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[D-ARG^1, D-PHE^5, D-TRP^{7,9}, LEU^{11}] SUBSTANCE P INDUCES APOPTOSIS IN LUNG CANCER CELL LINES IN VITRO

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The broad spectrum antagonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P has been shown previously to inhibit the growth of small cell lung cancer cells both in vitro and in vivo. To elucidate further the pathways involved in the growth inhibitory actions of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P we have examined the effect of this agent on cell viability and the induction of apoptosis in small cell and non-small cell lung cancer cells. Treatment of lung tumor cells with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P caused a concentration-dependent loss of cell viability which was accompanied by the onset of apoptosis, as defined by cytological criteria and DNA fragmentation. This effect occurred in both small cell and non-small cell lung cancer cells and was not dependent on *de novo* protein synthesis. Such findings indicate that the antiproliferative action of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P involves a signal transduction pathway for apoptosis.

The demonstration in recent years that small cell lung cancer (SCLC) growth is sustained, at least in part, by multiple neuropeptide growth factors (1) has led to the development of both ligand-specific and broad spectrum neuropeptide antagonists, as antiproliferatives. Peptides characterised as broad spectrum antagonists have the unusual and thus far unexplained property of interfering with the action of multiple Ca^{2+}-mobilising peptides (2). Both classes of antagonist have been shown to inhibit the in vitro and in vivo growth of SCLC cell lines (3,4), although broad spectrum antagonists are potentially more useful anticancer drugs given the mitogenic complexity of SCLC growth.

An important consideration in the application of antitumor agents which target mitogenic signals is whether their action results in cytostasis or cytoxicity. Although there is no ostensive basis on which to expect inhibitors of growth factor action to be cytotoxic rather than cytostatic, evidence suggests that some cells require growth factors for survival. Thus, several studies have demonstrated that growth factor deprivation in both normal and tumor cells (5-9) may activate a sequence of biochemical events that results in the cessation of cell proliferation and activation of a genetically determined program of cell death termed 'apoptosis' (10). Cells

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Abbreviations: SCLC-small cell lung cancer; LHRH-luteinizing hormone-releasing hormone; NSCLC-non-small cell lung cancer; FCS-fetal calf serum; MTT-[3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium.

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undergoing apoptosis display shrinkage, loss of cell-cell contact, chromatin condensation, and internucleosomal degradation of DNA. Apoptosis has been shown to occur in hormone-dependent tumors of the breast following estrogen ablation (6), or treatment with analogues of luteinizing hormone-releasing hormone (LHRH) or with somatostatin (7). LHRH analogues and somatostatin also trigger apoptosis in pancreatic carcinomas (8). Similarly, antiandrogens and androgen ablation have been shown to activate apoptosis in prostate cancers (9). Such findings raise the possibility that agents which intervene in the autocrine growth of lung tumor cells may also activate apoptosis in these cells. The present study therefore investigates the effect of the broad spectrum antagonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P on cell survival in human SCLC and non-small cell (NSCLC) cell lines *in vitro*.

METHODS

Peptides. [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P and substance P 1-9 were obtained from Peninsula Laboratories, Inc., Merseyside, U.K.

Cell lines. Human SCLC lines NCI-H69, COR-L88 and COR-L51, and NSCLC cell lines COR-L23 (large cell carcinoma), MOR (adenocarcinoma) and LUDLU-1 (squamous cell carcinoma) were grown routinely in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS), penicillin and streptomycin (all Gibco BRL, Paisley, Scotland).

Cell viability. Cells were disaggregated by brief exposure to 0.02% EDTA and were seeded into 96-well plates at densities of 300 (NSCLC) and 10^4 (SCLC) cells / well in RPMI 1640 medium containing 5 μg insulin/ml, 10 μg transferrin/ml, 30 nM sodium selenite, 2 mM glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml. The effect of increasing concentrations of antagonist was determined by treating cells with 1-100 μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P for 24 h at 37^{0}C. Control cultures were treated with 1-100 μM substance P 1-9. Cell viability was determined either or by the [3-(4,5-dimethylthiazol-2-yl)]-2,5-diphenyltetrazolium (MTT) assay (11) or by trypan blue exclusion. The influence of cycloheximide on [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P action, was investigated by treating cells with 30 μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P in the presence or absence of cycloheximide (10 μg/ml). Cell viability was then determined using the MTT assay and trypan blue exclusion.

