Background: Enfuvirtide is a potent inhibitor of systemic HIV-1 replication, but its penetration into the human central nervous system (CNS) has not been analysed. Here, we define cerebrospinal fluid (CSF) enfuvirtide pharmacokinetics and present a case illustrating the use of enfuvirtide as a probe to trace the origins of CSF HIV-1 quasispecies.

Methods: Enfuvirtide CSF pharmacokinetics were assessed in 18 CSF and plasma sample pairs from four HIV-1-infected individuals. Enfuvirtide levels were measured by liquid chromatography tandem mass spectrometry using known standards and controls that included spiked CSF samples from untreated, HIV-negative individuals. A segment of the gp41 coding region encompassing the heptad repeat HR-1 and HR-2 domains was amplified from selected CSF and plasma samples and independent clones sequenced to assess resistance-associated mutations.

Results: CSF and plasma samples obtained between 2 and 20 h after enfuvirtide injection showed plasma concentrations similar to previous reports (mean $3.68\pm1.82\text{mg/ml}$) with prolonged decay. By contrast, enfuvirtide in all CSF samples was below the assay detection limit of $0.025\text{mg/ml}$. In one individual, who developed a transient increase in CSF HIV-1 RNA, seven of seven CSF and plasma clones had identical enfuvirtide resistance-associated V38A mutations, suggesting that the CSF quasispecies derived from that of blood.

Conclusions: Enfuvirtide penetration into CSF is negligible; thus, in clinical settings, where direct CNS drug exposure is crucial, this drug is not likely to directly contribute to the local therapeutic effect. Enfuvirtide can be used as a tool to dissect the origin of the CNS virus.

Introduction

Enfuvirtide (T-20) is a 36 amino acid synthetic peptide that interferes with HIV-1 infection by inhibiting virion fusion and subsequent entry of the viral genome into the target cell [1]. It mimics the heptad repeat 1 (HR-1) region of the viral gp41 and, through interaction with the HR-2 region, prevents formation of the hairpin configuration essential to the fusion process [2]. Enfuvirtide differs from other classes of anti-HIV-1 drugs in several important properties, including extracellular site of action, absence of cross-resistance with other approved drugs, and minimal pharmacological interaction with other antiviral drugs [3]. Its principal disadvantage is that it must be given by injection to avoid degradation by proteases. Indeed, this is perhaps the major reason that the drug is now used almost exclusively by patients with multidrug HIV-1 resistance. Although HIV-1 can also develop resistance to enfuvirtide, this is related principally to mutations in the highly conserved HR-1 domain, which lies within the N-terminal region of gp41 [4–7] and is unrelated to resistance to other licensed antiretroviral drugs.

Although the chemical structure of enfuvirtide and the results of preclinical animal studies suggest that the drug would not reach effective antiviral concentrations...
in the brain, there have been no human studies to assess central nervous system (CNS) pharmacokinetics and effects. We therefore undertook the current study to measure whether enfuvirtide reached effective concentrations in the cerebrospinal fluid (CSF). Additionally, we describe a case example illustrating how the properties of enfuvirtide can be used to examine some broader issues of CNS infection and antiviral treatment.

Methods

Plasma and CSF sampling

CSF and blood samples for this study were obtained in the context of a longitudinal Sentinel Neurological Cohort (SNC) study evaluating the effects of HIV-1 infection on CSF; this study was approved by the University of California, San Francisco (UCSF) Committee on Human Research. In all cases CSF was obtained for study purposes rather than for clinical diagnosis and was processed in standardized fashion, as previously described [8]. Archived matched CSF and plasma samples that had been aliquoted and stored at -80°C were chosen for these analyses; CSF samples were centrifuged to remove cells before supernatants were stored. The time of drug administration, phlebotomy and CSF collection were all recorded; the midpoint of the CSF collection, which took 10–12 min, was used as the CSF sample time point for calculations. Plasma samples were obtained within 1 h before or after this time point. We selected all individuals who had been treated with enfuvirtide while participating in the SNC study. This initially designated six possible individuals for inclusion. All medications, including enfuvirtide, were prescribed by the patients’ caregivers and not determined by our observational CSF studies. None of the CSF samples had >10 red blood cells/μl.

