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Phase I study of *beta*-alanyl-melphalan as a potent anticancer drug*

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Summary. The dipeptide *beta*-alanyl-melphalan was synthesized and tested for its potential anticancer activity. It is shown to possess considerable toxicity toward Ehrlich ascites tumor cells and 3T3 mouse embryo cells in in vitro toxicity assays and is a potent anticancer agent when used in vivo in traditional phase I chemotherapy assays. The potential role for small peptide transport mechanisms in transportation of anticancer agents is discussed.

Introduction

Effective cancer chemotherapy is based on the premise that the anticancer agent can be targeted specifically to the cancer cells comprising the malignancy – the magic bullet of Ehrlich. One such means of targeting cells for potentially toxic compounds lies in the phenomenon of portage transport. This form of metabolite transport relies on the ability to couple normally impermeant molecules to permeant molecules, permitting their accumulation by cells, and the peptide transport systems described in microorganisms [2, 11, 12, 16, 21] and in mammalian cell systems [1, 4, 13, 18] afford a convenient means for toxic metabolite accumulation [17].

Peptide transport systems are biochemically and genetically distinct from amino acid transport systems and exhibit a requirement for a free alpha-amino group and a molecular structure resembling a peptide. The actual composition of the groups comprising the side chains and the presence or absence of a free carboxyl group appear to contribute little to the recognition of the molecule for transport.

A classical example of portage transport in bacteria utilized alafosfalin (L-analyl-1-aminoethylphosphonic acid) which is a phosphonate analogue of L-alanyl-L-alanine. Studies with this dipeptide have shown that the N-terminal L-alanine moiety serves purely as a carrier for aminoethylphosphonic acid (AEPA). When disguised as a peptide it is possible for the amino acid analogue AEPA to be transported into the bacterial cell where it is released by peptidase action. In this particular case the liberated AEPA appears to be toxic through its inhibition of the alanine racemase responsible for D-alanine synthesis, and thus blocks synthesis of D-alanyl-D-alanine, which is necessary for cell wall synthesis [14].

It is ironic that after the pioneering work of Christensen and Rafn over 34 years ago describing small peptide transport in Ehrlich ascites tumor cells [4], we are just rediscovering the potential for this transport mechanism in targeting cancer cells for toxic antimetabolites. The potential of peptides containing toxic metabolites was recognized in the work of Bergel [3] and Larionov [15] in which oligopeptides of sarcolysine [3-*p*-bis-(2-chloroethyl)aminophenyl-D,L-alanine] were studied for their anticancer activities. Research efforts were continued at the Istituto Sieroterapico Milanese Serafin Belfanti (I. S. M.) under the direction of Dr. De Barbieri and resulted in synthesis of hundreds of peptides, numerous publications on the subject [e. g., 5–8] and eventual development of a combination of the most effective peptides into a formulation known as Peptichemio [9].

In a recent publication [10], De Barbieri reported on the activity of a tripeptidic mustard, 3-(*p*-fluorophenyl)-L-alanyl-3-[*m*-bis-(2-chloroethyl)aminophenyl]-L-alanyl -L-methionine ethyl ester hydrochloride (PTT.119), as an effective anticancer drug and this work was quickly followed by reports of research conducted in the United States on the potential for PTT.119 in killing of cancer cells [23–25]. The research to be described here represents a continuation of this effort and describes the synthesis and evaluation of a dipeptide containing the toxic alkylating agent melphalan as the toxic antimetabolite. The amino acid residue *beta*-alanine is incorporated into the peptide as the N-terminal residue to reduce hydrolysis by peptidohydrolytic enzymes and the resultant dipeptide, *beta*-alanyl-melphalan, is shown to be a potent anticancer agent when used in traditional phase I chemotherapy studies.

Materials and methods

Cell cultures. The Ehrlich ascites tumor cell line utilized in this study was originally the generous gift of Dr. Margaret Neville and the University of Colorado (Denver) and has been maintained in the laboratory for the past 8 years. Propagation has been through serial transfer in the abdominal cavity of CF-1 albino mice by weekly transfer of approximately one million tumor cells.

For in vitro toxicity assays, tumor cells are harvested directly from the abdominal cavity of cervically dislocated mice into culture flasks (25 cm^2) containing 5 ml NCTC

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135 medium (Gibco) supplemented with 10% fetal calf serum (Gibco). The cultures are grown in a humidified CO_2 incubator gassed with 5% CO_2. The culture medium is routinely changed at 3- or 4-day intervals and toxicity assays are routinely performed on 1-week-old cultures. By this time the cultures are free of mouse red blood cells and are nearing confluency.

The Balb/3T3 clone A31 mouse embryo cell line was obtained from the American Type Culture Collection (ATCC CCL 163) and was routinely cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% calf serum. This cell line is routinely passaged by trypsinization (1X trypsin, Gibco) and splitting 1:6 just prior to confluency. Cultures become confluent within 1 week, and the passage number of cells utilized in toxicity assays was controlled by regular thawing of cells at a specific passage number.

