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Inhibitor of hematopoietic pluripotent stem cell proliferation: Purification and determination of its structure

(bone marrow/tetrapeptide/nuclear magnetic resonance/mass spectrometry)

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ABSTRACT **We report here a five-step purification procedure that led to the isolation from fetal calf bone marrow extract of a tetrapeptide, Ac-Ser-Asp-Lys-Pro (M_r 487), exerting a high inhibitory activity on the proliferation of hematopoietic pluripotent stem cells [defined here as spleen colony-forming units (CFU-S)]. The structure of this molecule was established from amino acid analysis, fast atom bombardment mass spectrometry, and ^1H nuclear magnetic resonance spectral data. This structure was confirmed by comparison with the corresponding synthetic molecule, which presents identical physicochemical characteristics and biological properties. Natural and synthetic peptides administered to mice (at a dose of 100 ng per mouse) after one injection of cytosine arabinonucleoside prevent CFU-S recruitment into DNA synthesis.**

Hemopoietic pluripotent stem cells, defined here as spleen colony-forming units (CFU-S), are responsible for the generation of the hematopoietic system. Its recovery after cell depletion following different types of stress, such as irradiation, drug administration, or bleeding, depends on the integrity of CFU-S. Within the last few years, it has become evident that CFU-S proliferation is under the control of regulators, some of which stimulate the quiescent stem cells (1–3), whereas others inhibit CFU-S entry into cell cycle (4–6). In normal, healthy mice, CFU-S are quiescent. This could be due to an equilibrium state between inhibitors and stimulators of CFU-S proliferation at a G_0 level that implies an excess of inhibitors. At this stage, CFU-S are invulnerable to phase-specific drugs. When normal conditions are perturbed, the above-mentioned equilibrium is disrupted and the net result will depend on the predominant factor.

We reported previously that fetal calf bone marrow contained a low molecular weight factor capable of preventing CFU-S recruitment into the cell cycle after irradiation or administration of a phase-specific cytostatic drug (7–9). This factor was able to protect CFU-S *in vivo* during a sequential treatment with cytosine arabinonucleoside (araC) (10) by keeping CFU-S out of the cycle and to increase the survival of mice given lethal doses of the same drug (11). Considering the fundamental interest of such an activity and its potential clinical applications, the purification of the inhibitory molecule was carried out and its structure was determined.

MATERIALS AND METHODS

Materials. Frozen fetal calf bone marrow and BALB/c SPF mice aged 2–3 months were obtained from Harlan Olac (Bicester, U.K.); araC (Aracytine) was from Upjohn. HPLC analyses were carried out on a Hypersil ODS column (Société Française Chromato Colonne, Neuilly-Plaisance, France) using equipment from Waters Associates.

Methods. *Extraction and purification procedure.* Large-scale extraction procedures and the first steps of purification were carried out as reported (7). Briefly, 5 kg of frozen calf bone marrow was homogenized in 10 mM phosphate buffer (pH 7.2) in the presence of 10 mM 2-mercaptoethanol and then centrifuged. Ultrafiltration through Sartorius membranes (no. 121 36; exclusion limit, 10^4 daltons) was used to separate the low molecular weight fraction from the bulk of the water-soluble molecules present in the marrow extract. The ultrafiltrate was then concentrated by flash evaporation (LUWA evaporator) and lyophilized. The dry residue was dissolved in 10 mM acetic acid and fractionated on a Bio-Gel P-2 (200–400 mesh, Bio-Rad) column (12.5 × 100 cm) using 10 mM acetic acid as an eluant. The active fraction eluted at V_e/V_o = 1.2–1.8 was lyophilized. Purification was achieved by passing this Bio-Gel P-2 fraction through octadecylsilyl silica cartridges (Sep-pak C_{18}, Waters). After washing with 3 ml of 0.1% aqueous solution of trifluoroacetic acid (TFA), the peptides absorbed to the C_{18} silica were eluted by 3 ml of 50% methanol in aqueous solution of 0.1% TFA, lyophilized, and then subjected to reverse-phase HPLC carried out on a Hypersil ODS C_{18} column (250 × 4 mm; particle size, 5 μm) at 25°C using 4.5% acetonitrile (CH_3CN) in an aqueous solution of 0.1% TFA as the solvent system at a flow rate of 1 ml/min. The absorbance was monitored at 215 nm. Aliquots corresponding to the single peaks were collected according to the absorption pattern, repurified using the same HPLC conditions, lyophilized, and assayed for inhibitory activity on CFU-S proliferation.

