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ORIGINAL ARTICLE

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Antitumour evaluation of dolastatins 10 and 15 and their measurement in plasma by radioimmunoassay

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Abstract Dolastatins 10 and 15 are small peptides isolated from the marine sea hare *Dolabella auricularia* that have been shown to interact with tubulin. Their growth-inhibitory properties were compared using panels of human ovarian and colon-carcinoma cell lines. Both agents were very potent inhibitors of cell growth, with dolastatin 10 being an average of 9.1-fold more potent than dolastatin 15 [mean 50% inhibitory concentrations (IC_{50} values) 2.3×10^{-10} and 2.1×10^{-9} *M*, respectively; $P < 0.05$] and more potent than paclitaxel or vinblastine. While neither dolastatin exhibited marked cross-resistance in cisplatin- or etoposide-resistant cell lines, contrasting effects were observed using an acquired doxorubicin-resistant (CH1doxR, 100-fold resistant, P-glycoprotein overexpressing) cell line. Resistance was significantly higher to dolastatin 15 (12.7-fold) than to dolastatin 10 (only 3.2-fold; $P < 0.05$) and was reversible in both cases by verapamil. In vivo, using a s.c. advanced-stage human ovarian carcinoma xenograft and equitoxic doses, greater activity was observed with dolastatin 10 (6.1-day growth delay) versus 0.4 days for dolastatin 15. A radioimmunoassay for dolastatin 10 (limit of detection in mouse plasma 5 ng/ml) was developed. The rabbit antiserum also cross-reacted by 65% with dolastatin 15. Comparative mouse pharmacokinetics following i.v. administration of 1 mg/kg showed that both compounds are rapidly eliminated, but with a shorter second-phase half-life ($t_{1/2\beta}$) being observed for dolastatin 15 (being detectable for only up to 4 h post-administration), the $t_{1/2\beta}$ being 3 times longer for dolastatin 10. In addition, areas under the plasma concentration-time curve (AUC values) were 1.6-fold higher for dolastatin 10 (333 versus 208 ng ml^{-1} h). Plasma binding of dolastatin 10 exceeded 90%. The highly sensitive RIA will be useful for pharmacokinetic studies in conjunction with the planned phase I clinical trials of these novel, extremely potent, tubulin-binding agents, of which dolastatin 10 appears to possess the more promising preclinical features.

Key words Dolastatins · Radioimmunoassay · Cytotoxicity · Xenograft · P-glycoprotein

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Introduction

Over the last 10 years a number of potent cytotoxic compounds have been isolated from the marine sea hare *Dolabella auricularia* [20]. Dolastatin 10 was first described in 1987 (Fig. 1) and is a linear polypeptide containing four unusual amino acids (dolavaline-valine-dolaisoleucine-dolaproline-dolaphenine [16]). The related compound dolastatin 15 (Fig. 1) is a depsipeptide composed of seven subunits containing five amino acids (dolavaline-valine-*N*-methylvaline-proline-proline) esterified to 2-hydroxy-isovalerate and a complex amide (dolapyrrolidone [17]). Complete syntheses of these compounds have been described elsewhere [15, 18, 19, 24]. Dolastatin 10 inhibits microtubule assembly and tubulin-dependent guanosine triphosphate (GTP) binding and acts as a potent non-competitive inhibitor of vincristine binding to tubulin ([1]; for a review see [4]). The interaction of dolastatin 15 with tubulin is weaker than that of dolastatin 10 [2], thought to be due to the absence of the dolaisoleucine residue that appears to be critical for the high activity of dolastatin 10.

These compounds have been shown to be highly growth-inhibitory by arresting cells in mitosis with induction of apoptosis in various leukaemia and lymphoma cell lines [2, 3, 26]. For example, in the L1210 murine leukaemia cell line the 50% inhibitory concentrations of dolastatins 10 and 15 are 0.4 and 3 n*M*, respectively, and in CHO cells these values are 0.5 and 5 n*M*, respectively [2]. Proliferation of

