Etravirine in CSF is highly protein bound

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Objectives: Etravirine has high affinity for plasma drug-binding proteins, such as albumin and α1-acid glycoprotein, which limits the amount of unbound etravirine available to enter the CNS. The objective of this study was to compare total and unbound etravirine concentrations in CSF with plasma concentrations and the in vitro median inhibitory concentration (IC50) for wild-type HIV (0.9 ng/mL).

Methods: Total and bound etravirine concentrations were measured in 17 CSF and plasma pairs by isotope-dilution liquid chromatography tandem mass spectroscopy, radioligand displacement and ultracentrifugation. Unbound etravirine concentrations were calculated from the bound fraction. The dynamic range of the assay was 7.8–2000 (plasma) and 0.78–200 (CSF) ng/mL.

Results: Subjects were mostly middle-aged (median 43 years) white (78%) men (89%). All CSF etravirine concentrations were above the limit of quantification. Total and unbound median etravirine concentrations in CSF were 9.5 (IQR 6.4, 26.4) and 0.13 (IQR 0.08, 0.27) ng/mL, respectively. Etravirine was 96% (IQR 94.5, 97.2) protein bound in plasma and 98.4% (IQR 97.8, 98.8) in CSF. Total etravirine in CSF was 4.3% (IQR 3, 5.9) of total and 101% (IQR 76, 160) of unbound etravirine in plasma. There were no significant correlations between unbound etravirine concentrations and concentrations of albumin in plasma or CSF. Unbound etravirine concentrations in CSF did not reach the wild-type IC50 in any of the specimens.

Conclusions: Unbound etravirine may not achieve optimal concentrations to inhibit HIV replication in the CNS.

Keywords: HIV, antiretroviral therapy, central nervous system, CNS, protein binding, CSF

Introduction

Antiretroviral (ARV) therapy significantly reduces the morbidity and mortality of systemic HIV disease and has had a major impact on the incidence of severe HIV-associated neurological disorder (HAND). Despite this, milder forms of HAND remain common. This could be due to the limited distribution of many ARV drugs into the CNS, which could allow HIV to continue to replicate in this protected compartment.1,2 Etravirine is a second-generation small molecule non-nucleoside reverse transcriptase inhibitor (NNRTI) that is active against wild-type and first-generation NNRTI-resistant HIV.3,4 In plasma, etravirine is reported to be 99.6% bound to albumin, and in vitro has 97.7–99.0% binding to α1-acid glycoprotein,5 which limits the amount of unbound etravirine available to enter the CNS.6 The objective of this study was to compare total and unbound etravirine concentrations in CSF with plasma concentrations and the in vitro median inhibitory concentration (IC50) for wild-type HIV (0.9 ng/mL).
glycoprotein (AAGP). This extensive protein binding has important implications for the CNS since only unbound drug is thought to distribute into this protected compartment. Based on this principle, etravirine may not reach therapeutic levels in the CNS and thus may be ineffective in terms of CNS virological control and HAND treatment as only unbound drug is available to inhibit HIV replication.

Despite these hypothetical limitations, etravirine may still be effective in the CNS since its inhibitory concentration against wild-type HIV is very low. For example, efavirenz has similar physicochemical properties to etravirine and yet its concentrations in CSF were found to exceed inhibitory concentrations by 10- to 20-fold. Furthermore, etravirine is not a substrate for P-glycoprotein (PGP), a multidrug molecular efflux transporter that is expressed on the luminal surface of brain microvascular endothelial cells, and thus is less likely to be actively excluded from the CNS. The objective of this study was to measure total and unbound etravirine in CSF to establish protein binding in the CNS and to compare these concentrations with total and unbound etravirine in plasma and with the in vitro inhibitory concentration for wild-type HIV.

**Methods**

**Subjects**

Subjects with HIV-1 infection were enrolled in observational cohort studies conducted at the University of California San Diego (UCSD) HIV Neurobehavioral Research Center (HNRC) between April 2007 and March 2009. The cohort studies included the CNS HIV AntiRetroviral Therapy Effects Research (CHARTER) and the California NeuroAIDS Tissue Network (CNTN) projects. Subjects were selected retrospectively if they met all the eligibility criteria: reported use of etravirine 200 mg orally twice daily; etravirine use for ≥2 weeks; reported adherence >95% in the 4 days preceding sampling; and available CSF and blood plasma stored at −80°C in the HNRC’s specimen repository. All parent studies were approved by the UCSD Human Research Protections Program and in accordance with the World Medical Association Declaration of Helsinki. All subjects were provided with and signed informed consent forms. For these parent studies, CSF had been obtained by lumbar puncture performed with aseptic techniques by experienced operators using 22 gauge pencil-point needles. Blood was obtained within 1 h of CSF by routine phlebotomy. All specimens were processed and frozen within 1 h of collection.

