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Formation of a defluorinated metabolite of a quinoxaline antiviral drug catalysed by human cytochrome P450 1A2

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Abstract

The in-vitro metabolism of GW420867X ((*S*)-2-ethyl-7-fluoro-3-oxo-3, 4-dihydro-2H-quinoxaline-1-carboxylic acid isopropyl ester), a quinoxaline drug for the potential treatment of HIV, has been studied with singly expressed human cytochromes P450 (CYP 450). No biotransformation of [^{14}C]GW420867X was evident in the presence of any of the CYP 450 isoforms, with the exception of CYP 450 1A2, where a single metabolite was observed in the HPLC radiochromatograms of enzyme incubations with the test compound. The structure of this metabolite was determined by nuclear magnetic resonance spectroscopy and mass spectrometry, and was shown to correspond to the replacement of the aromatic fluorine of GW420867X with a hydroxyl group. Thus, it appeared that CYP 450 1A2 catalysed the specific defluorination of GW420867X, presumably during formation of an arene oxide intermediate during aromatic hydroxylation.

Introduction

(*S*)-2-Ethyl-7-fluoro-3-oxo-3, 4-dihydro-2H-quinoxaline-1-carboxylic acid isopropyl ester (GW420867X, Figure 1) is a novel quinoxaline antiviral agent, which is currently under investigation for the treatment of HIV infection. The molecule possesses a fluorine substituent in the aromatic ring. The compound is extensively metabolized upon oral administration to laboratory animals and man. A metabolite was detected in the urine of mini-pigs where the fluorine had been lost from this position with subsequent hydroxylation and conjugation with glucuronic acid (Ismail et al 1999). This component was also detected in the urine and plasma of man following oral administration of GW420867X, as well as in the urine of rabbits and mice (Dear et al 2000). In addition to this 7-defluoro 7-hydroxyglucuronide of GW420867X, two additional metabolites were detected in human urine which were characterized as having undergone NIH shift rearrangement of the fluorine and hydroxylation and glucuronidation on the 7-carbon, which had originally borne the fluorine. These components (8-fluoro, 7-hydroxyglucuronyl GW420867X and 6-fluoro, 7-hydroxyglucuronyl GW420867X) were also detected in human plasma after GW420867X administration. To predict which cytochrome(s) P450 (CYP 450) might be involved in the phase 1 metabolism of GW420867X in man, the compound has been incubated with cDNA-derived single CYP 450 isozymes and the extent of biotransformation assessed. In addition, liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy were used to identify a metabolite generated in-vitro. Although GW420867X is

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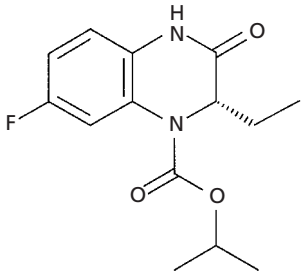


Figure 1 Structure of GW420867X.

extensively metabolized in man, the apparent elimination half-life of the parent drug following oral administration is in the region of 50 h (Moore et al 1999), and therefore the rate of in-vitro metabolism was expected to be low.

Materials and Methods

Singly expressed CYP 450s

Microsomes prepared from baculovirus singly expressing human CYP 450s 1A2, 2A6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1 and 3A4 (Supersomes) were supplied by Gentest Corporation. [^{14}C]GW420867X of specific activity 835 MBq mmol^{-1} and radiochemical purity $> 99\%$ was supplied by Isotope Chemistry, Glaxo Wellcome R&D. Non-radiolabelled GW420867X was supplied by Chemical Development Division, Glaxo Wellcome R&D. Isocitric dehydrogenase (purified from porcine heart) was supplied by Sigma Chemical. All other chemicals and reagents were of analytical grade or equivalent.

Microsomal incubations

Gentest Supersomes 1A2, 2A6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1 and 3A4 were incubated with [^{14}C]GW420867X (25 μM) at a final P450 concentration of 60 or 120 pmol mL^{-1}, in the presence of 2 mM NADPH in 50 mM Tris-HCl buffer, pH 7.4. An NADPH regenerating system (final concentration of 2.5 mM $MgCl_2$, 0.25 mM EDTA, 5 mM isocitric acid and isocitric dehydrogenase (approximately 1.25–3 units mL^{-1})) was used in the incubations. In addition, known substrates for the P450 isozymes (with the exception of CYP 450 2C8) were incubated with the appropriate singly expressed P450 to demonstrate catalytic activity under the conditions used. Incubations (0.5 mL) were carried out at 37°C for 60 min, and were terminated by the addition of 0.25 mL acetonitrile. The terminated incubations were centrifuged at 14 500 *g* for 5 min before analysis by radio-HPLC (system 1).

