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Research paper

Antiproliferative activity of synthetic tetrapeptides, analogs of AS-I phytotoxin, towards cancer cell lines

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The *in vitro* chemosensitivity of three cancer cell lines [HT29 (colon), HeLa (cervical) and T47D (breast)] to eight synthetic tetrapeptides, analogs of AS-I toxin, with phytotoxic effect on a series of plants was studied. Mouse fibroblast L929 cell line was also tested for chemosensitivity to these peptides. All cell lines were especially sensitive to Cys–Val–Gly–Glu tetrapeptide with IC_{50} values of 0.18, 0.3 and 0.63 mM for HT29, HeLa and T47D cells, respectively, whereas the IC_{50} value for the L929 cells was higher than 1 mM. Antiproliferative activity was also observed with peptides Tyr–Val–Gly–Glu and His–Val–Gly–Glu with IC_{50} values higher than those obtained for Cys–Val–Gly–Glu. For the rest of the peptides tested the IC_{50} values were found close to or higher than 3 mM. [© 1998 Rapid Science Ltd.]

Key words: Cytotoxic activity, HeLa (cervical), HT29 (colon), mouse fibroblast L929 cells, synthetic tetrapeptides, T47D (breast).

Introduction

Small peptides currently are under development as possible anti-tumor agents. Neuropeptides have been found to be of therapeutic value for small cell lung (SCLC), colorectal and pancreatic cancers.[1–3] [Arg6, Trp7,9, NMePhe8]-Substance P(6–11) represents the first broad spectrum antagonist of a number of neuropeptides shown to act as antitumor agents.[1] Very recently it has also been reported that H-Arg-D-Trp-NMePhe-D-Trp-Leu-Met-NH_2, a neuropeptide growth factor can be used for the treatment of small-cell lung cancer.[2] [DArg1, DTrp5,7,9, Leu11]-Substance P has been identified out of a panel of novel and known Substance P analogs as the most potent inhibitor of signal transduction and growth *in vitro* and *in vivo* in SCLC cells.[3]

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Antiproliferative activity has been reported by various other peptides as well. Dehydrodidemnin B (DDB), a novel depsipeptide isolated from Mediterranean tunicates,[4] was studied on Ehrlich carcinoma growing *in vivo* and in primary cultures. Daily administration of DDB almost doubled the animal life-span and the total number of tumor cells decreased by 70–90%.

The tetrapeptide acetyl-*N*-Ser-Asp-Lys-Pro, known as a negative regulator of hematopoiesis, has been reported as an inhibitor of hematopoietic pluripotent stem cell proliferation.[5] The tetrapeptide sequence is identical to the N-terminus of Thymosin β 4, from which it may possibly be derived. The peptide LUI03793 (NSC D-669356), a new synthetic derivative of dolastin 15, which has been isolated from the mollusk *Dolabella auricularia*,[6] is an antiproliferative compound. Treatment of tumor cell lines with this synthetic compound indicated a block in the G_2/M phase and it was suggested that LU103793 exerts its cytotoxic activity primarily through disruption of microtubule organization. In addition, several synthetic short chain analogs of luteinizing hormone-releasing hormone[7] were found to exert some cytotoxic effect on MCF-7 breast cancer cell lines as well.

Here we examined for antiproliferative activity a series of eight synthetic tetrapeptides, analogs of phytotoxin AS-I, most of which are known to have severe toxic effects on the leaves of a series of young plants.[8] All compounds were tested against three cancer lines and one normal cell line.

Materials and methods

All amino acid derivatives were purchased from Sigma

(Steinheim, Germany). Fmoc-amino acid derivatives and 2-chlorotrityl chloride resin were purchased from Biopharmaceutical Laboratories (Patras, Greece).

Abbreviations

Bu^t, tert butyl; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, *N,N'*-diisopropylethylamine; DMF, *N,N'*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; iPrOH, 2-propanol; RP-FCC reverse phase flash column chromatography; TFE, trifluoroethanol; TLC, thin layer chromatography; Trt, triphenylmethyl.

Synthesis of tetrapeptides

All peptides were synthesized either in solution or by the solid phase method as it follows. Peptides **I** and **II** were prepared as reported.[7] Peptides **III–V** were obtained according to the same procedure[8] as peptides **I** and **II**. Peptides **VI–VIII** were prepared as follows.