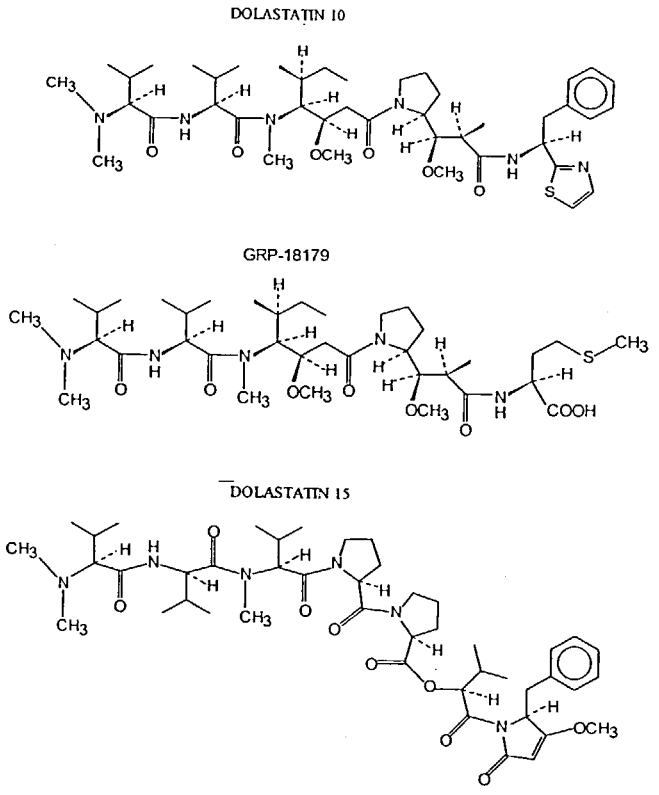


Fig. 1 The structures of dolastatin 10, dolastatin 15 and the hapten GRP-18179

human leukaemic cells was also inhibited at nanomolar or sub-nanomolar concentrations by the two compounds [26]. Multidrug-resistant human and murine leukaemia cell lines have been shown to be 10- to 20-fold cross-resistant to dolastatin 10 [28].

Potent in vivo antitumour activity for dolastatin 10 (i.p. administration) has been reported against a variety of i.p. implanted tumours, including murine P388 leukaemia and B16 melanoma and human melanoma xenografts in nude mice [16]. Antitumour activity following i.p. administration has also been demonstrated against the i.p. implanted M5076 sarcoma, human LOX melanoma and OVCAR-3 ovarian carcinoma [30].

Because of their potent growth-inhibitory and antitumour properties the dolastatins have been selected for clinical evaluation. Pharmacokinetic studies using different schedules of administration and dose levels during further preclinical development and in phase I trials will require an extremely sensitive method of analysis. Little information is available on the disposition of either dolastatin 10 or dolastatin 15 because a suitably sensitive assay is not available. The availability of a tritiated form of dolastatin 10 [10] has allowed the murine pharmacokinetics of an i.v. injection in mice to be evaluated [14]. High-performance liquid chromatography (HPLC) analysis has been developed to determine the purity of dolastatins 10 and 15 [21], but sufficient sensitivity to determine concentrations in biological samples has not been shown. A bioassay that depends on the growth-inhibitory activity of dolastatin 10 for L1210 cells [23] has been described and has a sensitivity of 0.7 n*M*. However, this assay does not distinguish between the parent drug and any cytotoxic species formed in vivo.

In this report we present in vitro growth-inhibitory data obtained for dolastatins 10 and 15 in panels of human colon and ovarian tumour cell lines (including cell lines selected for acquired resistance to the commonly used anticancer drugs cisplatin, doxorubicin and etoposide) and comparative data on their in vivo antitumour activity in a human ovarian xenograft. In addition we report the development of a radioimmunoassay for dolastatins 10 and 15 and its use to determine the pharmacokinetics of these compounds in mice.

Materials and methods

Generally labelled dolastatin 10 (1.6 Ci/mmol) was supplied by the United States National Cancer Institute through the Research Triangle Institute. Dolastatins 10 and 15 were supplied under the auspices of the United Kingdom Cancer Research Campaign (CRC) Phase I/II Clinical Trials Committee by Dr. D. Secher. Chemicals were obtained from Sigma unless stated otherwise, and solutions were prepared in Milli Q deionised water.

Cell culture

The SKOV-3, HX/62, CH1, A2780 and 41M human ovarian carcinoma cell lines and the BE, HT29, MAWI, LoVo, SW480 and SW620 colon-carcinoma cell lines were used. Biological and chemosensitivity details and sources for the ovarian carcinoma cell-line panel have previously been reported [6]; the colon lines were obtained from the American Type Culture Collection or the European Collection Animal Cell Cultures (ECACC, Porton Down, UK). In addition, lines selected (in vitro) for acquired resistance to cisplatin (CH1cisR, 6.5-fold resistant in terms of IC_{50} values [8]; A2780cisR, 15-fold resistant [9]), doxorubicin (CH1doxR, 90-fold resistant [25]) and etoposide (CH1etopR, 10-fold resistant [29]), were included. The A2780 pair of cell lines was kindly provided by Dr. T. Hamilton (Fox Chase Cancer Center, Philadelphia, Pa.).

All lines were grown as monolayers in Dulbecco's modified Eagle's medium containing 2 m*M* glutamine, 10% foetal calf serum (Imperial Laboratories), 50 μg gentamicin/ml 2.5 μg amphotericin B/ml, 10 μg insulin/ml and 0.5 μg hydrocortisone/ml in 10% CO_2/90% air. Cells were free of *Mycoplasma* contamination during this study.

Assessment of growth inhibition

This was performed using a 4-day sulforhodamine B (SRB) assay as previously described [13]. Briefly, cells were plated (at between 3 and 6×10^3 cells/well) in 160 μl medium in 96-well microtitre plates and, the following day, drugs were added at up to ten differing concentrations in quadruplicate wells. Dolastatins were initially dissolved in ethanol and stored as a stock solution of 1 m*M* at –20 °C; the final concentration of ethanol in contact with cells was 0.5% and was not inhibitory to the cells. Unless otherwise stated, drugs were left in contact with cells for 96 h before the assessment of cell numbers by staining with 0.4% SRB dissolved in 1% acetic acid as previously described [13]. Where the effect of the time of exposure on growth inhibition was investigated, drug was washed off the cells at the indicated times by aspiration, and warm phosphate-buffered saline was added before replacement with growth medium for the remainder of the 96-h period. In experiments using verapamil, the resistance modifier was added 2 h prior to the addition of dolastatins and

throughout the 96-h period at the previously determined highest non-toxic concentration of 6 μ*M* [25]. Growth inhibition was compared using IC_{50} values determined as described elsewhere [13].

