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In vitro and *in vivo* activity of LS 4477 and LS 4559, novel analogues of the tubulin binder estramustine

K.M. Nicholson[a], R.M. Phillips[b], S.D. Shnyder[b], M.C. Bibby[b,*]

[a]*Division of Cancer Studies, University of Manchester, Manchester M13 9TT, UK*
[b]*Cancer Research Unit, University of Bradford, Bradford BD7 1DP, UK*

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Abstract

LS 4477 and LS 4559, two of a series of *N*-acyl-aminoalkyl phenyl ethers, are rationally designed compounds based on the tubulin binder estramustine. This study investigated their mechanism of action and compared their effectiveness in relation to estramustine *in vitro* against a panel of human and murine cell lines and *in vivo* against two murine colon tumour models (MAC). At biologically relevant concentrations, LS 4477 and LS 4559 caused a 59.9 and 56% reduction in tubulin assembly, respectively, compared with a 28.4% reduction in tubulin assembly by estramustine. The analogues were approximately 100 times more potent in chemosensitivity tests *in vitro* than the parent compound. Both analogues were orally active against the MAC 15A murine tumour model, to a greater extent than estramustine, producing significant growth delays ($P<0.01$). Significant activity was also shown against the slower growing MAC 26 tumour for LS 4577 (the soluble pro-drug of LS 4559). The results presented in this study suggest these compounds warrant further development with a view to assessing their clinical activity.

Keywords: Estramustine analogues; *In vitro*; *In vivo*

1. Introduction

Tubulin and microtubules are structures ubiquitous to most eukaryotic cells and are the main components of spindles in the mitotic apparatus. In addition, microtubules are involved in other interphase functions such as cell motility, determination of cell shape, organisation and positioning of organelles and signalling between cell surface receptors and the nucleus [1]. The major role of microtubules during cell division makes them potentially useful targets for anti-cancer agents and amongst the most studied chemotherapy agents are the vinca alkaloids, such as vincristine and vinblastine, which are known to bind tubulin. A resurgence in interest centred on tubulin binders as effective anti-cancer agents occurred following the discovery of paclitaxel and the elucidation of its novel mechanism of action [2]. In contrast to other tubulin binders such as the vinca alkaloids that cause the depolymerisation of microtubules, paclitaxel causes the polymerisation and stabilisation of tubulin.

Estramustine (Estracyt) is another of the class of compounds termed antimicrotubule agents and its structure consists of an oestrogen linked to nor-nitrogen mustard via a carbamate ester linkage (Fig. 1). It was envisaged that estramustine would target hormone receptors before cytotoxicity was induced by release of the nitrogen mustard group following cleavage of the carbamate ester link. However, the true mechanism of action of estramustine was different to that expected since the carbamate ester link was excessively stable to enzymatic cleavage. Results from *in vivo* experiments with estramustine confirmed that it had different properties to those expected of alkylating agents or hormones. Although estramustine is metabolised to oestrogens both *in vitro*, and *in vivo*, it is also active in tissues and cell lines which lack oestrogen receptors [3]. It was also apparent that estramustine did not possess alkylating activity [4].

* Corresponding author. Tel. +44-1274-233227; fax: +44-1274-233234.
E-mail address: m.c.bibby@bradford.ac.uk (M.C. Bibby).

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However, on exposure to estramustine it was noted that cells were arrested in metaphase and the mitotic spindle was absent [5]. These results suggested that estramustine was acting via an interaction with microtubules to promote microtubule disassembly and it has since been confirmed that estramustine acts via a direct interaction with microtubule-associated proteins [6] and with tubulin [7,8]. A phosphate group was added at the 17-β position of the steroid D ring to increase the water solubility of the compound (Fig. 1). Estramustine phosphate sodium is immediately dephosphorylated to estramustine after administration and is then partially oxidised to estromustine, an agent with the same effects as estramustine before finally being broken down into oestrone and oestradiol [9].

As for its anticancer activity, estramustine has been shown to have moderate activity against hormone-refractory and metastatic prostate cancers. One reason for this is the presence of estramustine binding protein (EMBP) in the prostate which facilitates uptake of estramustine into these tissues [10,11]. Expression of EMBP has been shown to correlate with the levels of estramustine measured in prostate tumours suggesting EMBP is responsible for the retention of estramustine in these tumours [12]. Estramustine has also shown promising activity in combination chemotherapy regimens where it has been shown to improve the efficacy of other anticancer agents such as etoposide, paclitaxel and vinblastine in patients with hormone-refractory prostate cancer [13–17].

LS 4477 and LS 4559, two members of a series of *N*-acyl-aminoalkyl phenyl ethers were rationally designed compounds based on estramustine which were synthesised to maintain the tubulin binding properties of estramustine whilst removing the steroid moiety from the structures. LS 4477 and LS 4559 differ only in the addition of a fluorine atom to position 3 on LS 4559 (Fig. 2). In preliminary studies, LS 4477 and LS 4559 were found to be more effective than estramustine against the human prostatic DU 145, the rodent prostatic AT-1 and human ovarian SCOV-3 cell lines *in vitro* [18]. The phosphate esters of the compounds, synthesised to increase solubility and provide potentially orally active analogues, were shown to be active against the Dunning hormone-resistant AT tumour and the Walker 256 carcinoma tumours *in vivo* [18].

This study was designed to extend these preliminary studies into a different cancer type by investigating the activity of estramustine and the potential activity of two novel analogues of estramustine, LS 4477 and LS 4559 in models of colon cancer. The ability of the analogues and parent compound to interact with tubulin was evaluated and the potential activity of the analogues in relation to estramustine was determined *in vitro* and *in vivo* against two well-established models of colon cancer [19,20].

