In vitro metabolism and drug interaction potential of a new highly potent anti-cytomegalovirus molecule, CMV423 (2-chloro 3-pyridine 3-yl 5,6,7,8-tetrahydroindolizine 1-carboxamide)

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Aims To identify the enzymes involved in the metabolism of CMV423, a new anticytomegalovirus molecule, to evaluate its *in vitro* clearance and to investigate its potential involvement in drug/drug interactions that might occur in the clinic.

Methods The enzymes involved in and the kinetics of CMV423 biotransformation were determined using pools of human liver subcellular fractions and heterologously expressed human cytochromes P450 (CYP) and FMO. The effect of CMV423 on CYP probe activities as well as on indinavir and AZT metabolism was determined, and 26 drugs were tested for their potential to inhibit or activate CMV423 metabolism.

Results CMV423 was oxidized by CYP and not by FMO or cytosolic enzymes. The K_m values for 8-hydroxylation to *rac*-RPR 127025, an active metabolite, and subsequent ketone formation by human liver microsomes were $44 \pm 13 \mu\text{M}$ and $47 \pm 11 \mu\text{M}$, respectively, with corresponding V_{max}/K_m ratios of 14 and $4 \mu\text{l min}^{-1} \text{nmol}^{-1}$ P450. Inhibition with selective CYP inhibitors indicated that CYP1A2 was the main isoform involved, with some participation from CYP3A. Expressed human CYP1A1, 1A2, 2C9, 3A4 and 2C8 catalysed *rac*-RPR 127025 formation with K_m values of $<10 \mu\text{M}$, $50 \pm 21 \mu\text{M}$, $55 \pm 19 \mu\text{M}$, *circa* $282 \pm 61 \mu\text{M}$ and *circa* $1450 \mu\text{M}$, respectively. CYP1B1, 2A6, 2B6, 2C19, 2D6, 2E1 or 3A5 did not catalyse the reaction to any detectable extent. CYP1A1 and 3A4 also catalysed ketone formation from *rac*-RPR 127025. In human liver microsomes, CMV423 at 1 and $10 \mu\text{M}$ inhibited CYP1A2 activity up to 31% and 63%, respectively, CYP3A4 activity up to 40% ($10 \mu\text{M}$) and CYP2C9 activity by 35% (1 and $10 \mu\text{M}$). No effect was observed on CYP2A6, 2D6 and 2E1 activities. CMV423 had no effect on indinavir and AZT metabolism. Amongst 26 drugs tested, none inhibited CMV423 metabolism *in vitro* at therapeutic concentrations.

Conclusions CMV423 is mainly metabolized by CYP1A2 and 3A4. Its metabolism should not be saturable at the targeted therapeutic concentrations range ($C_{\text{max}} < 1 \mu\text{M}$). CMV423 will probably affect CYP1A2 and 1A1 activities *in vivo* to some extent, but no other drug–drug interactions are expected.

Keywords: allostery, cytosolic enzymes, drug metabolizing enzymes, drug–drug interactions, FMO, hetero activation, heterologous expression, homo activation, human, *in vitro/in vivo* predictions, metabolic clearance, P450

Introduction

Human cytomegalovirus (CMV) is a ubiquitous virus that infects 60–80% of people in industrialized countries. In

healthy individuals the virus is generally clinically silent, existing in equilibrium with the body's immune system. However, in subjects with an immature immune system or in immune-compromised patients, viral reactivation is very frequent and can lead to lethal disease [1]. CMV423 (2-chloro 3-pyridine 3-yl 5, 6, 7, 8-tetrahydroindolizine 1-carboxamide) is a novel molecule with high activity against CMV in assays *in vitro*, with IC_{50} values between 1 and 10 nM (Aventis, data on file). CMV423 is 100–90 000

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times more potent than the currently used anti-CMV drugs, ganciclovir, foscarnet and cidofovir [1] and acts by a novel mechanism, inhibiting human CMV replication at an early step of the viral replicative cycle. CMV423 is also active on clinical isolates resistant to the other anti-CMV drugs (Aventis, data on file). Therefore CMV423 is a good candidate for a new anti-CMV drug and identifying the enzyme(s) involved in its metabolism is essential to explain interindividual variability in pharmacokinetics and to predict metabolic drug interactions [2, 3]. It is particularly important to evaluate the potential for interactions between CMV423 and drugs administered in patients with autoimmune immunodeficiency syndrome. Preliminary metabolic studies *in vivo* in animals and *in vitro* (Aventis, data on file) have shown that CMV423 is mainly transformed to an active metabolite, *rac*-RPR 127025 (2-chloro 3-pyridine 3-yl 8-hydroxy 5,6,7-trihydroindolizine 1-carboxamide), by hydroxylation of its alicyclic ring. *Rac*-RPR 127025, in turn, is oxidized to an inactive ketone metabolite, RPR 123830 (2-chloro 3-pyridine 3-yl 8-keto 5,6,7-trihydroindolizine 1-carboxamide). Alternatively, CMV423 can be oxidized on its pyridine nitrogen to the inactive RPR 122701 (2-chloro 3-pyridine-*N*-oxide 3-yl 5,6,7,8-tetrahydroindolizine 1-carboxamide). The objectives of this study were to determine the enzymes involved in CMV423 metabolism, to estimate its intrinsic clearance *in vitro* and to predict the most likely drug–drug interactions *in vivo*.

