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Antiviral Research 25 (1994) 193–200

Antiviral Research

In vitro protein-binding characteristics of atevirdine and its *N*-dealkylated metabolite

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Received 7 March 1994; accepted 18 June 1994

Abstract

The in vitro protein-binding characteristics of atevirdine (ATV), a non-nucleoside reverse transcriptase inhibitor with activity against HIV-1, and its *N*-dealkylated metabolite (N-ATV) were studied using equilibrium dialysis. ATV and N-ATV were studied at concentrations of 5, 10, 20, and 30 μM in five protein-containing solutions [albumin 4%, plasma, serum, immune globulin (IgG) 1.5%, α_1-acid glycoprotein (AAG)] for 5 h at 37°C. All samples were analyzed by high-performance liquid chromatography. The free fraction of atevirdine in plasma, albumin, and serum was 0.01–0.02 over the range of drug concentrations studied. The fraction unbound (f_u) in these protein solutions statistically differed from IgG and AAG ($P < 0.05$), where the fraction unbound averaged 0.96 and 0.53, respectively. N-ATV had a similar binding profile as ATV with a fraction unbound of 0.04, 0.03, 0.03 in albumin, plasma and serum, respectively. A difference existed in N-ATV binding when compared to IgG and AAG with an average f_u of 0.87 and 0.59 ($P < 0.05$ vs. plasma). The potential clinical implications of the high degree of protein binding for ATV and N-ATV are discussed.

Keywords: Protein binding; Atevirdine; Reverse transcriptase inhibitor; HIV; Pharmacokinetics

1. Introduction

Nucleoside analogs such as zidovudine, didanosine, and zalcitabine are the mainstay of therapy for human immunodeficiency virus (HIV) infection. A number of alternative

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SSDI 0166-3542(94)00053-0

antiretroviral agents with various mechanisms of action are currently being investigated, as monotherapy and in combination with nucleoside analogs. One such class of compounds are the non-nucleoside reverse transcriptase inhibitors. These agents are potent inhibitors of HIV-1 reverse transcriptase, but do not cause HIV DNA chain termination (Vasudevachari et al., 1992). Atevirdine (ATV, U87201E, Upjohn Company, Kalamazoo, MI) and delavirdine (U90152S, Upjohn Company) are bis-heteroarylpiperazines (BHAPs) that represent one family of nonnucleoside reverse transcriptase inhibitors (NNRTIs). This class of drugs, unlike nucleoside analogs, do not require phosphorylation by the host cell and are not competitive with respect to deoxynucleoside triphosphates.

Preliminary pharmacokinetic data in dogs and rats indicate that one pathway for atevirdine biotransformation is via the cytochrome *P*-450 IIIA system to an *N*-dealkylated (N-ATV) metabolite, which lacks activity against HIV. The disposition of atevirdine in animals and healthy human volunteers appears to be capacity-limited. A phase I protocol (AIDS Clinical Trials Group Protocol 199) in HIV-infected patients has studied atevirdine at an initial dose of 600 mg every 8 h with maintenance doses adjusted to attain trough plasma concentrations between 5 and 10 μM (Reichman et al., 1994). This target was based on an ED_{100} for HIV-1 of 1–2 μM (U-87201E, Atevirdine Investigational Brochure, 1993). A second Phase I study (AIDS Clinical Trials Group Protocol 187) is utilizing a targeted trough concentration, dosage-escalation study design to examine antiviral activity and clinical tolerance during ATV monotherapy over a 12-week period while maintaining total trough ATV concentrations ranging from 5–13, 14–21 and 22–31 μM (Morse et al., 1994).

Preliminary studies of atevirdine in human plasma suggest a high degree of protein binding (Upjohn Co., data on file). Extensive protein binding of an antiretroviral may be important when treating HIV-infected patients in whom fluctuations in plasma protein fractions are common. HIV-infected individuals characteristically develop polyclonal B cell activation manifested by hypergammaglobulinemia (Lane et al., 1983) leading to increased serum concentrations of IgG, IgA, and IgD. On the other hand, profound weight loss due to malabsorption, esophageal lesions, diarrhea, and other disease-induced factors may contribute to the development of malnutrition and resulting hypoalbuminemia (Dworkin et al., 1990). Lastly, HIV disease is characterized by intermittent infections, malignancies and autoimmune events which may be associated with elevated α_1-acid glycoprotein concentrations (MacKichan, 1992).

