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Synthesis of Peptide and Pseudopeptide Amides Inhibiting the Proliferation of Small Cell and Epithelial Types of Lung Carcinoma Cells

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Abstract: Small cell lung cancer (SCLC) cell lines produce and secrete various peptide hormones, e.g. bombesin (BN)/gastrin releasing peptide (GRP) like peptides that are proposed to function as their autocrine growth factors. To inhibit the proliferative effect of these hormones we have synthesized short chain BN[7-14]-analogues replacing the C-terminal peptide bond by a methylene-amino (-CH_2NH-) unit and introducing D-Phe or D-Ser into position 12. As several substance P (SP) analogues were found to inhibit the growth of SCLC cells, some short chain SP-analogues have been synthesized. (Pseudo)octapeptides were synthesized in solution, by fragment condensation using the DCC/HOPfp method. Fragments and SP-analogues were synthesized stepwise using pentafluorophenyl esters. The resistance to hydrolysis of the reduced peptide bond made permitted exact quantification of the Leuψ(CH_2NH)Leu pseudopeptide in hydrolysates. The binding ability of both types of peptides to BN-receptors on Swiss 3T3 mouse fibroblast cells and their antiproliferative effect on NCI-H69 human SCLC cell line have been tested and compared with a short chain SP-antagonist pHOPA-D-Trp-Phe-D-Trp-Leu-Leu-NH_2 (**R**) previously described as a potent inhibitor of SCLC proliferation. While BN-analogues showed weak activity in inhibition of proliferation of SCLC cells, SP-analogues **6**: D-MePhe-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)-Leu-NH_2 and **7**: D-MePhe-D-Trp-Phe-D-Trp-Leu-MPA, in spite of greatly diminished affinity towards the BN-receptor, inhibited SCLC proliferation more effectively than **R** (**6**: $IC_{50} = 2$ μM, **7**: $IC_{50} = 5$ μM and **R**: $IC_{50} = 10$ μM). Moreover, **6** inhibited the respiratory activity of SK-MES 1 epithelial type of lung carcinoma cells in proliferating but not in the quiescent state, suggesting that the antiproliferative effect of these compounds is not due to simple cytotoxicity. These short chain analogues of SP might be promising candidates as therapeutic agents in the treatment of SCLC. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: substance P analogues; synthesis of pseudopeptide amides; small cell lung cancer; inhibition of proliferation

INTRODUCTION

Small cell lung cancer (SCLC) is a rapidly developing fatal malignancy, representing one of the most aggressive forms of cancer. Despite the initial response to chemotherapy, its metastatic spread is extensive and patients usually die within 1 year after diagnosis [1]. Production of various peptide hormones, such as gastrin releasing peptide (GRP), the mammalian counterpart of the amphibian bombesin (BN), neurotensin, insulin, vasopressin,

Abbreviations: SCLC, small cell lung cancer; BN, bombesin; GRP, gastrin releasing peptide; SP, substance P; pHOPA, 4-hydroxyphenyl-acetyl; MPA, 2-amino-3-methylpentane; cycloLeu, 1-amino-1-cyclopentane-carbonyl; D-MePhe, D-N-methyl-phenylalanyl; LAH, lithium aluminium hydride; MES, mercaptoethanesulfonic acid; DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; Dnp, 2,4-dinitrophenyl; HOPfp, pentafluorophenol.

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bradykinin, cholecystokinin was detected in different SCLC cell lines, which express receptors for these hormones [2,3,14]. The growth of some SCLC cell lines could be inhibited by anti-BN monoclonal antibody and BN-receptor antagonists *in vitro* and *in vivo* [4–6]. According to these observations, peptides of the BN/GRP family are considered as autocrine growth factors of SCLC, so their antagonists would be inhibitors of the proliferation of SCLC cells and potential therapeutic compounds [7,8].

Though substance P (SP) has minimal homology with BN and was not detected among peptides produced by SCLC cells [2], surprisingly the first synthetic peptide exhibiting BN-antagonist properties was the SP-antagonist [D-Arg^{1}-D-Pro^{2}-D-Trp^{7,9}-Leu^{11}]-SP [9,10]. Furthermore, several SP-analogues were reported to act as BN-antagonists and to inhibit SCLC clonal growth [11,12,22]. These peptides inhibit not only BN-stimulated but other neuropeptide induced mitogenesis, too. They are often characterized as 'broad spectrum' antagonists, hypothesizing that these peptides recognize a common domain of neuropeptide receptors or a separate protein influencing the activity of neuropeptide receptors [13–16]. Although, SP-analogues synthesized so far lack selectivity and behave irregularly in different test systems [17], SP-like peptides could be potentially more useful anticancer drugs than BN-specific antagonists due to the mitogenic complexity of SCLC growth [14,29,30].

In this paper we report some new short chain (pseudo)peptides of both the BN and the SP families. We have synthesized BN-related octapeptides with N-terminal deletion and introduction of D-Phe or D-Ser into position 12 or replacement of the C-terminal peptide bond by a methylene amino (-CH_2NH-) unit (Table 1). These modifications were based on the results of previous studies demonstrating that the C-terminal GRP octapeptide still retains the affinity for the GRP-receptor and can stimulate mitogenesis [18] and moreover that the introduction of D-Phe into position 12 or reduction of the C-terminal peptide bond within the BN molecule yields effective BN-receptor antagonists [19,20]. The synthesis of new SP-related molecules was based on the modification of the known short chain SP-antagonist **R** [21] that proved to be a remarkable BN-antagonist and could inhibit the proliferation of NCI-H69 SCLC cells [22].

