using these conditions, 4-hydroxydebrisoquine and [^{14}C]debrisoquine eluted with retention times of 10 and 21 minutes, respectively. [^{14}C]Testosterone incubation samples (20-40 µl aliquots) were analysed using a µBondapak C_{18} (10 µm), 300 x 3.9 mm i.d. column (Millipore Waters, Hemel Hempstead, Hertfordshire, UK) maintained at ambient temperature (12). Mobile phases A (methanol : water : acetonitrile (39 : 60 : 1, by volume) and B (methanol : water : acetonitrile (80 : 18 : 2, by volume) were pumped at a flow rate of 1.5 ml/min as a concave gradient (0-25 minutes): 90% A at initial conditions, falling to 0% A between 25-30 minutes and returning to initial conditions between 35-45 minutes. The eluate was monitored for UV absorbance at 254 nm.

Using these conditions, 6β-hydroxytestosterone and [^{14}C]testosterone eluted with retention times of 15 and 26 minutes, respectively. [^{14}C]Lauric acid incubation samples (40-50 µl aliquots) were analysed using a Supelcosil LC-8 (3 µm), 150 x 4.6 mm i.d. column (Supelchem, Saffron Walden, Essex, UK) maintained at 42°C (13). Mobile phases A (water containing 0.1% acetic acid) and B (methanol containing 0.1% acetic acid) were pumped at a flow rate of 1.0 ml/min as linear gradient: 50% A between 0-20 minutes and 0% A between 25-35 minutes with a 10-minute re-equilibration at initial conditions. Using these conditions, 12-hydroxylauric acid and [^{14}C]lauric acid eluted with retention times of 12 and 28 minutes, respectively.

Liquid chromatography with tandem mass spectrometric analysis

The 16-hydroxyprednisolone that was present following incubation of [^{14}C]des-CIC with HLM was quantified using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS), using dexamethasone as internal standard. An aliquot (100 µl) of internal standard working solution (1 µg/ml) was added to 100 µl of a methanolic extract derived from HLM samples after freeze-drying and reconstitution in 5 ml of water. The analyte and internal standard were extracted from the aqueous phase into methyl-*tert*-butyl ether (MTBE, 5 ml) and this extract was dried and reconstituted in 200 µl of MTBE for transfer to HPLC vials. The MTBE was evaporated once more before the samples were reconstituted in 50 µl of acetonitrile : water (40 : 60, by volume). Samples (20 µl) were analysed by reversed-phase HPLC using a Perkin Elmer series 200 pump with integral autosampler, a Perkin Elmer Sciex API III+ mass spectrometer (PE Sciex, Toronto, Canada) and a Columbus C_{18} (5 µm) 100 x 3.2 mm i.d. column (Phenomenex). Mobile phase (acetonitrile : water, 40 : 60, by volume) was pumped at 1 ml/min and the 16-hydroxyprednisolone and dexamethasone eluted with retention times of 1.1 and 2.4 minutes, respectively. The samples were ionised by atmospheric pressure chemical ionisation (APCI) using a heated nebuliser interface at 550°C. Nitrogen was used for both the sheath and auxiliary gases at 80 psi and 2 litres/minute, respectively, and the collision energy was 30 eV. The characteristic ion dissociation transitions of m/z 377.3→147.0 and 393.3→147.0 for 16-hydroxyprednisolone and dexamethasone, respectively, were used to detect peaks using MacQuan software (version 1.5, Perkin Elmer) collected at a scan rate of 1.3 scans/second and dwell time of 333 ms. The assay was linear ($r \geq 0.99$) between 0.5 ng/100 µl (lower limit of quantification) and 50 ng/100 µl of methanolic HLM extract.

Statistical analyses

The rates of formation of M2 and M3 were correlated with the activity data pertaining to the major isoforms of CYP supplied by XenoTech LLC after logarithmic transformation and linear regression analysis using SAS 6.11 software (SAS Institute, Cary, NC, USA). Significance of the correlation was determined by an *F* test at the 5% level.

