Pergamon

European Journal of Cancer, Vol. 34, No. 10, pp. 1602–1606, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00152-X

Original Paper

The Antitumour Activity of the Prodrug *N*-L-leucyl-doxorubicin and its Parent Compound Doxorubicin in Human Tumour Xenografts

K. Breistøl,[1] H.R. Hendriks,[2] D.P. Berger,[3] S.P. Langdon,[4] H.H. Fiebig[3] and Ø. Fodstad[1]

[1]The Norwegian Radium Hospital, Norway; [2]EORTC New Drug Development Office, University Hospital Vrije Univeriteit, Gebouw Zuid, Amstelveenseweg 601, NL-1081 JC, Amsterdam, The Netherlands; [3]Albert-Ludwigs Universität, Germany; and [4]ICRF Medical Oncology Unit, Western General Hospital, Edinburgh, U.K.

The antitumour activity of the investigational agent *N*-L-leucyl-doxorubicin (Leu-DOX) was compared with that of doxorubicin (DOX) in human tumour xenografts growing subcutaneously in athymic nude mice. Leu-DOX was developed as a prodrug of DOX, and may be converted into the clinically active parent compound by hydrolytic enzymes present in or on tumour cells. It has been suggested that a better therapeutic index with a reduced cardiac toxicity and higher efficacy might be obtained. Both compounds were administered intravenously weekly for 2 weeks, each at maximum tolerated doses of 8 mg/kg and 28 mg/kg for DOX and Leu-DOX, respectively. The panel of xenografts represented three different tumour types. Leu-DOX showed antitumour activity, defined as tumour growth inhibition > 50% and specific growth delay > 1.0, in 10 of the 16 tumours, including two of five breast, five of seven small cell and three of four non-small cell lung carcinomas. In comparison, DOX was active in one breast, four small cell lung and two lung adenocarcinoma xenografts. In all the DOX sensitive lung tumours, Leu-DOX showed higher efficacy than the parent compound. Based on the results of the present study, and since phase I clinical trials with Leu-DOX have already been performed, phase II clinical evaluation of Leu-DOX in patients with breast and lung cancer is recommended. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: antitumour activity, *N*-L-leucyl-doxorubicin, doxorubicin, human tumour xenografts
Eur J Cancer, Vol. 34, No. 10, pp. 1602–1606, 1998

INTRODUCTION

ANTHRACYCLINES REPRESENT antitumour drugs with a wide spectrum of activities in the clinical management of cancer patients. Doxorubicin (DOX) is predominantly used with high efficacy in the treatment of breast and small cell lung cancer (SCLC) patients. It is well known, however, that cumulative cardiotoxicity restricts its use to a limited number of treatment courses [1].

Many analogues of DOX have been developed with the intention of reducing the risk of cardiomyopathy and/or acute toxicity [2]. Unfortunately, none of the commercially available analogues have proven clearly advantageous to DOX, considering the overall efficacy, toxicity, and the spectrum of antitumour effects. In attempts to overcome the disadvantages of DOX, cardioprotective agents administered in conjunction with the drug [3], and reformulation of the drug in liposomes have been tested [4–8]. An alternative approach is the use of prodrugs which are converted into the active compound when reaching the tumour [9–15]. Several approaches have recently been described, including the use of agents activated by antibody-directed glucuronidases or lactamases [9, 10]. Another prodrug candidate is *N*-L-leucyl-doxorubicin (Leu-DOX). The compound is synthesised by linking one L-leucine amino acid to the amino group of DOX, resulting in an almost non-toxic conjugate from which DOX can be released by the activity of hydrolytic enzymes [16–18]. Such enzymes, like cathepsins, can be detected in and on tumour cells and it is suggested that they may cleave Leu-DOX into DOX intracellularly or in the pericellular space [19–21].

Correspondence to H.R. Hendriks.
Received 15 Sep. 1997; revised 30 Jan. 1998; accepted 18 Feb. 1998.