Cytology. Cells treated with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P were washed twice with phosphate buffered saline and cytospun onto glass slides. Cells were fixed using Cytospin Collection Fluid (Shandon Inc. Pittsburg, USA) and stained with haematoxylin and eosin. Control cells were cultured in serum-free medium and processed similarly.

Flow cytometry. Flow cytometric analyses were performed using the Cambridge dual laser flow cytometer as previously described (12). Briefy, 5x10^5 cells were cultured in serum-free medium in the presence or absence of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P. After various treatment times, cells were stained with ethidium bromide (50 μg/ml) plus 0.125% TritonX-100 and ribonuclease A (0.5 μg/ml) for 10 min prior to analysis.

Gel electrophoresis of DNA. DNA fragments were isolated using the SDS-proteinase K method with minor modifications (13). Briefly, 2x10^6 cells treated with 100 μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P for various times were lysed in 10 mM Tris, pH7.9, 5 mM EDTA, 100 mM NaCl, 0.5% SDS containing 0.5 mg proteinase K/ml, at 55^{0}C for 4 h. High molecular DNA was precipitated by the addition of NaCl to 1 M followed by incubation at 4^{0}C for 16 h. The lysates were then centrifuged at 57,000g for 20 min at 4^{0}C and the pellet containing high molecular DNA discarded. Low molecular weight DNA fragments in the supernatant were extracted with phenol and chloroform:isoamyl alcohol (24:1) and precipitated with ethanol. Then DNA was resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0 and electrophoresed on Visigel separation matrix (Stratagene, La Jolla, CA). DNA was visualised by ethidium bromide staining and UV transillumination.

RESULTS

Effects of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P on SCLC and NSCLC cell viability. Figure 1 shows that treatment of SCLC and NSCLC cells with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P resulted in a concentration-dependent cytotoxicity with the concentration required to reduce optical density to 50% of control being 30μM for both cell lines. Cytotoxicity was confirmed by a concentration-dependent increase in trypan blue positive cells following treatment with the antagonist. Treatment with the peptide fragment substance P 1-9 was not cytotoxic over the concentration range investigated for [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P. Cycloheximide treatment had no effect on the cytotoxic action of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P and was not cytotoxic when given alone (data not shown).

Cytology of lung cancer cells treated with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P. Following treatment with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P both SCLC and NSCLC cells appeared shrunken and showed extensive chromatin condensation (Figure 2) compared to untreated control cultures. These changes were concentration- and time-dependent occurring as early as two hours following treatment with 100μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P.

Flow cytometric analyses of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P-treated cells. Figure 3 shows that compared to untreated controls (panel A) nuclei from cells treated with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P (panel B) showed an increase in right angle scatter width (488RS WDTH), consistent with increased nuclear density and chromatin condensation. This change occurred as early as 2 h after treatment with 100μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P. It can be seen that the increase in right angle scatter width

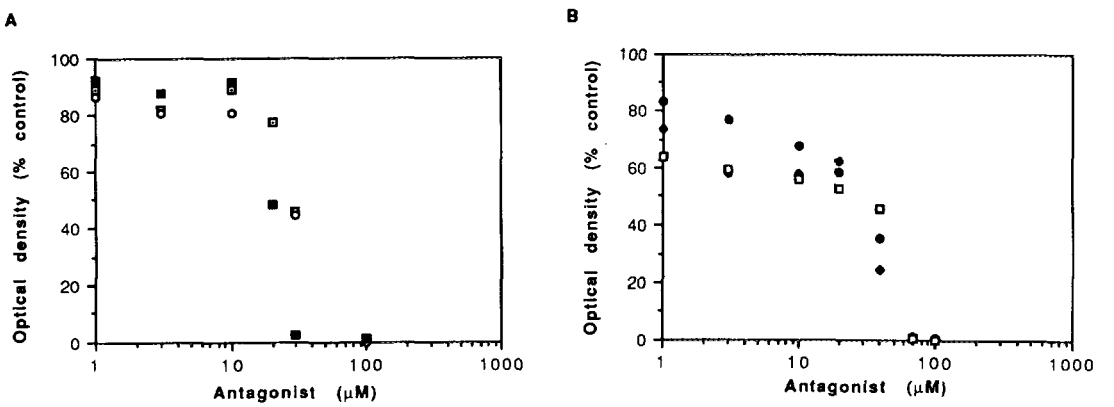

Figure 1. Effects of increasing concentrations of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P on cell survival following 24 hour treatment determined using the MTT assay. Optical density in the MTT assay has been previously shown to be proportional to final cell number. Points represent mean values from three replicate wells in a single experiment. Essentially similar results were obtained in three repeat experiments. Panel A: SCLC cell lines ⊡ COR-L88, ○ NCI-H69, ■ COR-L51; Panel B: NSCLC cell lines ● COR-L23, ◆ MOR, □ LUDLU-1.