Antitumour evaluation

The in vivo xenograft counterpart of the CH1 cell line (also named PXN/109T/C in previous publications [5, 9]) was grown s.c. as described previously [5] in female BALB/c mice. Animals bearing similarly sized tumours (typically being around 6–8 mm in diameter and having grown for 4–6 weeks post-implantation) were randomised to receive either dolastatin 10, dolastatin 15 (six animals) or no treatment (vehicle, ethanol/saline; ten control animals). Drugs were then given according to previously determined equitoxic (maximum tolerated) doses, schedules and routes of administration (kindly provided by Dr. J. Plowman, National Cancer Institute, Bethesda, Md., via Dr. D. Secher, CRC Phase I/II Committee). Dolastatin 10 was given at a single i.v. dose of 450 μg/kg on day 0, whereas dolastatin 15 was given i.p. at 5.25 mg/kg on days 0, 4 and 8. Although dolastatin 15 may also be injected i.v., for convenience with a multiple dosing schedule, i.p. administration was selected; previous studies using murine tumours showed comparable antitumour activity following drug delivery by the two routes.

Tumours were measured (in two dimensions using calipers) on days 4, 7, 14, 21 and 28, and mean tumour volumes were calculated as described elsewhere [5, 9] and normalised to their respective day-0 values to produce relative tumour volumes. Growth delays (the difference in time taken for control and treated tumours to double in volume) were then determined.

Radioimmunoassay

Preparation of immunogen

The dolastatin 10 analogue dolavaline-valine-dolaisoleucine-dolaproline-methionine (GRP-18179, Fig. 1) was conjugated to bovine thyroglobulin (Sigma) using an *N*-hydroxysuccinimide condensation reaction. Briefly 5 μmol GRP-18179 was dissolved in dry redistilled dimethylformamide (DMF) and 5 μmol *N*-hydroxysuccinimide and 15 μmol *N,N'*-dicyclohexylcarbodiimide were added (total volume 0.35 ml). The solution was mixed and left at room temperature until crystals of dicyclohexyl urea formed. The supernatant was added to a solution of bovine thyroglobulin (12.5 mg) in 1 ml water at a molar ratio of 200:1. Following a further overnight period of reaction at room temperature the conjugate was dialysed against deionised water (2 × 1 l), made up to a protein concentration of 5 mg/ml and stored at 4 °C in the presence of 0.1% sodium azide.

Immunisation

Three New Zealand White rabbits (approximately 3 months old) were immunised with the GRP-18179–thyroglobulin conjugate. The priming injection consisted of 1 mg conjugate and 0.1 ml bacille Calmette-Guérin (BCG) vaccine (Evans Pharmaceuticals) in 0.5 ml sterile saline emulsified with 1.2 ml non-ulcerative Freund's adjuvant (NUFA; Guildhay Ltd., Guildford, Surrey) and was injected i.m. into four to six sites. The animals were boosted in the same way, but without the addition of BCG, at approximately 3-month intervals. Blood was obtained from the central ear vein prior to immunisation and at appropriate intervals after injection.

Radioimmunoassay techniques

Conventional radioimmunoassay techniques were used. The assay diluent was 0.05 *M* phosphate-buffered saline (pH 7.4) containing 1 g gelatin/l. All dilutions and dispensing of standards and samples were made with a Dilutrend dilutor. Solutions were kept in iced water throughout the assay procedure. Samples were assayed in duplicate at three dilutions such that the results were read off the linear part of the standard curve. Dolastatins 10 and 15 were stored in aliquots as stock standards at –20 °C at a concentration of 10 μg/ml and were diluted in assay buffer for preparation of standards in the range of 2–200 ng/ml. The antiserum (WA1) was diluted 1/500 immediately prior to use in assay buffer. Tritiated dolastatin 10 was diluted in assay buffer prior to use such that approximately 2 ng was added to each tube (100 μl).

Diluted standards and samples (0.1 ml) were added in order to LP3 tubes with 0.3 ml assay buffer, after which 0.1 ml diluted antiserum and diluted radiolabel were added and the tubes were vortexed and left to stand in iced water for 1 h. Each assay included total-count and non-specific-binding tubes containing only the radiolabel and assay buffer as well as zero-binding tubes (B_0), which contained only radiolabel and antiserum. The antibody-bound ligand was separated from unbound ligand by the addition of ice-cold dextran-coated charcoal [2.5% (w/v) activated charcoal (Sigma) coated with 0.25% (w/v) Dextran T-70 (Pharmacia)] to all tubes except the total-count tubes for 10 min. Following centrifugation at 2500 rpm for 10 min at 4 °C, 500-μl aliquots of supernatant were taken for scintillation counting in 4 ml Ultima gold scintillant (Canberra-Packard). The concentration of dolastatin was determined from the appropriate standard curve using a data-reduction programme that utilised a four-parameter logistic plot (RiaSmart, Canberra-Packard).

