Auristatin PYE, a novel synthetic derivative of dolastatin 10, is highly effective in human colon tumour models

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Abstract. Despite promising early data, the natural product dolastatin 10 has not been successful as a single agent in phase II clinical trials. Herein the mechanism of action and efficacy of a synthetic analogue, auristatin PYE, was investigated in 2 human colon adenocarcinoma models, DLD-1 and COLO 205. *In vivo* efficacy was assessed in subcutaneous xenografts following intravenous administration. Mechanistic studies investigated effects of auristatin PYE on microtubule disruption using immunocytochemistry, whilst cell cycle effects were studied using flow cytometry. Possible effects on tumour functional blood vasculature were assessed in tumour-bearing mice. Auristatin PYE was less potent *in vitro* than dolastatin 10, but was significantly more effective ($p<0.01$) *in vivo* against both tumours. Significant effects on tumour blood vasculature were seen, with optimal shutdown at 6-h post-treatment. Extensive necrosis became more evident over time after treatment. Auristatin PYE caused severe disruption of normal microtubule structure at concentrations and times comparable with the IC_{50} data, and also instigated a G_2/M cell cycle block. Auristatin PYE was more effective in the DLD-1 and COLO 205 models than dolastatin 10, with anti-tumour effects mediated through vascular shutdown. These data suggest that auristatin PYE has good potential as an anti-cancer agent.

Introduction

The formation of novel tumour vasculature is an important contributor to tumour growth and metastasis (1). When this process is inhibited by administering agents which can suppress the growth of vascular endothelial cells, or disrupt the vascular structure, then tumour growth is severely restricted (1). One strategy in agent design is to interfere with microtubule function, which leads to a subsequent arrest of cells in mitosis. This means that rapidly proliferating immature tumour endothelial cells are a major target for such a strategy (2). A broad range of natural products and their synthetic analogues such as the *Vinca* alkaloids, taxanes and combretastatins which target tubulin as part of their mechanism of action have demonstrated good activity in the clinic, but the first two agents have toxicities which limit their potential usefulness (3).

Dolastatin 10 is a peptide originally isolated from the Indian Ocean sea hare *Dolabella auricularia* (4). Subsequently it was shown to be a potent disruptor of tubulin polymerisation (5), to inhibit the binding of *Vinca* alkaloids to tubulin in a non-competitive manner, and also to stabilise the binding of colchicines to tubulin (5,6). Dolastatin 10 has demonstrated potent activity in preclinical studies both *in vitro* and *in vivo* against a range of lymphoma, leukaemia and solid tumours (7-9).

In phase I clinical trials, dose limiting toxicities (DLT) were myelosuppression and phlebitis, with moderate peripheral neuropathy also seen in some patients, although this was not dose limiting (10,11). Phase II trials have been carried out in non-small cell lung, prostate, melanoma, colorectal, ovarian, breast and pancreatobiliary tumours, but all failed to demonstrate significant clinical activity in these tumours as a single agent (12-18).

The unimpressive results in phase II trials, together with problems of a complex chemical synthesis with low yields, and poor water solubility, have led to the development of dolastatin 10 analogues (19-21). One of these, TZT-1027 (auristatin PE), has progressed to clinical trials, with DLT in phase I studies of neutropaenia and infusion arm pain (22), and the compound is currently undergoing phase II studies. Other analogues auristatin E and monomethylauristatin E have been conjugated to anti-CD30 and anti-Lewis Y monoclonal antibodies and demonstrated good potency and selectivity to CD30 positive tumour models *in vivo* (23,24).

Auristatin PYE (Fig. 1) is a novel synthetic derivative of dolastatin 10 with a structural modification of a phenol to a pyridine from auristatin PE. Preliminary results have shown it to be a potent tubulin binder and have nM potency across the NCI 60 cell line panel (25). Thus, it was decided to investigate auristatin PYE further as a potential improvement on dolastatin 10. In this study, we evaluated the activity of

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auristatin PYE in human colon adenocarcinoma cell lines both *in vitro* and *in vivo*, in comparison with dolastatin 10. We also evaluated its mechanism of action as an anti-cancer agent with particular attention to the effects of auristatin PYE on the tumour vasculature.

