Antitumor activity of the alkylating oligopeptides J1 (L-melphalanyl-*p*-L-fluorophenylalanine ethyl ester) and P2 (L-prolyl-*m*-L-sarcolysyl-*p*-L-fluorophenylalanine ethyl ester): comparison with melphalan

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Peptichemio, a mixture of six short oligopeptides all comprising the alkylating amino acid *m*-L-sarcolysin, has shown clinical activity in several malignancies. Previous studies have suggested that activity mainly resides in one of the peptides, P2 (L-prolyl-*m*-L-sarcolysyl-*p*-L-fluorophenylalanine ethyl ester). In the present study the *in vitro* activity of P2 was further investigated and compared to melphalan and the novel alkylating dipeptide J1 (L-melphalanyl-*p*-L-fluorophenylalanine ethyl ester), which is structurally related to P2 and melphalan. Cytotoxic activity was studied using patient tumor cells in a non-clonogenic cytotoxicity assay, whereas cellular response, and kinetics thereof, were studied in the lymphoma cell line U-937 GTB. Cellular metabolism was studied using microphysiometry, kinetic effects on macromolecular synthesis by radiolabeled substrate incorporation and, finally, the microculture kinetic assay of apoptosis was used to monitor morphologic changes following drug exposure. The assays compared P2 favorably with melphalan. Interestingly J1 was even more cytotoxic, and produced more pronounced effects in the kinetic assays for macromolecular synthesis, metabolic activity and apoptosis. The results indicate that the delivery properties of J1 are improved compared to those of melphalan and P2. *Anti-Cancer Drugs* 14:617–624 © 2003 Lippincott Williams & Wilkins.

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Introduction

Peptichemio (PTC) is a mixture of six oligopeptides all containing *m*-[*bis*(2-chloroethyl)amino]-L-phenylalanine (*m*-L-sarcolysin, an isomer of melphalan). PTC was developed in the 1960s and has been extensively studied for its interesting cytotoxic profile. The main rationale for developing PTC was to create a drug with high cytotoxic activity due to the presence of an alkylating group and that at the same time provided a selective uptake into neoplastic cells [1]. PTC has shown clinical activity in several human malignancies both as a single agent (e.g. in advanced breast cancer [2] and ovarian cancer [3]) as well as part of combination therapy (e.g. in non-Hodgkin's lymphomas [4]). In addition, cytotoxic activity has also been demonstrated in tumors resistant to other alkylating agents, e.g. breast cancer resistant to cyclophosphamide [5] and plasma cell neoplasms previously treated with combination chemotherapy including melphalan [6] or melphalan/cyclophosphamide [7].

Studies on the individual oligopeptides of PTC showed that L-prolyl-*m*-L-sarcolysyl-*p*-L-fluorophenylalanine ethyl ester (P2) possessed higher activity than the other five oligopeptides. In fact it was several-fold more toxic to human melanoma cells than *m*-L-sarcolysin alone when tested in clonogenic [8] as well as non-clonogenic [9] cytotoxicity assays. This was, at least partly, explained by increased DNA cross-linking after P2 exposure [8]. Further *in vitro* studies of the peptides confirmed the higher cytotoxic effect of P2 compared to both the other oligopeptides in PTC and to the alkylating amino acid derivatives *m*-L-sarcolysin and melphalan. In addition, P2 was found to be more active against slow growing cell lines, was less affected by intracellular GSH levels, demonstrated low levels of cross-resistance with standard drugs and showed a positive *in vitro* therapeutic index [10].

In the present study we have further explored the pharmacological differences *in vitro* between P2, melphalan and a novel alkylating dipeptide. This dipeptide, L-melphalanyl-*p*-L-fluorophenylalanine ethyl ester, J1, was designed as an intermediate between melphalan and P2. The activity of the three compounds was

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compared in several assays and the previously observed advantages of P2 compared to melphalan were confirmed. However, J1 was shown to be even more active than P2 in all assays. The data presented here support the basic idea that peptide derivatives may provide a useful delivery strategy for introducing alkylating agents into tumor cells.