Mouse pharmacokinetics

Dolastatins 10 and 15 (1 mg/kg) were given i.v. to groups of BALB/c nude mice ($n = 3$). Blood samples were obtained by cardiac puncture at 0, 5, 10 and 30 min and at 1, 2, 4, 7 and 24 h and were collected into heparinised tubes. The blood was immediately separated by centrifugation and the plasma was stored at –20 °C until assayed. The extent of plasma protein binding of dolastatin 10 was determined by centrifugation of pooled mouse plasma in a Centrifree micropartition system (Amicon; 1800 rfc for 60 min). The drug concentration in the filtrate was determined by radioimmunoassay.

Statistical analysis

Where appropriate, statistical significance was evaluated using Student's two-tailed unpaired *t*-test or two-tailed alternate (Welch's approximate) unpaired *t*-test; a P value of <0.05 was considered significant.

Results

Growth inhibition

Table 1 shows the mean IC_{50} values obtained for each of the lines comprising the human ovarian and colon-carcinoma cell-line panels. Both dolastatins were very potent inhibitors of cell growth, with IC_{50} values generally being in the sub-nanomolar range. Dolastatin 10 (mean IC_{50} 2.3×10^{-10} *M* across all 11 cell lines) was significantly more potent ($P < 0.05$; 9.1-fold) than dolastatin 15 (mean IC_{50} 2.1×10^{-9} *M*). In addition, there was a trend for both drugs being more potent against the colon cell-line panel (e.g., mean IC_{50} values for dolastatin 10: 5.6×10^{-11} and 4.4×10^{-10} *M* for the colon and ovarian panels, respectively), although this did not reach statistical significance. The HX/62 human ovarian carcinoma cell line was relatively resistant to both drugs.

The effect of the time of agent exposure on growth inhibition was determined using the CH1 ovarian carcino-

Table 1 Comparative growth inhibition by dolastatin 10 and dolastatin 15 against panels of human ovarian and colon-carcinoma cell lines. Data represent mean values ±SE ($n \geq 3$)

Cell line	IC_{50} (n*M*) Dolastatin 10	Dolastatin 15
Colon:		
BE	0.035±0.018	1.0 ±0.29
HT29	0.018±0.004	0.16 ±0.05
MAWI	0.045±0.02	0.35 ±0.16
LoVo	0.031±0.011	0.17 ±0.07
SW480	0.16 ±0.06	2.24 ±1
SW620	0.046±0.006	0.12 ±0.03
Ovarian:		
A2780	0.052±0.003	0.061±0.006
CH1	0.046±0.013	0.15 ±0.009
SKOV-3	0.17 ±0.07	4.2 ±2.3
41M	0.17 ±0.6	5[a]
HX/62	1.8 ±0.78	~10[a]

[a] $n = 2$

ma cell line (Fig. 2). Results show that at all time points (and as reflected in Table 1), dolastatin 10 was more potent than dolastatin 15. Potency peaked for both agents following a 24-h exposure period. Dolastatin 10 was 24-fold more potent after 96 h of exposure than following a 2-h exposure period; the corresponding difference for dolastatin 15 was a factor of 13.2.

Growth-inhibitory effects on acquired drug-resistant cell lines

Figure 3A shows the effects of the dolastatins on two acquired cisplatin-resistant human ovarian carcinoma cell lines and a line selected for resistance to etoposide. Resistance factors (RF; IC_{50} resistant/IC_{50} parent) recorded for dolastatin 10 were <1.5 for both cisplatin-resistant lines,

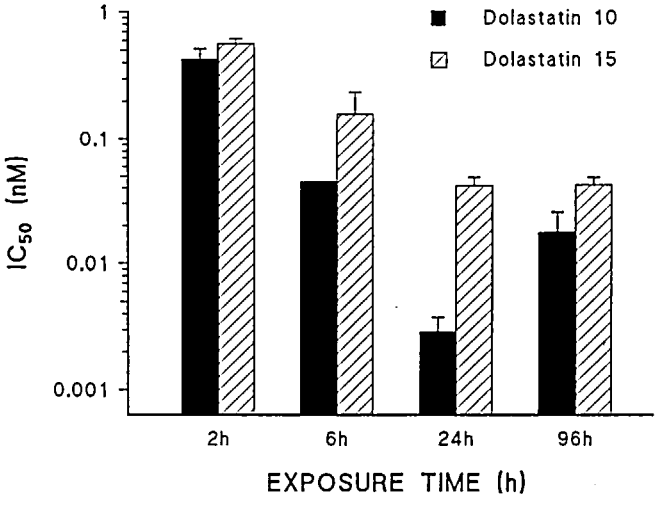
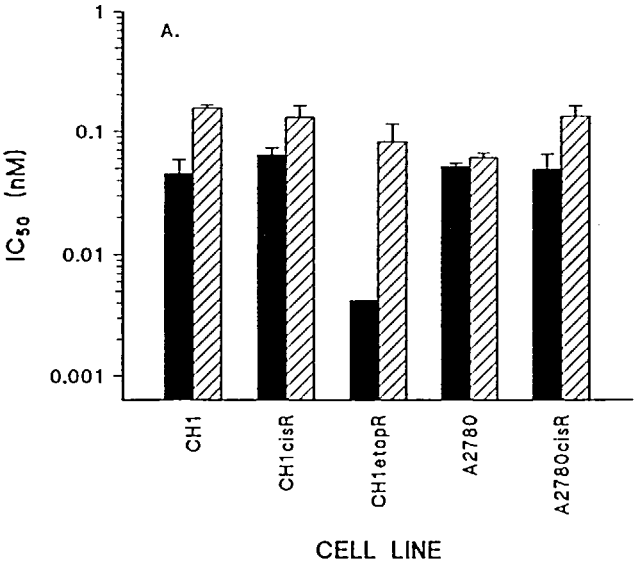