Materials and methods

Compounds. Auristatin PYE and dolastatin 10 provided by G.R.P. were initially dissolved in DMSO (Sigma, Poole, UK), and were then diluted to the appropriate concentration using sterile physiological saline for *in vivo* studies (10% DMSO), and cell culture medium for *in vitro* studies (≤0.1% DMSO). For *in vivo* studies compounds were administered as single doses injected intravenously at 0.1 ml injection volume per 10 g of body weight. Paclitaxel (Sigma) was initially dissolved in DMSO and then diluted to the appropriate concentration using cell culture medium.

Animals. Female CD1-Foxnl^{nu} immunodeficient nude mice (Charles River Laboratories, Margate, UK) aged 6-8 weeks were used. Mice received CRM diet (S.D.S., Witham, UK) and water *ad libitum*. Mice were kept in cages in an air-conditioned room with regular alternating light and dark cycles. All animal procedures were carried out under a project licence issued by the UK Home Office and UKCCCR guidelines (26) were followed throughout.

Cell lines. DLD-1 and COLO 205 human colon adenocarcinoma cell lines (both from LGC Promochem, Middlesex, UK) were cultured in RPMI-1640 cell culture medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine and 10% fetal bovine serum (all from Sigma). These lines were selected due to their good vascularisation when grown *in vivo* as subcutaneous xenograft tumours. Human umbilical cord endothelial cells (HUVECs) were isolated from umbilical cords from elective Caesarean sections performed at Bradford Royal Infirmary's Maternity Unit (27) and cultured on 0.2% gelatin-coated tissue culture vessels in medium M199 supplemented with 100 mM sodium pyruvate, 200 mM L-glutamine, 5000 IU/ml^{-1} penicillin, 5000 μg/ml^{-1} streptomycin (all from Sigma) and 10% human serum (28).

Growth inhibition assays. Tumour cell growth inhibition was assessed using the MTT assay (29). 1x10^4 DLD-1 or COLO 205 cells were inoculated into each well of a 96-well plate and incubated overnight at 37˚C in a humidified atmosphere containing 5% CO_2. Compounds were dissolved in DMSO and then diluted in complete cell culture medium to give a broad range of concentrations, such that the maximum final DMSO concentration was not >0.1%. Medium was removed from each well and replaced with compound or control solutions, and the plates then incubated for a further 1 or 96 h. For the 1-h plates, compound was removed after 1 h and fresh culture medium was added for the remaining 95 h. After 96-h, culture medium was removed and 200 μl of 0.5 mg/ml^{-1} MTT solution (Sigma) in complete medium added to each well. Following a further 4-h incubation, the solution was removed from each well and 150 μl of DMSO was added to solubilise the formazan crystals resulting from MTT conversion. Absorbance values for the resulting solutions were read at 550 nm on a microplate reader and cell survival was calculated as the absorbance of treated cells divided by the control. Results were expressed in terms of IC_{50} values (i.e. concentration of compound required to kill 50% of cells) and all experiments were performed in triplicate.

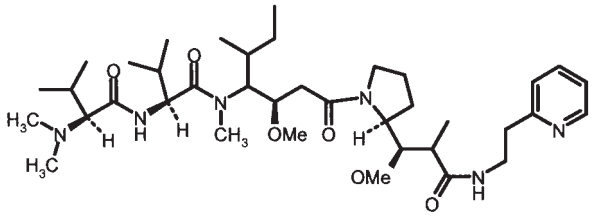

Figure 1. Structure of auristatin PYE.

Immunocytochemical analysis of microtubule disruption. DLD-1 cells (5,000) were seeded into each well of a Nunc 8-well chambered coverglass (Fisher Scientific, Loughborough, UK), or for analysis with HUVECs, 75,000 cells were seeded onto 2% gelatin-coated sterilised glass cover slips in 6-well plates. In both cases cells were left to adhere for 24 h under normal incubation conditions. Auristatin PYE at a range of concentrations was then added to each culture for varying incubation times, and then the medium was removed and fresh medium was added for a further incubation of 1 h. Paclitaxel was administered as a positive control compound. Medium was then removed and the cells were fixed in pre-cooled methanol at -20˚C for 30 min. After 2 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 in PBS for 30 min. After 3 further washes in PBS, the secondary antibody, TRITC-conjugated rabbit anti-mouse IgG (Dako, Ely, UK) was added at a dilution of 1:50 for 30 min. After 3 final washes, the cultures were mounted in Vectashield (Vector Laboratories, Peterborough, UK) and stored at 4˚C until analysis. Cells were analysed and images captured with a Zeiss LSM510 confocal system attached to an Axiovert 200 M inverted microscope using LSM510 software (all from Zeiss, Welwyn Garden City, UK).