Further incubations with Gentest Supersomes CYP 450 1A2 were carried out to investigate the product produced by this enzyme upon incubation with GW420867X. Gentest Supersomes 1A2 were incubated with [^{14}C]GW420867X (25 μM) at a final P450 concentration of 160 pmol mL^{-1} in the presence of 2 mM NADPH in 50 mM Tris-HCl buffer, pH 7.4. An NADPH regenerating system (as described above) was used in the incubations. Incubations (0.5 mL) were carried out at 37°C for 180 min, and were terminated by the addition of 0.25 mL acetonitrile. The terminated incubations were centrifuged at 14 500 *g* for 5 min. One incubation was characterized by radio-HPLC (system 2) and by LC-MS. The remaining four incubations were taken to dryness under a stream of nitrogen, resuspended in 150 μL acetonitrile/water (50/50, v/v), and injected onto the radio-HPLC (system 2, with the exception that an injection volume of 100 μL and an yttrium glass 400-μL solid scintillant cell were used). The eluate containing the major metabolite was collected from each run, pooled, taken to dryness under nitrogen, resuspended in a 50:50 mixture of acetonitrile and D_2O and analysed by proton nuclear magnetic resonance (^{1}H NMR).

Radio-HPLC analysis

System 1

Initial incubations with different singly expressed CYP 450s were analysed using a Lichrospher 100 RP-18 250 × 4 mm 5 μ column with a Lichrospher 100 RP-18 4 × 4 mm guard column. The mobile phase consisted of acetonitrile/water (50/50, v/v), at a flow rate of 1 mL min^{-1}. Detection was by use of a Berthold LB506 radiodetector with a 1 mL liquid admixture cell (scintillant flow of 3 mL min^{-1}). This system separated biotransformation products from parent GW420867X (retention time approximately 8 min), and was used to determine the total extent of biotransformation.

System 2

Further incubations with CYP 450 1A2 were analysed using a Hypersil ODS 250 × 4.6 mm 5 μ column. The mobile phase consisted of solvent A (0.1% v/v formic acid in acetonitrile) and solvent B (0.1% v/v formic acid in water). A linear gradient was used which altered the proportion of solvents: 0 min, 10% A; 8 min, 10% A; 8.1 min, 20% A; 25 min, 25% A; 37 min, 25% A; 37.1 min, 50% A; 42 min, 50% A; 42.1 min, 100% A; 43 min, 100% A. The flow rate was 1 mL min^{-1}. Detection was by use of a Berthold LB507 radiodetector with

a 1 mL liquid admixture cell (scintillant flow of 3 mL min^{-1}). This system had been developed to separate biotransformation products from parent GW420867X (retention time approximately 46 min) and from each other, and was used to determine the metabolite profile.

LC-MS

The LC conditions for LC-MS analysis of CYP 450 1A2 incubates were those described in system 2. LC-MS experiments were conducted on a Hewlett-Packard Model 1100 liquid chromatograph (Hewlett Packard, Walderbron, Germany) coupled in series to a Bruker Esquire ion trap mass spectrometer (Bruker-Daltoniks, Bremen, Germany), fitted with an atmospheric pressure ionization source (API). The column eluate was split 1 in 10, directing approximately 100 μL min^{-1} to a pneumatically-assisted electrospray interface. Nitrogen was used as the nebulizer gas at 60 psi, and as the drying gas at a flow rate of 11 L min^{-1} and a temperature of 250°C. The mass spectrometer was operated in positive ion mode with a scan rate of 13000 amu s^{-1} and a range of 100–310 m/z.