The affinity of peptides of both families for BN-receptor on Swiss 3T3 mouse fibroblast cells and their effect on ^{3}H-thymidine incorporation of NCI-H69 SCLC and SK-MES 1 epithelial type of lung carcinoma cells were tested and compared with reference compound **R**. Two novel short chain SP-analogues (**6** and **7**) proved to be more effective in inhibition of ^{3}H-thymidine incorporation but less potent BN-antagonists than **R**.

MATERIALS AND METHODS

Cell Lines and Peptides

NCI-H69 and SK-MES 1 cell lines were purchased from ATCC (Rockville, MD, USA). Swiss 3T3 mouse fibroblast cell line was from Flow Laboratories (Irvine, UK). Tyr^{4}-BN was obtained from Sigma (St. Louis, MO, USA). The structure of the new synthetic analogues are shown in Table 1.

Table 1 Amino Acid Sequences of Bombesin, Substance P and Synthetic Peptides Studied

Bombesin	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2
BN-analogues	
1	Glp-Trp-Ala-Val-Gly-His-Leuψ(CH_2NH)Leu-NH_2
2	Glp-Trp-Ala-Val-Gly-D-Phe-Leu-Leu-NH_2
3	Gln-Trp-Ala-Val-Gly-D-Ser-Leu-Leu-NH_2
5	Gln-Trp-Ala-Val-Gly-D-Ser-cycloLeu-Leu-NH-CH_3
SP-analogues	
R	pHOPA-D-Trp-Phe-D-Trp-Leu-Leu-NH_2
5	pHOPA-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2
6	D-MePhe-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2
7	D-MePhe-D-Trp-Phe-D-Trp-Leu-MPA
8	D-Tyr-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2
9	D-Tyr(Et)-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2

Analytical Procedures

Melting points were determined by a Büchi apparatus and are not corrected. Optical rotations were measured by a Perkin Elmer 241 polarimeter. For TLC analysis precoated plates of silica gels (DC-Fertigplatten, Merck) were used in the following solvent systems: S_1: EtOAc: stock solution = 1:9:1; S_2: EtOAc: stock solution = 4:1; S_3: EtOAc: stock solution = 7:3; S_4: EtOAc: n-BuOH: AcOH: water = 1:1:1:1; S_5: n-BuOH: AcOH: water = 4:1:5 (upper phase). The stock solution is a mixture of pyridine: AcOH: water = 20:6:11 (v/v).

Chromatograms were visualized by spraying the plates with ninhydrin followed by o-tolidine/KI after chlorination. Amino acid analysis (AAA) was performed by a Biotronik LC 5001 analyser coupled to a Shimadzu C-R3A Chromatopac integrator. Samples (1 mg) were hydrolysed both in l ml of 6 M HCl and in 3 M mercaptoethanesulphonic acid (MES) at 110°C for 24 h. NMR spectra were taken by a Varian VXR-300 NMR spectrometer in deuterated dimethylsulphoxide (DMSO-d_6) solution using tetramethylsilane (TMS) as internal standard at ambient temperature.

Preparative and Analytical Liquid Chromatographic Procedures. Chromatography on silica gel column was carried out using a mixture of $CHCl_3$/EtOH/water/AcOH (75:28:4:5) as eluent. Fractions were checked by TLC using the S_3 system.

HPLC purification of the peptides was performed on a Whatman Partisil C 18 10 μm column (22 × 500 mm). Samples of 150 mg of crude peptide in 1 ml 0.2% aqueous TFA were injected and gradient eluted with a mobile phase of 0.2% aqueous TFA/0% MeCN 0–40% in 180 min at a flow rate of 5 ml min^{-1}. Octapeptides with N-terminal Gln were eluted using A/B buffer gradient starting from 0 to 100% B buffer at linear rate within 3 h. (A buffer: 0.05 M NH_4OAc (pH 4.0)/MeCN (85:15) mixture; B buffer: 0.05 M NH_4OAc (pH 4.0)/MeCN (25:75) mixture).

Purity of the fractions and the final products was checked by analytical HPLC on a BST (Hungary) Nucleosil 300 C18 10 μm column (4 × 250 mm) using A/B buffer gradient elution starting from 30 to 80% of buffer B within 1 h at a flow rate of 1 ml min^{-1}. Detection was performed by spectrophotometric W detector at 254 nm.