RESULTS

Metabolism of ciclesonide by HLM

Analysis by radio-TLC of the reconstituted samples from incubations of [^{14}C]ciclesonide with pooled HLM indicated the formation of radiolabelled components that chromatographed with similar R_f values to authentic des-CIC, [^{14}C]M2 and [^{14}C]M3. A representative radiochromatogram is shown in Fig. 2. The two to three bands of radioactivity associated with M3 were collectively quantified as a 'family' of related metabolites. These had been previously identified as being ciclesonide derivatives that were isomers of the hydroxycyclohexane ciclesonide, designated M3a-e (14). Confirmation of the designation of the microsomal radiolabelled components as M2 and

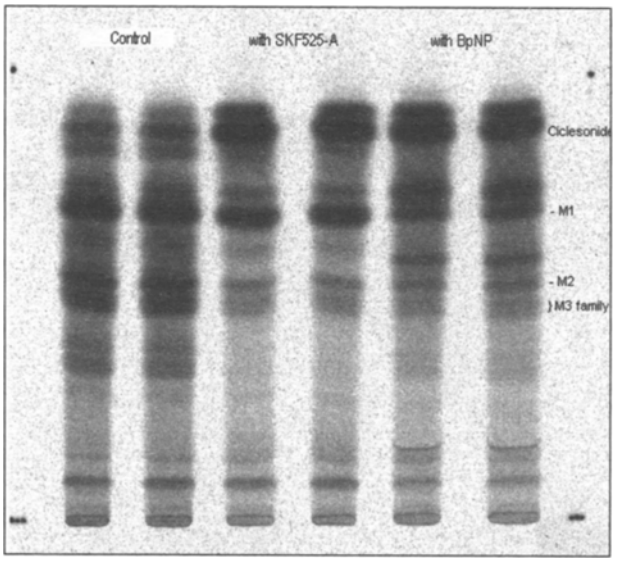

Fig. 2: Radiochromatogram obtained following incubation of [^{14}C]ciclesonide with pooled human liver microsomes in the presence of enzyme inhibitors.
[^{14}C]Ciclesonide (10 µM, in acetone) was incubated with pooled HLM (0.5 mg/ml) at 37°C for 15 minutes in the presence of either water (as solvent control), SKF 525-A (1 mM), or bis-*p*-nitrophenylphosphate (BpNP, 1 mM). Each incubation was conducted in duplicate. The reactions were stopped by rapid freezing in liquid nitrogen. The samples were then processed and chromatographed as described under *Materials and Methods*.

M3 was obtained by two-dimensional co-chromatography of a selected incubation sample with authentic [^{14}C]M2 and [^{14}C]M3 (data not shown).

The results of the initial incubations conducted with two concentrations (1 μM and 10 μM) of [^{14}C]ciclesonide, prepared as solutions in acetone, indicated that des-CIC was the primary metabolite of [^{14}C]ciclesonide metabolism, and that this served as a precursor to both M2 and M3 (Fig. 3). Both M2 and M3 were metabolised further, as evidenced by the time-dependent decrease in radioactivity associated with these components and the appearance of additional radiolabelled components in the lower part of the TLC plate (Figs. 2 and 3). Subsequent additional *in vitro* inter-species metabolism studies have tentatively identified additional Phase 1 and Phase 2 metabolites mainly based on M2 and M3 metabolites (14).

On the basis of these results, a substrate concentration of 10 μM [^{14}C]ciclesonide (and [^{14}C]des-CIC), dissolved in acetone (1% (v/v) final concentration) was selected for all of the subsequent incubations in order to provide an amount of radioactivity in the reconstituted samples that could be readily quantified. All later incubations used one or other of the time points included in the initial incubations to investigate different stages of the metabolic pathway. The results from the succeeding incubations with pooled HLM were very similar with respect to profiles and extents of metabolism to the results of the initial incubation performed under similar conditions. However, heat-denatured microsomes were used in all of the subsequent control incubations rather than incubation solutions from which microsomes were omitted, due to loss of radioactive material in the absence of added protein.