Based on molar values, Leu-DOX has been shown to be overall three times less toxic than DOX, and cardiotoxicity in mice and rabbits, comparable with that of DOX, was first induced at a four times higher dose of Leu-DOX [22–25]. The reduced cardiotoxicity may be attributed to a lower accumulation of DOX in heart tissue, as demonstrated in mice and rats [22, 25, 26].

Previously, Leu-DOX was found to be superior to DOX in inhibiting tumour growth in mice bearing subcutaneously grown L1210 leukaemia [16] and human ovarian cancer xenografts [27]. To investigate further the potential advantage of Leu-DOX, its antitumour efficacy at equitoxic doses was compared with that of DOX in panels of human tumour xenografts representing three different tumour types. The results indicate that Leu-DOX has a clinical potential in both DOX-sensitive and -resistant tumours that should encourage further clinical evaluation.

MATERIALS AND METHODS

Animals

Female nude mice, 5–8 weeks of age at the start of the experiment, were used in this study. The investigators used the strain available within their respective laboratories: NRMI nu/nu (Freiburg), Balb/c nu/nu (Oslo), nude of a mixed background (Edinburgh). The mice were maintained under specific pathogen-free conditions, and food and water were supplied *ad libitum*. Housing and all procedures involving animals were performed according to protocols approved by the animal care and use committee at the individual institution, in compliance with National Ethical Committee's guidelines on animal welfare.

Drugs and doses

DOX and Leu-DOX were supplied by Medgenix Group (Fleurus, Belgium). The drugs were dissolved in sterile water to obtain stock solutions of 12.5 mg DOX/ml and 20 mg Leu-DOX/ml. The stock solutions were kept at 4°C protected from light, and were further diluted with 5% glucose or 0.9% NaCl immediately before use.

Prior to the start of the study, dose-finding studies were carried out by one of the testing laboratories (Oslo) in order to determine the maximum tolerated dose (MTD) for Leu-DOX. The definition of MTD allows a median body weight loss of about 15% of the initial weight within 2 weeks after the first injection. Both drugs were administered intravenously weekly ×2 at the MTD.

Human tumour xenografts

The human tumour xenografts used in the experiments were selected on the basis of differences in histology, growth characteristics and, if known, sensitivity to DOX [28, 29].

Treatment and evaluation

Mice were implanted with fragments of human tumour xenografts subcutaneously in both flanks. Tumour growth was assessed once or twice weekly by caliper measurements of the tumour in two dimensions [28]. Treatment was started when the tumours had reached a median diameter of 5–6 mm. Prior to the start of treatment, mice were selected according to tumour volume and assigned to groups in order to achieve an equal distribution in the different groups. Thereafter, drug treatment and control groups were chosen randomly.

Tumour volume was calculated by the formula $0.5 \times \text{length} \times \text{width}^2$. Relative tumour volumes (RTV) were calculated for each individual tumour by dividing the tumour volume at a specific number of days after the start of treatment, day X, by the tumour volume at the start of treatment, day 0 multiplied by 100%:

$$\text{RTV} = \frac{\text{Volume tumour day } X}{\text{Volume tumour day 0}} \times 100\%.$$

Median RTV values were used to draw semilogarithmic growth curves and to calculate treatment efficacy. Tumour doubling time (TD) of test and control groups was defined as the period (days) required to double the median RTV value.

Treatment efficacy was assessed by three evaluation criteria: specific growth delay (SGD), optimal growth inhibition (T/C%) and tumour growth curves. The SGD was calculated over one and two doubling times, as follows:

$$\text{SGD} = \frac{\text{TD treated (days)} - \text{TD control (days)}}{\text{TD control (days)}}.$$

Optimal growth inhibition at a particular day within 5 weeks after the last drug administration was calculated from the median RTV values of treated versus control groups:

$$\text{T/C\%} = \frac{\text{RTV treated}}{\text{RTV control}} \times 100\%.$$

Based on the experience with the models used, a T/C value of $< 50\%$ *and* a SGD value of >1.0 were defined as standard criteria for antitumour activity [28].