NMR

^{1}H NMR experiments were performed on a Varian Inova 750, operating at 750.29 MHz. ^{1}H NMR spectra were acquired into 90048 data points over a spectral width of 15009 Hz (20 ppm), acquiring 10000 transients with dual suppression of the residual HOD and acetonitrile peaks using a one-dimensional nOesypresat sequence. All proton chemical shifts were referenced to acetonitrile at 2.0 ppm.

Results

The activity of singly expressed CYP 450 1A2, 2A6, 2C9, 2C18, 2C19, 2D6, 2E1 and 3A4 was confirmed by turnover of the probe substrates phenacetin (10 μM), coumarin (10 μM), diclofenac (25 μM), diclofenac (25 μM), mephenytoin (100 μM), bufuralol (10 μM), chlorzoxazone (200 μM) and midazolam (10 μM), respectively (data not shown). No probe substrate was available for CYP 450 2C8. Turnover was manifest as production of metabolite in the incubation mixture. No apparent biotransformation of [^{14}C]GW420867X at a concentration of 25 μM was observed with singly expressed CYP 450s 2A6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1 and 3A4. Biotransformation of 25 μM [^{14}C]-GW420867X was observed only with singly expressed CYP 450 1A2. A turnover of 5–6%, as measured by

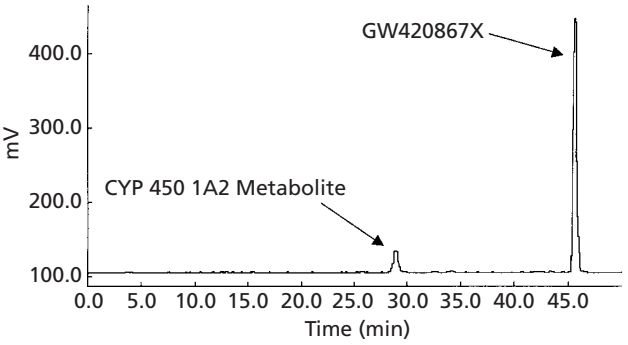


Figure 2 HPLC radiochromatogram of incubation of 25 μM [^{14}C]GW420867X for 3 h with singly expressed CYP 450 1A2 (160 pmol mL^{-1}).

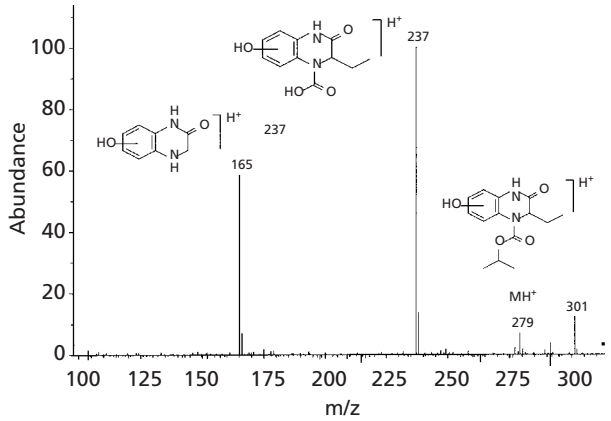


Figure 3 Mass spectrum of CYP 450 1A2 metabolite of GW420867X and proposed fragmentation scheme.

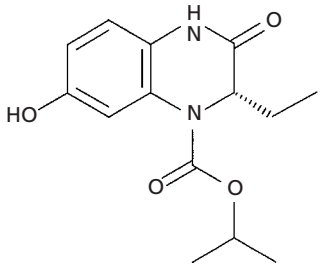
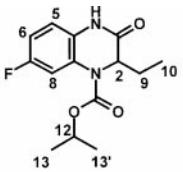
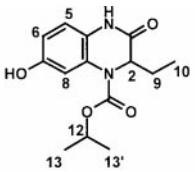
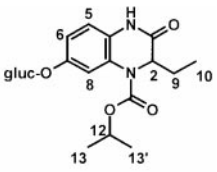


Figure 4 Structure of metabolite formed by human CYP 450 1A2-mediated biotransformation of GW420867X.

Table 1 ^{1}H NMR shifts of GW420867X, CYP 450 1A2 metabolite and M8 (previously observed glucuronide of CYP 450 1A2 metabolite).

