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Pharmacokinetics of the anti-HIV bicyclam SID791 (JM3100) in rabbits, as determined by both analytical and bio-assay methods

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Summary

The serum levels of the bicyclam derivative 1,1'-[1,4-phenylenebis(methylene)]-bis-1,4,8,11-etraazacyclotetradecane octahydrochloride dihydrate [SID791 (JM3100)], a potent inhibitor of HIV replication (De Clercq *et al.*, 1994) were determined in rabbits using two different methods. A method based on the UV-absorption of the Cu-complex of SID791 was used to analyse by HPLC the serum drug concentrations, and an antiviral activity bio-assay was performed to investigate whether the drug in the rabbit serum was in an available active form. After subcutaneous (sc) administration of SID791 to rabbits at 25 mg kg^{-1} of body weight, the compound was cleared from the serum in a bi-exponential manner (β1-half-lives: 67 and 69 min; β2-half-lives: 320 and 245 min; distribution volumes: 0.40 and 0.37 l; total body clearance: 0.30 and 0.29 l h^{-1}; and AUC: 83.3 and 86.2 h μg ml^{-1}, as determined by HPLC and bio-assay, respectively). Thus, very similar kinetic parameters were noted if serum drug concentrations were determined by HPLC analysis or bio-assay, suggesting *that in the rabbit serum the drug is present as an* antiviral active agent.

Key-words: bicyclam, bio-assay, HIV, pharmacokinetics

Introduction

Bicyclam derivatives which contain two monocyclam (1,4,8,11-tetraazacyclotetradecane) units, linked by an aliphatic bridge, have been identified as potent and selective inhibitors of the replication of human immunodeficiency virus (HIV) (De Clercq, 1992; De Clercq *et al.*, 1992; Yamamoto *et al.*, 1992). Further pursuing this lead, it was found that bicyclam derivatives in which the cyclam units are tethered by an aromatic linker [i.e. phenylenebis(methylene)] are even more potent and selective inhibitors of HIV replication *in vitro*. The prototype of this series [SID791 (JM3100)], has an EC_{50} (50% effective concentration) of 1 to 10 ng ml^{-1} in MT-4 cells without being toxic to the host cells at 500 μg ml^{-1} (De Clercq *et al.*, 1994). In primary T4 lymphocytes or primary monocytes, SID791 proved inhibitory to HIV-1(III_B) and several clinical HIV-1 isolates at an EC_{50} of less than 1 ng ml^{-1}. SID791 was also found to interfere directly with virus-induced syncytium formation, albeit at a higher concentration (1–2 μg ml^{-1}) than that required for inhibition of viral replication. Following subcutaneous injection of SID791 at 10 mg per kg of body weight to rabbits, serum drug levels exceeded for at least 6 h by >100-fold the EC_{50} required for inhibition of HIV replication *in vitro* (De Clercq *et al.*, 1994). The extensive binding of SID791 to plasma proteins (data not shown) had raised the concern that in the serum the drug may not be sufficiently available in an antivirally active form. In this study the pharmacokinetics of SID791, following subcutaneous administration to rabbits at 25 mg kg^{-1} was monitored by both HPLC analysis and antiviral activity (bio-assay) measurements.

Results

SID791 was not detected by either of the above methods in rabbit serum after peroral (po) administration of a dose of 25 mg kg^{-1}. Pharmacokinetic parameters and serum drug concentration profiles after sc administration of SID791 into rabbits are shown in Table 1 and Fig. 1. When SID791 was administered sc to rabbits at a dose of 25 mg kg^{-1}, the serum peak drug concentrations were 36.8 and 33.3 μg ml^{-1}, as determined by HPLC and bio-assay, respectively. The serum drug concentrations decreased to 1 μg ml^{-1} after 8 h. The 24 h values were near the detection limits of both methods, being 0.15 μg ml^{-1} for HPLC and 0.25 μg ml^{-1} for the bio-assay. Concentrations *vs* time data were fitted to a bi-exponential

