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The metabolic activation of abacavir by human liver cytosol and expressed human alcohol dehydrogenase isozymes

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Abstract

Abacavir (ZIAGEN®) is a reverse transcriptase inhibitor marketed for the treatment of HIV-1 infection. A small percentage of patients experience a hypersensitivity reaction indicating immune system involvement and bioactivation. A major route of metabolism for abacavir is oxidation of a primary βγ unsaturated alcohol to a carboxylic acid via an aldehyde intermediate. This process was shown to be mediated in vitro by human cytosol and NAD, and subsequently the αα and γ2γ2 human isoforms of alcohol dehydrogenase (ADH). The αα isoform effected two sequential oxidation steps to form the acid metabolite and two isomers, qualitatively reflective of in vitro cytosolic profiles. The γ2γ2 isozyme generated primarily an isomer of abacavir, which was minor in the αα profiles. The aldehyde intermediate could be trapped in incubations with both isozymes as an oxime derivative. These metabolites can be rationalized as arising via the aldehyde which undergoes isomerization and further oxidation by the αα enzyme or reduction by the γ2γ2 isozyme. Non-extractable abacavir protein residues were generated in cytosol, and with αα and γ2γ2 incubations in the presence of human serum albumin (HSA). Metabolism and residue formation were blocked by the ADH inhibitor 4-methyl pyrazole (4-MP). The residues generated by the αα and γ2γ2 incubations were analyzed by SDS-PAGE with immunochemical detection. The binding of rabbit anti-abacavir antibody to abacavir-HSA was shown to be dependent on metabolism (i.e. NAD-dependent and 4-MP sensitive). The mechanism of covalent binding remains to be established, but significantly less abacavir-protein residue was detected with an analog of abacavir in which the double bond was removed, suggestive of a double bond migration and 1,4 addition process.

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1. Introduction

Adverse drug reactions represent an area of increasing clinical concern for both prescribing physicians and pharmaceutical companies [1,2]. Approximately 25% of these are designated as idiosyncratic, or hypersensitivity reactions, in that they do not occur in most patients, and do not involve the known pharmacological properties of the drug [3]. There is substantial evidence of immune system mediation, which in turn may be initiated through metabolic activation of the drug to reactive species which covalently bind to proteins [4,5].

Abacavir (ZIAGEN®, Fig. 1) is a nucleoside reverse transcriptase inhibitor marketed in 1999 for the treatment of HIV-1 infection. Approximately 4% of