2. Materials and methods

2.1. Tubulin assembly assay

Porcine brain microtubules were isolated from fresh tissue immediately following slaughter of the animal and were purified by repeated cycles of assembly and disassembly [21] (kindly provided by Dr A.T. McGown, Paterson Institute for Cancer Research, Manchester, UK). Estramustine (Pharmacia), LS 4477 (Pharmacia) or LS 4559 (Pharmacia), dissolved in dimethyl sulphoxide (DMSO) (50 μl) to give a range of final concentrations from 0 to 40 μM, were added to Mes Buffer (750 μl) [0.1 M Mes 1 mM ethylene diamine tetra acetic acid (EDTA), 1 mM EGTA, 1 mM magnesium chloride and 1 mM 2-mercaptoethanol pH6.4], 10 mM guanidine triphosphate (GTP) (100 μl) and tubulin (100 μl) on ice. Control samples were prepared as above but with 50 μl of drug-free DMSO. Assembly was initiated by increasing the temperature to 35 °C and reading the absorbance of each sample at 350 nm over 30 min against sample blanks and control samples.

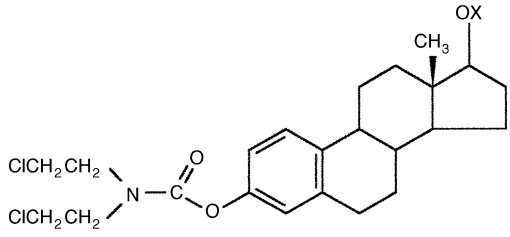

Fig. 1. Structure of estramustine (X = H) and its phosphate ester prodrug estramustine phosphate sodium ($X = PO_3Na_2H_2O$).

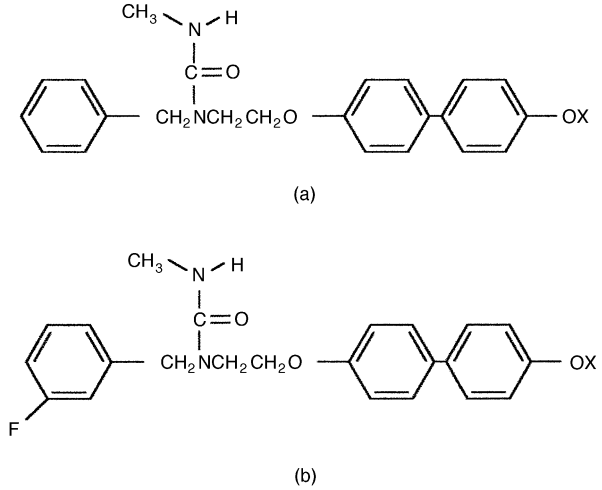

Fig. 2. (a) Structure of LS 4477 (X = H) and its phosphate ester prodrug LS 4578 ($X = PO_3Na_2$) and (b) Structure of LS 4559 (X = H) and its phosphate ester prodrug LS 4577 ($X = PO_3Na_2$).

2.2. Competitive binding assay

The ability of estramustine, LS 4477 and LS 4559 to compete with colchicine binding to tubulin was assessed using a spun column method [22]. Tubulin was incubated with colchicine (100 μl of 10 μM colchicine spiked with 20 nCi/ml [^{3}H]-colchicine, provided by Dr McGown) and test compound in Mes buffer to give a range of tubulin/test compound ratios. Samples were incubated for 90 min at room temperature in the dark then placed on ice for 5 min to terminate the reaction. Samples were added to G50 Sephadex columns and the eluent collected after centrifugation (900*g* for 2 min). The eluent (100 μl) was added to 5 ml of Ecoscint A and was analysed by liquid scintillation counting. All experiments were performed in triplicate.

2.3. Cell culture

A panel of human and murine cells was used in this study (see Table 1 for cell line characteristics). All cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with 25 mM HEPES buffer (Gibco), supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate and penicillin-streptomycin (50 IU/ml/50 μg/ml). Cells were grown as monolayer cultures, with the exception of K562 which grew as a suspension culture, at 37 °C in a humidified atmosphere of 95% air/5% CO_2. All cell lines were routinely tested for mycoplasma contamination and were found to be free of infection.

2.4. In vitro *chemosensitivity testing*

Estramustine was dissolved in DMSO and diluted in RPMI medium such that DMSO did not exceed a concentration of 1% (v/v). LS 4477 and LS 4559 were dissolved in DMSO and diluted in RPMI medium to give a final DMSO concentration of 0.3% (v/v). DMSO was not found to be cytotoxic to cells at these concentrations. Chemosensitivity was assessed following a continuous 96-h exposure to a range of drug concentrations by use of a standard dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay [23,24]. Cytotoxicity was expressed as an IC_{50} value which is the concentration of drug required to reduce cell survival by 50%.

2.5. Confocal microscopy of tubulin morphology

DLD-1 cells were seeded at 1×10^4 cells/ml in RPMI 1640 medium supplemented with 10% FBS onto eight-chamber slides (Iwaki) and left to attach and spread for 24 h (all incubations at 37 °C, 5% CO_2). The compound under test was then added to the appropriate well and the cells incubated for 90 min. After a wash with HBSS, fresh RPMI 1640 was added and the cells incubated for a further hour. Medium was then removed and the cells fixed in pre-cooled methanol at −20 °C for 30 min. After two washes in PBS (all incubations at room temperature from this stage), cells were incubated in the primary monoclonal antibody, mouse anti-α-tubulin (Sigma) at a dilution of 1:500 for 30 min. After three further washes in PBS, the secondary antibody, tetramethylrhodamine isocyanate-conjugated rabbit anti-mouse IgG (Dako) was added at a dilution of 1:50 for 30 min. After three final washes, the cultures were mounted in fluorescent mounting medium (Dako) and stored at 4 °C until they were analysed with a Biorad Microradiance Confocal Imaging System attached to a Nikon CM-800 microscope using LaserSharp 2000 software.

