Human Pharmacokinetics of a New Vinca Alkaloid S 12363 with Use of a Monoclonal Antibody-based Radio- or Enzyme Immunoassay


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ABSTRACT

S 12363 is a new Vinca alkaloid derivative, characterized by the grafting of an a-aminophosphonate, onto the Vinca nucleus, facilitating drug penetration and increasing intracellular drug retention. As a high cytotoxic activity had been demonstrated in vitro and in vivo models recommended by the National Cancer Institute, a phase I trial was initiated in cancer patients.

In order to quantify S 12363 systemic levels in humans, two monoclonal antibody-based immunoassays, RIA (radio-) and EIA (enzyme immunoassay) were developed. The γ-emitting probe used in the RIA, 125I-(deacetyl-O4-vinblastine)-tyramine, bound very tightly to the monoclonal antibody (dissociation constant, \(K_d = 2.5 \times 10^{-11}\) M), demonstrating a high affinity mainly directed toward the catharanthein fraction (vindeine, vincristine, vinblastine, 100% cross-reactivity; vinorelbe, 0.3% cross-reactivity). In the EIA, a deacetyl O4-vinblastine Ovalbumine conjugate was used as the competing antigen. Its binding to the monoclonal antibody was revealed by an anti-mouse immunoglobulin G conjugated to biotin which interacts with streptavidin labeled with alkaline phosphatase. This method permitted obtaining nearly the same sensitivity and reproducibility with EIA as with RIA, their respective minimum quantitation limits being 0.100 and 0.040 ng/ml (106 and 42 pM) of S 12363 in plasma. These assays allowed the study of S 12363 systemic pharmacokinetics in cancer patients during a phase I trial up to 72 h after dosing. As determined by RIA, the S 12363 plasma profile was triphasic with a terminal half-life; \(t_\text{1/2} = 49 \pm 16\) h, a plasma clearance, \(CL = 0.14 \pm 0.04\) liter/h/kg, and a volume of distribution at steady state, \(V_d = 5.0 \pm 2.8\) liter/kg.

The pharmacokinetics of S 12363 is linearly related to dose when increased from 0.08 up to 0.84 mg/m² in humans. Its plasma profile and pharmacokinetic parameters are close to those of other Vinca alkaloids with clearance and terminal half-life being intermediate between those of vinblastine and vincristine. Therapeutic doses are 4 to 10 times lower and should be a direct consequence of the higher uptake and retention by the cells of this new aminophosphonate Vinca alkaloid derivative.

INTRODUCTION

Vinca alkaloids are an important class of antitumor agents, on which minor chemical modifications led to molecules with different antitumor spectrum, potency, and toxicity. Recently, amino acid derivatives have been grafted onto vinblastine so as to facilitate transport of these large molecules.

In this way, S 12363 is an a-aminophosphonate derivative of vinblastine which has demonstrated a high preclinical antitumor activity against a spectrum of murine and human tumors (2). This new Vinca alkaloid compound was found to be 72- and 36-fold more cytotoxic in the in vitro panel system than were VBL and VCR, respectively, the in vivo antitumor activity being at least equivalent to that of VCR and VBL but at doses 10 to 40 times lower. By contrast, S 12363 which differs only by the configuration of the asymmetric carbon atom of the side chain was 300-fold less cytotoxic and 1000-fold less potent in vivo than S 12363. This result has been related to the stereoselective uptake of S 12363 demonstrated in human tumor cells (3), which, together with an increased intracellular retention, may improve the Vinca alkaloid activity critically (4).

In this paper we describe the characteristics of a RIA and an EIA which both used a monoclonal antibody (Mab) for the measurement of S 12363 systemic levels in cancer patients during a phase I clinical trial. The performances of both assays, in terms of sensitivity and specificity toward other analogue and potential metabolites, are discussed. The results of the preliminary pharmacokinetic study in humans are presented.

