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[D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] Substance P Inhibits the Growth of Human Small Cell Lung Cancer Xenografts *in vivo*

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We report the effect of substance P analogue, [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P (D-Phe^{5}SP), on the growth of human small cell lung cancer (SCLC) xenografts HC12 and ICR-SC112. Daily intraperitoneal (ip) administration (500 μg/day for 3 weeks) had no effect on HC12 growth rate. When administered by continuous 14-day subcutaneous (sc) infusion by osmotic minipump implanted adjacent to the tumour, D-Phe^{5}SP 2.1 μg/day, caused significant inhibition ($P < 0.05$) of the growth of HC12 and ICR-SC112 on day 7 and day 14 compared with phosphate buffered saline (PBS)-treated controls. HC12 and ICR-SC112 tumour volume remained at 53–67% of control for 14–21 days postinfusion. D-Phe^{5}SP 1 mg/day did not inhibit tumour growth, but dense fibrous capsules developed at the minipump outlet. Animals treated by sc infusion (but not ip) of PBS or D-Phe^{5}SP failed to gain weight, and some groups lost weight. D-Phe^{5}SP-treated animals had lower white blood counts than controls (not significant). These data suggest a potential clinical role for D-Phe^{5}SP in the treatment of SCLC.
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INTRODUCTION

SMALL CELL lung cancer (SCLC) is initially chemosensitive, but most patients relapse with chemoresistant disease and only 3% survive up to 7 years after diagnosis [1].

A number of mitogenic factors have been identified for SCLC growth *in vitro*. These include bombesin (or its mammalian equivalent gastrin-releasing peptide, GRP) [2], insulin-like growth factor (IGF)-1 [3] and transferrin [4]. The neuropeptides vasopressin, bradykinin and GRP cause Ca^{2+} mobilisation in SCLC (an early mitogenic event), which can be inhibited by analogues of substance P (SP) [5]. Analogues of SP were also found to inhibit the *in vitro* mitogenicity of bombesin, vasopressin [6] and bradykinin [7] in Swiss 3T3 cells. These inhibitory effects may be mediated by interaction of SP analogues with neuropeptide receptors, which share a common putative structure with seven hydrophobic transmembrane domains [8–12].

The antitumour activity of SP analogues has been assessed *in vitro*. [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P (D-Phe^{5}SP) is the most potent bombesin antagonist in Swiss 3T3 cells and inhibits the growth of SCLC cell lines [13]. We have shown that D-Phe^{5}SP causes inhibition of DNA synthesis and cell growth in a number of human tumour cell lines, including SCLC, non-SCLC, ovarian and cervical carcinoma cell lines, with lesser inhibition of normal human bone marrow and skin fibroblasts cells [14]. Our current study was designed to evaluate the *in vivo* effects of D-Phe^{5}SP on the growth of human SCLC xenografts in nude mice.

MATERIALS AND METHODS

Small cell lung cancer (SCLC) cells

Human SCLC cell line HC12 was kindly provided by Dr G.M. Duchesne (Institute of Cancer Research, Surrey, U.K.). ICR-SC112 was established in our laboratory from a lymph node aspirate taken from a previously untreated patient with SCLC. Both cell lines were initially established *in vivo* by injecting 2×10^6 viable cells subcutaneously (sc) into the flank of female athymic nude mice. Solid sc tumours grew at the site of inoculation with no evidence of metastasis. Xenograft tumour cells were examined cytogenetically to confirm human origin and the presence of the 3p deletion characteristic of SCLC [15]. Tumours were routinely passaged by implanting 1-mm^3 pieces of excised tumour sc in the flank of female athymic nude mice using a trocar. Animals were housed under sterile conditions in isolators.

D-Phe^{5}SP administration

Mice were anaesthetised with a halothane/oxygen mixture for all procedures. Preliminary experiments were performed on groups of three to five HC12 tumour-bearing mice, to assess the optimum dose of D-Phe^{5}SP. These experiments used mice with initial tumour volumes ranging between 22 and 352 mm^3. Tumour volume was measured three times weekly for 2 weeks to establish the pretreatment growth rate. HC12 tumour-bearing animals were treated with phosphate buffered saline (PBS) (controls) or D-Phe^{5}SP at 500 μg/day administered intraperitoneally (ip) for 3 weeks, or 0.21–1000 μg/day for 14 days by continuous sc infusion. Alzet osmotic minipumps (Model 1007D 7-day infusion; Alza Corporation, Palo Alto, California 94303, U.S.A.) were implanted sc for 1 week, then replaced with fresh pumps for a further week [16]. The minipumps were orientated such that the outlet was as near as possible to the tumour. On removal of the minipump tumour volume was measured for a further 7–14 days.