Enfuvirtide measurements

Enfuvirtide was measured in both plasma and CSF by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an adaptation of a previously described method [9]. The CSF assay was performed using calibration standards and quality control samples prepared in fluid collected from HIV-negative volunteers spiked with known concentrations of enfuvirtide. The performance of the LC-MS/MS method was characterized for plasma; however, characterization studies were not performed for CSF because of insufficient control matrix. Nonetheless, the same method was used for analysis of both matrices, and acceptable data for quality control samples assayed with patients’ samples indicated data should be reliable for CSF. We examined freeze/thaw stability and noted an approximate 20% loss of enfuvirtide in CSF at low concentrations upon freezing and thawing the spiked samples, perhaps related to a limited amount of enfuvirtide adhesion to the tubes. Whatever the cause, CSF results may underestimate the actual concentrations to this degree. The lower limit of quantitation of enfuvirtide in CSF and plasma was 0.025 μg/ml.

Virological and general laboratory methods

HIV RNA was measured in cell-free CSF and plasma by the Roche Amplicor Monitor assay (versions 1.0 and 1.5, Roche Diagnostic Systems, Inc., Branchburg, NJ, USA) using the standard and ultrasensitive extraction methods. Concurrent paired CSF and plasma samples were treated identically and run at the same time. Background determinations of CSF cell counts, differential counts, protein and albumin, along with blood CD4+ and CD8+ T-cell counts and blood albumin were performed in the San Francisco General Clinical Laboratories using routine methods. The CSF:blood albumin concentration ratio (CSF albumin/serum albumin ×10$^{-3}$) was used as an index of blood–brain barrier disruption with normal value of 4.6 ±1.3 (sd) [10].

Enfuvirtide resistance

Enfuvirtide resistance mutations were evaluated by sequence analysis of amplified cloned segments containing an approximately 400-nucleotide segment of the gp41-coding region encompassing the HR-1 and HR-2 domains derived from samples of CSF and plasma, using techniques previously described [6].

Statistical analysis

Statistics were performed using Prism 5.0 (GraphPad Software Inc, San Diego, CA, USA).

Results

Study participants

A total of six individuals were identified who had been prescribed enfuvirtide while participating in CSF cohort studies. In two of these individuals, enfuvirtide was not detected in the blood (or CSF) and they were therefore omitted from the pharmacokinetic analysis because they were presumed to be nonadherent to enfuvirtide. One of these two individuals was viraemic therefore omitted from the pharmacokinetic analysis and the other four individuals, 18 pairs of CSF and blood samples were available for analysis and were used to define CSF enfuvirtide levels. All individuals were male and between 45 and 54 years old at the first sampling interval (Table 1). These individuals exhibited a broad range of plasma and CSF HIV-1 RNA concentrations related to a limited amount of enfuvirtide adhesion to the tubes. Whatever the cause, CSF results may underestimate the actual concentrations to this degree. The lower limit of quantitation of enfuvirtide in CSF and plasma was 0.025 μg/ml.
and blood CD4+ T-cell counts. In general, CSF white blood cell counts (WBCs) were low, except for an episode in one patient (7044) described in more detail below. Likewise, CSF:blood albumin ratios were normal except for one mildly increased level (8.27) in this same patient.

Pharmacokinetics

Figure 1 shows the concentrations of enfuvirtide in CSF and plasma from the 18 paired samples. The overall mean plasma enfuvirtide concentration for all intervals was 3.69 μg/ml (SD 1.828 μg/ml), a level above the fifty percent effective dose (ED50) of wild-type HIV-1 and in line with previous observations [3,7,11]. Although most of the samples clustered within the first 4 h after enfuvirtide injection, more delayed sampling in one individual showed levels above 1.0 μg/ml, consistent with enfuvirtide’s long half-life and previous observations [3,11]. In contrast to plasma, CSF concentrations of enfuvirtide were below the level of quantitation in all samples, indicating negligible, subtherapeutic drug penetration into this compartment. The CSF concentrations were significantly different from plasma (P=0.0001, t=0.81, paired t test).