Fig. 2 The effect of exposure time on the growth-inhibitory properties of dolastatin 10 (*black bars*) and dolastatin 15 (*hatched bars*) against the CH1 human ovarian carcinoma cell line. Data represent mean values ±SD ($n = 3$)



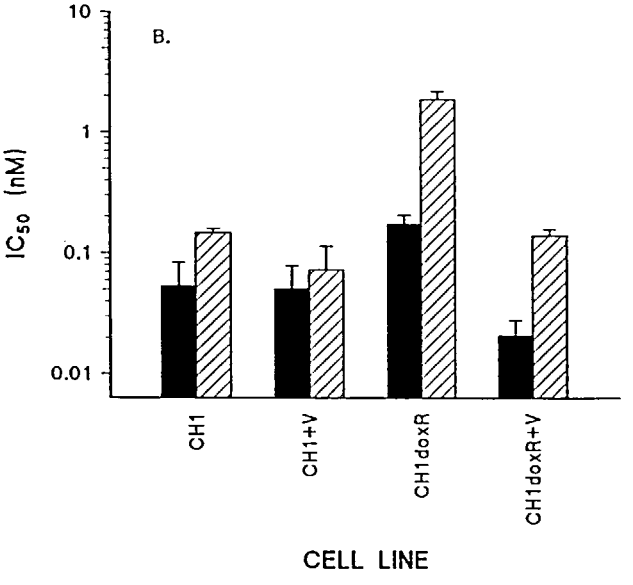


Fig. 3A, B Growth inhibition of dolastatin 10 (*black bars*) and dolastatin 15 (*hatched bars*) against A human ovarian carcinoma cell lines possessing acquired resistance to cisplatin (*cisR lines*) or etoposide (*etopR line*) versus the respective parent lines or B the CH1 human ovarian carcinoma cell line and a subline possessing acquired resistance (90-fold) to doxorubicin (*CH1doxR*) either alone or in the presence of 6 μ*M* verapamil (+*V bars*). Data represent mean values ±SD ($n = \geq 3$)

indicative of no cross-resistance. For dolastatin 15, no cross-resistance was observed with CH1cisR (RF 0.83), whereas low-level resistance (RF 2.1) was observed with A2780cisR. Interestingly, collateral sensitivity (RF <0.6) was observed to both agents with the acquired etoposide-resistant CH1etopR cell line.

The multidrug-resistance (*MDR*) phenotype mediated by the *mdr1* gene is commonly associated with anticancer drugs derived from natural products (e.g., paclitaxel, doxorubicin, vincristine, etoposide) [12]. Figure 3B shows the

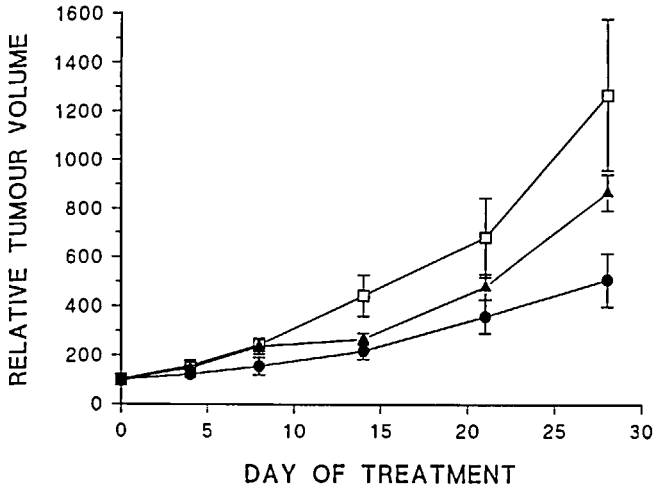


Fig. 4 In vivo activity of dolastatin 10 (450 μg/kg given i.v. × 1; *black circles*) and dolastatin 15 (5.25 mg/kg given i.p. q4dx3; *black triangles*) versus untreated controls (*white squares*) as determined using the advanced-stage CH1 human ovarian carcinoma xenograft. Data represent mean values ±SD ($n = 6$ for treated groups, $n = 10$ for controls)

