In vitro effects of substance P analogue [D-Arg¹, D-Phe⁵, D-Trp⁷, Leu¹] substance P on human tumour and normal cell growth

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Summary Analogues of the neurotransmitter substance P (SP) can interact with neuropeptide receptors, and are reported to inhibit growth of small cell lung cancer cell lines (SCLC CLs). We found [D-Arg¹, D-Phe⁵, D-Trp⁷, Leu¹] substance P (D-Phe⁵SP) significantly inhibited DNA synthesis by 10 10 human tumour CLs; six SCLC, one N-SCLC (squamous), two ovarian and one squamous cervical carcinoma, with inhibition to 50% control levels (IC₅₀) of 20–50 µM. There was dose dependent inhibition of colony formation efficiency (CFE) in 3 3 SCLC and 1 1 N-SCLC CL. IC₅₀ of 0.5–6.5 µM in 5% serum. Exposure of SCLC CL HC12 to 100 µM D-PheSP for 1–4 h caused a progressive fall in viable cell number; surviving cells, grown in the absence of peptide showed a decreased growth rate. During 1 week’s exposure of two SCLC CLs to 20 µM D-PheSP, growth was slower than control cultures, while 50–100 µM completely inhibited growth. These inhibitory effects were partially reversed by increasing serum concentration from 5 to 20%, but not by SP, vasopressin, bombesin or insulin-like growth factor 1. There was some inhibition of CFE by 3 3 normal human bone marrow cells. IC₅₀ of 30–80 µM, compared with 8 µM for HC12 in 20% FCS. Therefore D-PheSP appears to have more potent antiproliferative effects in tumour cells than normal cells, suggesting a role for this analogue in tumour treatment.

Small cell lung cancer (SCLC) accounts for 25% of primary lung cancers. While 70–80% of patients respond to conventional chemotherapy, fewer than 5% survive 5 years. SCLC synthesises a number of peptides, including bombesin (or its mammalian homologue gastrin releasing peptide GRP) (Cutitta et al., 1985) and insulin-like growth factor-1 (IGF-1) (Macaulay et al., 1988), which appear to operate as autocrine growth factors. Analogues which block the growth effects of these factors may provide new approaches to therapy.

Substance P (SP) was first isolated in 1931 (Euler & Gadum. 1931). It is a basic 11 amino acid secretory neurotransmitter belonging to the widely distributed tachykinin family. Tachykinins possess a common C-terminal tripeptide Gly-Leu-Met-NH₂. The family includes substance K (neurokinin A), neurokinin B, eldosin, physalaemin, kassinin, uperlein and phyllomedusin. Physalaemin-like peptides are produced by SCLC cell lines (CLs) (Lazarus et al., 1983), and have an inhibitory effect on lung cancer cell growth in vitro (Bepler et al., 1987).

Tachykinins have a variety of physiological effects including the contraction of smooth muscle, lowering blood pressure and stimulatory effects on spinal or sensory nerves. SP may also have a role in acute inflammation (Matsuda et al., 1989). SP analogues abolish the behavioural effects of SP (Lembeck et al., 1981), antagonise the effects of bombesin in vivo (Yachnis et al., 1984), and have potent local anaesthetic actions (Piercey et al., 1981; Post et al., 1985).

Analogues of SP were found to block the release of amylase from pancreatic acinar cells in vitro, by the competitive inhibition of peptides which interact with the bombesin receptor (Jensen et al., 1984). They also inhibited the mitogenic stimulation of Swiss 3T3 cells by bombesin, vasopressin and bradykinin (Corps et al., 1985; Woll & Rozengurt, 1988a). These peptides have no significant sequence homology with SP, and it has been suggested that SP analogues recognise a common domain in the receptors for these neuropeptides, which is not the ligand binding site (Woll & Rozengurt, 1988a).

Woll and Rozengurt (1988b) screened a panel of analogues for antiproliferative effects in Swiss 3T3 cells, and [D-Arg¹, D-Phe⁵, D-Trp⁷, Leu¹] substance P (D-Phe⁵SP) was found to be the most potent bombesin antagonist. The SP analogues were shown to inhibit the growth of SCLC CLs (Woll & Rozengurt, 1988b; Bepler et al., 1989; Woll & Rozengurt, 1990). We have studied the effect of D-PheSP on growth on ten human tumour cell lines and normal human cells.

