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RESEARCH PAPER

N-terminal valine adduct from the anti-HIV drug abacavir in rat haemoglobin as evidence for abacavir metabolism to a reactive aldehyde *in vivo*

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BACKGROUND AND PURPOSE

The aim of this study was to obtain evidence for the activation of the nucleoside reverse transcriptase inhibitor abacavir to reactive aldehyde metabolites *in vivo*. Protein haptenation by these reactive metabolites may be a factor in abacavir-induced toxic events.

EXPERIMENTAL APPROACH

The formation of *N*-terminal valine adducts from the abacavir-derived aldehydes was investigated in the haemoglobin of Wistar rats treated with eight daily doses (120 mg·kg^{-1}) of abacavir. The analyses were conducted by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry upon comparison with synthetic standards.

KEY RESULTS

An *N*-terminal valine haemoglobin adduct derived from an α,β-unsaturated aldehyde metabolite of abacavir was identified *in vivo* for the first time.

CONCLUSIONS AND IMPLICATIONS

This preliminary work on abacavir metabolism provides the first unequivocal evidence for the formation of an α,β-unsaturated aldehyde metabolite *in vivo* and of its ability to form haptens with proteins. The methodology described herein can be used to assess the formation of this metabolite in human samples and has the potential to become a valuable pharmacological tool for mechanistic studies of abacavir toxicity. In fact, the simplicity of the method suggests that the abacavir adduct with the *N*-terminal valine of haemoglobin could be used to investigate abacavir-induced toxicity for accurate risk/benefit estimations.

Abbreviations

ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; HIV, human immunodeficiency virus; HSR, hypersensitivity reaction; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry

Introduction

Abacavir ({(1*S*, 4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-cyclopent-2-en-1-yl}methanol, **1**, Figure 1) is a nucleoside reverse transcriptase inhibitor used for the treatment of human immunodeficiency virus (HIV) infection. Abacavir-based regimens have a significant role in HIV-treatment guidelines due to the antiretroviral efficacy of the

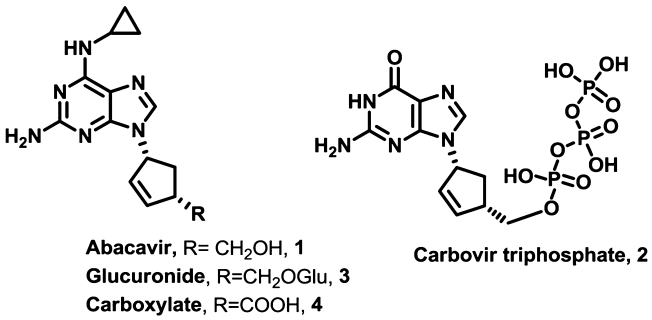

Figure 1

Structures of abacavir (**1**), its active metabolite carbovir triphosphate (**2**), and the two inactive metabolites, the glucuronide **3** and the carboxylate **4** (represented as the carboxylic acid).

drug and its availability as one-pill fixed dose combinations (Thompson *et al*., 2010). However, despite its clinical worth and although individual susceptibilities to adverse effects differ among patients, abacavir is associated with toxic events. Potentially life-threatening abacavir-induced hypersensitivity reactions (HSRs) usually occur within the first 6 weeks of treatment, albeit with an incidence of less than 5% (Clay, 2002), and result in discontinuation of the drug. Despite the availability of a prospective test to identify HLA-B*5701 allele-positive patients, who are at greatest risk for abacavir-induced HSR (Nolan, 2009), the test does not predict which patients will definitely develop HSR (Mallal *et al*., 2008). Moreover, although the association between long-term abacavir exposure and increased risk of myocardial infarction is still controversial (Costagliola *et al*., 2010), current guidelines recommend caution in abacavir administration to patients who are at a higher risk for cardiovascular disease (Thompson *et al*., 2010). Consequently, the establishment of accurate risk/benefit estimations requires both a thorough understanding of the mechanisms of abacavir-induced toxicity and the development of reliable toxicity biomarkers.

