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Antiproliferative Action of Valorphin in Cell Cultures

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Abstract: The antiproliferative effects of the haemoglobin β-chain fragment (33–39) (valorphin or VV-haemorphin-5) were studied in a panel of tumour cell lines and normal cells of different origin, using various methods of activity determination (trypan blue inclusion test, sulphorhodamine B staining, MTT staining, flow cytometry and clonogenic test). Valorphin suppressed the proliferation of tumour cells by 25%–95%, depending on the cell line. The maximal valorphin activity was detected in transformed cells of fibroblastic (L929) and epithelial (MCF-7) origin, transformed haematopoietic cells (K562, HL-60) being less sensitive. In normal cells, valorphin activity was several fold lower (10%–15%). A study of the dynamics of cell proliferation in L929 cells using a visual cell count and flow cytometry showed that valorphin induced reversible and relatively short (24 h) S-phase arrest of cell proliferation, accompanied by a reversible increase of cell size. The proliferation delay was followed by a comparatively long period of reversible resistance of the cells to the peptide (96 h) when the cells are dividing at normal rate. The same dynamics were demonstrated for A549, MCF-7 and primary murine breast carcinoma cells. On the basis of the data obtained, a pattern of regulation of cell growth by valorphin is suggested. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: valorphin; tumour cells; antiproliferative effect; cell cycle arrest; reversibility; resistance

INTRODUCTION

Valorphin (VVYPWTQ) belongs to the family of endogenous fragments of a haemoglobin β-chain segment (32–41) known as haemorphins which exhibit a set of *in vitro* and *in vivo* opioid effects mediated by interaction with opioid receptors [1–3].

Earlier it was demonstrated that, like 'classical' ligands of opiate receptors, haemorphins inhibit tumour cell proliferation in the absence of fetal serum. The inhibitory effect of haemorphins is due to both antiproliferative and cytolytic activity [4–7]. The high content of haemorphins in tissues [8], sufficient for antiproliferative action, the accumulation of haemorphins in lung carcinoma tissue [9], as well as an antitumour effect *in vivo* [10], together point to the ability of haemorphins to participate directly in the inhibition of malignant neoplasia at tissue level.

In the present work, the spectrum of *in vitro* inhibitory effects of valorphin on the growth of cell populations of different origin were studied.

In our preceding studies the ability of valorphin to inhibit the proliferation of L929 transformed cells after 24 h of incubation was demonstrated [4,6,7]. In the present study the specificity

Abbreviations: FBS, fetal bovine serum; SRB, Sulphorhodamine B.

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of valorphin action was studied in a panel of cells of different origin, including normal and tumour cells: L929 (transformed murine fibroblasts), M3 (murine melanoma), HL-60 (human promyelocytic leukaemia), K562 (human erythroid leukaemia), WEHI-3 (transformed murine myelomonocytes), MCF-7 (human breast carcinoma), A549 (human lung carcinoma), ECV304 (human epithelial-related umbilical cord cells expressing endothelial cell markers), EA.hy926 (hybridoma between human umbilical vein endothelial cells (HUVEC) and A549 cells, expressing all major markers of endothelial cells), MEF (murine embryonic fibroblasts), PMBC (primary murine breast carcinoma), murine red bone marrow cells and murine spleen cells. A cell adhesion assay conducted for L929 cells proved the absence of contribution from an anti-adhesive component in the overall cell number reduction induced by valorphin, as the peptide did not inhibit the cell adhesion to gelatin-coated plastic.

The effects of valorphin on long term incubation (72–144 h of incubation) were studied in the most sensitive cell lines: L929, MCF-7, PMBC and A549, by SRB staining (A549 and L929), visual cell count (all cell lines) and clonogenic test (A549 and MCF-7). Daily measurements of cell number changes (by visual cell count, using a microscope) allowed the dynamics of cell population growth in the presence of the peptide to be followed and demonstrated the reversible arrest of proliferation by valorphin and the acquisition of resistance to it by the cells. The parallel colorimetric determination of the effect of valorphin on cell population growth (staining with SRB) confirmed the data obtained by visual cell count. Further, the correlation between the two methods allowed the use of the less laborious method, i.e. the colorimetric method, to demonstrate the temporary character of the resistance. To prove the ability of valorphin to affect cell cycle progression, flow cytometry analysis was carried out. The induction of S-phase arrest by valorphin was demonstrated by measuring DNA content in target cells. The dynamics of cell number change were confirmed cytometrically. Also, the reversible enlargement of cell size accompanying cell cycle arrest induced by valorphin was demonstrated during cytometric analysis.

MATERIALS AND METHODS

Peptide Synthesis

VVYPWTQ was synthesized in the Laboratory of Peptide Chemistry of the Institute of Bioorganic Chemistry by standard symmetric anhydride solid phase Fmoc chemistry [11]. Fmoc deprotection was achieved with 25% piperidine in dimethylformamide. Purification was carried out by RP-HPLC on a Nucleosil 120–7C_{18} column. The homogeneity of the peptide was confirmed by analytical reversed phase HPLC (purity > 97%) and the molecular mass and structure were confirmed by MALDI mass-spectrometry and sequencing respectively.

Cell Culturing

L929, M3, HL-60, K562, WEHI-3, MCF-7, A549, ECV304 cells and MEFs were cultured in RPMI-1640 medium (Gibco BRL) enriched with 10% of FBS (Gibco BRL) and supplemented with 2 nM glutamine (Sigma), 10% of vitamin solution (Flow Laboratories), 100 nM penicillin and 100 μg/ml of streptomycin (Gibco BRL). For MCF-7s the medium was enriched with 0.2% of Spite medium supplement (Sigma). The cells were reseeded every 48 h. ECV304 and EA.hy926 cells were cultured in DMEM/F12 (1 : 1) medium enriched with 10% of FBS.

Preparation of Primary Murine Breast Carcinoma Cell Culture (PMBC)

Murine breast carcinoma cells were isolated from female BLRB mice. Mice were inoculated subcutaneously near the right flank with tumour cell suspensions (approximately 10^7 cells per 0.5 ml of PBS) prepared from syngenic spontaneous breast carcinoma tumours. Four weeks after the injection, the tumour tissues were isolated, cut into 5 mm^2 pieces and incubated in trypsin/versene solution (1/1) for 1 h. A cell suspension was obtained using a Potter homogenizer. The suspension was filtered to remove cell aggregates and the cells were pelleted by centrifugation (1000 × **g**, 10 min). The pellet was resuspended in a RPMI-1640 culture medium containing 10% of FBS and the cell suspension was centrifuged again under the same conditions. After that the cells were suspended again and placed in a 50 ml culture flask. After 2 days of incubation, the murine breast carcinoma cells obtained were re-seeded in assay plates.