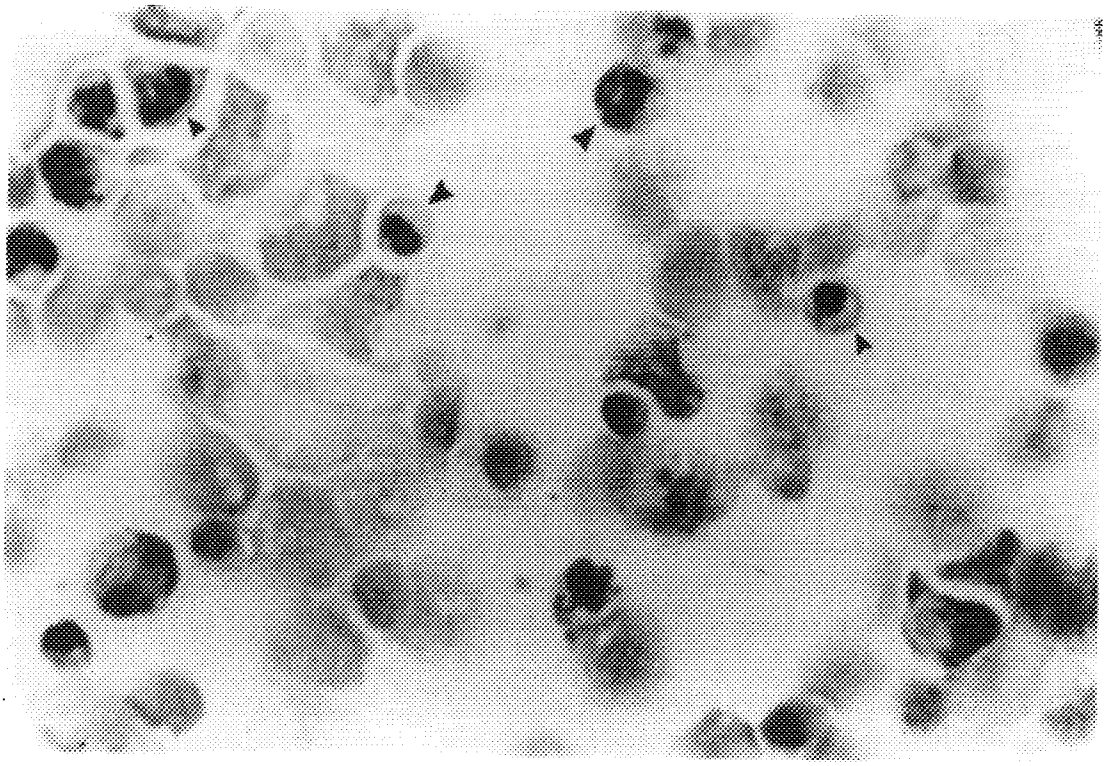

Figure 2. Apoptotic features induced by [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P in SCLC cell line NCI-H69. Cells were treated with 50μM [D-Arg^1, D-Phe^5, D- Trp^{7,9}, Leu^{11}] substance P for 4 hours. Nuclei of treated cells show chromatin condensation and nuclear fragmentation (arrows). Magnification x360.

occurred in nuclei in all phases of the cell cycle. Similar results were obtained for NSCLC cells treated with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P.

Induction of DNA degradation in SCLC and NSCLC cell lines by [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P. Figure 4 shows that treatment of both SCLC and NSCLC cell lines with

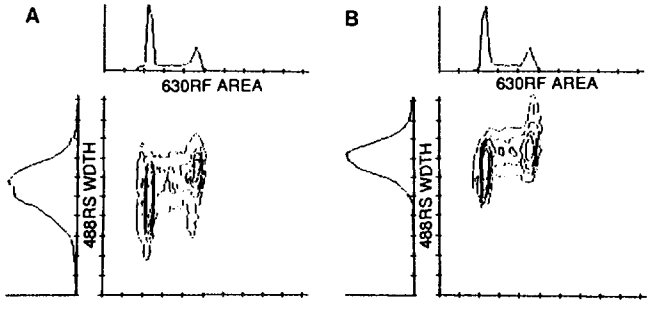

Figure 3. Flow cytometric analysis of SCLC NCI-H69 nuclei from untreated cells (panel A) and cells treated with 100μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P for 2 hours (panel B). Treatment with 100μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P caused a similar increase in right angle scatter width (488RS WDTH) in COR-L51, COR-L88, MOR, COR-L23 and LUDLU-1.