Metabolic activation to aldehyde metabolites is thought to play a significant role in the toxic responses elicited by the drug. Abacavir is a prodrug that undergoes conversion to the active carbovir triphosphate (**2**, Figure 1) via stepwise intracellular anabolism (Faletto *et al*., 1997). In conjunction with this activation process, abacavir is extensively metabolized in the liver via two pathways mediated by uridine diphosphate glucuronyltransferase (EC 2.4.1.17) and alcohol dehydrogenase (ADH, EC 1.1.1.1), to afford the glucuronide **3** and the carboxylate **4**, respectively, as the major metabolites (Figure 1). Walsh *et al*. (2002) have demonstrated that metabolism to the carboxylate involves a two-step oxidation process mediated *in vitro* by human ADH via an aldehyde intermediate (**5**, Figure 2). These authors also provided evidence for an ADH-promoted isomerization of aldehyde **5** to the α,β-unsaturated aldehyde **6**. More recently, using human haemoglobin as a model protein, we demonstrated that the α,β-unsaturated aldehyde is the electrophilic species primarily responsible for *in vitro* reaction with the protein (Charneira *et al*., 2011)

Protein haptenation is regarded as a critical step in the onset of drug-induced HSR. These allergenic responses are related to the ability of drugs and/or their metabolites to bind covalently to proteins. The resulting drug-protein adducts act as antigens, interacting directly with immune receptors (Park *et al*., 2011). Indeed, aldehyde-protein adducts are often implicated in allergenic hypersensitivity conditions (O'Brien *et al*., 2005). This, and the fact that ADH is present in epithelial tissues, including the skin (Lockley *et al*., 2005), strongly suggests the involvement of aldehyde metabolites in abacavir-induced skin rash. Likewise, although the molecular mechanisms of drug-induced cardiotoxicity are much less understood than those of HSR (Aberg and Ribaudo, 2010), several aldehydes have been associated with the inception of cardiovascular pathologies. For instance, acetaldehyde, the primary ethanol metabolite, is thought to be involved in the onset of alcoholic cardiomyopathy (Guo and Ren, 2010a), with genetic polymorphisms in ADH and aldehyde dehydrogenase (ALDH, EC 1.2.1.3) seemingly determining the susceptibility to alcoholism and alcohol-induced diseases (Tolstrup *et al*., 2009; Bian *et al*., 2010). An intriguing example is the observation of an exacerbated ethanol-induced myocardial contractile dysfunction, following acute ethanol exposure, in a murine model with cardiac-specific overexpression of ADH, suggesting that heart metabolism may play a role in drug-induced cardiotoxicity (Guo and Ren, 2010b). Similarly, direct exposure to the highly reactive α,β-unsaturated aldehyde acrolein, an endogenous product of lipid peroxidation and ubiquitous environmental pollutant, has been shown to cause myocardial dysfunction in mice possibly due to site-specific protein modification (Luo *et al*., 2007).

In this study we describe the detection and characterization of abacavir-specific adducts in the haemoglobin of Wistar rats treated with abacavir. These findings provide unequivocal evidence for the bioactivation of abacavir via aldehyde metabolites with haptenation ability *in vivo*.

Methods

Reagents

All commercially available reagents were acquired from Sigma-Aldrich Química, S.A. (Madrid, Spain), unless specified otherwise, and used as received. Adduct standards **7** and **8** were prepared as described in Charneira *et al*. (2011). Whenever appropriate, enzymes are classified according to the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Alexander *et al*., 2011).

Animal treatment

A group of Wistar rats (10–13 weeks old; three males and three females; 274–388 g) was obtained from the *vivarium* of the Faculty of Medical Sciences, New University of Lisbon. The animal handling protocol was approved by the Institutional Animal Care and Use Committee. The animals were kept under controlled temperature with 12/12 h light/dark cycles. They received a standard rodent diet and tap water *ad libitum*. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al*., 2010; McGrath *et al*., 2010).