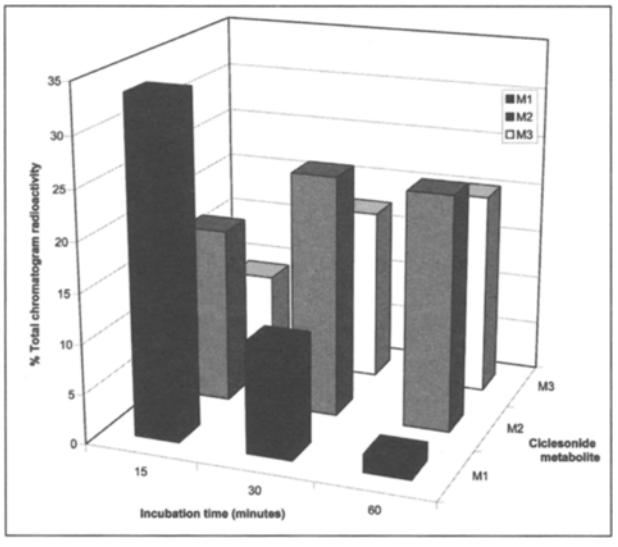

Fig. 3: Time-dependent change in the relative amounts of des-CIC (M1), M2 and M3 present in incubation samples following incubation of [^{14}C]ciclesonide with pooled human liver microsomes. [^{14}C]Ciclesonide (10 μM, in acetone) was incubated with pooled HLM (0.5 mg/ml) at 37°C for 15, 30 and 60 minutes. The reactions were stopped by rapid freezing in liquid nitrogen. The samples were then processed and chromatographed as described under *Materials and Methods*. Individual radiolabelled components of the TLC plates were evaluated and quantified using a bio-image analyser with proprietary software.

Enzymology of ciclesonide metabolism

[^{14}C]Ciclesonide was incubated with pooled HLM for 15 minutes in the presence of either BpNP (1 mM, a non-specific esterase inhibitor) or SKF 525-A (1 mM, a non-specific CYP inhibitor) and in the absence of NADPH. The data (Table I) indicate that BpNP had a similar effect (>80% inhibition) on the rates of formation of des-CIC, M2 and M3, while both SKF 525-A and the absence of NADPH had a greater inhibitory effect on the formation of M2 and M3 than on that of des-CIC. In parallel incubations, the rate of formation of 6β-hydroxytestosterone from testosterone was reduced by 10% and 73% by BpNP and SKF 525-A, respectively. These results suggest that des-CIC is formed from ciclesonide by the action of an esterase and is then further metabolised to M2 and M3 by CYP.

In order to identify the CYP involved in the formation of M2 and M3 from des-CIC, [^{14}C]ciclesonide was first incubated for 60 minutes with HLM prepared from 16 individual donors. The rate of formation of M2 was generally lower than that of M3, although the rate of M2

Table I: Effect of the inclusion of bis-p-nitrophenylphosphate and SKF 525-A and of the omission of NADPH on the metabolism of [^{14}C]ciclesonide by pooled human liver microsomes

Addition	Rate of metabolism (pmol/min/mg protein)			
	Ciclesonide	des-CIC	M2	M3 'family'
Water (control)	941	461	156	107
+ SKF 525-A (1 mM)	270 (71%)	237 (49%)	17 (89%)	14 (87%)
+ BpNP (1 mM)	361 (62%)	84 (82%)	25 (84%)	19 (82%)
NADPH omitted	845 (10%)	790 (0%)	16 (90%)	13 (89%)