Preparation of Murine Embryonic Fibroblasts (MEF)

The cells were obtained from 12 day murine embryos. After removing heads, limbs and tails, the bodies were minced and washed in a serum-free medium and then incubated in 0.25% trypsin solution for 18 h at 4 °C. The suspension obtained was

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separated from the solid and the cells were washed with culture medium and pelleted by centrifugation (rpm 2000, 7 min). The pelleted cells were resuspended in culture medium supplemented with 10% of FBS, and then placed in 50 ml culture flasks.

Preparation of Primary Murine Red Bone Marrow and Spleen Cell Cultures

Murine red bone marrow cells were isolated from five Balb/c mice femora and spleen cells were isolated from spleens of the same mice, using a Potter homogenizer. The cells were washed and suspended in RPMI-1640 medium containing 10% FBS and placed in 96-well plates (500 000 cells/well).

Evaluation of Cytotoxicity and Change of the Cell Number by the Trypan Blue Inclusion Method

Cell count in the presence of trypan blue dye. A cell count in the presence of trypan blue dye allows live cells to be distinguished from dead cells due to the ability of the dye to penetrate cells with damaged membranes. The method was utilized for evaluation of the change of live cell number in a sample relative to a control. This method also permits simultaneous determination of cytotoxicity.

L929, A549, MCF-7, M3, MEF, PMBC, HL-60, K562 and WEHI-3 cells (10 000 per well) were placed in 96-well assay plates (Flow) and incubated for 18 h. The culture medium was then replaced with one containing 1 μM valorphin and the cells were incubated for 18–24 h, depending on the cell line. Cells incubated in the absence of the peptide were used as a control. To desorb MCF-7, PMBC, L929 or MEF cells from assay plates, the culture medium was removed and the cells were treated consecutively with 20 μl of 0.25% trypsin (Sigma) solution (for 1 min) and 40 μl of 0.02% versene solution (for 10 min) (Gibco BRL). A549 cells were treated with 40 μl of 0.25% trypsin/0.02% versene (1 : 1) for 10 min. M3 and WEHI-3 cells were treated with 40 μl of 0.02% versene solution. The resultant cell suspensions were subjected to cell count. HL-60 and K562 cells were counted directly in the culture medium.

After addition of 2–3 μl of Trypan blue dye (Sigma) (0.1% solution in distilled water) to the sample under examination the dead (trypan blue-stained) and the live (non-stained) cell numbers were determined by visual count with a Diavert microscope and Garayev chamber. 200–300 cells were examined in each sample. The live cell concentration in each sample was calculated and the change of live cell number was determined according to Eqn (1)

$$[(E_o - E_x)/E_o] \times 100\% \quad (1)$$

where E_o is the live cell concentration in the control; E_x is the live cell concentration in the experimental sample.

Evaluation of cytotoxicity. The cytotoxic effect was evaluated simultaneously with the change of live cell number. In the course of the cell count, the number of dead (trypan blue stained) cells was determined in each sample and cytotoxicity was calculated according to Eqn (2)

$$\text{(Dead cell number in the sample)}/\text{(Total cell number in the sample)} \times 100\%. \quad (2)$$

Determination of the change of total cell number. The cells (L929, A549, PMBC or MCF-7) were placed in 96-well assay plates at a density of 2500–10 000 cells/well and incubated for 18 h. The culture medium was then replaced with one containing 1 μM valorphin. Cells incubated in the absence of peptide were used as a control. Samples co-incubated with 1 μM epirubicin were used as reference samples. After various incubation periods (24, 48, 72, 96, 120 or 144 h), the total cell concentration in each sample was determined as described above. The change of cell concentration was calculated according to Eqn (1).

Three or four samples for each experimental data point and for the controls were examined within the framework of each independent experiment. The statistical significance of the data obtained in 5–6 independent experiments was estimated as described below.

Evaluation of Antiproliferative Activity by Sulforhodamine B (SRB) Staining

This method for the evaluation of the amount of cell material in samples after a period of incubation corresponding to several proliferation cycles of the target cells is based on the ability of SRB to interact with cellular proteins. The absorption of the reacted dye solution at 540 nm depends linearly on the cell number in the sample in the range 5000–100 000 cells per well of a 96-well assay plate. The method was used for evaluation of cell population growth in experimental samples relative to controls after time intervals corresponding to more than two

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proliferative cycles of the target cells (M3, A549, L929, EA.hy926 and ECV304).

SRB staining. After completion of the experiment, the culture medium was removed from the plates and the cells were air dried. The dried cells were fixed with 100% methanol for 24 h at 4 °C. The methanol was then removed and the fixed cells were air dried and washed consecutively with distilled water (once, 200–300 μl/well for a 96-well plate, 2–3 ml/well for a 12-well plate) and with 1% acetic acid solution in distilled water (four times, 200–300 μl/well for a 96-well plate, 2–3 ml/well for a 12-well plate). Then the cells were incubated with SRB (0.4% solution in 1% acetic acid solution in distilled water): 100 μl/well for a 96-well plate and 1 ml/well for a 12-well plate, for 20 min at room temperature. The dye was then removed and the cells were washed with 1% acetic acid solution in distilled water (five times, 200–300 μl/well for a 96-well plate, 2–3 ml/well for a 12-well plate). Finally, the protein-associated dye was dissolved in 10 mM Tris solution in distilled water (100 μl of the solution for a 96-well plate and 1 ml for a 12-well plate).

Evaluation of antiproliferative activity. Optical densities at 540 nm were determined with a Multiscan MCC/340 spectrophotometer, and the antiproliferative activity (%) was determined according to Eqn (3)

$$[(OD_{540} \text{ of the control} - OD_{540} \text{ of the sample})/ OD_{540} \text{ of the control}] \times 100\%. \quad (3)$$

Twelve observations were made for each experimental point within the framework of each independent experiment. The statistical significance of the data obtained in five independent experiments was estimated as described below.

Evaluation of antiproliferative activity in L929, A549, MCF-7 and M3 cells. The cells were placed in 96-well plates (10 000 cells/well) and incubated for 18 h. The culture medium was replaced with a fresh one containing a test substance (1 μM valorphin or 1 μM epirubicin). Cells incubated in the absence of any test substance were used as a control; cells treated with epirubicin were used as reference samples. The samples were incubated for 96 h. After completion of the experiment, the activity was evaluated as described above.

Evaluation of antiproliferative activity in ECV304 and EA.hy926 cells. The cells were placed in 96-well plates (2000–2500 cells/well) and incubated for 3–4 h. Culture medium containing valorphin was then added so that the peptide concentration in experimental samples was 1 μM or 0.1 μM. Cells incubated without the peptide were used as a control, and cells treated with epirubicin (1 μM) were used as the reference samples. The experimental plates were incubated for 72 h. After completion of the experiment activity was evaluated as described above.

Evaluation of Antiproliferative Activity by MTT Staining

This method was used for the evaluation of live cell numbers in primary cultures of murine red bone marrow and spleen cells. It is based on the determination of intracellular enzymatic activity. The interaction of the major MTT component [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide] with intracellular enzymatic systems leads to the generation of formazan crystals. After the dissolution of the crystals the amount of formazan obtained in the samples is evaluated by photometry at 540 nm.