HPLC analysis. The active fraction was analyzed by HPLC on Hypersil ODS C_{18} column using the following solvents: (*i*) CH_3CN/0.1% TFA in H_2O (4.5:95.5), pH 2.5; (*ii*) CH_3CN/0.1% TFA in H_2O (20:80), pH 2.5; (*iii*) CH_3CN/0.01 M CH_3COONH_4 (5:95), pH 4.2, 3.5, and 3.2; (*iv*) CH_3CN/0.25 M H_3PO_4/$(C_2H_5)_3N$ (5:95), (3:97), and (1:99), pH 3.1. The flow rate was 1 ml/min; absorption was monitored at 215 nm.

Preparation of the various solvents: The three buffers at pH 4.2, 3.5, and 3.2 (see *iii*) were prepared by the classical technique by mixing the appropriate quantities of CH_3COOH and NH_4OH. Then these buffers were mixed with CH_3CN as mentioned. For *iv*, buffer was prepared from 0.25 M H_3PO_4 adjusted to pH 3.1 by addition of $(C_2H_5)_3N$. This buffer was then mixed with various quantities of CH_3CN as mentioned. All mixtures have the same pH, 3.1.

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Abbreviations: CFU-S, colony-forming unit(s), spleen; FAB, fast atom bombardment; araC, cytosine arabinonucleoside; TFA, trifluoroacetic acid; COSY, correlated spectroscopy; MIKE, metastable ion kinetic energy; CID-MIKE, chemical ion dissociation-MIKE.

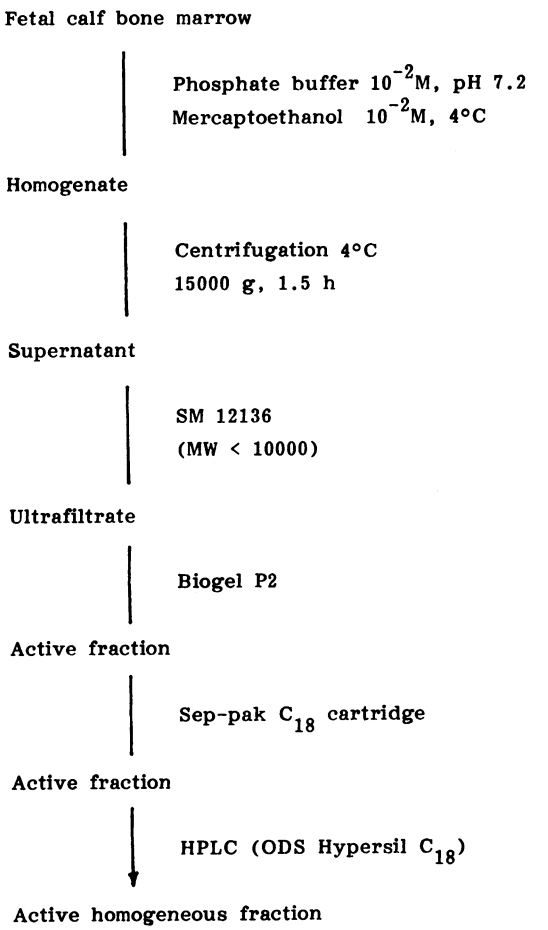

FIG. 1. Summary of purification of CFU-S proliferation inhibitor.

Amino acid analysis. Peptide hydrolysis was carried out for 24 hr at 110°C in a 0.1% phenol/5.7 M HCl solution in evacuated sealed tubes. Amino acid composition was determined with an LKB 4400 amino acid analyzer operated with a normal ninhydrin system.

UV spectra. Absorption spectra of active fractions (1 μg/ml of H_2O) were registered by using a Shimadzu UV 240 spectrophotometer.

Mass spectrometry (MS) studies. Fast atom bombardment (FAB) MS analyses were performed with a reverse geometry VG-ZAB 2F spectrometer (VG-Analytical, Manchester, U.K.) fitted with a FAB source and a xenon atom gun (Ion Tech, Teddington, U.K.). The acceleration voltage of the ions was 8 keV. The FAB gun also operated at +8 keV. The ions from the matrix (glycerol spiked with trichloroacetic acid) have been partially removed by subtraction of a blank spectrum.

The normal spectra were recorded by the VG Data System 2035 (VG-Analytical, Manchester, U.K.) based on a PDP-8 computer (Digital) at a speed of 10 sec per decade.

