Journal of Peptide Science
J. Peptide Sci. **7**: 284–293 (2001)
DOI: 10.1002/psc.322

Synthesis and Biological Evaluation of Analogues of the Tetrapeptide *N*-Acetyl-Ser-Asp-Lys-Pro (AcSDKP), an Inhibitor of Primitive Haematopoietic Cell Proliferation

JOSIANE THIERRY[a], CATHERINE GRILLON[a,*], SANDRINE GAUDRON[a], PIERRE POTIER[a], ANDREW RICHES[b] and JOANNA WDZIECZAK-BAKALA[a]

[a] Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France
[b] School of Biology and Medical Sciences, University of St Andrews, Scotland, UK

Received 20 December 2000
Accepted 15 January 2001

Abstract: The tetrapeptide *N*-Acetyl-Ser-Asp-Lys-Pro (AcSDKP), an inhibitor of haematopoietic stem cell proliferation, reduces *in vivo* and *in vitro* the damage to the stem cell compartment resulting from treatment with chemotherapeutic agents or ionizing radiations. In order to provide new molecules likely to improve the myeloprotection displayed by this tetrapeptide, we have prepared a set of analogues of AcSDKP. These compounds are derived from the parent peptide by substitution or modification of the *N*- or of the *C*-terminus, or substitution of side chains. We report here that almost all investigated analogues retain the antiproliferative activity reducing *in vitro* the proportion of murine Colony-Forming Units Granulocyte/Macrophage (CFU-GM) in S-phase and inhibiting the entry into cycle of High Proliferative Potential Colony-Forming Cells (HPP-CFC). This shows that the polar groups of Ser, Asp or Lys are critical for the expression of biological activity, but that the modification of the *N*- or *C*-terminus mostly yielded compounds still retaining antiproliferative activity and devoid of toxicity. The efficacy of AcSDKP analogues in preventing *in vitro* the primitive haematopoietic cells from entering into cycle makes these molecules new candidates for further *in vivo* investigations. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: AcSDKP; AcSDKP analogues; CFU-GM; HPP-CFC; proliferation inhibitor; pseudopeptides

INTRODUCTION

A complex and tightly regulated balance between the opposing effects of stimulatory and inhibitory growth signals regulates the cell cycle status of haematopoietic progenitors and stem cells. The tetrapeptide *N*-Acetyl-Ser-Asp-Lys-Pro (AcSDKP) originally isolated from foetal calf bone marrow has been identified as a physiological negative regulator of primitive haematopoietic cell proliferation [1]. It inhibits selectively *in vitro*, as well as *in vivo*, the entry into DNA synthesis of haematopoietic cells [1,2], protecting them from the toxicity of anticancer drugs [3–5].

The use of such haematopoietic growth inhibitors has been proposed in order to allow higher doses of chemotherapeutic agents, resulting, in turn, in increased tumour responses or cures. Accordingly, it has been reported that animals treated with lethal doses of cytotoxic agents showed significantly higher haematopoietic recovery when AcSDKP was given at an appropriate time relative to chemotherapy administration [6,7]. Promising preliminary

Abbreviations: ACE, angiotensin converting enzyme; Ant, anthranilyl; Coum, 2-(7-dimethylamino-2-oxo-2H-chromen-4-yl)-acetyl; IBCF, isobutyl chloroformate; PfpOH, pentafluorophenol; Suc, succinyl.

* Correspondence to: ICSN, CNRS, 91198 Gif-sur-Yvette Cedex, France; e-mail: Catherine.Grillon@icsn.cnrs-gif.fr

sults of the first clinical trials, which demonstrated a reduced neutropenia in cancer patients receiving AcSDKP and chemotherapeutic agents, have already been reported [8].

In our search for a better understanding of the relation between structure and activity, we have designed and studied structural analogues of the parent peptide, especially peptides with modified side chains [9] and peptide backbone in which the amide bond was reduced to methylene amino bond [10,11]. The current study was undertaken to further develop the structure-activity study of the peptide by exploring the *N*- or *C*-modification or replacement of some of the side chains (Figure 1).

The use of peptides as drugs is hampered by the fact that they have an extremely short half-life owing to the activity of degradative enzymes, and thus, this limits their efficacy and usefulness. This study is aimed at providing molecules with better biological properties.

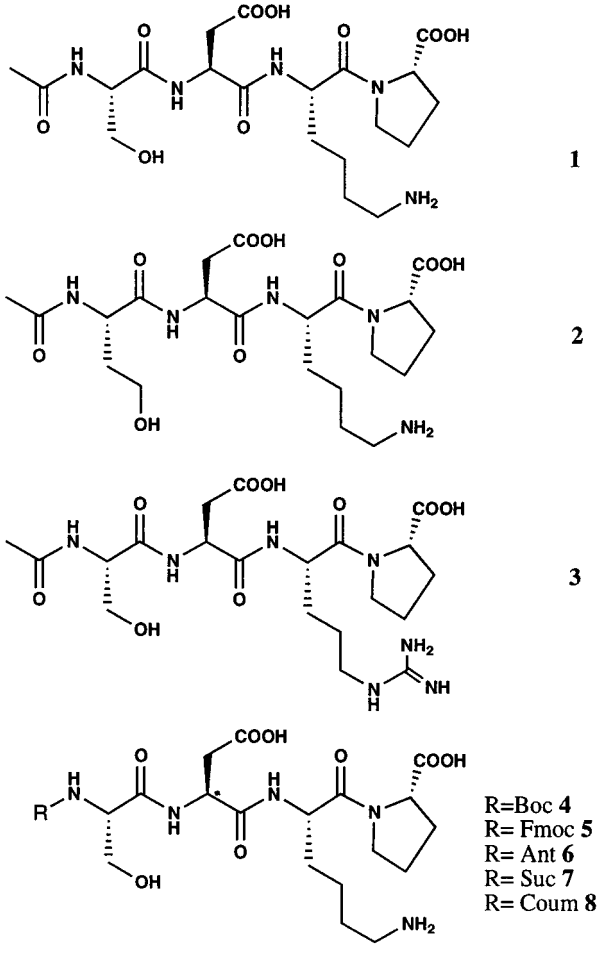

Figure 1 Analogue formulae.