Synthesis of beta-alanyl-melphalan. BOC-*beta*-alanine (10.598 g, 56 mmol) was dissolved in 80 ml methylene chloride and added to a solution of 5.786 g (28 mmol) dicyclohexylcarbodiimide dissolved in 240 ml methylene chloride. The resulting mixture was stirred at room temperature for 10 min and then cooled at 4 °C for 10 min. The precipitate was removed by filtration and was washed with two 80-ml methylene chloride washes. The washes and filtrate containing BOC-*beta*-alanine anhydride were combined. Melphalan (4.270 g, 14 mmol) was slowly added to stirring dimethylformamide (1120 ml). To this suspension, after stirring for 15 min, was added the methylene chloride solution of BOC-*beta*-alanine anhydride. One milliliter of diisopropylethylamine was added to the stirring solution, and stirring was continued for 4 h at room temperature. The reaction was monitored for ninhydrin-positive material. All ninhydrin tests proved negative.

The resulting yellow solution was evaporated under vacuum, yielding a thick yellow oil. The oil was dissolved in toluene and a white insoluble material filtered off. The insoluble material was washed several times with toluene and the washes were combined with soluble materials. The solution was evaporated, yielding a yellow oil. The oil was dissolved in ethyl ether, leaving an insoluble off-white solid. This solid was washed several times with ether and then dried under vacuum, again yielding an off-white solid. This material, BOC-*beta*-alanyl-melphalan (3.86 g, 58% yield), was homogenous by high-performance liquid chromatography and thin-layer chromatography (HPLC and TLC; see below), was ninhydrin-negative, and showed positive with nitrobenzylpyridine (test for melphalan). This form of the dipeptide was most stable for storage and was stored at −20 °C.

Prior to utilization in toxicity or chemotherapeutic studies, sufficient BOC-*beta*-alanyl-melphalan was converted to the dipeptide-HCl salt for usage over a 1- or 2-month period. For deblocking, the BOC-peptide was dissolved in 250 ml trifluoroacetic acid and reacted at room temperature for 20 min. After evaporation under vacuum, the resulting yellow-brown oil was ninhydrin-positive, nitrobenzylpyridine-positive, and homogenous by HPLC and TLC. The oil was treated with a three-fold molar excess of 2 *N* HCl with stirring at room temperature for 10 min. After evaporating the solution under vacuum, the resulting solid quickly became a yellow-brown oil. This compound was ninhydrin- and nitrobenzylpyridine-positive. HPLC analysis indicated this compound to be approximately 85% pure, containing two decomposition products comprising 6% and 9% of the total "peak areas".

TLC analysis of synthetic products. The products of peptide synthesis were monitored by TLC. Analysis was performed using Merck 10 × 10 cm HPTLC Silica Gel 60 and F 254 plates with a concentration zone. The developing solvent was chloroform/methanol (65/35, v/v). Detection was with nitrobenzylpyridine or ninhydrin.

HPLC analysis of synthetic products. The products of peptide synthesis were also monitored by HPLC. A Vydac C4 (0.4 × 25 cm) column was utilized in conjunction with the programmed gradient consisting of 90% solvent A/10% solvent B to 50% solvent A/50% solvent B over 20 min, holding at 50% solvent A/50% solvent B for 5 min, and returning to the original solvent composition over 5 min. Flow rate was maintained at 1 ml/min. Solvent A consisted of 0.15% trifluoroacetic acid in water and solvent B consisted of 0.15% trifluoroacetic acid in acetonitrile. Elution of solutes was monitored at 210 nm.

In vitro toxicity assays. For in vitro toxicity assays, Ehrlich ascites tumor cells or 3T3 mouse embryo cells (control) were seeded into appropriate culture media and incubated to a point where they were near to being confluent. Although the optimal times for cell growth have not been adequately determined, nor the individual cells within the population assessed for differential sensitivity to the target drugs, this culture time was chosen because it provided for the maximum numbers of cell that were not as yet quiescent, i. e., would still be replicating their DNA and would thus presumably still be sensitive to the action(s) of melphalan.

The Ehrlich ascites tumor cells were harvested directly from the abdominal cavity of a CF-1 mouse into NCTC-135 media (Gibco) supplemented with 10% fetal calf serum. The number of cells innoculated were sufficient to yield near-confluent cultures within 1 week. Culture medium was routinely changed once before the medium change with fresh medium containing the drug being tested. Following this latter medium change, the cultures were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Following this treatment the culture medium is poured into a centrifuge tube, the cells in the flask trypsinized (2 min at room temperature) using 1X trypsin (Gibco), the flasks vigoroursly agitated, and the trypsinized cells added to the centrifuge tube. The cells were centrifuged in an IEC tabletop centrifuge (3500 rpm) and the supernatant discarded. The cells were resuspended in Dulbecco's phosphate-buffered saline (DPBS) (8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4, and 0.1 g $MgCl_2$ per liter glass distilled water) supplemented with 1% bovine serum albumin (BSA; fraction V). The amount of DPBS/BSA added varied with the size of the cell pellet, but was normally 2.5–3.0 ml. An aliquot of this cell suspension (0.5 ml) was transferred to a new tube and 0.1 ml of trypan blue (0.4% trypan blue in PBS; Sigma Chemical Company) added. The cells were then transferred to a hemocytometer and counted. Cells staining blue were considered metabolically "dead" and their number, added to the number of "clear" cells, equaled total cell number.