Materials and methods

Cells

Primary cultures of human tumor cells from 27 patients were used to determine the cytotoxic activity of the test compounds. Fifteen hematological (five acute lymphocytic leukemias, three acute myelocytic leukemias, two chronic lymphocytic leukemias and five non-Hodgkin's lymphomas) and 12 solid tumor samples (three ovarian carcinomas, two mesotheliomas, and one each of adrenocortical cancer, breast cancer, corpus uteri cancer, non-small cell lung cancer, gastric cancer, pheochromocytoma and renal adenocarcinoma) were used to determine the dose–response relationship for J1, P2 and melphalan. Ten of the patients had previous chemotherapy. In addition, most of the samples were analyzed for sensitivity to some standard drugs of different mechanistic classes representing therapeutic alternatives for that group of diagnosis (one or two concentrations per drug, empirically derived cut-off concentration, see below). The tumor samples were obtained by bone marrow/peripheral blood sampling, routine surgery or diagnostic biopsy and this sampling was approved by the local ethics committee at the Uppsala University Hospital. Leukemic cells were isolated from bone marrow or peripheral blood by 1.077 g/ml Ficoll-Paque (Kabi-Pharmacia, Uppsala, Sweden) density gradient centrifugation [11]. Tumor tissue from solid tumor samples were minced into small pieces and tumor cells were then isolated by collagenase dispersion followed by Percoll (Kabi-Pharmacia) density gradient centrifugation [12]. Cell viability was determined by the Trypan blue exclusion test and the proportion of tumor cells in the preparation was judged by visual inspection of May–Grünewald–Giemsa-stained cytospin preparations by a cytopathologist. Cells were in general cryopreserved in a culture medium containing 10% dimethylsulfoxide (DMSO; Sigma, St Louis, MO) and 50% heat-inactivated fetal calf serum (FCS; HyClone, Cramlington, UK) by initial freezing for 24 h at –70°C, followed by storage in liquid nitrogen. Cryopreservation in this way does not affect drug sensitivity [13].

The histiocytic lymphoma cell line U-937 GTB [14] was used for studies of macromolecular synthesis, cellular metabolism and the microculture kinetic assay of apoptosis. The cell line was a kind gift from K. Nilsson (Department of Pathology, Uppsala University, Sweden). Cells were cultured in complete RPMI 1640 medium (see below), split twice weekly and harvested in log-phase. U-937 GTB cells are highly sensitive to the toxic actions of P2 [10].

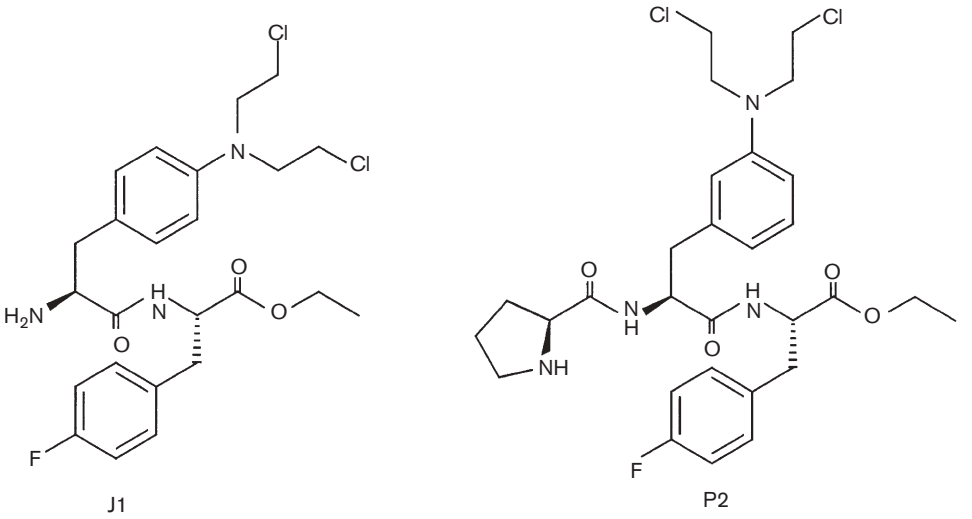
Reagents and drugs

Cell culture medium RPMI 1640 (HyClone) supplemented with 10% heat-inactivated FCS (HyClone), 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin was used for cytotoxicity and Cytostar experiments. A low buffering RPMI 1640 medium (National Veterinary Institute, Uppsala, Sweden) supplemented the same way was used for the Cytosensor experiments and for the microculture kinetic assay of apoptosis (MiCK) a complete supplemented medium without phenol red (Sigma) was used. Fluorescein diacetate (FDA; Sigma) was dissolved in DMSO to 10 mg/ml and kept frozen as a stock solution in the dark. [^{14}C]Thymidine (Amersham, Little Chalfont, UK; CFA.532l, 56 mCi/mmol, 50 μCi/ml) and [^{14}C]leucine (Amersham; CFB.183, 56 mCi/mmol, 50 μCi/ml) were handled according to the manufacturer's instructions and stored in refrigerated containers until use.

Melphalan (Alkeran; GlaxoWellcome London, UK) and all other standard drugs (cisplatin, cladribine, cytarabine, docetaxel, doxorubicin, etoposide, 5-fluorouracil, gemcitabine, topotecan, vincristine and vinorelbine) were obtained from commercial sources, and dissolved according to instructions from the manufacturer. The standard drugs were tested in the fluorometric microculture cytotoxicity assay (FMCA) at the empirically derived cut-off concentration, defined as the concentration that produces a significant scatter of survival index (SI) values among human tumor samples [11,15].