The *in vitro* biological study of these analogues was undertaken with two assays using two different populations of haematopoietic cells, such as the murine high proliferative potential colony-forming cells (HPP-CFC) and colony-forming units granulocyte/macrophage (CFU-GM) to select the best candidates for further *in vitro* and *in vivo* investigations.

MATERIALS AND METHODS

The synthetic AcSDKP was kindly provided by IPSEN-Biotech (Paris, France). AcS-D-DKP, where L-Asp was replaced by its enantiomer, was synthesized in our laboratory, as previously described [9]. The synthesis of the three pseudopeptides (AcSΨDKP, AcSDΨKP and AcSDKΨP), as well as those of the *C*-modified analogues (AcSDKPyr and AcSDKP-NH_2) have been reported previously [10], and the biological activities of four of them have been studied before [11].

Chemistry

All protected amino acids were purchased from Bachem AG or Novabiochem and are of L-configuration, except where otherwise stated. Thin-layer chromatography (TLC) were run on silica gel precoated plates (60 F-254, Merck). Protected peptides were purified by column chromatography on Merck silica gel 60 (40–63 μm), and characterized by their FAB or LSI mass spectrum. Mass spectra were obtained using a Kratos MS 80 mass spectrometer, using a xenon FAB gun with glycerol, thioglycerol, or nitrobenzyl alcohol as a matrix. High performance liquid chromatography (HPLC) purifications were performed on a C-18 Beckmann Ultrasphere column (5 μm, 10 × 250 mm), using either a gradient or an isocratic elution with a mixture of acetonitrile and water containing 0.1% TFA at a flow rate of 3 mL min^{-1}. Elution was monitored by recording absorbance at 215 nm. The fractions were pooled, concentrated and lyophilized. Pure peptides were characterized by their high resolution LSI mass spectrum recorded on a Fisons (VG ZabSpec-T) mass spectrometer. HPLC analysis for purity control was performed on a Waters Nova-Pak column C-18 (4 μm particle size, 3.9 × 150 mm), with a solvent system consisting of a binary system of water and acetonitrile containing 0.1% TFA at a 1 mL min^{-1} flow rate, with monitoring at 215 nm. The solvent programmes involved the following linear gradients: (1) 0–50% acetonitrile over 50 min,

(2) 0–80% acetonitrile over 40 min. k' values are reported in these two solvent systems.

Typical Procedures

Acidolysis. The protected peptide was dissolved in a 1/1 mixture of DCM and TFA (10–30 equivalent). After stirring for 1–2 h at room temperature for deprotection of Boc, and overnight at 4°C for final deprotection when Ser(OBu^t) needs to be cleaved, the solvents were removed in vacuum after dilution with DCM. The trifluoracetate salt was generally triturated with a mixture of dry ether and petroleum ether (1/3, v/v) and dried under vacuum for several hours.

Hydrogenolysis. The protected peptide was dissolved in either ethanol or methanol/water (9/1) for final deprotection, except for the Arg-3 analogue, where acetic acid had to be used to get the fully protected peptide into solution. To this solution was added 10% Pd/C (about 20% by weight). The reaction was stirred under an atmosphere of hydrogen at atmospheric pressure and room temperature for 5 h in the case of Z deprotection or overnight for final deprotection. The catalyst was removed by filtration through a pad of Celite and the filtrate concentrated under reduced pressure.

Coupling reactions. Mixed anhydride method. The *N*-protected amino acid was dissolved in tetrahydrofuran (5 mL/mmol). To this solution, cooled to -15°C, was added NMM (1 equivalent) and IBCF (1 equivalent). After 5 min, the temperature was lowered to -20°C, and the cooled solution of *C*-protected amino acid or peptide (1.1 equivalent), dissolved in a minimum amount of DMF or DCM, was added along with NMM (1.1 equivalent) in the case of the trifluoracetate salt. After 1 h at -10°C, the temperature was allowed to warm up to room temperature. Completion of the reaction was monitored by TLC. The reaction mixture was then concentrated under reduced pressure, the residue was taken up with ethyl acetate and 5% citric acid. The organic layer was washed with water, 5% sodium bicarbonate, water, brine and dried over sodium sulphate. Evaporation of the solvent yielded the crude product, which was either purified through column chromatography, or used in the next step when homogeneous by TLC.

Acetylation reaction. The *N*-deprotected peptide or its trifluoroacetate salt was dissolved in DMF (2 mL/mmol). Acetylimidazole (1.1 equivalent) was added to the solution, cooled on an ice bath. Triethylamine (1.1 equivalent) was added in the case of the salt. The reaction mixture was stirred at room temperature. Completion of the reaction was monitored by TLC. The reaction mixture was evaporated down, and the residue was taken up with 0.5 N HCl and ethyl acetate. The organic layer was washed with water until the pH was 7, with brine then was dried over sodium sulphate. Evaporation of the solvent yielded the crude product, which was purified through column chromatography before the last deprotection step.

Biology

Bone marrow from normal 9–11 week-old CBA/J mice was used as the source of haematopoietic bone marrow cells (BMC). BMC were obtained by flushing the content of tibias and femurs with Dulbecco's medium or α-medium (Gibco-Life Technology, Cergy Pontoise, France).

