Intracellular and plasma pharmacokinetics of efavirenz in HIV-infected individuals

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Objectives: The site of action of efavirenz is inside HIV-infected cells. Measurement of intracellular (IC) concentrations of efavirenz may therefore provide further understanding of therapeutic failure, especially where virological rebound occurs despite adequate plasma levels, and a lack of detectable viral resistance. Here, we determined IC and plasma pharmacokinetics of efavirenz and their relationship with plasma protein binding and P-glycoprotein (P-gp, an active drug efflux transporter) expression.

Patients and methods: Venous blood samples from 10 HIV-infected patients receiving efavirenz (600 mg once a day plus two nucleoside reverse transcriptase inhibitors) were collected over the 24 h dosing interval. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated. Plasma protein bound and unbound efavirenz were separated using ultrafiltration. IC (or cell-associated), total plasma and unbound plasma efavirenz levels were quantified using HPLC-UV. P-gp expression was measured by flow cytometry. Area under the concentration-time curves (AUC0–24) were then calculated using non-compartmental analyses and the IC accumulation expressed as a ratio of IC to plasma AUC0–24.

Results: The median (range) % unbound and IC accumulation ratio was 0.6% (0.4–1.5%) and 1.3 (0.7–3.3), respectively. There was a linear relationship between IC and total AUC0–24 ($r^2 = 0.59$, $P = 0.01$) but not unbound AUC0–24 ($r^2 < 0.01$, $P = 0.75$). An inverse correlation between IC AUC0–24 and % unbound was observed ($r^2 = 0.41$, $P = 0.05$). There was no relationship between IC AUC0–24 and P-gp expression on the cell surface ($r^2 < 0.01$, $P = 0.98$).

Conclusions: There was a direct relationship between % bound efavirenz in plasma and IC accumulation implying that the IC accumulation of efavirenz is related to binding to IC proteins or other cellular constituents. Studies investigating the unbound concentration of antiretrovirals inside the cell are now required.

Keywords: antiretrovirals, non-nucleoside reverse transcriptase inhibitors, NNRTIs

Introduction

Despite the success of highly active antiretroviral therapy in reducing morbidity and mortality amongst HIV-infected patients, therapeutic failure remains common. Although low plasma levels of efavirenz (<1000 ng/mL) have been linked to treatment failure, measurement of intracellular (IC) efavirenz concentrations may give a better indication of antiviral exposure at the site of action.

To date, there is only one study describing the IC accumulation of efavirenz. Rotger et al. used Bayesian calculations to predict plasma and IC AUCs from single randomly timed samples and related these to CYP2B6 genotype and CNS toxicity. IC (or more accurately cell-associated) concentrations of other antiretrovirals have also been determined and are thought to be the net result of a number of variables, such as the physiochemical properties of the drug, protein binding (plasma and IC) and active transport by influx and/or efflux proteins. As it is the unbound drug that is able to enter cells by passive diffusion, changes in the free fraction of efavirenz in plasma may alter its IC concentration. However, there are no data describing the free fraction of efavirenz over the dosing interval.
and generally few studies have related unbound concentrations of antiretrovirals to IC exposure.

The drug efflux pump, P-glycoprotein (P-gp), has been shown to limit IC concentrations of some protease inhibitors. However, there are relatively few data investigating active transport of the non-nucleoside reverse transcriptase inhibitors (NNRTIs), particularly efavirenz. Although efavirenz is not thought to be a substrate for P-gp, Fellay et al. showed differential immune reconstitution in patients taking efavirenz with allelic variations in MDRI (the gene encoding P-gp). The authors postulated that subjects with lower cellular P-gp expression, with the TT genotype, might have higher IC efavirenz concentrations giving better suppression of HIV replication.

The aim of this study was to quantify IC concentrations of efavirenz in vivo and to calculate the total IC exposure over the dosing interval (AUC<sub>0–24</sub>). IC exposure was then related to plasma efavirenz (total and unbound) AUC<sub>0–24</sub>, P-gp expression on the surface of lymphocytes and to the log P of efavirenz.