In subsequent experiments treatment was commenced when tumour size reached approximately 6 × 6 mm (113 mm^3 tumour volume). HC12 and ICR-SC112 tumour-bearing mice received a 14-day sc infusion of PBS, 0.21 or 2.1 μg/day D-Phe^{5}SP. At the end of treatment the minipumps were removed and a 25-μl blood sample was taken by tail vein puncture. The minipumps were flushed through with PBS and residual peptide

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was quantitated by HPLC (C-4 reverse phase column using a gradient of acetonitrile in 0.1% trifluoroacetic acid). Mice were weighed every 7 days, while weekly tumour measurements were taken throughout infusion and continued for a further 14 days (HC12 tumour-bearing mice) or 21 days (ICR-SC112 tumour-bearing mice) after minipump removal. ICR-SC112 has a slower *in vivo* growth rate than HC12, therefore, it was possible to measure the tumours until day 35. Animals were sacrificed by cervical dislocation on day 28 (HC12 tumour-bearing mice) and day 35 (ICR-SC112 tumour-bearing mice).

To assess the stability of the analogue, D-Phe^{5}SP 100 μmol/l in PBS (the concentration in the pump necessary to achieve a dose of 2.1 μg/day *in vivo*) was incubated at 37°C for 0, 2, 4, 7 and 14 days. Aliquots were taken at each time point and stored at −20°C. The bioactivity of fresh and pre-incubated D-Phe^{5}SP was compared in a double layer agar clonogenic assay containing 5% fetal calf serum (FCS). HC12 cells were seeded at 10^4/dish and grown for 2 weeks in the presence of 0–10 μmol/l fresh D-Phe^{5}SP or 10 μmol/l pre-incubated D-Phe^{5}SP.

Tumour measurements

Tumour volume was calculated according to the formula

$$V = \frac{\pi \times a \times b^2}{6}$$

where V = volume, a = largest diameter (mm) and b = diameter (mm) perpendicular to a. Tumour volume was expressed as a percentage of the initial (day 0) volume and the non-parametric Mann–Whitney U-test was used to calculate significant differences between tumour volumes of control and D-Phe^{5}SP-treated groups. The t-test on slopes was used to calculate and compare tumour growth rate of control and D-Phe^{5}SP-treated animals, and to assess weight change in mice. Blood counts of control and treated mice were compared using analysis of variance and the two-tailed Dunnetts test.

RESULTS

Antitumour effects

Our initial *in vivo* experiment used daily ip administration of D-Phe^{5}SP 500 μg/day, a total of 10.5 mg over 3 weeks. There was no effect on HC12 tumour growth (data not shown), but the therapy was tolerated with no treatment-related deaths.

Preliminary experiments using groups of three to five HC12 tumour-bearing mice were performed to establish effective doses of analogue. Tumour growth delay was observed using a 14-day continuous infusion of 0.21 and 2.1 μg/day D-Phe^{5}SP. At 21 μg/day infusion inhibition of tumour growth rate was no greater than that seen at 2.1 μg/day, and 1 mg/day infusion had no inhibitory effect. Growth rate inhibition was seen only in animals with tumours ≤ 285 mm^3 at the start of infusion.

Seven of nine HC12 tumour-bearing mice receiving the 14-day infusion of 1 mg/day D-Phe^{5}SP developed fibrous capsules containing inflammatory cells in the local area of D-Phe^{5}SP delivery. This fibrous/inflammatory reaction was not seen at other doses of analogue, nor in the control mice.