Synthesis of the Peptides. **Boc-Gln-Trp-Ala-Val-Gly-OBzl (10)**. Deprotection of Boc-Val-Gly-OBzl (3.25 g, 9 mmol) in 6 M HCl/dioxane (15 ml) and subsequent trituration with anhydrous ether yielded H-Val-Gly-OBzl·HCl. A solution of this salt in DMF (20 ml) was stirred overnight with Boc-Ala-OSu (2.7 g, 10 mM) and Et_3N (1.9 ml, 14 mM) at ambient temperature, the reaction mixture was evaporated and a solution of the residue in $CHCl_3$ (70 ml) was washed with 1 M HCl, 5% $NaHCO_3$ and water. After removal of the organic solvent Boc-Ala-Val-Gly-OBzl was isolated with EtOAc/n-hexane (1:2) mixture, followed by deprotection as above, to give H-Ala-Val-Gly-OBzl·HCl (3.0 g, 8 mM). A solution of the free tripeptide salt in DMF (30 ml) was stirred for 2 h with Boc-Trp-OPfp (4.3 g, 9 mM) in the presence of neutralizing Et_3N, the solvent was removed and a solution of the residue in EtOAc was washed as above. Boc-Trp-Ala-Val-Gly-OBzl was isolated with n-hexane, and then treated with 6 M HCl/dioxane (50 ml). The resulting suspension was filtered, and the residue was washed with ether. A solution of the free tetrapeptide salt in DMF (50 ml) was stirred 1 h with Boc-Gln-OPfp (3.2 g, 8 mM) and Et_3N, the reaction mixture was evaporated, the residue was solidified by rubbing with EtOH (40 ml) to give **10** (4.8 g, 71%, m.p. 217–219°, $R_f^3 = 0.55$).

Boc-Glp-Trp-Ala-Val-Gly-OBzl (11) was prepared the same way (1.25 g, 72%, m.p. 204° (dec.), $R_f^3 = 0.65$).

Boc-Gln-Trp-Ala-Val-Gly-OH (12). To a solution of **10** (3.5 g, 4 mM) in MeOH (200 ml) 10% Pd on charcoal was added (1 g), hydrogen gas was bubbled through the mixture for 1.5 h. Then the catalyst was removed, the solvent evaporated to give **12** (2.7 g, 90%, m.p. 184–186°, $R_f^3 = 0.30$, $[\alpha]_D = -10.7°$ (c = 1, DMF)).

Boc-Glp-Trp-Ala-Val-Gly-OH (13) was prepared from **11** in the same way (m.p. 215° (dec.), $R_f^3 = 0.20$, $[\alpha]_D = -1.2°$ (c = 1, DMF)).

Boc-Glp-Trp-Ala-Val-Gly-His-Leuψ(CH_2NH)Leu-NH_2 (16). Diethylamine (14.6 g, 200 mM) in n-hexane (50 ml) and then, after 10 min stirring at 0°C, a solution of Z-Leu-OCH_3 (21.3 g, 76 mmol) in n-hexane/THF (4:1) were added portionwise to a chilled suspension of LAH (3.6 g, 100 mmol) in anhydrous THF (30 ml), the mixture was stirred 1.5 h at 0°C, diluted dropwise with EtOAc (70 ml) and acidified with 6 M HCl. Evaporation of the organic layer gave an oily aldehyde Z-Leu-H. A portion of it (18.6 g, 71.2 mM), and H-Leu-NH_2·HCl (11.6 g, 70 mM) were dissolved in a mixture of MeOH/AcOH (99:1,150 ml) and a solution of $NaBH_3CN$ (4.8 g, 80 mM) in THF (40 ml) was added dropwise. The reaction mixture was stirred for 30 min at 0°C and for 2 h at ambient temperature and then evaporated. A solution of the

residue in EtOAc was washed with 5% $NaHCO_3$ and water. The solvent was evaporated, and recrystallization of the residue from EtOAc gave Z-Leuψ(CH_2NH)Leu-NH_2 (11 g, 40%, m.p. 120–122°, $[\alpha]_D = -27.8°$ (c = 1, MeOH)).

Z-Leuψ(CH_2NH)Leu-NH_2 (7.2 g, 20 mM) in MeOH (120 ml) was hydrogenated with 10% Pd on charcoal (0.5 g) and in the presence of 6 M HCl/dioxane (5 ml) 2 h, the catalyst was removed by filtration, and the solution was evaporated. Trituration of the residue with a mixture of EtOH/ether (1:5) gave H-Leuψ(CH_2NH)Leu-NH_2 HCl (**14**) (5.1 g, 85%, m.p. 110° (dec.), $[\alpha]_D = +5.5°$ (c = 1, MeOH).

Coupling **14** with Boc-His(Dnp)-OPfp under the usual conditions yielded Boc-His(Dnp) Leuψ(CH_2NH)Leu-NH_2 (**15**) (90%, m.p. 58° (dec.), $[\alpha]_D = +8.7°$ (c = 1, MeOH)). **15** (1.9 g, 3 mM) was deprotected in the ususal way. A mixture of **12** (1.2 g, 1.9 mM), HOPfp (1.2 g, 6.5 mM) and DCC (0.84 g, 4 mM) in DMF (25 ml) was treated with a solution of the free peptide hydrochloride salt in DMF (25ml), the mixture was stirred for 15 min at 0°C, and Et_3N (0.8 ml) was added to adjust pH to 8. After stirring for 30 min at 0°C and for 2 h at ambient temperature the DCU content was removed by filtration and the solvent was evaporated. The residue was dissolved in a DMF/$CHCl_3$ (1:4) mixture and then washed with water. Subsequent to evaporation the residue was triturated with EtOH/ether (1:4) to give Boc-Glp-Trp-Ala-Val-Gly-His(Dnp)-Leuψ(CH_2NH)-Leu-NH_2 (1.6 g, 74%). A solution of the octapeptide (1.6 g, 1.4 mM) in DMF (10 ml) was stirred 1.5 h with 2-mercaptoethanol (2.5 ml, 35 mM), the mixture was diluted with ether and the precipitate was filtered off and washed with EtOH/ether (1:4) mixture to give **16** (0.9 g, 57%, m.p. 155° (dec.), $[\alpha]_D = -30.8°$ (c = 1, MeOH), $R_f^3 = 0.15$).