MATERIALS AND METHODS

Drugs and Reagents. S 12363, the (1S)-1-(3-deacetyl-3-demethoxy carbonylvincaleukoblastinylcarbonylamino)-2-methylpropylphosphonic acid diethylester sulfate (Fig. 1); S 12362, the (1R) epimer (5), were provided by the Institut de Recherche Servier (Suresnes, France), as well as the synthesis intermediates, impurities, and potential metabolites.

Chemicals were purchased from Sigma Chemical Co. (St Louis, MO) or Merck (Darmstadt, Germany), and were all of analytical grade.

Immunoassay. The hapten of S 12363 (deacetyl-O4-vinblastinoyl-23)carboxyhydrate, was synthesized as described by Bhushana Rao et al. (1), and then conjugated to BSA according to Rahmani et al. (6). This immunogen (Fig. 1) was purified by gel filtration on Sephadex G25 (Pharmacia PD10, Uppsala, Sweden). By spectrometric analysis, the molar ratio of hapten molecules to BSA was calculated to be 6:1. Two 6-week-old BALB/c mice received i.p. injections of 0.1 mg of conjugate at days 0, 8, 41, 53. Then, another 0.1 mg conjugate i.p. injection was repeated 4 days before cell hybridization.

Monoclonal Antibody. Immunospleen cells were fused with P3/NS-1/Ag4-1 mouse myeloma cells according to conventional procedures (7). Culture supernatants were screened for production of anti-S 12363 antibody by EIA, coating the deacetyl-O4-vinblastine/ovalbumin conjugate (Fig. 1) onto the solid phase; the specific binding was revealed by horseradish peroxidase-conjugated anti-mouse immunoglobulin (Amersham, England). Among the 3 positive monoclonal hybridomas, which were subsequently grown as ascites in pristane-primed BALB/c mice, one was selected for further immunoassay development.

Apparatus. EIA was performed by using a Biomek 1000 automated laboratory workstation (Beckman Instrument, Palo Alto, CA) which includes a plate washer, a liquid handling facility, a photometer, and a PC-driven control unit (IBM, Greenock, United Kingdom). To optimize RIA and to prepare plasma standards or controls by serial dilutions, a processor-controlled sample distributor TECAN 505 (B. A L Berthold, Elancourt, France) was used.

Radioligand. To increase the sensitivity of this RIA, a 125I-labeled probe with a high specific activity was chosen. S 12363 itself cannot be directly labeled with iodine because of the lack of a phenolic group in the molecule. Therefore, a hapten derived from S 12363, the (deacetyl-O4-vinblastinoyl-23)-carboxyhydrate, was reacted with a tyramine, as described by Rahmani et al. (9). The resulting deacetyl-O4-vinblastine/tyramine conjugate was then purified by high pressure liquid chromatography on a C8 Nucleosil column (5 \(\mu\)m), and the elution was performed with trifluoroacetic acid/water/methanol (0.2:45:55, v/v) solution.

This conjugate was iodinated with chloramine T, according to the method of Hunter and Greenwood (10). Na125I (Amersham): 37 MBq (1 mCi) were added to 20 \(\mu\)l (4 \(\mu\)g) of deacetyl-O4-vinblastine/tyramine conjugate and 10 \(\mu\)l (5 \(\mu\)g) of chloramine-T in 0.5 \(\mu\)l phosphate buffer (pH 7.5). The reaction was stopped after 1 min by adding 10 \(\mu\)l (50 \(\mu\)g) of sodium metabisulfite solution.

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The abbreviations used are: VBL, vinblastine; VCR, vincristine; RIA, radioimmunoassay; EIA, enzyme immunoassay; Mab, monoclonal antibody; BSA, bovine serum albumin; L, low level; H, high level; AUC, area under the curve; CL, plasma clearance; CV, coefficient of variation; HPLC, high performance liquid chromatography.
phosphate buffer. The reaction mixture was chromatographed in trifluoroacetic acid/water/methanol (0.2:45:55, v/v) solution on a C₁₈ Nucleosil column (5 μm). The fractions containing the monoiodinated derivative were stored in methanol (90 μCi/ml) at −80°C for up to 2 months.