The cells were incubated with 1 μM valorphin for 96 h and then stained with MTT dye. Cells incubated in the absence of peptide were used as a control, and cells treated with 1 μM epirubicin were used as reference samples. 20 μl of the stock solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (2.5 mg/ml of PBS) were added to each well. The plates were incubated at 37 °C for 2.5 h, then the formazan crystals formed were pelleted by centrifugation and dissolved in 100 μl of 100% dimethylsulphoxide. Finally, 10 μl of 20% aqueous SDS was added to each well. Optical densities in the wells were measured using a Multiscan MCC/340 spectrophotometer at 540 nm. The activity of the substances tested was calculated according to Eqn (3). The statistical significance of the data obtained in five independent experiments was estimated as described below.

L929 Cell Adhesion Assay

Gelatin-coated 96-well plates were prepared. Aliquots (50 μl) of 1% aqueous gelatin solution were added to the wells and immediately removed. The open plates were incubated for 1 h in UV light. Then 100 μl of PBS was added to the wells and the plates were incubated in a CO_2 incubator at 37 °C for 1 h. The PBS was then removed and the wells were washed once with 200 μl of PBS. Then 20 000 cells were seeded in each well, and simultaneously

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0.1 mM valorphin was added to the experimental samples. The wells without peptide were used as negative controls, and wells where 0.02% of EDTA (versene, Sigma) was added were used as positive controls. The plates were incubated for 40 min, then the wells containing cells were washed three times with the culture medium. The samples were then stained with MTT as described above. The antiadhesive activity (%) was calculated according to Eqn (4)

$$[(\mathrm{OD}_{540} \text{ of the control} - \mathrm{OD}_{540} \text{ of the sample})/\mathrm{OD}_{540} \text{ of the control}] \times 100\%. \tag{4}$$

The statistical significance of the data obtained in three independent experiments was estimated as described below.

Statistical Processing of the Results

The statistical significance of the data obtained in 5–6 independent experiments was assessed by the confidence interval: $\mathrm{CI}_{0.01} = st_{0.01,n-1}/\sqrt{n}$, where $\mathrm{CI}_{0.01}$ is the confidence interval with a $p = 0.01$ significance level for a given mean value, s is the corresponding standard deviation, n is the number of the experiments, $t_{0.01,n-1}$ is the coefficient of Student's t-distribution for a probability $p = 0.01$ and number of degrees of freedom $n - 1$. Values exceeding the corresponding confidence interval were assumed statistically significant at the $p < 0.05$ probability level.

Clonogenic Test

A549 or L929 cells were placed in 96-well plates (Flow) (50 000/well) and incubated for 18 h. Valorphin (1 μM) or epirubicin (1 μM) was then added. Cells incubated in the absence of the peptide and epirubicin were used as controls. After 24 h of incubation, cell suspensions were obtained as described above. The average cell concentration was determined for control samples. All the suspensions were diluted 1000 fold. Equal portions of each sample were transferred to the wells of 12-well plates (Nunc). The portion volume corresponded to the volume of the diluted control suspension containing approximately 100 cells. The cells were incubated in 3 ml or RPMI-1640 media supplemented with 10% FBS. The number of colonies containing more than eight cells was determined by microscopic examination at day 6 beginning from the transfer of the cells to the 12-well plates, using a microscope. The percentage of the colonies formed in experimental samples was calculated according to Eqn (5)

$$\text{Colony formation (\%)} = (N_{\text{experiment}}/N_{\text{control}}) \times 100\% \tag{5}$$

where N is the number of colonies consisting of more than eight cells.

Flow Cytometry Analysis of DNA Content in L929 Cells

L929 cells (300 000/flask) were placed in 50 ml culture flasks and incubated for 18 h in 5 ml of RPMI-1640 supplemented with 10% FBS. The medium was then replaced with one containing 1 μM valorphin or 1 μM epirubicin. The control cells were incubated with a fresh portion of medium containing neither peptide nor epirubicin. After 24, 48 and 120 h of incubation, the culture medium was removed and the cells were detached from the plastic as described above and transferred to 15 ml tubes containing 6 ml of PBS. The samples were centrifuged for 7 min at 1500 × **g**, the supernatant was removed, and the cells were resuspended in 3 ml of PBS and centrifuged again under the same conditions. After supernatant removal, the cells were resuspended in PBS (the final volume of each sample was 1 ml) and fixed with 2 ml of 96% ethanol. The samples were stored at 4 °C. Before the beginning of the procedure, the cells in the samples were pelleted at 1500 × **g** and washed three times with 2 ml of PBS by repeated centrifugation/resuspension. After the last resuspension, 0.5 ml of propidium iodide was added to each sample (10 μg/ml propidium iodide dissolved in 50% PBS/50% RNAase H (RNAase H concentration 1 μg/ml)). The samples were incubated for 15 min and subjected to flow cytometry analysis. For reliable and reproducible results, at least 2000 cells were analysed in each sample. Flow cytometry analysis was performed on an EPICS Elite flow cytometer (Coulter Electronics Inc., USA). The intensity of fluorescence of propidium iodide intercalated into DNA molecules at wavelengths over 620 nm was used for determination of DNA content. The intensity of the forward angle light scatter (FALS) was used for cell size (relative diameter) evaluation. The raw data were processed with MultiCycle and MultiGraph software. The cell volumes were calculated according to Eqn (6)

$$V = (4/3)\pi R^3 \tag{6}$$

where R is the radius in arbitrary units.

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The difference between the volume of control cells and the volume of valorphin-treated cells was calculated according to Eqn (7)

$$\Delta V(\%) = [(\text{mean control volume} - \text{mean treated volume}) \times 100\%] : (\text{mean control volume}) \quad (7)$$

Cell Count by Flow Cytometry

L929 cells (100 000/flask) were placed in 50 ml culture flasks and incubated for 18 h in 5 ml of RPMI-1640 supplemented with 10% FBS. The cell culture medium was then replaced with one containing 1 μM valorphin. The control cells were incubated with a fresh portion of culture medium containing no peptide. After 24, 48, 72, 96 and 120 h of incubation the culture medium was removed and the cells were suspended in 1 ml of trypsin/versene solution (1 : 1,v/v) and transferred to 15 ml tubes. After gentle stirring, 0.04 ml of cell suspension was injected into the flow cytometer (EPICS Elite, Coulter Electronics Inc., USA) and a cell count was performed accompanied by determination of FALS intensity. The raw data were processed with MultiCycle and MultiGraph software.