2.6. Animals

Pure strain, male NMRI mice (B and K, Hull) aged 6–8 weeks used in this study were exposed to regular cycles of light and dark and were given unlimited access to food and water. Experiments were performed under a project licence approved by the Home Office and UK official guidelines were observed [25].

Table 1
In vitro chemosensitivity results following exposure of cells to estramustine, LS 4477 or LS 4559 for 96 h as assessed by an MTT assay[a]

Cell line	Cell line characteristics	$IC_{50}\pm$S.D. (μM)		
		Estramustine	LS 4477	LS 4559
MAC 15A	Ascitic murine adenocarcinoma of the colon [27]	2.40±0.36	0.01±0.001	0.02±0.003
MAC 26	Well differentiated murine adenocarcinoma of the colon [27]	2.10±0.22	0.01±0.002	0.01±0.001
DLD-1	Human adenocarcinoma of the colon [28]	3.07±0.42	0.02±0.003	0.03±0.001
HCT 18	Human adenocarcinoma of the colon	2.27±0.17	0.07±0.001	0.03±0.002
HRT 18	Human primary rectal adenocarcinoma [29]	1.57±0.21	0.03±0.002	0.03±0.001
K562	Human chronic myelogenous leukaemia [30]	0.80±0.10	0.03±0.001	0.03±0.002
HCLO	Human adenocarcinoma of the colon	2.53±0.32	0.04±0.007	0.12±0.026

MTT, dimethylthiazolyl-2,5-diphenyltetrazolium bromide; IC_{50}, concentration required for 50% reduction in survival.
[a] Results are expressed as an IC_{50} value (μM)±standard deviation of three independent experiments.

2.7. Tumour models

MAC 15A subcutaneous (s.c.) tumours were established by implantation of ascitic MAC 15A cells grown in the peritoneal cavity of donor NMRI mice into the flank of male NMRI mice. Treatment commenced on day 3 post-cell inoculation when tumours had reached a measurable size. MAC 26 tumours were routinely maintained as solid tumours grown subcutaneously in NMRI mice. Tumours were removed from donor mice and placed in 0.9% sterile saline before tumour fragments (1–2 mm) were implanted subcutaneously into the flank of male NMRI mice. Treatment commenced approximately 17 days post-implantation when tumours reached a measurable size.

2.8. Chemotherapy

Estramustine phosphate sodium (EMPS), the water soluble pro-drug of estramustine (Pharmacia) was prepared in 0.9% sterile saline. For intravenous (i.v.) administration EMPS was prepared in 0.9% sterile saline with 12% bovine serum albumin (BSA). LS 4578 (Pharmacia) and LS 4577 (Pharmacia), the soluble pro-drugs of LS 4477 and LS 4559, respectively, (Fig. 2) were dissolved in 0.9% sterile saline. EMPS was administered to mice as 0.1ml/10g body weight via i.v., intraperitoneal (i.p.) or oral routes. All test compounds were administered at the specified dose daily for 5 days. The new analogues were administered orally. The effect of each drug on tumour volume was assessed by serial two-dimensional caliper measurements. Tumour volume was calculated using the formula $a^2 \times b/2$ where a is the smaller and b the larger value [26]. Relative tumour volume (RTV) was calculated taking day 0 values to represent a tumour volume of 1.0. The significance of tumour growth delay was assessed using a Mann–Whitney U test on the time taken by each tumour in control and treated groups to reach a RTV of 2.0.

Table 2
Effect of estramustine, LS 4477 and LS 4559 on the ability of purified tubulin to assemble[a]

	% Change in absorbance		
Concentration (μM)	Estramustine	LS 4477	LS 4559
Control	100	100	100
10.0	89.5	45.9	47.6
20.0	79.1	43.2	44.0
40.0	71.6	40.1	44.0

[a] Tubulin assembly in the presence and absence of each drug was followed spectrophotometrically (350 nm) at 35 °C over 30 min. Results are expressed as % change in absorbance assuming change in absorbance for control (drug-free) samples to be 100%.

3. Results

3.1. Tubulin assembly assay

The ability of estramustine, LS 4477 and LS 4559 to disrupt microtubule assembly was assessed by following the assembly of purified porcine brain tubulin in the presence and absence of each drug. The effect of each concentration was compared with assembly of control (DMSO) samples, assuming the change in absorbance of these samples to represent 100% (see Fig. 3 and Table 2). The results show that estramustine was only slightly effective at inhibiting tubulin assembly at all the concentrations tested, although a concentration dependent effect was apparent. Maximum inhibition of assembly occurred upon the addition of 40 μM estramustine which corresponded to a 28.4% inhibition of tubulin assembly. Estramustine had little effect on the dynamics of tubulin assembly since the initial rapid phase of assembly and the time for equilibrium to be achieved were similar in control and estramustine-treated samples (Fig. 3). LS 4477 inhibited tubulin assembly with maximum effects relating to 59.9% inhibition occurring with a concentration of 40 μM. Addition of less than 5 μM had little effect on tubulin assembly (data not shown). Concentrations exceeding 5 μM caused a reduction in the time for tubulin assembly to reach a plateau. The concentration of LS 4477 required to reduce tubulin assembly by 50% was calculated as 5.2 μM which was similar to that observed with vinblastine (data not shown). LS 4559 was also effective at inhibiting tubulin polymerisation. However, the effect was not dependent on concentration since similar levels of inhibition occurred over the range of concentrations tested. The concentration of LS 4559 required to inhibit tubulin polymerisation by 50% was calculated as 3.5 μM, a value less than that calculated for LS 4477 or vinblastine.