The following octapeptides were prepared similarly:

- **Boc-Glp-Trp-Ala-Val-Gly-D-Phe-Leu-Leu-NH_2 (17)**, 67%, m.p. > 250° (dec.), $R_f^3 = 0.30$.
- **Boc-Gln-Trp-Ala-Val-Gly-D-Ser(O^{t}Bu)-Leu-Leu-NH_2 (18)**, 60%, m.p. > 230° (dec.), $[\alpha]_D = -23.6°$ (c = 0.5, DMF), $R_f^2 = 0.45$.
- **Boc-Gln-Trp-Ala-Val-Gly-D-Ser(O^{t}Bu)-cycloLeu-Leu-NH-CH_3 (19)**, 77%, m.p. = 240–242°, $[\alpha]_D = +1.8°$ (c = 0.5, DMF), $R_f^2 = 0.45$.

Final Deprotection (General Procedure). *t*-Butyl types protecting groups were removed by treatment with TFA for 20 min at ambient temperature. The crude peptides were obtained by precipitation with anhydrous ether, then purified with RP-HPLC.

Glp-Trp-Ala-Val-Gly-His-Leuψ(CH_2NH)Leu-NH_2 (1). **16** (0.75 g, 0.75 mM) was dissolved in TFA (10 ml) and after 20 min stirring at ambient temperature the mixture was diluted with ether. The precipitate was filtered off and purified by RP-HPLC to give **1**, 26%, $R_f^4 = 0.6$, $R_f^5 = 0.25$, $[\alpha]_D = -26.9°$ (c = 0.5, 10% AcOH), AAA (HCl): Glu 0.96 (1), Gly 0.95 (1), Ala 1.06 (1), Val 1.05 (1), His 0.98 (1), Trp 03 (1), Leuψ(CH_2NH)Leu 0.9 (1).

Glp-Trp-Ala-Val-Gly-D-Phe-Leu-Leu-NH_2 (2), 55%, $R_f^3 = 0.70$, $R_f^4 = 0.60$, $[\alpha]_D = -41.0°$ (c = 0.5, 90% AcOH), AAA (HCl): Glu 0.98 (1), Gly 0.97 (1), Ala 0.99 (1), Val 0.99 (1),Leu 2.03 (2), Phe 1.04 (1), Trp 0.4 (1).

Gln-Trp-Ala-Val-Gly-D-Ser-Leu-Leu-NH_2 (3), 34%, $R_f^3 = 0.1$, $R_f^5 = 0.35$, $[\alpha]_D = -18.5°$ (c = 0.5, water), AAA (MES): Glu 1.00 (1), Gly 1.00 (1), Ser 0.89 (1), Val 0.98 (1), Leu 2.02 (2), Trp 1.13 (1).

Gln-Trp-Ala-Val-Gly-D-Ser-cycloLeu-Leu-NH-CH_3 (4), 36%, $R_f^3 = 0.10$, $R_f^5 = 0.30$, $[\alpha]_D = +5.0°$ (c = 0.5, water) AAA (MES): Glu 1.00 (1), Ser 0.88 (1), Ala 1.00 (1), Val 1.00 (1), Trp 0.94 (1), cycloLeu not quantified.

H-Leu-MPA (20). Et_3N (9.4 ml, 67 mM), then pivaloyl chloride (9.0 ml, 73 mM) and then after 15 min a solution of 2-amino-3-methyl pentane (6.7 g, 74 mM) in THF (20 ml) was added portion-wise to an ice cold solution of Z-Leu-OH (15.9 g, 60 mM) in anhydrous THF (160 ml). The mixture was stirred for 30 min at 0°C and for 3 h at room temperature, then evaporated. A solution of the residue in EtOAc was washed with 1 M HCl, 5% $NaHCO_3$ and water, then the solvent was removed. A solution of the oily residue in MeOH (200 ml) was treated with a stream of hydrogen gas for 2 h in the presence of 10% Pd on charcoal (2.5 g). Then the catalyst was removed by filtration and the solvent evaporated. The residue was chromatographed on a silica gel column, using EtOAc/MeOH/n-hexane (6:1:3) mixture as eluent. The purity of the fractions was checked by TLC. The following two components were obtained: component A **(20)**: 4.2 g (19.5 mM), oil, $R_f = 0.30$ (eluent), $[\alpha]_D = +11.6°$ (c = 1, MeOH), $[\alpha]_D = -20.4°$ (c = 1, EtOAc); component B: 3.1 g (14.5 mM), oil, $R_f = 0.15$ (eluent), $[\alpha]_D = +9.5°$ (c = 1, MeOH), $[\alpha]_D = -9.7°$ (c = 1, EtOAc).

Further resolution failed, so **20** as a mixture of two diastereomers was used for the synthesis of **7**.

Z-D-MePhe-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2 (21) was synthesized stepwise using our method with protected amino acid pentafluorophenyl esters starting from **14**, 75%, m.p. = 120° (dec.), $[\alpha]_D = -6.5°$ (c = 1, MeOH), $R_f^2 = 0.8$.

Z-D-MePhe-D-Trp-Phe-D-Trp-Leu-MPA (22) was prepared from **20**, 56%, m.p. = 109° (dec.), $[\alpha]_D = -22.9°$ (c = 1, MeOH), $R_f^1 = 0.7$.