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Method	C_{max} ($\mu g\ ml^{-1}$)	t_{max}	$t_{1/2}\beta1$ [a] (min)	$t_{1/2}\beta2$ [b] (min)	V_D (l)	Cl_t (l serum h^{-1})	AUC (h $\mu g\ ml^{-1}$)
HPLC	36.8	30	67	320	0.40	0.30	83.3
Bio-assay	33.3	60	69	245	0.37	0.29	86.2

Table 1. Pharmacokinetic parameters of SID791 (JM3100) after sc administration at a dose of 25 mg kg^{-1} to rabbits

Pharmacokinetic parameters were calculated from the serum concentrations measured by HPLC analysis and by an anti-HIV activity bio-assay. SID791 concentrations in rabbit *vs* time after administration were fitted to bi-exponential functions ($C = A.e^{-\beta1.t} + B.e^{-\beta2.t}$) using log-linear regression analysis (Niazi, 1977; Naesens *et al.*, 1992). The elimination rate constants ($\beta1$ and $\beta2$) were derived from the slopes of these linearized curves; the half-life ($t_{1/2}$), distribution volume (V_D), total body clearance (Cl_t) and AUC (area under the curve) were calculated from the formulae: $t_{1/2} = 0.693/\beta$, V_D = Dose/A+B, $Cl_t = (\beta1 + \beta2) \times V_D$ and AUC = Dose/Cl_t.
[a]Half-life of the $\beta1$ elimination phase.
[b]Half-life of the $\beta2$ elimination phase.

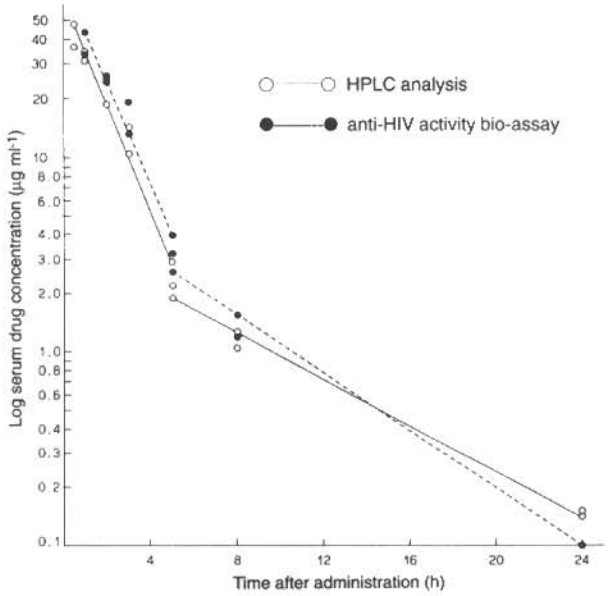

Fig. 1. Serum drug concentration following subcutaneous administration of SID791 (JM3100) (25 mg kg^{-1}) to rabbits. Serum SID791 concentrations were determined by HPLC analysis and by measuring their inhibitory effect of HIV-1 (NL43)-induced cytopathicity in MT-4 cells (anti-HIV activity bio-assay). Data represent mean values of two and three separate HPLC and bio-assay determinations, respectively. Fitted HPLC analysis data (○—○), fitted anti-HIV activity bio-assay data (●—●).

function ($C = A.e^{-\beta1.t} + B.e^{-\beta2.t}$) to calculate the elimination rate constants, serum half-lifes, distribution volume, total body clearance and area under the curve (AUC). The serum concentrations of SID791 after sc administration showed a bi-exponential elimination phase and were detectable up to 24 h after administration (Fig. 1). The half-lives of the $\beta1$ elimination phase analysed by HPLC and bio-assay were 67 and 69 min, respectively. The distribution volumes were 0.40 and 0.37 l, with total body clearance of 0.30 and 0.29 l serum h^{-1}, determined by HPLC and bio-assay, respectively. The AUC for SID791 after a sc dose of 25 mg kg^{-1} to rabbits were 83.3 and 86.2 h $\mu g\ ml^{-1}$, as determined by HPLC and bio-assay, respectively. The data for sc SID791 administration fitted well to bi-exponential functions, the correlation coefficient of the linear regression curves being higher than 0.959.