3.2. Competitive binding assay

Control samples (drug/tubulin, 0/1) had a mean count of 11343.7±1024.2 dpm. Samples containing [^{3}H]-colchicine with neither test compound or tubulin (ratio 0/0) had a mean count of 97.8±62.9 dpm which related to 0.9±0.5% of the control counts. This negligible value suggested that [^{3}H]-colchicine does not bind to Sephadex columns in the absence of tubulin, therefore all radioactivity can be assumed to result from [^{3}H]-colchicine remaining bound to tubulin. As such, results were expressed as a percentage of control counts and accordingly the percentage of bound [^{3}H]-colchicine.

Estramustine, at all drug/tubulin ratios, was comparatively ineffective at competing with colchicine for binding to tubulin (Fig. 4 and Table 3) with a minimum of 65.9±3.2% of colchicine remaining bound to tubulin

at the highest drug/tubulin ratio. LS 4477 showed a concentration-dependent inhibition of colchicine binding with maximum mean displacement of 90.8% colchicine (9.2±3.4% colchicine remaining bound) with a drug/tubulin ratio of 100/1 (Table 3 and Fig. 4). LS 4559 caused an almost complete displacement of [^{3}H]-colchicine at all drug/tubulin ratios of more than 1:1. Maximum inhibition occurred with a ratio of 100/1 and accounted for the mean displacement of 93.3% colchicine (6.7±0.9% remaining bound). At all drug/tubulin ratios tested, LS 4559 was more effective than the related compound, LS 4477, at binding competitively with colchicine to tubulin.

3.3. In vitro *chemosensitivity studies*

The effect of estramustine on a panel of cell lines mainly derived from the colon is shown in Table 1. All cell lines with the exception of K562, the only leukaemia cell line in the panel, exhibited similar levels of sensitivity to estramustine. K562 cells were the most sensitive and DLD-1 the most resistant. Cells were also exposed to LS 4477 and LS 4559 for 96 h (Table 1). MAC 15A and MAC 26 were the most sensitive to the effects of LS 4477 and HCT 18 the most resistant. LS 4559 was also most effective against MAC 26 and MAC 15A cells, but least effective against HCLO cells. DLD-1 cells were also exposed to the prodrugs LS 4578 and LS 4577. For 96 h exposures, IC_{50} values were 0.94 and 0.90 μM, respectively. Much higher concentrations were required to kill DLD-1 cells in a shorter 90-min exposure (IC_{50} 640 and 585 μM, respectively).

The comparative IC_{50} values produced for each agent have shown that the pattern of cell sensitivity and resistance is not consistent for each agent tested. A trend appeared such that the cell lines derived from murine adenocarcinoma of the colon, MAC 26 and MAC 15A, were most sensitive to the analogues and the human-derived cell lines were more resistant. The results showed that the cell panel was approximately 100 times more sensitive to the analogues, LS 4477 and LS 4559, than estramustine.