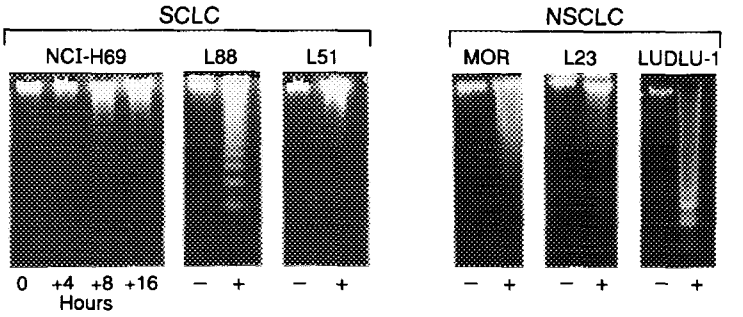

Figure 4. [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] Substance P-induced DNA fragmentation in SCLC and NSCLC cell lines. (-) DNA from untreated controls; (+) DNA from cells treated with 100μM antagonist for 8 hours except where indicated for NCI-H69.

100μM [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P resulted in DNA fragmentation typical of apoptosis. The kinetics of DNA fragmentation following treatment with the antagonist are shown for the SCLC cell line NCI-H69. It can be seen that DNA fragmentation can be detected within 4 h of treatment.

DISCUSSION

Previous studies have shown that the 'broad spectrum' antagonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P inhibits the growth of SCLC cell lines in vitro in a concentration-dependent manner (14). For NCI-H69 the concentration reducing growth to 50% of control is reported to be 24μM. On the basis of mechanistic studies performed to date, the antiproliferative action of this and other broad spectrum antagonists is thought to result from direct, or indirect, inhibition of neuropeptide receptor signalling resulting in cytostasis (2,4,14,15). However, the findings of the present study demonstrate that treatment of both SCLC and NSCLC cells with this antagonist results in cell death and that death is by apoptosis. Thus, treated cells show loss of cell-cell contact and nuclear condensation, and analysis of DNA from dying cells revealed fragmentation of chromatin into nucleosome ladders. In both SCLC and NSCLC cells the concentration effecting 50% cell death was 30μM. Importantly, the peptide fragment substance P1-9 which has no receptor binding properties was not cytocidal at any of the concentrations tested.

The finding that [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P is active against both SCLC and NSCLC cells is consistent with a previous report describing the antiproliferative effects of the substance P analogue [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}] substance P in the NSCLC cell line A549 (16). Although the involvement of neuropeptides in the autocrine growth of NSCLC is less well documented than that for SCLC, we and others have demonstrated the presence of functional neuropeptide-receptors on NSCLC cells (17,18), including receptors for gastrin-releasing peptide, bradykinin, vasopressin and cholecystokinin, and a role for the bombesin-like peptide neuromedin B, in the autocrine growth of these cells

(19). Since [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P has been shown to inhibit the mitogenic actions of these neuropeptides (2,4,13,14) it is possible that the cytocidal effects of [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P are related to the disruption of neuropeptide signalling in both types of lung tumour cell and that this in turn leads to cell death.

The rapid onset of apoptosis in SCLC and NSCLC cells treated with [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P (within 2 hours of treatment), together with the failure of cycloheximide to protect against this process, suggests that cell death is a direct result of antagonist action. This is in contrast to the findings of several reports which show that apoptosis in other systems is an active process requiring *de novo* protein synthesis (20-22). However, similar observations to those of the present study have been reported for the induction of apoptosis in B-chronic lymphocytic leukemic and normal lymphocytes following treatment with autologous or heterologous serum in the presence of cycloheximide (23). Similarly, a rapid onset for apoptosis has been descibed following serum deprivation in fibroblasts with deregulated c-*myc* expression where the first deaths were observed within 30 minutes of serum withdrawal (24). On the basis of the findings presented here, it seems likely that the antiproliferative action [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P involves the induction of apoptosis via the activation of preformed effectors. Studies are now in progress to define further the signalling pathways involved in [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P-induced cell death.

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