CFU-GM assay. The assay was carried out according to slightly modified procedures described previously by Wierenga and Konings [12]. BMC were incubated in α-medium (3×10^6 cells mL^{-1}) for 7 h at 37°C in a 5% CO_2 humidified atmosphere, either with culture medium as control or with AcSDKP or analogues (2×10^{-9} M final concentration). AcS-D-DKP was used systematically as a negative control. Hydroxyurea (HU, 200 μg/mL final concentration) or culture medium was then added to a duplicate culture for a 1-h incubation. Cells were washed twice and plated out for the colony-forming assay. BMC (4×10^4) in 1 mL of complete medium (α-medium containing 30% foetal calf serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin) supplemented with 0.8% methylcellulose, 10% pokeweed mitogen-stimulated spleen-conditioned medium as source of growth factors were plated in 35-mm polystyrene culture dishes. Quadruplicate cultures were incubated for 8 days at 37°C in a fully humidified atmosphere with 5% CO_2. The colonies were counted using an inverted microscope. Groups of more than 50 cells were counted as colonies. For each condition, the proportion of cells in S-phase was estimated from the difference in the number of colonies between cells treated with HU and those treated without it and the percentage of inhibition in the presence of AcSDKP, or the analogue was calculated. The whole experiment was repeated three times. The effect of the addition of AcSDKP or analogues versus control was evaluated through an analysis of variance performed on the proportion of cells in S-phase, taking into account the matching of data arising from the same

experiment, the intra- and inter-experiment variations of the data and the number of comparisons performed through Dunnett's *t*-test. The potential cytotoxicity of the analogues on CFU-GM was evaluated by comparing the number of CFU-GM derived from BMC incubated with or without the molecule.

HPP-CFC assay. This assay was carried out according to the procedure described by Robinson *et al.* [13]. BMC were incubated in Dulbecco's medium (5×10^6 cells mL^{-1}) for 2 h at 37°C, either with culture medium as control or with 3-h conditioned medium of bone marrow cells from mice sublethally irradiated 7 days before as a crude source of stimulators. AcSDKP or analogues (2×10^{-9} M final) or medium, as stimulation control, was added at the beginning of the incubation. Cytosine arabinoside (AraC, 50 $\mu g\,mL^{-1}$ final concentration) or culture medium was then added for a 1-h incubation. Cells were washed twice prior to HPP-CFC assay. Two mL of complete medium (Dulbecco's medium containing, 20% horse serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 $\mu g\,mL^{-1}$ streptomycin) supplemented with 10% conditioned medium from the WEHI 3B myelomonocytic leukemic cell line, 10% conditioned medium from L929 fibroblast cell line as source of growth factors, and 0.5% melted agar (Bactoagar, Difco, Detroit, Michigan, USA) were distributed into 55 mm diameter non-tissue culture grade plastic petri-dishes as the underlayer. BMC (6×10^4) in 2 mL of complete medium, supplemented with 0.3% melted agar, were then plated over the prepared underlayers. Quadruplicate cultures were incubated for 14 days at 37°C in a fully humidified atmosphere, with 5% CO_2. Twelve hours before the end of the culture, cells were stained by adding 1 mL of a 1 mg mL^{-1} 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT, Sigma, Saint Quentin Fallavier, France) solution in saline. HPP-CFC macroscopic colonies defined as those in excess of 1 mm were scored. For each condition, the proportion of cells in S-phase was estimated from the difference in the number of colonies between cells treated with AraC, and those treated without it and the percentage of inhibition in the presence of AcSDKP or the analogue was calculated. The whole experiment was repeated three times. The effect of stimulating medium versus control and of the addition of AcSDKP or analogues versus stimulating medium alone, was evaluated through an analysis of variance performed on the proportion of cells in S-phase, using Dunnett's *t*-test. The potential cytotoxicity of the analogues on HPP-CFC was evaluated from independent experiments by comparing the number of HPP-CFC derived from BMC incubated with or without the molecule, but in absence of conditioned medium from regenerating BMC.

RESULTS

Synthesis of Peptides (Figure 2)

All the syntheses have been realized step by step using mainly the mixed anhydride methodology [14], except for the dimers, where two tetrapeptide fragments were assembled. α-amino groups were protected with Boc, the side chain groups and the *C*-terminus with benzyl alcohol-derived protecting groups in the case of **2**, **3**, **4** (Figure 2(A)); α-amino groups were protected with Fmoc; side chain groups and *C*-terminal carboxy groups were protected with *tert*-butyl alcohol-derived protecting groups in the case of **5**, **6**, **7** (Figure 2(B)).

The homoserine was introduced as the benzyl ether derivative [15]. The acetylation of the *N*-teminal amino group was efficiently and selectively performed, as already described [10] by treatment with acetylimidazole in DMF. Final deprotection through hydrogenolysis with 10% Pd/C in MeOH yielded **2**. A stepwise synthesis of the Arg-3 analogue was realized, with its side chain being protected by nitration, the final hydrogenolysis step was run in neat acetic acid as the protected peptide was poorly soluble in alcohol.

The synthesis of CoumSDKP (2-(7-dimethylamino-2-oxo-2H-chromen-4-yl)-Acetyl-SDKP) **8** has been described in detail in [16]. The incorporation of the anthranilyl residue was accomplished by treating the partially protected tetrapeptide with its hydroxybenzotriazolylester as described in [17] (Figure 2(B)). The dimer head-to-tail **9** resulted from the coupling of the tetrapeptide **III** with the compound **II** bearing Fmoc protection on the *N*-terminus, benzyl alcohol-derived protecting groups on the side chains, the *C*-terminus, of which being activated through its pentafluorophenylester (Figure 2(C)). This pentafluorophenylester was prepared by treatment with a complex obtained from DCCI and PfpOH [18]. The dimer head-to-head **10** results from the DCCI-coupling of the tetrapeptide **III** with the tetrapeptide **V**, the protected precursor of **7**, followed by acidolysis of the compound by TFA (Figure 2(D)).