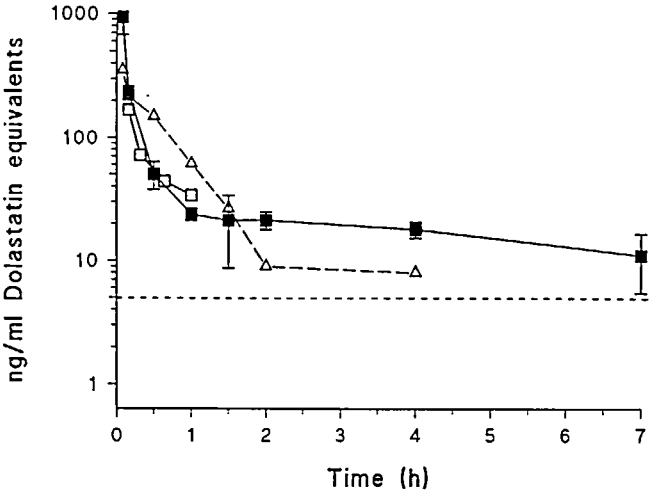


Fig. 6 The plasma levels of immunoreactive dolastatin 10 and dolastatin 15 measured in mice ($n = 3$) following the i.v. administration of 1 mg/kg. Dolastatin 10 (*white squares*) samples were collected for 1 h only; dolastatin 10 (*black squares*) and dolastatin 15 (*white triangles*) samples were collected for up to 24 h. The limit of detection of the assay is shown by the *dotted line*. For clarity the standard deviation between mice at each time point is shown for dolastatin 10 (24-h profile) only, a similar variation being observed for the other groups

growth-inhibitory effects of the dolastatins against an acquired doxorubicin-resistant cell line (CH1doxR, 90-fold resistant) previously shown to overexpress the *mdr1* gene product P-glycoprotein (P-gp) and to be fully cross-resistant to paclitaxel and vinblastine [22, 25]. It was noteworthy that the doxR line was only 3.2-fold cross-resistant to dolastatin 10 but was significantly more resistant (12.7-fold; $P < 0.05$) to dolastatin 15. As in our previous studies with the CH1doxR cell line [22], the resistance modifier verapamil (highest non-toxic concentration 6 μ*M*) was used to attempt circumvention of dolastatin resistance in the CH1doxR cell line. Whereas verapamil induced only small differences in the growth-inhibitory effects of the dolastatins against the parental CH1 line, the modifier completely overcame the cross-resistance to both dolastatin 10 and dolastatin 15 in CH1doxR.

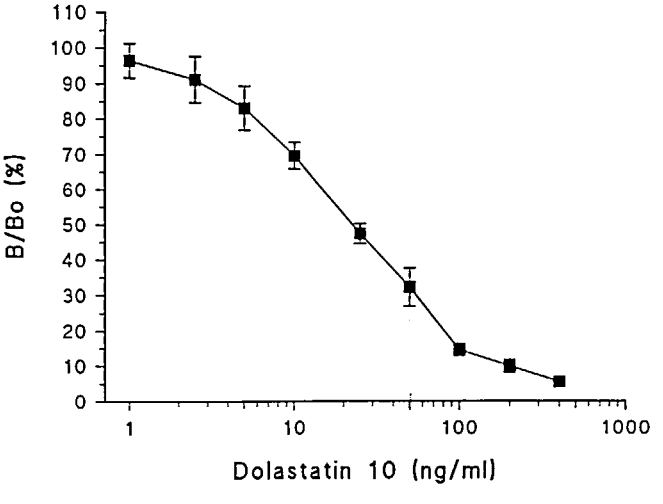


Fig. 5 The mean (±SD) of 5 successive standard curves generated for dolastatin 10, expressed as the percentage of drug bound over the binding in the absence of added standard (*B/Bo*)

In vivo antitumour testing

Using previously obtained information (courtesy of the United States National Cancer Institute), we evaluated the dolastatins at equitoxic doses and optimal schedules in nude mice bearing advanced-stage CH1 human ovarian carcinoma xenografts, the in vivo counterpart of a cell line shown to be sensitive to the effects of the two agents (Table 1). Dolastatin 10 (450 μg/kg given i.v. on day 0) induced a tumour growth delay of 6.1 days as compared with 0.4 days for dolastatin 15 (5.25 mg/kg given i.p. on days 0, 4, 8; Fig. 4). Unfortunately, a lack of adequate drug supplies precluded further evaluation.

Radioimmunoassay

All three animals produced antisera following the prime injection, the highest level of binding being achieved at 7 weeks after the priming immunisation. The highest-titre antiserum (WA1) was used for these studies at a dilution of 1/500. Subsequent blood samples obtained following booster injections showed similar or higher-level binding of [^{3}H]-dolastatin 10.

Using the protocol described, we obtained a standard curve for dolastatin 10 that covered the range of 0–200 ng/ml. Figure 5 shows the mean (±SD) of 5 successive standard curves. A 50% inhibition of binding occurred at

Table 2 Comparative pharmacokinetic data (±SE) estimated for dolastatin 10 and dolastatin 15 in mice using the period 0–4 h. Two-compartmental analysis using PCNONLIN was employed to evaluate the plasma profiles

	Dolastatin 10 0–24 h	Dolastatin 10 0–4 h	Dolastatin 15 0–4 h
$t_{1/2\alpha}$ (h)	0.04± 0.001	0.04± 0.002	0.09± 0.18
$t_{1/2\beta}$ (h)	2.4 ± 1.25	1.6 ± 0.83	0.52± 0.32
AUC (ng ml^{-1} h)	364.5 ±53	333 ±38	208 ±41
r^2	1.0	1.0	0.97

22±2.6 ng/ml. The assay is sensitive to the addition of 1.25 ng dolastatin 10/ml as determined from fall of a 2–3 SD in binding from that of the Bo (binding in the absence of standard). The addition of undiluted plasma caused a matrix effect, which could be avoided by dilution of plasma samples at least four times prior to assay. This gives a final limit of detection for plasma samples of 5.0 ng/ml. The recovery of dolastatin 10 from plasma was complete (102.3±20.7%, n = 10, at 100 ng/ml). The within-assay variation determined with one plasma sample was 11.1% (702±78 ng/ml) and the between-assay variation of a control sample was 12.1% (97.1±11.8 ng/ml).