Materials and methods

Cell lines

We are grateful to Dr G. Duchesne, Institute of Cancer Research (ICR), Surrey, UK for SCLC CLs HX149 and HC12, and to Dr A.F. Gazdar, National Cancer Institute, Bethesda, USA for NCI-H226 (squamous lung carcinoma). SCLC CLs lnc ICR-SCL12, ICR-SCL13, ICR-SC65 and ICR-SCL17 were established in our laboratories (Everard et al., 1990). Fresh tumour cells (ICR-SCL155) were obtained by fine needle aspirate from a previously untreated SCLC patient. The cells were incubated overnight in RPMI-1640 medium supplemented with 5% foetal calf serum (FCS) for use in a [H]-thymidine incorporation assay. Excess cells were later established as a cell line. All lung cultures were maintained at 37°C in RPMI-1640 supplemented with 5% FCS in 10% CO₂ in air, and were characterised biochemically and or morphological as previously described (Carney et al., 1985).

Dr L. Kelland. ICR, Surrey, UK kindly provided ovarian carcinoma cell lines SKOV3 (originally from the American Type Culture Collection) and CH1 (Hills et al., 1989). HX155 (cervical squamous carcinoma cell line) (Kelland et al., 1987) and SF1 (normal human skin fibroblast cells). These were grown in RPMI-1640 medium supplemented with 10% FCS. Normal human bone marrow samples were obtained from bone marrow donors attending the Royal Marsden Hospital, Belmont, Surrey.

Peptides

[D-Arg¹, D-Phe⁵, D-Trp⁷, Leu¹] substance P (D-Phe⁵SP) substance P (SP) and bombesin were purchased from Peninsula Laboratories Europe Ltd. St Helens, UK. [Arg⁷]vasopressin was from Sigma Chemical Co Ltd. Dorset, UK.

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1H-thymidine was kindly donated by Drs W. Markii and K. Scheibii. Ciba-Geigy Ltd, Basel, Switzerland. Human glycosylated recombinant human insulin-like growth factor 1 (rh-IGF1) was kindly donated by Drs D. Gillespie and M. Arden Jones of Sandsoz, UK.

**Growth assays**

All assays were performed in RPMI-1640 medium supplemented with 5% FCS unless stated otherwise.

**DNA synthesis**

DNA synthesis was measured by 3H-thymidine incorporation (Rozengurt & Heppell, 1975). Cells were inoculated at 6,000 cells well in RPMI-1640 supplemented with 5%–20% FCS and D-Phe5SP 1–100 µM alone or in combination with SP 1–100 µM, IGF-1 0.1–70 nM, vasopressin 1–1,000 nM or bonealb 1–1,000 nM. Control cells received an equivalent volume of phosphate buffered saline (PBS). After 24 h incubation (37°C, 10% CO2 in air) cells were labelled with 0.4µCi 3H-thymidine well to give a final volume of 200 µl well and were harvested on an Inotech cell harvester following a further 24 h incubation.

**Cell growth**

Cultures of SCLC CLs HC12 and ICR-SC155 were grown at 2 x 105 cells ml in the presence of 0, 20, 50 or 100 µM D-Phe5SP. After 1 week cell number was determined by Coulter Counter, and viability assessed by trypan blue exclusion on a haemacytometer. Normal human skin fibroblasts (SF1) at 105 cells ml were grown in RPMI-1640 medium supplemented with 10% FCS, which was replaced after 1 week with RPMI-1640 supplemented with 5% FCS and 0, 50 or 100 µM D-Phe5SP. After 3 days the monolayer was trypsinised and viable cells were counted as above.

To assess the effect of short term exposure to the analogue aliquots of 105 HC12 cells were treated with PBS (control) or 100 µM D-Phe5SP. At hourly intervals control and treated cells were washed (centrifuged at 90 g for 5 min in RPMI-1640 medium with 5% FCS) and viable cells were counted on a haemacytometer. The cells were then cultured in fresh medium without the analogue, and viable cells were counted after 1 week.

**Clonogenic assay**

Lung tumour cells were seeded at 5 x 102–2 x 103 plate in 0.5 ml 0.3% agar, and layered over an overlay of 1 ml 0.5% agar containing D-Phe5SP 0–100 µM or SP 0–100 µM. After 2–4 weeks incubation colonies of ≥ 50 cells were counted.