Discussion

The bicyclam SID791 (JM3100) is a potent inhibitor of HIV replication *in vitro* with a selectivity index ≥100 000. The pharmacokinetic properties of SID791 in rabbits by HPLC analysis and by an antiviral activity bio-assay was investigated. The extensive binding of SID791 to plasma protein (data not shown) raised the concern that sufficient drug might not be available in serum to block virus relication. These studies show that sc administration of SID791 to rabbits at a 25 mg kg^{-1} dose resulted in very similar kinetics, whether the serum drug concentrations were determined by HPLC analysis or bio-assay, thus suggesting that the drug appears in the rabbit serum in a biologically active form within the detectable concentration range.

Antiviral activity was detected in the serum at drug levels that exceeded the EC_{90} by > 35-fold and the EC_{50} by > 100-fold for at least 8 h (Fig. 1). The serum concentrations of SID791 after sc bolus injection in rabbits showed a bi-exponential elimination phase with a $\beta1$ elimination half-life of approximately 1 h. This could expand to significantly longer time in man. SID791 was still detectable up to 24 h after administration. In addition to the half-lifes, the distribution volumes, the total body clearance and AUC for SID791 (following sc administration of a 25 mg kg^{-1} dose to rabbits), were calculated. Most of AUC was due to the $\beta1$ elimination phase (Fig. 1). The pharmacokinetic parameters were very similar whether determined by HPLC or bio-assay. Thus, the kinetic parameters of the drug, as determined by HPLC analysis, reflected those obtained by the antiviral activity bio-assays. These data indicate that, despite the extensive binding of SID791 to plasma protein, the compound remains fully available for effecting its anti-HIV activity.

In contrast with the data obtained for sc administration, SID791 could not be detected by HPLC or bio-assay in the rabbit serum following po administration of a 25 mg kg^{-1} dose. This observation points to the low oral bio-availability of SID791. Further structural modifications of this class of compounds will be attempted to improve the oral bio-availability.

Materials and Methods

Compound

The bicyclam derivative 1,1'-[1,4-phenylenebis-(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride dihydrate (molecular weight, 830) [SID791 (JM3100)] was synthesized at Johnson Matthey (Pharmaceuticals Research, Pennsylvania, USA), following procedures that are described elsewhere (Bridger *et al.*, 1995).

Cells

MT-4 cells [human T-lymphoblastoid cells carrying human T-lymphotropic virus type 1 (HTLV-I) (Harada *et al.*, 1985)] were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and gentamicin (20 μg ml^{-1}).

Virus

A molecular clone of HIV-1 (NL43) was obtained from the culture supernatant of an infected MT-4 cell line and stored in small aliquots at –80 °C until used. Virus stock titres were 10^5 $CCID_{50}$ (50% cell culture infective dose) per ml.

Rabbit experiments

SID791 was administered subcutaneously (sc) or perorally (po) at a single dose of 25 mg (based on the molecular weight of the salt form) per kg body weight to rabbits (Leuven Animal Production Center, Leuven, Belgium). At different times after injection (0.25, 0.5, 1, 2, 3, 5, 8 and 24 h) the blood was collected from the ear vein at room temperature and kept at 4 °C for a period of 12 h, after which the serum was collected. The samples were stored at –20 °C until assayed.