Formal cross-reactivity experiments were limited by the supply of dolastatin 10 analogues and metabolites. For this reason the results of the radioimmunoassay are expressed as dolastatin equivalents (i.e., immunoreactivity). Dolastatin 15 cross-reacted in the assay by approximately 65%, and can thus also be measured using the WA1 antiserum as long as the dolastatin 15 standards are included in the assay. Unrelated compounds such as halichondrin B do not cross-react with the antibodies.

Pharmacokinetics

No immunoreactivity was detected in plasma from untreated mice. The plasma levels of immunoreactive dolastatin 10 and dolastatin 15 measured following i.v. administration of 1 mg/kg are shown in Fig. 6. Initially plasma samples were taken for only 1 h following dolastatin 10 administration. As there was adequate sensitivity to measure immunoreactivity at this time point, longer periods were included in subsequent experiments. Good agreement between experiments were observed (Fig. 6). For dolastatin 10, immunoreactivity was detected in all three animals at 7 h, but by 24 h it was possible to detect the drug in only two of three animals. For dolastatin 15, immunoreactivity was detectable only for up to 4 h following administration. Plasma binding of dolastatin 10 exceeded 90%. A two-compartmental analysis of the pharmacokinetic data covering up to 4 h was undertaken using PCNONLIN, and the results are shown in Table 2. The second-phase half-life ($t_{1/2\beta}$) of dolastatin 10 was approximately 3 times longer than that of dolastatin 15. The area under the concentration-time curve (AUC) was 1.6-fold greater for dolastatin 10 than for dolastatin 15 (333±38 and 208±41 ng ml^{-1} h, respectively).

Discussion

There is currently considerable interest in the group of compounds known as the dolastatins as they have been shown to be extremely potent growth inhibitors of leukaemia and lymphoma cell lines [2, 3, 26]. The high potency of the drugs was confirmed in this study, but using human tumour cell lines derived from the "solid" tumours of ovarian and colon carcinoma. In common with the leukaemia and lymphoma data [2, 3], dolastatin 10 was more potent than dolastatin 15 (across the colon and ovarian panels, by 9.1-fold). This may relate to their respective binding affinities for tubulin; IC_{50} values recorded for inhibition of glutamate-induced polymerisation of tubulin were 23 μ*M* for dolastatin 15 as compared with 1.2 μ*M* for dolastatin 10 [2]. Furthermore, against the five human ovarian carcinoma cell lines, dolastatin 10 was 17-fold and 2-fold more potent than the tubulin-interacting drugs paclitaxel and vinblastine, respectively [7, 22]. The HX/62 human ovarian carcinoma cell line was relatively resistant to both dolastatins, especially dolastatin 10. The mechanism(s) underlying this is presently unknown, although HX/62 is known not to overexpress P-gp (data not shown; see below).

Our experiments in which the time of drug exposure was varied (2, 6, 24 and 96 h) indicate that the drugs were 24- and 13.2-fold less potent after the 2-h exposure period and 2.5- and 3.7-fold less potent following the 6-h exposure period as determined for dolastatin 10 and 15, respectively (as compared with the IC_{50} values obtained following 96 h of exposure).

Hence, it appears that for maximal growth inhibition, both dolastatins must be in contact with the tumour cells for at least 6 h. This is in agreement with other studies using leukaemia and lymphoma cell lines [3, 26], where the inhibitory effects of both dolastatins (added at 0.1 ng/ml) were reversible if cells were washed free of drug after a 0.5-, 1- or 4-h period of exposure. However, by 8-h drug exposure it was not possible to reverse the inhibitory effects of either agent [3].

The dolastatins showed no marked cross-resistance in human ovarian carcinoma cell lines selected for resistance to the commonly used anticancer drugs cisplatin and etoposide. Indeed, there was evidence of collateral sensitivity to the dolastatins (especially dolastatin 10) with the etoposide-resistant cell line CH1etopR. Mechanistic studies with the recently established etoposide-resistant cell line are ongoing but do not appear to involve elevated P-gp levels [29].

Previous studies have shown that leukaemia cell lines expressing a multidrug-resistance (MDR) phenotype (selected for resistance to vincristine) are cross-resistant to dolastatin 10 and that such resistance is reversible by verapamil [28]. Our data, obtained using the CH1 human ovarian carcinoma cell line selected for resistance to doxorubicin (90-fold) and known to overexpress P-gp [25], show that dolastatin 15 is a significantly better substrate for P-gp than is dolastatin 10; resistance factors were 12.7-fold to dolastatin 15 but only 3.2-fold to

dolastatin 10. Resistance to both agents was reversible by the P-gp resistance modifier verapamil.