Materials and methods

Materials

Lymphoprep was purchased from Nycomed Pharma AS (Oslo, Norway). PBS tablets were purchased from Gibco Life Technologies Ltd (Paisley, UK). CellFIX was purchased from Becton Dickinson (Oxford, UK). Anti-IgG2a negative control antibody and goat anti-mouse IgG2a R-phycocyanin conjugated secondary antibody were purchased from Serotec (Oxford, UK). U1C2 antibody was obtained from Immunotech (Marseille, France). Amicon Centrifuge Filter Systems were purchased from Millipore Corporation (Bedford, MA, USA). HPLC grade acetonitrile, methanol, ethyl acetate and n-octanol were purchased from Sigma–Aldrich Company Ltd. (Oxford, UK). Ammonium acetate and potassium carbonate were purchased from Fisher Scientific (Leicestershire, UK). Buffco and blank plasma were purchased from the National Blood Transfusion Service (Liverpool, UK). Internal standard (Ro31-9564) was obtained from Roche (Basel, Switzerland). Efavirenz was obtained from BMS Service (Liverpool, UK). Internal standard (Ro31-9564) was obtained from Roche (Basel, Switzerland). Efavirenz was obtained from BMS (DuPont (Wallingford, USA)).

Determination of the partition coefficient of efavirenz

The traditional n-octanol–water shake flask method was used to determine the lipophilicity of efavirenz. This was consistent with previous investigations with the protease inhibitors and therefore allowed comparisons between the drugs to be made. This method determines the relative distribution of a given drug into either n-octanol saturated water (lipid bilayer-like) or n-octanol (lipid-like).

Equal volumes of analytical grade n-octanol and distilled water were mixed (24 h) before the phases were allowed to separate (12 h). The top layer (n-octanol) was discarded leaving the n-octanol saturated water. A stock solution of efavirenz was prepared in n-octanol, which was added to equal volumes of n-octanol saturated water to yield final concentrations of either 5 µM (1332 ng/mL) or 10 µM (2663 ng/mL). Samples were mixed on a mechanical shaker (30 min) and left to stand (2 h) to allow separation of the two phases. Three aliquots (of varying volumes) of each phase were collected carefully to avoid contamination. Samples were evaporated to dryness using a rotary evaporator and drug levels quantified using IC methods described below.

The partition coefficient (P) was calculated as a ratio of the concentration of drug residing in the organic phase (n-octanol) divided by the concentration of drug in the aqueous phase (n-octanol saturated water). Log P was calculated as the log<sub>10</sub> (partition coefficient).

Subjects

Ten subjects (6 male, 4 female; 7 Caucasian, 3 Black-African) with a median age of 35 years (range 23–59 years) were enrolled into this study. Routes of HIV infection (number of patients) were heterosexual transmission (n = 5), homosexual transmission (n = 4) and via injecting drug use (n = 1). Each subject provided written informed consent to participate in the study, which was approved by the local ethics committee. All subjects were receiving dual NNRTIs plus efavirenz (600 mg once a day) for a median duration of 24 months (range 6–48 months). Concurrent NNRTIs (number of patients) were lamivudine (n = 7), zidovudine (n = 6), stavudine (n = 4) and didanosine (n = 1).

Exclusion criteria were: age <18 years, pregnancy or breast feeding, evidence of pre-existing impairment of liver (ALT >5 times upper limit of normal) or renal function (creatinine >300 µmol/L), anaemia (Hb <10.0 g/dL), acute bacterial/viral infection (other than HIV), chronic hepatitis B or hepatitis C, and whether receiving the following or not: cytotoxic chemotherapy, rifampicin, verapamil, macrolide antibiotics or non-steroidal anti-inflammatory drugs within the previous 2 weeks, and α-interferon or IL-2 within the previous 2 months.

To allow laboratory analysis of samples throughout the profile, all patients were switched from evening doses of efavirenz to a morning dose. This was done over a period of 3 days to create minimum alterations in exposure to efavirenz (day 3, 600 mg once a day in the morning; day 2, 200 mg in the morning plus 400 mg in the evening; day 1, 400 mg in the morning dose plus 200 mg in the evening; day 0, 600 mg once a day in the morning—pharmacokinetic sampling day).

After the study day evening doses were resumed, using the same 3 day strategy in reverse.