Esterification of Fmoc-Glu(Bu^t)OH with the resin.[9] To 1 g of the 2-chlorotrityl chloride resin (1.6 mmol active chloride) in 15 ml DCM Fmoc-Glu(Bu^t)OH (1 mmol, 424.52 mg) and DIEA (4 mmol, 0.7 ml) were added, and the reaction mixture was stirred for 25 min at room temperature. Then 2 ml mixture of MeOH:DIEA (9:1 v/v) was added in the reaction vessel in order to destroy excess active chloride on the resin and stirring was continued for an additional 15 min. The resin was filtered, washed with $2 \times$ DCM:MeOH:DIEA] (92:5:3 v/v/v), $3 \times$ DCM, $2 \times$ iPrOH and $2 \times$ Et2O, and dried *in vacuo* for 24 h. Yield 1.31 g (80%). Substitution 0.67 mmol amino acid/g resin according to the ninhydrin method.[10]

Cleavage of the Fmoc group.[11] The cleavage of the Fmoc group was performed with 20% piperidine in DMF for 5 min followed by a second treatment with the same solution for 25 min.

Activation and coupling procedure.[11] Fmoc-amino acid (2.01 mmol, 3-fold molar excess of the resin substitution) and HOBt (0.5×2.01 mmol) were dissolved in DMF (3 ml) and cooled at 0 C. DIC (0.1×2.01 mmol) was then added, and the resulting solution was stirred for 15 min at 0 C and for a further 10 min at 20 C. The precipitated DIU was filtered, washed with DMF and the combined filtrates containing the benzotriazolylester of the Fmoc-amino acid were transferred to the solid phase reactor. After shaking for 24 h the washing procedure was applied. Completion of the acylation reactions was monitored by Keiser's test. After incorporation of each amino acid, resin samples were treated with the solvent system DCM:TFE:AcOH (7:2:1, v/v/v) for 2 min and the intermediate peptide that was cleaved from the resin was checked for purity by TLC in the solvent system toluene:AcOH:MeOH (7:1:1.5, v/v/v) or AcCN:H_2O (5:1, v/v). A double coupling was performed in a few cases of slightly positive Keiser's test.

Washing procedure.[11] After each coupling or removal of the Fmoc group the resin was washed with 10 ml/g resin of the following solvents: $3 \times$ DCM, $2 \times$ iPrOH, $2 \times$ DMF, $2 \times$ iPrOH and $1 \times Et_2O$. Additional washings with DCM (3×15 ml/g resin) were performed before cleavage of the desired protected peptide from the resin.

Cleavage of the protected peptides from 2-chlorotrityl resin.[11] The peptide-resin ester (1 g) was treated with a mixture (15 ml) of DCM:TEE:AcOH (7:2:1, v/v/v) (solvent A) for 2 h or DCM:TFE (8:2, v/v) (solvent B) for 30 min at room temperature. The resin was separated by filtration and washed three times with the used splitting mixture. The combined filtrates were concentrated *in vacuo*, and the crude peptide was precipitated by addition of ether, collected by filtration, washed with ether on the filter and dried under vacuum.

Fmoc-HN-Tyr(Bu^t)-Val-Gly-Glu(Bu^t)-O-Resin **(VIIa)**. This protected peptide-resin ester was synthesized on a sample of H_2N-Glu(Bu^t)-2-chlorotrityl resin ester (1.5 g, 1 mmol) following the above described general procedure. Treatment of **VIIa** with solvent A yielded 530 mg (96%) of crude H_2N-Tyr(Bu^t)-Val-Gly-Glu(Bu^t)-COOH (**VIIb**). The crude peptide was subjected to RP-FCC using AcCN:H_2O (5:1) as eluent. The column used (40×2.3 cm) was packed using a slurry (45 g) of RP-silica gel in AcCN:H_2O (5:1) and applying a gentle pressure on the top of the column. After application of the sample (120 mg) dissolved in the minimum amount of mixture AcCN:H_2O (5:1 v/v), the column was eluted and fractions of 1 ml were collected and checked by TLC. The fractions containing pure **VIIb** fractions were combined, concentrated *in vacuo* and lyophilized. Yield 110 mg (90%), m.p. 221–223 C, $[\alpha]_D^{25}$+16.7, Rf_1=0.40, Rf_2=0.46 and Rf_3=0.62.

HCl·H_2N-Tyr-Val-Gly-Glu-OH (**VIIc**). The side protected tetrapeptide VIIb (200 mg, 0.34 mmol) was treated with 1.2 M HCl:CH_3COOH containing 10% anisole and allowed at room temperature 2 h. The solution was evaporated at reduced pressure and the residue was treated with anhydrous diethyl-ether to provide the corresponding tetrapeptide hydrochloride **VIIc** as a white powder. Yield 150 mg (95%), m.p. 240 C [decomp., (soft 152 C)], $[\alpha]_D^{25}$+13.7, (c1, AcOH), Rf_1=0.17, Rf_2=0.28 and Rf_3=0.38.