Crude peptides were purified by preparative reverse phase HPLC using mixtures of water and

acetonitrile containing 0.1% TFA as an eluant. The purified peptides were characterized by their HR-FAB mass spectrum, and their purity were checked by HPLC analysis in two systems of solvents (Table 1). It is worth noting that most of the modifications yielded compounds with similar retention times, that is to say that they have the same polarity as AcSDKP, but the introduction of a bulky lipophilic group such as Boc, Fmoc and Coum gave compounds (**4**, **5** and **8**) with quite a different retention time.

Biological Activity

As previously reported, AcSDKP inhibits *in vitro*, at nanomolar concentrations, the entry into S-phase of cell cycle of two types of murine primitive haematopoietic cells: CFU-GM and HPP-CFC [12,13]. Thus, the biological activity of AcSDKP analogues was investigated by measuring their effect on the proliferative state of these cells. In order to make a more complete discussion, we have compiled in Tables 2 and 3 the activities of the analogues described here as well as those of the analogues reported in [11]. These molecules were designed to be resistant to ACE hydrolysis and differ from the parent peptide by exhibiting modifications of the backbone (methylene amino bonds: AcSΨDKP, AcSDΨKP and AcSDKΨP), or amidation of the *C*-terminus (AcSDKP-NH_2). We also included the activity of the analogue with D-Asp-2, which was always used as a negative control [9,11,19].

Both *C*-terminus modified peptides, AcSDKPyr and AcSDKP-NH_2, significantly reduced the proportion of CFU-GM in S-phase (56 and 61%) (Table 2), and were also found to be potent in the inhibition of HPP-CFC entry into cell cycle (57 and 51%) (Table 3).

All the *N*-terminus substituted peptides, BocSDKP, FmocSDKP, AntSDKP, SucSDKP and CoumSDKP were found to be active in both experimental assays. A significative decrease of CFU-GM proportion in S phase, similar to that measured for AcSDKP, and equal to 56, 53, 66 and 68% was reported for BocSDKP, FmocSDKP, AntSDKP and SucSDKP (Table 2). Similarly, 51, 59, 52 and 53% of inhibition of HPP-CFC entry into S phase was observed respectively for BocSDKP, FmocSDKP, AntSDKP and SucSDKP (Table 3). However, a slight loss of efficacy was observed for the compound in which the acetyl has been substituted for the fluorescent coumaryl residue (CoumSDKP) (42% for CFU-GM and 37% for HPP-CFC compared with 69% for AcSDKP in both assays).

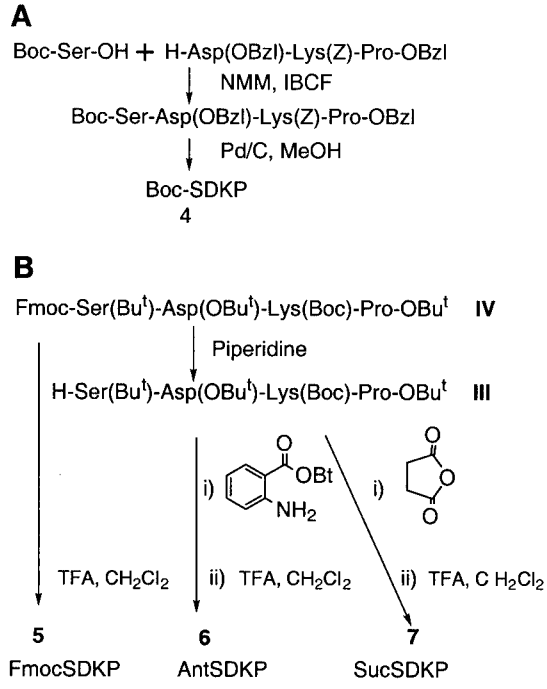

Figure 2 Synthesis of the analogues. (A) synthesis of Boc-SDKP; (B) synthesis of Fmoc-, Ant- and Suc-SDKP; (C) synthesis of dimer **9**; (D) synthesis of dimer **10**.