Methods

Chemicals

CMV423 (2-chloro 3-pyridine 3-yl 5, 6, 7, 8-tetrahydroindolizine 1-carboxamide), *rac*-RPR 127025 (2-chloro 3-pyridine 3-yl 8-hydroxy 5,6,7-trihydroindolizine 1-carboxamide) and its enantiomers RPR 130863 (R, dextro-rotary) and RPR 130866 (S, levorotatory), RPR 123830 (2-chloro 3-pyridine 3-yl 8-keto 5,6,7-trihydroindolizine 1-carboxamide), RPR 122701 (2-chloro 3-pyridine-*N*-oxide 3-yl 5,6,7,8-tetrahydroindolizine 1-carboxamide), and indinavir were synthesized at the Centre de Recherche de Vitry/Alfortville, Aventis Pharma, France. Alpha-naphthoflavone (ANF), 3'-azido-3'-deoxythymidine (AZT), 3'-azido-3'-deoxythymidine β -D-glucuronide (AZT-glucuronide), diethyldithiocarbamate (DDC), reduced nicotinamide adenine dinucleotide phosphate (β -NADPH), nicotinamide adenine dinucleotide (NAD^+), paracetamol, quinidine, sulphaphenazole, Tris HCl, troleandomycin (TAO) and uridine 5'-diphosphoglucuronic acid (UDPGA) were purchased from the Sigma Chemical Company (St Louis, USA). Furaflavine, was from Ultrafine Chemicals (Manchester, UK), ketoconazole from Biomol Research Laboratories (Plymouth

Meeting, USA) and omeprazole from Astra Mölndal (Sweden).

Stock solutions

Stock solutions of RPR molecules and cytochrome P450 (CYP) inhibitors were made by dissolving the compounds in dimethylsulphoxide (DMSO). The final concentration of DMSO in subsequent incubations was 0.5% (v/v), except if otherwise stated. The indinavir stock solution was 200 μM in methanol and the final concentration of methanol in subsequent incubations was 0.5% (v/v).

Biological material and preparation of microsomes

Human liver tissue was obtained from 'Assistance Publique – Hôpitaux de Paris (AP-HP)' and used in accordance with French Law (article L.672–1 in the 'Code de la Sante Publique'). Samples originated from donors including four men and one woman, 15–50 years old. No other clinical details were available. Subcellular fractions were prepared as previously described [4]. Different microsomal preparations were used for the various experiments. Our strategy is to use a pool of microsomes at the beginning of each new drug development programme, in order to optimise the likelihood of identifying all the enzymes involved in a single series of experiments. Inter-individual metabolism will be studied using microsomes from individual livers. Several pools of microsomes were used in this study because different laboratories were involved, and over a considerable period of time. The human liver microsome preparations were: Pool 1 (3 livers) for the kinetic studies, pool 2 (5 livers) for the CYP inhibitor study, pool 3 (3 livers) for the inhibition of CYP activities by CMV423 and pool 4 (3 livers) for the experiments studying the effect of CMV423 on indinavir metabolism and that of other drugs on CMV423 metabolism. A single liver preparation was used for the experiments on AZT glucuronidation. Pools 1 and 4 contained 0.37 and 0.25 nmol P450 mg^{-1} protein, respectively. Microsomes from yeast cells (*Saccharomyces cerevisiae*) expressing the human CYP isoforms 1A1, 1A2, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 and 3A5 were obtained from the Centre de Recherche de Vitry/Alfortville, Aventis Pharma, France. Microsomes from lymphoblasts expressing human CYP1B1, 2A6, 2B6 and FMO3 were from the Gentest Corporation (Wolburn, USA). Anti-mouse CYP2a-5 antibody was a gift from Professor P. Maurel (Montpellier, France) and antirat CYP3A1 antibody was obtained from Human Biologics (Scottsdale, USA).

Incubations with human liver subcellular fractions

Incubations were carried out in 100 mM potassium phosphate buffer, pH 7.4 in a shaking water bath under

aerobic conditions. The solubility of CMV423 in the human microsomal preparations was first assessed by injecting aliquots of the incubation mixture, without NADPH, directly onto the h.p.l.c. without addition of solvent or prior centrifugation. CMV423 was soluble up to the highest tested concentration of 100 μM . In enzyme kinetic studies, CMV423 and *rac*-RPR 127025 concentrations were 10, 20, 30, 50, 80 and 100 μM , except where stated otherwise. The concentration was 10 μM in the experiments with expressed enzymes, CYP inhibitors and the studies of the effect of other drugs on CMV423 metabolism. We selected the concentration of 10 μM because (1) it was below the K_m for CMV423 metabolism in human liver microsomes, maximizing the potency of competitive inhibitors, and (2) it was the maximum C_{max} targeted for humans at that time. Incubations contained 1 mg ml⁻¹ of protein for the S9 (9000 g supernatant) fraction and 3 mg ml⁻¹ for the cytosolic (105 000 g supernatant) fraction. Human liver microsome incubations contained 0.75, 1.0 and 0.5 mg ml⁻¹ of protein in the enzyme kinetic experiments, those with CYP inhibitors and studies of the effect of other drugs on CMV423 metabolism, respectively. After a 3–5 min equilibration at 37°C, the reactions were started by addition of NADPH to a final concentration of 1 mM. Additional incubations with the cytosolic fraction were carried out using 1 mM NAD⁺ instead of NADPH, to test for alcohol dehydrogenase activity. In the kinetic studies, aliquots were taken at 0.1, 5, 10, 20, 40 and 60 min after starting the reaction, and mixed with an equal volume of methanol. After centrifugation for 15 min at 10 000 g the supernatants were analysed by high performance liquid chromatography (h.p.l.c.) as described below. Kinetic studies were performed in duplicate and inhibition studies in triplicate, except noted otherwise. Linearity with time, as well as first order parameters were systematically determined. The initial formation rate of *rac*-RPR 127025 was linear in the range 0.25–1.25 mg ml⁻¹ of protein.