Case study with enfuvirtide resistance in CSF and plasma

The following case illustrates how enfuvirtide resistance mutations can be used to explore the ontogeny of the CSF quasispecies and, more speculatively, how limited drug penetration might modify changes in CSF compared with plasma HIV-1 concentrations.

Patient 7044 exhibited three ‘spikes’ in CSF HIV-1 RNA levels over the course of observation (Figure 2). During one of these episodes (circled in the figure) he experienced mild flu-like symptoms, although these were sufficiently mild as to only be elicited in retrospect when the CSF results showed a cell count of 260 WBC/l. Despite ongoing treatment with multiple drugs (in addition to enfuvirtide, the patient was taking lamivudine, abacavir, tenofovir, efavirenz and lopinavir/ritonavir), the CSF HIV RNA rose to 171,000 copies/ml at this visit, while plasma HIV RNA levels were 5,440 copies/ml. Extensive evaluation by the primary physician failed to identify a cause and, although sustained (and even increased) at a subsequent visit 4 months later, the CSF cells and virus eventually remitted spontaneously. This patient reported excellent adherence to medications during this time. Whether this episode related to an intercurrent viral infection, a change in therapy (from stavudine to tenofovir 7 weeks earlier) or another cause, was not established. The two other increases in CSF HIV-1 RNA levels were not accompanied by CSF pleocytosis; the last of these developed 10 weeks after switching from lopinavir/ritonavir to atazanavir/ritonavir, but

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age, years</th>
<th>Number of sample pairs</th>
<th>Timespan of samples, years</th>
<th>Plasma HIV, log10 copies/ml</th>
<th>CSF HIV, log10 copies/ml</th>
<th>Blood CD4+ T-cell count, cells/μl</th>
<th>CSF WBC count, cells/μl</th>
<th>CSF/blood albumin ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>7044</td>
<td>45.7</td>
<td>7</td>
<td>2.5</td>
<td>&lt;20–145</td>
<td>&lt;20–4,370</td>
<td>256–549</td>
<td>0–643</td>
<td>3.74–8.27</td>
</tr>
<tr>
<td>7071</td>
<td>47.2</td>
<td>5</td>
<td>2</td>
<td>&lt;20</td>
<td>&lt;20–69</td>
<td>105–189</td>
<td>0–3</td>
<td>4.02–6.43</td>
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<tr>
<td>7102</td>
<td>54.1</td>
<td>4</td>
<td>1.5</td>
<td>&lt;20–30,100</td>
<td>&lt;20</td>
<td>71–89</td>
<td>0–4</td>
<td>4.08–6.05</td>
</tr>
<tr>
<td>7105</td>
<td>48.6</td>
<td>2</td>
<td>0.5</td>
<td>88,700, 260,000</td>
<td>435, 1340</td>
<td>7, 44</td>
<td>1, 1</td>
<td>4.58, 6.21</td>
</tr>
</tbody>
</table>

CSF, cerebrospinal fluid; WBC, white blood cell.
also resolved without further changes in medication. At the times of the concurrent CSF and plasma HIV-1 RNA elevations during the first three years, the patient exhibited high-level phenotypic and genotypic resistance to all the other antiretroviral drugs in his regimen, and the same major reverse transcriptase and protease resistance mutations were identified in both CSF and plasma with the exception of preserved tenofovir phenotypic susceptibility (1.4-fold in plasma and 1.5-fold in CSF) in the presence of genotypic resistance in both fluids by PhenoSense assay (Mongram Biosciences, South San Francisco, CA). The individual remains well 5 years after the circled episode.

Clonal sequence analysis of the gp41 segment containing the HR-1 and HR-2 coding regions was applied to the CSF and plasma samples obtained at the interval circled in Figure 2. The results of both bulk sequencing of CSF and plasma and analysis of seven individual CSF and seven plasma clones all showed the valine to alanine mutation at position 38 of the HR-1 region of the gp41 coding sequence (V38A), a change known to confer enfuvirtide resistance [4–6], while background sequences within this 125-nucleotide region were also nearly identical.