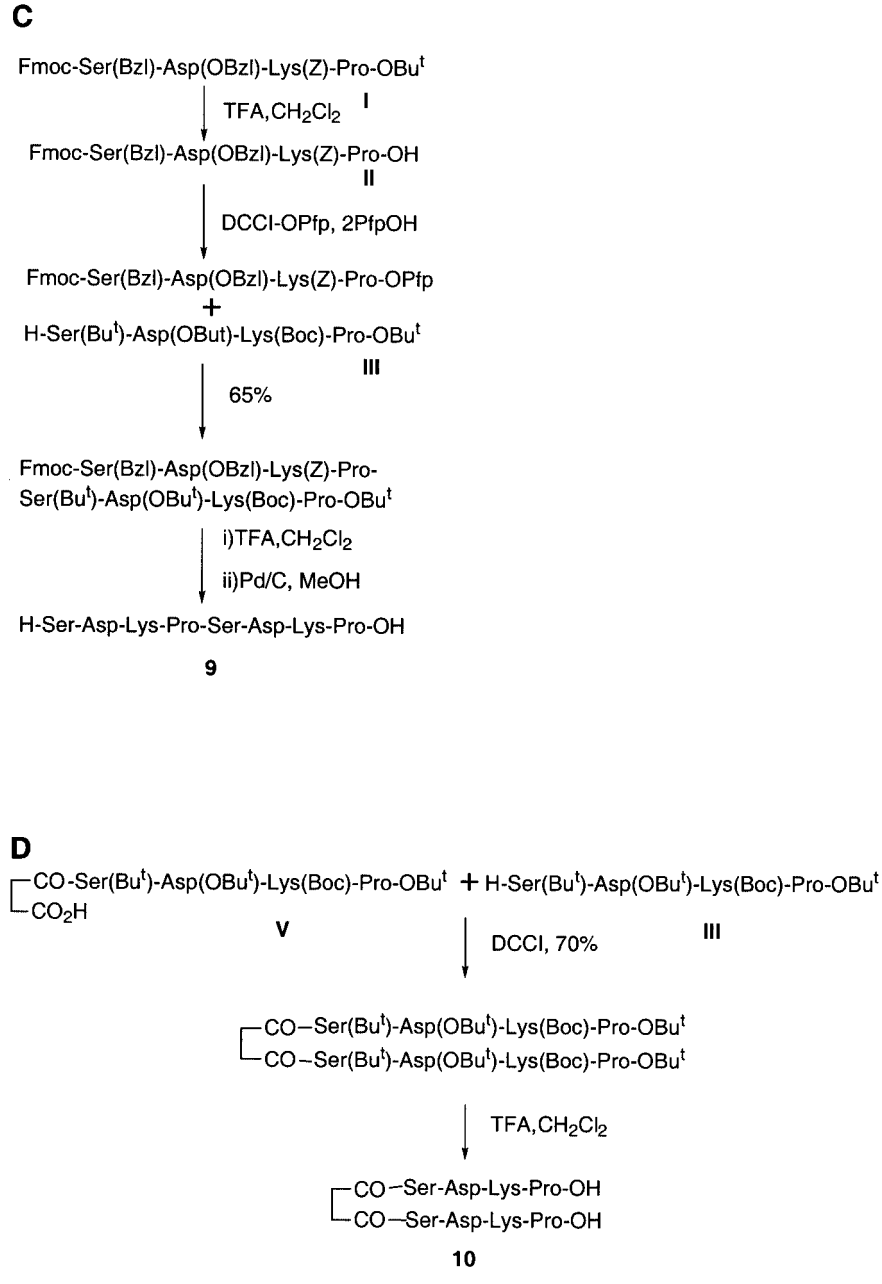

Figure 2 (*Continued*)

The analogue in which the serine residue has been replaced by homoserine was shown to retain the inhibitory activity reducing proliferation to 61% and 69% for respectively CFU-GM (Table 2) and HPP-CFC (Table 3).

The modification of charge and length of the side chain of lysine-3 achieved by the replacement of this residue by arginine maintained the biological activity of the resulting compound (AcSDRP) in the HPP-CFC assay (Table 3), while AcSDRP was found to be less efficient to inhibit the entry into S phase of CFU-GM (Table 2).

The dimer **10** (head-to-head) and dimer **9** (head-to-tail) were shown to exhibit a significant inhibition of HPP-CFC proliferation (74% and 86%, respectively). These inhibitory potencies were

Table 1 Analytical Properties of the Analogues

Compound		Formula	MS[a]		HPLC[d]	
			Found	Calc.[b]	$k'1$	$k'2$
AcSDKP	**1**	$C_{20}H_{33}N_5O_9$			10.4	9.5
AcHseDKP	**2**	$C_{21}H_{35}N_5O_9$	502.2519	502.2513	10.4	9.5
AcSDRP	**3**	$C_{20}H_{33}N_7O_9$	516.2429	516.2418	11.6	9.7
BocSDKP	**4**	$C_{23}H_{39}N_5O_{10}$	546.2775	546.2775	17.5	12.7
FmocSDKP	**5**	$C_{33}H_{41}N_5O_{10}$	668.2921	668.2931	30.2	19.0
AntSDKP	**6**	$C_{25}H_{36}N_6O_9$	565.587[c]	565	13.6	10.9
SucSDKP	**7**	$C_{22}H_{35}N_5O_{11}$	546.2412	546.2411	11.0	9.3
CoumSDKP	**8**	$C_{31}H_{42}N_6O_{11}$	674.71[c]	675	19.1	13.6
Dimer	**9**	$C_{36}H_{60}N_{10}O_{15}$	874.4396	872.4239	12.6	10.0
Dimer	**10**	$C_{40}H_{64}N_{10}O_{18}$	974.4556	972.4400	14.5	10.8

[a] LSI-HRMS; [b] calculated for $(M+H)^+$, except for **9** and **10** where $(M+2H)^{2+}$ was observed; [c] $(M+H)^+$ and $(M+Na)^+$ FAB-MS; [d] C-18 RP-HPLC in two conditions, cf. Materials and Methods.

Table 2 Effect of AcSDKP Analogues on the Proportion of CFU-GM in S-phase

Analogues		CFU-GM analysis					
		Control	With various analogues			With AcSDKP	
		% cells in S-phase	% cells in S-phase	Inhibition (%)	p	% cells in S-phase	Inhibition (%)
AcS-D-DKP	(a)	33.2	36.2	−9*	NS	12.8	61
AcHseDKP	**2**	33.0	12.9	61	0.01	9.3	72
AcSDRP	**3**	35.3	20.8	41	NS	10.6	70
BocSDKP	**4**	38.5	16.9	56	0.01	11.8	69
FmocSDKP	**5**	38.5	18.2	53*	0.01	11.8	69
AntSDKP	**6**	38.5	13.0	66	0.01	11.8	69
SucSDKP	**7**	38.5	12.5	68	0.01	11.8	69
CoumSDKP	**8**	38.5	22.3	42*	0.01	11.8	69
AcSDK-Pyr		36.0	15.8	56	0.01	10.2	72
AcSDKP-NH_2	(a)	36.0	13.9	61	0.01	10.2	72
AcSΨDKP	(a)	33.2	9.8	70	0.01	12.8	61
AcSDΨKP	(a)	33.2	7.3	78	0.01	12.8	61
AcSDKΨP	(a)	33.2	9.9	70	0.01	12.8	61
Dimer	**9**	35.3	18	49	NS	10.6	70
Dimer	**10**	35.3	8.5	76	0.05	10.6	70