RESULTS

Selectivity of Valorphin Action in Cell Cultures of Various Origin

Several series of experiments aimed at the determination of the selectivity of valorphin action in cell cultures were carried out, using alternative methods of the activity determination. The trypan blue inclusion method for determination of the short term effect of the peptide was applied by us previously for the determination of endogenous peptides activity [4,6,7]. It was applied in most cases (in the cells with a relatively rapid proliferation rate) in the present work as a rapid means of detecting the effect in L929 transformed murine fibroblasts, WEHI-3 transformed murine myelomonocytes, HL-60 human promyelocytic leukaemia, M3 murine melanoma, K562 human erythroid leukaemia, A549 human lung carcinoma, MCF-7 human breast carcinoma cells; two primary cell cultures — murine breast carcinoma (PMBC) and murine embryonic fibroblasts (MEF) — were also included in the study. The ability of the peptide to suppress the live cell number compared with the negative control was determined after 24 h of co-incubation in the presence of 10% FBS. As the results in Table 1 (Figure 1) show, valorphin reduced the cell number in all the transformed cell lines tested, the maximal effect being detected in L929 and MCF-7 cells (64% and 55% reduction of live cell number, respectively). The high activity detected is suggestive of a cytotoxic component in the overall decrease of the cell number. The transformed cells of haematopoietic origin (HL-60 and K562) were significantly less sensitive to the action of the peptide. The data obtained indicate a partial selectivity of valorphin for the cell type. On the other hand, the effect of valorphin on murine embryonic fibroblasts (MEF) was notably weaker than in transformed fibroblasts L929, i.e. in the case of cells with the same phenotype the transformed cells seem more sensitive to the action of the peptide.

Generally, the results obtained demonstrate that valorphin inhibits tumour cell growth significantly, being much less potent in normal cells. The partial selectivity of valorphin action in cell tumour cultures of different origin is also demonstrated.

In the next experimental series the prolonged effect of valorphin was estimated. As with the previous test, the activity was studied both in tumour and normal cell cultures. The most sensitive tumour cell lines (L929, MCF-7, A549 and M3) were chosen for the experiment. After the application of 1 μM valorphin the cells were incubated for 96 h and then stained with SRB. Epirubicin, a

Table 1 Activity of 1 μM Valorphin in Cell Cultures of Various Origin

Cell line	Activity (%)
L929 murine transformed fibroblasts	64 ± 8^a
MCF-7 human breast carcinoma	55 ± 3^a
A549 human lung carcinoma	47 ± 6^a
M3 murine melanoma	42 ± 6^a
Primary murine breast carcinoma (PMBC)	42 ± 9^a
WEHI-3 murine transformed myelomonocytes	38 ± 7^a
K562 human erythroid leukaemia	27 ± 5^a
HL-60 human promyelocytic leukaemia	23 ± 3^a
Murine embryonic fibroblasts (MEF)	13 ± 6

The cells were co-incubated with valorphin for 18–24 h, then the activity was evaluated by the trypan blue inclusion method.

[a] Values with CV < 30%.

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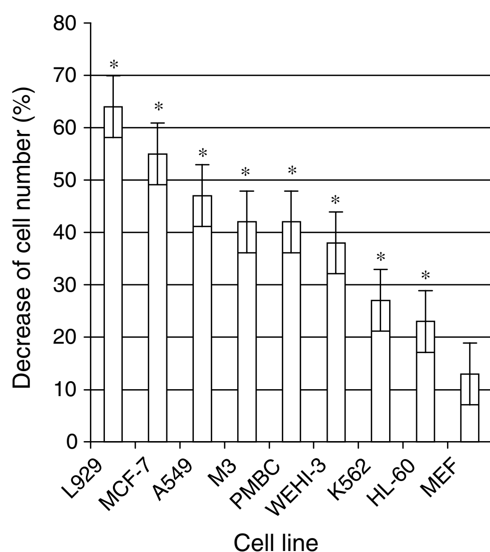

Figure 1 The activity of 1 μM valorphin in cell cultures: L929 transformed murine fibroblasts, MCF-7 human breast carcinoma, A549 human lung carcinoma, M3 murine melanoma, WEHI-3 transformed murine myelomonocytes, K562 human erythroid leukaemia, HL-60 human promyelocytic leukaemia, primary murine breast carcinoma (PMBC) and murine embryonic fibroblasts (MEF). The cells were co-incubated with valorphin for 18–24 h, then the activity was evaluated by the trypan blue inclusion method. *Values with CV < 30%.

standard cytotoxic drug, was used as a reference. The results obtained are shown in Figure 2A. As in the previous test, the most sensitive cells were L929 cells (antiproliferative effect 68%). In MCF-7, A549 and M3 cells, the activity was lower (48%, 48% and 42%, respectively), which also corresponds to the sensitivity of these cells to the action of the peptide demonstrated by trypan blue inclusion test. The SRB test demonstrated not only the correlation of the alternative methods but also the prolonged action of valorphin.

Using the SRB assay, the activity of valorphin was also tested in cell lines expressing endothelial cell markers, which was not included in the earlier trypan blue assay — the cell lines were ECV304 human epithelial-related umbilical cord cells expressing some endothelial cell markers, and EA.hy926 cells, a hybridoma of HUVEC and A549 epithelioma which expresses all major endothelial cell markers. The latter cell line has been used extensively as an endothelial cell model [12–14]. The permanent line ECV304 was first described in 1990 as a spontaneously transformed human umbilical vein endothelial cell (HUVEC) line [15,16].

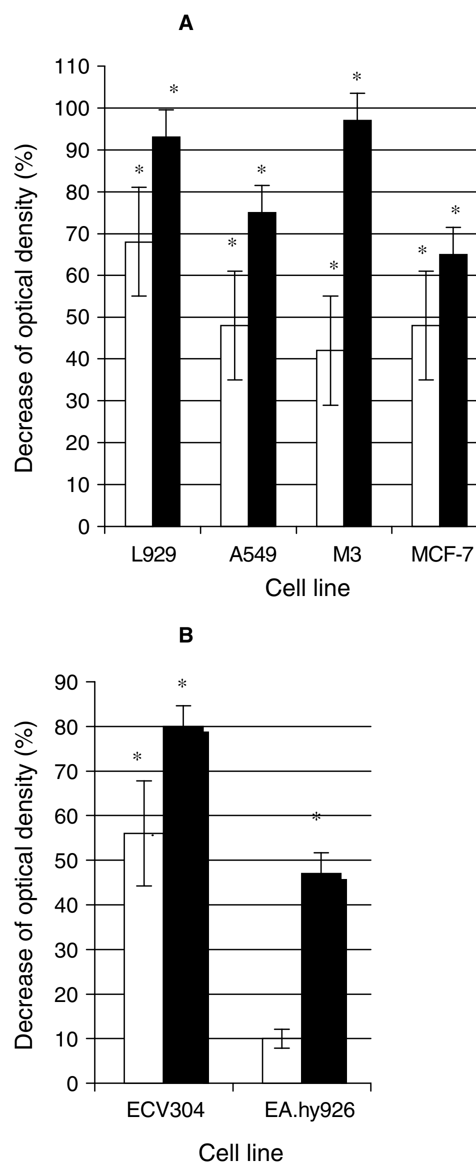

Figure 2 The long-term effect of 1 μM valorphin (light columns) and 1 μM epirubicin (dark columns) in tumour cells, evaluated by SRB staining. (A) Effects in L929, A549, M3 and MCF-7 cells. The cells were co-incubated with the test substances for 96 h. (B) Effect of valorphin in ECV304 human epithelial-related umbilical cord cells expressing endothelial cell markers EA.hy926 hybridoma (HUVEC/A549) cells. The cells were co-incubated with the test substances for 72 h. $^{\dagger}p < 0.01$.

