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Phase I clinical study of LL-D49194α1 with retrospective pharmacokinetic investigations in mice and humans

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Summary. LL-D49194α1 is a new cytotoxic antibiotic selected for clinical phase I study because of its impressive pre-clinical anti-tumour activity and its low toxicity profile in experimental animals. A total of 15 patients were treated in centres in Glasgow and Amsterdam at doses ranging from 0.25 to 4 mg/m^2. One minor response was noted in a patient with colonic carcinoma. The study was suspended following the discovery of unexpected cardiotoxicity. As this toxicity was not consistent with the standard (EORTC) European Organisation for Research and Treatment of Cancer toxicology profile, we chose to investigate the pharmacokinetics of LL-D49194α1 in mice and humans in more detail to try to explain this phenomenon. A major difference in plasma protein binding was discovered between mice and patients, with a suggestion of non-linear kinetics being noted at higher doses in humans. It is likely that these differences in drug handling account for the unexpected and serious toxicity encountered in this trial.

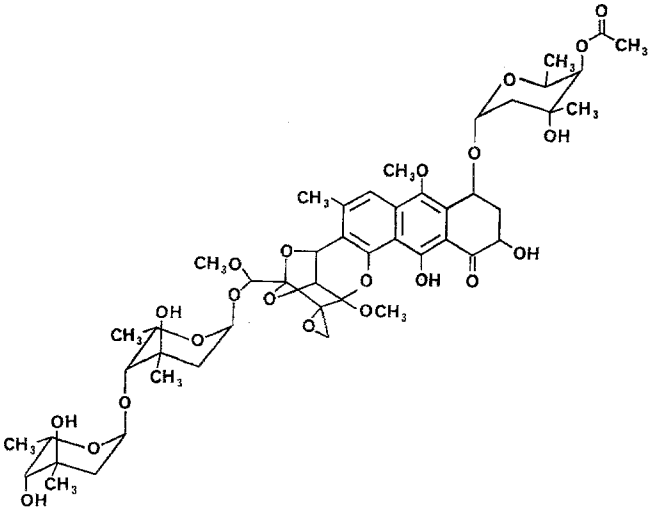

Fig. 1. Molecular structure of LL-D49194α1

Introduction

LL-D49194α1 (Fig. 1) is a new cytotoxic antibiotic recovered from a fermentation broth of *Streptomyces vinaceus drappus*. Although the mode of action of LL-D49194α1 has not been fully characterised, it has been associated with a potent inhibition of DNA and RNA synthesis [6]. The drug has shown significant activity against the human colon-carcinoma lines WiDr and CA 61 in vitro [6] and has exhibited activity against P388, B16, M5076 and MX-1 transplantable murine and human tumours in vivo. No schedule dependency was observed in these studies. The particularly broad range of the agent's experimental activity and its novel structure justified its further development through pre-clinical and into clinical phase I studies.

Toxological investigations in mice treated by i.v. bolus administration indicated that deaths occurred at between 13 and 19 days after dosing. The toxicities encountered included tail necrosis, weight loss, haematuria, testicular atrophy and signs of extra-medullary haematopoiesis. Haematological analysis indicated a degree of marrow suppression, particularly in the erythroid lineage. A fairly steep dose-lethality relationship was observed in these studies, with the dose lethal to 10% of the mice (LD_{10}) being 0.89 mg/kg and that lethal to 50% of the mice (LD_{50}) being 1.09 mg/kg (confidence intervals, 0.5–0.9 and 0.94–1.23 mg/kg, respectively). The assays available at that time for LL-D49194α1 in plasma [2] did not prove to be sufficiently sensitive for the evaluation of full pre-clinical pharmacokinetics; however, preliminary data indicated that the plasma level in mice fell below the limit of detection within 30 min of i.v. administration.

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This paper describes the clinical phase I study of LL-D 49194α1 carried out jointly in Glasgow and Amsterdam and co-ordinated through the European Organisation for Research and Treatment of Cancer-N New Drug Development Office (EORTC-NDDO). The primary purpose of the study was to determine the maximum tolerated dose in humans using a schedule of i. v. administration once every 3 weeks. In addition, this report describes more detailed murine pharmacokinetics studies performed in Glasgow utilising a new and more sensitive high-performance liquid chromatographic (HPLC) assay.