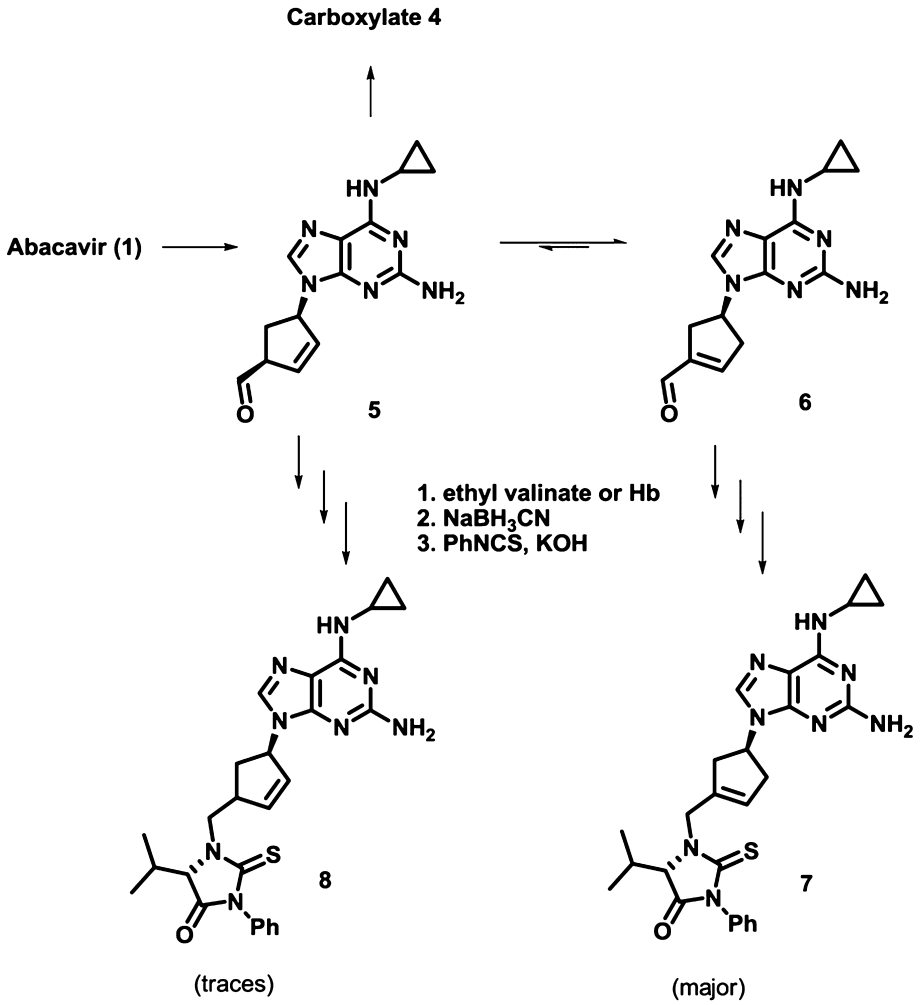

Figure 2

Two-step oxidation of abacavir (**1**) to the carboxylate **4**, involving aldehyde **5**, which isomerizes (*in vitro* and *in vivo*) to the α,β-unsaturated aldehyde **6** (Walsh *et al.*, 2002; Charneira *et al.*, 2011). Both aldehydes reacted *in vitro* with ethyl valinate and human haemoglobin to produce adduct **7** as the major product upon stabilization by reduction and *N*-alkyl Edman degradation with phenyl isothiocyanate (Charneira *et al.*, 2011).

Abacavir was suspended in methyl cellulose (0.2% in water) with 5% of methanol. The rats were administered eight daily i.p. doses of 120 mg abacavir kg^{-1} body weight. An extra group of each gender (two males and two females) received the vehicle alone. Two hours after the last treatment, the rats were killed by administration of an overdose of anaesthetic (60 $mg \cdot kg^{-1}$ pentobarbital). The chest cavity was opened, blood was collected by cardiac puncture into tubes containing EDTA, and blood was separated into blood cells and plasma by centrifugation (10 min, 3000× *g*, 4°C). The pellet containing erythrocytes was stored at –80°C until haemoglobin isolation.

Haemoglobin isolation

Rat haemoglobin was isolated according to the methodology described by Törnqvist (1994). Briefly, the cells were washed three times with 1 volume of 0.9% NaCl. To each mL of haemolysate (red blood cells diluted with 1.5 volumes of distilled water), 50 mM HCl in cold 2-propanol (6 mL) were added. After centrifugation (approximately 3000× *g*, 10 min, 4°C) and removal of cell membranes, the acidified globin was precipitated with cold ethyl acetate (5 mL), added slowly with mixing. The precipitate was washed twice with ethyl acetate, followed by pentane, and dried under a nitrogen stream.

Serum albumin isolation

The methodology described by Lindstrom *et al.* (1998) was followed with small modifications. Briefly, rat serum albumin was isolated from thawed plasma by dropwise addition of a saturated ammonium sulfate solution until a final concentration of 50% was reached. After centrifugation (900× *g*, 30 min, 4°C) to remove the immunoglobulins, the supernatant was filtered through an *Amicon*® filter unit (Millipore, Billerica, MA, USA, 4 mL, 30 000 MWCO) with centrifugation (3800× *g*, 20 min, 4°C) and then dried under reduced pressure.