For pharmacokinetic analysis, five blood samples were obtained from each participant over the 24 h dosing period. The first sample was provided immediately prior to their morning dose (0 h, 35 mL) and then four samples of 25 mL at 2, 6, 12 and 24 h post dose. The total blood volume donated by each subject over the study period was therefore 135 mL. Blood samples were collected into lithium heparin tubes.

Isolation of PBMCs

At each time point, blood (5 mL) was centrifuged (700 g, 6 min, 4°C) to yield plasma. Plasma was then stored at −20°C prior to analysis. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from the remaining blood sample using density gradient centrifugation (700 g, 25 min, 4°C) and cell density was determined using a haemocytometer. Isolated PBMCs were then washed in ice-cold PBS and centrifuged (700 g, 4 min, 4°C) three times to remove extracellular drug before extraction in methanol (1 mL, 60% v/v, 12 h). This initial extraction procedure was incorporated into the assay to ensure adequate removal of cell debris. Prior to methanol extraction, an aliquot of PBMCs (4–7 × 10<sup>6</sup> cells) was removed for determination of P-gp expression. Importantly, the time between blood sampling and methanol extraction was consistently ≤1 h and all sample processing within this time was carried out under ice-cold conditions to prevent drug loss. These samples were then centrifuged (2772 g, 4 min, 4°C) and the supernatant poured into glass tubes and dried using a rotary evaporator. Samples were stored as dried extracts at −20°C until analysis.

Total and unbound drug separation

Ultrafiltration was used to separate protein bound and unbound drug in plasma. Heat-inactivated plasma (800 µL) was injected into an Amicon Centrifree Filter System and centrifuged (1800 g; 60 min). A temperature of 37°C was maintained during centrifugation to prevent alterations in drug protein binding. The resulting filtrate (~220 µL/sample), which contained only the unbound drug, was removed for analysis.
Quantification of efavirenz

Quantification of total, unbound and IC efavirenz was carried out using HPLC with UV detection (HPLC-UV). Internal standard (IS; 20 μL, 10 μg/mL) was added to dried PBMC extracts (reconstituted in 200 μL distilled water), ultrafiltrate samples (200 μL) and plasma standards (200 μL, range 5–1000 ng/mL; note that the matrix did not alter the slope of the standard curve). IS (20 μL, 250 ng/mL) was added to heat inactivated (58°C, 40 min) plasma samples (200 μL) and standards (range 100–8000 ng/mL; 200 μL), Potassium carbonate (100 μL, 1 M) and ethyl acetate/n-hexane (50:50 v/v; 3 mL) were added to each tube and the samples tumbled for 30 min using a rotary mixer. Following centrifugation (3291 g; 6 min), the aqueous layer was frozen using a cryogenic bath, the organic layer transferred into clean glass tubes and evaporated to dryness. Dried extracts were then reconstituted in mobile phase (150 μL), vortexed thoroughly and transferred into autosampler vials ready for injection (100 μL) in the HPLC system. Recovery of efavirenz, after extraction from plasma, using this method is 98%.

Efavirenz and IS were resolved on a (5 μm; 250 × 4.6 mm) Hypersil Elite C18 column (Thermo Hypersil-Keystone, Runcorn, UK) with a pre-column guard (Si 60 × 5 μm; Merck, New Jersey, USA) with an HPLC system (Kontron Instruments Ltd, Hertfordshire, UK) using the mobile phase (10 mM ammonium acetate buffer/acetonitrile/methanol; 40/52/8; by vol.) at a flow rate of 1.2 mL/min. Efavirenz was eluted at 4.8 and 13.9 min, respectively. The peak areas for efavirenz and IS were quantified using the Chromelone (Version 6.5) data acquisition system ( Dionex Corporation, California, USA). The lower limit of quantification (LLQ) of efavirenz in plasma and ultrafiltrate were taken as the lowest point on the standard curves (100 and 5 ng/mL for the two standard curves). Intra-assay and inter assay coefficients of variation at 5 ng/mL were 10.8 and 14.9%, respectively. The quantification if IC efavirenz is dependent on cell number, therefore a minimum number of 20 × 10^6 cells were required from each sample. The laboratory also participates in an external quality assurance programme (KKG, The Netherlands).