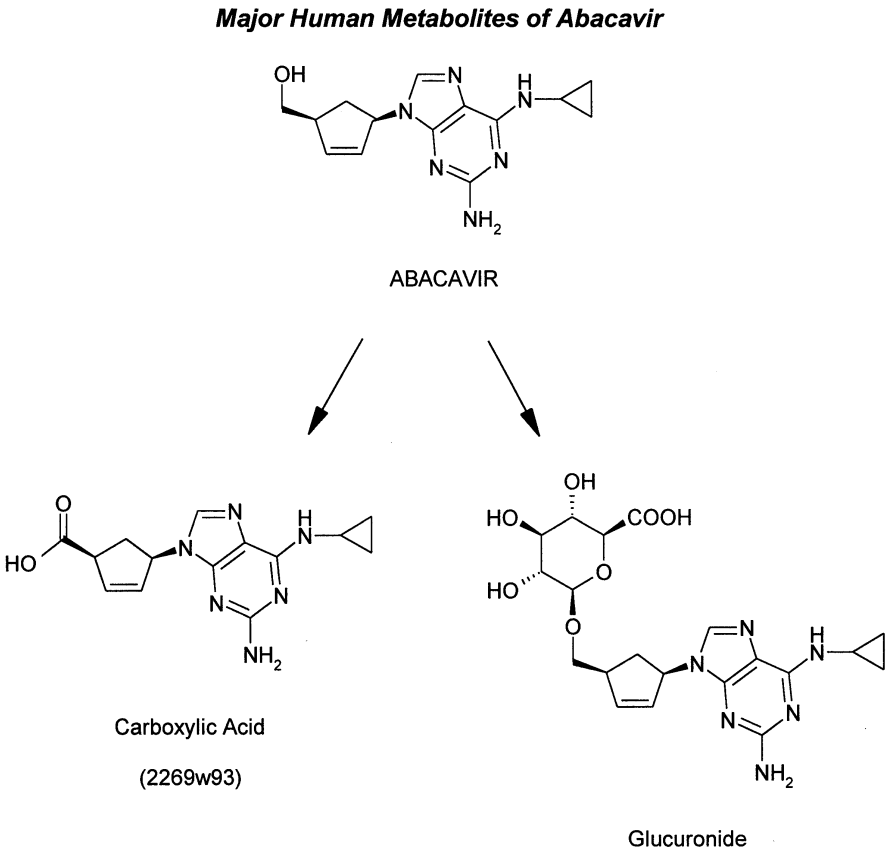

Fig. 1. Major routes of metabolism of abacavir in humans. Following a 600 mg oral dose of ^{14}C-abacavir, 83% of the radioactivity was excreted in the urine, and 16% in the feces. In urine, the acid metabolite (2269W93) accounted for 30% of the dose and the glucuronide for 36%, with 1% unmetabolized abacavir. The remaining 16% of dose was comprised of numerous minor metabolites [8]. A number of these have been characterized, and include two isomers of 2269W93. The fecal component was comprised primarily of 2269W93 and abacavir.

patients experience a hypersensitivity reaction. The symptoms are varied, but fever and rash are the most common [6], and a small number of fatalities have also been reported. The clinical presentation is consistent with immune mediation, and hence bioactivation is likely to play a role in its origination.

As part of broader investigations to understand the etiology of this hypersensitivity, metabolic investigations were performed to identify potential bioactivation pathways for abacavir, and the enzyme systems involved. While many factors are likely to play a role in idiosyncratic toxicities [7], genetic polymorphisms in drug metabolizing enzymes are factors that can potentially be identified.

The major routes of metabolism of abacavir in humans are conjugation to an ether glucuronide, and oxidation to a carboxylic acid metabolite designated as 2269W93 [8], (Fig. 1). Approximately 15% of the dose is comprised of a number of minor metabolites, but from structural considerations, none of those identified to date indicate the potential for reactive intermediates. In contrast, the metabolism of abacavir to 2269W93 involves a two step oxidation process via an aldehyde intermediate. This aldehyde has not been observed directly as a metabolite of abacavir, and attempts to synthesize it have been unsuccessful due to its apparent instability.

A number of aldehyde metabolites have been previously implicated as reactive and capable of covalent binding to proteins, and this in turn has been suggested to underlay clinical adverse events of the parent compounds. In these cases, the underlying reactivity can be ascribed to one of two mechanisms: Schiff base formation, or a 1,4 addition process. Schiff base formation has been proposed for the aldehyde metabolite of Sorbinil, which shows an incidence of hypersensitivity [9], and for ethanol, in which acetaldyde formation is proposed to underlay the immune hepatitis observed with chronic alcohol consumption [10]. The reversibility of this Schiff base formation has been proposed to be limited either by reduction by ascorbate [11], or for acetaldehyde, cyclization with an amide nitrogen of the protein to form a stable imidazolidinone [12]. Thus mechanisms may exist in vivo for the stabilization of these somewhat unstable adducts. A more widely encountered mechanism is observed for αβ unsaturated aldehydes (Michael type acceptors) and numerous examples exist in the literature of proposals for this type of reactive intermediate being associated with covalent binding and adverse clinical events [13–15].

As discussed below, both mechanisms might be possible for abacavir. The purposes of the studies described here were to utilize in vitro methods to determine if oxidation of abacavir to the carboxylic acid via an aldehyde intermediate could lead to protein covalent binding, to investigate the mechanism, and to identify the enzyme systems involved.

2. Materials and methods

2.1. Chemicals

Human liver microsomes and cytosol were obtained from Xenotech, (Kansas City, KS). NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NAD, 4-MP, methoxylamine, horse liver alcohol dehydrogenase (HLADH), HSA, triethylamine, toluene, isobutylchloroformate, and 7-ethoxycoumarin were obtained from Sigma-Aldrich (St Louis, MO). Laemmli sample buffer was obtained from Bio-Rad (Hercules, CA). Ultima-Flo M was obtained from Packard (Meriden, CT). Gelcode Blue Reagent, SuperBlock Blocking Buffer, SuperSignal West Pico Chemiluminescent Substrate, horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L chains), BupH Tris Buffered Saline Packs, Blue Ranger pre-stained protein standards, and keyhole limpet hemocyanin (KLH) were obtained from Pierce (Rockford, IL). Immobilized recombinant protein A IPA-400HC was purchased from RepliGen (Needham, MA). Abacavir, ^{14}C- abacavir, 2269W93, and dihydro-abacavir were synthesized at GlaxoSmithKline.

Human ADH isozymes were obtained as a gift from Dr.Tom Hurley, Dept of Biochemistry and Molecular Biology, Indiana University Medical School.