[^{14}C]Ciclesonide (10 μM) was incubated at 37°C for 15 minutes with pooled HLM (0.5 mg/ml) in the presence of inhibitors or with NADPH (2 mM) omitted from the reaction mixture. The reactions were stopped by rapid freezing in liquid nitrogen. The samples were then processed and chromatographed as described under *Materials and Methods*. Individual radiolabelled components of the TLC plates were evaluated and quantified using a bio-image analyser with proprietary software.
BpNP bis-*p*-nitrophenylphosphate
(%) data in parenthesis are % inhibition with the modification, compared to control activity

formation varied 10-fold among the 16 HLM (6-65 pmol/min/mg protein), compared to a 1.5-fold variation in M3 formation (43-65 pmol/min/mg protein; Fig. 4). In all samples numerous other metabolite bands were present in the lower portion of the TLC plates corresponding to less lipophilic metabolites. Quantifiable amounts of des-CIC were present only in the samples from incubations with microsomes from Donors 14, 19, 20 and 24 and with pooled HLM, performed in parallel. These samples also contained the largest quantities of M2. The formation of M2 was statistically significantly negatively correlated with activities of CYP3A4/5 (testosterone 6β-hydroxylation: r = -0.96, p = 0.0001), CYP2C8 (paclitaxel 6α-hydroxylation: r = -0.71, p = 0.0019) and CYP2B6 (7-ethoxy-4-trifluoromethylcoumarin deethylation: r = -0.71, p = 0.0019, and S-mephenytoin N-demethylation: r = -0.79, p = 0.0003). M3 formation statistically significantly correlated only with CYP4A9/11 activity (lauric acid 12-hydroxylation: r = 0.47, p = 0.065); none of the other correlations were statistically significant at the 10% level.

The possible involvement of CYP2C8, CYP2D6, CYP3A4/5 and CYP4A9/11 in the production and/or utilisation of M2 and M3 were further investigated using chemical inhibitors of these enzymes and the cDNA-expressed enzymes. Data from the incubation of [^{14}C]ciclesonide in the presence of chemical inhibitors of CYP are shown in Table II. These indicate that the greatest extent of inhibition of M2 and M3 formation (approximately 85% of each) from des-CIC was effected by the selective CYP3A4/5 inhibitor, TAO (100 μM) following a 15-minute pre-incubation with microsomes and NADPH. DEDC (300 μM) also inhibited these reactions by approximately 45%. The chemical inhibitors

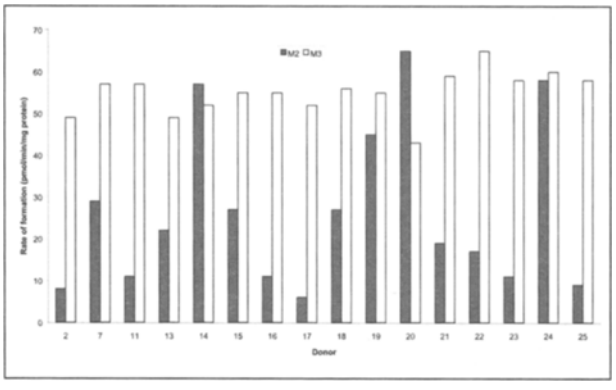

Fig. 4: Variation of rate of M2 and M3 formation among 16 individual donor HLM
[^{14}C]Ciclesonide (10 μM) was separately incubated with HLM (0.5 mg/ml) prepared from 16 individual donors at 37°C for 60 minutes. The reactions were stopped by rapid freezing in liquid nitrogen. The samples were then processed and chromatographed as described under *Materials and Methods*. Individual radiolabelled components of the TLC plates were evaluated and quantified using a bio-image analyser with proprietary software.

of CYP2C8 (sulphaphenazole, 20 μM) and CYP2D6 (quinidine, 10 μM) had negligible effects on the rates of metabolism of M2 and M3. None of these inhibitors caused any notable changes in the rates of metabolism of either parent ciclesonide or of des-CIC. All of the chemical inhibitors used were effective in reducing the metabolism of the probe substrates of each respective CYP (data not shown).