Incubations with expressed enzymes

Microsomes from yeast expressing CYP1A1, 1A2, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4 or 3A5 and control microsomes were incubated at 50–100 pmol P450 ml⁻¹ or 5 mg protein ml⁻¹ (CYP2C19 and 2E1) in 50 mM TrisHCl-KOH pH 7.4 containing 1 mM EDTA at 25°C. Cytochrome b5 (200 pmol), purified from rabbit liver [5], was added to the CYP3A4 and 2C8 recombinants. Incubations were as described above with a 5 min preincubation period before the addition of NADPH. Microsomes from lymphoblast expressing CYP were incubated according to the manufacturer's protocol. In the screening experiments, the incubation times with yeast and lymphoblasts microsomes were 30 and 15 min,

respectively. In enzyme kinetic experiments, the determination of linearity with respect to incubation times and protein concentrations was as described for human liver microsomes.

Enzyme kinetic data analysis

Each set of data points was initially visually inspected. Rate parameters and catalytic constants were then calculated by iterative nonlinear regression, using GraFit software (Sigma). The CV of the initial rate of CMV423 metabolism to RPR 127025 in the same pool of human liver microsomes preparation was 4.2% ($n=4$). The enzymatic models tested were: one and two Michaelis–Menten functions, with and without inhibition by substrate excess, a Hill function, each with different weighting (constant or proportional and robust or non-robust). The best fit was selected by *F*-tests on the chi squared values of the fits.

Effect of selective CYP inhibitors on CMV423 and rac-RPR 127025 metabolism

Benzylimidazole 1 mM, coumarin 10 μM , sulphaphenazole 50 μM , omeprazole 50 μM , quinidine 1 μM or ketoconazole 1 μM were added to the incubation mixture simultaneously with CMV423 or *rac*-RPR 127025. Fura-fylline 10 μM , DDC (diethyldithiocarbamate) 100 μM and TAO 20 μM were preincubated for 15 min with human liver microsomes and NADPH before the addition of CMV423 or *rac*-RPR 127025. Inhibitors were also incubated with microsomes with NADPH in the absence of CMV423 and *rac*-RPR 127025 to establish that no interference occurred during HPLC analysis. Anti-CYP2a-5 and anti-CYP3A1 antibodies were used at concentrations of 1.0 and 2.5 mg IgG ml⁻¹, respectively.

H.p.l.c. analysis of rac-RPR 127025 and RPR 123830

Supernatants from metabolic and kinetic studies with CMV423 and *rac*-RPR 127025 were analysed using a Symmetry C8 column (Merck), (5 μm i.d., 250 $\times$ 4.6 mm), maintained at 30°C. The injection volume was 80 μl and isocratic separation was achieved with a mobile phase of 25 mM KH₂PO₄: CH₃CN, 80:20 (v/v) at a flow rate of 0.8 ml min⁻¹. Peaks were detected at 239 nm for CMV423 and *rac*-RPR 127025 and 316 nm for RPR 123830. *Rac*-RPR 127025 and RPR 123830 concentrations were determined by interpolation from calibration curves using authentic standards, unless otherwise noted. RPR 122701 concentrations were calculated as CMV423 equivalents. Within different incubations of 10 μM CMV423 and 10 μM RPR 127025, the coefficients of variation (CV) of the u.v. h.p.l.c. area of the metabolite

peaks were 6.2% ($n=14$) and 5.2% ($n=12$), respectively. The retention times of CMV423 and metabolites varied by less than 1% between studies. The quantitative detection limit of CMV423 and each of its metabolites was 0.2 μM .

Effect of CMV423 and rac-RPR 127025 on CYP marker activities

The protocols and strategy were as described previously [4], except that diclofenac 4'-hydroxylase [6] and midazolam hydroxylase [7] were used as CYP2C9 and 3A4 marker activities, respectively, and a single substrate concentration, close to the published K_m values, was used for each marker activity. These concentrations were: phenacetin 40 μM [8], coumarin 0.5 μM [9], diclofenac 5.6 μM [6], S-mephenytoin 100 μM [10], bufuralol 15 μM [11], chlorzoxazone 40 μM [12] and midazolam 5 μM [13].