Boc-D-Tyr-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2 (23), 71%, m.p. = 136° (dec.), $[\alpha]_D = -19.2°$ (c = 1, MeOH), $R_f^1 = 0.55$.

Boc-D-Tyr(Et)-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)-Leu-NH_2 (24), 58%, m.p. = 114°(dec.), $[\alpha]_D = -23.4°$ (c = 1, MeOH), $R_f^1 = 0.70$, was prepared similarly as **21**. Deprotection of **21** and **22** was carried out by catalytic hydrogenolysis in MeOH under the conditions described above. Deprotection of **23** and **24** was performed with HCl/dioxane. Column chromatography on silica gel of the crude substances gave the following peptides:

pHOPA-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2 (5), 42%, m.p. = 132–137°, $[\alpha]_D = 21.9°$ (c = 1, 50% AcOH), $R_f^2 = 0.40$, AAA HCl): Phe 1.08 (1), Leuψ(CH_2NH)Leu 0.92 (1), Trp 1.58 (2).

D-MePhe-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2 (6), 68%, m.p. = 90° (dec.), $[\alpha]_D = -22.8°$ (c = 1, 10% AcOH), $R_f^3 = 0.25$, AAA HCl): Phe 1.10 (1), Leuψ(CH_2NH)Leu 0.98 (1), Trp 1.20 (2), MePhe not quantified.

D-MePhe-D-Trp-Phe-D-Trp-Leu-MPA (7), 78%, m.p. = 153–155°, $[\alpha]_D = -41.0°$ (c = 1, EtOH), $R_f^1 = 0.20$, AAA (MES): Leu 0.90 (1), Phe 1.10 (1), MePhe 1.00 (1), Trp 1.56 (2).

D-Tyr-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2 (8), (44%, m.p. = 105° (dec.), $[\alpha]_D = 17.1°$ (c = 1, MeOH), $R_f^3 = 0.45$, AAA (HCl): Tyr 1.00 (1), Phe 1.10 (1), Leuψ(CH_2NH)Leu 1.12(1),Trp 0.28(2).

D-Tyr(Et)-D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2 (9), (48%, m.p. = 166°(l (dec.), $[\alpha]_D = -35.2°$ (c = 1, MeOH), $R_f^3 = 0.40$, AAA (MES): Tyr 0.95 (1), Phe 1.05 (1), Leuψ(CH_2NH)Leu 1.12 (1), Trp 1.22 (2).

Biological Assays

Cell Culture. Stock culture of NCI H-69 cell line was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2/95% air at 37°C. SK-MES 1 cells were grown in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal calf serum in humidified atmosphere of 5% CO_2/95% air at 37°C.

Proliferation Assay on NCI H-69 Cells. For determination of ^{3}H-thymidine incorporation NCI H-69 cells were washed by centrifugation (1200 rpm, 4°C, 5 min) and incubated in 100 µl of RPMI 1640-HITESA (hydrocortisone 10 nM, insulin 5 mg/ml, transferrin 10 mg/ml, estradiol 10 nM, sodium selenite 30 nM, bovine serum albumin (BSA) 0.1%) medium containing different concentrations of peptides previously dissolved in EtOH. The final EtOH concentration was 2.5% (v/v). After 72 h incubation in humidified atmosphere at 37°C, 37 kBq ^{3}H-thymidine (Amersham International) was added in 10 µl RPMI 1640 and cells were incubated for further 24 h. Cells were solubilized with 10 µl of 20% sodium dodecyl sulfate (SDS) solution, harvested onto Whatman 3 filter paper, washed three times with 5% ice cold trichloroacetic acid and finally with 96% EtOH. The radioactivity in acid precipitable material was measured with LKB Wallac 1211 liquid scintillation counter. Values for ^{3}H-thymidine incorporation were calculated as percentage of the control experiment when cells were treated with vehicle only.

Proliferation Assay on SK-MES 1 Cells. SK-MES 1 cells were suspended by treatment with 0.25% trypsin and in a cell concentration of 5×10^5 cell/ml 100 µl/well of cell suspension was distributed into a 96 well tissue culture plate. After incubation for 48 h the medium was replaced with 100 µl of D-MEM-HITESA containing different amounts of compounds (1–100 µM) dissolved in EtOH. The final EtOH concentration was 2.5% (v/v). ^{3}H-Thymidine incorporation was determined as described previously.

Measurement of Binding Inhibition. For binding experiments Tyr^4-BN was radiolabelled with Na^{125}I according to the chloramine-T method. Swiss 3T3 cells were seeded into 24 well tissue culture plates and were grown to confluence. For determination of binding inhibition confluent and quiescent Swiss 3T3 cells were washed three times with binding buffer (24 mM N-2-hydroxiethylpiperazine-N′-2-ethane-sulfonic acid (HEPES), 0.2% BSA in D-MEM, (pH 7.4) and incubated in 250 µl of binding buffer containing ^{125}I-Tyr^4-BN (1 nM) and various concentrations of peptides at 4°C. Three hours later cells were washed twice with ice cold binding buffer and three times with ice cold phosphate buffered saline (PBS, pH 7.4) and the quantity of cell associated ^{125}I-labelled BN was measured in a γ-counter. Non-specific binding was determined in the presence of 10 µM unlabelled BN. Values are expressed as percentage of specific binding obtained in the presence of vehicle only.