An experiment was considered to be evaluable if the number of mice per treatment or control group was more than five, and sufficient to yield at least six evaluable tumours. At the start of the treatment, the minimum tumour diameter was 4 mm equivalent to a volume of 30 mm^3. If a mouse died within 2 weeks after the final injection, this was considered as toxic death and the animal was excluded from further calculations. Treatment groups with more than two of six toxic deaths, or more than 15% body weight loss were considered not evaluable for antitumour efficacy.

RESULTS

Dose-finding studies

Prior to the start of the therapy experiments, dose-finding studies were carried out to establish the MTD for Leu-DOX given intravenously weekly ×2 in non-tumour-bearing female Balb/c or NIH-III nude mice. The compound was injected at several different dose levels to groups of four mice each. After dosing, body weight and toxic death were recorded. For the different groups the median body weight was determined at each day of measurement. Leu-DOX at doses of 30–40 mg/kg caused a maximum median body weight loss of 4–7% during the first week, before the mice gained weight at the end of the second week. However, irreversible cachexia occurred after the third week from start of treatment. Therefore, the dose of Leu-DOX was reduced to 28 mg/kg in tumour-bearing mice, a dose that was well tolerated.

The MTD of DOX at this schedule was previously determined to be 8 mg/kg [27, 30]. In the present study, this dose was also well tolerated, inducing a median body weight loss in tumour-bearing mice which was comparable to that observed with Leu-DOX.

Table 1. *Efficacy of doxorubicin (DOX; 8 mg/kg) and* N-L-*leucyl-doxorubicin (Leu-DOX; 28 mg/kg) in human tumour xenografts, intravenously days 0 and 7*

	No of tumours			Lethality		DOX				Leu-DOX			
Tumour line	Control	DOX	Leu-DOX	DOX	Leu-DOX	T/C%	SGD1–2	SGD1–4	Activity*	T/C%	SGD1–2	SGD1–4	Activity*
Breast cancer													
MAXF 401	12	13	14	0/7	0/7	59.5	0.2	0.6	–	44.9	0.3	0.8	(+)
MAXF 449†	10	10	9	1/7	1/7	9.5	4.17	NA	++++	15.7	3.53	1.79	+++
MAXF 583	11	12	8	0/7	2/7	47.4	0.04	0.62	(+)	11.0	5.12	>2.52	+++
MAXF 857	11	9	9	1/6	1/6	39.1	0.23	0.22	(+)	28.1	0.93	0.98	(+)
MAXF 1162‡	13	9	8	2/7	2/7	59.9	0.60	0.28	–	43.7	0.90	0.78	(+)
Small cell lung cancer													
H-128	14	12	17	0/6	0/8	64.1	0.16	0.28	–	68.7	0.19	0.18	–
H-146	9	8	6	0/5	0/5	24.2	4.66	1.71	+++	7.1	6.22	NA	++++
DMS 53	10	11	9	0/8	0/8	73.0	0.09	0.01	–	52.4	−0.15	0.37	–
PJD	7	6	8	0/5	0/6	48.8	1.64	NA	+	31.7	NA	NA	++
LXFS 650	12	11	11	0/7	0/7	27.8	3.99	1.70	++	10.3	5.90	2.37	+++
LXFS 538	8	8	8	0/6	0/6	61.8	0.5	NA	–	25.8	2.9	NA	++
WX322	14	14	14	0/8	0/8	43	1.1	NA	+	29.0	1.6	NA	++
Non-small cell lung cancer													
HXFE 397	13	14	12	0/7	1/7	53.6	0.73	0.73	–	48.9	1.27	0.74	+
LXFA 629	10	9	11	2/7	1/7	40.8	1.62	0.79	+	37.9	1.90	0.98	++
AHXOL	12	12	12	0/6	0/6	55.0	0.4	NA	–	47.6	0.5	NA	(+)
EKVX	9	8	9	0/6	0/7	41.6	1.2	0.6	+	22.6	>2.5	NA	+++
Active/total									7/16 (44%)				10/16 (63%)