Cell cycle analysis. DLD-1 cells in exponential growth were treated with a range of concentrations of auristatin PYE or paclitaxel as a positive control for G_2/M cell cycle block for 6 h. Following further incubation in drug-free medium for 24 h, cells were processed for analysis to check progression through the cell cycle using a method based on those of Ormerod (30). Cell cycle profile was then obtained with a Becton Dickinson flow cytometer (Oxford, UK).

Tumour system. Tumours were excised from a donor animal, placed in sterile physiological saline containing antibiotics and cut into small fragments of ~2 mm^3. Under brief general inhalation anaesthesia, DLD-1 and COLO 205 fragments respectively were implanted in left and right flank of each mouse using a trocar. Once the tumours could accurately be measured by calipers (mean tumour volume of 32 mm^3), the

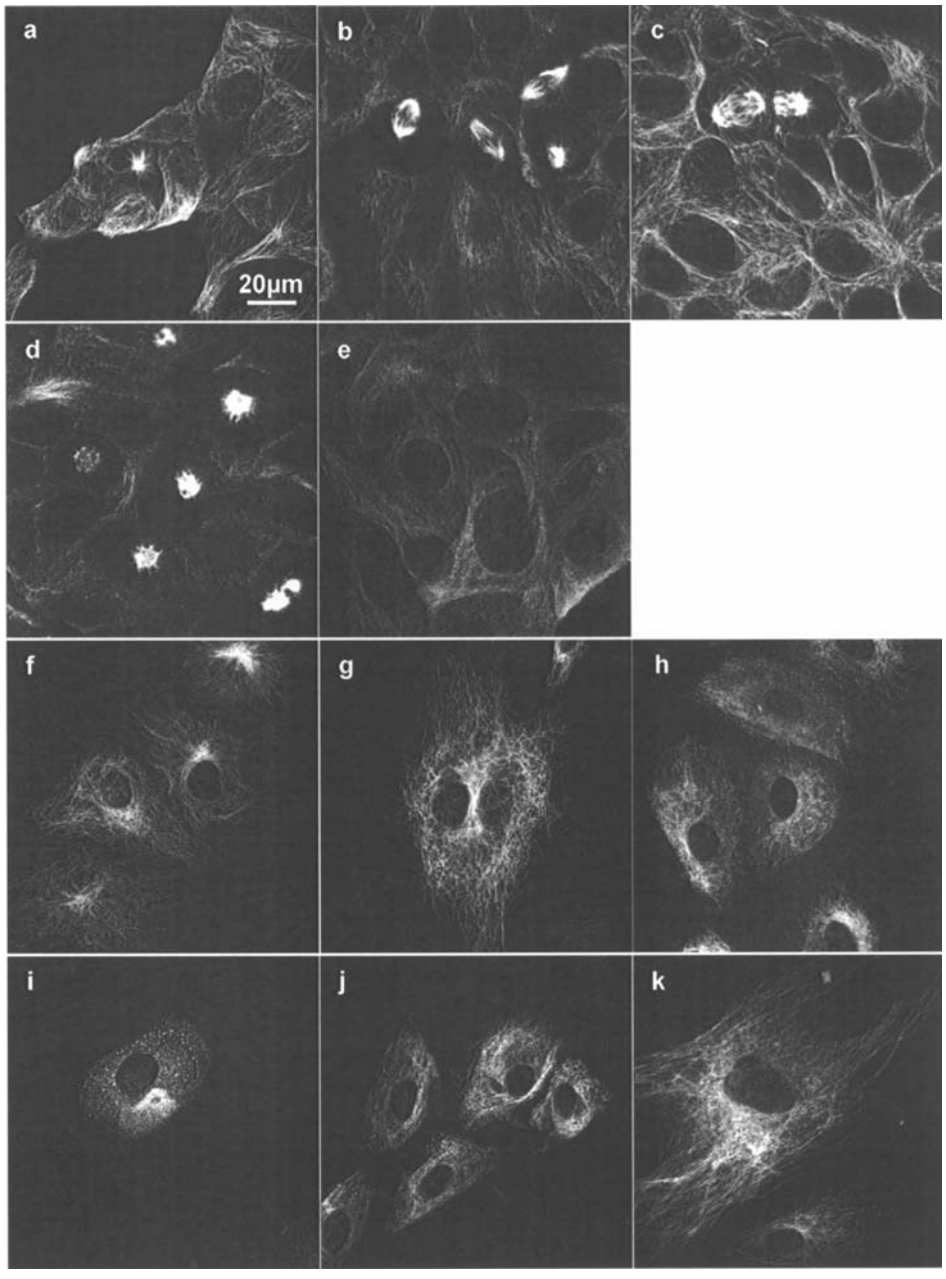

Figure 2. The effects of auristatin PYE on microtubule structure. (a-c) DLD-1 cells exposed to 7 nM of auristatin PYE for 1, 6 and 24 h showing an increase in the disruption of normal microtubule structure as seen in untreated cells (e). Disruption is also seen with the positive control compound paclitaxel at 440 nM (d). Increasing disruption is seen in HUVECs exposed to 7 nM of auristatin PYE for 1, 6 and 24 h (f-h) showing an increase in the disruption of normal microtubule structure as seen in untreated cells (k). Similar disruption is also seen with dolastatin 10 (i), and the positive control compound paclitaxel at 440 nM (j).