P2 (L-prolyl-*m*-L-sarcolysyl-*p*-L-fluorophenylalanine ethyl ester, hydrochloride salt, 632.06 g/mol) was a kind gift from Istituto Sieroterapico, S. Belfanti, Milan, Italy. The synthesis of J1 (L-melphalanyl-*p*-L-fluorophenylalanine ethyl ester, hydrochloride salt, 534.93 g/mol) will be described elsewhere. Molecular structures of P2 and J1 are given in Figure 1. For initial cytotoxicity experiments the peptides were dissolved in ethanol acidified with 10% concentrated HCl (aq.) to 8.20 mM (corresponding to 2.50 mg/ml melphalan, 5.18 mg/ml P2 and 4.38 mg/ml J1). These solutions were further diluted 10 times with sterile water before plating, giving a final highest concentration of 82 μM (corresponding to 25 μg/ml melphalan) when cell suspension was seeded to the plates. Due to the high toxicity in the initial samples the maximum concentration was decreased to one-fifth (i.e. 16.4 μM) by dilution with sterile water. For all other experiments the peptides were dissolved in absolute ethanol to 4.0 mM and further diluted with sterile water. The maximum ethanol concentrations did not produce any effects in the assays used (not shown). Time in

Fig. 1



Structures of the melphalan-containing dipeptide J1 [containing the *bis*(2-chloroethyl)amino-group in the *para* position of the aromatic ring] and the *m*-L-sarcolysin-containing tripeptide P2 [containing the *bis*(2-chloroethyl)amino group in the *meta* position of the aromatic ring].

aqueous solution was always kept minimal to limit the influence of mustard hydrolysis (J1, P2 and melphalan).

FMCA

The FMCA has been presented in detail previously [11]. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were prepared with 20 µl drug solution at 10 times the desired concentration and stored for up to 2 months at −70°C. The three test compounds were tested at five different concentrations (obtained by 5-fold serial dilution) in triplicate and six wells with 20 µl phosphate buffered saline (PBS) served as control. At day 0 of the experiment $50–100 \times 10^3$ cells in 180 µl fresh medium was added to the wells of the thawed plate; six blank wells received cell medium only. After 72 h incubation the cells were washed once with PBS and 100 µl of fluorescein diacetate (10 µg/ml) in a physiological buffer was added. After another 40 min incubation the generated fluorescence (ex. 485; em. 528 nm) was measured in a 96-well scanning fluorometer (Fluoroscan II; Labsystems, Helsinki, Finland). The generated fluorescence is proportional to the number of living cells, and data are presented as survival index (fluorescence in test wells in percent of control wells with blank values subtracted) and as IC_{50} values (inhibitory concentration 50%). Quality criteria for a successful assay include the following: a coefficient of variation less than 30% in the blank, control and test wells, a control signal more than 5 times the blank and, finally, more than 70% tumor cells in the cell preparation, having an initial cell viability of more than 90% as judged by the Trypan blue exclusion test.

Measurement of DNA and protein synthesis

The effects on DNA and protein synthesis were monitored in a Cytostar-T plate, (available in the *In situ* mRNA Cytostar-T assay kit; Amersham) using ^{14}C-labeled thymidine and leucine. The Cytostar-T plate is a 96-well microtiter plate with scintillants molded into the transparent polystyrene bottom. When labeled compound is absorbed into the intracellular compartment of the cells adherent to the bottom of the wells, the radioisotope is brought into proximity with the scintillant and thereby generates a detectable signal. Free radiolabeled compound in the supernatant is unable to stimulate the scintillant [16,17].

U-937 cells were suspended in fresh medium containing 111 nCi/ml thymidine (for DNA experiments) and 222 nCi/ml leucine (for protein experiments) yielding a final radioactivity in the wells of 20 and 40 nCi, respectively. Cell suspension (50×10^3 cells in 180 µl) was added to each well, blank wells received isotope-containing medium only. Drugs and PBS in test and control wells were added in duplicates (20 µl/well) 2 h after cell seeding when the measured radioactivity in cell containing wells was at least double compared to blank wells. Radioactivity was measured with a computer controlled 1450 MicroBeta trilux liquid scintillation counter (Wallac, Turku, Finland) immediately after addition of the cell suspension and at different time points up to 72 h. Between measurements, the plates were stored in an incubator at 37°C. During measurement, the plates were

covered with a plate sealer to inhibit microbiological contamination.

Measurement of extracellular acidification

The Cytosensor silicon microphysiometer (Molecular Devices, Sunnyvale, CA) was used to study the effect of the different alkylating compounds on cellular metabolism. Extracellular acidification originates from excreted acidic byproducts of cellular respiration, such as lactic acid and carbon dioxide. The Cytosensor offers a continuous on-line measurement of the metabolic response to the compounds of interest and it contains two four-channel workstations, allowing eight separate test conditions to be run in parallel.