Based on these observations we used doses of 0.21 or 2.1 μg/day D-Phe^{5}SP, starting treatment when individual tumours had reached approximately 113 mm^3, thereby standardising the initial tumour volume. Figure 1 shows the results of the subsequent experiment where HC12 tumour-bearing animals (14 or 15 per group) were treated with PBS or D-Phe^{5}SP 0.21 or 2.1 μg/day. A 14-day infusion of 2.1 μg/day caused

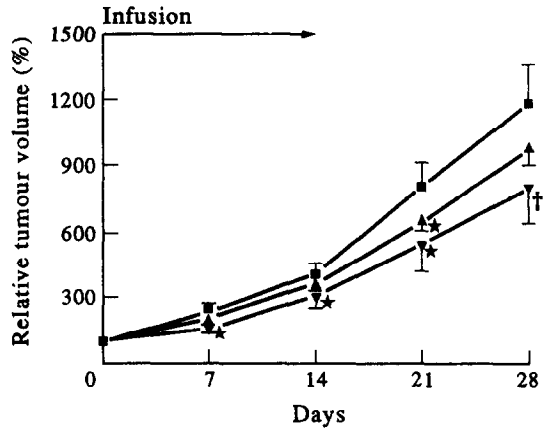

Fig. 1. Growth of HC12 xenografts in nude mice treated with PBS (control group; 15 animals ■), D-Phe^{5}SP 0.21 μg/day (14 animals ▲) or D-Phe^{5}SP 2.1 μg/day (15 animals ▼) by continuous sc infusion adjacent to the tumour from day 0 to day 14. Tumour volumes expressed as mean ± SEM. ★ $P < 0.5$ Mann–Whitney U test. † $P = 0.052$.

significant inhibition (day 7: 67 ± 6% control; day 14: 75 ± 12%, both $P < 0.05$ by the Mann–Whitney U test) of HC12 tumour volume compared to control animals. In this experiment the lower dose of 0.21 μg/day D-Phe^{5}SP had no significant effect on tumour growth rate compared to controls, although inhibition of tumour volume was transiently seen at day 21 ($P < 0.05$). The lack of overall growth inhibition at this dose contrasted with inhibition of growth rate seen in individual animals in the preliminary experiment. This discrepancy may be due to increased numbers of animals and standardised pre-infusion tumour volumes in the main experiment compared with the preliminary study. Consequently, a dose of 2.1 μg/day was evaluated in ICR-SC112 tumour-bearing mice (Fig. 2). Significant tumour growth inhibition (day 7: 67 ± 5% control; day 14: 61 ± 4%, both $P < 0.05$) was again seen in D-Phe^{5}SP-treated animals, with one tumour showing transient regression on day 7 with regrowth by day 14.

We continued to monitor tumour volume after removal of minipumps. HC12 tumour volume remained significantly lower in the D-Phe^{5}SP 2.1 μg/day-treated group than in the PBS-

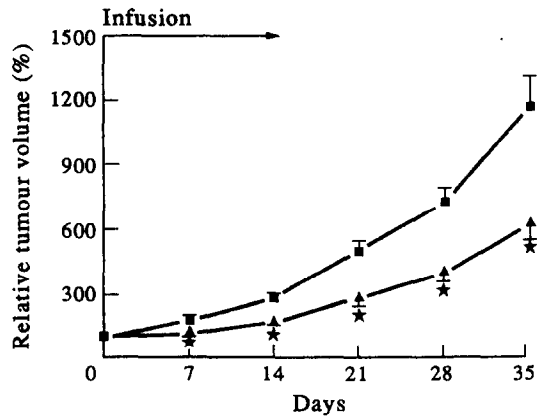

Fig. 2. Growth of ICR-SC112 xenografts in nude mice treated with PBS (control group; 8 animals ■) or D-Phe^{5}SP 2.1 μg/day (9 animals ▲) administered by continuous sc infusion from day 0 to day 14. Tumour volume expressed as mean ± SEM. ★ $P < 0.05$ Mann–Whitney U test.

Table 1. Effect of a continuous infusion of D-Phe⁵SP (day 0–14) on the weight of (a) HC12 and (b) ICR-SC112 tumour-bearing nude mice