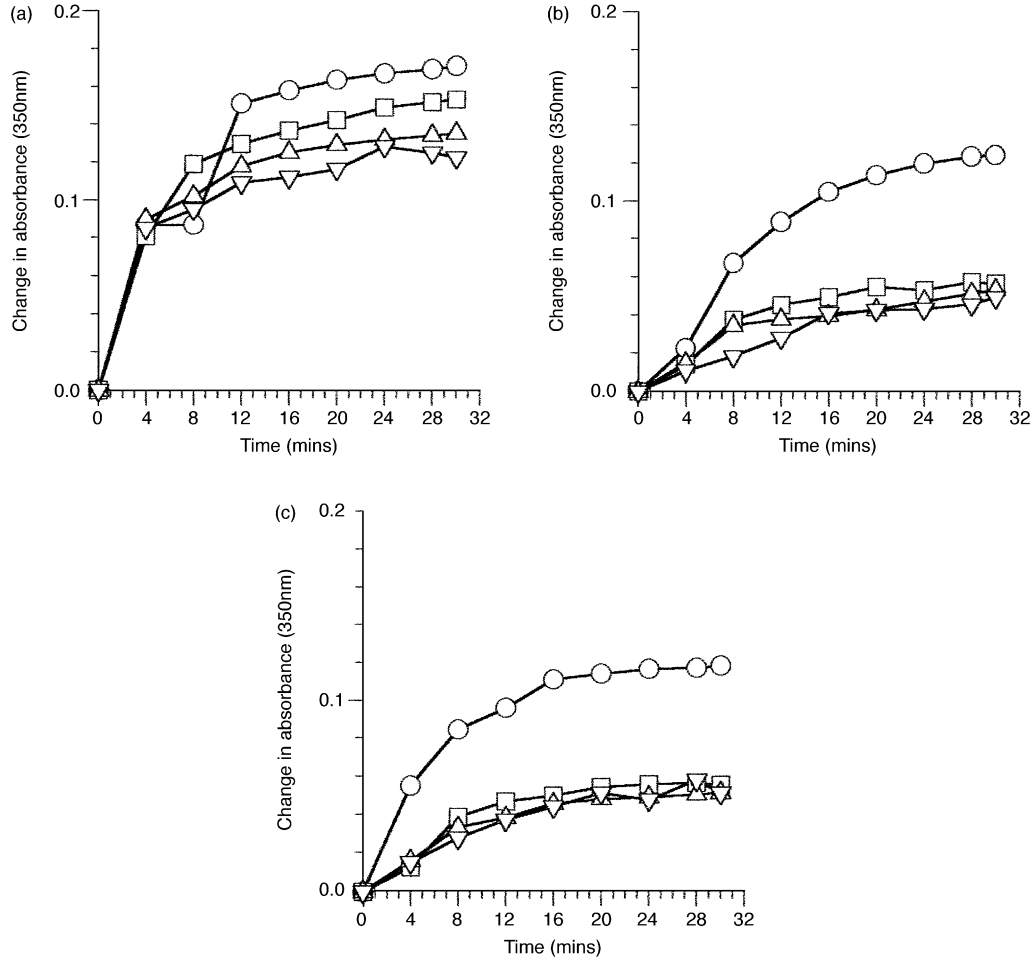

Fig. 3. Effect of (a) Estramustine, (b) LS 4477 and (c) LS 4559 on the ability of purified tubulin to assemble. Tubulin assembly in the absence of drug (○) or in the presence of 10 μM (□), 20 μM (Δ) and 40 μM (▽) drug was followed spectrophotometrically (350 nm) at 35 °C for 30 min. Each graph represents change in absorbance against time (min).

3.4. Confocal microscopy of tubulin morphology

The appearance of the tubulin cytoskeleton following exposure of DLD-1 cells to EMPS, LS 4577 or LS 4578 are shown in Fig. 5. Images show the effects of exposure to the compound at one tenth the IC_{50} or IC_{50} concentration for 90 min. The control image (Fig. 5a) demonstrates a well-defined cytoplasmic tubulin network and a single dividing cell with a normal spindle. At one tenth of the 90 min IC_{50} concentrations all three compounds caused cells to round up and the tubulin took on an amorphous appearance. In the case of EMPS some normal spindle formation was apparent (Fig. 5b) whereas both analogues caused more severe damage. Although LS 4578 resulted in the appearance of aberrant spindles (Fig. 5c) the damage was clearer in the LS 4577 exposed cells (Fig. 5d). After exposure to the IC_{50} concentration of the two analogues, DLD-1 cells were devoid of properly formed microtubules and α tubulin fluorescence was limited to the centrosome (Fig. 5e and f).

Table 3
Effect of estramustine, LS 4477 and LS 4559 on the binding of [^{3}H]-colchicine to tubulin[a]

	% of control dpm		
Drug:tubulin ratio	Estramustine	LS 4477	LS 4559
0:1	100	100	100
0.5:1	80.8±1.9	41.9±13.4	20.1±3.8
1:1	72.2±3.2	17.2±1.8	9.1±4.7
10:1	72.1±3.2	16.3±3.6	7.9±0.8
100:1	65.9±3.2	9.2±3.4	6.7±0.9

dpm, disintegrations per minute.

[a] Results are expressed as a % of control dpm (i.e. percentage of residual colchicine remaining bound to tubulin) from triplicate experiments±standard deviation. Background levels were 0.9±0.5% of the control counts.

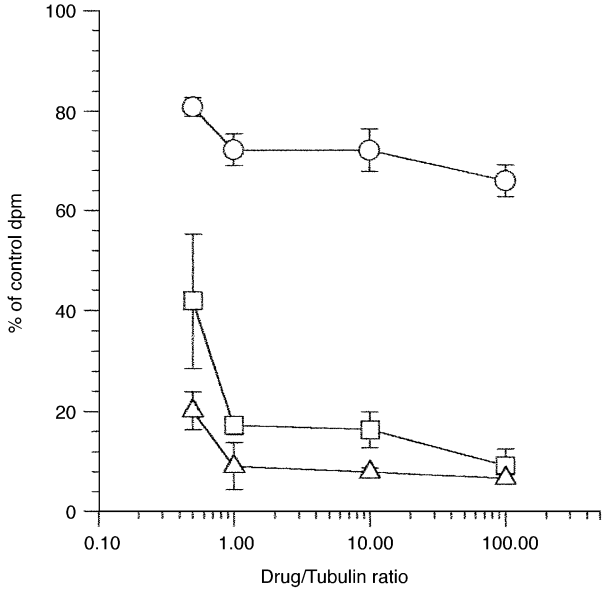

Fig. 4. Effect of estramustine (○), LS 4477 (□) and LS 4559 (△) on the binding of [^{3}H]-colchicine to purified tubulin over a range of protein/drug ratios using a spun column method. Results are expressed as a percentage of control dpm±standard deviation ($n=3$) as assessed by liquid scintillation counting.

3.5. In vivo *chemotherapy*

The antitumour effects of EMPS, the soluble prodrug of estramustine; and the novel analogues of estramustine were assessed against two members of the mouse adenocarcinoma of the colon (MAC) series of tumours. Data are summarised in Table 4. MAC 15A tumours showed a mean doubling time in the range 0.4–0.9 days. Estramustine phosphate sodium was administered to mice bearing s.c. MAC 15A tumours by i.p. injection or orally, each day for 5 days. Doses in the range of 20–50 mg/kg i.p. daily for 5 days failed to significantly increase the time for MAC 15A tumours to reach a relative tumour volume of 2.0 in comparison with untreated tumours. In addition, EMPS administered at a maximum tolerated dose of 100 mg/kg orally, daily ×5 did not result in a significant growth delay. However, when EMPS was given as an i.p. injection at this higher dose a small growth delay ($P<0.05$) was apparent.

LS 4577, the water-soluble phosphate ester prodrug of LS 4559 was administered orally to MAC 15A-bearing mice at doses ranging between 10 and 40 mg/kg, daily for 5 days as with the parent compound. Modest effects ($P<0.05$) were observed with doses of 20 and 30 mg/kg (Fig. 6a) with growth delays of 1.0 and 1.6 days, respectively. Oral administration of 40 mg/kg resulted in a significant growth delay ($P<0.01$) of 2.0 days (Fig. 6b) with no apparent toxic effects in the form of body weight loss. Higher doses were not tolerated.