The purpose of this study was to determine the protein-binding characteristics of atevirdine and its *N*-dealkylated metabolite in a variety of protein solutions employing an equilibrium dialysis technique.

2. Materials and methods

Atevirdine, its *N*-dealkylated metabolite, and U88352 were supplied as analytical grade powder by the Upjohn Company. Human serum albumin (A-3782; essentially fatty acid and globulin-free) and α_1-acid glycoprotein (G-9885, purity 99%) were

purchased from Sigma Chemical Company (St. Louis, MO). Phosphate-buffered saline was prepared in the laboratory, (K_2HPO_4 0.18%, Na_2HPO_4 0.95%).

Plasma and serum were collected from two healthy volunteers after informed consent was signed. The plasma was assayed for total protein and albumin concentration at the Erie County Medical Center clinical chemistry laboratory. Blood samples were collected in Vacutainer (Becton Dickinson, Rutherford, NJ) blood collection tubes, promptly centrifuged, and the plasma or serum removed. Collection tubes for serum samples did not contain anticoagulant, while collection tubes for plasma samples contained EDTA as an anticoagulant.

Protein binding was determined by equilibrium dialysis using plexiglas dialysis cells with a 400 μl total capacity in each chamber. A Spectra/Por 2 molecular porous dialysis membrane (Spectrum Medical Industries, Los Angeles, CA) with a molecular weight cut-off of 12 000 to 14 000 daltons was used. All experiments were performed in triplicate at 37°C for 5 h in a water bath. The protein solutions consisted of serum, plasma, human serum albumin (HSA) 4% solution, α_1-acid glycoprotein (AAG) 100 mg% solution and immune globulin intravenous (human) solution 1.5% (Gammimune N, Cutter/Miles Elkhart,IN).

Both human serum albumin and α_1-acid glycoprotein were dissolved in 2 ml of phosphate-buffered saline. Immune globulin 5% was diluted to 1.5% using phosphate-buffered saline. Prior to the addition of drug to each solution, the pH was adjusted to 7.4 using microliter quantities of 2 N HCl or 3 N NaOH diluted 1:10. Atevirdine was added to each solution to attain a concentration of 5, 10, 20, and 30 μM. Similar experiments were repeated with the same concentrations of N-ATV. The opposite side of the dialysis cell contained 400 μl of 0.1 M phosphate-buffered solution at a pH of 7.4. After equilibrium was reached, the entire volume from both sides of the dialysis apparatus were removed with a microliter syringe and the volume was recorded. All studies were performed in a darkened room due to the light sensitivity associated with this drug (Upjohn Co., personal communication) and all samples were stored at -20°C until time of assay. To assess atevirdine degradation in the dialysis chamber, 20 μM ATV and N-ATV was prepared in all five protein solutions, maintained in the water bath at 37°C for 5 h, and assessed for changes in concentration.

Samples removed from the protein-containing chamber of the dialysis cell were assayed for atevirdine and N-ATV concentrations using an HPLC assay developed by the Upjohn company (Howard and Schwende, 1993). Briefly, the procedure includes the addition of 150 μl of acetonitrile with internal standard (1 mg U-88352 per 100 ml). The sample is then vortexed and centrifuged and an aliquot of the supernatant is diluted 1:1 with buffer. The sample (10 μl) is then injected into the HPLC with an automatic injector (Waters 710B), 510 Solvent Pump (Waters Assoc., Milford, MA) and eluted using a mobile phase of 48% acetonitrile and 52% ammonium phosphate buffer (20 mM) at a flow rate of 1.3 ml/min. The drug, metabolite, and internal standard were separated with a Zorbax chromatographic column (RX-C8, 4.6 mm $\times$ 25 cm, Dupont Co., Maryland) and detected by monitoring fluorescence at 450 nm at an excitation wavelength of 295 nm (Hitachi 1040 Fluorescence Detector, Hitachi Ltd., Tokyo, Japan). Quantitation of drug and metabolite was achieved by calculating peak height ratios relative to the internal standard. The plasma standard curve ranged from 1 to 40

μM with quality controls at 4 μM and 40 μM. The peak height of ATV, N-ATV and the internal standard were evaluated with a weighted $(1/x^2)$ linear regression for calibration standards. The interday coefficient of variation ($n = 5$ days) was 4.5% and 3.3% for the high and low ATV quality controls, and 6.3% and 3.7% for the N-ATV quality controls, respectively.