Patients and methods

Drug administration

LL-D 49194α1 was supplied by American Cyanamid Company. Pre-formulation studies have been reported elsewhere [1]; in brief, the drug was formulated by freeze-drying in 5-ml vials containing 1.1 mg LL-D 49194α1 and 8.8 mg mannitol. The contents of the vials were reconstituted immediately prior to their use by the addition of 2.2 ml 0.9% sterile sodium chloride. For human administration, the required volume was further diluted in 10 ml 0.9% sodium chloride and given as a 5-min infusion into the side arm of a free-flowing drip. Treatment was repeated at 3-week intervals in the absence of disease progression or significant toxicity.

Dosage and dose escalation

According to current EORTC practice, the starting dose for patients was chosen as one-tenth of the murine LD_{10} dose, i.e. 0.25 mg/m^2. Three patients were enrolled at each dose level, and dose escalations of up to 100% were carried out at the discretion of the responsible clinicians in the absence of significant toxicity. Pharmacokinetically guided dose escalation [3] was not feasible due to the lack of a sufficiently sensitive assay and, hence, of pre-clinical data prior to the phase I investigations.

Patient selection

Patients with histologically confirmed metastatic cancer refractory to conventional treatment were entered into the study after fully informed consent had been obtained. Eligibility criteria included an adequate performance status (WHO grades 0–2) as well as adequate pre-treatment bone marrow values (total WBC, $>4\times10^9$ cells; platelet count, $>100\times10^9$ cells) and hepatic (bilirubin level, <25 μM) and renal (creatinine value, <120 μM) function.

Pre-treatment evaluation included a complete physical examination, chest radiograph, 12-lead electrocardiogram, urinalysis and creatinine clearance estimation as well as measurement of standard biochemical and haematological indices. In addition, measurements of the tumour burden were made using ultrasound examination, computer-assisted tomography and isotope scans as deemed appropriate. During therapy, patients were seen at least once weekly for toxicity assessment and repeat biochemical and haematological tests. Evaluation of the tumour response was carried out every 3 weeks by physical examination and plain radiology; more complex imaging studies were carried out, when appropriate, at 6-week intervals.

Clinical pharmacology studies

At study entry, pharmacokinetic sampling was carried out on all consenting patients. Blood samples (2–5 ml) were taken at 0, 2, 4, 6, 8, 10, 15, 20, 30, 45, 60 and 90 min and 2, 4, 6, 8 and 24 h after dosing, and the plasma was stored at −20° C until analysis. Pharmacokinetics studies were subsequently performed following the development of the HPLC method described below.

Drug extraction. LL-D 49194α1 was extracted from plasma using 3-cm^3 C2-Bond Elut cartridges pre-conditioned with 2 ml methanol followed by 2 ml de-ionised water. Plasma contaminants were eluted to waste with 1 ml methanol/water (1 : 19, v/v) and the analyte was eluted in 0.6 ml methanol. Aliquots of the methanolic extract were diluted with 200 μl KH_2PO_4 (5 mM, pH 7) to make the samples compatible with the mobile phase.

HPLC methodology. The extracted samples were assayed by HPLC (injection volume, 25–250 μl) using a 15- × 0.46 cm C6 (5 μM) reverse-phase analytical column. The drug was eluted isocratically in methanol/acetonitrile/KH_2PO_4 (1 : 1 : 2, by vol.; 5 mM, pH 7) at a flow rate of 1 ml/min (retention time, 7.9 min) using UV (270 and 405 nm) and fluorescence detection (excitation wavelength, 405 nm; emission wavelength 480 nm).

Assay validation. The recovery of LL-D 49194α1 from plasma over the concentration range of 0.1–250 ng/ml was 88% ± 8% ($r = 0.999$), and the limit of detection was 0.1 ng/ml. The identity of LL-D 49194α1 extracted from patients' plasma samples was confirmed using photodiode array detection (scanning range, 200–500 nm). The extracted samples co-eluted with a known reference standard (retention time, 7.9 min) and yielded an identical UV-visible spectrum (absorbance maxima, 402, 270 and 232 nm; data not shown).