mice were allocated into groups of 8 by restricted randomisation to keep group mean tumour size variation to a minimum.

Chemotherapy studies. Compounds were administered by a single intravenous injection, with the day of therapy designated as day 0. The maximum tolerated doses (MTDs) of auristatin PYE and dolastatin 10 were established in the CD1-Foxnl^{nu} model at 2 mg/kg^{-1} and 0.6 mg/kg^{-1}, respectively. The efficacy of auristatin PYE was compared with dolastatin 10 at their MTDs versus DLD-1 and COLO 205. The effects of therapy were assessed as previously described (31). Briefly, daily 2-dimensional caliper measurements of the tumours were taken, with volumes calculated using the formula (a^2 x b) / 2, where a is the smaller and b the larger diameter of the tumour. Tumour volume was then normalised to the respective volume on day 0, and semi-log plots of relative tumour volume (RTV) versus time were made. Mann-Whitney U tests were performed to determine the statistical significance of any differences in growth rate (based on tumour volume doubling time) between control and treated groups, and between the 2 compounds.

Assessment of vascular shutdown and tumour necrosis. In order to further investigate the mechanism of action of auristatin PYE *in vivo*, the effects of treatment on the functional vasculature and development of necrosis in DLD-1 tumours

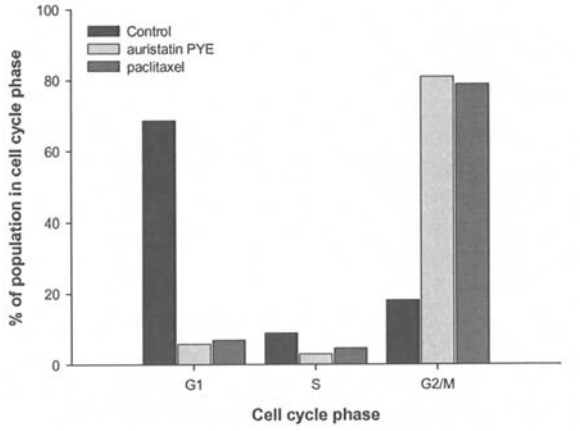

Figure 3. Cell cycle analyses of DLD-1 cells exposed to auristatin PYE showing G_2/M stage block, with paclitaxel as a positive control compound.