Oral treatment with 40, 60 or 80 mg/kg LS 4578 (the water-soluble phosphate ester prodrug of LS 4477), daily for 5 days failed to significantly alter growth rates of s.c. MAC 15A. Increasing the dose given orally to 100 mg/kg daily for 5 days (Fig. 6c) caused a significant effect on the time to RTV 2.0 in relation to control tumours ($P<0.05$). Increasing the dose to 120 mg/kg (Fig. 6d) resulted in a significant growth delay ($P<0.01$), while no body weight loss was observed. Due to the lack of potency of this analogue higher doses were not examined.

MAC 26 is a second member of the series of s.c. MAC tumour models. It is a well-differentiated slower growing tumour than MAC 15A. Significant antitumour effects were apparent with EMPS doses of 100 mg/kg administered i.v. (growth delay of 3.8 days, $P<0.01$). LS 4577 was administered orally to mice bearing s.c. MAC 26 tumours at 40 mg/kg daily for 5 days, a dose that showed activity against the MAC 15A s.c. tumour. A growth delay of 2.5 days ($P<0.05$) was observed

Table 4
In vivo activity of estramustine (as the water-soluble phosphate sodium), LS 4577 and LS 4578 against murine adenocarcinoma of the colon

Tumour	Treatment	Dose (mg/kg)	Route	Statistical significance of antitumour effects[a]
MAC 15A	EMPS	20×5	i.p.	NS
		30×5	i.p.	NS
		40×5	i.p.	NS
		50×5	i.p.	NS
		100×5	i.p.	$P<0.05$
		100×5	Oral	NS
		250[b]	i.p.	Toxic
MAC 15A	LS 4577	10×5	Oral	NS
		20×5	Oral	$P<0.05$
		30×5	Oral	$P<0.05$
		40×5	Oral	$P<0.01$
MAC 15A	LS 4578	40×5	Oral	NS
		60×5	Oral	NS
		80×5	Oral	NS
		100×5	Oral	$P<0.05$
		120×5	Oral	$P<0.01$
MAC 26	EMPS	100×5	i.p.	$P<0.05$
		50×5	i.v.	NS
		100×5	i.v.	$P<0.01$
	LS 4577	40×5	Oral	$P<0.05$

NS, non significant; i.p., intraperitoneal; i.v., intravenous; EMPS, estramustine phosphate sodium.
[a] Determined by Mann–Whitney analysis of tumour growth delay.
[b] Single dose only.

against the MAC 26 tumour model with no associated body weight loss at this dose level.

4. Discussion

This study has investigated the mechanism of action and the activity, both *in vitro* and *in vivo* of two novel analogues of estramustine, LS 4477 and LS 4559 as anticancer agents. It has been shown that in accordance with the rational design of these compounds they are effective at causing tubulin depolymerisation. More importantly, this study has demonstrated that both analogues have potent cytotoxic activity against a panel of cell lines and have significant antitumour activity when administered orally against well-characterised tumour models *in vivo*.

In the mechanism-based studies, LS 4559 was more effective than LS 4477 in causing tubulin to depolymerise, especially at lower concentrations. Since these two compounds differ only in a single substitution of a fluorine atom at position 3 in LS 4559, it may be that this fluorine atom in some way affects the interaction and binding between tubulin and LS 4559 to increase its potency. These findings are in agreement with similar studies that have also identified LS 4477 and LS 4559 as effective agents at causing a dose-dependent inhibition of tubulin assembly [18].

Estramustine was relatively ineffective at causing tubulin to disassemble in the assay system used. Estramustine is known to bind to microtubule-associated proteins, such as MAP 2 and the tau proteins which cover the microtubule structure, and to tubulin [6–8]. Microtubule-associated proteins are essential for tubulin assembly; thus, it is believed that disruption of their function will have a deleterious effect on tubulin assembly and other microtubule-dependent processes. However, the results of this assay suggest that estramustine may be limited in producing these effects.

From the competitive binding assay employed in this study, it was apparent that estramustine was ineffective at inhibiting the colchicine binding property of tubulin. It has been previously reported that estramustine at concentrations up to 100 μM was unable to displace colchicine from tubulin [8]. The results from this study demonstrated that the two analogues of estramustine were able to substantially compete with colchicine for binding to tubulin, indicating that the binding site on tubulin for the two analogues is close to or at the binding site for colchicine and distinct from that of estramustine. Consistent with the assembly assay data, LS 4559 was the more effective of the two analogues over all tubulin: test compound ratios and it appears that the binding to tubulin at the colchicine site on the beta subunit of tubulin is influenced by the addition of the fluorine atom in LS 4559. The ineffectiveness of estramustine to displace colchicine from its binding site may be a consequence of a poor binding affinity of estramustine or the presence of a distinct binding site on tubulin for estramustine. Laing and colleagues [8] discovered that two classes of binding sites for estramus-