The 3T3 cells are routinely maintained in culture. The appropriate number of culture flasks were trypsinized and

split 1:6 into Dulbecco's modified Eagle's minimal medium supplemented with 10% calf serum. The cells were incubated for 2–3 days or until the cells were nearly confluent. The incubation medium was changed for fresh medium supplemented with the drug being tested. The cells were incubated an additional 24 h and then harvested and counted as described above for the Ehrlich ascites tumor cells.

Chemotherapy assays. For the chemotherapy assays, Ehrlich ascites tumor cells were harvested fresh from the abdominal cavity of a mouse into hypotonic buffer (0.1 *M* Tris-HCl, 0.01 *M* NaCl, 0.0015 *M* MgCl2). Treatment with hypotonic buffer was repeated until the tumor cells were essentially free of red blood cells and then the tumor cells were transfered to DPBS and injected into the abdominal cavities of mice (approximately one million tumor cells per mouse). After 24 h, the drug being tested was injected intraperitoneally at the desired concentration. Control mice having been injected with tumor cells received a sham injection of the solvent containing the drugs. Since all "drugs" tested in this report were soluble in DPBS, this solvent was utilized in all assays. Additional controls consisted of mice receiving a sham injection of tumor cells (DPBS) and the same drug treatment as the mice receiving the tumor cells. The mice were weighed daily and their water and food consumption monitored (data not shown). The survival after injection of each mouse in the assay was recorded.

Results

Synthesis of the dipeptide beta-*alanyl-melphalan*

The synthesis of the dipeptide utilized in this study involved the condensation of BOC-*beta*-alanine with the bifunctional alkylating agent melphalan. The melphalan and BOC-*beta*-alanine were purchased from Sigma Chemical Company. Analysis of these two reactants by HPLC revealed that they were essentially pure, as determined by absorbance at 210 nm. The melphalan contains a minor contaminant which elutes early and constitutes less than 10% of the 210-nm absorbing material (Fig. 1). Analysis of melphalan by TLC revealed an Rf value of 0.04 (data not shown).

Following condensation of the reactants (see Methods for details), the dipeptide BOC-*beta*-alanyl-melphalan was analyzed for purity and completeness of reaction. The reaction was continuously monitored for ninhydrin-positive materials during the course of the reaction and analysis by HPLC of the final product revealed the presence of only minor traces of materials absorbing at 210 nm that were not at the position, 21.2 min, at which the BOC *beta*-alanyl-melphalan eluted. As demonstrated in Fig. 2, the product was essentially pure, and the major portion of the synthetic product was stored frozen (−20 °c) in a dessicator. The derivitized dipeptide was a white powdery material that could be easily weighed and aliquoted. As we were to learn later, deblocking the peptide would result in the conversion of a powdery residue into an oily residue which was difficult to weight. Consequently, only a sufficient quantity of dipeptide was deblocked at any one time to permit testing over a period of 1–2 months.

Following deblocking, the TFA salt of *beta*-alanyl-melphalan was converted to the HCl salt form prior to analy-

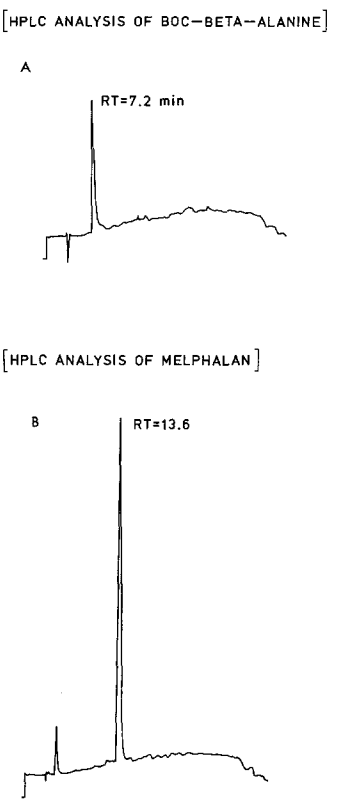

Fig. 1 A, B. HPLC analysis of reactants in the synthesis of *beta*-alanyl-melphalan. **A** BOC-*beta*-alanine; **B** melphalan. *RT*, retention time (minutes) of materials absorbing at 210 nm

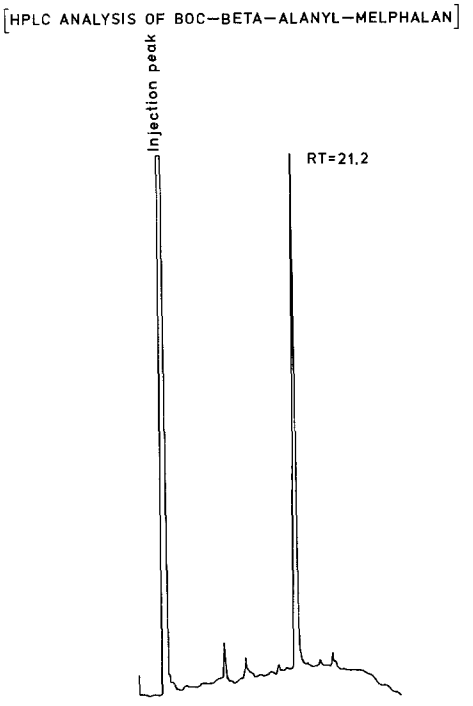

Fig. 2. HPLC analysis of immediate product of peptide synthesis, BOC-*beta*-alanyl-melphalan. *RT*, retention time (minutes) of materials absorbing at 210 nm