The amino acid sequence was established by metastable ion kinetic energy (MIKE) (a) and chemical ion dissociation-MIKE (CID-MIKE) (b) spectra of the protonated molecular ion at m/z 488. However, the width of the signal does not allow mass measurements better than ±1 atomic mass unit.

The MIKE spectra were collected in the continuum mode, using 20–40 accumulated 10-sec kinetic energy scans, which were directly converted into intensity vs. mass spectra by the MIKE software. In the CID-MIKE mode, the pressure measured at the outside of the collision cell was $1.5\ 10^{-6}$ mbar (1 bar = 100 kPa).

NMR studies. The 400-MHz ^1H NMR spectra were acquired on a Bruker WM 400 spectrometer. For amide-region analysis, 150 μg of the isolated peptide was dissolved in 400 μl of water (pH 3.0, 19°C). The spectrum was obtained by using the "jump and return" sequence (12). High-field-region analysis was performed in ^2H_2O. The JR2D experiment from a correlated spectroscopy (COSY) spectrum in H_2O was analyzed by using the JR2D pulse sequence (13). Two-hundred fifty-six time domain points were acquired in t_1, with 256 free induction decays acquired at each t_1. The spectrum was Fourier transformed in both dimensions using a sine-bell window function.

Biological assay. Experiments were carried out on BALB/c mice housed under specific pathogen-free conditions. They were randomly divided into donor mice (treated and untreated controls) and recipient mice.

The biological activity of different fractions was studied *in vivo* by inhibition of the entry of CFU-S into the cell cycle following a single administration of araC dissolved in saline and injected i.p. at a dose of 800 mg/kg in order to trigger the normally quiescent CFU-S into the cell cycle. Six hours later the fraction dissolved in saline was injected i.p.; the control group received the same volume of saline. Six hours later—that is, 12 hr after the araC injection—mice were sacrificed and the bone marrow was harvested. CFU-S were studied according to the Till and McCulloch technique (14) and their percentage in DNA synthesis was determined by the [^3H]thymidine suicide technique of Becker *et al* (15).

RESULTS AND DISCUSSION

The molecule that prevents hematopoietic stem cell (CFU-S) proliferation was purified to homogeneity from fetal calf bone marrow according to the procedure described in Fig. 1. The purified molecule was shown to be effective in preventing CFU-S entry into the cell cycle in araC-treated mice at the dose of 100 ng per mouse (Table 1).

The homogeneity of the active molecule was assessed by HPLC reverse-phase chromatography using seven different solvent systems. In all cases the examined sample behaved as a homogeneous compound eluted as a single symmetrical peak (Table 2).

Table 1. Inhibition of CFU-S entry into DNA synthesis by Ac-Ser-Asp-Lys-Pro isolated from fetal calf bone marrow and by the corresponding synthetic molecule

	Natural peptide		Synthetic peptide	
Mouse treatment	CFU-S, no. per 10^5 cells	% CFU-S in S phase	CFU-S, no. per 10^5 cells	% CFU-S in S phase
Control	15.76 ± 0.56	0 ± 5.8	15.83 ± 0.56	7.55 ± 5.3
araC	20.83 ± 0.60	44.31 ± 2.9	21.15 ± 0.55	36.57 ± 2.9
araC + peptide*	18.19 ± 0.53	15.17 ± 3.9†	17.91 ± 0.46	14.61 ± 3.4†

Results are expressed as mean ± SEM; $n = 10$ experiments. Student's t test was used to determine the statistical significance of the inhibitory effect.
*100 ng of peptide.
†$P < 0.001$.

Table 2. HPLC analysis of CFU-S proliferation inhibitory peptide

Solvent system	No. of peaks	Retention time, min
CH_3CN/0.1% TFA in H_2O (4.5:95.5), pH 2.5	1	18
CH_3CN/0.1% TFA in H_2O (20:80), pH 2.5	1	6
CH_3CN/0.01 M CH_3COONH_4 (5:95)		
pH 4.2	1	3
pH 3.5	1	10
CH_3CN/0.25 M $H_3PO_4/(C_2H_5)_3N$, pH 3.1		
5:95	1	6
3:97	1	9
1:99	1	16

The UV absorption spectra of the active sample measured at pH 7 showed no characteristic absorption between 250 and 400 nm. This excluded the presence of aromatic amino acids as well as nucleoside residues. The inhibitory fraction appeared to be devoid of any sugar content, as assessed by colorimetric determination (16).