Toxicity studies in mice

The toxicity of LL-D 49094α1 was studied in female BALB/c mice (weight 18–20 g) following a single i. v. bolus injection at 0.9, 1.0, 1.1, 1.25 and 1.4 mg/kg (ten mice per group). The animals were observed and weighed daily for 28 days, and LD_{10} and LD_{50} values were determined by probit transformation of the mortality data.

Pharmacokinetics studies in mice

The pharmacokinetics of LL-D 49194α1 were examined in BALB/c mice at one-tenth of the LD_{10} value (0.1 mg/kg), at one-half of the LD_{10} (0.5 mg/kg) and at the LD_{10} of 1.0 mg/kg to check for linear pharmacokinetics. At various times (2–120 min) following a single i. v. bolus injection, blood was taken by cardiac puncture from mice under ether anaesthesia (three to five animals per time point) and the plasma was pooled and stored at −20° C until analysis. Aliquots (0.5–1.25 ml) of plasma were assayed by HPLC as described above.

The plasma protein binding of LL-D 49194α1 and doxorubicin was compared in mouse and human plasma. Aliquots (1 ml) of BALB/c mouse or human plasma were incubated in triplicate with either LL-D 49194α1 (50 μm) or doxorubicin (85 μm) for 30 min at 37° C. Standards were also prepared at the same concentration in 0.01 m TRIS/HCl buffer (pH 7.4). The plasma samples were transferred to micropartition filters (Amicon Corp., Gloucestershire, UK) and centrifuged for 15 min at 3000 *g* (21° C) using a fixed-angle rotor. The ultrafiltrate (25 μl) was assayed by HPLC for LL-D 49194α1 using the method detailed above. Doxorubicin was analysed using a 25- × 0.4-cm C18 μBondapak analytical column and was eluted at 2.5 ml/min with 30% acetonitride/70% 5 mM ammonium formate (adjusted to pH 3.6 with formic acid). Doxorubicin was detected at 480 nm and showed a retention time of 4.8 min.

The extent of plasma protein binding was determined by comparing the peak area of the plasma ultrafiltrate to that of the unfiltered standard. Correction for non-specific binding of doxorubicin and LL-D 49194α1 was made by determining the peak area of the appropriate drug standard before and after ultrafiltration.

Table 1. Patient's characteristics

Sex (M/F)	Age (years)	Diagnosis	Previous therapy (excluding surgery)	Centre
F	46	Ovarian cancer	Yes	G
M	61	Colorectal cancer	Yes	G
F	58	Colorectal cancer	Yes	G
M	71	Colorectal cancer	Yes	A
F	57	Colorectal cancer	Yes	G
F	73	Unknown primary	No	G
F	56	Colorectal cancer	Yes	A
F	69	Colorectal cancer	Yes	A
F	63	Ovarian cancer	Yes	G
M	70	Hepatocellular cancer	Yes	A
F	68	Colorectal cancer	No	G
F	60	Colorectal cancer	Yes	G
M	53	Pancreatic cancer	No	G
M	53	ACUP	Yes	G
F	45	Ovarian cancer	Yes	G

ACUP, Adenocarcinoma of unknown primary site; G, Glasgow; A, Amsterdam

Table 2. Toxic effects[a]

Dose level (mg/m^2)	Number of courses	N/V					Cardiac				
		0	1	2	3	4	0	1	2	3	4
0.25	9	5	2	2	0	0	8	1	0	0	0
0.5	6	0	0	2	4	0	6	0	0	0	0
1.0	3	0	0	0	3	0	2	1	0	0	0
2.0	5	1	0	1	3	0	2	2	1	0	0
4.0	3	0	1	0	1	1	1	1	0	0	1

[a] Maximal grade of toxicity for each dose level according to WHO grade; N/V, Nausea and vomiting

Results

A total of 15 patients (11 in Glasgow and 4 in Amsterdam) were entered into the study; their characteristics are summarised in Table 1. All were considered to be evaluable for toxicity. A total of 26 courses of LL-D49194α1 were given, with 3 patients being treated at each dose level of 0.25, 0.5, 1, 2 and 4 mg/m^2.

Toxic effects

The two commonest manifestations of toxic effects in this phase I study are summarised in Table 2. Nausea and vomiting were observed at all dose levels; in all but two patients treated at the highest dose level, this was easily controlled by the prophylactic use of i.v. metoclopramide. Isolated non-dose-related episodes of phlebitis, headache, diarrhoea, anaemia and thrombocytopenia were observed. No evidence was found of dose-dependent myelosuppression.