Isozymes were analyzed by SDS-PAGE and each shown to be comprised of a single major protein. The identities of the αα and γ2γ2 isozymes were independently confirmed by LC/MS/MS analysis of tryptic digests using MASCOT protein database searching [16].

2.2. Microsomal incubations

^{14}C-Abacavir (3 μg/ml; 0.5 μCi/ml) was incubated with 1 mg/ml pooled human liver microsomes in 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM NADP, 5 mM $MgCl_2$, 10 mM glucose-6-phosphate, and 0.5 Units/ml glucose-6-phosphate dehydrogenase. Incubations containing no NADP were used as negative controls. After incubating the samples in duplicate for 1-20 h in a 37 °C shaking incubator, 50 μl of each incubation was removed and added to 50 μl of cold acetontrile. The samples were vortexed and centrifuged in an Eppendorf model 5314 microcentrifuge at 10,000 rpm for 5 min. An aliquot of 20 μl was analyzed by HPLC for metabolite profiling. The remainder of the incubations were analyzed for non-extractable residue formation as described below. Metabolic viability of the microsomes was confirmed using 7-ethoxycoumarin.

2.3. Cytosol and expressed ADH incubations

^{14}C-Abacavir (3 μg/ml; 0.5 μCi/ml) was incubated with 1 mg/ml of pooled human liver cytosol in 50mM sodium pyrophosphate buffer, pH 7.4 or pH 8.8, containing 7.5 mM NAD. Incubations containing no NAD were used as negative controls. When used, 4-MP was added as an aqueous solution at the start of the incubation, to give a final concentration of 0.6 mM. Incubations were run in duplicate for 2-20

hours in a 37 °C shaking incubator, and then placed on ice for 5 min prior to filtering a 100 μl aliquot through a 0.45 μm AcroPrep GHP filter plate using a Waters Alliance filtration system. Aliquots of ~20 μl were analyzed by HPLC and LC/MS for metabolite profiling.

Incubations with human ADH isozymes were performed as described for cytosol, in 50 mM sodium pyrophosphate buffer, pH 7.4 and pH 7.8 (αα isozyme), or pH 8.8. Screening for metabolic activity with abacavir was performed with the different ADH isozymes using constant units of activity (0.025 Units/ml each) with variable enzyme protein level, and at constant enzyme protein level (17 μg/ml) with a variable number of units. Incubations were analyzed at 1, 2, 3, 4 and 20 hours by HPLC. When present, HSA was at a final concentration of 20 mg/ml. Incubations with dihydro-abacavir were performed under the same conditions. ADH inhibition by 4-MP was performed as described for cytosolic incubations. For trapping experiments with methoxylamine, an aqueous solution was added at the start of each incubation such that the final concentration was 10 mM.

All human ADH isozymes were assayed for viability using either ethanol (αα, β1β1, β2β2, γ2γ2, π and σ) or pentanol (χ) as substrates. Metabolism was assessed by measuring initial rates of NADH formation spectrophotometrically, and expressed as units of activity (μmol NADH formed/min) as previously described [17].

2.4. Measurement of Non-Extractable Protein Residues

Incubations were placed on ice for 5 min and the proteins precipitated by adding 3 ml cold acetonitrile. The samples were vortexed and centrifuged at 2500 rpm for 5 min at 4-8 °C. The supernatants were removed and the pellets resuspended in 3 ml cold acetonitrile, vortexed, and centrifuged as before for a total of 5 washes or until the radioactivity counted in the washes were < 2 times background. The pellets were dissolved in 1 ml 1%SDS and 0.8 ml was counted in a liquid scintillation counter. Some samples were further analyzed by SDS-PAGE/Western blot analysis.

2.5. HPLC and LC/MS analyses

Incubation supernatants were analyzed using a Waters Alliance 2690 HPLC system. The eluate was monitored using a Waters 996 photo diode array detector ($\lambda = 295$nm) and a Berthold Model LB 507A radioactivity flow monitor (EG & G Berthold, Nashua, NH), equipped with a 1 ml flow cell. The HPLC eluate was mixed with 3 volumes of Ultima-Flo M scintillation cocktail using a Waters 510 pump prior to entering the flow cell. HPLC analyses were performed on a Kromasil C18, 5 μm column (3.2 × 150mm; Phenomenex, Torrance, CA) with a mobile phase consisting of 0.025 M ammonium acetate buffer (pH 4) containing 5% methanol (A) and acetonitrile (B) delivered as a linear 50 min gradient of 0-13% B, at a flow rate of 0.7 ml /min.

While optimum chromatographic resolution of metabolites was achieved with the chromatographic conditions described above, this gave poor mass spectral response. For LC/MS analyses, the same column was used with a mobile phase consisting of

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