Amino acid analysis of the purified molecule (Table 3) suggested the following stoichiometric composition: 1 Asp, 1 Ser, 1 Lys, 1 Pro.

All attempts at sequencing the intact active peptide by classical Edman degradation were unsuccessful, suggesting an N-terminal blocked molecule. The primary structure of the CFU-S proliferation inhibitor was established by MS and NMR studies.

The FAB mass spectrum of the CFU-S inhibitory factor exhibited a protonated molecular ion, MH^+, corresponding to a molecular weight of 487 (Fig. 2), a value that might correspond to an acetyl-substituted tetrapeptide.

In the MIKE spectrum (Fig. 3*a*) of metastable ions the base peak was found at m/z 373. It corresponded to the loss of 115 mass units from the pseudomolecular ion. This mass loss was attributed to a terminal proline group. The peak, centered at m/z 244, corresponded to a mass difference of 129 ± 1 mass units from the preceding one. This value could be attributed either to a lysine (128 mass units) or to an acetyl-serine (129 mass units) residue. The next peak was found at m/z 129. The mass difference from the preceding one was 115 mass units. It corresponded to an aspartate residue.

Two different sequences could be therefore proposed from this spectrum: Ac-Ser-Asp-Lys-Pro or Lys-Asp-Ac-Ser-Pro.

The collisional activation (CID-MIKE) spectrum (Fig. 3*b*) was more complex and showed some additional features. The more characteristic ones were the loss of 32 mass units from MH^+ attributed to the cleavage of the side chain of serine and a peak centered at m/z 83.5 corresponding to the imminium ion from an anhydro-*N*-acetyl-serine (m/z 84). Thus, this spectrum indicates a free hydroxyl group on the serine residue.

Confirmation of amino acid composition and complementary information concerning the structure of the peptide were obtained by NMR studies.

A MQT-COSY spectrum (where MQT indicates multiple quanta technique) (17) (not shown) established the coupling pattern of single proline, aspartic acid, lysine, and serine and

Table 3. Amino acid composition of CFU-S proliferation inhibitor isolated from calf bone marrow

Residue	Ratio
Aspartic acid	1.11
Serine	1.00
Lysine	0.95
Proline	0.79

Contaminants inferior to 0.3 are omitted. Results are expressed relative to the value for serine, which was taken as 1.00.

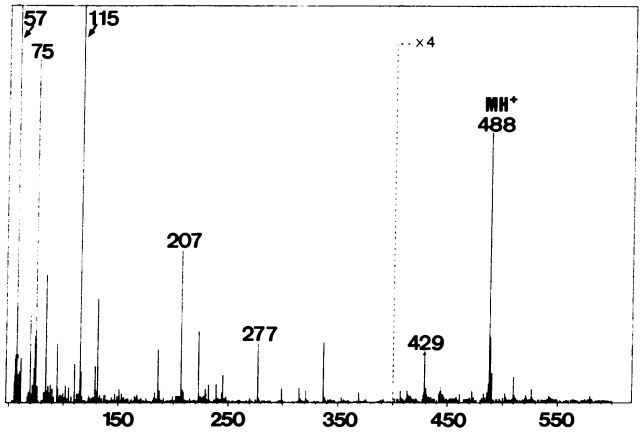

FIG. 2. FAB mass spectrum of CFU-S proliferation inhibitor.

allowed the attribution in the 1D spectrum (Fig. 4*B*). The attribution in the amide region (Fig. 4*A*) resulted from the JR2D spectrum. Observation in the JR2D experiment (Fig. 4 *C–E*) of three cross-peaks between α-CH groups and amide resonances that correspond in *A* to one proton integration indicate the presence of α-amino monosubstituted residues for lysine (8.2 ppm), serine (8.4 ppm), and aspartic acid (8.6 ppm), whereas the amino group of the lysine side chain (7.6 ppm) remains unsubstituted, as shown by a two-proton integration. In the 1D high-field region (Fig. 4*B*), the chemical shifts of the β protons of serine (3.83 ppm) and ε protons of lysine (3.03 ppm) indicate that the amino group of the lysine side chain as well as the hydroxyl group of the serine side chain remain unsubstituted. The presence of a singlet at 2.09 ppm is attributed to an acetyl residue that must be linked to the N-terminal position of the peptide.