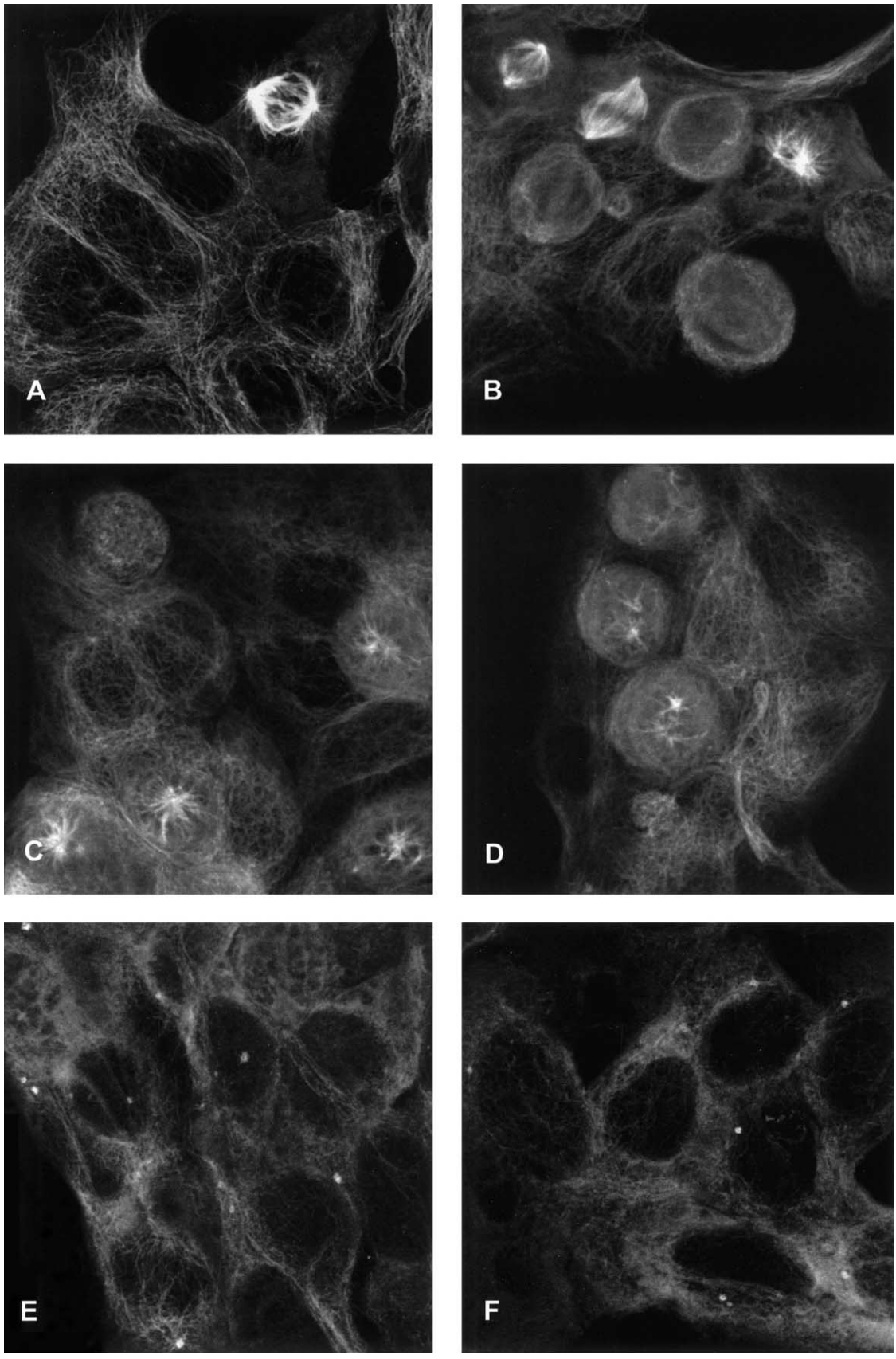

Fig. 5. Confocal microscope images of tubulin structure in DLD-1 cells exposed to test compounds for 90 min: (a) control cells, (b) estramustine (0.1 times IC_{50}); (c) LS 4578 (0.1 times IC_{50}); (d) LS 4577 (0.1 times IC_{50}); (e) LS 4578 at IC_{50}; and (f) LS 4577 at IC_{50}.