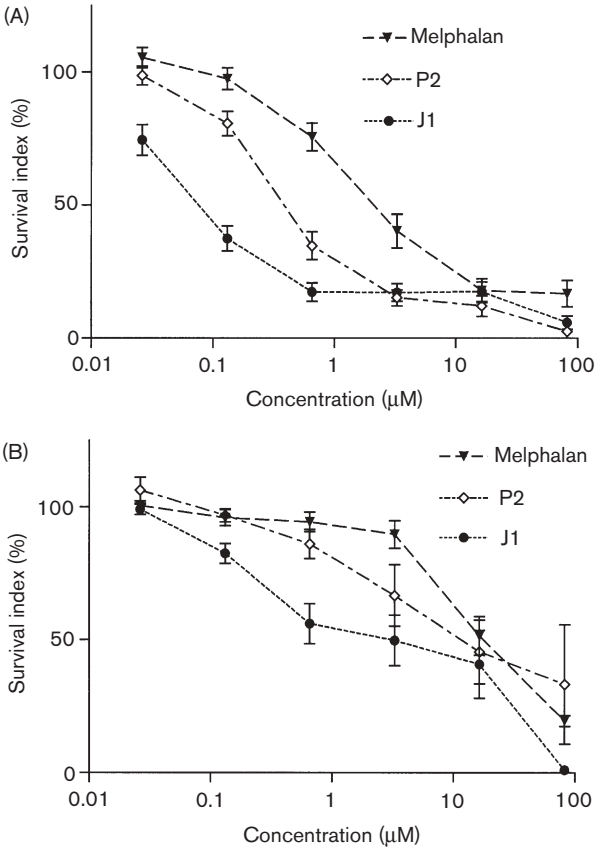
The Cytosensor microphysiometer method has been used for the study of cytotoxic drug effects in U-937 cells previously [18,19]. Decreased acidification rate measured by the Cytosensor correlates well with reduced viability as judged by microscopic evaluation of May–Grünewald–Giemsa-stained cell preparations [19].

U-937 cells were suspended in agarose matrix (25% agarose and 75% cell suspension) and placed in flow chambers. The flow chambers were perfused with complete low buffered cell medium and every 90 s the flow was stopped for 30 s and the extracellular acidification rate was measured with a light-sensitive potentiometric sensor [20]. In order to establish a baseline for the experiment, cells were exposed to medium only during the first hour of the assay. Cells in two of the channels, one per workstation, in each experiment were kept unexposed and served as controls. Freshly prepared drug solution was added directly to the room tempered medium container at the start of drug exposure. The concentration indicated (i.e. 4.0 μM) refers to this initial concentration and does not take mustard or peptide hydrolysis in the medium into consideration. Each experiment was run for 24 h with continuous drug exposure. The acidification rate was calculated by the Cytosoft program as –μV/s and was later normalized to a percent value, percent of control base line [18].

The MiCK assay of apoptosis

Apoptotic changes in cell morphology, such as membrane blebbing and chromatin condensation, can be measured by optical changes in a cell suspension as previously described by Kravtsov *et al.* [21,22]. Control cultures of proliferating cells show a steady increase in optical density versus time, whereas apoptotic cultures show a steep increase followed by a plateau (corresponding to cellular morphology in different phases of apoptosis). Necrotic cultures, on the contrary, display a decrease in absorbance as cells disintegrate. The method has been demonstrated to correlate with morphological and cytochemical parameters of apoptosis in several cell systems, both established cell lines (including U-937) and in primary cultures of human tumor cells, as well as for a wide range of cytotoxic drugs representing different mechanistic classes [21,22]. Flat-bottomed microtiter plates (Nunclon; Nalge Nunc, Roskilde, Denmark) were prepared with quadruplicate wells of 20 μl drug solution, eight wells received 20 μl PBS to serve as control (four) and blank (four). U-937 cells in log-phase were washed and resuspended in a phenol red free complete medium to a cell density of 1.1×10^6 cells/ml. Cell suspension (180 μl) was added to the wells of the microtiter plate; four PBS wells received 180 μl cell medium only to serve as blank. To allow gas and temperature equilibration the plate was placed in a standard incubator (37°C with a humidified atmosphere containing 5% CO_2) for 30 min after which each well was layered with 50 μl of mineral oil (Sigma). Absorbance at 600 nm was measured automatically every 5 min for 24 h in a SpektraMAX Plus microtiter plate spectrophotometer (Molecular Devices) at 37°C. The experiment was performed 3 times.

Fig. 2



Concentration–response curves for the test compounds in hematological (A, $n=15$) and solid (B, $n=12$) patient tumor samples. Error bars indicate SEM. Mean IC_{50} values in these samples were 1.5 and 19 μM for melphalan, 0.33 and 16 μM for P2, and 0.055 and 3.0 μM for J1 (hematological and solid tumors, respectively).

Statistical analysis

The cytotoxic IC_{50} for each patient sample was determined from concentration effect curves in GraphPad Prism using non-linear regression analysis. Comparison of activity in these samples was made with a paired *t*-test (GraphPad Prism). Inhibition of macromolecular synthesis is presented as the mean of three experiments, whereas results from the other assays are presented as one representative experiment.