One toxic death occurred in this study. A 45-year-old women with recurrent ovarian carcinoma and no history of cardiac disease or of previous exposure to known cardiotoxic drugs was treated at 4 mg/m^2. At approximately 41 h post-dosing, she developed hypotension and dyspnoea but showed no clear evidence of left ventricular failure. An electrocardiogram (ECG) revealed an elevation in ST waves across all infero-lateral leads that was suggestive of myocardial infarction. Symptomatic treatment was given, but the patient died approximately 12 h later. Autopsy examination revealed normal coronary arteries with no macroscopic evidence of myocardial infarction or pulmonary thromboembolism. Histological examination of the myocardium revealed inflammatory-cell infiltration (mostly neutrophil polymorphs), intercellular oedema, swelling and homogeneous eosinophilic change in some myocardial cells and early changes in myocardial-cell nuclear degeneration. These alterations were found in the myocardium of all four cardiac chambers, suggesting pancarditis rather than a focal pathological change. It was felt that these changes were best explained by a toxic effect of the drug (G. Lindop, personal communication).

This fatality prompted a closer examination of the cardiac status of the other patients on study, which revealed asymptomatic changes in ST-T waves in patients treated at lower doses (see Table 2). One patient treated at the lowest dose level showed ECG changes following his fifth drug infusion, suggesting that chronic exposure to low doses may also be cardiotoxic. In view of the evidence of cardiotoxicity observed at most of the dose levels, with a fatal event occurring at the highest dose, we decided that the study should be terminated.

Therapeutic effects

One man with previously untreated colonic carcinoma showed a minor response (i.e. a reduction of less than 50% in tumour volume) after completing five courses at 0.25 mg/m^2. The ultrasound measurements of a right iliac fossa mass changed from 8.6×6.6 (pre-treatment) to 7.6×5 cm (post-treatment). This minor response was associated with a major improvement in performance status that was sustained for 6 months. No other objective response was observed.

Murine toxicity

The dose-lethality curve in mice was fairly steep as reflected in the close relationship between the LD_{10} and the LD_{50} values (Table 3). However, this is not an unusually steep dose-response relationship and is comparable with the toxicity data previously obtained in BALB/c mice using other cytotoxic drugs, including the anthrapyrazole CI-941 [4] and the anti-tumour antibiotic rhizoxin [5]. A dose of 1.4 mg/kg was universally lethal, with deaths occurring at between 2 and 14 days post-treatment. The toxicities encountered included body weight loss, tremor and abdominal swelling (three of ten mice treated at 1.25 mg/kg).

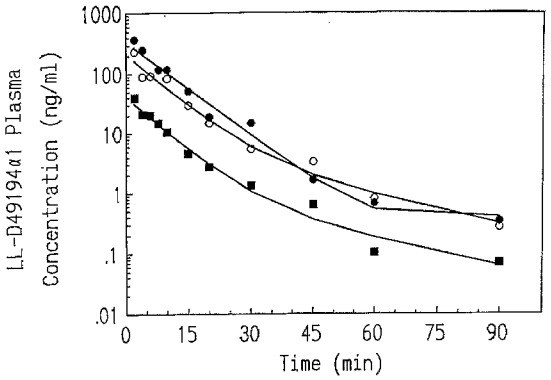

Fig. 2. Plasma concentration versus time curve for LL-D49194α1 in BALB/c mice given 0.1 (*squares*), 0.5 (*open circles*) or 1.0 mg/kg (*filled circles*) by i.v. bolus injection