**Assays**

Total etravirine was measured by isotope-dilution liquid chromatography tandem mass spectrometry. Etravirine was extracted with methyl-tert-butyl ether (MTBE) after addition of internal standard (d4-efavirenz). d4-Efavirenz was used as internal standard since stable isotopically labelled etravirine was not available, and etravirine and efavirenz have similar chromatographic properties. Criteria for internal standard suitability when using d4-efavirenz for assay of total etravirine in CSF and plasma were satisfied according to guidelines established by Matuszewski et al. MTBE extracts were dried under a stream of purified nitrogen before reconstitution in a minimal volume of organic mobile phase prior to injection. The dynamic range of these assays was 7.8–2000 (plasma) and 0.78–200 (CSF) ng/mL. For total etravirine assays, recovery from both matrices was 94% or greater. Direct quantification of unbound etravirine was not possible since unbound concentrations were below the lower limit of quantification. Therefore, unbound etravirine concentrations were calculated from the percentage of etravirine protein binding determination (equivalent to bound concentrations). Protein binding was determined by measuring the displacement of bound etravirine by [14C]etravirine in the unbound fraction of matrix generated by ultracentrifugation as previously described. Ultracentrifugation was used to generate unbound fractions for protein binding measurement since non-specific binding of etravirine to ultrafiltration membranes used to generate unbound fractions was >99.9%. Experiments were performed to verify that unbound etravirine remained in the uppermost supernatant layers following ultracentrifugation by utilizing protein-free artificial plasma or CSF, previously dosed with known amounts of radiolabelled etravirine. In addition, experiments were performed to ensure that the uppermost supernatant was free of binding proteins such as AAGP and albumin, which would be spun down because of their molecular weight. ELISA was used to verify that there were <3 ng/mL AAGP and <6.25 ng/mL albumin in diluted aliquots of ultracentrifuged upper-phase supernatant. Samples tested were naive plasma or CSF from three distinct lots, which were previously spiked with 25 ng/mL AAGP and 25 µg/mL albumin, plus three CSF samples and three plasma samples from the current study. HIV RNA was quantified by reverse transcription PCR using a TaqMan RealTime assay (Roche Diagnostics) with a lower limit of quantification of 50 copies/mL. Blood T cell subsets were counted using routine clinical flow cytometry. Albumin was measured in CSF (n=13) using an immunonephelometry assay and in plasma (n=17) using a standard colorimetric assay with normal ranges 0–35 mg/dL (CSF) and 3.5–5.2 g/dL (plasma) by ARUP Laboratories (Salt Lake City, UT, USA).

**Statistical analysis**

Descriptive and bivariate statistics were generated using standard methods. Drug concentrations in CSF were compared with drug concentrations in plasma and with an in vitro, protein-free 50% inhibitory concentration (IC50) for wild-type HIV-1 (0.9 ng/mL). Spearman’s non-parametric correlation method was used to compare plasma and CSF concentrations using the R version 2.13.0 statistical software package.

**Results**

Seventeen CSF–plasma pairs were obtained from nine subjects with three subjects providing a single pair, four subjects providing two pairs and two subjects providing three pairs. Subject demographics and baseline characteristics are presented in Table 1. Subjects were predominantly middle-aged (median, 43 years; range, 33–51), white (78%) men (89%). All subjects were taking concurrent ARVs: emtricitabine (89%), tenofovir (78%), lamivudine (11%), darunavir/ritonavir (78%), raltegravir (56%), enfuvirtide (33%), maraviroc (22%) and didanosine (11%). Median duration of etravirine use was 8.0 months (IQR 5.1, 9.7). The last etravirine dose prior to sampling was taken with food in 94% of samples. All subjects had undetectable HIV RNA in CSF.