Proton position	GW420867X	CYP 450 1A2 metabolite	Metabolite M8
			
2	4.78 (m)	4.77 (m)	4.76 (m)
5	6.98 (dd, ^{2}J ^{19}F-^{1}H = 5.1 Hz, ^{1}J H5-H6 = 8.8 Hz)	6.86 (d, ^{1}J H5-H6 = 8.7 Hz)	6.94 (d, ^{2}J H5-H6 = 8.8 Hz)
6	6.94 (dt, ^{1}J ^{19}F-^{1}H = 8.8 Hz, ^{1}J H5-H6 = 8.8 Hz, ^{2}J H6-H8 = 2.2 Hz)	6.66 (dd, ^{1}J H5-H6 = 8.8 Hz, ^{2}J H6-H8 = 2.4 Hz)	6.90 (dd, ^{1}J H5-H6 = 8.8 Hz, ^{2}J H6-H8 = 2.2 Hz)
8	7.38 (broad)	n/o	7.35 (broad)
9	1.60 and 1.35	obs	1.59 and 1.36
10	0.85 (t, J = 7.3 Hz)	obs	0.86
12	4.96 (m)	4.98 (m)	4.96 (m)
13, 13′	1.29 and 1.22	obs	1.29 and 1.23
H1-gluc	-	-	4.93 (broad)
H2, 3, 4-gluc	-	-	3.63-3.46
H5-gluc	-	-	3.97

n/o, not observed (signal too broad); obs, signal obscured by endogenous components; d, doublet; dd, doublet of doublets; dt, doublet of triplets; t, triplet; m, multiplet.

radioactive peak area, was detected in incubations containing 60 pmol P450 mL^{-1} following incubation over 60 min. In an incubation containing 120 pmol P450 mL^{-1} there was 9 % turnover. In additional incubations with Gentest CYP 450 1A2 at a concentration of 160 pmol mL^{-1}, 15 % turnover following 180 min incubation was observed.

Radio-HPLC profiling (system 2) of incubations of 25 μM [^{14}C]GW420867X with Gentest CYP 450 1A2 (160 pmol mL^{-1}) indicated the presence of one major biotransformation product with a retention time of approximately 29 min (Figure 2). Isolation and characterization of this component was carried out by HPLC followed by LC-MS and ^{1}H NMR.

LC-MS analysis of the major metabolite in Gentest CYP 450 1A2 incubations yielded a protonated molecular ion (MH^{+}) of m/z = 279 (compared with GW420867X m/z = 281), together with a sodium adduct ion at m/z 301. In-source fragmentation was consistent with the loss of the fluorine from the aromatic portion of the molecule and subsequent hydroxylation. The MS spectrum and rationalization of the resulting in-source fragment ions are shown in Figure 3. Structural elucidation of relevant fragment ions was based on comparison with an equivalent spectrum of authentic parent drug.

Further evidence of the structure of the metabolite (Figure 4) was provided by rationalization of its ^{1}H NMR spectrum. Comparison of chemical shifts and coupling constants of the metabolite with that of GW420867X and the previously characterized glucuronide of the defluorinated-7-hydroxylated component (M8) enabled the location of the position of hydroxylation.

The proton-fluorine couplings observed in the NMR spectrum of GW420867X for aromatic H5 and H6 protons were not observed in the metabolite indicating the loss of the fluorine atom. These diagnostic signals, which appeared at 6.98 ppm as a doublet of doublets (H5) and at 6.94 ppm as a doublet of triplets (H6) in

GW420867X, were simplified in the NMR spectrum of the metabolite where only evidence of proton-proton couplings were observed and these signals were reduced to a doublet (H5) at 6.86 ppm and a doublet of doublets (H6) at 6.66 ppm.

The ^{1}H NMR spectrum of the metabolite was consistent with a defluorinated 7-hydroxylated species. Therefore it is unsurprising that the aromatic substitution pattern and chemical shifts observed for the metabolite were similar to those observed for the glucuronide conjugate M8, which has been isolated and characterized by Dear et al (2000). The differences in the chemical shifts of the aromatic protons (H5 and H6) seen between the metabolite and M8 were consistent with the loss of the glucuronide moiety.