[^{14}C]des-CIC (10 μM) rather than [^{14}C]ciclesonide was used as the substrate in experiments performed with Supersomes®, as the co-expression of any esterase activity along with the CYP isoforms under investigation was not assured and could have led to anomalous conclusions being drawn. Incubations of [^{14}C]des-CIC with 20 or 100 pmoles of CYP2C8, CYP2D6, CYP3A4 or CYP4A11 proceeded for up to 60 minutes. The highest rates of M2 and M3 formation from [^{14}C]des-CIC were achieved with CYP3A4 Supersomes®, where the biotransformation of des-CIC was almost complete within 20 minutes at both CYP concentrations used (Fig. 5A). There was also evidence for the further metabolism of both M2 and M3 in these incubations (Fig. 5B and 5C, respectively). The metabolism of des-CIC by CYP2D6, particularly to M3, was observed while the involvement of CYP2C8 was less apparent. CYP4A11 showed no capacity to catalyse the metabolism of [^{14}C]des-CIC. Each Supersomes® used in these incubations were effective in catalysing the metabolism of their selective CYP substrate (tolbutamide, debrisoquine, testosterone and lauric acid, respectively) in incubations conducted in parallel to those with [^{14}C]des-CIC (data not shown).

Formation of 16-hydroxyprednisolone

The potential formation of 16-hydroxyprednisolone from [^{14}C]des-CIC by pooled HLM was separately investigated by incubating for 60 minutes. As any 16-hydroxyprednisolone present was non-radiolabelled (Fig. 1), quantification of this potential metabolite was performed by LC-MS/MS. Small amounts of 16-hydroxyprednisolone were formed, which accounted for a maximum of 2% of the original substrate concentration (Table III). As such, 16-hydroxyprednisolone represented a relatively minor metabolite of des-CIC when compared to M2 and the M3 'family'. No attempt was made to identify the enzyme(s) responsible for 16-hydroxyprednisolone formation from [^{14}C]des-CIC.

DISCUSSION

It is generally accepted that the major hepatic isoforms of cytochrome P450 that are involved in xenobiotic

Table II: Metabolism of [^{14}C]ciclesonide (10 μM) by pooled human liver microsomes after either 15 or 30 minutes incubation, in the presence of chemical inhibitors of cytochromes P450

Addition	Associated CYP	Rate of metabolism (pmol/min/mg protein)				
		Ciclesonide	des-CIC	M2	M3	
Ethanol (control)*		644	163	135	121	
Sulphaphenazole (20 μM)*	2C8, 2C9	(0%)	648 (1%)	161 (6%)	127 (0%)	131
Acetone (control)*		637	171	124	107	
Quinidine (10 μM)*	2D6	644 (0%)	178 (0%)	145 (0%)	99 (7%)	
Acetone (control)†		948	473	163	145	
TAO (100 μM) †	3A4/5	824 (13%)	738 (0%)	24 (85%)	25 (83%)	
Water (control)*		634	190	130	103	
DEDC (300 μM)*	2A6, 2B6, 2C8, 2E1, 4A9/11	621 (2%)	384 (0%)	74 (43%)	55 (47%)	

[^{14}C]Ciclesonide (10 μM) was incubated at 37°C for either 15 (†) or 30 (*) minutes with pooled HLM (0.5 mg/ml) in the presence of inhibitors at the concentrations stated. The reactions were stopped by rapid freezing in liquid nitrogen The samples were then processed and chromatographed as described under *Materials and Methods.* Individual radiolabelled components of the TLC plates were evaluated and quantified using a bio-image analyser with proprietary software.
DEDC diethyldithiocarbamate
TAO troleandomycin
(%) data in parenthesis are % inhibition by the chemical inhibitor, compared to the respective control activity

Table III: Relative amounts of M2, M3 'family' and 16-hydroxyprednisolone formed from [^{14}C]des-CIC in incubations with pooled HLM.

Incubate	Incubation time (minutes)	Relative amount of des-CIC and metabolites (% of total des-CIC-derived material detected)			
		des-CIC	M2	M3	16-hydroxyprednisolone
With microsomes	0	97	0	0	0
	15	50	15	21	1
	30	36	17	20	1
	60	31	16	17	2
Without des-CIC	60	0	0	0	0
With denatured HLM	60	75	1	1	0
Without NADPH	60	76	1	1	0