Measurement of Cytotoxicity. 100 µl/well of SK-MES1 cells suspension (10^6 cell/ml) was distributed into a 96-well tissue culture plate. Ten microlitres of **6** in different concentrations was added promptly or after 4 days of culturing when cells were in quiescent

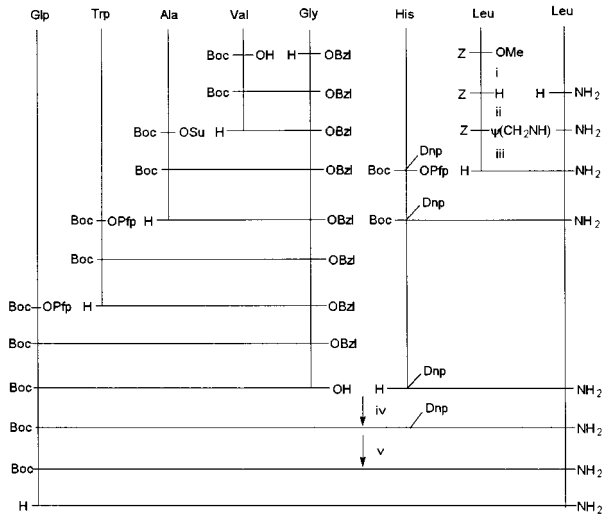

Figure 1 Synthesis of [Glp[7], Leu[13]ψ(CH$_2$NH)Leu[14]] BN[7-14]. (i) LAH/DEA, (ii) NaBH$_3$CN, (iii) H$_2$/Pd-C, (iv) DCC/HOPfp7, (v) 2-mercaptoethanol.

state. After a further 3 day culturing the MTT based cytotoxicity test was performed [33].

RESULTS AND DISCUSSION

Synthesis of the Peptides

BN-related octapeptides were synthesized in solution by coupling the N-protected pentapeptide acids, Boc-Glx-Trp-Ala-Val-Gly-OH to the C-terminal tripeptide amides using the DCC/HOPfp method. The N-protected pentapeptides were synthesized in solution, stepwise, starting from Boc-Val-Gly-OBzl using pentafluorophenyl esters [23,24] (Scheme 1). The octapeptides were deprotected using TFA, then purified by RP-HPLC. SP-related compounds were synthesized using pentafluorophenyl esters, starting from pseudopeptide amide Leuψ(CH$_2$NH)Leu-NH$_2$·2HCl or Leu-MPA. Couplings were performed with 10% excess of active ester. Acylation of secondary amine in methylene amino group was not observed during the synthesis. The methylene-amino bond was formed by reductive alkylation of Leu-NH$_2$·HCl with Z-Leu-H aldehyde prepared according to the LAH/DEA method [25]. Due to the hydrophobic character of the deprotected substances, RP HPLC gave poor recovery, so chromatography on a silica gel column was used for purification of SP-related compounds.

Purity of the final products was checked by analytical HPLC in gradient systems and found to exceed 95% in most cases; but **3** and **4** were obtained merely in 90% purity. In the latter cases the impurity was the corresponding Glp-octapeptide that appeared during the RP-HPLC purification procedure even under mild conditions – in MeCN/ammonium acetate buffer (pH 4.0) gradient system.

For amino acid analysis the samples were hydrolysed using 6 M HCl or 3 M MES. Hydrolysis with MES gave better recovery for Trp and Tyr. The ether bond of Tyr(Et) was stable under these conditions and Tyr(Et) was eluted between Phe and His. Leuψ(CH$_2$NH)Leu pseudodipeptide remained intact in both types of hydrolysis and could be detected in the hydrolysates (Figures 1 and 2).

The signal of the CH$_2$ moiety of the -CH$_2$NH- group appeared in the ^{1}H-NMR spectrum as a multiplet close to 2.4 ppm, whereas the signal of the CH$_2$ moiety showing a triplet multiplicity was found close to 52 ppm in the ^{13}C-NMR spectrum.

Biological Characteristics of the Peptides

BN antagonizing effect of the compounds was measured as their ability to displace radiolabelled BN (^{125}I-Tyr^4-BN) from BN-receptors on Swiss 3T3 mouse fibroblast cells (Figure 3). Surprisingly, new

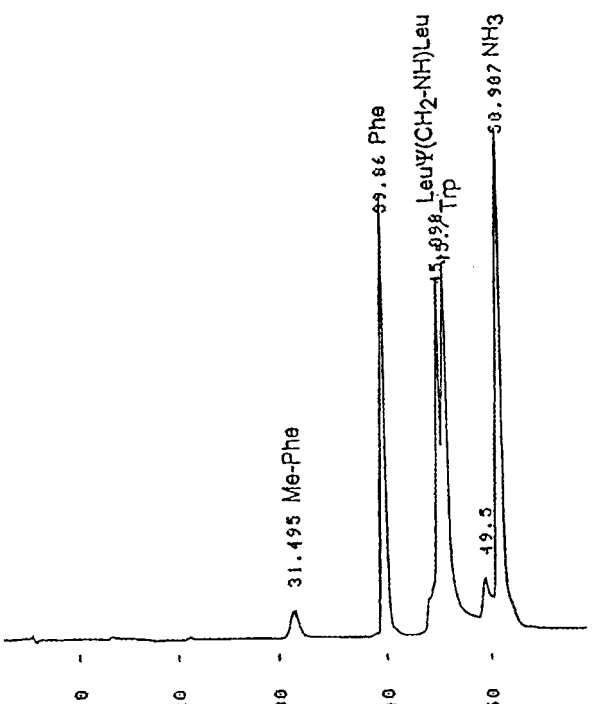

Figure 1 Amino acid analysis of **6** using the standard program. Hydrolysis in 6 N HCl. MePhe of secondary amine represents a small peak at 570 nm.