Samples removed from the phosphate-buffered saline side of the dialysis chamber were measured with the same HPLC method with slight modifications. The procedure did not include protein precipitation using acetonitrile. The injection volume of sample was 50 μl and the standard curve was prepared in phosphate-buffered saline with a range of 0.065 to 1 μM. The interday coefficient of variation for the low ATV quality control (0.1 μM) and high (0.4 μM) quality controls were 7.2% and 5.4%, respectively. The coefficient of variation for N-ATV was 3.4% and 5.4% for low and high controls. Plasma and buffer standard curves were only acceptable if both quality controls were within 15% of their targeted values.

After equilibrium dialysis both the free and total drug concentration of atevirdine were determined. The concentration of drug in the protein solution after dialysis was completed was total drug (D_T). Free drug (D_F) was represented by the concentration of drug in the buffer side of the chamber. Drug bound (D_B was equal to (D_T-D_F). To determine the fraction of drug that was unbound (f_u), the following equation was used: $f_u = D_F/(D_F + D_B)$.

Due to increased osmotic pressure exerted by the presence of protein, volume shifts often occur across the dialysis membrane. This commonly results in a larger volume on the protein-containing side of the chamber. This fluid transfer does not affect the unbound concentration, but may decrease the bound concentration (Tozer et al., 1983). To correct for this dilution the equation proposed by Boudinot and Jusko was employed (Boudinot and Jusko, 1984):

$$f_b = \frac{(D_T - D_F) \cdot (V_{pe}/V_{pi})}{((D_T - D_F) \cdot (V_{pe}/V_{pi})) + D_F}$$

D_F, D_B, and D_T are representative of free, bound, and total drug concentrations. V_{pi} and V_{pe} are the volumes obtained initially and after equilibrium was reached.

One way analysis of variance (ANOVA) was used to compare the mean fraction unbound of ATV and N-ATV in plasma, albumin, serum, IgG and AAG. Tukey's multiple range test was used for multiple comparisons. A difference was considered significant at $P < 0.05$.

3. Results

The volunteer plasma albumin concentration was 4.7 g/dl and total protein was 7.7 g/dl. Recovery of the initial amount of ATV added to the dialysis system using plasma, albumin, and serum resulted in an average recovery of 92.4, 90.8, and 94.7% respectively. Similar recovery values were noted for N-ATV (Table 1). However, mean recovery from binding studies of ATV and N-ATV using immunoglobulin were 78.0 and 77.8 and in α_1-acid glycoprotein 72.4 and 74.4%, respectively. Volume shifts at 5,

Table 1
Percent of total ATV (top) and N-ATV (bottom) recovered during equilibrium dialysis studies

Conc. μM	Albumin 4%	Plasma	Serum	IgG 1.5%	AAG 100 mg%
5	87.7 (13.6)	81.7 (14.0)	–	78.3 (16.5)	83.3 (5.86)
10	96.5 * (2.12)	98.0 * (46.7)	–	75.3 (4.62)	68.5 * (3.54)
20	91.3 (16.9)	98.2 (1.75)	98.4 (2.71)	90.1 (8.44)	67.7 (7.51)
30	87.6 (2.65)	91.6 (3.52)	91.0 (2.72)	68.2 (0.551)	70.1 (1.84)
Average recovery (S.D.)	90.8 (4.19)	92.4 (7.75)	94.7 (5.23)	78.0 (9.13)	72.4 (7.33)
Conc. μM	Albumin 4%	Plasma	Serum	IgG 1.5%	AAG 100 mg%
5	83.0 (2.65)	93.7 (6.66)	NS	79.0 (4.24)	67.7 (5.51)
10	99.6 (0.577)	87.3 (2.08)	NS	74.3 (4.16)	73.1 (9.34)
20	89.0 (2.83) *	87.0 (4.00)	85.3 (4.04)	81.3 (5.51)	78.6 (3.21)
30	88.7 (3.79)	82.5 (3.53) *	90.5 (6.36)	76.7 (1.53)	78.3 (4.73)
Average recovery (S.D.)	90.1 (6.92)	87.6 (4.61)	87.9 (3.68)	77.8 (3.01)	74.4 (5.14)

* 1 Cell excluded due to leakage.
NS, serum not studied at 5 and 10 μM.