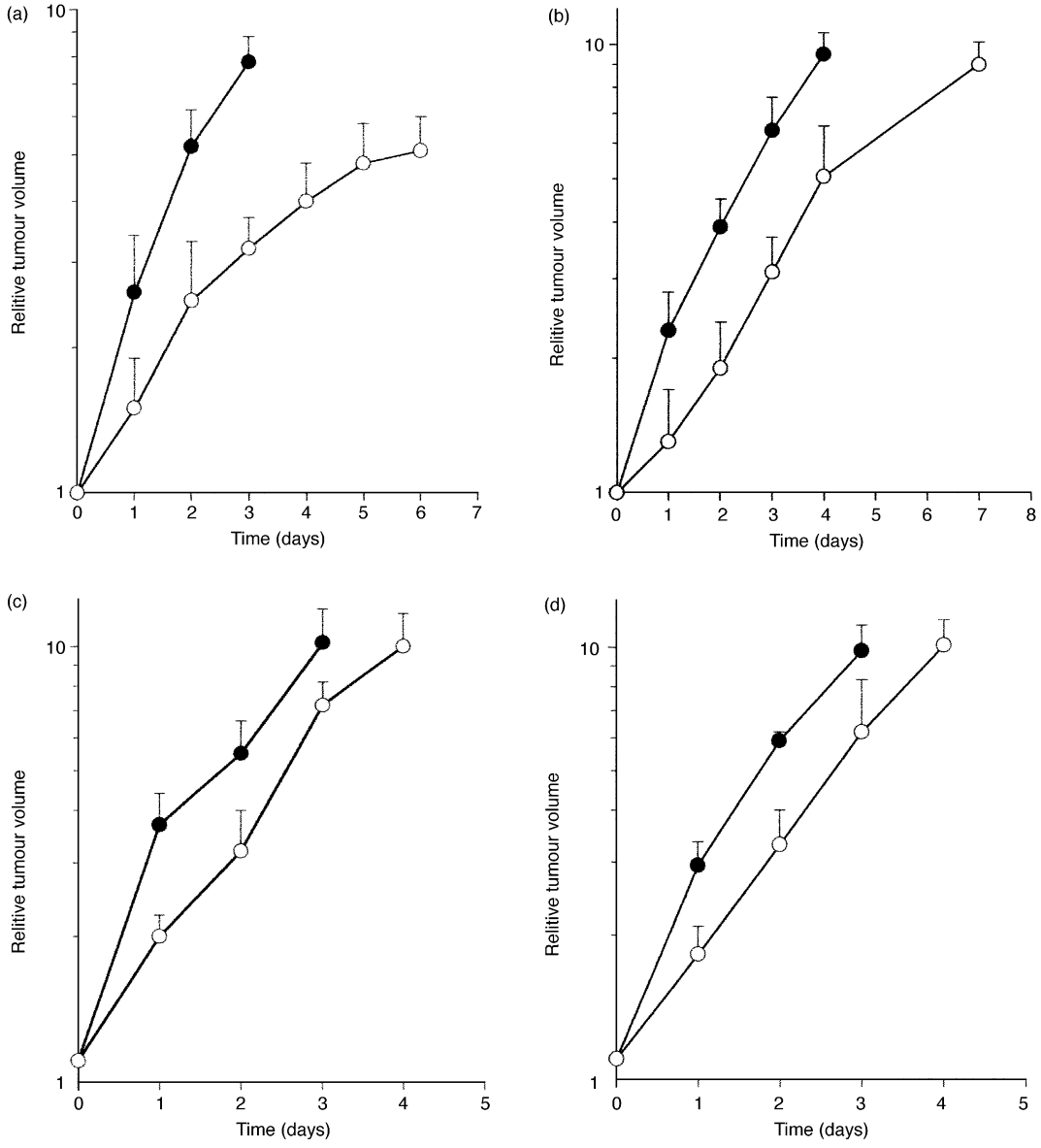

Fig. 6. Growth curves (mean±standard deviation (S.D.)) of MAC 15A tumours after daily ×5 oral treatments with: (a) 30 mg/kg LS 4577; (b) 40 mg/kg LS 4577; (c) 100 mg/kg LS 4578; (d) 120 mg/kg LS 4578 (●, control, ○, treated).

tine were present on tubulin: one with high affinity for estramustine and one with low affinity which on further evaluation were shown to be distinct from the colchicine binding site. The results from direct photolabelling of tubulin isotypes with estramustine suggested that estramustine bound covalently to both the alpha and beta subunits of tubulin. In contrast, the binding sites for colchicine [31], GTP [32] and paclitaxel [33] were found to be on the beta subunit of tubulin although vinblastine was also shown to bind to both alpha and beta subunits [34]. However, it has been shown that estramustine does not bind to each of these sites [8].

LS 4477 and LS 4559 were approximately 100 times more potent than the parent compound, estramustine, in the *in vitro* chemosensitivity studies, a response which correlates with the mechanism-based studies. Although these IC_{50} values are down in the nM range whereas inhibition of tubulin is in the μM range this can be easily explained if concentration versus time of exposure values are considered. Confocal microscopy revealed significant effects on tubulin structure in DLD-1 cells after 90-min exposure to the prodrugs at IC_{50} and one-tenth of IC_{50} concentrations, suggesting tubulin to be a major target for these agents in cells.

The demonstration that LS 4477 and LS 4559 were exerting their effects via an interaction with tubulin and that they exhibited substantial *in vitro* activity, lead to the inclusion of these compounds in *in vivo* tests. This study investigated antitumour activity against the MAC tumour models grown in syngeneic mice. These tumours have been used for preclinical evaluation of potential new agents for a number of years [19,20]. MAC 15A is a

rapid growing solid tumour established from ascitic cells implanted subcutaneously in NMRI mice. Estramustine, administered i.p. as the prodrug EMPS at a dose of 100 mg/kg daily for 5 days caused a significant growth delay and was well tolerated by the animals. At the same dose EMPS was ineffective when administered orally. In contrast, LS 4577, the soluble *in vivo* equivalent of LS 4559, was highly active when administered orally at doses of 40 mg/kg daily for 5 days. In order to obtain the same response with LS 4578, the *in vivo* equivalent of LS 4477, a dose 3 times greater (120 mg/kg orally daily $\times 5$) had to be administered. Thus the minor difference in the *in vitro* activity of the two compounds relating to the single substitution of a fluorine atom on LS 4559 has translated into a large difference in doses required for the same effect *in vivo*. Both analogues were well tolerated by the animals. LS 4577 was also tested against the slower growing MAC 26 tumour and a significant growth delay was produced. Estramustine produced a significant growth delay against this tumour model only when administered intravenously.

Both analogues were found to be active against the Dunning hormone-insensitive AT-1 rat prostatic tumour. Whilst this tumour model was resistant to doxorubicin, vinblastine and estramustine, the new analogues LS 4577 and LS 4578 caused a dose-dependent inhibition of tumour growth when administered either orally or intravenously (data not shown). Of the two analogues, LS 4577 was the most effective causing a 50% inhibition of AT-1 tumours and a 63% inhibition of Walker 256 mammary carcinoma, a trend consistent with the results presented for the MAC tumours.

In conclusion, LS 4559 consistently showed greater activity than LS 4477 in all assays. The only difference between the analogues is in a single fluorine atom added to position 3 in LS 4559, but this addition has had beneficial effects on *in vitro*, and to a greater extent, *in vivo* activity. However, the apparent increase in activity of the analogues in relation to the parent compound coupled with the fact that they are active *in vivo* when administered orally with no obvious toxicity, suggests that they warrant further development.

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