D-Phe⁵SP (μg/day)	n	Weight (g)* Day 0	Day 7	Day 14	Day 21	Day 28	Day 35
(a) HC12							
0†	15	20.6 ± 0.3	20.0 ± 0.4	19.3 ± 0.4	19.5 ± 0.6	19.4 ± 0.5	—
0.21‡	14	20.6 ± 0.5	19.6 ± 0.4	19.2 ± 0.5	18.9 ± 0.5	18.4 ± 0.4	—
2.1	15	19.8 ± 0.6	19.0 ± 0.6	18.7 ± 0.6	19.0 ± 0.7	18.7 ± 0.7	—
(b) ICR-SC112							
0	8	19.1 ± 0.7	19.5 ± 0.6	18.9 ± 0.7	19.8 ± 0.8	19.9 ± 0.7	20.1 ± 0.8
2.1	9	18.0 ± 0.4	18.0 ± 0.4	17.5 ± 0.4	18.1 ± 0.4	18.0 ± 0.3	18.0 ± 0.4

n, no. of mice in group. *Mean ± S.E.M. †$P < 0.05$ (*t*-test on slopes). ‡$P < 0.001$ (*t*-test on slopes).

treated controls after cessation of treatment (day 21: 67 ± 15% control, $P < 0.05$; day 28: 67 ± 13%, of borderline significance $P < 0.052$, Fig. 1). One of the 15 HC12 tumours showed a transient regression, with a reduced tumour volume on day 21 but regrowth by day 28. Similarly, D-Phe⁵SP-treated mice bearing ICR-SC112 xenografts showed persistent reduction in tumour volume compared with PBS controls after the end of the infusion (day 21: 56 ± 7% control; day 28: 55 ± 6%; day 35: 53 ± 6%, $P < 0.05$, Fig. 2). However, the *t*-test on slopes showed that the postinfusion growth rate of D-Phe⁵SP-treated tumours was the same as the control tumours. Thus, there was no further tumour growth inhibition once D-Phe⁵SP infusion had ceased, but neither was there any rebound growth.

In order to assess the possibility that the peptide was undergoing degradation in the minipumps, we tested the growth inhibitory effects of D-Phe⁵SP after incubation at 37°C for 0–14 days. HC12 cells grown in the presence of 10 μmol/l fresh or preincubated D-Phe⁵SP showed equivalent inhibition of colony formation to approximately 10% of controls (data not shown). This indicates that bioactivity was retained for up to 14 days incubation at 37°C. Minipumps removed from the mice were also assessed for residual peptide by flushing with PBS. HPLC analysis showed a recovery rate of 0.1–0.5% of the initial concentration of D-Phe⁵SP, confirming that most of the peptide had been released during implantation.

Toxicity

Toxicity was assessed by weight loss (Table 1) and haematological parameters (Table 2). Mice would be expected to gain 2–3 g over a 4–5-week period (i.e. the duration of these experiments). The expected weight gain was observed in HC12 tumour-bearing animals receiving daily ip injections of D-Phe⁵SP 500 μg/day for 3 weeks. However, no weight gain was observed in any group receiving continuous sc infusion by minipump, including control animals receiving an infusion of PBS. Most groups of animals showed slight weight loss compared to the pretreatment means. This loss was statistically significant in the HC12 tumour-bearing mice treated with an infusion of PBS (6 ± 2% pretreatment weight, $P < 0.05$) and those receiving D-Phe⁵SP 0.21 μg/day (11 ± 2%, $P < 0.01$). However, no significant weight loss was seen in the HC12 tumour-bearing mice treated with the higher dose of D-Phe⁵SP 2.1 μg/day. The pretreatment mean in the latter group was lower despite random allocation of animals to the three groups, but this was not statistically significant. Neither group of ICR-SC112 tumour-bearing mice showed significant weight loss (Table 1).

Table 2. Effect of 14-day infusion of D-Phe⁵SP on WBC, RBC, haemoglobin (Hb) and platelets (Plts) in (a) HC12 and (b) ICR-SC112 tumour-bearing nude mice

D-Phe⁵SP (μg/day)	n	WBC (×10⁹/l)	RBC (×10¹²/l)	Hb (g/l)	Plts (×10⁹/l)
(a) HC12					
0	5	8.6 ± 1.4	11.3 ± 0.6	192 ± 6	1234 ± 125
0.21	5	7.6 ± 0.6	12.1 ± 0.4	200 ± 8	1042 ± 109
2.1	5	6.4 ± 0.9	12.1 ± 0.7	198 ± 8	970 ± 64
(b) ICR-SC112					
0	4	8.5 ± 0.6	11.4 ± 0.7	191 ± 6	1291 ± 103
2.1	4	6.5 ± 1.6	10.9 ± 0.3	178 ± 5	1125 ± 177

Data expressed as mean ± S.E.M. *n*, number of mice/group. Blood samples taken day 14.