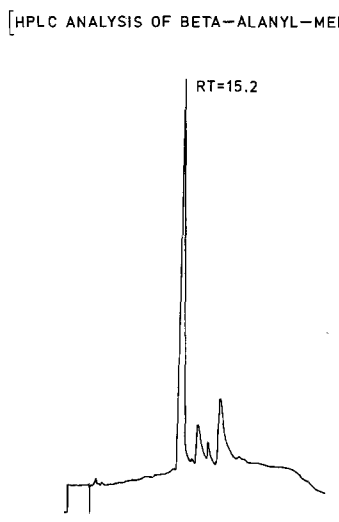

Fig. 3. HPLC analysis of the HCl salt of *beta*-alanyl-melphalan. *RT*, retention time (minutes) of materials absorbing at 210 nm

sis and utilization in toxicity testing. Analysis of the deblocked dipeptide by HPLC revealed the presence of at least three 210-nm absorbing compounds comprising less than 14% of the total 210-nm absorbing materials. The absence of these "contaminant" peaks in the TFA salt form of the dipeptide suggests that they are the result of the generation of the HCl salt form. The major peak of 210-nm absorbing materials (*beta*-alanyl-melphalan) eluted at 15.2 min and comprised over 85% of the available materials eluting from the column (Fig. 3). The retention time of *beta*-alanyl-melphalan HCl was significantly different from the retention times of melphalan (13.6 min) and BOC-*beta*-alanine (7.2 min).

In vitro toxicity testing of melphalan

The objective of the toxicity-testing program was to assess the ability of melphalan, *beta*-alanine, and *beta*-alanyl-melphalan to damage the metabolic viability of the Ehrlich ascites tumor cells and the mouse embryo 3T3 cells utilized as a "control" noncancerous cell line. An effort was made to test each cell line at a point just prior to the cell cultures reaching confluency. Thus, the Ehrlich ascites tumor cells

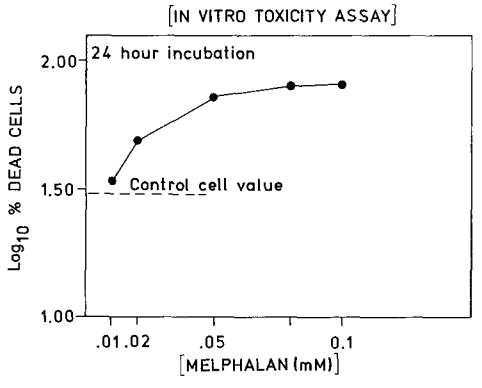

Fig. 4. In vitro toxicity assay of melphalan to Ehrlich ascites tumor cells. Near-confluent cultures were incubated for 24 h with melphalan at the concentrations indicated. Metabolic viability was assessed by trypan blue exclusion

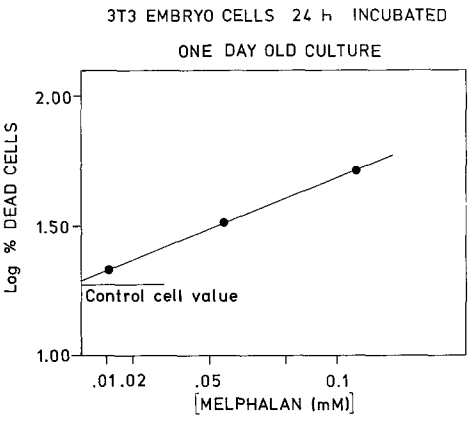

Fig. 5. In vitro toxicity assay of melphalan to 3T3 mouse embryo cells. Near-confluent cultures were incubated for 24 h with melphalan at the concentrations indicated. Metabolic viability was assessed by trypan blue exclusion

were isolated from the abdominal cavity of a mouse directly into culture media. At the end of 1 week (with a media change after 3 or 4 days), the near-confluent cultures were exposed for 24 h to the test drug at the concentrations indicated. Cell viability was assessed by vital dye (trypan blue) exclusion. Figure 4 reveals that concentrations of melphalan at 0.1 m*M*, result in approximately 80% "death" of Ehrlich ascites tumor cells. As the concentration of melphalan is reduced, toxicity diminishes in a nonlinear manner until at 0.01 m*M*, cell viability approaches that of nontreated tumor cells (30% cell death).

Analysis of toxicity of melphalan to the 3T3 mouse embryo cells over the same concentration range utilized against the tumor cells reveals a reduction in cell viability of 48% at melphalan concentrations of 0.1 m*M*. Melphalan concentrations 0.01 m*M* provide for cell viability near to that of the control nontreated cells (18%). Unlike the toxicity melphalan concentration study with the tumor cells, the data plot for the 3T3 cells reveals a linear correlation of toxicity with melphalan concentrations between 0.1 and 0.01 m*M* (Fig. 5).