Determination of P-gp expression

The aliquot of PBMCs saved for P-gp analysis was centrifuged (2772 g, 4 min, 4°C) and the cells fixed (CellFIX 1:10, 1 mL, 30 min, 4°C). PBMCs were then centrifuged (700 g, 4 min, 4°C) and the resulting pellet resuspended in PBS to give a cell density of 2 × 10^6 cells/mL. Aliquots of cell suspension (200 μL; 400 000 cells) were then incubated (30 min, 4°C) with primary antibodies; two aliquots with the monoclonal P-gp specific antibody, UIC2, (directed to an extracellular epitope18 2.4 μg/mL), two with IgG2a isotype control antibody (4.8 μg/mL) and one left as cells alone. Following primary antibody incubation, cells were washed (1 mL PBS, 4°C) and centrifuged (700 g, 4 min, 4°C) twice before incubation with the R-phycocerythrin-conjugated anti-IgG2a secondary antibody (2.0 μg/mL; 30 min, 4°C). Cells were then washed (1 mL PBS, 4°C) and centrifuged (700 g, 4 min, 4°C) twice, resuspended (CellFIX 1:10, 0.5 mL) and analysed by flow cytometry. It is important to note that validation experiments showed efavirenz did not alter UIC2 binding.

A Coulter Epics XL-MCL flow cytometer was used for sample analysis. Forward and side scatter were detected on a linear scale and fluorescence in the FL-2 channel detected on a logarithmic scale. A clear population of lymphocytes was electronically gated from the total PBMC population, using light scatter properties. For each sample 3000 events were collected within this gate, generating a histogram of FL-2 fluorescence. P-gp expression was analysed by subtracting the histogram generated with the isotype control antibody from that generated with the P-gp-specific antibody, using FCS express software. This was then expressed as % P-gp-positive cells.

Validation experiments

To estimate loss of cell-associated efavirenz during the cell isolation procedure, PBMCs from three single-donor buffy coats were incubated in efavirenz (10 μM (2663 ng/mL), 37°C 5% CO2, 18 h), then transferred into drug-free medium and maintained at either 4°C or 37°C for up to 60 min. PBMCs were then washed, incubated in methanol and dried as described above.

Drug loss resulting from efavirenz binding to the filter was quantified using filtered plasma from healthy volunteers, which was spiked with different concentrations of efavirenz. Efavirenz was quantified in these spiked ultrafiltrates before and after a second filtration step. The % recovery was then calculated.

To reduce the risk of HIV transmission in the laboratory, all plasma samples are heat inactivated (58°C, 40 min) prior to storage (–20°C) and analysis. The effect of heat inactivation and a single freeze–thaw cycle on total and unbound plasma concentrations and on the % unbound was investigated using blank plasma spiked with four concentrations of efavirenz (500, 1500, 3000 and 5000 ng/mL).

Patient samples were pooled, split into six aliquots, then filtered and assayed as six independent samples to assess variability associated with both filtration and efavirenz quantification. This was termed inter spin column variation.

Data analysis

IC concentrations (expressed as ng/mL) were calculated using the cell density of the sample and taking 0.4 μL as the volume of a cell (as determined by Gao et al.19).

The AUC0–24 was calculated over the dosing interval for total, unbound and IC exposure, using non-compartmental analysis (Topfit, Version 2.0; Gustav Fischer Verlag, Stuttgart, Germany). Owing to the long half-life of efavirenz, the time points over the dosing interval did not allow for accurate determination of half-life. To account for variability in efavirenz plasma concentrations, IC penetration of efavirenz was calculated as a ratio of IC AUC0–24 to plasma AUC0–24. The % unbound efavirenz was calculated as (unbound AUC0–24,total AUC0–24) × 100. Interindividual variability (% coefficient of variation; CV) was calculated as (standard deviation/mean) × 100.

Data exhibited no evidence of non-normality (Shapiro–Wilk test). Differences in drug adsorption to filters were tested using an ANOVA test with Bonferroni correction. This test was also used to investigate differences in % unbound efavirenz over the dosing interval.

Relationships between IC efavirenz and protein binding or P-gp expression were investigated using simple linear regression. All statistical analyses was carried out using the Arcus Quickstat Biomedical Software Version 1.1 (©1997).

