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Research paper

Proline prodrug of melphalan, prophalan-L, demonstrates high therapeutic index in a murine melanoma model

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Abstract

The therapeutic efficacy of prophalan-L, the L-proline prodrug of melphalan that demonstrated prolidase-dependent bioactivation to melphalan, was examined *in vivo* in a mouse melanoma model. Prophalan-L exhibited 2- to 2.5-fold higher hydrolytic and cytotoxic activity than prophalan-D, the D-analog, in B16-F10 murine melanoma cells *in vitro*. Prophalan-L cytotoxicity in B16-F10 cells was lower ($GI_{50} = 221$ μM) than that of melphalan ($GI_{50} = 173$ μM). The tumor growth profiles in C57BL/6J mice injected with B16-F10 cells and treated with melphalan (5.5 μg/g i.p.) and equimolar concentrations of the prodrugs demonstrated significant difference between the control (buffered saline) and melphalan or prophalan-L but no significant difference between control and prophalan-D or between melphalan and prophalan-L. Prophalan-L was significantly less toxic than melphalan, while no significant difference was observed in toxicity, measured as percent weight loss, between the prodrugs and saline control. Tumor reduction efficacy at high doses (12 μg/g i.p.) was similar for melphalan and prophalan-L; however, fatal toxicity was associated with melphalan while prophalan-L exhibited significantly lower systemic toxicity. An excellent correlation between GI_{50} and tumor reduction efficacy was observed for the tested drugs ($r^2 = 0.95$). Prophalan-L thus demonstrates higher therapeutic index than melphalan in the murine melanoma model.

Keywords: Prodrug; Melphalan; Mouse melanoma model; Prolidase; Therapeutic efficacy

1. Introduction

Melanoma ranks amongst the top five cancers in both men and women. The incidence of melanoma has steadily increased over the last few decades and in 2004 approximately 55,000 new cases will have been diagnosed and almost 10,500 deaths will have occurred [1]. Detection and treatment of early-stage disease can cure most patients but a majority of patients with deep primary tumors or tumors that metastasize to regional lymph nodes succumb to distant metastases. Median survival after the onset of distant metastases is only 6–9 months, and the 5-year survival rate is less than 5% [2–4].

For the last five decades, chemotherapy has been used with varying success. Dacarbazine was approved by FDA to be used alone or in combination for treating melanoma but has not shown good response rates [5,6]. Recently, melphalan has been used for various malignancies including melanoma but its use has been limited due to severe side-effects [7]. The side-effects associated with chemotherapy include unacceptable damage to normal cells and organs, a narrow therapeutic index, and a relatively poor selectivity for neoplastic cells [8–10].

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Enzyme–prodrug targeting is one of the strategies implemented to address the problems associated with chemotherapy. Prodrug strategies for melphalan have been incorporated using various non-human enzymes such as penicillin amidase [11,12], β-lactamase [13], and with endogenous enzymes such as prolyl endopeptidase [14] using an antibody-directed enzyme prodrug therapy (ADEPT) approach. Since, the ADEPT approach has not been clinically successful due to absence of specific tumor antigens, questions about uptake/transport of active drug once it is released outside the tumor cells, and certain adverse immune reactions causing unwanted effects, endogenous enzymes have gained more attention. Endogenous enzymes such as collagenase [15] and prolidase [16–18] have also been targeted using melphalan prodrugs.

The microarray and Affymetrix genechip expression data in the NCI-60 cancer cell lines have shown that prolidase, a highly specific dipeptidase, is overexpressed in melanoma. We previously demonstrated that the hydrolytic as well as cytotoxic activity of prophalan-L (*p*-di(2-chloroethyl)amino-L-phenylalanyl-L-proline), the L-proline prodrug of melphalan, in human cancer cells *in vitro* correlated well with mRNA expression of prolidase while the uptake of the two prodrugs, prophalan-L and its D-proline analog, prophalan-D-(*p*-di(2-chloroethyl)amino-L-phenylalanyl-D-proline), was not significantly different from each other and from that of the parent drug [19,20]. The viability of prolidase targeting in melanoma cells *in vitro* with prophalan-L warranted preliminary studies *in vivo* in an animal model.