Radioimmunoassay Procedure. The anti-S 12363 antibody solution was diluted to 1:150,000 or 1:30,000. H-RIA or L-RIA), in 5 ml polypropylene tubes. All samples were assayed in triplicate.

Enzyme Immunoassay Procedure. The hapten of S 12363 [(deacetyl-O⁻⁴-vinblastinoyl-23) carboxyhydrazide] was covalently coupled to ovalbumin by using the procedure previously described for the preparation of the immunogen; the molar ratio of hapten molecules to ovalbumin was calculated to be 2:1. The resulting conjugate (Fig. 1) was used as the solid phase-bound antigen for the EIA. Microtiter plates (Maxisorp, NUNC, Denmark), were coated, just before use, with 200 ng of this conjugate in 200 μl of 0.05 M bicarbonate buffer (pH 9.6) per well, for 18 h at 37°C. Plates were then washed (washing solution: 0.9% NaCl/0.05% Tween 20; 300 μl/well, 4 consecutive times) and filled with 5% gelatin in 0.1 M phosphate buffer (pH 7) in order to block unoccupied sites. After 1 h of incubation at 37°C and washing, 50 μl of standard, blank, or unknown sample were dispensed into the wells, together with 50 μl of anti-S 12363 antibody at the working dilution, 1:250,000-fold or 1:100,000-fold, respectively, for L-RIA or H-RIA, in 0.5% BSA/0.1 M phosphate buffer (pH 7). The plates were incubated for 18 h at 4°C and washed before the biotinylated sheep anti-mouse immunoglobulin conjugate (Amersham) diluted in 0.5% BSA/0.1 M phosphate buffer (pH 7) (1:1000, 100 μl/well) was added, and incubation continued for 1 h at 37°C. After washing, the conjugate: streptavidin-alkaline phosphatase (Amersham) diluted in 0.1 M Tris/0.5% BSA buffer (pH 8) (1:1000 for L-EIA or 1:5000 for H-EIA; 100 μl/well) was added and the plates were incubated for 1 h at 37°C. Then the plates were washed and the enzymatic substrate, p-nitrophenyl phosphate solution (1 mg/ml, 100 μl/well) in 0.1 M diethanolamine buffer (pH 9.8) was dispensed into the wells. The reaction was stopped after 45 min at 37°C by adding 100 μl of NaOH (2N)/well and the absorbance at 415 nm (reference, 540 nm), was measured (Biomek 1000, Beckman Instruments).

Two standard curves were prepared for low and high level radio- and enzyme immunoassay procedures. The standard solutions were daily prepared from a S 12363 aqueous stock solution (0.1 mg/ml) by appropriate dilutions with citrated blank human plasma (Centre de Transfusion Sanguine, Orléans, France). The S 12363 concentration in the standard solutions ranged from 0.025 to 1 ng/ml for L-RIA or L-EIA, from 1 to 20 ng/ml for H-RIA and from 1 to 10 ng/ml for H-EIA.

The nonspecific binding was measured in the absence of the antibody and subtracted from bound (B) and from control binding (B₀). The standard curves representing the B-NSB/B₀-NSB ratio versus the logarithm of the concentration of S 12363 (ng/ml) were smoothed spline log-fit in order to overcome the oscillating behavior to exact or interpolating splines (RIA, Cobra software, Packard Instrument Co., CT; EIA, Immunofit, Beckman Instruments).

Patients. Patients entered in the multicenter phase I trial were between 18 and 75 years old, with a proven malignant disease which had not responded to conventional therapy. Patients were free from previous treatments for a period of at least 4 weeks, although for some specific treatments with Vinca alkaloids, nitrosoureas, cisplatin, or radiotherapy, a minimum period of 6 weeks was used. Each patient had a detailed medical history taken and a thorough examination. Each one gave signed informed consent before entry into the study.