Fmoc-HN-Cys(Trt)-Val-Gly-Glu(Bu^t)-O-Resin (**VIa**). This protected peptide-resin ester was synthesized starting from H_2N-Glu(Bu^t)-2-chlorotrityl resin ester (1.5 g, 1 mmol). Treatment of **VIa** with solvent A yielded 650 mg (92%) of crude H_2N-Cys(Trt)-Val-Gly-Glu(Bu^t)-COOH (2b). This was purified by repeated precipitations from DMF:H_2O. Yield 89%, m.p. 169-171 C, $[\alpha]_D^{25}$+2.1, (c1, DMF), Rf_1=0.66, Rf_2=0.60 and Rf_3=0.67.

HCl·H_2N-Cys-Val-Gly-Glu-OH (**VIc**). A portion of peptide VIb was treated as described for peptide **VIIb**. Yield 90%, m.p. 117-119 C (94 C soft), $[\alpha]_D^{25}$+11, (c1, AcOH), Rf_1=0.23, Rf_2=0.13 and Rf_3=0.18.

Fmoc-HN-His(Trt)-Val-Gly-Glu(Bu^t)-O-Resin (**VIIIa**). This protected peptide-resin ester was synthesized starting from H_2N-Glu(Bu^t)-2chlorotrityl resin ester (1.5 g, 1 mmol). Treatment of **VIIIa** with solvent B yielded 670 mg (90%) of crude H_2N-His(Trt)-Val-Gly-Glu(Bu^t)-COOH (**VIIIb**). It was purified by repeated precipitations from DMF:H_2O. Yield 85%, m.p. 161-165 C, $[\alpha]_D^{25}$ −19.7, (c1, DMF), Rf_1=0.57, Rf_2=0.49 and Rf_3=0.65.

HCl·H_2N-His-Val-Gly-Glu-OH (**VIIId**). A portion of peptide **VIIIb** treated with TFA yielded the peptide TFA.H_2N-His-Val-Gly-Glu-OH (**VIIIc**). This was purified by RP-FCC as described for **VIIb**, using H_2O:MeOH (9:1, v/v) as eluent (the fractions containing pure **VIIIc** were combined, concentrated *in vacuo* and lyophilized twice by 1.2 M HCl:AcOH, and yielded the correspondent hydrochloride salt). Yield 90%, m.p. 143-145 C (117 C soft), $[\alpha]_D^{25}$ −14.2, (c1, AcOH), Rf_1=0.12, Rf_2=0.10 and Rf_3=0.20.

TLC solvent systems. 1, AcCN:H_2O 3:1 (v/v); 2, BuOH:CH_3COOH:H_2O 4:1:1 (v/v/v); 3, BuOH:CH_3.COOH:Pyr/H_2O 4:1:1:2 (v/v/v/v).

Cell lines and culture conditions

Cell lines used were HT29 (human colon cancer), HeLa (human cervical cancer), T47D (human breast cancer) and L929 (mouse fibroblasts).

Adherent cells at the logarithmic growth phase were plated (100 μl per well) in 96-well flat-bottom microplates at densities of 5000-10000 cells per well and left at 37 C for 24 or 48 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin and streptomycin.

Bioassay

Cytotoxicity after 48 or 72 h evaluated by an optimized sulforhodamine B (SRB) assay.[12] Results were expressed as percentage of untreated control cells and dose-response curves were plotted. The potency of the experimental agents was expressed by the IC_{50} values (50% inhibitory concentration). Cytotoxic activities were expressed as IC_{50} values (concentration in mM which gives 50% of inhibition). Experiments were carried out in triplicate.