*Efficacy criteria: (+) T/C < 50% or SGD > 1.0; + T/C < 50% and SGD > 1.0; ++ T/C < 40% and SGD > 1.5; +++ T/C < 25% and SGD > 2.0; ++++ T/C < 10% and SGD > 3.0. †Schedule: intravenous day 0. ‡Schedule: intravenous days 0 and 14. NA, not applicable because a relative tumour volume of 400% (i.e. 4 times that of the control) was not reached during the experiment. T/C%. optimal growth inhibition; SGD, specific growth delay.

Antitumour activity

Applying the standard criteria for antitumour activity (T/C < 50% and SGD > 1.0) antitumour activity was achieved in 10 of 16 human tumour xenografts with Leu-DOX: two of five breast cancer, five of seven SCLC and three of four non small cell lung cancer (NSCLC) (Table 1). In comparison, DOX activity was observed in seven of the 16 xenografts: one of five breast cancer, four of seven SCLC and two of four NSCLC. DOX induced only minimal antitumour activity in PJD, WX322 (SCLC), LXFA 629 and EKVX (NSCLC) xenograft lines, whereas moderate to high antitumour activity was observed in MAXF 449 (breast), H-146 and LXFS 650 (SCLC). In the latter two xenografts, DOX induced tumour reduction for a short period, after which regrowth occurred.

Leu-Dox showed a higher efficacy than DOX in most DOX-responsive xenografts (Table 1). Thus, Leu-DOX caused a temporary regression of LXFS 650 and EKVX tumours as compared with the tumour volume at the start of treatment, whereas DOX only induced reduced tumour growth rate (Figure 1(a)). Notably, Leu-DOX showed considerable activity in three DOX-resistant tumours: MAXF 583 breast cancer, LXFS 538 SCLC, and LXFE 397 NSCLC xenografts (Table 1, Figure 1(b)), and also marginal effects in three other DOX-resistant lines (MAXF 401, MAXF 1162 and AHXOL). In two lines, H-128 and DMS 53, both drugs were inactive.

DISCUSSION

The antitumour activity of Leu-DOX, a prodrug of DOX, was compared with the parent compound in panels of subcutaneous human tumour xenografts in nude mice. At equitoxic dose levels, the amount of DOX was almost 3-fold higher on a molar basis administered as Leu-DOX compared with when DOX was given as a free drug. The treatment results showed that Leu-DOX had superior activity to DOX in six of seven DOX-responsive xenografts. In addition, Leu-DOX was active in three of nine DOX-resistant xenografts. According to the efficacy criteria used, Leu-DOX was active in 63% and DOX in 44% of the xenografts tested.

Boven and colleagues [27] have reported results from comparative activity studies of Leu-DOX and DOX in four human ovarian cancer xenografts. In their study, the prodrug had higher efficacy than DOX in three DOX-sensitive xenografts, whereas one tumour was resistant to both drugs. The data from our study not only confirm the differential activity of the compounds in three other tumour types, but also that Leu-DOX was superior to the parent drug in DOX-resistant xenografts.

There is evidence suggesting that Leu-DOX is converted into the parent compound in human tumour tissue by hydrolytic enzymes such as cathepsins. These enzymes have been demonstrated to be present in or on the surface of human tumour cells in elevated amounts [19–21].

Comparative pharmacokinetic studies of equimolar doses of Leu-DOX and DOX in Colon 26 tumour-bearing mice showed that 26, 30 and 16% of Leu-DOX was converted into DOX in plasma, heart and tumour, respectively. On the basis of the area under the curve (AUC) for the first 48 h, the authors concluded that the values obtained for DOX in this insensitive murine tumour cannot explain the enhanced antitumour activity shown by Leu-DOX in sensitive human tumour xenografts [22].