Bone marrow samples for clonogenic assay were layered onto Ficoll-Hypaque and centrifuged at 550 g for 20 min. Nucleated cells were collected from the interface, washed and counted on a Coulter Counter. Cells were plated at 103 dish in a double layer assay as above, with a final concentration of 20% FCS and 100 ng ml-1 GM-CSF. HC12 cells were set up in clonogenic assay under identical conditions.

**Statistics**

Results represent mean ± s.e.m. of a minimum of three replicates and are expressed as % control. Analysis of variance and the 2-tailed Dunnett's test were used to determine the significance of differences between control and treated groups, and Tukey's test was used to test for significant differences between treated groups.

**Results**

Inhibition of 1H-thymidine incorporation in human cancer cell lines and normal tissue

The substance P analogue D-Phe5SP was tested against six SCLC CLs, one fresh SCLC tumour sample, one squamous lung carcinoma CL, two ovarian carcinoma CLs and one squamous cervical carcinoma CL. The minimum concentration required to cause significant inhibition (P < 0.01) of DNA synthesis varied with the cell line. Five of ten CLs were inhibited by 10 µM D-Phe5SP (80–90% control). 4 x 10 by 20 µM (70–83% control), and 1 x 10 by 50 µM (58 ± 2% control). Two of ten CLs (CHI and HX155) showed significant stimulation (P < 0.01) at 1 µM (115–116% control). Fresh SCLC tumour cells (ICR-SC155) were inhibited by 10 µM D-Phe5SP (67 ± 3% control). D-Phe5SP concentrations above these minimum inhibitory levels led to increased inhibition and 1H-thymidine incorporation was negligible at 100 µM D-Phe5SP in all cell lines. Figure 1 shows representative results in SCLC CL ICR-SC112.

Inhibition to 50% of control (IC50) in 5% FCS was also variable: 20–30 µM in six SCLC cell lines, 20 µM in fresh SCLC tumour cells, 40 µM in a squamous lung carcinoma cell line, 20–50 µM in two ovarian carcinoma cell lines and 30 µM in a cervical carcinoma cell line (Table 1). In HC12 (SCLC CL), increasing the serum concentration from 5% to 10% to 20% FCS resulted in IC50 of approximately 20, 40

![Figure 1](https://example.com/figure1.png)

**Table 1** The concentration of D-Phe5SP (µM) required to inhibit H-thymidine incorporation into DNA to approximately 50% of control (IC50)

| Cell line | D-Phe5SP IC50 (µM) | SP |
|-----------|--------------------|----|
| HC12 (SCLC) | 20 | NS |
| HX149 (SCLC) | 25 | 100 (86 ± 5%) |
| ICR-SC112 (SCLC) | 20 | NS |
| ICR-SC132 (SCLC) | 30 | 100 (80 ± 2%) |
| ICR-SC65 (SCLC) | 30 | ND |
| ICR-SC17 (SCLC) | 30 | ND |
| ICR-SC155 (SCLC) | 30 | ND |
| NC1-H226 (NSCLC) | 40 | NS |
| SKOV 3 (Ovarian) | 50 | NS |
| CHI (Ovarian) | 20 | 100 (72 ± 2%) |
| HX155 (Cervix) | 30 | NS |
| SF1 (Fibroblasts) | 50 | NS |

The lowest concentration of substance P (SP 1–100 µM) which caused significant inhibition (P < 0.01) of H-thymidine incorporation is also shown (mean ± s.e.m. of control). All assays performed in the presence of 5% FCS. NS = not significant. ND = not done.
and 60 µM respectively (Figure 2). Normal human skin fibroblasts (SF1) showed stimulation (132–155% control P<0.01) of DNA synthesis at 1–20 µM D-Phe5SP, however ≥40 µM caused inhibition (65 ± 4% control P<0.01). The IC50 was 50 µM (Table 1).

Substance P (SP) itself had no significant growth inhibitory effect in 5/8 cell lines, while 3/8 showed inhibition (70–90% control P<0.01) at 100 µM. SP 1–100 µM had no significant effect on DNA synthesis in normal human skin fibroblasts (SF1) (Table 1).

SP (100 µM), bombesin (1 µM), vasopressin (1 µM) and IGF-1 (70 nM) were tested in combination with D-Phe5SP (1–100 µM), to see if they were able to reverse the inhibitory effect of the analogue. No reversal was seen in 3.3 SCLC CLs or 1.1 squamous lung carcinoma CL. This was further investigated in SCLC CL, HX149 where inhibition at 20 µM D-Phe5SP in RPMI-1640 supplemented with 5% FCS (56 ± 5% control) and 5 µM in unsupplemented RPMI-1640 (68 ± 2% control) was not reversed by bombesin (1–1000 nM), vasopressin (1–1000 nM) or SP (1–100 µM) (data not shown).