Results and discussion

All peptides were fully deprotected and used in the assays as free bases adjusted to the same pH 7.0 in phosphate-buffered saline. Peptides were tested against three cancer cell lines HeLa, HT29 and T47D,

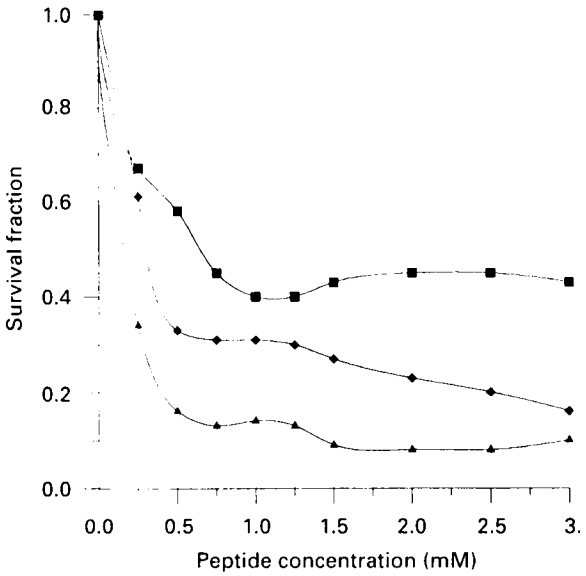

Figure 1. Chemosensitivity of three human cancer cell lines towards peptide **VI**. Cell inoculum density 10000 cells/well for HT29 and T47D cell lines, respectively, and 5000 cells/well for HeLa cell line. Plating period 48 h, exposure period 48 h. HeLa (◆), HT29 (▲) and T47D (■).

and one normal cell line of mouse fibroblast cells. The strongest antiproliferative activity was expressed by peptide **VI** (Figure 1). The estimated IC_{50} values were 0.18, 0.33 and 0.63 mM for HT29, HeLa and T47D cell lines, respectively. Figure 1 shows that HT29 cells were the most sensitive to this peptide. Peptide **VII** (Figure 2A) exhibited less inhibitory activity than peptide **VI** in all cell lines used. After 48 h treatment with the peptide **VI**, IC_{50} values remained constantly above 2.5 mM. The antiproliferative activity presented by peptide **VIII** (Figure 2B) was also very small, at least in HeLa and T47D cells. Peptides **I-IV** were found to be the least active with IC_{50} values higher than 2.5 mM. Figure 3 shows the chemosensitivity of peptides **I** and **IV** towards HeLa cells.

To test the cytotoxicity of the peptides to normal cells, the L929 mouse fibroblast cell line was used. Figure 4 shows the chemosensitivity of the most potent peptide **VI**. This potency seems to be lower in the case of L929 cells, with an IC_{50} value higher than 1 mM.

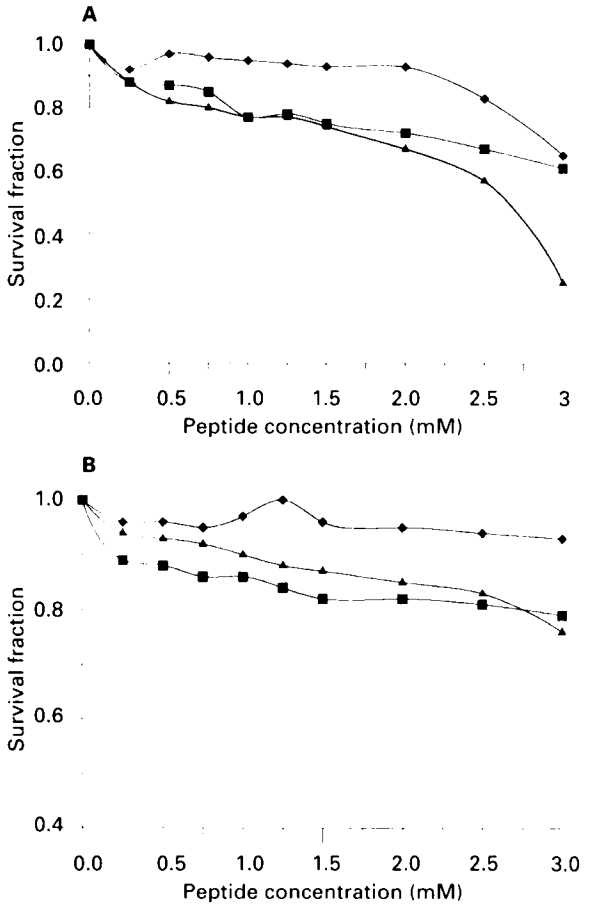

Figure 2. Chemosensitivity of the three human cancer cell lines towards peptide **VII** and **VIII**. Experimental conditions as in Figure 1.

Table 1 summarizes the results from the examination of these eight peptides on all tested cell lines. Only peptide **VI** shows significant antiproliferative activity on these cell lines.