Effect of CMV423 on indinavir and AZT metabolism

Indinavir was incubated at 0.1 and 1 μM with 1 mg ml⁻¹ of human liver microsomal protein. In the presence of NADPH, h.p.l.c. analysis showed the appearance of two major peaks, M1 and M2, eluting at 19.2 min and 24.5 min, respectively. Indinavir eluted at 27.4 min. Mass spectrometry analysis showed that M1 corresponded to the mass of indinavir without the methylpyridine moiety and with hydroxylation on the indane group. M2 corresponded to two metabolites, one that had lost the methylpyridine moiety and the other that was hydroxylated on the pyridine ring. These metabolites have been identified previously [14, 15]. CMV423 was coincubated with indinavir and NADPH in the microsomes and, in a separate experiment, preincubated for 15 min with NADPH and microsomes before addition of indinavir. Control tubes contained no CMV423. With 0.1 μM indinavir, reactions were stopped at 0.1 and 5 min and with 1 μM indinavir they were stopped at 0.1, 5, 10, 15, 20, 30 and 60 min. Indinavir and its metabolites were separated using a Purosphere C18 column (Merck), (5 μm i.d., 125 $\times$ 3 mm). Solvent A was 20 mM NH₄CH₃CO₂: CH₃CN:CH₃CO₂H 90:10:1 (v/v/v) and solvent B was 20 mM NH₄CH₃CO₂: CH₃CN:CH₃CO₂H 10:90:1 (v/v/v). A gradient was applied consisting of 0–5% solvent B over 5 min followed by 5–50% solvent B from 5 to 40 min at a flow rate of 0.5 ml min⁻¹. Detection was by fluorescence with an excitation wavelength of 250 nm and an emission wavelength of 560 nm. Indinavir concentrations were calculated using standard curves generated from the authentic compound and the concentrations of the metabolites monitored were calculated as indinavir equivalents.

Incubation with AZT was carried out in 50 mM Trisbase-HCl, pH 7.4 and contained 5 mM MgCl₂,

4 mg ml⁻¹ of human liver microsomes, AZT at 0.25 mM or 1 mM and CMV423 at 1 μM or 10 μM . After a 5-min equilibration at 37°C, glucuronidation was initiated with UDPGA (5 mM final concentration) and the reactions were stopped after 60 min by addition of an equal volume of CH₃CN and placing the samples on ice. After centrifugation at 10 000 g for 10 min, the supernatants were analysed by h.p.l.c., as described previously [16]. Control incubations contained either AZT alone or 0.5 mM chloramphenicol as a positive control for the inhibition of glucuronidation [17]. Authentic AZT and AZT glucuronide were used as standards to quantify the reaction rate.

Effect of selected drugs on CMV423 and rac-RPR 127025 biotransformation in human liver microsomes

Drugs were added at the same time with CMV423 or rac-RPR 127025 or was incubated with the microsomal preparation for 15 min in the presence of NADPH before the addition of CMV423 or rac-RPR 127025. After a 10 min incubation, reactions were stopped with methanol and, after centrifugation, the supernatants were analysed by h.p.l.c. Inhibitors were also incubated with the microsomes containing NADPH in the absence of CMV423 and rac-RPR 127025 to establish that no interference occurred during h.p.l.c. analysis. The approach used was to screen each drug at a single high concentration, namely at its limit of solubility, in order to be able to classify rapidly the effects of the 26 selected drugs. When more than 50% inhibition was observed in coincubation experiments, the drugs concerned were then tested at several concentrations, encompassing their C_{max} in human plasma, to estimate the inhibitory potency at therapeutic concentrations. In time-dependent experiments drugs were used at concentrations near to or above their C_{max} .

Results

CMV423 biotransformation in human liver subcellular fractions

Figure 1 shows the h.p.l.c. profiles after a 10 min incubation of 10 μM CMV423 (b) or 10 μM rac-RPR 127025 (a) with 1 mg of human liver microsomes in the presence of NADPH. The incubation with CMV423 (retention time 39 min) led to the formation of two metabolites, rac-RPR 127025 (retention time 17 min) and RPR 122701 (retention time 7 min) whose structures are shown in Figure 3. Rac-RPR 127025 is, in turn, oxidized to the ketone metabolite, RPR 123830 (retention time 9 min). In the absence of NADPH, there was no metabolism of either CMV423 or rac-RPR 127025

Figure 1 H.p.l.c. profiles of CMV423 biotransformation in human liver microsomes. Analysis of the supernatants obtained after incubating 10 μM CMV423 (b) or 10 μM *rac*-RPR 127025 (a) for 10 min with human liver microsomes (1 mg ml⁻¹) in the presence of NADPH (1 mM).

(data not shown). CMV423 was also metabolized to *rac*-RPR 127025 and RPR 122701 in the human liver S9 fraction in the presence of NADPH, but not by the human liver cytosolic fraction in the presence of either NADPH or NAD⁺ (data not shown). Similar results were obtained for the formation of RPR 123830 from *rac*-RPR 127025. The kinetics of CMV423 and *rac*-RPR 127025 biotransformation in human liver microsomes are shown in Figure 2 and the enzymatic constants are given in Table 1. The best fit of the data by nonlinear regression was obtained with the single Michaelis-Menten enzyme model, using a constant weighting. The mean $\pm$ s.d. K_m values for hydroxylation and ketone formation were 44 ± 13 μM and 47 ± 11 μM , respectively. The mean intrinsic clearance values (V_{max}/K_m) were 14 and 4 $\mu\text{l min}^{-1} \text{nmol P450}^{-1}$ for *rac*-RPR 127025 and RPR 123830 formation, respectively.