Results

Cytotoxicity in primary tumor cultures

Concentration–response curves of melphalan, P2 and J1 in hematological and solid tumors (Figure 2A and B), showed the highest cytotoxic activity for J1. Statistically significant differences between IC_{50} values for J1 and melphalan/P2 ($p < 0.01$), and between P2 and melphalan ($p < 0.01$ in hematological and $p < 0.05$ in solid samples) were observed. Furthermore, a correlation analysis of $logIC_{50}$ values in these cells yielded a high similarity between J1 and P2 (Pearson's correlation = 0.92; not shown) indicating a similar mode of action. Exposure to standard chemotherapeutic drugs yielded a variety of responding and non-responding samples (from survival index at the empirically derived cut-off concentrations). The potency of J1, on a molar basis, is comparable to or higher than that of the studied drugs (not shown).

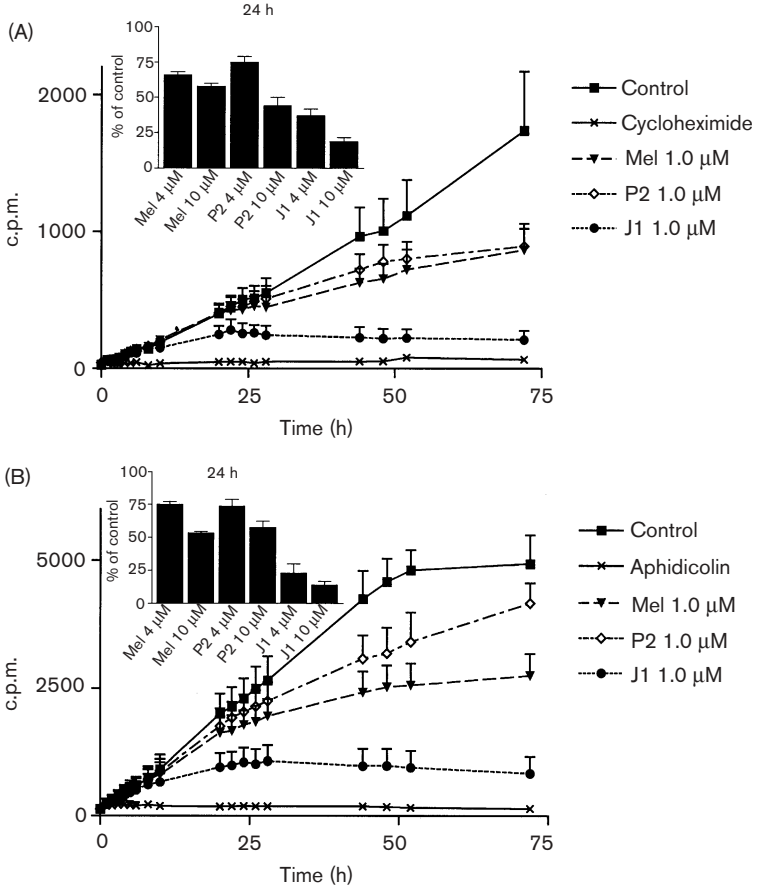
Inhibition of macromolecular synthesis

Inhibition of both DNA and protein synthesis in U-937 cells was observed within the first 24 h for all test compounds (Figure 3). The response was more rapid for J1 than for P2 and melphalan at equimolar concentrations. Furthermore, cells exposed to J1 appeared incapable of macromolecular synthesis after 24 h, whereas melphalan and P2 exposed cells retained some synthetic activity.

Decreased extracellular acidification

All the test compounds decreased extracellular acidification measured by the Cytosensor. The most pronounced effect was observed for J1-treated cells, for which

Fig. 3



Effects on protein (A) and DNA (B) synthesis in U-937 cells following exposure to 1.0 μM of the test compounds. Mean of three independent experiments ± SEM. Cycloheximide (10 μg/ml) was used as a positive control for inhibition of protein synthesis and aphidicolin (5 μg/ml) for DNA synthesis. Effects were dose dependent; inset shows substrate incorporation (% of control) at 24 h for 4 and 10 μM of the test compounds.

acidification abruptly decreased after a few hours of exposure (Figure 4).

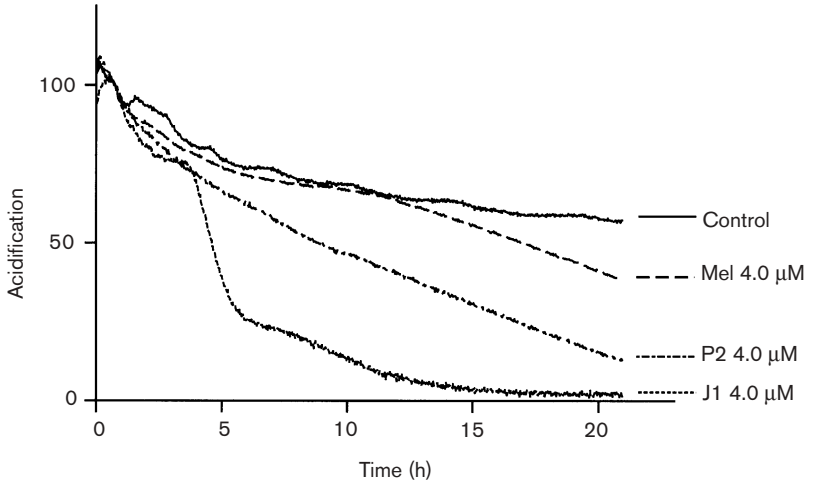
J1 exposure results in apoptotic MiCK curves