Previously, in vivo activity has been reported for dolastatin 10, but predominantly using i.p. administration to i.p. implanted tumours [16, 30]. In vivo activity against the s.c. implanted human ovarian xenograft CH1 was observed in this study (growth delay 6.1 days). The CH1 tumour was originally selected on the basis of its good sensitivity to cisplatin (growth delay around 40 days with cisplatin and carboplatin [5]). However, activity lower than that observed with dolastatin 10 has recently been obtained with paclitaxel (0.7-day growth delay) and doxorubicin (1.7-day growth delay; data not shown). The administration protocols used for the dolastatins were based on those employed at the NCI during their evaluation of these compounds. At equitoxic doses and optimum schedules, dolastatin 15 appeared to have less antitumour activity than dolastatin 10, even though it was given in a multiple dose schedule. However, this drug has a shorter half-life than dolastatin 10 (see below), and antitumour activity would ideally need to be compared under schedules ensuring similar drug exposure for the two compounds. However, a lack of drug supplies prevented a more extensive study of drug doses and schedules.

Antibodies to other natural-product cytotoxic compounds have previously been described [11, 27] and used to develop immunological methods for their analysis. The hapten used in our study was relatively immunogenic, producing antisera suitable for assay development in all three animals immunised. The radioimmunoassay has a limit of detection of 5 ng/ml, which is unfortunately higher than that obtained in many other radioimmunoassays for small molecules, and this is thought to be due primarily to the relatively low specific activity of the radiolabeled dolastatin 10.

The antibodies used in the radioimmunoassay (RIA) developed for the measurement of dolastatin 10 cross-react extensively with dolastatin 15. This is to be expected when the structures of the hapten and dolastatins 10 and 15 are compared. The most immunogenic part of the hapten is that which is furthest away from the point of attachment to the carrier protein. Four amino acid residues are common to dolastatin 10 and the hapten, but only the first two residues of dolastatin 15 are shared with the hapten, resulting in reduced recognition by the antiserum.

Dolastatin 10 is rapidly metabolised by liver homogenate or microsomal extracts [14] to more polar species, which are relatively long-lived in the plasma. If the antibodies used in the RIA had cross-reacted to a great extent with these products, results similar to those obtained when dolastatin 10-associated radioactivity was specifically measured would not have been obtained. One of these putative metabolites is dihydroxydolastatin. However, as the dolastatin breakdown products and metabolites have not yet been characterised and their interaction with the antibody is not known, the results obtained with the antibody are reported as dolastatin 10 or dolastatin 15 equivalents (immunoreactivity). When such metabolites are available, e.g. from liver microsomal preparations, formal specificity studies will be carried out. The precise cytotoxic nature of dolastatin metabolites is also not known.

The pharmacokinetic results obtained for dolastatin 10 in this study are comparable with those obtained using radiolabeled drug [14] and L1210 bioassay [23]. This suggests that interference due to metabolite cross-reactivity may not be significant. Using plasma levels determined over 24 h, these studies show that there is a rapid distribution phase (approx. 2 min) followed by a more prolonged second phase of elimination (2.4 h; Table 2). Correcting for the dose used and assuming linear kinetics, the C_{max} (940±264 ng/ml) and $AUC_{0-7\ h}$ values (364.5±53 ng ml^{-1} h) were similar to those measured using radiolabel (1116 ng/ml and 270 ng ml^{-1} h, respectively, corrected to 1 mg/kg i.v.), where the dose was approximately 4 times lower than that used in this study. The elimination $t_{1/2}$ value (2.4 h) obtained by RIA is similar to that obtained with radiolabeled drug (5.6 h) but is considerably shorter than that obtained with the bioassay (15 h).

The pharmacokinetics of dolastatin 15 have not previously been described. The slightly faster elimination half-life and lower AUC value found for dolastatin 15 may partly account for the poorer antitumour activity observed in vivo.

Because of the high potency of dolastatins 10 and 15, it is essential that a highly sensitive assay be available for both pre-clinical and clinical studies. The RIA offers a convenient high-throughput alternative to the more laborious bioassay for measurement of these compounds in biological fluids. In the absence of a higher-specific-activity radiolabel, it is expected that an enzyme-linked immunosorbent assay (ELISA) would be capable of providing superior sensitivity. Indeed, preliminary work in our laboratory has shown that at least a 5-fold increase in sensitivity can be achieved using an ELISA. However, until drug metabolism and the cytotoxic properties of any metabolites have been more fully described, the results obtained with the present antibodies cannot be considered to be specific for the parent compound or any cytotoxic species. The use of an immunoassay with a separation method, e.g. HPLC, would provide a means of overcoming this problem.

The RIA described in this study will be useful for the determination of the pharmacokinetics of dolastatin 10 (and 15) during phase I clinical trials. From the pre-clinical antitumour data obtained in this study, dolastatin 10 would appear to possess antitumour properties more favourable than those of dolastatin 15 (viz., more in vitro potency, less susceptibility to MDR, more activity against a s.c. implanted human ovarian carcinoma xenograft). Clinical trials are expected to begin during 1995.

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