Dosing, Collection of Samples, and Analysis. The i.v. doses of S 12363 were given as a bolus dissolved in isotonic saline over a period of not more than 5 min at increasing dose levels (Fibonacci scale) of between 0.12 and 0.84 mg/m². Blood samples were collected at selected time intervals (from 3 min to 72 h) into heparinized tubes. Plasmas were stored at −20°C until analysis and S 12363 concentrations were measured by using the RIA described above.

Pharmacokinetic Analysis. Each of the plasma profiles was fitted to a polynoexponential equation, using a weighted interactive least squares curve fitting program (11) written for the Hewlett Packard 1000 microcomputer. The selection for the 3-compartment model over the others was performed according to the criterion of Akaike et al. (12).

The plasma half-lives (t₁/₂a, t₁/₂B, t₁/₂y) of S 12363 were calculated by direct integration and extrapolation to infinity, the plasma clearance (CL) and the initial (V₁) and steady state (Vₙ) volumes of distribution were determined. Dose dependence was tested by linear regression of AUC versus dose and the slope of the regression line was tested for significance from zero.

Fig. 1. Chemical structures of S 12363, immunogen, radioligand, and ligand used for immunoassay development.
RESULTS
The monoclonal antibody issued from the screening demonstrated a high affinity for the S 12363 analogue used as the radioligand, with a dissociation constant $K_d$ of $2.5 \times 10^{-11}$ M. The binding inhibition of the radio- or enzymolabeled probes to the Mab by various Vinca alkaloid derivatives (vincristine, vinblastine, vindesine, and vinorelbine) and by a series of compounds structurally similar to S 12363 was investigated (Table 1). As expected, all modifications on the catharanthine moiety (13) resulted in a considerable loss of immunoreactivity (vinorelbine, Y 269: N-oxide metabolite of S 12363, Y 295, Y 296). By contrast, modifications on the vindoline moiety (vinblastine, vincristine, vindesine) and on the $\alpha$-aminophosphonate chain in position C 23 (S 12362, Y 346, Y 347), had much lower effects. These results tend to demonstrate that the Mabs epitope is mainly directed toward the catharanthine moiety of the molecule. This is easily explained by the structure of the immunogen obtained by activation of S 12363 in position C 23 before conjugation to BSA. These results are in accordance with the previous studies of Rahmani et al. (6, 9), Hacker et al. (14), and Pontarotti et al. (15), using the same strategy for obtaining the immunogen.

Both methods allowed obtaining a working range from 0.025 to 10 or 20 ng/ml, the quantitation limit being 0.040 ng/ml (CV = 12.8%; n = 6) and 0.100 ng/ml (CV = 11.3%; n = 8) of S 12363, with RIA and EIA methods, respectively. Precision and accuracy have been demon-

Table 1 Cross-reactivity of S 12363 analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Nature of substituent</th>
<th>Cross-reactivity factor* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 12363</td>
<td>$\text{N} \quad \text{COOCH}_3$</td>
<td>$\text{H}$</td>
</tr>
<tr>
<td>Y 269</td>
<td>$\text{N}$</td>
<td>$\text{H}$</td>
</tr>
<tr>
<td>Y 295</td>
<td>$\text{N}$</td>
<td>$\text{COOCH}_3$</td>
</tr>
<tr>
<td>Y 296</td>
<td>$\text{N}$</td>
<td>$\text{H}$</td>
</tr>
<tr>
<td>S 12362</td>
<td>$\text{N}$</td>
<td>$\text{COOCH}_3$</td>
</tr>
<tr>
<td>Y 346</td>
<td>$\text{N}$</td>
<td>$\text{COOCH}_3$</td>
</tr>
<tr>
<td>Y 347</td>
<td>$\text{N}$</td>
<td>$\text{COOCH}_3$</td>
</tr>
</tbody>
</table>

* The compounds were tested with the anti-S 12363 by RIA and ELISA and their 50% inhibitory concentration values were compared with that of S 12363.

* Known in vitro N-oxide metabolite of S 12363.