Drug toxicity to 3T3 cells of different culture ages

Because of the possibility that cell density, doubling times, etc. might contribute to the sensitivity of cell populations to the toxic effects of melphalan and *beta*-alanyl-melphalan, we decided to examine melphalan and *beta*-alanyl-melphalan toxicity to different culture ages of 3T3 cells. One flask of mouse 3T3 cells was split into six culture flasks and melphalan added for 5 consecutive days. The cell viabilities were determined after 24 h treatment. The viability of the control cells varied from approximately 15% ($\log_{10}$ = 1.26) dead cells at day one to less than 5% ($\log_{10}$ = 0.69) dead cells at day 5. The cells treated with 0.1 m*M* or 0.01 m*M* melphalan were not significantly different at different cultures ages of 3T3 cells. All data points of cell viabilities were corrected for the differences from control cell viabilities. In addition, we compared two different concentrations of melphalan treatment. We found that 0.1 m*M* melphalan (1.40 ± 0.08, the average of $\log_{10}$% dead cells from day 1 through day 5) was significantly more toxic to the 3T3 cells than 0.01 m*M* melphalan (1.09 ± 0.07, the average of $\log_{10}$% dead cells from day 1

Table 1. Log_{10} % dead 3T3 cells versus time since inoculation into culture flask

Day	Concentration of melphalan (mM)							
	None log_{10} %	%	0.01 log_{10} %	%	0.01[a] log_{10} %	0.1 log_{10} %	%	0.1[a] log_{10} %
1	1.26	(18.20)	1.31	(20.42)	1.04	1.68	(47.86)	1.33
2	1.02	(10.47)	1.13	(13.49)	1.11	1.56	(36.31)	1.53
3	1.00	(10.08)	1.00	(10.10)	1.00	1.31	(20.42)	1.31
4	0.77	(5.89)	0.86	(7.24)	1.12	1.10	(12.59)	1.43
5	0.69	(4.90)	0.83	(6.76)	1.20	0.98	(9.55)	1.42
Day	Concentration of *beta*-alanyl-melphalan (mM)							
	None log_{10} %	%	0.01 log_{10} %	%	0.01[a] log_{10} %	0.1 log_{10} %	%	0.1[a] log_{10} %
1	0.89	(7.76)	1.50	(31.62)	1.69	1.83	(67.61)	2.06
2	0.86	(7.24)	1.49	(30.90)	1.73	1.84	(69.18)	2.14
3	0.85	(7.08)	1.56	(36.31)	1.84	1.86	(77.44)	2.19

Trypsinized cells were split 1 : 6 and seeded into 25 cm^2 culture flasks. At the days indicated, the cultures were treated for 24 h with melphalan and *beta*-alanyl-melphalan at the concentration indicated and counted for viable cells
Each data point represents an average of 3 to 5 assays
[a] Values adjusted such that the "none" treatment equals a log_{10} value of 1.00 for all days

through day 5; Table 1). Equivalent testing of *beta*-alanyl-melphalan toxicity to 3T3 cells revealed that there is also no difference in sensitivity of 3T3 cells of different culture ages. Concentration of *beta*-alanyl-melphalan of 0.1 m*M* yielded a log_{10} value of 2.13 ± 0.06 (the average of log_{10}% dead cells from day 1 through day 3). This concentration was found to be significantly more toxic to 3T3 cells than the 0.01 m*M* *beta*-alanyl-melphalan (1.75 ± 0.07, the average of log_{10}% dead cells from day 1 through day 3). *Beta*-alanyl-melphalan treatment was also observed to be more toxic to 3T3 cells, at all concentrations tested, than melphalan. From this study we concluded that although the viability of the 3T3 cells improved with culture age, sensitivity to melphalan and *beta*-alanyl-melphalan were not significantly different at different culture ages.

In vitro toxicity testing of beta-*alanyl-melphalan*

Toxicity of the dipeptide containing melphalan to tumor cells over the concentration range of 0.1 m*M* to 0.01 m*M* (Figs. 6) revealed a linear correlation of log % cell death vs concentration of the dipeptide, i. e., 0.1 m*M* dipeptide yields a log_{10} value of 1.17 (51% cell death) and 0.01 m*M* dipeptide yields a log_{10} value of 1.17 (15% cell death). At all concentrations tested, the dipeptide was less toxic than the corresponding free melphalan (refer to Fig. 4). The conditions for testing were identical to those utilized for toxicity testing of melphalan to the tumor cells.

Equivalent testing of the dipeptide for toxicity to mouse 3T3 cells (Fig. 7) revealed that over the concentration range 0.01 m*M* to 0.1 m*M*, the dipeptide was dramatically more toxic to the 3T3 cells than to the cancer cells (Fig. 6) i. e., 0.1 m*M* concentrations of the dipeptide yielded a log_{10} value of 1.83 (67% cell death) and a 0.01 m*M* concentration gave a value of 1.35 (22% cell death). Nontreated cells exhibited a log_{10} value of 0.89 (8% cell death). In addition, the log % cell viability was linear over the concentration range tested and concentrations less than 0.01 m*M* were necessary to obtain cell viabilities equal to those of control (nontreated) cells.