At a concentration of 10 μM only J1 yielded a MiCK curve consistent with apoptotic morphology, as described by Kravtsov *et al.* [17,22]. The curves from P2 showed a very modest apoptotic tendency, whereas the apoptotic effect of melphalan was negligible during the 24-h assay (Figure 5). A V_{max} value may be calculated from the steepest portion of the apoptotic curve (i.e. the increase in optical density) using SoftMax Pro software. This value correlates to the extent of apoptosis (i.e. percentage of apoptotic cells in the suspension), according to the equation presented by Kravtsov *et al.* [22]. Values from the experiments in Figure 5 thus correspond to a maximum of 28 (J1), 5 (P2) and 0 (melphalan) % apoptotic cells, respectively. These results were confirmed by microscopic examination of cytospin slides from cells exposed during 3 h (not shown).

Discussion

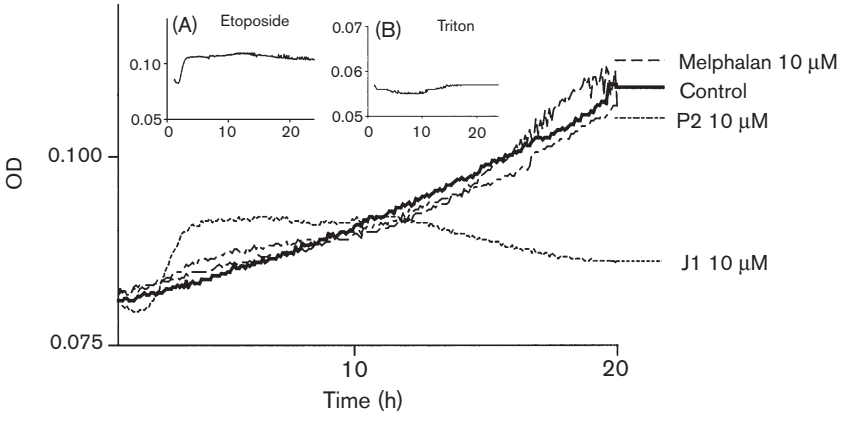
PTC, a mixture of six alkylating peptides, has previously been extensively studied and clinical activity has been reported in a wide range of human malignancies [2–7]. More recent *in vitro* studies have focused on P2 as the

Fig. 4



Measurement of extracellular acidification in U-937 cells following exposure to 4.0 μM of the test compounds. One typical experiment out of three is shown. Effects were dose dependent and at lower concentrations the response was less obvious for all compounds.

Fig. 5



MiCK curves (optical density versus time) for the studied drugs at 10 μM. Inset shows typical curves for apoptosis (A: etoposide, 25 μM) and necrosis (B: Triton X-100, 0.1%), respectively. One typical experiment out of three is shown. Effects were dose dependent and at lower concentrations the J1 curves reflected only inhibition of proliferation.

most active peptide in PTC, with properties such as high alkylating capacity [8], high cytotoxic activity against cell lines [8–10] and primary cultures of human tumor cells [10], as well as low dependence on proliferation and glutathione levels [10].

In the present study the characteristics of the cytotoxic activity of the *m*-L-sarcolysin-containing tripeptide P2 and melphalan is further explored. In addition, a novel melphalan containing dipeptide, J1, representing an intermediate between melphalan and P2, is included in the assays. Previous cell line studies of the alkylating amino acids melphalan and *m*-L-sarcolysin using the FMCA method have shown an almost identical cytotoxic activity for the two compounds, making J1 an interesting test compound [10]. Surprisingly, in the present study J1 was found considerably more active than both melphalan and P2 in the primary human tumor cells. Correlation analysis suggest a similar mode of action of the test compounds, but of different potency. Based on mean IC_{50} values, J1 was 5 (solid)- to 6 (hematological)-fold more potent than P2, which in turn was several-fold more potent than melphalan. Interestingly, J1 compares favorably with P2 and melphalan considering not only potency, but also in the kinetics of action, an important factor considering the short expected half-life of the peptides [23].

The tested compounds, melphalan, P2 and J1, are all nitrogen mustard derivatives. Melphalan is derived from a single aromatic amino acid (L-phenylalanine), J1 is a dipeptide and P2 a tripeptide, each containing one *bis*(2-chloroethyl)amino group as a bifunctional alkylating moiety. Despite having equal 'alkylating capacity', the drugs display different properties considering both potency and kinetics of their actions on tumor cells. Such differences are probably a result of differences in both physicochemical (e.g. hydrophobicity, stability) as well as biochemical (e.g. interaction with membrane transporters, ability to alkylate target molecules) characteristics of the molecules.