Haematological toxicity was assessed at the end of the 14-day infusion of PBS or D-Phe⁵SP (Table 2). White blood cell (WBC) counts fell by approximately 25% in animals treated with 2.1 μg/day D-Phe⁵SP, but this was not statistically significant. Nor was there a significant difference between red blood cell (RBC), haemoglobin or platelet counts in animals receiving D-Phe⁵SP (0.21 or 2.1 μg/day) compared to control animals. There were no treatment-related deaths.

DISCUSSION

We report an evaluation of the *in vivo* effect of D-Phe⁵SP on the growth of human SCLC xenografts. Tumour growth was significantly inhibited during continuous infusion of D-Phe⁵SP 2.1 μg/day. On cessation of the infusion, treated tumours remained at 53–67% of the volume of control tumours, with a growth rate equivalent to that of controls. Similar results were reported by Mahmoud *et al.* [17] who were the first group to demonstrate that SCLC growth can be inhibited *in vitro* and *in vivo* by a bombesin antagonist. In contrast to our study they used an analogue of bombesin itself, [Psi^{13,14}, Leu^{14}]BN. As in our study, growth inhibition was seen using sc administration close to the tumour. Xenograft growth was slowed during 2–5 weeks of daily injections to approximately 50–70% compared with PBS-treated controls.

The absence of an antiproliferative effect with 1 mg/day continuous sc infusion may be because the dose–response curve is not linear *in vivo*, as reported with some other biological therapies [18, 19]. However, we found no evidence of this *in vitro* [14]. A more likely explanation is that *in vivo* fibrous capsule

formation in the local area of high-dose D-Phe[5]SP infusion was physically limiting access of the analogue to the tumour. The development of fibrous capsules containing inflammatory cells may reflect the role of SP in acute inflammation [20].

Our previous *in vitro* study had shown that D-Phe[5]SP causes potent dose-dependent inhibition of SCLC growth, with complete abolition of colony formation at 10–100 μmol/l [14]. The antiproliferative effect was not as potent *in vivo* as that observed *in vitro* and the increased *in vitro* sensitivity of ICR-SC112 over HC12 was not seen. We excluded peptide degradation or retention within the minipump as reasons for the *in vivo* effect being less than that predicted by *in vitro* studies, and several other reasons could account for the decreased *in vivo* potency of D-Phe[5]SP. This small peptide probably has a short half-life, which may account for the lack of effect of daily ip injections. Endogenous SP circulates in blood bound to high molecular weight plasma proteins which protect it from enzymatic degradation, while unbound SP is rapidly degraded [21]. Other possible factors are rapid renal clearance, peptidase activity or poor tumour penetrance, although here small size would be an advantage [22]. Our inability to demonstrate an effect with D-Phe[5]SP administered ip contrasts with the recently published results of Langdon *et al.* [23], who reported that the hexapeptide SP antagonist [Arg[6], D-Trp[7,9], MePhe[8]] SP inhibited the growth of SCLC xenografts when administered locally or ip.

Both SP and bombesin have physiological effects on the nervous system and the gut. SP analogues antagonise the *in vivo* effects of SP [24] and bombesin [25], and have local anaesthetic [26] and analgesic [27] effects in rodents. Potential inhibition of SP-mediated pain transmission by D-Phe[5]SP might be advantageous clinically, but other antagonist effects could be deleterious. In this study we attempted to assess the toxicity of D-Phe[5]SP by haematological parameters and weight loss. The absence of weight loss in ip-treated animals suggests that the failure to gain weight/weight loss observed in HC12 tumour-bearing mice may be attributed to the procedure of minipump implantation (i.e. the stress of the surgical procedure and/or the effect of weekly doses of anaesthetic) or to the presence of the pump itself, and not directly to D-Phe[5]SP toxicity. A similar degree of weight loss in minipump-treated controls was observed in a study of the effects of adenosine dialdehyde in murine neuroblastoma [16].

This study demonstrates that [D-Arg[1], D-Phe[5], D-Trp[7,9], Leu[11]]SP has *in vivo* antitumour activity against human SCLC xenografts when delivered by continuous sc infusion. We conclude that D-Phe[5]SP may have a place in the clinical management of patients with SCLC, either in combination with or after chemotherapy.

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