In this study, we evaluate the *in vivo* tumor reduction efficacy of the prodrugs in comparison to melphalan in a C57BL/6J mouse melanoma model. A B16-F10 mouse melanoma cell line derived from C57BL/6J mice was used as the tumor model. The B16-F10 mouse melanoma cells were evaluated *in vitro* for hydrolysis, uptake, and cytotoxicity of the prodrugs and melphalan. B16-F10 cells were injected subcutaneously in C57BL/6J mice and the effects of treatment with melphalan and the two prodrugs and saline control compared *in vivo* with respect to tumor growth profile and toxicity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin–EDTA were obtained from Gibco-BRL (Grand Island, NY). Cell culture supplies were purchased from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). The C57BL/6J-derived B16-F10 murine melanoma cell line clone was a generous gift from Dr. K.L. Rock (University of Massachusetts, Worcester, MA). L-Proline, glutathione (reduced form), glacial acetic acid, *o*-phosphoric acid, ninhydrin, melphalan (4-[bis(2-chloroethyl)amino]-L-phenylalanine), XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate), PMS (*N*-methyldibenzopyrazine methyl sulfate), and manganese chloride tetrahydrate were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Benzyloxycarbonyl L-proline (Cbz-PRO) was purchased from Novabiochem (San Diego, CA). All other chemicals and reagents used were of analytical or HPLC grade.

2.2. Cell culture and hydrolysis of melphalan prodrugs in B16-F10 homogenates

B16-F10 cells were cultured at 37 °C in 5% CO_2 and 90% relative humidity in DMEM supplemented with 10% FBS and 1% non-essential amino acids. The proline prodrugs of melphalan were synthesized as described earlier [19]. The hydrolysis of the substrate prodrug, prophalan-L, and the negative control prophalan-D was determined in B16-F10 cells in order to assess the extent of prodrug activity in the cells. Briefly, B16-F10 cells were grown as described above and the passage number and growth time of the cells were noted. The cells were then washed with 0.15 M NaCl and collected by scraping. The cells were re-suspended in 0.15 M NaCl and then centrifuged at 3000 rpm for 5 min. The cell pellet was re-suspended in 0.05 M Tris–HCl buffer (pH 7.8), and sonicated for 10 s at 0 °C three times. The sonicated suspension was centrifuged at 18,000*g* for 30 min at 4 °C. The supernatant was then used in prodrug hydrolysis studies and to determine protein content. The protein assay was carried out using the method by Lowry et al. [21]. The protein content was adjusted to approximately 1000 μg/mL by appropriate dilutions before use in hydrolysis studies. For enzyme activation, 1 mL of the supernatant was incubated with 1 mL of 0.05 M Tris–HCl, pH 7.8, buffer containing 2 mM $MnCl_2$ for 2 h at 37 °C. Following incubation, the hydrolysis (prolidase) reaction was initiated by adding 0.1 mL of the pre-incubated mixture to 0.1 mL solution of 0.05 M Tris–HCl, pH 7.8, buffer containing 2 mm prophalan-L or 2 mM prophalan-D. Mixtures of 0.1 mL solutions of 0.05 M Tris–HCl, pH 7.8, buffer containing 2 mm prophalan-L or 2 mM prophalan-D with 0.1 mL of 0.05 M Tris–HCl buffer at pH 7.8, containing 2 mm $MnCl_2$ served as controls. The mixtures were incubated at 37 °C for 60 min. After 60 min the reaction was quenched by withdrawing 150 μL of the reaction mixture and adding it to 150 μL of cold 10% TFA solution. The quenched mixture was centrifuged at 1500 rpm for 20 min at 4 °C and 100 μL of the supernatant was withdrawn for colorimetric assays of proline content. Thus, the initial hydrolysis rates (<10% substrate hydrolysis) were calculated for the two prodrugs.