(a) Results from Gaudron *et al.* [11].
* Significantly different from AcSDKP, $p<0.05$; NS, non-significant.

found to be significantly higher than the one measured for the parent tetrapeptide (Table 3). The dimer **10** showed also a good inhibitory effect on CFU-GM, whereas dimer **9** proved to be less efficient as the 49% inhibition extent is lower and not significant, probably owing to the large dispersion of the number of colonies between the three experiments.

The analogue AcS-D-DKP, in which the L-Asp has been replaced by D-Asp was shown to be completely devoid of activity in the two assays.

Moreover, none of the analogues incubated with BMC induced any change in the number of colonies in both assays derived from treated BMC (Table 4). This indicates the absence of cytotoxic activity of all these compounds.

Table 3 Effect of AcSDKP Analogues on the Entry into S-phase of HPP-CFC

Analogues		HPP-CFC analysis					
		Control	With various analogues			With AcSDKP	
		% cells in S-phase	% cells in S-phase	Inhibition (%)	p	% cells in S-phase	Inhibition (%)
AcS-D-DKP	(a)	30.9	26.6	14*	NS	16.1	48
AcHseDKP	**2**	33.8	10.6	69	0.01	9.0	73
AcSDRP	**3**	30.7	11.3	63	0.01	17.1	44
BocSDKP	**4**	25.2	12.4	51	0.01	14.5	42
FmocSDKP	**5**	26.0	10.6	59	0.01	10.8	58
AntSDKP	**6**	26.0	12.5	52	0.01	10.8	58
SucSDKP	**7**	26.0	12.3	53	0.01	10.8	58
CoumSDKP	**8**	34.4	21.8	37*	0.01	10.5	69
AcSDK-Pyr		34.4	14.7	57	0.01	10.6	69
AcSDKP-NH_2	(a)	34.4	16.9	51	0.01	10.6	69
AcSΨDKP	(a)	30.0	11.8	61	0.01	16.0	47
AcSDΨKP	(a)	33.8	11.5	66	0.05	11.6	66
AcSDKΨP	(a)	33.8	15.1	55	0.01	11.6	66
Dimer	**9**	34.4	4.8	86*	0.01	10.6	69
Dimer	**10**	30.7	8.0	74*	0.01	17.1	44

(a) Results from Gaudron *et al.* [11].
* Significantly different from AcSDKP, $p<0.05$; NS, non-significant.

Table 4 Lack of Effect of AcSDKP Analogues on the Number of HPP-CFC and CFU-GM Colonies

Analogues		HPP-CFC number/ 3×10^4 plated BMC		CFU-GM number/ 4×10^4 plated BMC	
		Control	With analogue	Control	With analogue
AcSDK-Pyr		29.9 ± 1.9	28.5 ± 2.5	68.6 ± 1.5	65.2 ± 3.0
AcHseDKP	**2**	27.5 ± 1.0	27.4 ± 1.3	60.9 ± 2.6	57.9 ± 1.9
AcSDRP	**3**	25.1 ± 1.1	24.8 ± 2.5	62.8 ± 4.2	55.8 ± 7.1
BocSDKP	**4**	26.2 ± 1.1	24.1 ± 1.2	61.7 ± 0.9	61.5 ± 3.8
FmocSDKP	**5**	26.5 ± 0.7	26.5 ± 3.2	61.7 ± 0.9	61.6 ± 3.2
AntSDKP	**6**	26.5 ± 0.7	26.5 ± 3.5	61.7 ± 0.9	58.3 ± 3.0
SucSDKP	**7**	26.5 ± 0.7	25.2 ± 2.6	61.7 ± 0.9	64.1 ± 4.0
CoumSDKP	**8**	29.9 ± 1.9	27.5 ± 1.8	61.7 ± 0.9	58.3 ± 2.5
Dimer	**9**	29.9 ± 1.9	25.1 ± 2.2	62.8 ± 4.2	53.5 ± 3.2
Dimer	**10**	25.1 ± 1.1	23.7 ± 1.4	62.8 ± 4.2	52.8 ± 1.2

Colony numbers were determined after 8 or 14 days of culture respectively for CFU-GM and HPP-CFC.
Values represent the mean $\pm$ S.E. of three or four separate experiments.

DISCUSSION

Efficacy of chemotherapy regimens is limited by two inter-related problems, namely drug toxicity and multi-drug resistance. Myelosuppression increases the patient's susceptibility to severe infections and compromises the achievement of the planned chemotherapy. Attempts to increase dose intensity, therefore, require effective haematopoietic support to ensure prompt restoration of normal haematopoiesis. The use of inhibitors of haematopoiesis could allow the development of new therapeutic strategies concerning myeloprotection during treatments of cancer. In fact, the quiescence of normal

haematopoietic stem cells induced by their proliferation inhibitor protects them from the toxicity of anticancer drugs, and consequently, provides reduction of marrow failure. The tetrapeptide AcSDKP belongs to the family of negative regulators of haematopoiesis. Its myeloprotective properties against cytotoxic drugs are well-established models, suggesting its clinical use as a marrow protector during cancer therapy.