10, 20, and 30 μM in plasma and serum were minimal, averaging 30 to 40 μl. The mean total volume recovered from the dialysis cells averaged 790 μl (99%). Degradation studies of ATV in the different protein solutions also yielded minimal loss of drug in plasma ($< 5.0\%$), albumin ($< 0.5\%$), immunoglobulin ($< 7\%$), and α_1-acid glycoprotein (3%).

Each original protein solution that was assayed for atevirdine was added at varying concentrations, 5 μM to 30 μM, to assess the accuracy of the stock solutions. Concentrations differed by an average of 6% of the targeted spike value with a range of (2%–10%).

Results of atevirdine-binding studies determined by equilibrium dialysis indicated a high degree of binding to albumin, plasma, and serum, with a f_u of 0.01–0.02 noted consistently in each solution. Serum was studied only at 20 and 30 μM, due to similar binding as in plasma. The mean f_u of these solutions was significantly lower than the solutions containing α_1-acid glycoprotein or immune globulin ($P < 0.05$) (Table 2). Over the range of concentrations studied, 5 μM to 30 μM, there was no change in the unbound fraction of ATV in plasma. To determine if a variation in albumin concentration had an effect on the fraction bound of atevirdine, a 20 μM sample was studied at an albumin concentration of 3, 4, and 5%. All samples resulted in a 99% fraction bound independent of the albumin concentration. Atevirdine in immunoglobulin 1.5% solution resulted in a minimal amount of binding with a mean fraction of unbound drug ranging from 0.86 to 1.0 (Table 2). α_1-Acid glycoprotein studies resulted in varying fractions of unbound drug ranging from 0.34 to 0.82 among the concentrations studied (Table 2).

N-ATV had a similar high-binding profile as atevirdine. When tested in albumin 4%, plasma and serum a mean free fraction of 0.04, 0.03, 0.03 was noted, respectively (Table 2). The fraction unbound was greater in both immune globulin and AAG with an average of 0.87 and 0.59. The mean fraction unbound was significantly greater in the immune globulin and α_1-acid glycoprotein than serum, plasma and albumin ($P < 0.05$). There was a trend toward a decrease in fraction bound in the immune globulin with increasing concentrations. Similar to atevirdine, the fraction of N-ATV recovered after

Table 2
Fraction of unbound ATV (top) and N-ATV (bottom) in albumin, plasma, serum, IgG, and AAG [a]

ATV fraction unbound (S.D.)					
Conc. μM	Albumin 4%	Plasma	Serum	IgG 1.5%	AAG 100 mg%
5	0.0168 (0.002)	0.0189 (0.003)	NS	1.000	0.343 (0.032)
10	0.0140 (0.003)	0.0168 (0.0001)	NS	1.000	0.824 (0.707)
20	0.0111 (0.002)	0.0118 (0.0001)	0.0101 (0.0001)	0.968 (0.038)	0.428 (0.006)
30	0.0105 (0.0003)	0.0108 (0.0004)	0.0096 (0.0002)	0.855 (0.059)	0.531 (0.030)
Mean	0.0131 (0.003)	0.0146 (0.004)	0.0098 (0.0004)	0.956 * (0.069)	0.532 * (0.209)
N-ATV fraction unbound (S.D.)					
Conc.	Albumin 4%	Plasma	Serum	IgG 1.5%	AAG 100 mg%
5	0.0410 (0.003)	0.0511 (0.0007)	NS	1.000 (0)	0.524 (0.076)
10	0.0370 (0.004)	0.0262 (0.002)	NS	0.820 (0.027)	0.680 (0.080)
20	0.0455 (0.0007)	0.0256 (0.0004)	0.0329 (0.001)	0.899 (0.0981)	0.585 (0.0278)
30	0.0485 (0.001)	0.0313 (0.002)	0.0345 (0.002)	0.759 (0.032)	0.583 (0.0141)
Mean	0.0430 (0.005)	0.0336 (0.012)	0.0337 (0.002)	0.870 * (0.104)	0.593 * (0.065)

[a] Results expressed as mean ($\pm$S.D.).
* $P < 0.05$ vs. albumin, serum, plasma (Tukey's test).
NS, serum not studied at 5 and 10 μM.

the completion of equilibrium dialysis was lower in the AAG and immune globulin experiments. The recovery averaged 74 and 78% in these two groups compared with 90, 88 and 88% (Table 1) in the albumin, plasma, and serum groups. Over the concentration range studied, the degree of protein binding for ATV and N-ATV was not concentration-dependent. When atevirdine was combined with N-ATV at 20 μM of each, there was no significant change in the fraction unbound for either compound.