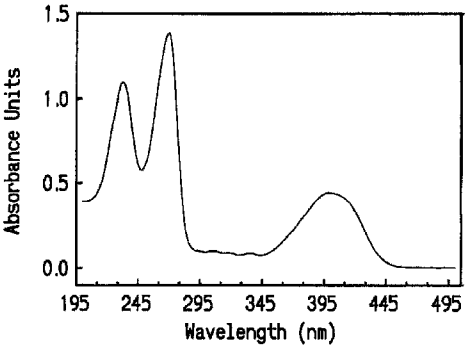

Fig. 3. UV absorbance spectrum of LL-D49194α1 extracted from BALB/c mouse plasma

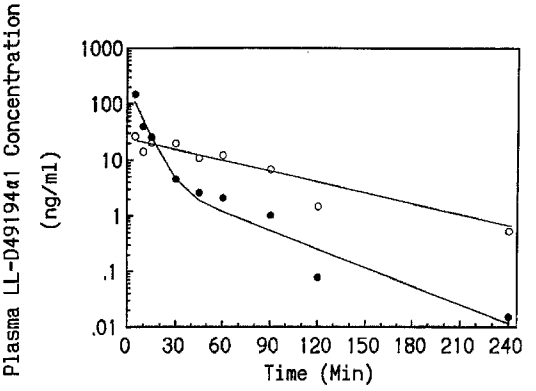

Fig. 4. Plasma concentration versus time profile of LL-D49194α1 (filled circles) and its major metabolite (*open circles*) in a patient treated with 4 mg/m² LL-D49194α1

Murine pharmacokinetics

Figure 2 illustrates the plasma concentration versus time curves for the doses tested in mice. LL-D49194α1 was rapidly cleared from the plasma, with levels lying below the limit of detection (0.1 ng/ml) at 90 min post-administration. The plasma pharmacokinetics of this drug is likely to follow a bi-exponential open model; however, as levels fell below the limit of detection at 90 min after treatment, an accurate estimation of this phase could not be made. Hence, the distribution phase was estimated (0–30 min) using the equation $C = Ae^{-\alpha t}$, where C is the plasma LL-D49194α1 concentration at time t, A is the concentration constant for the first phase and a is the first-order rate constant [7] from which the elimination half-life ($t_{1/2}$) is determined. Values for the area under the concentration-time curve (AUC) were calculated using the trapezoidal rule [7]. The pharmacokinetics of LL-D49194α1 were linear at doses between one-tenth of the LD_{10} and the LD_{10} itself, and the AUC value at 1.0 mg/kg (3 mg/m²) was 3270 ng ml⁻¹/min. Table 4 summarises these results.

A metabolite was detected in mouse plasma (retention time, 3.5 min) at levels exceeding those of the parent drug (assuming the same extinction co-efficient) at approximately 30 min post-administration. Spectral analysis of the purified metabolite revealed that the characteristic absorbance of LL-D49194α1 at 402 nm (Fig. 3) had been lost. This feature may be indicative of a ring-opened species that subsequently loses the chromophore. Confirmation of the metabolite's structure by mass spectrometry is awaited.

Table 3. Mortality data for BALB/c mice

LD_{10}	0.96 mg/kg (0.55–1.37 mg/kg)	2.88 mg/m²
LD_{50}	1.06 mg/kg (0.68–1.44 mg/kg)	3.20 mg/m²

95% confidence limits are shown in parentheses

Table 4. Murine pharmacokinetics

Dose (mg/kg)	$t_{1/2\alpha}$ (min)	AUC (ng ml⁻¹/min)
0.1	5.71±0.46	341
0.5	5.49±0.51	1942
1.0	5.69±0.75	3269

Human pharmacokinetics

These studies showed that the drug was rapidly cleared from the plasma and that the data could be fitted to a bi-exponential open model, with the $t_{1/2\alpha}$ value being 3.2 ± 1 min and the $t_{1/2\beta}$ value being 18 ± 2 min following a dose of 1 mg/m². Table 5 summarises the results obtained in all of the patients in Glasgow, and Fig. 4 illustrates a typical concentration versus time profile. A major metabolite with spectral and chromatographic properties identical to those of the metabolite isolated from mouse plasma was also found in the patient's samples (data not shown) at levels exceeding those of the parent drug (Fig. 4).

The AUC values obtained at the starting dose (12, 21 and 30 ng/ml min) were substantially lower than the value resulting from a dose of one-tenth of the LD_{10} in mice (namely, 342 ng ml⁻¹/min). Following a dose of 4 mg/m², which is associated with cardiotoxicity in humans, the AUC values were 385 and 1994 ng ml⁻¹ min, both of which were nonetheless substantially lower than the AUC observed at the LD_{10} in mice (3270 ng ml⁻¹/min).