2.3. Cell proliferation assays of melphalan, prophalan-L, and prophalan-D in B16-F10 cells

Cell proliferation assays were conducted with B16-F10 cells to determine and compare the cytotoxic activities of the prodrugs, prophalan-L and prophalan-D, and the parent drug melphalan. The mouse melanoma cells were

plated overnight in a 96-well cell culture plate at a density of 5000 cells/well per 0.1 mL. Stock solutions (1 mm) of the prodrugs, prophalan-L and prophalan-D, and the parent drug, melphalan, were prepared in RPMI-1640 phenol red-free media supplemented with FBS. Stock solutions were serially diluted to obtain a total of six drug concentrations; 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 mM, for cell proliferation studies. After 24 h, the media in the 96-well plate were aspirated and replaced with drug solutions in the phenol red-free media. Growth media alone (phenol red-free) served as controls. The cells were then incubated at 37 °C and 5% CO_2 for 48 h. After 48 h, 50 μL of XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) labeling mixture (5 mL of 1 mg/mL XTT in RPMI-1640 phenol red-free media mixed with 100 μL of 0.383 mg/mL PMS in phosphate-buffered saline) was added to each well. The color development, due to formation of formazan dye by metabolically active cells, was monitored for four hours after which the plates were read at 490 nm (805 nm as the reference wavelength) with a precision microplate reader (Emax, Molecular Devices). The percent cell viability, at different drug and prodrug concentrations, relative to control was then plotted as a function of drug/prodrug concentration to compute the GI_{50} values for melphalan, prophalan-L, and prophalan-D.

2.4. Uptake of melphalan, prophalan-L, and prophalan-D in B16-F10 cells

Uptake studies in B16-F10 cells were performed to determine if uptake or hydrolysis or both determine the cytotoxic effect of the melphalan prodrugs. For the uptake study, around 300,000 cells were seeded per well in a 6-well plate. After the cells were confluent, the media were aspirated and the cells were washed with uptake buffer at room temperature. The uptake buffer contained 145 mm NaCl, 3 mm KCl, 1 mm NaH_2PO_4, 1 mm $CaCl_2$, 0.5 mM $MgCl_2$, 5 mm D-glucose, and 5 mm MES (pH 6.0). The cells were then incubated with 0.5 mm drug/prodrug solution prepared in uptake buffer for 20 min on a shaker at room temperature. After 20 min, the drug solution was removed and the cells were washed with cold PBS (phosphate-buffered saline, pH 7.4). The PBS was aspirated and 0.1% SDS solution was added to cells and shaken vigorously for 1 h to detach and dissolve the cells. The cell suspension was then transferred to an Eppendorf tube and TFA (final concentration of 5%) was added to precipitate protein. The mixture was then briefly sonicated and the tube was spun down at 10,000 rpm for 5 min after which the supernatant was filtered and analyzed by HPLC. The cell pellet was dissolved in 1% SDS and assayed for protein content.