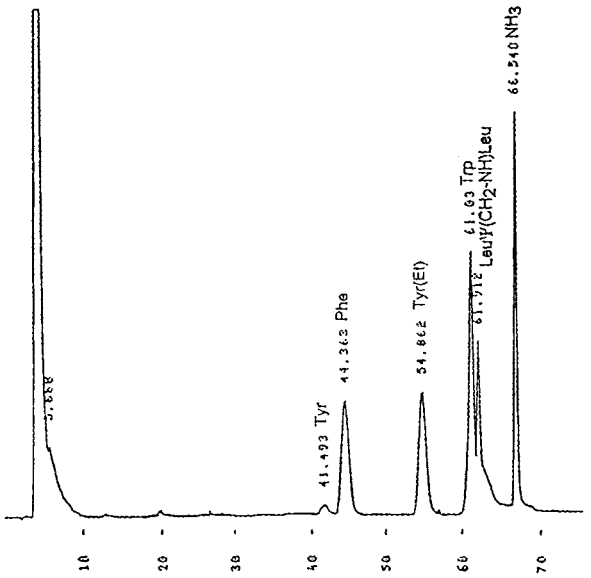

Figure 2 Amino acid analysis of **9**. Hydrolysis in 3 M mercaptoethanesulphonic acid.

synthetic BN-analogues as well as the SP-related compounds lost their affinity for BN-receptors characteristic for the reference peptide **R**. The BN-analogues could not inhibit the binding of 1 nM ^{125}I-Tyr^4-BN to 100 μM. Among the SP-related peptides only **8** was able to inhibit BN-binding, giving a 50% inhibition (IC_{50}) at 45 μM.

The loss of BN-receptor binding affinity with BN[7-14] analogues with reduced peptide bond was already observed on Swiss 3T3 cells [26]. Modification of the BN-octapeptide in position 12 gave the same result. In the case of SP-analogues our observation is in accordance with those results obtained by investigations of other pseudopeptide SP-antagonists – the incorporation of reduced peptide bond between position 10 and 11 of [D-Arg^1-D-Pro^2-D-Trp^{7,9}-Leu^{11}]-SP increased the selectivity of the compound for SP-receptors compared with BN-receptors of pancreatic acini [27].

The effect of the synthesized peptides on proliferation was monitored by determination of ^{3}H-thymidine incorporation of NCI-H69 SCLC cells. While BN-analogues had weak inhibitory effect on ^{3}H-thymidine incorporation of NCI-H69 SCLC cells, SP-related peptides **6** and **7** proved to be more effective than the reference peptide **R** giving an IC_{50} at concentrations of 2, 5 and 10 μM, respectively. Besides the loss of affinity for BN-receptor, in contrast with BN-analogues, our SP-analogues preserved the antiproliferative feature of **R**. Surprisingly, even C-terminal segments, containing two additional amino acids and having a reduced peptide bond between position 10 and 11 were active (Table 2). Furthermore, in contrast to reference peptide **R**, **6** could effectively inhibit the proliferation of SK-MES1 epithelial type of lung carcinoma cells, too (Figure 4).

To investigate whether the antiproliferative effect of these compounds is due to cytotoxicity, we examined the effect of **6** on the respiratory activity of SK-MES1 epithelial type of lung carcinoma cells in proliferating and in quiescent state. **6** effectively inhibited the respiratory activity of cells in proliferation but it had no effect on the intensity of respiration of cells in confluent and quiescent states (Figure 5). This observation suggests that the antiproliferative effect of **6** is not a simple cytotoxic