ECV304 cells were shown to have most of the properties of HUVEC, including the expression of different surface and secreted molecules [16–18]. According to reports from cell banks in Europe and the USA in Spring 2000, the ECV304 cell line was found identical to the T-24 human urinary

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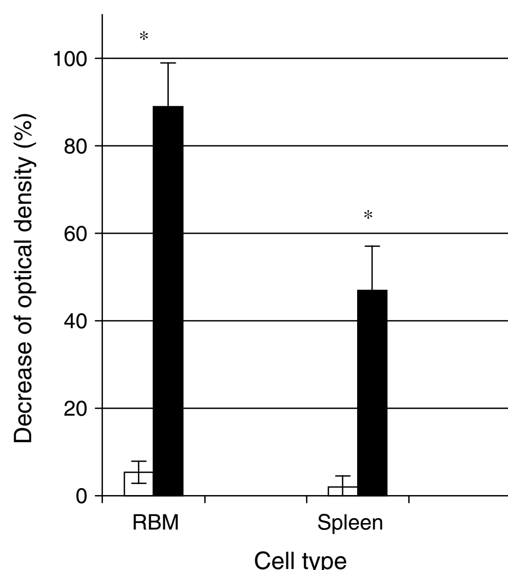

Figure 3 The activity of 1 μM valorphin (light columns) and 1 μM epirubicin (dark columns) in primary cultures of normal cells: murine red bone marrow (RBM) and murine spleen cells. The cells were co-incubated with the tested substances for 72 h, then the activity was evaluated by MTT staining. $^{*}p < 0.01$.

bladder carcinoma line, but researchers continue to find the biomarkers of typical endothelial cells when studying the ECV304 line which implies that this line could be, like the EA.hy926 cell line, considered as a model of human endothelial cells. In the both cell lines, the effect was measured after 72 h of co-incubation with valorphin. As seen in Figure 2B, the reduction of ECV304 cell number was 56%, i.e. the tested line was also sensitive to the action of the peptide, while in EA.hy926 cells the peptide exhibited no activity, both cell lines being sensitive to epirubicin action. The observed difference in sensitivity of the tested cells to the action of valorphin points to a lack of correspondence of ECV304 cells to the endothelial cell phenotype. The latter fact should lead to the restriction of ECV304 cell line involvement in antiangiogenic assays.

Finally, the activity of valorphin in primary cultures of murine red bone marrow and spleen cells was determined by MTT staining after 72 h of co-incubation, as another example of its action on normal cells. Figure 4 shows the comparison of 1 μM valorphin activity in red bone marrow cells and spleen cells with the activity of 1 μM epirubicin. In contrast to epirubicin, valorphin did not significantly affect cell viability/proliferation.

Summarizing, tumour cells appear to be much more sensitive to valorphin than normal cells. In contrast, epirubicin has comparable toxicity towards tumour and normal cells.

The Absence of an Antiadhesive Component in the Action of Valorphin

In all the above experiments, the cell number reduction induced by valorphin may be at least partially ascribed to the inhibition of cell adhesion to the well bottom, as the culture medium was removed prior to the cell count or staining with SRB. To study the ability of valorphin to influence cell adhesion, the adhesion of L929 cells to gelatin-coated plastic was tested in the presence of the peptide. Gelatin-coated plastic was used because of its greater sensitivity compared with non-coated plastic. Valorphin (0.1 mM) was added to the cell suspension (200 000 cells/ml) as the cells were placed in the wells (20 000 cells/well). A cell suspension containing 0.02% EDTA was used as a positive control. After 40 min of incubation, the wells containing cells were washed with medium and fresh medium was added. The effect was determined by MTT staining. It was found that valorphin had no effect on adhesion to gelatin-coated plastic: the average effect was 4% ± 4% compared with the negative control (in the samples with 0.02% of EDTA the adhesion was inhibited by 90% ± 2%). Thus, it seems that an inhibition of adhesion did not contribute to the detected overall cell number decrease induced by valorphin.

The Anticlonogenic Potential of Valorphin

A clonogenic test was carried out in L929 and A549 cell lines in order to study the prolonged valorphin action in more detail. The clonogenic potential of the cells subjected to short-term treatment with 1 μM valorphin (24 h, after which the peptide was removed) was determined and compared with the non-treated cells (control). The total number of colonies and the percentage of colonies with a normal proliferation index (more than eight cells in the colony), were determined by direct counting using a microscope. The data obtained are summarized in Tables 2,3. The results demonstrate a reduction of the number of cells able to form colonies on the one hand, and a suppression of proliferation in the colonies after the peptide removal on the other (because there is a reduced cell number per colony). The latter points to a prolonged action of the peptide. The data obtained allow the

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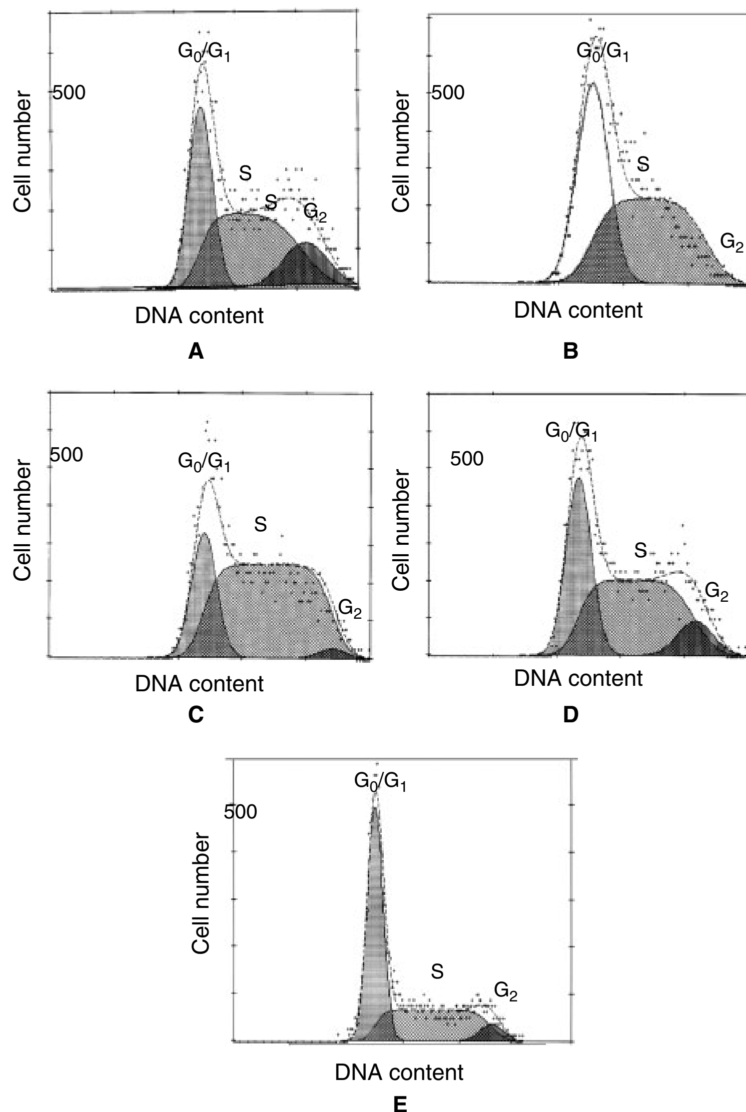