[^{14}C]des-CIC (10 μM) was incubated with pooled HLM (0.5 mg/ml) at 37°C for up to 60 minutes. The reactions were stopped by rapid freezing in liquid nitrogen. The samples were then processed and chromatographed as described under *Materials and Methods.* des-CIC, M2 and M3 were quantified by radio-TLC, while 16-hydroxyprednisolone was quantified by LC-MS/MS, as described in *Materials and Methods.* The data are expressed as the percentage of each identified component relative to the total amount of des-CIC-derived material detected, including radioactive bands on the TLC plates that were present in addition to des-CIC, M2 and M3.

biotransformations in human are CYP1A2, 2C9/10, 2C19, 2D6 and 3A4 (15), although others, such as CYP2B6 and 2C8 contribute to the metabolism of certain compounds. When investigating the disposition of a drug candidate in human, it is therefore important to determine if the compound is capable of interacting with these isoforms,

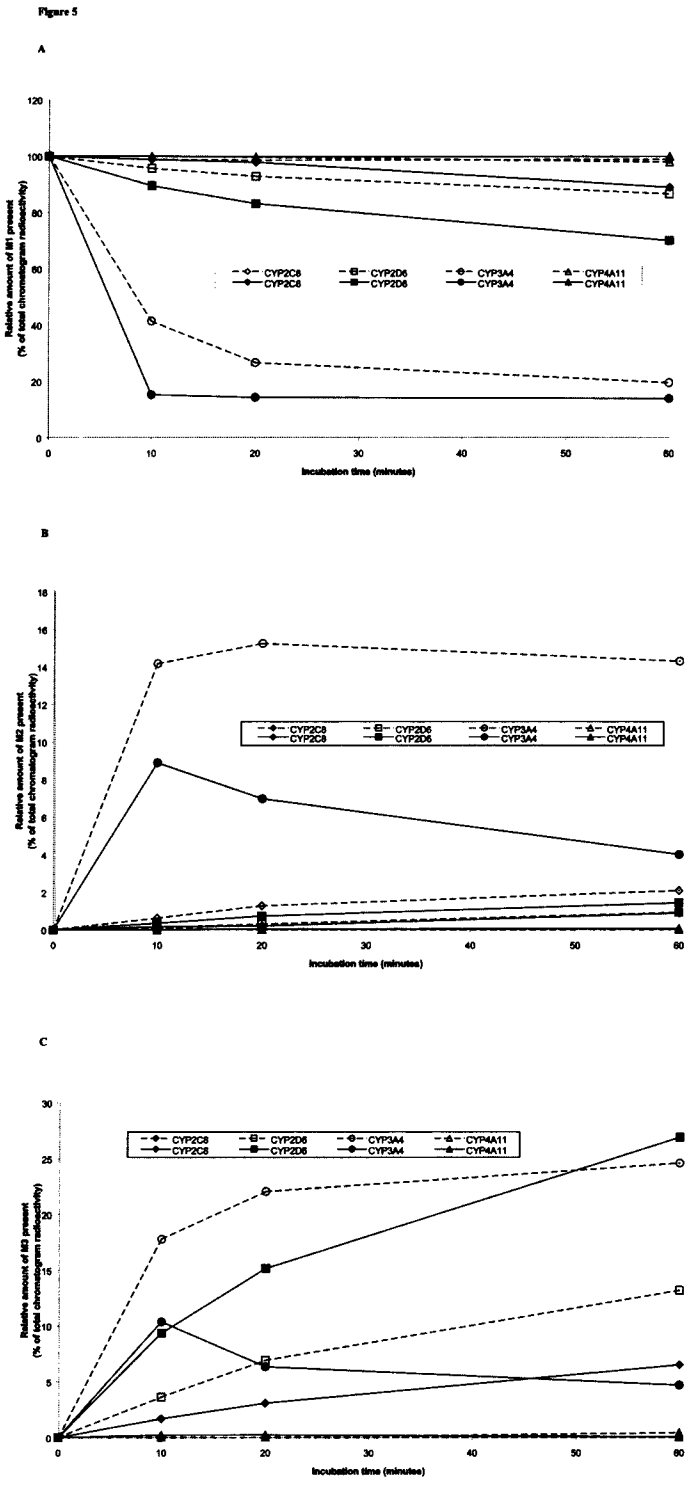

Fig. 5 : Amounts of des-CIC (A), M2 (B) and M3 'family' (C) present in samples following incubation of [^{14}C]des-CIC with CYP2C8, 2D6, 3A4 and 4A11 Supersomes®