Etravirine plasma and CSF concentrations as well as sampling information are displayed in Figure 1 and pharmacokinetic aggregate data are summarized in Table 2. CSF post-dose sampling intervals were well distributed over the dosing interval. All 17 plasma and CSF samples had detectable total and unbound etravirine concentrations. Total etravirine in CSF exceeded the IC50 for wild-type HIV-1 (0.9 ng/mL) in all 17 specimens, but unbound etravirine in CSF was below the IC50 in all specimens. Median CSF fractional penetration (i.e. the relative concentration of drug reaching the CSF compartment) was 4.3% (IQR 3.0–5.9%) of total plasma and 101% (IQR 76%–160%) of unbound plasma concentrations. There was no statistically significant
correlation between fractional penetrance and CSF-to-plasma-protein ratios, including albumin and AAGP. Correlations between unbound etravirine in CSF and total \( r=0.58, P=0.017 \) or unbound \( r=0.44, P=0.076 \) etravirine in plasma were comparable (Figure 2). There were also similar correlations between total etravirine in CSF and total \( r=0.72, P=0.001 \) or unbound \( r=0.62, P=0.008 \) etravirine in plasma. Protein binding in CSF (median 98.4%; IQR 97.8%, 98.8%) was slightly greater than of that in plasma (median 96.0%; IQR 94.5%, 97.2%), but not at a significant level \( P=0.35 \). Concentration-dependent binding of etravirine was seen in the plasma \( r=0.79, P=0.0001 \) with an increasing unbound fraction of etravirine with higher total plasma etravirine concentrations (Figure 3). There were no significant correlations between unbound etravirine concentrations and concentrations of albumin and AAGP in plasma or CSF (Table 3). Unbound to total CSF and plasma ratios were not correlated with post-dose sampling interval.

### Discussion

In order to exert its pharmacological effect, etravirine must be unbound from plasma and CSF proteins in order to enter cells and inhibit HIV replication. In the presence of binding proteins, greater drug concentrations are needed to inhibit viral replication. Experiments show a strong relationship between percent human plasma (which contains binding proteins) and in vitro median inhibitory concentrations of ARV drugs.\(^{14}\) ARV drugs that are highly bound to plasma proteins also encounter

\[ \text{IC}_{50} \]

\[ \text{Time post-dose (h)} \]

\[ \text{Etravirine (ng/mL)} \]

**Figure 1.** Total and unbound etravirine (ETR) concentrations in plasma and CSF.
problems entering the CNS as only unbound drug can cross the blood–brain barrier. Poor ARV distribution into the CNS may allow continued HIV replication in the CNS and may be associated with detectable and higher HIV RNA in CSF.16 In our project, the majority of specimens had plasma HIV RNA <50 copies/mL, indicating that these patients had good virological control. All 17 specimens had adequate levels of unbound etravirine in plasma, with concentrations above the IC₅₀ of 0.9 ng/mL. The unbound fraction of etravirine in plasma was 4.0%, which is much higher than reported by the manufacturer (0.1%).5 With a total CSF-to-unbound plasma ratio of ~1, essentially all of the unbound plasma etravirine was in equilibrium with the CNS. This finding is consistent with the physicochemical characteristics of etravirine, including its relatively low

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**Figure 2.** Relationships between unbound CSF and plasma (unbound and total) etravirine concentrations. Filled circles indicate unbound CSF etravirine and unbound plasma etravirine concentrations, while open circles represent unbound CSF etravirine and total plasma etravirine concentrations.
molecular weight and high lipophilicity. Total etravirine in CSF exceeded the IC₅₀ for wild-type HIV-1 in all 17 specimens, but unbound etravirine in CSF failed to reach the IC₅₀ in any of the specimens. Even though the concentrations of binding proteins were ~1000-fold lower in CSF than in plasma, the percentage of etravirine bound to proteins was similar in both compartments. In addition, unbound CSF etravirine concentrations were not correlated with albumin or AAGP concentrations.

This is the first study reporting both total and unbound etravirine concentrations in plasma and CSF. The few studies reporting unbound concentrations of ARV in CSF have consistently shown mostly unbound drug, which is likely due to the low concentrations of binding proteins in the CNS. For example, even though the protease inhibitor darunavir is highly protein bound in plasma (97%), it remains mostly unbound in CSF (6.8%). Another protease inhibitor, indinavir, shows similar characteristics with 58% of drug found to be bound in plasma and only 5.7% bound in CSF. However, etravirine appears to be different in that it has a very high affinity for binding proteins and remains predominantly bound even in the CNS. Interestingly, a report on another NNRTI, efavirenz, showed a CSF bound fraction of 59%. Tiraboschi et al. previously observed that total etravirine levels in plasma and CSF both exceed IC₅₀ concentration, and thus suggested etravirine to be effective against viral replication in the CNS. However, our results suggest caution in using the traditional assumption that total CSF antiretroviral concentrations are likely effective if they exceed wild-type HIV-1 median inhibitory concentrations, as etravirine remains highly protein bound.
in the CSF and thus unbound etravirine concentrations are well below the IC50.