To investigate the structural requirements enabling AcSDKP-derived compounds to inhibit the proliferation of primitive haematopoietic cells, we have altered different sites of the molecule namely: the *C*-terminus or *N*-terminus, and some of the side chains. We also investigated the effect of dimerization on the activity as dimerization sometimes results in different activity.

The intact biological activity of the two analogues of AcSDKP modified on the *C*-terminal residue of proline (AcSDKP-NH_2 and AcSDKPyr), shows clearly that this amino acid is not essential for the antiproliferative activity. These data are in complete agreement with our former results, showing that removal of the *C*-terminal proline did not affect biological activity [9,19].

Considering the small size of the molecule, it was thought important to study the influence of varying the size as well as the polarity of the *N*-substituent, as we needed fluorescent and conjugated analogues used as tools for studying the molecular mechanism of the peptide. Results from Tables 2 and 3 concerning compounds **4–7** indicate that none of the structural modifications at the *N*-terminus abolished the biological activity. Substitution of the *N*-terminal acetyl by bulky lipophilic *N*-amino protecting groups such as Boc or Fmoc had no significant influence upon activity. The same was found for anthranilyl- and succinyl-substituted SDKP although the molecules have a different net charge. However, replacement of the acetyl residue by the lipophilic fluorescent coumaryl residue resulted in partial loss of inhibitory activity. These results showed that most of the substitutions were tolerated yielding compounds still active.

The two dimers **9** and **10** display surprisingly inhibitory activity as the parent peptide. In fact, it is well known that the dimerization of biological factors leads often to some modification of their activity. Thus, the pentapeptide GlpGluAspCysLys, an another inhibitor of primitive haematopoietic cell proliferation, yields upon dimerization a compound exhibiting very potent opposite stimulatory effect [20]. Moreover, it has been reported that only the monomeric form of macrophage inflammatory protein-1α (MIP-1α) belonging to the family of haemoregulatory factors is able to inhibit haematopoietic stem cell proliferation [21]. In our case, the inhibitory activity is retained with a slightly better potency. These dimers can be viewed as either *N*- or *C*-substituted analogues, so these findings further confirm the previous ones, that is to say, that neither the *C*-terminal proline nor the *N*-terminal acetyl group are crucial for biological activity.

All these biological data obtained with the compounds resulting from structural modifications of *C*- and *N*-terminus of AcSDKP are consistent with our previous studies, which have shown that the tripeptide sequence SDK play a pivotal role in the expression of biological activity [9,19].

The lack of inhibitory effect of the isomeric analogue AcS-D-DKP shows the importance of the aspartic residue. It has been previously reported [11] that the modification of AcSDKP peptide backbone by introducing aminomethylene in place of amide group yielded pseudopeptides: Ac-Ser-Ψ(CH_2-NH)-Asp-Lys-Pro (AcSΨDKP), Ac-Ser-Asp-Ψ(CH_2-NH)-Lys-Pro (AcSDΨKP), Ac-Ser-Asp-Lys-Ψ(CH_2-N)-Pro (AcSDKΨP), still retaining full antiproliferative activity and displaying metabolic stabilility towards Angiotensin Converting Enzyme involved in the catabolism of AcSDKP. Further modification of the backbone by introducing an increasingly popular β-homo amino acid (3-aminopentanedioic acid) in place of Aspartic acid gave a compound with an extra methylene between the alpha carbon and the carboxy group. The corresponding peptide was found completely inactive in the CFU-GM assay (36.1% CFU-GM in S-phase with this analogue versus 34.5% in the control). These results suggest that the expression of the activity results from a given relationship between the hydroxy, carboxy and amino functions borne by the side chains of Ser, Asp and Lys.

To ensure that the results obtained were not biased by a toxic effect of the studied compounds, the analysis of the number of colonies derived from bone marrow exposed to the studied molecules was performed. It appeared that all AcSDKP analogues are devoid of toxic effect at the concentration which effectively inhibits the proliferation of CFU-GM and HPP-CFC.

Taken together, the present study reveals the significant antiproliferative activity of various AcSDKP analogues with substantially various structural parameters. Consequently, some of these newly developed active analogues of AcSDKP could turn out to be useful therapeutic agents offering suitable clinical efficacy.

Acknowledgements

The authors thank Dr J.Y. Mary for his valuable help in statistical analysis and Ipsen-Biotech for its financial support.