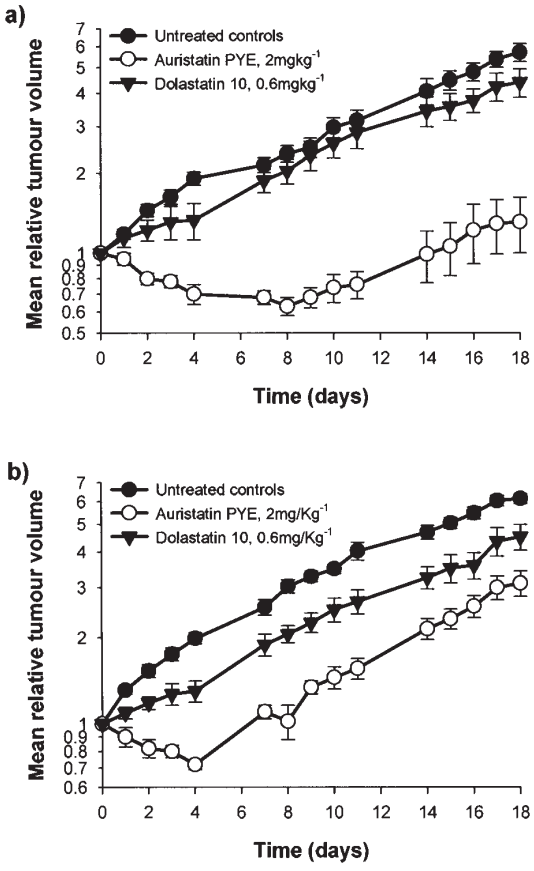

Figure 4. Comparison of the efficacy of auristatin PYE and dolastatin 10 administered at their respective MTDs as a single dose i.v. against s.c. implanted tumour xenografts. (a) DLD-1, (b) COLO 205. Points represent mean ± SD, (n=8).

was assessed as previously described (32). Tumours were set up in both flanks of 12 mice and treatment with auristatin PYE at MTD was carried out once the tumours had reached a minimum diameter of 7 mm to ensure that an established tumour vasculature was in place. At 1, 6 and 24 h after treatment 3 mice were taken for assessment, with the final 3 mice serving as a control group. Hoechst 33342 (bisBenzimide, Sigma, Poole, UK) was used to assess the functional tumour vasculature (33,34). Hoechst 33342 was dissolved in sterile saline and injected intravenously by the tail vein at 40 mg/kg^{-1}. One minute after injection the mice were euthanised by cervical dislocation and the tumours carefully and rapidly excised. One tumour from each mouse was then wrapped in aluminium foil and immediately immersed in liquid nitrogen and stored at -80˚C until ready for ultracryotomy, whilst the other tumour was immersion fixed in 10% neutral-buffered formalin for 24 h and processed for paraffin embedding.

Frozen 10-μm sections were taken at ~100 μm intervals through the tumour. Five random fields from each of 5 random sections were examined for each tumour under UV illumination using a Leica DMRB microscope, with images captured digitally through a JVC 3-CCD camera and processed using AcQuis (Synoptics, Cambridge, UK) software. Functional vasculature was assessed by placing a cm^2 grid over the captured digital image and counting the number of points on the grid which overlay fluorescently stained cells. Comparisons were made between percentage vasculature in control and treated tumours. For each animal 5-μm thick paraffin sections were taken and stained with haematoxylin and eosin to assess for hemorrhagic necrosis. Digital images were captured using the same system as above but with bright-field illumination.

Results

In vitro growth inhibition. Auristatin PYE was less potent than dolastatin 10 when tested *in vitro* against both DLD-1 and COLO 205 cell lines. For the DLD-1 cell line, IC_{50s} in nM for 1- and 96-h drug exposures were 31.0±7.0 and 4.4±1.3, respectively for auristatin PYE compared with 2.0±1.1 and 0.3±0.1 for dolastatin 10. For COLO 205 the IC_{50s} in nM for 96-h drug exposures were 1.2±0.6 for auristatin PYE and 0.3±0.01 for dolastatin 10.

Microtubule disruption. Immunocytochemical observation of microtubule structure in the auristatin PYE-treated DLD-1 cells showed considerable disruption of the normal tubulin cytoskeletal structure with the presence of asters (star-shaped formations of tubulin adjacent to the chromosomes) characteristic of cell cycle block seen at doses and times comparable with the IC_{50} data (Fig. 2a), with pronounced disruption seen at 3 h. This rapid disruption of the tubulin cytoskeleton was also seen in HUVECs with a more diffuse punctate pattern of immunolabelled α-tubulin seen even at 1 h (Fig. 2b), although there seemed to be a reduced effect at 7 nM compared with the DLD-1 cells.

Cell cycle analyses. At all concentrations of auristatin PYE assayed, a considerable accumulation of cells in G_2/M phase was seen compared with the untreated control samples (Fig. 3). A similar cell cycle profile was seen for the positive control compound paclitaxel (Fig. 3).