[^{14}C]des-CIC (10 μM) was incubated with 20 pmoles (open symbol, dashed lines) or 100 pmoles (closed symbol, full line) of CYP2C8 (diamond), CYP2D6 (square), CYP3A4/5 (circle) and CYP4A9/11 (triangle) at 37°C for 10, 20 and 60 minutes. The reactions were stopped by rapid freezing in liquid nitrogen. The samples were then processed and chromatographed as described under *Materials and Methods.* Individual radiolabelled components of the TLC plates were evaluated and quantified using a bio-image analyser with proprietary software.

either as substrate, inducer or inhibitor, as this may affect both the safety and efficacy of co-administered drugs, or render prodrugs pharmacologically ineffective. Although most interactions that can occur are manageable, usually by appropriate dosage adjustment, a few may be potentially life threatening (2).

In the present study, the human hepatic enzymology and, in particular, the CYP involved in the metabolism of ciclesonide was investigated *in vitro*. On the basis of the results of initial incubations, a substrate concentration of 10 μM [^{14}C]ciclesonide (or [^{14}C]des-CIC) was used in all subsequent microsomal incubations in order to achieve a radioactive concentration that could be readily quantified by the method of radio-TLC. This is approximately 100-fold higher than the C_{max} in human serum following an inhalative dose of 640 μg ciclesonide (16).

The initial step in the metabolism of ciclesonide has been shown to be de-esterification to yield des-CIC (Fig. 1) as seen in the present *in vitro* experiments. Thus, it was hypothesised that this reaction was catalysed by an esterase. Again, it was conjectured that the metabolism of des-CIC by hydroxylation to M2 and M3, and possibly the further biotransformation of these metabolites, for which evidence was obtained here (Fig. 2), was catalysed by one or more CYP enzymes. Evidence supporting both of these assumptions was obtained in the present investigation from the results of incubations of [^{14}C]ciclesonide with pooled HLM in the presence of BpNP (1 mM) and SKF 525-A (1 mM), respectively. BpNP is known to inhibit unspecific liver microsomal carboxylesterases (17), while SKF 525-A has been shown to bring about significant inhibition *in vitro* of the major CYP isoforms (18). Further investigation of the formation of des-CIC has identified carboxylesterase and cholinesterase as the enzymes involved in this process (19).

In order to identify the CYP enzymes involved in the metabolism of des-CIC to M2 and M3, a three-pronged approach was adopted, involving regression analysis, chemical inhibitors of CYP and Supersomes® (5, 6). The rates of M2 formation in incubations of [^{14}C]ciclesonide with individual HLM from 16 donors negatively correlated with CYP3A4/5, 2B6 and 2C8 activities. This negative correlation phenomenon is unusual for this type of experiment, but might be expected if the same CYP was also responsible for the further metabolism of M2. The most statistically significant, albeit a weak correlation of the rate of M3 formation with a CYP activity, was found in the case of CYP4A9/11. CYP2B6 was not investigated further as at the time this study was conducted selective probe substrates and inhibitors of this isoform were not available. Despite having not been implicated in des-CIC metabolism by the regression analysis data, CYP2D6 was included in the list of CYP enzymes for further investigation.