Detachment of N-terminal valine adducts from haemoglobin

After haemoglobin isolation from the red blood cells, each sample (30–200 mg) was subjected to *N*-alkyl Edman degradation essentially as described by Törnqvist (1994), but replacing the derivatizing agent with phenyl isothiocyanate. Briefly, each 50 mg sample was dissolved in N,N-dimethylformamide (1.5 mL), followed by the addition of 1 M NaOH (50 μL) and phenyl isothiocyanate (10 μL). A slight excess of derivatizing agent was used as compared with the original method in order to ensure quantitative yields. The samples were subsequently stirred for 2 h at 37°C and then for 1.5 h at 45°C. Upon cooling to room temperature, water (2 mL) was added, and adducts were extracted with ethyl acetate (2 × 1 mL). The organic phase was dried under reduced pressure, and the contents were analysed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

Hydrolysis of serum albumin to amino acids

Serum albumin samples (10 mg) were dissolved in PBS (3.5 mL) and Pronase E (EC 3.4.24.31, 190 μL, 530 μg·mL^{-1}) and leucine aminopeptidase M (EC 3.4.11.2, 80 μL, 130 μg·mL^{-1}) were added. The solution was incubated at 37°C overnight. The enzymatic hydrolysate was concentrated in a C-18 Sep-Pak cartridge (Waters Associates, Milford, MA, USA). The cartridge was conditioned with methanol (3 mL), followed by water (6 mL). The sample was then loaded, and the cartridge was rinsed with water (1 mL) and methanol (2 mL). The methanolic eluate was dried under reduced pressure, redissolved in 60 μL acetonitrile/0.1% aqueous formic acid (1:1) and analysed by LC-ESI-MS/MS.

LC-ESI-MS/MS

LC-ESI-MS/MS analyses were performed with a ProStar 410 autosampler, two 210-LC pumps, a ProStar 335 diode array detector, and a 500-MS ion trap mass spectrometer, with an ESI ion source (Varian, Inc., Palo Alto, CA, USA). Data acquisition and processing were performed using Varian MS Control 6.9.3 software (Varian, Inc.). The samples were injected onto the column via a Rheodyne injector with a 20 μL loop. Separations were conducted at 30°C using a Luna C18 (2) column (150 mm × 2 mm, 3 μm; Phenomenex, Torrance, CA, USA). The mobile phase was delivered at a flow rate of 200 μL·min^{-1}. *N*-terminal valine adducts from haemoglobin were analysed using a 15 min linear gradient from 0 to 100% acetonitrile in 0.1% formic acid, followed by a 2 min isocratic elution with acetonitrile and an 8 min linear gradient back to 100% of 0.1% formic acid. Modified amino acids released from serum albumin were analysed using a 5 min isocratic elution with 5% acetonitrile in 0.1% aqueous formic acid, followed by a 30 min linear gradient from 5 to 70% acetonitrile, a 2 min linear gradient to 100% acetonitrile, and an 8 min isocratic elution with acetonitrile. The mass spectrometer was operated in the positive ESI mode; the optimized operating parameters were: ion spray voltage, +5.2 kV; capillary voltage, 80 V; and radio frequency loading, 70%. Nitrogen was used as the nebulizing and drying gas at pressures of 310 and 69 kPa, respectively; the drying gas temperature was 350°C. MS/MS spectra were obtained with an isolation window of 2 Da, an excitation energy of 1.7 V and an excitation time of 10 ms.

Results

We recently reported the preparation of synthetic standards of the two putative aldehyde metabolites (**5** and **6**) derived from abacavir and demonstrated that they both react *in vitro* with ethyl valinate via Schiff base formation (Charneira *et al*., 2011). Adduct **7** (Figure 2) was obtained in both instances as the major product after stabilization by reduction and *N*-alkyl Edman degradation. In the same study, we demonstrated that, under similar conditions, adduct **7** is also formed *in vitro* by reaction at the *N*-terminal valine of human haemoglobin. To obtain evidence that metabolically generated abacavir-derived aldehydes have haptenation ability *in vivo*, we treated three male and three female Wistar rats with eight daily doses of abacavir (120 mg·kg^{-1} body weight) and investigated the formation of covalent adducts with the *N*-terminal valine of haemoglobin by LC-ESI-MS/MS.