4. Discussion

ATV is one among a new group of non-nucleoside reverse transcriptase inhibitors that is undergoing clinical trials in patients with HIV infection. In contrast to nucleoside analog reverse transcriptase inhibitors, NNRTIs tend to have more complex pharmacokinetic characteristics which include capacity-limited metabolism, hepatic enzyme inducing and/or inhibition potential, and a considerable degree of binding to plasma proteins. These pharmacokinetic aspects contribute to a variable and prolonged plasma half-life for ATV (Morse et al., 1993).

The results of this in vitro study indicate that ATV is highly bound (> 98%) to plasma protein, primarily to albumin and minimally to AAG. Phase I clinical studies of ATV conducted by the AIDS Clinical Trials Group (ACTG Protocol 199 and 187) have employed a study design which has sought to adjust maintenance doses to attain targeted trough total ATV plasma concentrations ranging from 5–30 μM. The results of this in vitro study suggest that 1–2% of the total plasma concentration is free ATV, and thus available for intracellular distribution in patients. Since the site of action is within infected CD4 + cells, the high degree of binding could have an impact on antiretroviral activity in two ways. On one hand, the extensive binding to plasma proteins may serve as a reservoir for drug thus maintaining a source of antiretroviral suppression for a

prolonged period of time. Alternatively, the high degree of plasma protein binding may impede drug diffusion, decreasing the amount of ATV which reaches the intracellular site for RT inhibition. Since in vitro tissue culture experiments which determine inhibitory concentrations (i.e., IC_{50}) may not contain physiologic concentrations of albumin, it remains for clinical trials to assess the relevance of the extensive binding of ATV to plasma protein noted in this study. Consistent with the latter scenario, preliminary results of an in vitro study of the blood distribution of ATV suggests that a lower concentration of ATV and N-ATV were observed in the mononuclear cell supernatant compared to plasma (O'Donnell et al., 1993). In addition, high plasma protein binding may decrease the intracellular atevirdine concentrations, thus possibly contributing to the emergence of resistant strains.

These data also suggest that the use of targeted ATV plasma concentrations which measure total drug may not provide the best correlation with antiretroviral effects. Instead, determination of the free drug concentration from clinical specimens may provide a better indication of exposure to the active drug and thus be more useful in interpreting surrogate marker changes, susceptibility changes or adverse effect relationships (Levy and Moreland, 1984). Lastly, the low degree of binding to AAG suggest that the fluctuation of AAG during intermittent episodes of opportunistic infection or malignancy should not influence the free ATV concentration on plasma (Pike et al., 1984).

Cachexia and malnutrition are common in HIV-infected and AIDS patients. The Centers for Disease Control recognize an "HIV wasting syndrome" as one AIDS-indicating condition for the surveillance case definition. Postulated mechanisms for this decrease in body weight include elevated tumor necrosis factor levels, gastrointestinal malabsorption, poor oral intake, and altered absorption and metabolism (McCorkindale, 1990; Dworkin et al., 1990). These factors, either alone or combined, contribute to a decrease in serum albumin concentrations. In a study by Chlebowski et al., 1989, the nutritional status of 71 AIDS patients was reviewed and it was noted that 98% of patients experienced profound weight loss and 83% had hypoalbuminemia (< 3.5 g/dl). This relationship has also been studied in AIDS, AIDS-related complex and HIV-positive patients (Dworkin et al., 1990). Mean albumin concentrations in these subgroups were 3.5, 4.2, and 4.1 (g/dl), respectively. A report of altered phenytoin protein binding in a patient with AIDS has also been described (Tohler et al., 1990).

The NNRTIs represent a potential advance in the battle against HIV. However, the optimal therapeutic use of these agents, particularly the highly protein bound BHAPs, will require an appreciation for their complex pharmacokinetic characteristics and possibly correlation of free drug concentrations with clinical and virologic outcomes. Further in vitro studies are underway to evaluate the impact of hypoalbuminemia as well as the potential for displacement of ATV from plasma protein by other commonly prescribed antiinfectives.

The work was supported by a grant from the Upjohn Company.

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