Results

The mean ± SD percentage recovery of efavirenz following ultrafiltration was 71.0 ± 3.9, 70.6 ± 2.3, 74.9 ± 7.9 and 75.2 ± 4.3% for plasma concentrations of 500, 1500, 3000 and 5000 ng/mL, respectively. There was no difference in drug adsorption at these concentrations (P > 0.1 for all comparisons). In keeping with other protein binding studies,10,21,22 unbound concentrations were not corrected for adsorption. Heat inactivation, one freeze–thaw cycle or a combination of heat inactivation followed by a freeze–thaw cycle caused no alteration in total, unbound or % unbound efavirenz. Inter spin column variation was 12.1%. Heat inactivation and a single freeze–thaw cycle caused no alteration in total concentrations, unbound concentrations or % unbound efavirenz at plasma concentrations of 500, 1500, 3000 and 5000 ng/mL (P > 0.05 for...
Plasma and intracellular efavirenz in vivo

Figure 1. Retention of IC efavirenz (EFV) following incubation in drug-free medium at 4°C (open squares) and 37°C (open triangles). Data are expressed as mean percentage drug retained ± SD (n = 3).

Figure 2. Total (open squares), unbound (open triangles) and IC (open circles) concentrations of efavirenz (EFV) over the 24 h dosing interval. Data are expressed as mean ± SD (n = 10).

Table 1. Summary of parameters analysed

<table>
<thead>
<tr>
<th>Disease markers</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL (copies/mL)</td>
<td>1550 (1110–3330)</td>
</tr>
<tr>
<td>CD4 (cells/mm³)</td>
<td>279 (181–1057)</td>
</tr>
<tr>
<td>Efavirenz AUC₀₋₂₄</td>
<td>55 569 (36 805–131 913)</td>
</tr>
<tr>
<td>unbound efavirenz (ng·h/mL)</td>
<td>443 (213–607)</td>
</tr>
<tr>
<td>IC efavirenz (ng·h/mL)</td>
<td>74 421 (29825–176 920)</td>
</tr>
<tr>
<td>Efavirenz IC accumulation ratios</td>
<td></td>
</tr>
<tr>
<td>IC:total efavirenz</td>
<td>1.3 (0.7–3.3)</td>
</tr>
<tr>
<td>IC:unbound efavirenz</td>
<td>213 (49–618)</td>
</tr>
<tr>
<td>% Unbound efavirenz</td>
<td>0.6 (0.4–1.5)</td>
</tr>
<tr>
<td>weight (kg)</td>
<td>70 (51–88)</td>
</tr>
<tr>
<td>serum albumin (g/dL)</td>
<td>4.4 (3.7–4.9)</td>
</tr>
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VL, viral load; AUC₀₋₂₄, area under the concentration-time curve (from 0–24 h); IC, intracellular.
*Only 3/10 patients had detectable VL (>50 copies/mL).

association between IC efavirenz AUC₀₋₂₄ and P-gp expression (r² < 0.01, P = 0.98; Figure 3d). Similarly, no correlation was observed between P-gp expression and either the IC:total plasma efavirenz AUC₀₋₂₄ ratio (r² = 0.17, P = 0.24) or the IC:unbound efavirenz AUC₀₋₂₄ ratio (r² = 0.07, P = 0.47).

Discussion

As the site of action of efavirenz is inside the host cell, investigations of factors which may impact IC concentrations of efavirenz are important. The median IC accumulation ratio was 1.3, indicating that efavirenz concentrations are slightly higher inside (or associated with) the cell compared with in plasma. This is comparable to the accumulation ratio of 0.9 observed by Rotger et al.² Indeed, when comparing the values for total and IC AUC₀₋₂₄ determined in this study with those predicted using Bayesian calculations³ the data are similar, despite the methodological differences (HPLC-UV with a LLQ of 5 ng/mL versus LC-MS/MS with LLQ of 0.4 ng/mL).

Here we show that efavirenz is lipophilic (log P of 2.07) and highly plasma protein bound (median % unbound of 0.63%). Studies investigating the IC concentrations of the protease inhibitors have found a general hierarchy of IC accumulation, which is comparable to the hierarchy for lipophilicity and protein binding, suggesting that the physiochemical properties of a drug dictate its IC accumulation. Based on protease inhibitor data it might have been expected that efavirenz would accumulate to a similar extent as nelfinavir (5-fold) or saquinavir (3-fold), whereas efavirenz accumulation is actually similar to that reported with ritonavir (accumulation ratio of 1.3³).