Inhibition of growth as assessed by cell number

One week’s exposure to 20, 50 or 100 µM D-Phe5SP resulted in the growth inhibition of SCLC CLs HC12 and ICR-SC155. Increase in cell number was significantly inhibited (P<0.01) by 20 µM (78 ± 1% and 32 ± 0.4% control respectively), while 50 and 100 µM caused cell number to fall below the initial inoculum (Figure 3). In contrast 50 µM D-Phe5SP had no effect on the cell number of confluent cultures of normal human skin fibroblasts, however 100 µM caused inhibition to 83 ± 2% control (P<0.01) (Figure 3).

Effect of short term exposure to 100 µM analogue

HC12 viable cell number dropped on exposure to 100 µM D-Phe5SP for 1–4 h. After 1 h the treated cell number fell to 55 ± 4% of the initial inoculum, and by 4 h it was 1 ± 0.1% (Figure 4a). The control cell number recoverable over 4 h was 78 ± 4–90 ± 9% of the initial inoculum.

Surviving treated and control cells were washed and cultured in fresh medium without D-Phe5SP. After 1 week the control cells had grown to 1706 ± 51–2530 ± 59% of the initial cell number. Cells pre-exposed to D-Phe5SP for 1 h showed similar growth to controls. However, cells which had survived 2, 3 and 4 h exposure had a significantly (P<0.01) reduced ability to grow, with cell number increases of 647 ± 20%, 224 ± 4% and 335 ± 7% respectively (Figure 4b). The same effects were found in two separate experiments, both carried out on the HC12 cell line.

Figure 2 D-Phe5SP (1–100 µM) effect on DNA synthesis of HC12 (SCLC CL) in the presence of 5% FCS (■), 10% FCS (▲), and 20% FCS (▼). Results expressed as % control incorporation (mean ± s.e.m.).

Figure 3 Inhibition of growth of SCLC CLs HC12 and ICR-SC155 after 1 week in the presence of 20, 50 or 100 µM D-Phe5SP. Effect of 3 days exposure to 50 and 100 µM D-Phe5SP on cell number of confluent cultures of normal human skin fibroblasts (SF1). Results expressed as % control cell number (mean ± s.e.m.). *Significant inhibition P<0.01.

Figure 4 a. HC12 viable cell number recovered after 1–4 h exposure to PBS (▲) or 100 µM D-Phe5SP (■), expressed as % of initial inoculum of 106 cells (mean ± s.e.m. of triplicate counts). Surviving cells from each time point were seeded at 8.5 x 103 ml−1 and grown for 1 week in the absence of analogue. b. Histogram showing growth of control (C) and treated (T) cells after 1, 2, 3 or 4 h pre-exposure to 100 µM D-Phe5SP. Cell counts expressed as % growth increase from original cell number (mean ± s.e.m. of triplicate counts). *Significant inhibition (P<0.01) compared to control.
D-Phe^5 SP caused dose dependent inhibition of colony formation (P<0.01) in 3 SCLC CLs and 11 squamous lung carcinoma CL, while substance P had no effect (Table II). Significant inhibition was seen at D-Phe^5 SP concentration as low as 1 μM.

Three normal human bone marrow samples showed variable inhibition (P<0.01) of colony formation in response to D-Phe^5 SP. Two of three had an IC₅₀ of 30 μM, while the third had an IC₅₀ of 80 μM. However the degree of inhibition was less than that seen in HC12 under the same growth conditions (Table II). In 20% FCS with GMCSF, 1 μM D-Phe^5 SP had no effect on the CFE of HC12, whereas this concentration had caused inhibition (31 ± 2%) P<0.01 in medium with 5% FCS.

Discussion

This study confirms previous reports of the potent antiproliferative effects of [D-Arg^1, D-Phe^2, D-Trp^5, Leu^7] substance P (D-Phe^5 SP) on small cell lung cancer (SCLC) cell lines (Woll & Rozengurt, 1986b; Woll & Rozengurt, 1990) (Figure 1). In addition, we have demonstrated equivalent inhibition of DNA synthesis in fresh SCLC tumour cells from a previously untreated patient, a squamous lung carcinoma cell line, a squamous cervical carcinoma cell line, and two ovarian carcinoma cell lines (Table I).