REFERENCES

1. Lenfant M, Wdzieczak-Bakala J, Guittet E, Prome JC, Sotty D, Frindel E. Inhibitor of hematopoietic pluripotent stem cell proliferation: purification and determination of its structure. *Proc. Natl. Acad. Sci. USA* 1989; **86**: 779–782.
2. Jackson JD, Ozerol E, Yun Y, Ewel C, Talmadge JE. Activity of Acetyl-Ser-Asp-Lys-Pro (AcSDKP) on human hematopoietic progenitors in short-term and long-term bone marrow cultures. *J. Hematother. Stem Cell Res.* 2000; **9**: 489–496.
3. Grillon C, Bonnet D, Mary JY, Lenfant M, Najman A, Guigon M. The tetrapeptide Acetyl-*N*-Ser-Asp-Lys-Pro (Seraspenide), a hematopoietic inhibitor, may reduce the *in vitro* toxicity of 3′-azido-3′-deoxythymidine to human hematopoietic progenitors. *Stem Cells* 1993; **11**: 455–464.
4. Aidoudi S, Guigon M, Drouet V, Caen JP, Han ZC. The tetrapeptide AcSDKP reduces the sensivity of murine CFU-MK and CFU-GM progenitors to aracytine *in vitro* and *in vivo*. *Int. J. Hematol.* 1996; **68**: 45–155.
5. Genevay MC, Mormont C, Thomas F, Berthier R. The synthetic tetrapeptide AcSDKP protects cells that reconstitue long-term bone marrow stromal cultures from the effects of mafosfamide (Asta Z 7654). *Exp. Hematol.* 1996; **24**: 77–81.
6. Bogden AE, Moreau JP, Gamba-Vitalo C, Deschamps de Paillette E, Tubiana M, Frindel E, Carde P. Goralatide (AcSDKP), a negative growth regulator, protects the stem cell compartment during chemotherapy, enhancing the myelopoietic response to GM-CSF. *Int. J. Cancer* 1998; **76**: 38–46.
7. Massé A, Ramirez LH, Bindoula G, Grillon C, Wdzieczak-Bakala J, Raddassi K, Sainteny F, Deschamps de Paillette E, Mencia-Huerta JP, Potier P, Carde P. The tetrapeptide Acetyl-*N*-Ser-Asp-Lys-Pro (Goralatide) protects mice from doxorubicin-induced toxicity: an improvement in both survival and hematological protection for early marrow stem cells and progenitors. *Blood* 1998; **9**: 441–449.
8. Carde P, Chastang C, Goncalves E, Mathieu-Tubiana N, Vuillemin E, Delwail V, Corbion O, Vekhoff A, Isnard F, Ferrero JM, Garcia-Giralt E, Gimonet JF, Stoppa AM, Leger-Picherit E, Frindel E, Monpezat JP, Munck J-N, Domenge C, Khayat D, Guilhot F, Monnier A, Zittoun R, Brun B, Namer M, Maraninchi D, Deschamps de Paillettte E, Guigon M, Najman A. Seraspenide (Acétyl-SDKP): étude en phase I-II d'un inhibiteur de l'hématopoïèse la protégeant de la toxicité de monochimiothérapies aracytine et ifosfamide. *C. R. Acad. Sci. Paris, série III* 1992; **315**: 545–550.
9. Thierry J, Papet MP, Saez-Servent N, Plissonneau-Haumont J, Potier P, Lenfant M. Synthesis and activity of *N*-Ac-Ser-Asp-Lys-Pro analogues on cellular interactions between T-cell and erythrocytes in rosette formation. *J. Med. Chem.* 1990; **33**: 2122–2127.
10. Gaudron S, Adeline MT, Potier P, Thierry J. NAcSDKP analogues resistant to angiotensin-converting enzyme. *J. Med. Chem.* 1997; **40**: 3963–3968.
11. Gaudron S, Grillon C, Thierry J, Riches A, Wierenga PK, Wdzieczak-Bakala J. *In vitro* effect of Acetyl-*N*-Ser-Asp-Lys-Pro (AcSDKP) analogues resistant to angiotensin I-converting enzyme on hematopoietic stem cell and progenitor cell proliferation. *Stem Cells* 1999; **17**: 100–106.
12. Wierenga P, Konings AWT. Goralatide (AcSDKP) selectively protects murine hematopoietic progenitors and stem cells against hyperthermic damage. *Exp. Hematol.* 1996; **24**: 246–252.
13. Robinson S, Lenfant M, Wdzieczak-Bakala J, Melville J, Riches A. The mechanism of action of the tetrapeptide Acetyl-*N*-Ser-Asp-Lys-Pro (AcSDKP) in the control of haematopoietic stem cell proliferation. *Cell Prolif.* 1992; **25**: 623–632.
14. Vaughan JR. Acylcarbonates as acylating agents for the synthesis of peptides. *J. Am. Chem. Soc.* 1957; **73**: 3547.
15. Turan A, Manning M, Haldar H, Sawyer WH. Synthesis and pharmacological properties of (4-homoserine)-oxytocin. *J. Med. Chem.* 1977; **9**: 1169–1172.
16. Cheviron N, Rousseau-Plasse A, Lenfant M, Adeline MT, Potier P, Thierry J. Coumarine-Ser-Asp-Lys-Pro, a fluorescent substrate for determination of angiotensin-converting enzyme activity via high-performance-liquid chromatography. *Anal. Biochem.* 2000; **280**: 58–64.
17. Stewart FHC. Synthetic approach to peptides of o- and p-aminobenzoic acid. *Aust. J. Chem.* 1983; **36**: 1629–1638.
18. Kovacs J, Kisfaludy L, Ceprini MQ. On the optical purity of peptide active esters prepared from *N,N′*-dicyclohexylcarbodiimide and 'complexes' of *N,N′*-Dicyclohexylcarbodiimide-pentachlorophenol and *N,N′*-dicyclohexylcarbodiimide-pentafluorophenol. *J. Amer. Chem. Soc.* 1967; **89**: 183–185.
19. Robinson S, Lenfant M, Wdzieczak-Bakala J, Riches A. The molecular specificity of action of the tetrapeptide *N*-Acetyl-Ser-Asp-Lys-Pro (AcSDKP) in the control of hematopoietic stem cell proliferation. *Stem Cells* 1993; **11**: 422–427.
20. Paukovits WR, Paukovits JB, Moser MH, Konstantinov S, Schulte-Hermann R. Activated granulocytes oxidize the endogenous stem cell inhibitory peptide pGlu-Glu-Asp-Cys-Lys (pEEDCK) to the stimulatory dimer: a redox-mediated mechanism for demand-induced hematopoietic regulation. *Exp. Hematol.* 1998; **26**: 851–858.
21. Mantel C, Kim YJ, Cooper S, Kwon B, Broxmeyer HE. Polymerization of murine macrophage inflammatory protein 1α inactivates its myelosuppressive effects *in vitro*: the active form is a monomer. *Proc. Natl. Acad. Sci. USA* 1993; **90**: 2232–2236.