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Table 2: Reproducibility interassay of RIA and EIA methods on human plasma control samples (n = 8)

<table>
<thead>
<tr>
<th>Theoretical concentrations of plasma control samples (ng/ml)</th>
<th>Method</th>
<th>Mean (ng/ml)</th>
<th>CV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.100</td>
<td>L-EIA</td>
<td>0.097</td>
<td>11.3</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>L-RIA</td>
<td>0.100</td>
<td>16.0</td>
<td>0</td>
</tr>
<tr>
<td>0.400</td>
<td>L-EIA</td>
<td>0.404</td>
<td>11.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>L-RIA</td>
<td>0.429</td>
<td>12.6</td>
<td>7.3</td>
</tr>
<tr>
<td>2</td>
<td>H-EIA</td>
<td>1.98</td>
<td>9.1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>H-RIA</td>
<td>1.95</td>
<td>14.4</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>H-EIA</td>
<td>3.95</td>
<td>8.4</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>H-RIA</td>
<td>4.23</td>
<td>13.7</td>
<td>5.8</td>
</tr>
<tr>
<td>6</td>
<td>H-EIA</td>
<td>5.74</td>
<td>12.2</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>H-RIA</td>
<td>8.43</td>
<td>17.4</td>
<td>5.4</td>
</tr>
</tbody>
</table>

The RIA and EIA methods were then compared to a selective HPLC method using fluorimetric detection (quantitation limit, 0.300 ng/ml). The concentrations of S 12363 measured on the 19 different clinical samples simultaneously by HPLC, RIA, and EIA are not statistically different at the 5% threshold level (one way analysis of variance with repeated measurements, P = 0.14). When the three analytical methods were compared, regression lines and correlation coefficients were the following: HPLC/RIA = 1.081 ± 0.018 × −0.125 ± 0.238, R = 0.998; HPLC/RIA = 1.285 ± 0.042 × −0.580 ± 0.553, R = 0.991; EIA/RIA = 0.83 ± 0.025 × −0.47 ± 0.41, R = 0.993. In the three cases correlation coefficient was significantly different from zero (P < 0.001).

The i.v. dosing of very low doses of S 12363 (0.08 to 0.84 mg/m²) and its fast and extensive distribution within the body lead to very low plasma levels which were difficult to quantify in the last decay phase. Of 30 administrations, 28 plasma profiles were best fitted to a triphasic model of decay and 2 to a biphasic one. A rebound of the concentrations was frequently noticed 1 to 2 h after the i.v. bolus of S 12363 and/or 24 h after the dosing and prevented the modeling for 2 of the administrations. Overall, the plasma profile of S 12363 was triphasic (Fig. 2) with a terminal half-life of about 50 h. The mean pharmacokinetic parameters and standard deviations calculated from the profiles resulting from blood sampling up to a minimum of 72 h after dosing (Table 3) were the following: t½α = 0.05 ± 0.02 h; t½β = 2.0 ± 1.1 h; t½γ; 49 ± 16 h; CL = 153 ± 59 ml/min (0.14 ± 0.04 liter/h/kg); Vdss = 316 ± 149 liter.

Dose dependence was assessed by linear analysis following 16 administrations (first course) using a ln/ln linear model and gave the linear regression equation ln(AUC) = 1.26 ± 0.25 ln(dose) + 1.98 ± 0.10. The regression coefficient R of 0.81 demonstrated that AUC and dose are dependent (P < 0.001) with the slope not being significantly different from unity. Thus the pharmacokinetics appear to be linear within the range of doses studied (0.08 to 0.84 mg/m²).

DISCUSSION

We have described here the production and characterization of a monoclonal antibody, which has been used successfully in the EIA and RIA techniques developed for the measurement of S 12363 systemic levels in cancer patients.