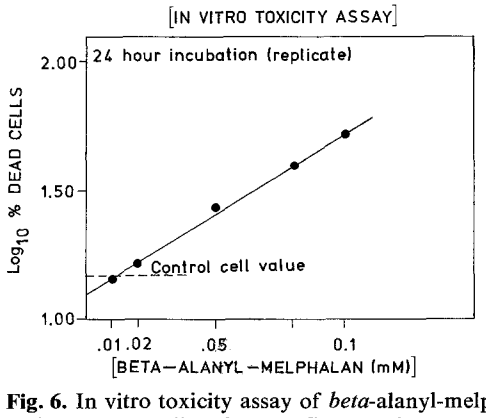

Fig. 6. In vitro toxicity assay of *beta*-alanyl-melphalan to Ehrlich ascites tumor cells. Near-confluent cultures were incubated for 24 h with the dipeptide at the concentrations indicated. Metabolic viability was assessed by trypan blue exclusion

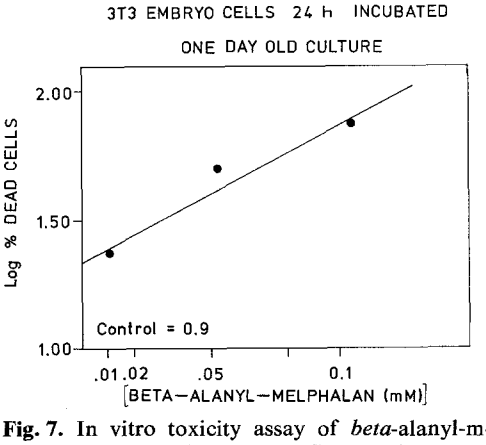

Fig. 7. In vitro toxicity assay of *beta*-alanyl-melphalan to 3T3 mouse embryo cells. Near-confluent cultures were incubated for 24 h with the dipeptide at the concentrations indicated. Metabolic viability was assessed by trypan blue exclusion

Chemotherapy assays

Traditional chemotherapy assays were performed in an effort to assess the ability of these two compounds, melphalan and *beta*-alanyl-melphalan, to inhibit the growth of tumor cells in situ. Both compounds had been demonstrated to be toxic to the tumor cells in the in vitro assays, and since melphalan had already been shown to be toxic to the mouse when utilized at concentrations greater than 15 mg/kg, we chose to test melphalan at a maximum concentration of 10 mg/kg. One million tumor cells are normally injected i. p. and followed 24 h later by the drug treatment. The average survival time of the mice is recorded and the T/C values recorded for each treatment. The data presented in Table 2 represent two separate chemotherapeutic assays performed over 2 consecutive months with each test group containing five or more animals. An average T/C ratio of 104 was found for mice treated with concentrations of *beta*-alanine from 10 to 50 mg/kg. As expected, this amino acid did not significantly extend the survival time of cancerous mice. The dipeptide *beta*-alanyl-alanine also did not significantly extend the survival time of cancerous mice. Equivalent treatment of cancerous mice with melphalan at 5 mg/kg (the lowest concentration tested) yielded an average T/C ratio of 180 ± 6 and treatment with 10 mg melphalan/kg gave an average T/C ratio of 194 ± 8. Such values for T/C ratios indicate considerable activity of melphalan in prolonging the survival time of cancerous mice. As indicated in the table, the T/C ratios are arbitrarily low, since cancerous mice treated with melphalan routinely survive beyond the 30-day "cut-off" time for termination of the assay. Mice surviving beyond 30 days are arbitrarily declared "dead" at day 30 in order that a T/C ratio may be calculated. Treatment of cancerous mice with *beta*-alanyl-melphalan concentrations of 5 mg/kg, 10 mg/kg, 20 mg/kg, 30 mg/kg, and 40 mg/kg gave average T/C ratios of 126 ± 7, 125 ± 7, 144 ± 21, 140 ± 12, and 153 ± 16 respectively. Although the T/C ratios appear to exhibit a general upward trend, only those for the 5 mg/kg and 40 mg/kg concentrations of dipeptide are significantly different. It is of interest that the three highest concentrations of dipeptide utilized were greater than the concentration of melphalan shown to be toxic to the mouse [10]. We never observed any toxic reactions to the dipeptide, at any concentration tested, by tumor-bearing mice or by mice having received a sham injection (buffer only instead of tumor cells). We monitored food and water consumption, autopsied selected mice for visible damage to organs, i. e., liver, and observed behavior patterns. All control animals, i. e., those receiving no tumor cells and a full chemotherapy regime, appeared normal.

Table 2. Calculated T/C values from chemotherapy assays

Treatment	Dosage (mg/kg)	T/C ratio
Control	None	100.0
beta-Alanine	10	103.6 ± 2.8
beta-Alanine	25	99.4 ± 16.8
beta-Alanine	50	108.1
beta-Alanine-alanine	25	89.8 ± 12.4
Melphalan	5	179.9 ± 6.2[a]
Melphalan	10	193.9 ± 8.0[a]
beta-Alanyl-melphalan	5	125.8 ± 6.8
beta-Alanyl-melphalan	10	125.5 ± 8.6
beta-Alanyl-melphalan	20	144.0 ± 21.0
beta-Alanyl-melphalan	30	139.6 ± 12.1
beta-Alanyl-melphalan	40	152.5 ± 16.2[a]

Mice are injected i.p. with one million tumor cells, and after 24 h, injected i.p. with the drug indicated. Controls receive equal volumes of solvent (DPBS). The time after injection that the "mouse" dies is recorded and the average days survival of all mice calculated