Alkylating agents in general are believed to act mainly by covalent conjugation to DNA, the N^7 of guanine has proven to be the strongest nucleophile [24], although adducts can form with several other nucleophilic entities of the nitrogen bases (nucleotides) of DNA. There is a rough correlation between antitumor efficacy and the ability to induce mutations and inhibit DNA synthesis [25]. The high activity of PTC, and P2 in particular, compared to other nitrogen mustards has, at least in part, been suggested to originate from a more efficient DNA cross-linking [8,9]. Both DNA and protein synthesis rapidly decreased following J1 exposure and after 24 h no additional incorporation of radiolabeled substrates could be detected. Equimolar concentrations of melphalan and P2 showed more modest effects, with continuous substrate incorporation of approximately 50–75% of control cultures. Thus, the early and pronounced inhibition of protein synthesis by J1 is probably the result of alkylation of nucleophilic entities of cytoplasmic mRNA, thereby inhibiting the translation, in addition to reactive destruction of cellular proteins (by alkylation of hydroxyl and sulphydryl groups). As a consequence the intracellular metabolism, measured by extracellular acidification in the Cytosensor, also displayed a rapid and pronounced decrease following J1 exposure. In addition, cell culture suspensions exposed to the J1 showed rapidly increasing MiCK curves representing a high proportion of apoptotic cells. The different kinetics of action between the test compounds might result from differences in drug delivery. Mechanisms for this may include active membrane transport and/or ion trapping by intracellular esterases/peptidases. Indeed, an increased expression of such proteins has been described for various tumors providing a potential for tumor selectivity [26–28].

Melphalan containing dipeptides have been studied previously. For example, a series of dipeptides of D- and L-melphalan with L-glutamic acid or L-valine (and their methyl or ethyl esters) was studied by Kupczyk-Subotkowska *et al.* These derivatives appear to be highly cytotoxic against two tested cell lines, although the assay was only performed at considerably higher (1.6 mM) concentrations than used in the present study (less than 10 μM) [29]. Melphalan-containing dipeptides with aromatic amino acids were synthesized by Bergel and Stock during their work with cytoactive amino acids and peptides in 1954–1962. Although similar to J1, all these 'bis-aromatic' dipeptides have the melphalan moiety in the C-terminus and activity in preliminary *in vivo* studies appeared to be similar to melphalan itself [30].

In summary, this study confirms the superior activity of P2 compared to melphalan as indicated in previous publications [8–10]. In fact, in all *in vitro* assays used, P2 compared favorably with melphalan. More importantly, the dipeptide J1, which can be considered as a structural intermediate between melphalan and P2, possessed an even higher cytotoxic activity in patient tumor samples, as well as a higher potency in the other assays and different kinetics of activity. The molecular bases for these observations are currently under investigation

Acknowledgments

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References

1 De Barbieri A. Peptichemio. A synthesis of pharmacological, morphological, biochemical and biomolecular investigations. In: *Proc Symp on Peptichemio*. Milan, Italy: 1972, pp. 1–46.

2 Buzdar AU, Legha SS, Blumenschein GR, Hortobagyi GN, Yap HY, Schell FC, *et al.* Peptichemio versus melphalan (L-PAM) in advanced breast cancer. *Cancer* 1982; **49**:1767–1770.

3 Paccagnella A, Tredese F, Salvagno L, Brandes A, Sileni VC, Daniele O, *et al.* Peptichemio in pretreated patients with ovarian cancer. *Cancer Treat Rep* 1985; **69**:17–20.

4 Carella AM, Bacigalupo A, Santini G, Giordano D, Damasio EE, Fusco FA, *et al.* Peptichemio: a new oncolytic drug in combination with vincristine and prednisolone in the treatment of non-Hodgkin lymphomas. *Acta Haematol* 1977; **57**:211–224.

5 Hug V, Hortobagyi GN, Buzdar AU, Blumenschein GR, Grose W, Burgess MA, *et al.* A phase II study of peptichemio in advanced breast cancer. *Cancer* 1980; **45**:2524–2528.

6 Paccagnella A, Salvagno L, Chiarion-Sileni V, Bolzonella S, De Besi P, Frizzarin M, *et al.* Peptichemio in pretreated patients with plasma cell neoplasms. *Eur J Cancer Clin Oncol* 1986; **22**:1053–1058.

7 Merlini G, Gobbi PG, Riccardi A, Riva G, Sardi C, Perugini S. Peptichemio induction therapy in myelomatosis. *Cancer Chemother Pharmacol* 1982; **8**:9–16.

8 Hansson J, Lewensohn R, Ringborg U. Cytotoxicity and DNA cross-linking induced by peptide conjugated *m*-L-sarcolysin in human melanoma cells. *Anticancer Res* 1991; **11**:1725–1730.

9 Lewensohn R, Ehrsson H, Hansson J, Ringborg U. Increased toxicity and DNA cross-linking by peptide bound *m*-L-sarcolysin (Peptichemio) as compared to melphalan and *m*-L-sarcolysin in human melanoma cell lines. *Anticancer Res* 1991; **11**:321–324.