Effect of CYP inhibitors on CMV423 and *rac*-RPR 127025 metabolism by human liver microsomes

Benzylimidazole, a potent nonspecific inhibitor of CYP but not FMO enzymes [18] inhibited the metabolism of CMV423 by 94%. Furafylline inhibited 71% of control

Figure 2 Rate vs substrate concentration plots of CMV423 and *rac*-RPR 127025 metabolism in microsomes from human liver and from yeast expressing human CYPs. Human liver microsomes (HLM): 0.75 mg ml⁻¹, pool 1. Expressed CYPs: 200 pmol ml⁻¹, except CYP1A1 100 pmol ml⁻¹. DMSO: 0.5%, except CYP3A4 1.9%. The data were fitted by non linear regression. Inserts: Eadie Hofstee plots.

Table 1 Kinetic constants for the biotransformation of CMV423 and *rac*-RPR 127025 *in vitro*.

Enzyme source	Pathway	K_m (μM)	V_{max}^a ($\text{pmol min}^{-1} \text{ pmol}^{-1} \text{ P450}$)
Human liver microsomes ^b	Hydroxylation ^c	44 ± 13^d	0.63 ± 0.08
	Ketone formation ^c	47 ± 11	0.20 ± 0.02
Yeast microsomes expressing:			
human CYP1A1	Hydroxylation	< 10	1.14 ± 0.12
	Ketone formation	nt	$0.63 \pm 0.06^{d*}$
human CYP1A2	Hydroxylation	50 ± 21	0.18 ± 0.04
	Ketone formation	nt	nd
human CYP2C8	Hydroxylation	$1450^{f\&}$ (Hill: 1.3)	$1.03^g \pm 0.26$
	Ketone formation	nt	nd
human CYP2C9	Hydroxylation	55 ± 19	0.13 ± 0.02
	Ketone formation	nt	nd
human CYP3A4	Hydroxylation	$282^g \pm 61$	$0.29^g \pm 0.03$
	Ketone formation	nt	$0.04 \pm 0.002^{d*}$

^aExcept for ketone formation by the expressed enzymes, where the data correspond to v at $200 \mu\text{M}$ *rac*-RPR 127025; ^bthis pool of microsomes contained $0.37 \text{ nmol P450 mg}^{-1} \text{ protein}$; ^c8-hydroxylation of CMV423; ^dstandard errors from the fit of the data, except ^{d*}s.d. of duplicates; ^eincubation of *rac*-RPR 127025; ^f S_{50} value; ^gestimated, because a high concentration of DMSO (1.9%) had to be used; nt=not tested; nd=metabolite not detected, even at a substrate concentration of $200 \mu\text{M}$.

activity, TAO and the anti-CYP3A antibody 33% and 40%, respectively. In contrast, ketoconazole ($1 \mu\text{M}$) had no effect on *rac*-RPR 127025 formation. No detectable inhibition was obtained with coumarin, anti-CYP2a-5 antibody, sulphaphenazole, omeprazole, quinidine and DDC. The transformation of *rac*-RPR 127025 to RPR 123830 was inhibited by furafylline by 60%. The other inhibitors showed no significant effects (<20% inhibition).

CMV423 and rac-RPR 127025 biotransformation by recombinant CYPs and FMO

Biotransformation of CMV423 ($10 \mu\text{M}$) to *rac*-RPR 127025 was observed with human CYP1A1, 1A2, 2C8, 2C9 and 3A4 but not by CYP1B1, 2A6, 2B6, 2C19, 2D6, 2E1, 3A5 or FMO3. Only CYP1A1 catalysed the formation of RPR 123830 from $10 \mu\text{M}$ *rac*-RPR 127025 and only CYP2C8 catalysed the formation of RPR 122701 from $10 \mu\text{M}$ CMV423. However, with $200 \mu\text{M}$ *rac*-RPR 127025, CYP3A4 also catalysed RPR 123830 formation, whereas CYP1A2 did not. In addition, in the presence of $10 \mu\text{M}$ α -naphthoflavone (ANF), CYP3A4 showed an increased (at least 5.5-times) ability to oxidize the low concentration of *rac*-RPR 127025 ($10 \mu\text{M}$) to RPR 123830. ANF ($10 \mu\text{M}$) was also found to increase the CYP2C8 and CYP3A4 catalysed metabolism of CMV423 by 2-fold and 3-fold, respectively, without having any effect on CYP3A5 activity. The enzyme kinetics obtained with expressed enzymes are shown in Figure 2, and kinetic constants are given in Table 1. With CYP1A1, 1A2, 2C9 and 3A4, the best fit of