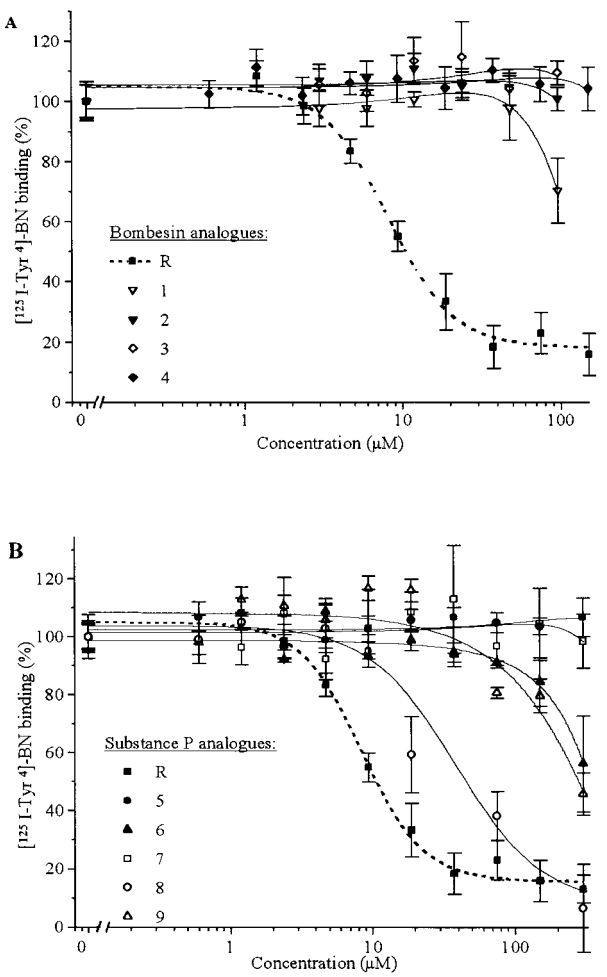

Figure 3 Inhibition of ^{125}I-Tyr^4-BN binding on Swiss 3T3 mouse fibroblast cells. **A**: BN-analogues (**1**–**4**); **B**: SP-analogues (**5**–**9**). Values were calculated as percentage of the control treated with vehicle only. Each point represents the mean ± SD (bars) of three determinations.

Table 2 Effectiveness of Synthetic Peptides on Inhibition of ^{3}H-Thymidine Incorporation of NCI-H69 Human SCLC Cell Line

	IC_{50} (μM)
R	10 ± 2
BN analogues	
1	no effect up to 300
2	84 ± 11
3	108 ± 143
4	124 ± 72
SP analogues	
5	9 ± 2
6	2 ± 0.3
7	5 ± 1
8	9 ± 2
9	8 ± 0
Fragments of **6**	
Leuψ(CH_2NH)Leu-NH_2	42 ± 4
D-Trp-Leuψ(CH_2NH)Leu-NH_2	28 ± 2
Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2	15 ± 1.5
D-Trp-Phe-D-Trp-Leuψ(CH_2NH)Leu-NH_2	12 ± 1.3

effect and new pseudopeptides may act on one or more of the processes participating in cell proliferation.

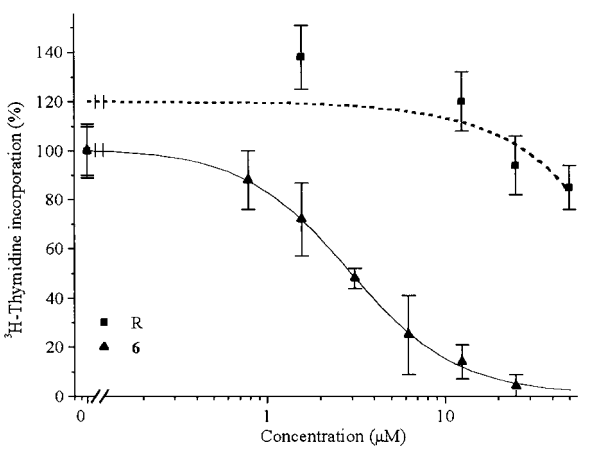

Figure 4 Inhibition of ^{3}H-Thymidine incorporation of SK-MES1 human epithelial type of lung carcinoma cell line. Values were calculated as percentage of the control treated with vehicle only. Each point represents the mean ± SD (bars) of three determinations.

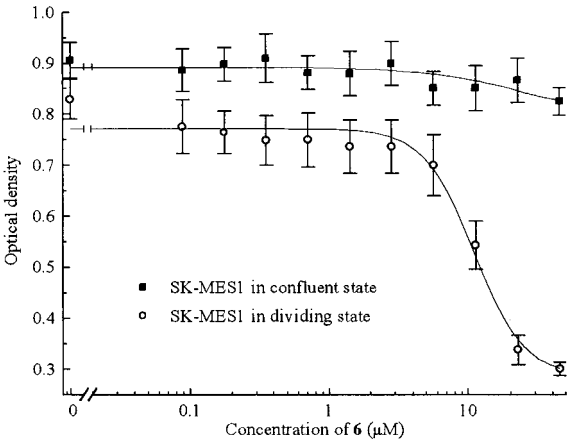

Figure 5 Effect of **6** on the respiratory activity of SK-MES1 human epithelial type of lung carcinoma cell line in proliferative and in quiescent state. Values were calculated as percentage of the control treated with vehicle only. Each point represents the mean ± SD (bars) of eight determinations.

CONCLUSIONS

Though GRP C-terminal octapeptide is known to be a minimal ligand for GRP-receptors on Swiss 3T3 cells [18], and a C-terminal sequence longer than a heptapeptide is required for BN-receptors [31], our modified BN-octapeptides have no affinity for BN-receptors on Swiss 3T3 cells and are weak inhibitors in proliferation assay. On the other hand, our results demonstrate that incorporation of reduced peptide bond into short chain SP-analogues increases their antiproliferative activity on SCLC cells despite the loss of BN-receptor binding affinity.

In our previous study we observed specific binding of radiolabelled **R** on NCI-H69 cells, and affinity of SP, **R** and **6** for this binding site was stronger than that of BN [22]. According to these results recognition of BN-receptor does not appear to be necessary for efficiency of these new short chain SP-analogues in inhibition of proliferation of NCI-H69 SCLC cells. To elucidate whether the above mentioned specific binding site plays any role in the antiproliferative action of these pseudopeptides and whether these compounds, similarly to SP-antagonist [D-Arg^1-D-Phe^5-D-Trp^{7,9}-Leu^{11}]-SP [28,32], are able to induce apoptosis in SCLC cell lines further investigations are needed. In spite of the unanswered questions the effectiveness of these new short chain SP-analogues makes them promising candidates for development of new therapeutic agents in the treatment of SCLC.

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