After blood collection, haemoglobin was precipitated and submitted to *N*-alkyl Edman degradation (Törnqvist, 1994) using phenyl isothiocyanate as the derivatization agent. This approach was originally developed for GC-MS analysis, which explains the common use of a fluorinated phenyl isothiocyanate, but its application to polar, thermolabile and high-molecular-weight adducts was limited. However, the successful LC-ESI-MS/MS analysis of acrylamide-derived valine adducts derivatized with phenyl isothiocyanate (Chevolleau *et al*., 2007) has broadened the scope of this methodology to the analysis of low volatility products such as the abacavir-derived phenylthiohydantoins (e.g. **7**) reported herein. Indeed, LC-ESI-MS/MS analysis of the ion at *m/z* 503 (corresponding to the protonated molecule of the expected adducts, **7** and/or **8**) allowed the unequivocal identification of the *N*-terminal valine adduct **7** in the ethyl acetate extract obtained from two (one male and one female) out of the six rats analysed in the current study. Adduct **8** was not detected in any of the rat samples. The assignment was based upon identical retention times and indistinguishable mass spectra (Figure 3) when compared with the synthetic standard of adduct **7**, prepared and fully characterized as described in Charneira *et al*. (2011). Specifically, under the chromatographic conditions used, the two positive rat samples and the synthetic adduct standard **7** displayed a signal eluting at 9.7 min and consistent with the protonated adduct molecule (*m/z* 503). Under the same conditions, the synthetic adduct standard **8** eluted approximately 5 min later. The corresponding MS/MS spectra consistently presented three fragment ions, stemming from loss of the cyclopropylaminopurine moiety (*m/z* 313), loss of the abacavir moiety (*m/z* 235), and cleavage of the purine-cyclopentene bond, with protonation on the purine moiety (*m/z* 191). These signals were absent from all the control rat samples (not shown). Based upon calibration with the synthetic standard, we estimate a limit of quantification of 2.8 pmol of adduct **7** on column, and adduct levels of *c*. 0.70 and 7.14 pmol·mg^{-1} haemoglobin in the positive male and female rats, respectively. These results, and the fact that only 1/3 of

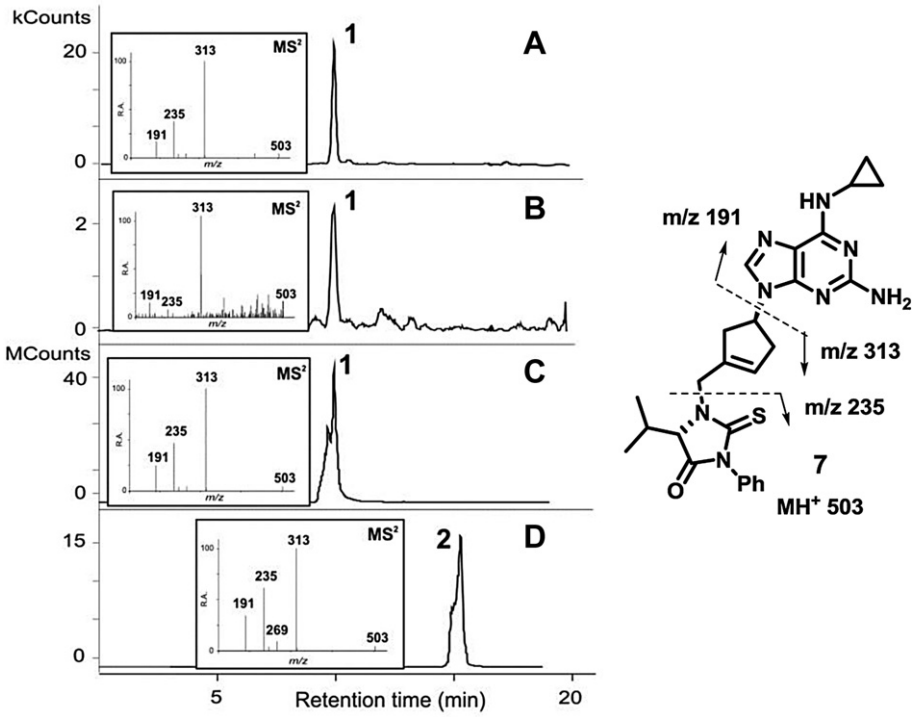

Figure 3

Extracted ion chromatogram (*m/z* 313) obtained following LC-ESI-MS/MS analysis of the ion at *m/z* 503 (protonated molecule) of the ethyl acetate extracts from the haemoglobin of abacavir-treated rats. The rat haemoglobin was subjected to *N*-alkyl Edman degradation as described in Methods: (A) female Wistar rat; (B) male Wistar rat; and LC-ESI-MS/MS chromatogram (*m/z* 503) of: (C) synthetic adduct standard (**7**); (D) putative adduct standard **8**. The elution conditions are outlined in Methods.

the rats analysed tested positive, suggest a high degree of inter-individual variability in abacavir activation/detoxification, which is reminiscent of the differential susceptibility of human patients to abacavir-induced toxicity (Clay, 2002; Mallal *et al.*, 2008).

In order to search for other potential targets of abacavir-derived aldehyde(s), the isolated rat serum albumin was hydrolysed to free amino acids by an adaptation of reported methodologies, using a combination of pronase E and leucine aminopeptidase M, to ensure the endo- and exopeptidase activities required for complete hydrolysis (Tsao and Otter, 1999). Following concentration of the enzymatic hydrolysate in a C-18 Sep-Pak cartridge, the methanolic eluate was analysed by LC-ESI-MS/MS for the presence of plausible adducts with amino acids containing nucleophilic side chains. Specifically, ions at *m/z* 406, 408, 415, 422 and 536, corresponding to the protonated molecules of adducts **9**, **10**, **11**, **12** and **13**, respectively (Figure 4), which would be indicative of reaction with cysteine or lysine, were searched. However, no adducts were detected in the rat albumin hydrolysate.