Figure 4 The DNA content of L929 cells measured by flow cytometry: (A) control: normally proliferating cells in the presence of 10% FBS; (B) after 24 h of co-incubation with 1 μM epirubicin; (C) after 24 h of co-incubation with 1 μM valorphin; (D) after 48 h of co-incubation with 1 μM valorphin; (E) after 120 h of co-incubation with 1 μM valorphin. The *x*-axis shows the linear fluorescence intensity of propidium iodide in arbitrary units corresponding to the DNA content in a cell. Minimal DNA content corresponds to cells containing non-duplicated DNA (cells in G0 or G1 phase), intermediate DNA content corresponds to cells containing duplicating DNA (S phase) and maximal DNA content to the cells containing duplicated DNA (G2/M phase). The *y*-axis shows the cell count (the number of cells with respective fluorescence).

suggestion that the inhibitory effect of valorphin on the growth of transformed cell cultures may be due to the two processes — to cytolysis as demonstrated earlier for L929 cells [7], and to the inhibition of proliferation, a prolonged effect, which was demonstrated in the clonogenic test as a reduced proliferation index of the cells in the colonies formed by cells treated with the peptide. On the other hand, the cell number in the colonies formed by the valorphin-treated cells was notably higher than in epirubicin-treated samples; i.e. valorphin-induced inhibition of proliferation seems to be reversible.

The Reversibility of Valorphin-induced Proliferation Inhibition and the Acquisition of Reversible Resistance by the Target Cells

To study the long-term dynamics of cell proliferation in the presence of valorphin, a series of experiments

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Table 2 Effect of 1 μM Valorphin in Clonogenic Test in L929 Cells

Sample	Number of cells/colony			Total	Colony formation (%)
	>28	12–28	8–11		
Negative control	15 ± 2	51 ± 2	27 ± 2	93 ± 6	100
1 μM valorphin	2 ± 1	13 ± 1	9 ± 1	24 ± 3	26
1 μM epirubicin	0	0	0	0	0

The number of colonies containing the corresponding number of cells per colony is given. Colony formation was calculated as a percentage relative to control, Eqn (4).

Table 3 Effect of 1 μM Valorphin in Clonogenic Test in A549 Cells

Sample	Number of cells/colony			Total	Colony formation (%)
	>16	8–15	4–7		
Negative control	13 ± 3	16 ± 4	12 ± 3	41 ± 6	100
1 μM valorphin	5 ± 2	7 ± 2	8 ± 3	20 ± 5	49
1 μM epirubicin	0	0	0	0	0

The number of colonies containing the corresponding number of cells per colony is given. Colony formation was calculated as a percentage relative to control, Eqn (4).

was carried out in which transformed cells (L929, MCF-7, PMBC or A549) were incubated in the presence of 1 μM valorphin for 96–120 h. After each 24 h interval, the culture medium in all samples was replaced with fresh medium containing no peptide in the control samples and freshly dissolved 1 μM valorphin in the experimental samples. A cell count was performed using a microscope after each day of the experiment, both in control and experimental samples. This approach allowed the determination of the dynamics of target cell proliferation in the presence of the peptide. The results are presented in Tables 4–7.

In all cell lines, the first application of the peptide induced the arrest of cell proliferation for approximately 24 h. After 48 and 72 h of co-incubation, the difference in cell number between the control and the experimental samples remained unchanged, indicating that the cells in the control and the experimental samples proliferated with similar rates. In other words, in all cell lines studied valorphin induced a reversible and relatively short-lived inhibition of cell proliferation. Repeated applications of the peptide did not affect the proliferation rate. In L929 cells, the re-application of valorphin after 96 h again decreased the proliferation rate in the experimental samples, pointing to the temporary character of the resistance acquired by the target cells.

The results obtained demonstrate that the delay of proliferation in transformed cells induced by valorphin was relatively short-term. The delay was accompanied by the acquisition of resistance to the further action of the peptide. The two effects were observed in all the cell lines tested. The prolonged co-incubation with L929 cells revealed the temporary character of the resistance to the action of the peptide. These conclusions are also in accordance with the results obtained for A549 and L929 cells by SRB staining after 96 h co-incubation with valorphin: the correlation between the results obtained after the single peptide application (SRB staining) and after regular refreshment of the

Table 4 L929 Cell Proliferation in the Presence of 1 μM Valorphin

Incubation time (h)	Cell number/well		Decrease of cell number (%)
	Control	Valorphin	
24	9350 ± 1220	5400 ± 100	42
48	27 080 ± 540	12 640 ± 2150	53
72	48 200 ± 2900	31 107 ± 4360	36
96	97 800 ± 17 600	56 030 ± 2250	43
120	168 000 ± 23 520	66 500 ± 16 630	60
144	210 400 ± 40 000	85 200 ± 2560	59

The effect was determined by visual cell counting in a Garayev chamber. The initial cell density was 2500–3000 cells/well of a 96-well assay plate.

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Table 5 A549 Cell Proliferation in the Presence of 1 μM Valorphin

Incubation time (h)	Cell number/well		Decrease of cell number (%)
	Control	Valorphin	
24	24 450 ± 1470	11 640 ± 100	52
48	40 240 ± 2410	18 640 ± 2050	54
72	69 800 ± 4900	33 760 ± 340	52
96	107 940 ± 8640	64 000 ± 640	41

The effect was determined by visual cell counting in a Garayev chamber. The initial cell density was 5000 cells/well of a 96-well assay plate.

Table 6 MCF-7 Cell Proliferation in the Presence of 1 μM Valorphin

Incubation time (h)	Cell number/well		Decrease of cell number (%)
	Control	Valorphin	
24	9490 ± 2800	2440 ± 700	74
48	12 430 ± 3200	3120 ± 1100	74
72	17 960 ± 2610	4580 ± 850	74

The effect was determined by visual cell counting in a Garayev chamber. The initial cell density was 5000 cells/well of a 96-well assay plate.

Table 7 Primary Murine Breast Carcinoma (PMBC) Cell Proliferation in the Presence of 1 μM Valorphin

Incubation time (h)	Cell number/well		Decrease of cell number (%)
	Control	Valorphin	
24	6580 ± 1000	3890 ± 920	41
48	8630 ± 450	2760 ± 770	68
72	11 840 ± 1200	4030 ± 120	66

The effect was determined by visual cell counting in a Garayev chamber. The initial cell density was 7000 cells/well of a 96-well assay plate.

peptide portion in the case of visual counting, points to the action of valorphin being confined to the short period following its application.