The median % unbound efavirenz observed here in vivo is similar to that stated in the summary of product characteristics.²⁰ There was no relationship between % unbound efavirenz and serum albumin concentration (data not shown), although all albumin concentrations were within the normal range. It is possible that marked changes in albumin concentration, such as those associated with liver injury, may cause changes in the free fraction. Although there was a trend relating total and unbound plasma AUC₀₋₂₄, this
was not statistically significant. The % unbound of some highly protein bound protease inhibitors (lopinavir and amprenavir) have been reported to be concentration dependent in vivo, altering over the dosing interval.\textsuperscript{21,22} Here we present the first data describing the unbound fraction of efavirenz in vivo and suggest that efavirenz binding is not concentration dependent within the range of plasma levels observed over the dosing interval.

Protease inhibitors are weak bases and preferentially bind to $\alpha_1$-acid glycoprotein, whereas the NNRTIs are weakly acidic and predominantly bind to albumin.\textsuperscript{23} Total IC efavirenz concentrations, as measured in this study, are likely to be a composite of the drug that is both bound and unbound to IC proteins. However, characterization of IC binding proteins is needed and any differential binding of protease inhibitors and NNRTIs to IC proteins remains unclear. The ratio of IC efavirenz $\text{AUC}_{0-24}$ to unbound efavirenz $\text{AUC}_{0-24}$ was 213, indicating that over 200-fold more drug resides in the cellular compartment compared with the unbound efavirenz in plasma. It is likely that the unbound efavirenz in plasma equilibrates with unbound efavirenz inside the cell, thereby suggesting a high degree of IC binding. As yet there are no available techniques able to distinguish between bound and unbound drug within the cell.

Simple linear regression identified a statistically significant correlation between total plasma efavirenz $\text{AUC}_{0-24}$ and IC efavirenz $\text{AUC}_{0-24}$. A limitation of this study is its small sample size, and much larger studies are needed to address the crucial question of the relationship between IC concentrations of antiretrovirals and clinical response. However, this is difficult with full IC profiles given the amount of blood required at each time point. Rotger et al.\textsuperscript{3} chose to use one random time point to predict IC and plasma AUCs.

\[ r^2 = 0.59, \ P = 0.009 \]

\[ r^2 = 0.13, \ P = 0.75 \]

\[ r^2 = 0.51, \ P = 0.02 \]

\[ r^2 < 0.01, \ P = 0.98 \]

Figure 3. Relationship between (a) total efavirenz (EFV) $\text{AUC}_{0-24}$, (b) unbound efavirenz $\text{AUC}_{0-24}$, (c) % unbound efavirenz and (d) P-gp expression with IC efavirenz $\text{AUC}_{0-24}$. Data analysed by simple linear regression ($n = 10$).
Plasma and intracellular efavirenz in vivo

which makes a larger study population much more practical. However, there are potential problems when using ratios of single time points. Indeed, consensus statements following a round table discussion of sanctuary sites concluded that single time points may not be adequate to assess drug exposure in sanctuary sites and that the ratio of AUCs (sanctuary site:total plasma) is preferable. However, as we observed, similar findings to those predicted by Rotger et al., such as Bayesian calculations or the use of limited sampling strategy models, may be useful for further mechanistic studies.

Of interest is the complete contrast that is observed when considering the IC pharmacokinetics of the two NNRTIs, efavirenz and nevirapine. We recently reported a low IC accumulation ratio (0.005) of nevirapine, whereas here we observe an IC accumulation ratio of efavirenz that is ~250 times higher (1.3), even though similar lipophilicities were observed. It is therefore likely that these drugs have different affinities for active efflux and influx transporters and for binding proteins within the cell.

In summary, this study measured total, unbound and IC efavirenz AUCs, and the first data describing unbound efavirenz in plasma in vivo are presented. Future studies should aim to (i) develop a method for determining the IC localization of efavirenz, (ii) quantify the unbound efavirenz concentration within the cell and (iii) relate IC efavirenz concentrations to antiviral activity to give some indication of the amount of drug that is required inside the cell in order to inhibit viral replication.

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Transparency declarations

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References