We conclude from our results that peptides from Table 1 can be divided in three categories. The first category, peptides **I-III**, which are tetrapeptide analogs with the same amino acids in different order, show the least antiproliferative activity (IC_{50} values for all cell lines used higher than 2.5 mM). The second category, peptides **IV-VI**, have serine, the N-terminal amino acid of tetrapeptide I (synthetic AS-I toxin), substituted by other aliphatic amino acids and specifically by homoserine, threonine or cysteine. These three peptides express better antiproliferative

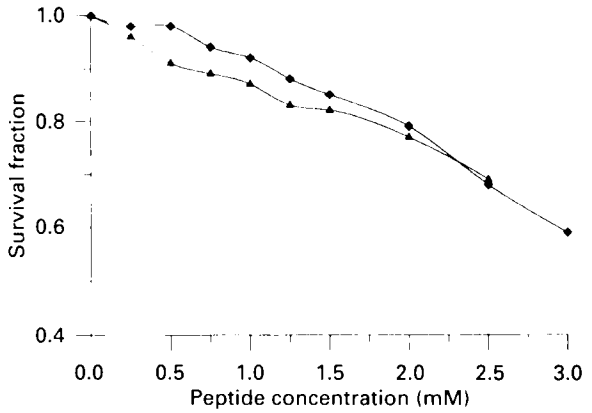

Figure 3. Chemosensitivity of HeLa cells against peptides **I** (▲) and **IV** (◆). Cell inoculum density 5000 cells/well, plating period 48 h, exposure period 48 h.

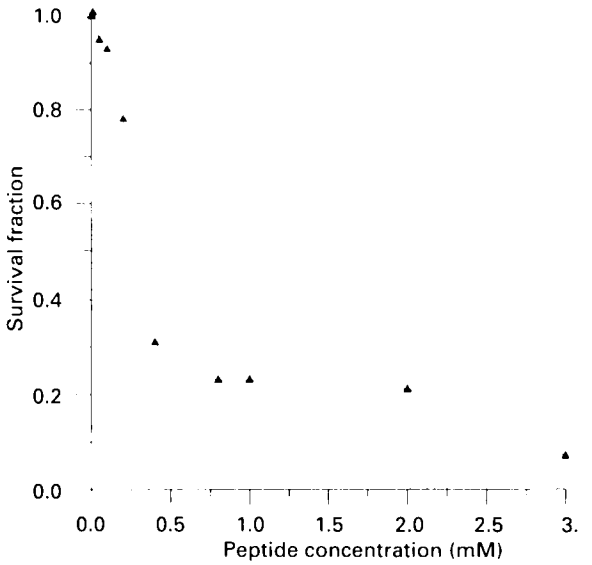

Figure 4. Chemosensitivity of L929 (mouse fibroblast) cell line towards peptides **VI**.

Table 1. IC_{50} values (in mM) of all tested peptides after 48 or 72 h treatment against a panel of cultured cell lines

Compound	Cell lines			
	HT29	HeLa	T47D	L929
Ser–Val–Gly–Glu (**I**)	>2.5	>2.5	>2.5	NT
Ser–Val–Glu–Gly (**II**)	>2.0	>2.5	>2.5	NT
Val–Gly–Ser–Glu (**III**)	>2.5	>2.5	>2.5	NT
Hse–Val–Gly–Glu (**IV**)	>2.0	>2.5	>2.5	0.3
Thr–Val–Gly–Glu (**V**)	<2.5	>2.5	>3.0	NT
Cys–Val–Gly–Glu (**VI**)	0.18	0.33	0.63	>1
Tyr–Val–Gly–Glu (**VII**)	1.26 (72 h) >2.5 (48 h)	>2.5	>2.5	0.5
His–Val–Gly–Glu (**VIII**)	~3	>3	>3	0.4

NT, not tested.

activities than the previous three peptides (**I–III**), with peptide **VI** showing the highest antiproliferative activity towards all cell lines tested here. The third category, peptides **VII** and **VIII**, where serine is substituted by tyrosine and histidine, respectively, shows similar antiproliferative activity as the analogs of the first and second category, with the exception of the cysteine analog (peptide **VI**).

Conclusions

The results obtained in this pilot study show that the C-terminal moiety -Val-Gly-Glu is crucial for the appearance of antiproliferative activity, whereas substitution of serine by cysteine (thio amino acid) gives analogs with better cytotoxic activity in terms of IC_{50} values. It is obvious that more general and accurate conclusions will have to be made by synthesizing more analogs and investigating their biological effect on cancer cell lines. In addition we plan to administer the potent peptide **VI** with known anticancer agents, such as naphthoquinone derivatives, cisplatin, etc., to see if a synergistic effect can be achieved.

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