2.5. Animals and tumor treatment

Female C57BL/6J black mice (6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were conducted using protocols approved by the University Committee On Use And Care Of Animals (UCUCA), University of Michigan, and the animals were housed and handled according to the University of Michigan Unit for Laboratory Animal Medicine guidelines. B16-F10 cells, cultured and maintained at $\leqslant$90% confluence, were suspended by trypsinization and washed once with complete media, re-suspended in complete media and allowed to recover at 37 °C for 4 h, followed by three washes with PBS, pH 7.4. The cells were then counted using a hemacytometer, and diluted to give a final concentration of 1.0×10^6 cells/mL in PBS, pH 7.4. The mice were bilaterally injected (day 0) subcutaneously (s.c.) in the dorsal rear hindquarters with 100 μL (1.0×10^5 cells) of B16-F10 cells per site. Treatment was begun 3 days after tumor injection (day 3). Mice were treated by intraperitoneal (i.p.) injection daily for 12 days (low dose) or 13 days (high dose) with 200 μL of (i) PBS with 1.5% DMSO (low dose, $n = 3$ mice) or 3% DMSO (high dose, $n = 6$ mice) as control; (ii) melphalan in low dose (5.5 μg/g body weight, $n = 3$ mice) or high dose (12 μg/g body weight, $n = 6$ mice); (iii) prophalan-L in equimolar concentration to low ($n = 3$ mice) or high dose ($n = 6$ mice) melphalan; or (iv) prophalan-D in equimolar concentration to low ($n = 3$ mice) or high dose ($n = 6$ mice) melphalan. Tumor volume was calculated from the two longest orthogonal axes using Vernier-type calipers assuming a spherical solid tumor mass with a diameter equal to the mean of the measured axes. Mice were monitored for weight and general health and animals exhibiting first signs of distress (loss of $\geqslant$20% body weight, lethargy, ataxia) or obvious discomfort due to infection or ulceration of the tumor were euthanized by CO_2 asphyxiation as per the End-Stage Illness Policy guidelines of UCUCA, University of Michigan. The averages of the tumor volumes were plotted for all the treatment groups as a function of days post-tumor injection. Data are shown for each treatment category. The weight of the animal in various treatment groups was also plotted as a function of days post-tumor injection. The toxicity of melphalan and prophalan-L was compared through the survival curve plotted for the various treatments.

2.6. Analytical methods

2.6.1. Colorimetric analysis of proline using Chinard's reagent

The extent of hydrolysis of substrates by prolidase was determined by assaying the amount of proline released according to the method described by Myara et al. [22] utilizing Chinard's reagent (25 g ninhydrin in 600 mL glacial acetic acid and 400 mL of 6 M *o*-phosphoric acid). Briefly, to 100 μL of the test sample, 200 μL each of Chinard's reagent and glacial acetic acid was added and the mixture was incubated at 90 °C for 10 min. Two-hundred microliters of the mixture was then pipetted into a flat-bottomed 96-well plate and the absorbance read at 495 nm using a precision microplate reader (Emax, Molecular Devices).

The amount of released proline was then calculated using standard curves generated using proline solutions. The standard curves generated showed excellent linearity over the proline concentration range 30 μM to 2 mm examined, with a limit of detection of ~10 μM.

2.6.2. HPLC analysis

The concentrations of melphalan and its prodrugs were determined using a Waters HPLC system (Waters Inc., Milford, MA). The HPLC system consisted of two Waters pumps (Model 515), a Waters auto-sampler (WISP model 712), and a Waters UV detector (996 Photodiode Array Detector). The system was controlled by Waters Millennium™ 32 software (Version 3.0.1). Samples were injected onto a Waters Xterra C_{18} reversed-phase column (5 μm, 4.6 × 250 mm) equipped with a guard column. The compounds were eluted using a gradient method. The flow rate was 1 mL/min; the injection volume was 30 μL. The aqueous mobile phase (Solvent A) was 0.1% (v/v) heptafluorobutyric acid (HFBA) in distilled water and the organic mobile phase (Solvent B) was 0.1% (v/v) heptafluorobutyric acid in acetonitrile. The prodrugs and parent drug were eluted with a linear gradient of 25–100% of Solvent B over 15 min at 260 nm. The standard curves generated for each of the two prodrugs and melphalan were used to calculate their concentrations in the test samples.

3. Results

3.1. Hydrolysis of melphalan prodrugs in B16-F10 cells

The hydrolytic activity for the prodrugs determined in B16-F10 cell homogenates yielded a specific activity of 0.83 ± 0.08 pmol/min/μg protein for prophalan-L and 0.35 ± 0.08 pmol/min/μg protein for prophalan-D. Thus, bioactivation of prophalan-L was roughly 2.4-fold higher than that for prophalan-D in the mouse melanoma cell homogenates. The hydrolysis of prophalan-L in B16-F10 cell homogenates was inhibited by about 60% in the presence of a specific inhibitor of prolidase, Cbz-PRO.