In vivo efficacy studies. Auristatin PYE was compared with dolastatin 10 in the DLD-1 and COLO 205 tumour models, with both compounds administered as a single i.v. injection at their respective MTDs. Both agents had negligible toxicity,

Table I. Comparison of the *in vivo* activity of auristatin PYE and dolastatin 10 administered i.v. as a single dose.

Tumour model	Compound and dose ($mgkg^{-1}$)	Mean tumour doubling time (days)	Maximum % weight loss (day)
DLD-1	Control	5.8	1.3 (10)
	Auristatin PYE, 2.0	30.0[a]	10.9 (3)
	Dolastatin 10, 0.6	8.9[b]	5.9 (3)
COLO 205	Control	4.4	1.3 (10)
	Auristatin PYE, 2.0	14.3[a]	10.9 (3)
	Dolastatin 10, 0.6	8.2[a,b]	5.9 (3)

[a]P<0.01, significantly greater growth delay compared with control tumours as determined by the Mann-Whitney U test. [b]P<0.01, significantly less growth delay for dolastatin 10-treated tumours compared with auristatin PYE-treated tumours as determined by the Mann-Whitney U test.

with the observed maximum weight loss well within the normal limits ($p<0.05$ for both compounds). Auristatin PYE was seen to induce a significantly larger growth delay than dolastatin 10 for both tumour models ($p<0.01$) (Fig. 4 and Table I), with differences in mean tumour doubling time of 21.1 days for DLD-1, and 6.1 days for COLO 205.

Assessment of vascular shutdown and tumour necrosis. The amount of functional vascular elements (as determined by the incorporation of the Hoechst 33342 dye into the nuclei of functioning endothelial cells) was significantly affected by administration of auristatin PYE, with shutdown already evident after 1 h (2.0% functional vascular elements compared with 8.1% seen in the control). Peak vascular shutdown was seen at 6 h (Fig. 5a-d) (0.9% functional vascular elements), and at this time the functional vasculature appeared to be confined to the periphery of the tumour. Some recovery in vascular function was observed at 24 h (6.6% functional vascular elements).

Histological evaluation of haematoxylin and eosin stained sections showed an increase in the amount of necrosis seen in DLD-1 tumours with exposure to auristatin PYE (Fig. 5e-h), with 27, 37 and 47% necrosis seen in tumour sections at 1, 6 and 24 h respectively following treatment, compared with 11% in the control. Residual viable tissue was found mainly at the periphery of the tumour at the 6 and 24 h time-points.

Discussion

Despite the huge progress made in target-driven anticancer drug development, the majority of agents approved for use in the clinic are still either natural products or their synthetic analogues. This particularly holds true in the area of agents targeting tubulin where the most successful compounds are still the *Vinca* alkaloids and the taxanes. However, these agents have considerable dose limiting toxicities, the main one being peripheral neuropathy (35,36), and hence there is still justification for searching out similar acting natural products or analogues which could give an improved pharmacological profile.

Dolastatin 10 is such an agent and has shown to be very promising in preclinical studies (7-9), with no neuropathy seen in phase I clinical trials (10,11). Unfortunately subsequent phase II clinical trials failed to demonstrate any activity when administered as a single agent (12-18), and the compound has a problematic low-yield chemical synthesis (37). The mechanism of action and favourable toxicity profile of dolastatin 10 has lead to the synthesis and evaluation of >200 analogues (19,38). Of the most active in initial screens, auristatin PE is currently in phase II trials (22). Auristatin PYE is another synthetic analogue that was selected for further evaluation due to promising preliminary data that showed it to have similar activity to auristatin PE in cancer cell line screens (25).

In terms of *in vitro* activity against the 2 cancer cell lines used in the study, auristatin PYE, although highly potent, did not inhibit growth as much as dolastatin 10. This is similar to previous findings for both auristatin PE and PYE (25), and seems to give the molecule an advantage over dolastatin 10 in that a higher concentration was tolerated when MTD was evaluated *in vivo* with increased efficacy.