From MS and NMR studies the following structure appeared as the most probable: Ac-Ser-Asp-Lys-Pro.

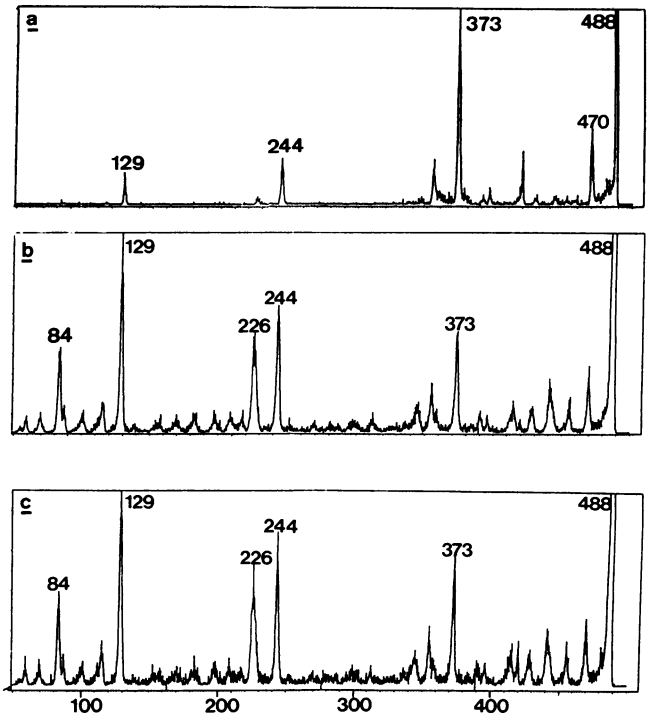

FIG. 3. Collisional activated mass spectra of the CFU-S proliferation inhibitory molecule. (*a*) MIKE spectrum. (*b*) CID-MIKE spectrum. (*c*) CID-MIKE spectrum of synthetic molecule.

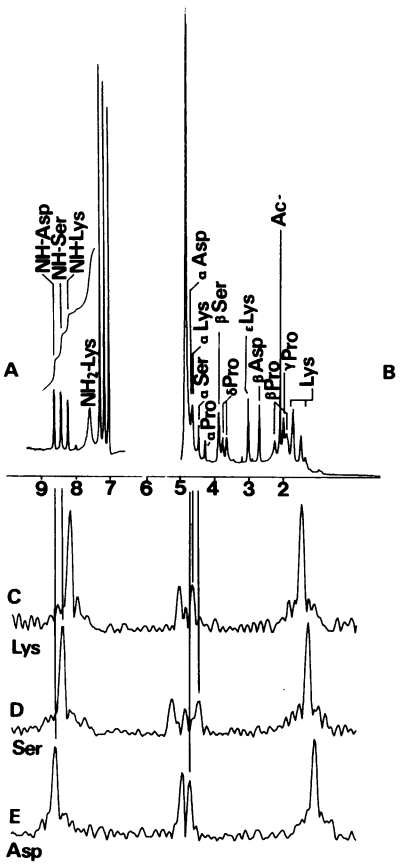

FIG. 4. The 400-MHz ^1H NMR spectra of CFU-S proliferation inhibitor. (*A*) Amide region (H_2O). (*B*) High-field region (^2H_2O). (*C–E*) Cross sections along ω_1 from a COSY spectrum in H_2O.

The proposed structure was confirmed by the identity of physicochemical and biological studies of the corresponding synthetic molecule (unpublished data). As shown in Table 1, the synthetic molecule compared to the natural one inhibits the entry of CFU-S into DNA synthesis *in vivo* at the dose of 100 ng per mouse. The possibility that the tetrapeptide could derive from a high molecular weight precursor undergoing degradation during the purification procedure cannot be excluded. However, a comparative study carried out on fetal calf liver using an acetone powder acidic extraction procedure, known to prevent proteolytic degradation, led to the isolation of a similar low molecular weight semipurified active factor (18). This suggested that the tetrapeptide might be of physiological significance. So far, thymosin β-4, tumor necrosis factor α, and rat liver phenylalanine hydroxylase are the only mammalian proteins known to contain the reported sequence. It is worth pointing out that in thymosin β-4 the sequence we have determined is present as an Ac-Ser-Asp-Lys-Pro terminal fragment. The hypothesis that the CFU-S proliferation inhibitor derived *in vivo* through proteolytic maturation of thymosin β-4 is attractive.

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