Discussion and conclusions

Our previous *in vitro* experiments (Charneira *et al.*, 2011) indicated that although both abacavir aldehyde metabolites can coexist in solution, aldehyde **5**, which is the product of direct abacavir oxidation, rapidly isomerizes to the thermodynamically more stable α,β-unsaturated aldehyde **6**; this species is the electrophilic intermediate mainly responsible for reaction with nucleophiles (Figure 2). Assuming that a similar situation will occur *in vivo*, we hypothesized that aldehyde **6** is the abacavir metabolite primarily involved in covalent adduct formation with biomacromolecules. Consequently, a causal relationship between the generation of aldehyde **6** and the onset of abacavir-induced toxic events is plausible and worth investigating. Abacavir-derived aldehydes have so far eluded detection *in vivo*. Indeed, as short-lived species *in vivo*, biomonitoring of aldehyde derivatives is extremely difficult; therefore, the establishment of direct correlations between aldehyde levels and the induction of specific pathologies is not straightforward. The rationale for the approach presented herein was based on the fact that, as reactive species, aldehydes bind covalently to biological macromolecules, affording covalent adducts which can be quantified in fluids and tissues (O'Brien *et al.*, 2005).

The abundant blood protein, haemoglobin, is often used as an easily accessible model for indirect biomonitoring of reactive metabolites, particularly by investigating covalent modification at the *N*-terminal valine residues, which are primary sites of reaction with several classes of electrophiles, including aldehydes (Davies *et al.*, 2009). Given that the pKa values of the α-amino groups from the *N*-terminal valine residues in proteins (*c.* 7.80) are close to that of the blood pH (Moll and Elfarra, 1999; Törnqvist *et al.*, 2002), a large fraction of these residues are unionized and thus have

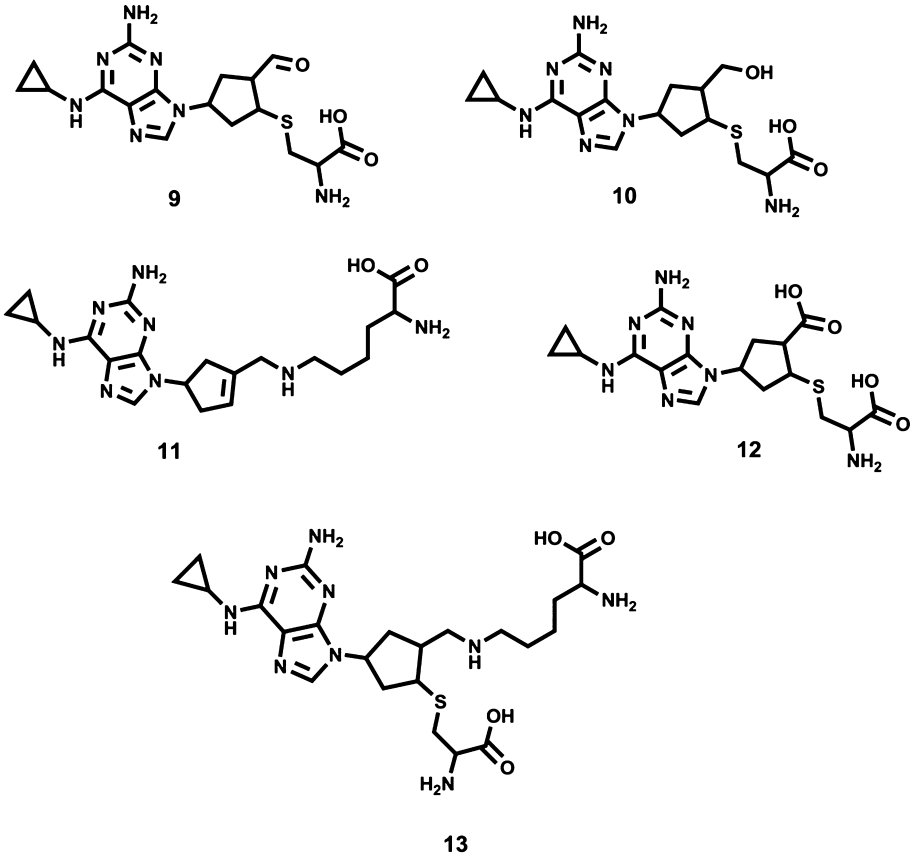

Figure 4

Structures of the covalent adducts expected upon (i) Michael addition of cysteine to the conjugated double bond of abacavir aldehyde **6**, without subsequent redox processes (**9**), with subsequent aldehyde reduction (**10**), or with subsequent aldehyde oxidation (**12**); (ii) lysine addition to the carbonyl group with Schiff base formation, followed by reduction (**11**); and (iii) putative cross-link product (**13**), formed from the cysteine adduct **9** by subsequent addition of a lysine residue to the carbonyl group with Schiff base formation, followed by reduction.