3.2. Cell proliferation studies with melphalan and prodrugs in B16-F10 cells

The two melphalan prodrugs, prophalan-L and prophalan-D, and parent drug melphalan were evaluated for their antiproliferative activity in B16-F10 cells. Fig. 1 shows the plot of percent cell viability as a function of prodrug or drug concentration in the B16-F10 cell lines. The profiles suggest that prophalan-L is slightly less cytotoxic than melphalan under the conditions of the cell proliferation study, exhibiting a GI_{50} value of 220.7 μM compared to 173.2 μM for melphalan, while prophalan-D was relatively ineffective at comparable concentrations (GI_{50} = 452 μM).

3.3. Uptake of melphalan, prophalan-L, and prophalan-D in B16-F10 cells

The uptake of melphalan, prophalan-L, and prophalan-D was determined in B16-F10 cells. The results indicated that the differences between the uptake rate of prophalan-L and prophalan-D in B16-F10 cells are not significantly different from each other and that the prodrugs exhibited higher uptake rate than melphalan. The uptake rate for melphalan, prophalan-L, and prophalan-D in the B16-F10 cells was 0.26 ± 0.01, 0.39 ± 0.04, and 0.39 ± 0.04 pmol/min/μg protein, respectively.

3.4. Treatment and effect on tumor growth

Tumor volume profiles following treatment low dose (5.5 μg/g i.p) melphalan or prodrugs are shown in Fig. 2.

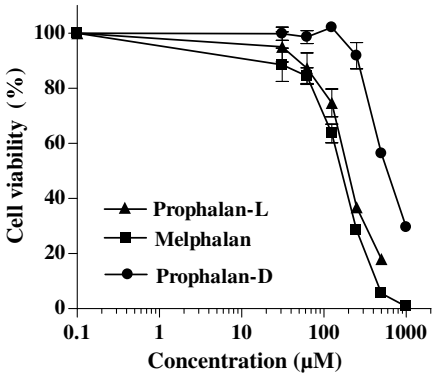

Fig. 1. Cell proliferation assays of melphalan, prophalan-L, and prophalan-D after 48-h incubation with B16-F10, murine melanoma cell line. The percent cell viability is plotted against the concentration of drug/prodrug in each treatment. The data are expressed as means ± SD (n = 3) for each treatment at each concentration.

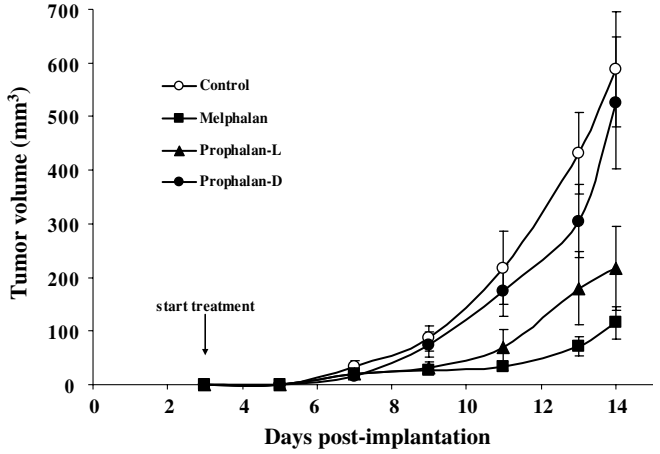

Fig. 2. Comparison of tumor growth inhibition profiles of control, prophalan-D, prophalan-L, and melphalan (5.5 μg/g i.p.) (mean ± SEM; n = 3). The tumor volume for each treatment group is plotted against the days after tumor implant. The treatment was begun on day 3 after tumor implant.

The profiles indicate that the tumor-reducing efficacy of prophalan-L on day 14 (tumor volume = 216.6 ± 78.2 mm^3) was significantly better compared to the corresponding value obtained with prophalan-D (tumor volume = 524.8 ± 122.8 mm^3) and appears to be consistent with their relative hydrolysis and cytotoxicity profiles obtained *in vitro* in B16-F10 cells. The tumors in animals treated with prophalan-D were not significantly different from control treatment (tumor volume = 588.2 ± 107.9 mm^3 on day 14). Further, tumor volume on day 14 for animals treated with melphalan (115.4 ± 30.8 mm^3) was not significantly different from that obtained with prophalan-L. However, significant weight loss in the animals (⩾10%) was observed with melphalan-treated mice while the prodrug and control treatment groups did not exhibit any weight loss (Fig. 3).