Our study is limited by the small number of subjects (n=9) and specimens (n=17), the heterogeneity of their ARV regimens and the virological suppression in plasma. While our results suggest that unbound etravirine concentrations may be subtherapeutic in the CNS, they are in contrast with some anecdotal clinical findings. Couzigou et al.21 reported a case of complete resolution of acute meningoencephalitis in the setting of HIV and hepatitis C coinfection in a 37-year-old ARV treatment-experienced woman with the addition of etravirine to a regimen including tenofovir, abacavir, lopinavir/ritonavir and saquinavir. This observation does not necessarily indicate that etravirine penetration in the CNS is sufficient to halt viral replication. It is possible that the addition of etravirine to her ARV regimen inhibited systemic viral replication and thus improved systemic HIV disease, leading to recovery from CNS symptoms. Furthermore, in the case of meningoencephalitis, disruption of the blood–brain barrier in this patient may have led to higher levels of etravirine and other ARVs in the CNS than in patients without CNS inflammation.

One interesting finding is the discrepancy between plasma protein binding of etravirine, measured at 96%, and the manufacturer's data of 99.6%. This difference may be due to different methodologies used in the determination of protein binding. Isolation of the unbound drug is typically performed via ultrafiltration of the matrix through a molecular weight cut-off membrane, for exclusion of proteins with molecular weight greater than that specified by the membrane characteristics. Our attempts to isolate unbound etravirine via ultrafiltration were thwarted by extensive etravirine binding to all ultrafiltration membranes tested. Despite the application of several procedural techniques known to effectively eliminate or reduce non-specific binding (pre-saturation of membrane with naïve matrix, modification of ionic composition, changes in pH and addition of solubilizing agents), extensive non-specific binding to ultrafiltration membranes could not be averted. An alternative method, equilibrium dialysis, was considered but not pursued due to similar drug binding properties of dialysis and ultrafiltration membranes. Ultimately, ultracentrifugation (without filtration), a method well documented and validated in the literature, was chosen to isolate the unbound drug fraction.13,14 While the methods (ultra-centrifugation, ultrafiltration or equilibrium dialysis) best suited to the study of protein binding in plasma may be debatable, we found ultracentrifugation to be the only workable solution due to the non-specific binding characteristic of etravirine.22

Our findings provide evidence that etravirine is highly bound in the CSF, and measurement of total CSF concentrations alone does not provide an accurate estimate of unbound CSF concentrations of etravirine. Although a relatively low concentration of etravirine is needed to inhibit HIV replication in the CNS, the unbound concentrations of etravirine were well below the IC50. However, it is important to mention that binding of drug to proteins is a dynamic equilibrium process. As unbound drug moves across biological membranes and out of the extracellular compartment, the equilibrium will shift, essentially drawing the drug away from the protein to replenish the depleted unbound drug fraction. This new free drug is also able to move across biological membranes. Therefore, the intracellular amount of drug may be adequate to inhibit viral replication despite a low unbound drug concentration in the CSF compartment. The adequate intracellular amount of drug and resulting virologic control may be supported by the fact that all subjects in this study had undetectable levels of HIV RNA in CSF, even in those subjects with detectable HIV RNA in plasma, although the effect of an individual antiretroviral cannot be established with certainty given that combination antiretroviral therapy is the standard of care and all subjects were on one or more antiretrovirals with good CNS penetration. The findings may have implications in selecting antiretrovirals to prevent or treat HAND, but additional research, including measurement of the unbound fraction of other antiretrovirals, as well as a prospective clinical trial using etravirine for the treatment of HAND, will be required to confirm the significance of the findings presented here.

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S. M. was involved in data collection and analysis, and discussion of the results. J. A. M. was involved in data collection and analysis, and discussion of the results. B. G. was involved in data collection and analysis, and discussion of the results. A. C. C. was involved in data collection and analysis, and discussion of the results. D. Croteau was involved in data analysis and discussion of the results. S. L. was involved in data analysis and discussion of the results. J. M. was involved in the conception and design of the study, data analysis, discussion of the results and manuscript preparation.

### Disclaimer

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