the data by nonlinear regression was obtained with the single Michaelis–Menten enzyme model, using a constant weighting. With CYP2C8, the best fit model was with a Hill function. The hydroxylation reaction occurred with K_m values of < $10 \mu\text{M}$ for CYP1A1, $50 \pm 21 \mu\text{M}$ for 1A2, $55 \pm 19 \mu\text{M}$ for 2C9, and approximately $282 \pm 61 \mu\text{M}$ for 3A4 and $1450 \mu\text{M}$ for 2C8 (S_{50} value). With the latter, the reaction rate increased exponentially with increasing CMV423 concentrations up to $100 \mu\text{M}$. Substrate concentrations up to $750 \mu\text{M}$ had to be used to estimate the half-saturating concentrations for CYP2C8 and CYP3A4, and therefore up to 1.9% DMSO was used to solubilize CMV423. Unfortunately, DMSO was slightly inhibitory at this concentration and therefore the kinetic parameters obtained are only estimates. For CYP2C8, the estimated Hill coefficient was 1.3. Oxidation of *rac*-RPR 127025 was found to be stereoselective. In human liver microsomes, oxidation of $100 \mu\text{M}$ RPR 130866 (the (–) isomer) was four times more rapid than the oxidation of $100 \mu\text{M}$ RPR 130863 (the (+) isomer, 75 vs $18 \text{ pmol min}^{-1} \text{ mg}^{-1}$). CYP1A1 mainly metabolized RPR 130863 (0.50 vs $0.08 \text{ pmol min}^{-1} \text{ pmol P450}^{-1}$), CYP3A4 metabolized RPR 130866 ($0.04 \text{ pmol min}^{-1} \text{ pmol P450}^{-1}$) but not RPR 130863, and CYP1A2 did not metabolize either isomer.

Effect of CMV423 and rac-RPR 127025 on CYP marker activities

Analyses were performed in duplicate, and the variability was less than 20%. CMV423 at $1 \mu\text{M}$ or $10 \mu\text{M}$ did not inhibit the activities of CYP2A6, 2D6 or 2E1, but

Table 2 Effect of CMV423 and *rac*-RPR127025 on *in vitro* indinavir oxidation and AZT glucuronidation. Experiments were performed as described in Methods. Values are mean of duplicate assays. CVs were below 6%.

Substrate (μM)	Inhibitor ($10\ \mu\text{M}$)	Design	Effect on substrate biotransformation (%)
Indinavir 0.1	CMV423	Co-incubation	-3
		Pre-incubation	-2
Indinavir 1	CMV423	Co-incubation	-24
		Pre-incubation	+2
Indinavir 1	<i>rac</i> -RPR 127025	Co-incubation	0
		Pre-incubation	+12
AZT 250	CMV423	Co-incubation	0
AZT 1000	CMV423	Co-incubation	-11

inhibited CYP1A2 activity by 11% and 53% after coincubation, and by 31 and 63% after preincubation, respectively. CYP2C9 activity was inhibited 35% at both 1 and $10\ \mu\text{M}$ CMV423, after coincubation. The inhibition decreased to 8 and 21%, respectively, after preincubation. CYP2C19 activity was inhibited by 25% at $10\ \mu\text{M}$ CMV423 in the coincubation experiment, but no inhibition was observed after preincubation. Under both incubation conditions, CYP3A4/5 activity was unaffected by $1\ \mu\text{M}$ CMV423, but was inhibited by 40% at a concentration of $10\ \mu\text{M}$. *Rac*-RPR 127025 at either $1\ \mu\text{M}$ or $10\ \mu\text{M}$ had no effect on the activity of CYP2A6, 2C9, 2C19, 2D6 or 2E1, but 30% inhibition of CYP1A2 and CYP3A4/5 activities were observed at a concentration of $10\ \mu\text{M}$.

Effect of CMV423 and rac-RPR 127025 on indinavir oxidation and AZT glucuronidation in vitro

As shown in Table 2, CMV423 and *rac*-RPR 127025 had no significant effect on the *in vitro* oxidative metabolism of indinavir or on the conjugation of AZT.

Effect of drugs on CMV423 biotransformation in vitro

These drugs were first tested at a single very high concentration. The assumption in this design is that no activation occurs that could counteract any inhibition, and *vice versa*. The results are shown in Table 3. From the results obtained, these drugs have been divided into 4 groups. The first group, causing less than 25% inhibition, consisted of acyclovir, AZT, cyclosporin A, desogestrel, didanosine, foscarnet, lamivudine, nelfinavir, norgestimate, saquinavir and stavudine. The second group, causing between 25 and 50% inhibition, was composed of ethinyl estradiol, indinavir, itraconazole, methadone, paracetamol, ritonavir, theophylline and warfarin. The third group,

causing more than 50% inhibition, consisted of cimetidine, enoxacin and imipramine. The fourth group activated the metabolism of CMV423 by more than 60% and consisted of chlormadinone, ethynodiol, norethindrone and (-) norgestrel. The most potent inhibitors, cimetidine, enoxacin and imipramine, were tested at several lower concentrations, encompassing their therapeutic ranges. Table 4 shows that, after coincubation, none of these three drugs inhibited CMV423 metabolism at concentrations up to 75, 5 and 75 times their C_{max} , respectively. Drugs showing a significant inhibition after preincubation in the screening were tested using several preincubation times. Table 5 shows that no significant metabolism-dependent inhibition was observed with any of these drugs at therapeutic concentrations. Finally, chlormadinone at $1\ \mu\text{M}$ still increased the formation of *rac*-RPR 127025, by 39% (data not shown).