When the dose of melphalan and prophalan-L was escalated to challenge the therapeutic window, a higher reduction in tumor growth was evident in both melphalan-treated (12 μg/g i.p.) and prophalan-L-treated (equimolar dose) mice. Thus, at the higher dose, the average tumor volume measured for melphalan-treated mice was 69.3 ± 18.5 mm^3 on day 14, and 139.6 ± 30.8 mm^3 for prophalan-L-treated mice on day 16 (Fig. 4). Severe toxicity (⩾20% loss in weight) was observed with melphalan-treated mice starting at day 9 (day 6 of treatment), with all mice ($n = 6$) succumbing to toxicity by day 14 (day 11 of treatment). In contrast, prophalan-L-treated mice exhibited signs of toxicity (⩾20% loss in weight) only after day 15 (day 12 of treatment). However, there appears to be no improvement in survival of prophalan-L-treated mice compared to controls. The survival curve for the three treatment groups at the higher dose is plotted in Fig. 5.

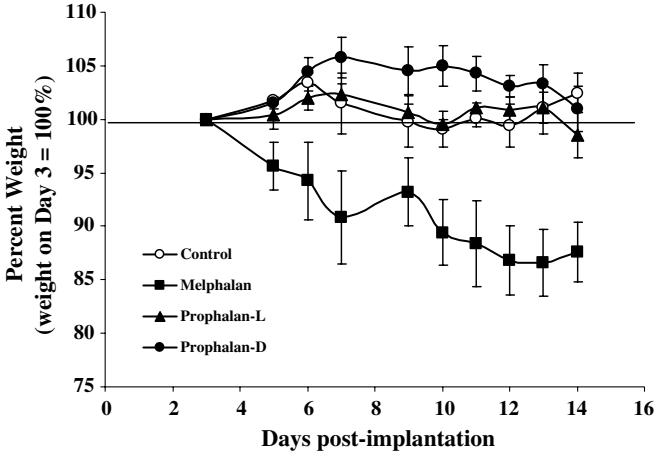

Fig. 3. Animal weight profile for the tumor bearing mice treated with control, prophalan-D, prophalan-L, and melphalan (5.5 μg/g i.p.) (mean ± SEM; $n = 3$). The weight, represented as percent initial weight, is plotted against the days after tumor implant. The treatment was begun on day 3 after tumor implant.

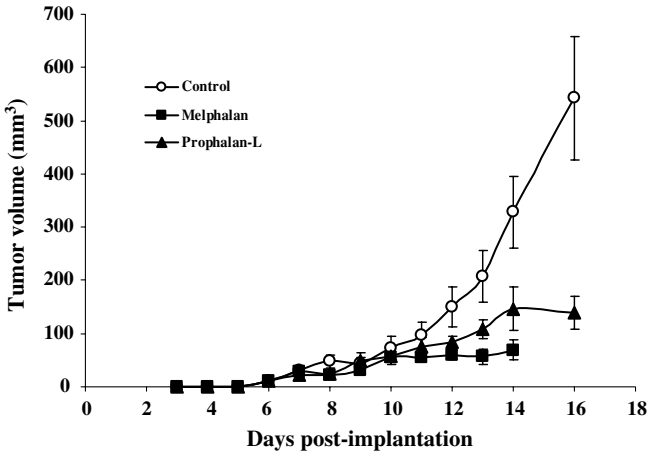

Fig. 4. Comparison of tumor growth inhibition profiles of control, prophalan-L, and melphalan (12 μg/g i.p.) (mean ± SEM; $n = 6$). The tumor volume for each treatment group is plotted against the days after tumor implant. The treatment was begun on day 3 after tumor implant.