Sulphaphenazole, quinidine, TAO and DEDC were used as chemical inhibitors of CYP2C8, CYP2D6, CYP3A4/5 and CYP4A9/11, respectively. Both quinidine and TAO are known to be selective for CYP2D6 and 3A4/5 at the concentrations used in the present study (9, 15). Sulphaphenazole is often used as a selective inhibitor of CYP2C9 *in vitro* (15), but it has been shown to also inhibit CYP2C8 (18, 20). Although DEDC was used as an inhibitor of CYP4A9/11 activity in the present study, it is known that this is not a selective inhibitor of the enzyme. Inhibitory activity has also been shown towards CYP2A6, 2B6, 2C8 and 2E1, among others (15, 18, 20) and, indeed, DEDC is a relatively weak CYP4A inhibitor. However, the use of DEDC was justified here with these caveats, on the basis that a widely available selective chemical inhibitor of CYP4A9/11 has not yet been established. TAO strongly inhibited the formation of M2 and M3 from [^{14}C]ciclesonide, whilst des-CIC formation was unaffected. The metabolite bands of radioactivity in the lower portion of the TLC plates were also noticeably absent in incubations with TAO. This suggests that CYP3A4/5 activity is responsible not only for the formation of M2 and M3, but also for the formation of other metabolites generated therefrom. Neither quinidine nor sulphaphenazole had any significant effect on the rate of formation of M2, M3 'family' or other metabolites, which is consistent with CYP2C8 and CYP2D6 having a relatively minor role in the formation of these metabolites. DEDC brought about marked inhibition of M2 and M3 formation, but this may have been as a result of its effect on other CYP enzymes, rather than on CYP4A9/11.

Further evidence for the predominant involvement of CYP3A4/5 in the formation of M2 and M3 'family' metabolites from des-CIC was provided by the results of the incubations with cDNA-expressed enzymes. In incubations with CYP3A4 Supersomes®, the formation of M2 and M3 was not only rapid and extensive, but the production of the unidentified metabolites that chromatographed in the lower portion of the TLC plate lanes was also catalysed. CYP2D6 and CYP2C8 Supersomes® formed only small quantities of M2 and M3. The involvement of CYP4A9/11 in the formation of M3, which was suggested by the regression analysis, was not borne out in the cDNA-expressed enzyme experiments, where negligible metabolism occurred.

The formation of 16-hydroxyprednisolone from des-CIC by HLM was progressive over the time-course used here and was apparently catalysed by an unidentified CYP isoform. Since the formation of 16-hydroxyprednisolone accounted for less than 2% of the overall metabolism of [^{14}C]des-CIC, the enzymes which catalyse the acetal

splitting were not investigated. The formation of 16-hydroxyprednisolone has been indirectly confirmed in *in vitro* metabolism studies using hepatocytes of rats, rabbits and humans where the formation of hippuric acid was observed as a minor metabolite (14). This is in contrast to the human hepatic *in vitro* metabolism of budesonide, a structurally similar synthetic glucocorticosteroid, where 16-hydroxyprednisolone, along with 6β-hydroxybudesonide (equivalent to the M2 metabolite of ciclesonide), are the major metabolites (21). Interestingly, CYP3A was also found to catalyse this metabolism.

Collectively, the results of this study of [^{14}C]ciclesonide human Phase I metabolism *in vitro* indicate that formation of des-CIC is catalysed by an esterase, and that formation of the secondary metabolites M2 and M3 is mediated by CYP. The enzyme with the greatest capacity for catalysing these latter reactions is CYP3A4/5, although CYP2D6 and CYP2C8 might also be involved but to a smaller degree. Furthermore, the involvement of CYP2B6 cannot be precluded on the basis of these data.

Although these results indicate that there may be some potential for ciclesonide to interact with drugs whose clearance is mediated primarily by CYP3A, subsequent experiments have shown that ciclesonide at therapeutic serum concentrations is not likely to induce the four CYP isoforms CYP1A2, CYP2C9, CYP2C19 and CYP3A4, or inhibit the seven CYP isoforms CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (22). In addition, an in vivo drug-drug interaction study has been performed to investigate whether systemic exposure to des-CIC and erythromycin are affected by combined administration of erythromycin (500 mg oral) and ciclesonide (640 μg via hydrofluoroalkane metered-dose inhaler). Combined administration of ciclesonide and erythromycin did not alter the pharmacokinetics of the drugs and lack of ciclesonide/erythromycin interaction was demonstrated. Therefore, systemic exposure to ciclesonide or erythromycin is not increased in patients receiving concomitant therapy (16). As a result, it is unlikely that significant drug-drug interactions will occur for patients taking ciclesonide with other concomitant medication.

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