Discussion

CMV423 was metabolized in the microsomal but not in the cytosolic fraction of human liver. The almost complete inhibition observed with benzylimidazole demonstrated the involvement of CYP but not FMO enzymes [18]. The potent effect of the CYP inhibitor furafylline suggested that CYP1A2 was the major isoform hydroxylating CMV423. Yeast expressed human CYP1A2 catalysed this reaction efficiently, with a K_m value similar to that in human liver microsomes ($50\ \mu\text{M}$ and $44\ \mu\text{M}$, respectively). The effects of TAO and anti-CYP3A antibody indicated some participation of CYP3A in CMV423 metabolism. Yeast expressed human CYPs showed a low affinity involvement of CYP3A4 but not CYP3A5. The CYP3A inhibitor ketoconazole did not inhibit this reaction in human liver microsomes, but it was recently shown that the concentration of $1\ \mu\text{M}$ we used may not be sufficiently high [19]. Expressed CYP1A1, 2C8 and 2C9 were also able to metabolize CMV423 into *rac*-RPR127025. CYP1A1 is expressed at extremely low levels *in vivo*, and it is not expected to play a significant role in CMV423 metabolic clearance. Our data indicate that CYP2C8 activity *in vivo* towards CMV423 should also be negligible at therapeutic concentrations of CMV423 (less than $1\ \mu\text{M}$). Because of the absence of detectable inhibition of sulphaphenazole, the involvement of CYP2C9 is likely to be minor *in vivo*.

The metabolism of *rac*-RPR 127025 metabolite was studied because it is pharmacologically active. CYP1A2 was the major isoform responsible for the transformation of *rac*-RPR 127025 to RPR 123830 in human liver microsomes, based on the potent inhibition of the reaction by furafylline. However, yeast-expressed human CYP1A2 did not metabolize *rac*-RPR127025, or its enantiomers.

Table 3 Effect of drugs on the biotransformation of CMV423 by human liver microsomes. Twenty-six drugs likely to be coadministered with CMV423 in the clinic, were tested in coincubation and preincubation experiments at a single high concentration (as described in the Methods). The *in vivo* C_{max} of each drug is given, in parenthesis, for comparison. The results are expressed as a percentage of controls (vehicles only). The drugs have been classified in four groups. 1): drugs giving less than 25% inhibition and no activation. 2): drugs giving between 25% and 50% inhibition. 3): drugs giving more than 50% inhibition and 4): drugs giving more than 60% activation. In each group, drugs are listed in alphabetical order. Each value represents the mean of a triplicate assay. Coefficients of variation were below 20%.

Drug name	Tested concentration (C_{max} in vivo) in μM	CMV423 (10 μM)	RPR 127025 (10 μM)		
		Effect on RPR 127025 formation (%)	Effect on RPR 123830 formation (%)		preincubation
		coincubation	preincubation	coincubation	
Group 1					
Acyclovir	1000 (10)	+20	+18	+23	+19
AZT	1000 (7)	+8	−2	−2	−13
Cyclosporin A	62.5 (0.5)	−8	−6	+10	−13
Desogestrel	125 (unknown)	+5	+8	+8	+54
Didanosine	1000 (5)	−10	−13	+38	+22
Foscarnet	1000 (46)	+4	0	−24	+5
Lamivudine	1000 (6)	−21	+17	+3	+6
Nelfinavir	125 (4)	+19	−4	+26	−17
Norgestimate	125 (unknown)	+34	−22	+28	+14
Saquinavir	62.5 (0.4)	+11	−23	−9	+21
Stavudine	1000 (4)	−1	+6	+5	+40
Group 2					
Ethinyl estradiol	62.5 (0.0005)	−9	−27	−7	−38
Indinavir	1000 (122)	−34	−21	−29	−24
Itraconazole	62.5 (1)	−17	−15	−23	−34
Methadone	250 (4)	+4	−39	+34	+60
Paracetamol	1000 (500)	−2	−32	−15	+7
Ritonavir	1000 (8)	−40	−28	−43	−36
Theophylline	1000 (110)	−21	−34	−31	−26
Warfarin	1000 (30)	−23	−41	−27	−12
Group 3					
Cimetidine	1000 (4)	−50	−65	−53	−51
Enoxacine	1000 (6)	−52	−48	−20	−17
Imipramine	1000 (0.4)	−82	−90	−77	−81
Group 4					
Chlormadinone	62.5 (unknown)	+81	+126	+207	+187
Ethinodiol	62.5 (unknown)	+113	+80	+136	+110
Norethindrone	250 (0.05)	+114	+85	+58	+81
(−) Norgestrel	250 (0.008)	+72	+4	+151	+171

Since expressed CYP1A1 clearly oxidized *rac*-RPR 127025, we looked for the presence of active CYP1A1 in our microsomes, using riluzole as a probe, which was previously shown to have different metabolic profiles when metabolized by CYP1A2 or CYP1A1 [4]. We did not detect any CYP1A1 activity, suggesting that the formation of RPR 123830 in human liver is not CYP1A1- but CYP1A2-dependent. Therefore, the lack of metabolism of *rac*-RPR 127025 by yeast expressed human CYP1A2 may be due to an intrinsically low enzyme activity. The CYP3A inhibitors TAO and ketoconazole did not inhibit *rac*-RPR 127025 oxidation to a significant level in pool 2 liver microsomes, but ethinyl estradiol, indinavir, itraconazole and ritonavir

caused moderate inhibition in pool 4 microsomes, indicating some contribution from CYP3A. Yeast-expressed CYP3A4 but not CYP3A5 catalysed this reaction. The formation of RPR 122701 seemed to be catalysed only by CYP2C8, but this remains to be confirmed by inhibition studies. Overall, CYP1A2 is expected to be the major enzyme involved in CMV423 metabolism *in vivo*, with a contribution from CYP3A4. The presumed metabolic pathways of CMV423 in human liver are summarized in Figure 3, together with the individual enzymes thought to be responsible for product formation.