HPLC analysis

Sample preparation. Serum samples (200 μl) were mixed with 50 μl saturated NaOH solution, and extracted with 1 ml water-saturated t-butylmethylether in a 2.1 ml polypropylene centrifuge vial, under frequent mixing over a period of 2 h. These were kept overnight and centrifuged. Then, 900 μl of the ether phase were transferred to a second 2.1 ml vial and backextracted for 1 h under frequent shaking with 900 μl 2% trifluoracetic acid (TFA). Of the TFA phase 800 μl were transferred to a 1.2 ml centrifuge vial and dried in a SpeedVac centrifuge. The residue was dissolved in 50 μl water and 10 μl 0.25 M copper(II) acetate and heated at 65 °C for 15 min, to convert the compound into its stable and strongly UV-absorbing copper complex. Aliquots of 50 μl were injected manually into the HPLC system.

The HPLC system. The HPLC system consisted of the following components: column Merck LiChrospher 60 RP18 Select B 5 μm, 250 × 4 mm + 4 × 4 mm precolumn (Merck, Darmstadt, Germany), Waters 590 pump, Waters U6K injector, Kratos Spectroflow 757 UV photometer and a Carbo Erba Mega series computing integrator. Solvent A was HPLC grade acetonitrile, solvent B a 0.1% (v/v) solution of trifluoroacetic acid in HPLC grade water. The mobile phase consisted of a (v/v) mixture of 13.5% solvent A and 86.5% solvent B. Isocratic runs of 15 min were made with a flow of 1 ml min^{-1} at ambient temperature. SID791 was detected as its copper complex by UV at 266 nm, 0.005 absorption unit full scale (AUFS). The complex eluted as a group of four incompletely resolved peaks after 5.6–6.7 min, the peak height of the largest (2nd) peak of the group was used for quantitation. The system was calibrated by a series of external standards. Blank rabbit plasma samples (200 μl) were spiked with stock solutions of SID791 in the range of 0.2–50 μg ml^{-1} or 0.1–20 μg ml^{-1}, and then extracted and analysed by the described procedure. Either plasma or serum can be used for the preparation of the standards, giving similar results within the precision of the method. Calibration curves for SID791 were calculated by linear regression analysis. SID791 extraction yields from serum samples were about 30%, the detection limit in 200 μl serum samples was about 0.1 μg ml^{-1}. The relative standard deviation (RSD) of the HPLC system precision (manual injection, quantitation by peak height) was 3–6%, determined for injections in the range of 20 to 10 μg ml^{-1}. The RSD of SID791 after complete sample work up and analysis (method precision) was in the range of 8–10% at concentrations of 1–50 μg ml^{-1}, 12% at 0.5 μg ml^{-1} and 22% at 0.2 μg ml^{-1}. The standard curve was linear ($r^2 = 0.997$), with standard deviation of regression = 1.23, y-intercept a = –0.4 with standard deviation 0.64 (95% confidence limits include zero), regression coefficient b = 0.0108 with standard deviation 0.0003.

Antiviral assay of serum samples

The compound concentrations in the serum were determined by a bio-assay based on the 50% effective concentration (EC_{50}) of SID791 required to inhibit HIV-1 (NL43)-induced cytopathicity in MT-4 cells (Witvrouw *et al.*, 1990) with an EC_{50} of 0.01 μg ml^{-1}. Briefly, 50 μl containing 100 $CCID_{50}$ of HIV-1 (NL43) were transferred to microtitre tray wells containing various dilutions of the serum samples (highest dilution 1/50). MT-4 cells were suspended in culture medium at 6×10^5 cells ml^{-1} and added to microtitre tray wells (50 μl cell suspension per well). After a 5 day incubation at 37 °C, the number of viable cells was assessed by the MTT [3'-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method as previously described (Pauwels *et al.*, 1988). Concentrations of SID791 in the rabbit serum samples were determined by comparison of their EC_{50} with the EC_{50} values of the compounds tested as such or in the presence of approximate dilutions of control serum. The presence of rabbit serum had no significant influence on the recovery of biologically active drug (data not shown).

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