Previous studies have focused on the ability of the SP analogues to antagonise interaction of neuropeptide growth factors, particularly bombesin, with cell surface receptors (Woll & Rozengurt, 1988a; Woll & Rozengurt, 1988b; Takuwa et al., 1990). Squamous lung carcinoma cell line NCI-H226 lacks detectable bombesin receptors (Moody et al., 1983) and appears to be slightly less sensitive to the analogue than the SCLC cell lines. However growth was undoubtably inhibited (Table II). Exogenous bombesin was not able to reverse D-Phe^5 SP inhibition of DNA synthesis in the three SCLC cell lines examined, which confirms previous suggestions that the analogue is not working solely by competition for binding to the bombesin receptor (Takuwa et al., 1990). This is in contrast with results in Swiss 3T3 cells, where the inhibitory effects of SP analogues can be reversed by excess gastrin releasing peptide (GRP) (Woll & Rozengurt, 1988b), and suggests that the mechanism of inhibition is different in the SCLC cells. Similarly, we saw no reversal of inhibition in the presence of vasopressin or GIP. Increasing serum concentration does partially reverse the inhibitory effect of the analogue, however even in the presence of 20% FCS DNA synthesis and cell growth are almost completely blocked (Figure 2 and Table II).

Table II *4 colony formation (mean ± s.e.m. of control) in the presence of 0–100 μM D-Phe^5 SP and 0–100 μM substance P (SP)

| Cell line | D-Phe^5 SP μM | SP μM |
|----------|---------------|--------|
| 0 | 5 | 7 |
| 10 | 12 | 14 |
| 50 | 100 | 110 |

| HC12 | 100±14 | 31±2 | 5±2 | 2±1 | 0 | NS |
| ICR-S112 | 100±11 | 8±1 | 2±1 | 0 | 0 | NS |
| HX149 | 100±2 | 93±14 | 9±2 | 0 | 0 | NS |
| NCI-H226 | 100±13 | 102±16 | 16±2 | 20±4 | 7±2 | NS |
| BM 1 | 100±5 | 120±12 | 85±5 | 83±2 | 30±1 | ND |
| BM 2 | 100±5 | 98±9 | 70±8 | 31±3 | 20±3 | ND |
| BM 3 | 100±5 | 96±5 | 87±4 | 11±3 | 7±2 | ND |
| HC12 | 100±8 | 123±6 | 19±1 | 2±0.6 | 0.6±0.6 | ND |

Assays performed in RPMI 1640 medium with 5% FCS except bone marrow (BM) and HC12 assays (■) which were in RPMI 1640 supplemented with 20% FCS and 100 ng GM-CSF ml⁻¹. Significant inhibition P<0.01; *Significant stimulation P<0.05; NS = not significant; ND = not done.

SP analogue [D-Arg^1, D-Pro^2, D-Trp^5, Leu^7] substance P (DAPTL-SP) is reported to have a cytostatic effect in SCLC CLs. With reversal of growth inhibition after washing and reculturing in medium without the analogue (Woll & Rozengurt, 1988b). We observed a significant reduction in viable cell number following brief exposure to D-Phe^5 SP 100 μM, and the growth potential of the surviving cells was not completely restored on removal of the analogue (Figure 4b). This difference in reversibility may simply reflect the reported increased potency of D-Phe^5 SP over DAPTL-SP (Woll & Rozengurt, 1988b), or they may be inhibiting growth through different mechanisms.

In general D-Phe^5 SP was less potent when tested against normal human skin fibroblasts (Figure 3) and human bone marrow cells (Table II) than tumour cells. Comparing the growth inhibitory effects of D-Phe^5 SP against HC12 and human bone marrow samples which were grown under identical assay conditions, colony formation was consistently higher by the bone marrow than the tumour cells, even at 100 μM. This differential effect between normal and tumour cells could be due to the number or type of receptors expressed on the cell membrane.

In summary D-Phe^5 SP shows antitumour effects against several different tumour cell types, including SCLC, squamous lung carcinoma, ovarian and squamous cervical carcinoma, with less growth inhibition seen against normal human skin fibroblasts and bone marrow. There is substantial cell death after 2–4 h exposure to 10 μM D-Phe^5 SP, and surviving cells exhibit growth inhibition. These results suggest that D-Phe^5 SP merits further study as a potential novel anti-tumour agent. We are currently investigating the mechanism of action and in vivo activity.

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