Each treatment protocol contained at least five mice per assay and each treatment was repeated 2–5 times

[a] T/C ratios are arbitrarily low in that mice still alive after 30 days were declared "dead" for purposes of calculation of the ratio

Discussion

The research described above came about as a result of previous research on the ability of the eukaryotic fungus *Neurospora crassa* to transport and utilize small peptides as sources of nitrogen. The presence of a transport mechanism in this microorganism for small tri- to pentapeptides had been suggested [10, 20], and it was demonstrated that *Neurospora* was capable of growing on peptides as a sole source of leucine only so long as they were small enough to be transported by this peptide transport mechanism. In a later study [22], it was demonstrated that *Neurospora* could grow on all sizes of peptide provided it was forced to utilize the peptides as a sole source of nitrogen *and* leucine. Analysis of the culture media for peptidohydrolytic enzymes revealed the interesting observation that growth on the larger (nontransportable) peptides required the production and secretion of extracellular peptidohydrolytic enzymes, and so long as these peptides were too large to be transported by the peptide transport system, the peptides served to "induce" the hydrolytic enzymes. Growth on a transportable peptide precluded induction and secretion of hydrolytic enzymes. In short, the ability of a peptide to be transported precluded its ability to act extracellularly in the induction of a physiological response by the microorganism, i. e., hydrolytic enzyme production.

As a logical extension to cancerous cells, it had been reported that Ehrlich ascites tumor cells were capable of transporting small peptides whereas noncancerous cells were incapable of small peptide transport [4]. Indeed, only two other types of cells, those lining the small intestine and the kidney proximal tubules-both noncancerous – have been reported as being capable of small peptide transport [1, 13]. Demonstration of a peptide transport mechanism in one type of mammalian cell suggested that the genetic potential for peptide transport was present in all mammalian cells and that if it was not expressed it must be repressed by some undefined regulatory mechanism. Derepression of repressed genetic functions in cancer cells is not uncommon, and the assumption that noncancerous cells might routinely be incapable of small peptide transport, whereas transformation to the cancerous state might result in the derepression of this genetic capability, led to this study. Since small peptide hormones are accepted as normal regulators of cell function, and cancerous cells appear to be altered in their responses to peptide hormones, we reasoned that the ability of cancer cells to transport small peptides might preclude the ability of these peptides, present in the extracellular environment, to induce a physiological response – a response similar to that observed for *Neurospora*.

Should this hypothesis be correct, it would mean that cancerous cells might be the only cells capable of small peptide transport (with specific exceptions), and thus that small peptides would be an efficient means of targeting toxic amino acid analogues to these cells. Although it has still not been clearly demonstrated that cancer cells transport small peptides, it is suggested by this study and by previous studies in this area that the incorporation of toxic amino acid analogues into small peptides represent a viable approach to the synthesis of a new group of anticancer drugs.

Both melphalan and the dipeptide-containing melphalan are toxic to mammalian cells grown in culture. Melphalan is clearly more toxic to tumor cells than *beta*-alanyl-melphalan, whereas the dipeptide is more toxic to the 3T3 cells than melphalan. We do not as yet fully understand the in vitro toxicity data presented, and in retrospect we suggest that the mouse embryo cell line may not have represented the best control cells for study. We suspect that the embryo cells may be capable of small peptide transport, since they represent a "nondifferentiated" cell line (somewhat analogous to cancerous cells). The results of the tumor cell toxicity studies are clearly analogous to those described previously [10, 23, 24], and we suggest that the inclusion of the amino acid residue *beta*-alanine in the N-terminal position reduces the susceptibility of *beta*-alanyl-melphalan to hydrolysis by peptidohydrolytic enzymes (manuscript in preparation) and may therefore enhance the body half-life of this particular peptide. We suggest that the reduction in hydrolyzability of the dipeptide explains (1) the lower T/C ratios found for *beta*-alanyl-melphalan treated mice compared with melphalan treatment and (2) the lower toxicity of the dipeptide to the mouse; 40 mg dipeptide/kg mouse weight does not appear toxic to the mouse, whereas 15 mg melphalan/kg has been reported as toxic [10]. Increasing concentrations of the dipeptide yield increasing T/C ratios, yet the values at 5 mg/kg (10 mg/kg) and 40 mg/kg are the only two concentrations, giving T/C ratios that differ significantly from each other.

The data presented in this manuscript clearly suggest that incorporation of a toxic metabolite into a small peptide molecule renders it less toxic to the animal carrying the cancer cells without dramatically reducing its ability to inhibit growth of the tumor cells. Future research in this area will concentrate on characterization of the peptide transport system(s) present in tumor cells and of the peptidohydrolytic enzymes produced.