Auristatin PYE caused disruption of microtubule structure in a concentration- and time-dependent manner in DLD-1 tumour cells. These effects were similar to those seen for paclitaxel in this study, and those reported for dolastatin 10 (39), symplostatin 1 (40) and auristatin PE (41), which suggests that the structural modifications of auristatin PYE do not effect the tubulin-binding ability of the molecule. This is also reflected in the very similar IC_{50s} for the inhibition of tubulin polymerization which are 1.2 μM for auristatin PYE (G.R. Pettit, personal communication), and 1.3 μM for dolastatin 10 (19). Since dolastatin 10 and its analogues have been shown to work at least partly by targeting the tumour vasculature, we also investigated the effects of auristatin PYE on microtubule structure in HUVECs, since these proliferating endothelial cells are seen as a good *in vitro* model for tumour endothelial cells (28). As with the tumour cells, disruption of microtubule structure was seen, suggesting that the tumour endothelial cells are a valid target for auristatin PYE.

The presence of asters characteristic of cell cycle block in the immunocytochemical studies was further investigated by cell cycle analysis using flow cytometry. G_2/M cell cycle blocks were seen at similar compound concentrations that resulted in aster formation. Again these effects were similar to those seen for paclitaxel in this study, and those reported for dolastatin 10

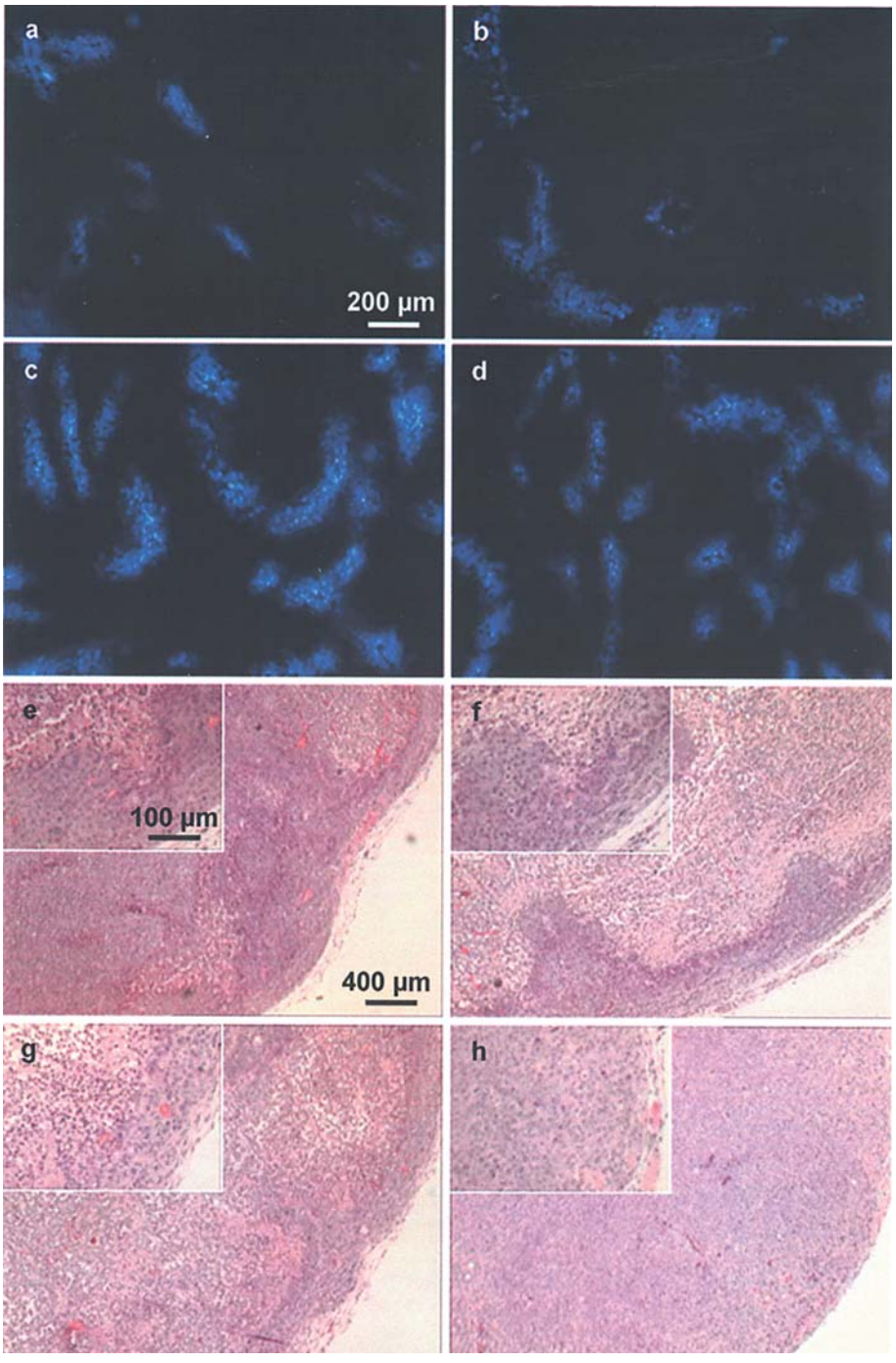

Figure 5. (a-d) Images of Hoechst 33342-stained functional vascular elements in cryosections of auristatin PYE-treated DLD-1 tumour 1 h (a), 6 h (b), 24 h (c) after treatment compared with untreated tumour (d). Functional vasculature elements seen only in the periphery of the tumour at 6 h, with function almost restored by 24 h. (e-h) Hematoxylin and eosin-stained images of auristatin PYE-treated DLD-1 tumour 1 h (e), 6 h (f), 24 h (g) after treatment compared with untreated tumour (h) (insets: high power). Increasing necrosis seen centrally with time in the treated tumour.

(39), symplostatin 1 (40) and auristatin PE (42). This once more would suggest that the structural modifications of auristatin PYE do not affect the dolastatin 10-like mechanism of action of the molecule.

In vivo efficacy studies were carried out to compare the activity of auristatin PYE with dolastatin 10 at their respective MTDs in mice bearing the DLD-1 and COLO 205 tumours. In both models auristatin PYE was seen to be a significantly more effective compound. This increased effectiveness of the synthetic analogue in the colon tumours over dolastatin 10 could be due to differences in pharmacokinetics and metabolism. Similar increases in activity were seen with auristatin PE (which has a comparable MTD to auristatin PYE) *in vivo* with a variety of human solid and haematological xenografts including the Colon 26 adenocarcinoma (43,44).