In order to confirm the temporary character of the resistance acquired by the target cells, SRB staining was used to compare the effect of the peptide measured after two various periods of incubation: (1) the incubation period exceeding the resistance period (96 h) determined by visual cell count for L929 cells; (2) the incubation period being shorter than the resistance period. A549 and MCF-7 cells were incubated with 1 μM valorphin or 1 μM epirubicin for 96 or 144 h. As seen from Table 8, in both cell lines the effect of valorphin was significantly higher 144 h after the first peptide application than after 96 h. For instance, in the case of A549 cells the effect was 83% and 48%, respectively; at the same time, the effect of 1 μM epirubicin used as a reference substance reached its maximal value after 96 h of co-incubation, and did not change subsequently. Valorphin induced the temporary resistance to itself and the duration of this resistance was not less than 96 h after the first peptide application. Being observed in all cell lines tested, the effect seems to be independent of cell line origin.

The Influence of Valorphin on Cell Cycle and Size of Transformed Cells Analysed by Flow Cytometry

Flow cytometry was used to investigate the pattern of cell cycle disturbance by valorphin. The following parameters were determined: the fraction of apoptotic cells, cell size and DNA content. The DNA content was measured using the intercalating fluorescent dye propidium iodide, fluorescence in the samples being measured at wavelengths > 620 nm. The proportions of DNA forms (duplicated, non-duplicated and duplicating DNA) in a sample correlate with the proportions of cells in the cell cycle phases G2, G1/G0 and S, respectively. Using a logarithmic scale, the share of cells carrying fragmented DNA (apoptotic cells) was determined. Forward angle light scatter was measured in the

Table 8 Effects of 1 μM Valorphin and 1 μM Epirubicin in A549 and MCF-7 Cells after 96 and 144 h of Co-incubation

Test substance	Antiproliferative effect (%)			
	A549		MCF-7	
	96 h	144 h	96 h	144 h
Valorphin	48 ± 7	83 ± 8	48 ± 10	75 ± 5
Epirubicin	91 ± 1	97 ± 0	65 ± 22	75 ± 5

The effect was determined with SRB staining as the decrease of extinction at 540 nm in the samples relative to the control. All values are statistically significant ($p < 0.05$).

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cell samples in order to obtain the distribution of cells according to their diameter in arbitrary units.

L929 cells were co-incubated with 1 μM valorphin for 120 h, with daily refreshment of the valorphin dose. Cytometric measurement of DNA content was carried out at 24, 48 and 120 h after the first application of peptide. The results obtained are summarized in Figure 4. As follows from diagram (C) obtained after 24 h, valorphin induced cell cycle arrest in the S phase, the cell number in this phase in the samples containing valorphin being 22% higher than in the corresponding control [diagram (A), characteristic for normally proliferating cells]. The diagram obtained after 48 h (D) points to the restoration of proliferation in the presence of valorphin; so the peptide delayed cell cycle progression only for 24 h beginning from its first application and then the cells became resistant to further action of the peptide. The diagram obtained at 120 h (E) demonstrates that the proliferation was again disturbed, though to a lesser extent than in the case of 24 h co-incubation. Thus, after completion of the relatively long refractory period, 5 days after the first application of the peptide, the cells again became sensitive to it. Epirubicin used as a reference substance, also induced cell cycle arrest in the S phase, this arrest being irreversible [diagram (B), 24 h].

Comparison of the number of apoptotic cells detected by cytometry after a 24 h co-incubation with valorphin (22% ± 5%, data not shown), with the cytotoxicity determined after the same time period by the trypan blue inclusion method (34% ± 8%) indicated the involvement of two processes in the total cell number decrease induced by the peptide, namely cytotoxicity and inhibition of proliferation.

In a parallel group of experiments, cell numbers and cell sizes in the samples were determined daily, the cytometrically obtained data being accompanied by visual cell counts. Cytometry (Figure 5) showed that the decrease of L929 cell number 24 h and 120 h after the first peptide application was 57% ± 8% and 76% ± 7%, respectively (Table 9), i.e. the dynamics of cell population growth in the presence of valorphin was confirmed cytometrically.

Finally, the cytometric analysis of cell size in the samples in both groups of experiments demonstrated an increase in the mean relative cell volume by 20% in the presence of valorphin after 24 h from the first peptide application, and again after 120 h (Figure 6). During the 48–96 h from the first peptide application the cell size corresponded to that in the control. So the arrest of cell division by valorphin was accompanied by an increase of mean cell size; after restoration of the ability to divide, the cell size returned to normal.

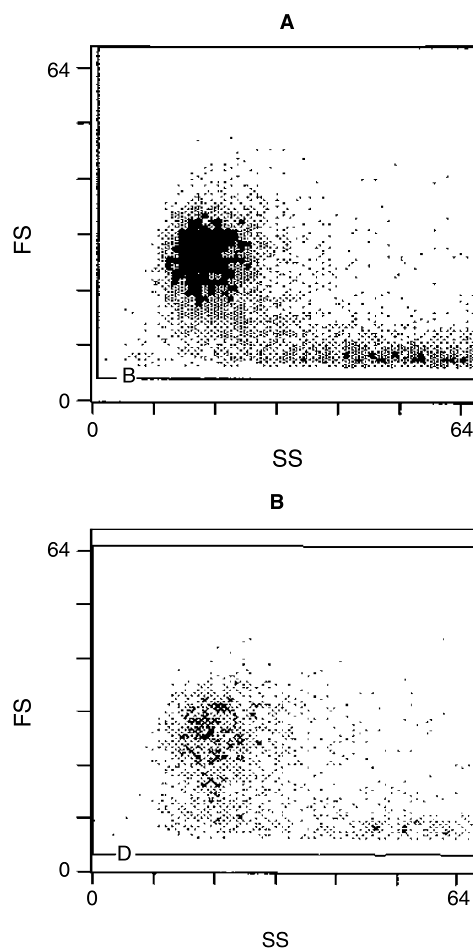

Figure 5 Flow cytometry L929 cell counts. (A) control; (B) cells incubated with 1 μM valorphin for 24 h. The x-axis shows the side scatter intensity (SS) in arbitrary units, which correlates with the granularity of the cell surface. The y-axis shows the forward scatter intensity (FS) in arbitrary units, which correlates with cell size. Each point on the plot corresponds to an individual cell.

Taken together, the results obtained by flow cytometry confirm that one of the most active components of tissue-specific peptide pools — valorphin — induces reversible inhibition of cell proliferation and reversible resistance to its further action of relatively long duration.