References

1. Addison JM, Burston D, Matthews DM (1973) Carnosine transport by hamster jejunum in vitro and its inhibition by other di- and tripeptides. Clin Sci Mol Med 45: 3
2. Becker JM, Naider F, Katchalski E (1973) Peptide utilization in yeast. Studies on methionine and lysine auxotrophs of *S. cerevisiae*. Biochim Biophys Acta 291: 388
3. Bergel F, Burnop VGE, Stock JA (1955) Cytoactive amino acid and peptide derivatives. Part II: Resoltuion of *para*-substituted phenylalanines and synthesis of *p*-di-(2-chloroethyl)-amino-DL-phenyl-[beta-14C]-alanine. J Chem Soc 1223
4. Christensen HN, Rafn ML (1952) Uptake of peptide by a free cell neoplasm. Cancer Res 12: 495
5. De Barbieri A, Di Vittorio P, Maugeri M, Mistretta AP, Perrone F, Tassi GC, Temelcou O, Zapelli P (1970) Investigations with synthetic antitumor peptides. Progress in Antimicrobial Anticancer Chemotherapy (Proc 6th Intern Congress Chemotherapy), Tokyo, p 146
6. De Barbieri A, Di Vittorio P, Golferini A, Maugeri M, Mistretta AP, Perrone F, Tassi GC, Temelcou O, Zapelli P (1972) Investigations on the mixture of antitumor peptides and mixture of same. Boll Chim Farm 111: 216
7. De Barbieri A (1972) Azione antitumorale di peptidi sintetici. Terapia 57: 123
8. De Barbieri A, Di Vittorio P, Golferini A, Maugeri M, Mistretta AP, Perrone F, Tassi GC, Temelcou O, Zapelli P (1973) Investigations of the effect of antitumor peptides and mixture of same. In: Leukämien und Maligne Lymphome. Pathophysiologie Klinik, Chemo- und Immunotherapie. Ed. Urban & Schwarzenberg, Munich, p 20
9. De Barbieri A, Chiappino G, Di Vittorio P, Golferini A, Maugeri M, Mistretta AP, Perrone F, Tassi GC, Temelcou O, Zapelli P (1972) Peptichemio: a synthesis of pharmacological, morphological, biochemical & biomolecular investigations. Proc Symp Peptichemio, p 3
10. De Barbieri A, Dall'Asta L, Comini A, Spingolo V, Mosconi P, Coppi G, Bekesi G (1983) Synthesis, acute toxicity and chemotherapeutic anticancer activities of a new tripeptidic mustard. Farmaco 38 (4): 205
11. De Felice M, Guardiola J, Lamberti A, Jaccarino M (1973) *E. coli* K12 mutants altered in the transport systems for oligo- and dipeptides. J Bacteriol 116: 751
12. Fickel TE, Gilvarg C (1973) Transport of impermeant substances in *E. coli* by way of oligopeptide permease. Nature 241: 161
13. Ganapathy V, Leibach FH (1983) Role of pH gradient and membrane potential in dipeptide transport in intestinal and renal brush-border membrane vesicles from the rabbit. J Biol Chem 258: 14189
14. Hassall CH, Atherton FR, Hall MJ, Lambert RW, Lloyd WJ, Ringrose PS (1982) Phosphonopeptides, novel inhibitors of bacterial cell-wall biosynthesis. In: Blaka K, Malon P (eds) Peptides. De Gruyter, Berlin, p 607
15. Larinov KF, Khokhlov AS, Shkadinskaia EN, Vasina OS, Trovsheikina VI, Novikova MA (1955) Studies on the antitumor activity of *p*-di-(2-chloroethyl)-amino-phenylalanine (sarcolysine) Lancet ii: 169
16. Payne JW, Gilvarg C (1971) Peptide transport. Adv Enzymol 35: 187
17. Ringrose PS (1980) Peptides as antimicrobial agents. In: Payne JW (ed) Microorganisms and nitrogen sources. Wiley, New York, p 641
18. Rubino A, Field M, Shwachman H (1971) Intestinal transport of amino acid residues of dipeptides: influx of the diglycine residues of glycyl-L-proline across mucosal border. J Biol Chem 246: 3542
19. Wolfinbarger L Jr, Marzluf GA (1974) Peptide utilization by amino acid auxotrophs of *N. crassa*. J Bacteriol 119: 371
20. Wolfinbarger L Jr, Marzluf GA (1975) Size restriction on utilization of peptides by amino acid auxotrophs of *N. crassa*. J Bacteriol 122: 949
21. Wolfinbarger L Jr, Marzluf GA (1975) Specificity and regulation of peptide transport in *N. crassa*. Arch Biochem Biophys 171: 637
22. Wolfinbarger L Jr, Snyder F, Costellano J (1983) Peptide utilization by nitrogen-starved *Neurospora*. J Bacteriol 153: 1567
23. Yagi MJ, Bekesi JG, Daniel MD, Holland JF, De Barbieri A (1983) Increased cancericidal activity of PTT.119, a new synthetic bis-(2-chloroethyl)-amino-L-phenylalanine derivative with carrier amino acids. I. In vitro cytotoxicity. Cancer Chemother Pharmacol 12: 76
24. Yagi MJ, Zanjani M, Holland JF, Bekesi JG (1984) Increased cancericidal activity of PTT.119, a new synthetic bis-(2-chloroethyl)amino-L-phenylalanine derivative with carrier amino acids II. In vivo bioassay. Cancer Chemother Pharmacol 12: 77
25. Yagi MJ, Chin SE, Scanlon KJ, Holland JF, Bekesi JG (1985) PTT.119, *p*-F-phe-*m*-bis-(2-chloroethyl)amino-L-phe-met-ethoxy HCl, a new chemotherapeutic agent active against drug-resistant cell lines. Biochem Pharmacol 34: 2347

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