The signals for the protons at positions H9, H10, H13 and H13′ were obscured by coincidental signals arising from endogenous material. However, as for the parent compound GW420867X, the signals for protons H2 and H12 of the metabolite were multiplets, indicating that the ethyl and isopropyl side chains were intact. For GW420867X, the signal from the proton at H8 was broad due to the restricted rotation of the carboxylic acid isopropyl ester side chain. Upon heating of the parent compound this broad signal sharpened such that a chemical shift could be assigned. The metabolite, however, was not heated, and thus the expected signal from proton H8 was too broad to observe.

The ^{1}H NMR shifts and coupling constants of the metabolite, GW420867X and for the glucuronide of the defluorinated 7-hydroxylated component (M8) are given in Table 1.

Discussion

Defluorination of xenobiotics has been demonstrated in-vivo and in-vitro. The loss of fluorine has been postulated to occur via a number of different mechanisms, depending upon the substrate in question and the biological system used to study its biotransformation. Enzymatic defluorination has been shown to occur widely in volatile fluorinated anaesthetics such as enflurane (Thummel et al 1993), sevoflurane (Kharasch & Thummel 1993) and the sevoflurane degradation product fluoromethyl-2,2-difluor-1-(trifluoromethyl)vinyl ether (FDVE) (Kharasch & Hankins 1996). The major human enzyme catalysing defluorination of these small aliphatic compounds was CYP 450 2E1, although FDVE can undergo non-enzymatic defluorination (possibly due to addition to protein thiols) with human liver microsomes (Kharasch & Hankins 1996). Although CYP 450 2E1 was the principal enzyme involved in the biotransformation of methoxyflurane, there was also evidence for the involvement of P450s 1A2, 2C9/10, and 2D6 (Kharasch & Thummel 1993). In addition to the defluorination observed in volatile anaesthetics, work has been performed on the defluorination of small aromatic amines. Analysis of rat urine following intraperitoneal dosing of 4-fluoroaniline indicated that oxidative defluorination accounted for approximately 10 % of urinary components detected (Scarfe et al 1999). This loss of fluorine, and replacement by a hydroxyl group in 4-fluoroanilines has been postulated to be mediated in rat by cytochrome P450 and – even more efficiently – by flavin monooxygenases (Boersma et al 1993). It was proposed that this C4-defluorination/hydroxylation of secondary amines such as 4-fluoro N-methylaniline may be initiated by an initial N-oxidation rather than by direct aromatic hydroxylation.

These results indicated that singly expressed human CYP 450 1A2 catalysed the oxidative defluorination of the quinoxaline GW420867X, with hydroxylation occurring on the carbon on which the fluorine had been present. This was the only biotransformation product observed in CYP 450 1A2 incubations. Biotransformation was not evident in incubations with other singly expressed CYP 450s. The apparent discrepancy between the single metabolite produced in-vitro and the extensive biotransformation in man in-vivo (Dear et al 2000) may be due, in part, to the relatively short length of viability of expressed enzymes, coupled with low catalytic rates (as evidenced by the long plasma half-life of GW420867X in man). The metabolite produced by CYP 450 1A2 has not been observed in-vivo; however, the subsequent glucuronide conjugate has been detected in mini-pig, rabbit, mouse and human urine and in human plasma. Additionally, two NIH shift metabolites of GW420867X were observed in human urine, where the fluorine migrated to either position 6 or position 8 on the molecule, with hydroxyglucuronidation on the 7 position in each case. This migration is postulated to proceed via arene oxide formation followed by subsequent NIH shift rearrangement and conjugation with glucuronic acid, but other potential mechanisms for NIH rearrangement have been postulated, such as via a ketone intermediate (Vannelli & Hooper 1995). Similar NIH shift rearrangements for fluorinated aromatics have been reported in dog for the spirohydantoin aldose inhibitor, imirestat (Gilbert et al 1992), and in rat and dog for the antipsychotic, sertindole (Sakamoto et al 1995).

Rat liver CYP 450 1A2 has been implicated in the oxidative and reductive dehalogenation of chlorinated

hydrocarbons (Yanagita et al 1997), and human CYP 450 1A2 has been implicated in the defluorination of methoxyflurane (Kharasch & Thummel 1993). However, often there are alternative products formed or more than one enzyme involved in the catalytic process. This specific biotransformation of GW420867X by human CYP 450 1A2 offers the possibility of further work to study mechanisms of aromatic oxidative defluorination.

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