With tumour growth inhibition for auristatin PYE administered at MTD being sustained for 8 days before a subsequent growth rate similar to the control tumour, this would suggest that the compound would benefit from a repeat dose schedule in order to maintain tumour inhibition.

The intermittent shutdown of tumour vasculature which is characteristic of many agents that target the microtubules (reviewed in ref. 45) was observed here for auristatin PYE, with optimal shutdown seen at 6 h. This coincides with the time when microtubule damage was seen for the HUVECs, suggesting that the agent works by disrupting the cytoskeleton of the tumour endothelial cells leading to congestive thrombi in the tumour microvasculature. This in turn results in leakage of substances from the vessels leading to necrosis in the surrounding tumour due to oxygen and nutrient depletion, and a high localised concentration of the auristatin PYE, increasing its toxicity on the tumour cells. Morphological examination of the tumours provided further evidence of this as there was a progressive increase in the amount of necrosis seen with time after compound administration. Surprisingly tumour necrosis was seen to appear as quickly as 1 h after treatment suggesting an active process. Epithelial disruption has been described previously as early as 2 h after treatment with the vascular disrupting agent flavone acetic acid (46), and the mechanism of these effects requires further evaluation. As is typical of many vascular targeting agents, a rim of viable tumour cells was seen at the periphery of the tumour where cells can obtain nutrients from unaffected blood vessels in surrounding normal host tissues. Although quite significant cell killing is seen, this may not result in any real clinical benefit, as the tumour will continue to grow from the remaining viable cells at the periphery of the tumour. Thus, a strategy of giving auristatin PYE in combination with a therapy which could remove the remaining viable tumour rim will be investigated, as has proved successful preclinically with other vascular targeting agents such as the *Vincas*, combretastatins and ZD6126 (31,32,47,48).

In conclusion, auristatin PYE has proven to be a more effective agent than dolastatin 10 in the preclinical setting. We have confirmed that its mechanism of action is similar to dolastatin 10, with strong effects on the tumour vasculature seen as well as potent activity against tumour cells. Thus, the data suggest that auristatin PYE has good potential as an anti-cancer agent and further investigations are warranted.

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