The dissociation constant of the antibody used here is similar to those obtained by Pontarotti et al. (15) with “anti-Vinca” Mabs derived from mouse × rat hybridoma, using the same haptenic structure and a similar radiolabeled ligand. The strong affinity of this antibody for the Vinca alkaloid nucleus allowed the quantitation of S 12363 in plasma when administered as an i.v. bolus (dose, 0.24 to 0.84 mg/m²), even during the last plasma decay phase, with, as a consequence, a satisfactory determination of the terminal half-life of the molecule. The minimum quantitation limits being, respectively, 0.040 and 0.100 ng/ml, for RIA and EIA, are similar to those previously reported by Rahmani et al. (9), using a rabbit antiserum together with a γ-emitting probe for vindesine sulfate or VBL assay.

Table 3: Pharmacokinetic parameters of S 12363 in cancer patients during phase I trial (sampling ≥ 72 hours)

<table>
<thead>
<tr>
<th>Patient</th>
<th>TOU</th>
<th>DUF</th>
<th>MEG</th>
<th>GENI</th>
<th>MOR</th>
<th>MORL</th>
<th>LOR</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCo (ng.h/ml)</td>
<td>42.2</td>
<td>98.2</td>
<td>48.8</td>
<td>68.4</td>
<td>45.3</td>
<td>146.4</td>
<td>150.8</td>
<td></td>
</tr>
<tr>
<td>CL (ml/min)</td>
<td>82</td>
<td>205</td>
<td>146</td>
<td>236</td>
<td>102</td>
<td>145</td>
<td>153 ± 59</td>
<td></td>
</tr>
<tr>
<td>CL (liter/h/kg)</td>
<td>0.096</td>
<td>0.177</td>
<td>0.179</td>
<td>0.170</td>
<td>0.080</td>
<td>0.116</td>
<td>0.14 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Vl (liter)</td>
<td>0.7</td>
<td>7.1</td>
<td>3.6</td>
<td>1.3</td>
<td>6.2</td>
<td>9.6</td>
<td>4.3 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Vdss (liter)</td>
<td>120</td>
<td>317</td>
<td>491</td>
<td>467</td>
<td>177</td>
<td>321</td>
<td>316 ± 149</td>
<td></td>
</tr>
<tr>
<td>Vds5 (liter/kg)</td>
<td>2.4</td>
<td>4.6</td>
<td>10.0</td>
<td>5.6</td>
<td>2.3</td>
<td>4.8</td>
<td>5.0 ± 2.8</td>
<td></td>
</tr>
</tbody>
</table>
The specificity of the antibody is mainly directed toward the catharanthine moiety of the Vinca nucleus, as described for the rabbit antiserum, previously used in Vinca alkaloid RIAs (9, 16). The correlation of the RIA and EIA Mab based assays with a specific HPLC method developed for the measurement of S 12363 plasma levels, indicates its suitability for S 12363 drug monitoring. Despite this, the potential metabolic products issuing from vindoline substitutions, especially those at positions 1, 3, or 4, could not be distinguished from the parent drug with these RIA or EIA methods, the cross-reactivity factor being about 100% with vincristine, vindesine, and vinblastine. Moreover, beyond 6 h following injections, when plasma levels are below the limit of quantitation by the HPLC method, the specificity of RIA and EIA could not be fully validated regarding hypothetic late-appearing metabolites. However, Y 269 (the known in vitro metabolite of S 12363) does not cross-react. Elsewhere, the cross-reactivity with other classical Vinca alkaloids is not a true limitation to the use of these assays for human pharmacokinetic monitoring of these compounds, different Vinca alkaloids never being associated in a polychemotherapy.

The human pharmacokinetics of S 12363 are very similar to those of vincristine and vindesine with a relatively long terminal half-life. This long \( t_{\text{1/2}} \) of S 12363 fails to support the hypothesis postulating that neurotoxicity exhibited by some of the Vinca alkaloids is related to their long half-life (17), since S 12363 shows very minor neurotoxicity. In fact, the dose-limiting toxicity observed here is haematological, and taking advantage of the linear relationship between dose and AUC, a pharmacodynamic \( E_{\text{max}} \) model could be developed (18).

In that way, the RIA and EIA developed here, together with the pharmacokinetic study included in the phase I trials, provide a valuable tool for helping in dosing adjustment in future studies.

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