**Discussion**

The failure to detect enfuvirtide in CSF, in the face of therapeutic levels in blood that are >100-fold higher than the limit of detection, indicates that this therapeutic peptide is excluded from crossing the blood–brain and blood–CSF barriers and does not appreciably enter the CSF. Our reconstruction studies with spiked samples suggest that there may be as much as a 20% loss of the compound in CSF with storage, freezing and thawing at low concentrations. Results might also have been influenced by differences in protein binding in blood and CSF, as enfuvirtide is about 90% bound to plasma proteins, chiefly albumin [3], and CSF binding is therefore presumed to be much lower (not directly measured). Even correcting for these factors, the CSF levels were uniformly low and cell exposure was likely to be below the effective therapeutic range. Our findings are paralleled by those of an earlier report demonstrating failure of enfuvirtide to cross the blood–testis barrier and reach therapeutic concentrations in semen [12].

The **in vivo** efficacy of some antiretroviral drugs can be influenced by additional factors compared with findings in cell culture; for example, in the case of some nucleosides, differences in intracellular metabolism and substrate competition [13], cell type [14] or activation [15] can be important. By contrast, enfuvirtide is active at the cell surface and, therefore, the extracellular concentration determines its antiviral activity. Hence, to the extent that local antiviral effect is important in reducing CSF (and, more broadly, CNS) HIV-1 infection, enfuvirtide is predicted not to contribute directly to viral suppression in this compartment, although it is important to caution that neither drug access to nor infection of the CSF space and brain parenchyma are identical. Likewise, without appreciable concentrations of the drug in the CSF, this compartment is less likely to serve as a site for the selection of resistance mutations; however, it is possible that low, but undetectable, levels in CSF might have contributed to local selection. Also, although concern about CNS toxicity of antiretroviral drugs has increased, if brain parenchymal penetration is similar to that of the CSF, enfuvirtide is unlikely to have potential for causing neurotoxicity.
Enfuvirtide in cerebrospinal fluid

Here, we report a case in which virus rebounded to a greater extent in CSF than in the plasma. The virus that emerged during this time exhibited genotypic evidence of resistance to enfuvirtide. Notably, the sequences that showed characteristic resistance mutations were identical in the plasma and CSF, suggesting a common source. Given that the enfuvirtide concentrations were measurable only in plasma, this ‘spike’ in CSF HIV-1 RNA in the setting of CSF pleocytosis is likely to have resulted from temporally proximate (almost certainly some time after starting enfuvirtide, and perhaps close to the time of the spike) seeding (transitory infection) and local amplification by activated CD4+ T-cells among the CSF cell reaction. This would be consistent with the analysis of Harrington and colleagues using the heteroduplex tracking assay that suggested the origin of CSF HIV-1 in short-lived cells [16].

Another of the four individuals (7071) exhibited a similar isolated CSF spike (CSF HIV-1 RNA 8,320 copies/ml, CSF WBC to 33 cells/μl, and plasma HIV RNA levels of 26 copies/ml); genotypic resistance testing was not successful in this individual. These two cases, in which virus appeared to replicate better in CSF than plasma while on a stable regimen, were highly unusual in our experience in individuals without neurological disease. In the longitudinal study from which these samples were taken, out of 435 total evaluations (36 with individuals taking enfuvirtide) in 101 individuals on antiretroviral therapy, we found only 13 examples where CSF HIV-1 RNA levels exceeded those of plasma. Seven of these 13 were from patients 7044 and 7071 included in this report, indicating a high association with enfuvirtide treatment, suggesting a common source. Given that the enfuvirtide concentrations were measurable only in plasma, this ‘spike’ in CSF HIV-1 RNA in the setting of CSF pleocytosis is likely to have resulted from temporally proximate (almost certainly some time after starting enfuvirtide, and perhaps close to the time of the spike) seeding (transitory infection) and local amplification by activated CD4+ T-cells among the CSF cell reaction. This would be consistent with the analysis of Harrington and colleagues using the heteroduplex tracking assay that suggested the origin of CSF HIV-1 in short-lived cells [16].

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Disclosure statement

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References


2. Wild CT, Shugar DC, Greenwell TK, McDanal CB, Matthews TJ. Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. Proc Natl Acad Sci U S A 1994; 91:9770–9774.


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