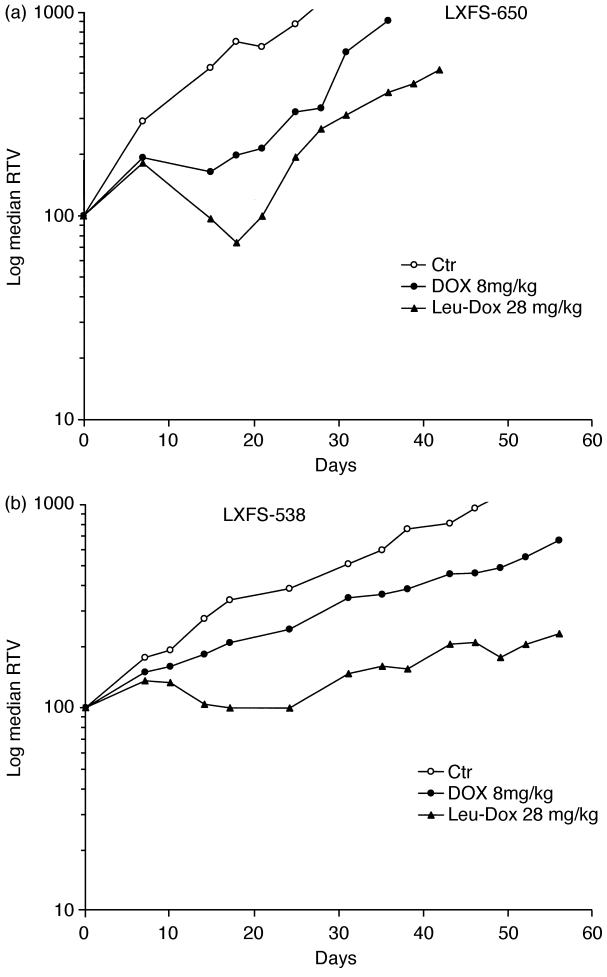

Figure 1. Growth curves of human tumour xenografts in nude mice: (a) small cell lung cancer LXFS 650; (b) non small cell lung cancer LXFS 538. Treatment was given i.v. on days 0 and 7.

In contrast, however, data recently obtained (not shown) showed that after bolus injection of equitoxic doses of the drugs into nude mice carrying LOX or THX human malignant melanoma tumour xenografts, Leu-DOX is converted into DOX at a slow rate, but the level in the tumour tissue was maintained for a longer period, resulting in higher DOX AUC values after treatment with Leu-DOX as compared with DOX. Similar relationships between the tumour tissue pharmacokinetics and the antiproliferative effects of a number of anthracyclines were recently observed in A2780 human ovarian xenografts in nude mice [31].

Leu-DOX has been tested in phase I clinical trials and the MTD was determined to be 225 mg/m^2, 3-fold higher than that of DOX given alone. In a concurrent pharmacokinetic study, the prodrug nature of Leu-DOX was confirmed, as the AUC for DOX correlated much better with the observed myelotoxicity than that of Leu-DOX [32, 33], i.e. DOX is the active and toxic metabolite of Leu-DOX.

Human tumour xenografts transplanted subcutaneously in nude mice have been shown to retain the histology and the chemosensitivity of the tumour tissue of the patient [34]. Since each tumour has its own characteristics, testing in tumour panels for each of several histological types is suggested for predicting the clinical potential of new anticancer drugs [35]. The majority of the human tumour xenograft lines used in this study were also employed in two large preclinical phase II studies previously performed [28, 29]. From these studies, it appeared that both panels reasonably well reflected the response to clinically active (DOX and cisplatin) and inactive agents. The effects of DOX in the present study were similar to those seen in the earlier phase II preclinical study, suggesting that the present Leu-DOX effects may be predicitive for the clinical situation. The results encourage phase II clinical trials to be carried out in patients with breast and lung cancer [27].

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