DISCUSSION

Valorphin belongs to a group of endogenous agonists of opiate receptors. The ability of naloxone to inhibit

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Table 9 Change of L929 Cell Number and of Mean Cell Volume During Co-incubation with 1 μM Valorphin

Incubation time (h)	Increase of mean cell volume relative to the control (%)[a]	Decrease of cell number relative to the control (%)[b]
0	0	—
24	>20	56 ± 1
48	<2	47 ± 2
72	<2	52 ± 4
96	>12	57 ± 11
120	>20	76 ± 7

[a] The diameter of the cells (D) in arbitrary units was determined by flow cytometry analysis and the volumes were calculated according to Eqn (4), where $R = D/2$.
[b] The given values are the average of the activity determined by the visual cell count and by flow cytometry analysis.

valorphin cytotoxicity points to the involvement of opiate receptors in its action on tumour cells [7].

In spite of the rather low affinity of valorphin towards opiate receptors [19], this peptide, like classical opioid peptides, effectively inhibits tumour cell growth. Our detailed study of the effect of valorphin on a panel of cell cultures of different origin shows that this peptide is more potent in reducing the number of transformed cells of fibroblastic and epithelial origin, than it is in reducing transformed haematopoietic cells. On the other hand, there is a notable difference between the effect of valorphin in transformed and normal cells, the effect being more pronounced in cells with a transformed phenotype. The data obtained earlier have demonstrated that haemorphins are present in lung, heart, brain and spleen in amounts (3–44 nmol/g of tissue, dependently on tissue type) sufficient for manifestation of antiproliferative and cytotoxic activity in cell cultures [8]. The data on the *in vivo* content of haemorphins, together with their effects in cell cultures speak of participation of this family of peptides in cell growth inhibition. The demonstrated restriction of growth inhibitory effects of valorphin on cells with a transformed phenotype may point to its direct involvement in antitumour defence *in vivo*.

The experiments carried out with L929 cells allowed the action of valorphin to be described as shown in Figure 7. After the first application the peptide induced cell cycle arrest in the S phase, for a period approximately corresponding to one proliferative cycle. This arrest was reversible, as the cells recovered their ability to divide. At the same time, the proliferating cells became non-sensitive to the further action of the peptide. The induced resistance may be due to the desensitization of

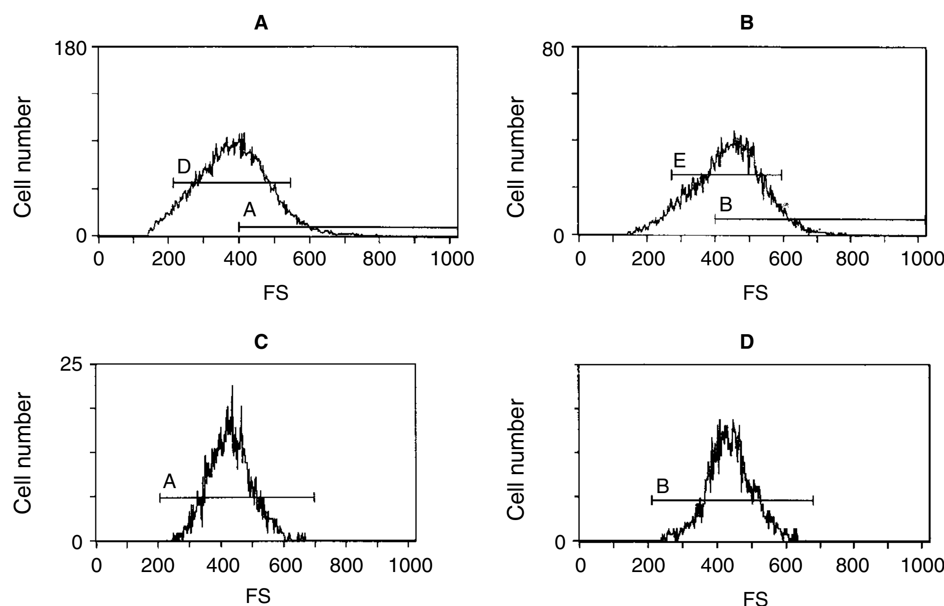

Figure 6 Cytometric measurement of forward angle light scatter for L929 cells. (A, C) — control; (B) cells incubated with 1 μM epirubicin for 24 h; (D) cells co-incubated with 1 μM valorphin for 24 h. The x-axis shows the forward scatter intensity (FS) in arbitrary units.

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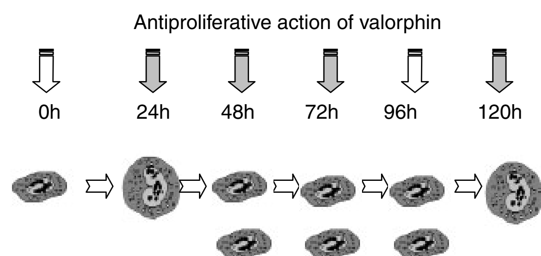

Figure 7 Induction of reversible proliferation arrest and reversible resistance in transformed cells by valorphin during the course of 120 h incubation with the peptide. The arrows illustrate the scheme of the peptide application, the dark arrows correspond to the periods of resistance.

certain stages involved in the transduction of cell cycle arresting signals.

It should be noted that the induction of reversible cell cycle arrest has been demonstrated for some classical opiate agonists: [Met^5]-enkephalin reversibly depresses the growth of HT-29 human colon cancer cells and human neuroblastoma SK-N-SH, the action being dose-dependent and not cytotoxic [20,21]. However, this phenomenon, i.e. the induction of reversible cell proliferation arrest, is not confined to peptide opiate agonists. In the last decade, a family of oligopeptide growth inhibitors which have several common features has been identified [22–24]. All the peptides have a substituted *N*-terminus (which makes them more resistant to aminopeptidases), a very low optimal active dose range (usually picomolar doses when given both *in vivo* and *in vitro*), bell-shaped dose-response curves, and, like valorphin, distinctive cell/tissue preferences [24]. Like valorphin, these peptides reversibly inhibit the proliferation of both tumour and normal cells [22,23,25–28]. The peptides which are 3–5 amino acid residues in length can be generated from a great number of precursors and subjected to further *N*-terminal modification, while the longest peptide (pyroEDDSDEEN) is identical to a *C*-terminal fragment of the largest subunit of RNA polymerase II. These or very similar sequences are present in a great number of transcription factors, protooncogenes and tumour-suppressive proteins. For some of them it has been shown that, unlike valorphin, they penetrate the cell and interact with intracellular targets [22, 28]. The properties of two peptides of this group, namely pyro-EEDSG and pyro-EHG, are even closer to that of valorphin: the treatment of target cells with these peptides is followed by a refractory period during which repeated treatment with the same peptide has no effect [23,24]. In the case of valorphin, for the first time the duration of the resistance period has been precisely determined.

We believe that the effects described here for valorphin are common for several other peptides earlier defined by us as 'components of tissue-specific peptide pools' [5,29,30] which arise in the course of endogenous degradation of functional proteins. The action of such peptides might include suppression of excessive proliferation of normal cells. In this case, such 'soft' regulation does not lead to significant cell loss. On the other hand, if malignant neoplasia is present, such peptides may be directly involved in the inhibition of tumour growth.

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