Table 5. Human pharmacokinetics

Dose (mg/m^2)	Patient number	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	AUC (ng ml^{-1}/min)
0.25	1	ND	ND	21.6
	2	1.87	39.2	30.46
	3	6.78	ND	12.91
0.5	4	ND	ND	28.8
1.0	5	3.06	15.88	66.2
2.0	6	2.33	12.96	567.0
	7	2.68	14.36	303.3
4.0	8	6.45	29.52	385.4
	9	3.39	25.5	1994.0

ND, Not determined

These preliminary data suggest that the pharmacokinetics of LL-D40194α1 is linear up to a dose of 1 mg/m^2 but may become non-linear at that of 2 mg/m^2. The cause of the non-linearity may involve the saturation of a clearance mechanism, possibly renal or faecal elimination or tissue uptake, and may contribute to the cardiotoxic effects in patients. The non-linear pharmacokinetics and the mechanisms underlying the cardiotoxicity of the drug are the subjects of a current investigation.

Plasma protein binding

Doxorubicin was extensively bound to plasma protein in both mouse and human plasma (89% and 84%, respectively). In contrast, marked inter-species differences were observed in the binding of LL-D49194α1 to protein in mouse and human plasma (42% and 8.5%, respectively).

Discussion

LL-D49494α1 shows a promising and wide range of activity in experimental tumour systems, and its mechanism of action has been associated with a strong inhibition of DNA and RNA production [6].

Pre-clinical (murine) toxicology studies commissioned by the EORTC-NDDO failed to predict the serious cardiac problems that were encountered in humans (myelosuppression was the predicted toxicity). At least three factors may be of relevance to this finding. Firstly, animals were killed for necroscopy and histology on days 14 and 28 post-dosing and no histological evidence of cardiac damage was found. Organs obtained on day 7 have been retrospectively examined for cardiac changes, and three of five showed signs of myocarditis consistent with a drug effect. Secondly, retrospective pharmacokinetics studies have shown that the drug exhibits linear pharmacokinetics in mice at doses of up to 3 mg/m^2, whereas there may be non-linear handling in humans at doses exceeding about 1 mg/m^2. Thirdly, and probably of most importance, a major difference in the plasma protein binding of LL-D49194α1 was found between mice and humans, which could effectively increase the free (active) plasma concentration by at least 4 times in humans. From our toxicology and pharmacokinetics studies in mice, it seems unlikely that the availability of a more sensitive HPLC analytical method prior to the study would have helped us to avoid the fatality due to cardiac toxicity. Clearly, if any suggestion of species differences in drug handling or the potential for cardiac damage had been recognised at an earlier stage, we would have been more cautious in carrying out dose escalation and in monitoring the cardiac status of our patients. The pre-clinical toxicology more clearly predicted a degree of myelotoxicity for this compound, but as detailed in the preceding section of this paper, no clear demonstration of this side effect was possible at the dose levels explored.

Another possible factor contributing to the observed cardiotoxicity may involve differences in metabolism between species. We were incapable of quantitating metabolite levels because analytical standards of unknown peaks have not been synthesised. This factor may be further elucidated in future studies of the same compounds.

It is noteworthy that although the activity of LL-D49194α1 apparently did not show schedule dependency, the cardiotoxicity was clearly time-related, being evident on day 7 and having resolved on day 14. In retrospect, histological examination of day-7 necroscopy material may have allowed us to avoid the clinical problems with cardiotoxicity. A further problem highlighted in this study was co-ordination between international centres. The ECG changes seen were subtle and, in themselves, not sufficient to cause concern, but they might have been deemed more significant if they had occurred in consecutive patients in the same clinical centre. Retrospective pharmacokinetics studies in mice also illustrated that on the basis of the assumption that the LD_{10} in mice was approximately equivalent to the maximum tolerated dose in humans, the plasma AUC was substantially lower (range, 12%–60%) in humans than in mice.

In conclusion, the results show that following a short-term infusion, cardiotoxicity rather than myelosuppression is dose-limiting in patients. Investigations are under way to investigate continuous-infusion schedules, and we are attempting to identify by mass spectrometry a major metabolite (possibly a ring-opened species) to elucidate its role in the toxicity and/or efficacy of the drug. Further potential development of this compound includes the derivation of less cardiotoxic analogues, and future clinical studies may require determinations of the effect of genetic polymorphisms on the drug's metabolism and of the influence of various rates of infusion on its toxicity.

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