The intrinsic clearance (V_{max}/K_m ratio) of *rac*-RPR 127025 oxidation in human liver microsomes was 3.5-times lower than the intrinsic clearance of *rac*-RPR

Table 4 Concentration-dependent effects of cimetidine, enoxacin and imipramine on CMV423 metabolism by human liver microsomes following co-incubation. The results are expressed as a percentage of controls (vehicles only). Each value represents the mean % change calculated from duplicate experiments. Coefficients of variation were below 15%.

Drug	Concentration (μM)	Effect on RPR 127025 formation (%)	Effect on RPR 123830 formation (%)
Cimetidine	3	0.0	+2
	10	-6.0	+10
	30	-6.0	+19
	100	-12.0	+5
	300	-16.0	-20
Enoxacin	3	-4.0	+7
	10	-5.0	0
	30	-12.0	+1
	100	-24.0	-25
	300	-36.0	-31
Imipramine	0.3	+3.0	-1
	1	-2.0	-6
	3	-7.0	-7
	10	+2.0	-7
	30	-12.0	-10
	100	-25.0	-29

Table 5 Time-dependent effects of cimetidine, enoxacin, ethinyl estradiol, imipramine and methadone on CMV423 metabolism by human liver microsomes. The results are expressed as percentage of controls (vehicles only). Each value represents the mean of a triplicate assay. Coefficients of variation were below 15%.

Drug (μM)	Pre-incubation time (min)	Effect on RPR 127025 formation (%)	Effect on RPR 123830 formation (%)
Cimetidine (10)	0	+4	-15
	10	-6	+16
	20	+1	-6
	30	-3	-13
Enoxacin (20)	0	-5	-9
	10	-23	-4
	20	+2	+2
Ethinylloestradiol (1)	30	-1	-1
	0	+11	+11
	10	+5	+23
	20	+9	+4
Imipramine (1)	30	+2	-9
	0	+7	+7
	10	-1	+8
Methadone (10)	20	-1	-4
	30	-4	-5
	0	-4	not analysed
	10	-1	not analysed
	20	-8	not analysed
	30	11	not analysed

Figure 3 The metabolism of CMV423 by human liver. The formation of *rac*-RPR 127025 and RPR 12830 are the major routes. Rank order of quantitative involvement of individual CYPs: bold > regulars > italic text.

127025 formation from CMV423. This indicates that, unless it is rapidly excreted, *rac*-RPR 127025 could be a major circulating species *in vivo*. In addition, since the targeted $C_{\max}$ of CMV423 in human plasma is 1 μM , the high K_m values for metabolism by CYP1A2 and CYP3A4 indicate that no saturation of CMV423 metabolism would be expected *in vivo* in man. The results of a phase I, oral dose escalation study of CMV423 support these *in vitro* data. The mean $C_{\max}$ and areas under the curve (AUCs) of both CMV423 and *rac*-RPR 127025 varied proportionally with the dose administered (5–100 mg). The mean AUC of *rac*-RPR 127025 was 4.3-fold higher than that of the parent compound, and the apparent elimination half-life of *rac*-RPR 127025 was only 50% shorter than that of CMV423 (Aventis, data on file).

CMV423 caused slight inhibition of CYP2C9 and CYP3A marker activities *in vitro*, but its greatest effect was on CYP1A2 activity. CMV423 did not inhibit indinavir phase I metabolism *in vitro*, which is mediated by CYP3A [15]. This is in agreement with the low K_m (1.3 μM) [15] and low K_i (0.2 μM) [20] values for indinavir with respect to CYP3A metabolism and inhibition, indicating that it should not be displaced from the active site of the enzyme by CMV423, which has a much higher K_m (282 μM). CMV423 also had no effect on AZT glucuronidation *in vitro*. Therefore, coadministration of CMV423 with indinavir and AZT, two drugs widely used in the treatment of AIDS, should not result in any deleterious metabolic interaction. The effects of 26 selected drugs on CMV423 biotransformation *in vitro* are generally in good agreement with the known enzymology of these drugs. Thus the metabolic clearance of CMV423 appears to be relatively insensitive to a large number of possible coadministered drugs, and CMV423 itself has a negligible impact on CYP activities *in vitro*, with the exception of CYP1A2.

Activation of CMV423 metabolism *in vitro* was observed in the presence of the contraceptives chlormadinone, desogestrel, ethynodiol, norethindrone and norgestrel. Steroids have been shown previously to activate CYP3A activities *in vitro* [21], and heteroactivation by flavone *in vivo* in the rat was reported in 1983 [22]. However, the relevance of this phenomenon in humans is unknown.

In conclusion, the metabolic clearance of the new anticytomegalovirus molecule CMV423 is mainly mediated by human liver CYP1A2 and, to a lesser extent, by CYP3A4. CMV423 metabolism should not be saturable within the therapeutic concentration range and is insensitive to inhibition by a large number of drugs with which it might be coadministered. CMV423 is not expected to alter the metabolic clearances of indinavir and AZT *in vivo*, and should only affect the activity of CYP1A2.

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