10 Larsson R, Dhar S, Ehrsson H, Nygren P, Lewensohn R. Comparison of the cytotoxic activity of melphalan with L-prolyl-*m*-L-sarcolysyl-L-*p*-fluorophenylalanine in human tumor cell lines and primary cultures of tumor cells from patients. *Br J Cancer* 1998; **78**:328–335.

11 Larsson R, Kristensen J, Sandberg C, Nygren P. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia, using a fluorometric microculture cytotoxicity assay (FMCA). *Int J Cancer* 1992; **50**:177–185.

12 Csoka K, Larsson R, Tholander B, Gerdin E, de la Torre M, Nygren P. Cytotoxic drug sensitivity testing of tumor cells from patients with ovarian carcinoma using the fluorometric microculture cytotoxicity assay (FMCA). *Gynecol Oncol* 1994; **54**:163–170.

13 Nygren P, Kristensen J, Jonsson B, Sundstrom C, Lonnerholm G, Kreuger A, *et al.* Feasibility of the fluorometric microculture cytotoxicity assay (FMCA) for cytotoxic drug sensitivity testing of tumor cells from patients with acute lymphoblastic leukemia. *Leukemia* 1992; **6**:1121–1128.

14 Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* 1976; **17**:565–577.

15 Nygren P, Fridborg H, Csoka K, Sundstrom C, de la Torre M, Kristensen J, *et al.* Detection of tumor-specific cytotoxic drug activity *in vitro* using the fluorometric microculture cytotoxicity assay and primary cultures of tumor cells from patients. *Int J Cancer* 1994; **56**:715–720.

16 Graves R, Davies R, Brophy G, O'Beirne G, Cook M. Noninvasive realtime method for the examination of thymidine uptake events: application of the method to V-79 cell synchrony studies. *Anal Biochem* 1997; **248**:251–257.

17 Harris DW, Kenrick MK, Pither RJ, Anson JG, Jones DA. Development of a high-volume *in situ* mRNA hybridization assay for the quantification of gene expression utilizing scintillating microplates. *Anal Biochem* 1996; **243**:249–256.

18 Ekelund S, Nygren P, Larsson R. Microphysiometry: new technology for evaluation of anticancer drug activity in human tumor cells *in vitro*. *Anticancer Drugs* 1998; **9**:531–538.

19 Liminga G, Jonsson B, Nygren P, Larsson R. On the mechanism underlying calcein-induced cytotoxicity. *Eur J Pharmacol* 1999; **383**:321–329.

20 McConnell HM, Owicki JC, Parce JW, Miller DL, Baxter GT, Wada HG, *et al.* The cytosensor microphysiometer: biological applications of silicon technology. *Science* 1992; **257**:1906–1912.

21 Kravtsov VD, Fabian I. Automated monitoring of apoptosis in suspension cell cultures. *Lab Invest* 1996; **74**:557–570.

22 Kravtsov VD, Greer JP, Whitlock JA, Koury MJ. Use of the microculture kinetic assay of apoptosis to determine chemosensitivities of leukemias. *Blood* 1998; **92**:968–980.

23 Ehrsson H, Lewensohn R, Wallin I, Hellstrom M, Merlini G, Johansson B. Pharmacokinetics of peptichemio in myeloma patients: release of *m*-L-sarcolysin *in vivo* and *in vitro*. *Cancer Chemother Pharmacol* 1993; **31**:265–268.

24 Lawley PD, Brookes P. Further studies on the alkylation of nucleic acids and their constituent nucleotides. *Biochem J* 1963; **89**:127–138.

25 Niculescu-Duvâz I, Baracu I. Alkylating agents. In: Wilman DEV (editor): *The Chemistry of Antitumor Agents*. Glasgow: Blackie; 1990, pp. 63–130.

26 Bosmann HB, Hall TC. Enzyme activity in invasive tumors of human breast and colon. *Proc Natl Acad Sci USA* 1974; **71**:1833–1837.

27 Gonzalez DE, Covitz KM, Sadee W, Mrsny RJ. An oligopeptide transporter is expressed at high levels in the pancreatic carcinoma cell lines AsPc-1 and Capan-2. *Cancer Res* 1998; **58**:519–525.

28 Martinez JM, Prieto I, Ramirez MJ, Cueva C, Alba F, Ramirez M. Aminopeptidase activities in breast cancer tissue. *Clin Chem* 1999; **45**:1797–1802.

29 Kupczyk-Subotkowska L, Tamura K, Pal D, Sakaeda T, Siahaan TJ, Stella VJ, *et al.* Derivatives of melphalan designed to enhance drug accumulation in cancer cells. *J Drug Target* 1997; **4**:359–370.

30 Bergel F, Stock JA. Cyto-active amino-acids and peptides. Part VIII. *N*(alpha)-acyl, amide, ester and peptide derivatives of melphalan. *J Chem Soc* 1960; 3658–3669.