nucleophilic character. The relatively long and well-controlled lifespan of the protein is also an important reason for choosing haemoglobin adducts as biomarkers of reactive electrophilic compounds (Törnqvist *et al.*, 2002). Moreover, the availability of a mild, simple and sensitive post-modification procedure capable of selectively detaching valine adducts from the protein as hydantoin derivatives (the *N*-alkyl Edman procedure) underlies the extensive use of haemoglobin valine adducts as biomarkers of exposure to toxicants (Boysen *et al.*, 2007; Chevolleau *et al.*, 2007).

As part of a programme aimed at elucidating the significance of abacavir metabolism to reactive aldehydes (and their haptenation to relevant proteins) in abacavir-induced toxicity, we investigated the feasibility of using *N*-terminal valine adducts in haemoglobin as biomarkers of abacavir activation *in vivo*. To this end, we treated Wistar rats with eight daily doses of abacavir (120 mg·kg^{-1} body weight), subjected their haemoglobin to *N*-alkyl Edman degradation, and searched for the presence of abacavir adducts with the *N*-terminal valine by LC-ESI-MS/MS. Additionally, the rat albumin was hydrolysed enzymatically to amino acids and analysed by LC-ESI-MS/MS in a search for potential products of (i) Michael addition of cysteine to the conjugated double bond of aldehyde **6**, either direct (**9**, Figure 4) or followed by reduction (**10**) or oxidation (**12**); (ii) lysine addition to the carbonyl group of aldehyde **6**, with Schiff base formation and subsequent reduction (**11**); and/or (iii) cross-linked products stemming from sequential addition of cysteine and lysine (**13**). Although the selected dose is *c.* 10-fold higher than the expected systemic exposure in humans administered abacavir, it was chosen in order to maximize the likelihood of obtaining a detectable response in a short time span. This approach has been followed in other reports, such as a 2 year study in which rats subjected to systemic abacavir levels *c.* 6- to 32-fold higher than the human exposure at the recommended dose (600 mg·day^{-1}) showed signs of myocardial degeneration and had increased incidences of malignant and non-malignant tumours (ViiV Healthcare ULC, 2010). As indicated above, comparison with a thoroughly characterized synthetic standard allowed us to detect unambiguously the abacavir adduct ***7*** in the haemoglobin from one rat of each gender treated with the drug. This observation represents the first unequivocal evidence of both the *in vivo* involvement of the α,β-unsaturated aldehyde intermediate **6** in the metabolic activation of abacavir and of its haptenation ability. Moreover, given that adduct **8** was not detected *in vivo*, these data are consistent with our observation *in vitro* that the α,β-unsaturated aldehyde **6** is the abacavir-derived electrophilic

intermediate primarily involved in reactions with bionucleophiles (Charneira *et al.*, 2011).

In our previous study, we also observed that the α,β-unsaturated aldehyde **6** reacts with N-acetylcysteine through Michael addition to the conjugated double bond (Charneira *et al.*, 2011). This observation was consistent with the known propensity of conjugated carbonyls to undergo 1,4-addition by soft nucleophiles, which explains why sulfhydryl groups are considered toxicologically relevant targets for α,β-unsaturated aldehydres (LoPachin *et al.*, 2008). Therefore, we also searched for cysteinyl–abacavir adducts in serum albumin hydrolysates from the abacavir-treated rats by means of an LC-ESI-MS/MS methodology similar to the one used earlier in our group for the successful detection and characterization of a nevirapine-cysteine adduct formed in human serum albumin *in vitro* (Antunes *et al.*, 2010). However, despite the fact that human and rat albumins both contain a free, solvent-accessible sulfhydryl group (Cys-34), expected to be ionized (i.e. as a highly nucleophilic thiolate) at physiological pH (Dooley *et al.*, 2007), we were unable to identify any cysteine-abacavir adducts in the enzymatic hydrolysate. Nonetheless, we cannot exclude the possibility that initially formed Michael adducts with cysteinyl residues underwent subsequent Schiff base formation through the carbonyl moiety, producing cross-linked products (e.g. adduct **13**, Figure 4) (Zhang *et al.*, 2003) that may not have been hydrolysed efficiently. It should be noted that we also searched for lysine-abacavir adducts (e.g. adduct **11**, Figure 4) in the albumin of the abacavir-treated rats due to the key role of lysine residues as a target for reactive aldehydes (Uchida, 2003) and our preliminary evidence of lysine-abacavir adduct formation *in vitro* (Charneira *et al.*, 2011). However, despite our previous evidence that the α,β-unsaturated aldehyde **6** reacts with nitrogen nucleophiles *in vitro* by 1,2-addition to the carbonyl group (Charneira *et al.*, 2011), we did not detect such lysine adducts in the rat samples. The negative results for cysteine and lysine adducts suggest that a lower sensitivity of the analytical method for these adducts, when compared with adduct **7**, may have been a factor. Indeed, a recognized advantage of analysing *N*-terminal valine adducts in haemoglobin is the simplicity of sample treatment, combined with the selective extraction of these adducts to an organic solvent, which minimizes matrix interferences and allows significant adduct enrichment, thereby enabling high levels of sensitivity in MS-based analytical methods.

The toxicological significance of aldehyde intermediates has long been recognized (O'Brien *et al.*, 2005). Representative examples of therapeutic drugs activated via aldehyde metabolites are the anticancer drug misonidazole (Heimbrook and Sartorelli, 1986), the anticonvulsant felbamate (Kapetanovic *et al.*, 2002), which has restricted use due to its toxicity, the antiviral acyclovir, used to treat herpes infections and associated with nephrotoxicity (Gunness *et al.*, 2011), and the aldose reductase inhibitor sorbinil (Maggs and Park, 1988). Given that exposure to aldehydes, either xenobiotic molecules or endogenous intermediates, has been associated with induced allergenic responses (O'Brien *et al.*, 2005) and cardiovascular pathologies (Luo *et al.*, 2007; Guo and Ren, 2010a) through protein haptenation, our data suggest a role for the α,β-unsaturated aldehyde metabolite **6** in abacavir-induced toxic responses. The presence of ADH, the enzyme primarily responsible for abacavir metabolism to aldehydes, in the skin (Lockley *et al.*, 2005) and heart (Nagasawa and Alexander, 1976) further supports this hypothesis.

Although glutathione conjugation represents a possible detoxification process for α,β-unsaturated aldehydes (O'Brien *et al.*, 2005), it is noteworthy that HIV-infected patients are reported to have depleted glutathione levels (Townsend *et al.*, 2003). Thus, efficient detoxification of the α,β-unsaturated aldehyde metabolite is unlikely in these patients, and the metabolite may alternatively react with sulfhydryl groups (or other nucleophilic side chains) in proteins, eliciting toxic responses. Also significant for the possible toxicological relevance of the α,β-unsaturated aldehyde **6** is chronic exposure to the parent drug under steady therapeutic regimens, which will result in potentially high systemic concentrations of the reactive metabolite. Moreover, inter-individual variability of aldehyde metabolizing enzymes (e.g. ADH and ALDH) is anticipated to have a crucial role in the detoxification/toxicity of the aldehyde metabolite, similar to that observed with other aldehydes, such as acetaldehyde (Tolstrup *et al.*, 2009; Bian *et al.*, 2010).

Even though the clarification of the role of the α,β-unsaturated aldehyde **6** in abacavir-induced toxicity still requires much effort in the analysis of clinical samples, the work presented herein supports the feasibility of using abacavir adducts with the *N*-terminal valine of haemoglobin as surrogate biomarkers of metabolic activation. By comparison, no adducts were detected in rat serum albumin, even though cysteine adducts were specifically searched and despite the fact that this protein contains a highly reactive cysteine thiolate. Therefore, regardless of the potential toxicological relevance of abacavir-derived adducts with cysteine, efficient monitoring of such adducts *in vivo* appears unlikely at the present stage. On the other hand, the availability of a synthetic standard of the abacavir adduct with the *N*-terminal valine of haemoglobin, combined with the simplicity and sensitivity of the analytical LC-ESI-MS/MS methodology, provides a robust strategy for the assessment of this prospective biomarker of abacavir-induced toxicity in HIV-positive patients. Moreover, this approach has the potential to become an important tool to help clarify the uncertainties in the molecular basis for abacavir-induced toxicity and allow more accurate risk/benefit estimations. Thus, the methodology described in the present manuscript is anticipated to be valuable in the search for potential correlations between adduct levels and biochemical markers of disease, and ultimately to establish whether or not abacavir activation through the α,β-unsaturated aldehyde **6** underlies abacavir-induced toxicity.

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Conflict of interest

The authors state no conflict of interest.

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