4. Discussion

Enzyme–prodrug targeting strategy is being used for a variety of anticancer agents with the prodrugs designed for either (a) increasing bioavailability of antitumor drugs or (b) increasing local delivery of antitumor drugs [23]. Prodrugs that have been designed for increasing the local delivery of antitumor drugs are expected to achieve high tumor concentrations of the drug and decrease the systemic toxicity associated with chemotherapy [10,24,25]. Prodrug strategies for melphalan have been incorporated using various non-human enzymes (penicillin amidase and β-lactamase; ADEPT approach) as well as endogenous enzymes such as prolyl endopeptidase (ADEPT approach), collagenase and prolidase [11–15,17,18]. Collagenase, a matrix metalloprotease (MMP), has been reported to have higher activity in cancer tissues relative to normal counterparts [26] and is related to malignant phenotypes since degradation of basement membrane components, such as type IV

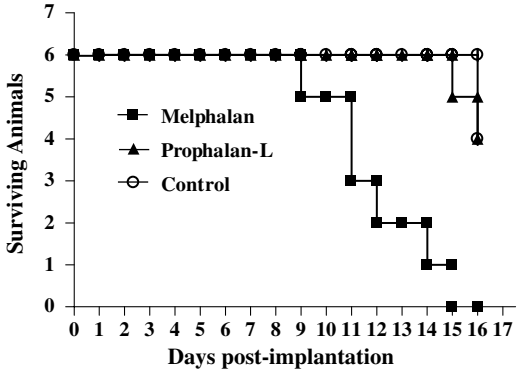

Fig. 5. Survival curve for the tumor bearing animals treated with control, prophalan-L, and melphalan (12 μg/g i.p.) ($n = 6$). The number of surviving animals in each treatment group is plotted against the number of days after tumor implant. The treatment was begun on day 3 after tumor implant.

collagen, is necessary for metastasis [27,28]. A melphalan hexapeptide (MHP) was designed as a prodrug for activation by collagenase and proposed to be an efficient way to deliver melphalan with increased cytotoxic activity in tumor cells. Prolidase which acts downstream in collagen metabolism and plays an important role in recycling proline has also been evaluated as a prodrug-activating enzyme for melphalan prodrugs [16–18]. The melphalan prodrug synthesized by Chrzanowski et al. [16–18], designated as MEL-PRO and consisting of a proline moiety linked to the amino group of melphalan by a carbonyl linker, exhibited lower cytotoxicity compared to melphalan when evaluated in MCF-7 breast carcinoma cells. We had previously demonstrated the feasibility of targeting prophalan-L to prolidase in melanoma cells *in vitro* using bioactivation, uptake, and antiproliferative assays [20]. The much lower bioactivation and cytotoxicity of prophalan-L in human foreskin fibroblasts, used as a model for non-cancerous skin cells, compared to melanomas (and other cancer cell lines with high prolidase expression) clearly suggested the potential for prophalan-L in achieving enhanced therapeutic efficacy in the treatment of melanomas. In this study, we describe the results of antitumor studies of melphalan and its proline prodrugs using a well-established B16-F10 murine melanoma model in C57BL/6J mice.

In vitro bioactivation and antiproliferative assays conducted using B16-F10 cells indicated that prophalan-L exhibited a 2.4-fold higher activity in B16-F10 cells compared to prophalan-D. The roughly 60% inhibition of prophalan-L hydrolysis in the presence of the specific inhibitor of prolidase, Cbz-PRO, corroborates the role of prolidase in bioactivation of prophalan-L in B16-F10 cell homogenates. The cytotoxicity of the two prodrugs in the B16-F10 murine melanoma cells (Fig. 1) reflects their bioactivation profiles, with prophalan-L being approximately twofold more cytotoxic than prophalan-D. Thus, the bioactivation of the prodrugs to parent melphalan most likely is the cytotoxicity-determining step since the uptake of the two prodrugs was not significantly different in the B16-F10 cells. Further, prophalan-L and prophalan-D were 1.3- and 2.6-fold less cytotoxic than melphalan, respectively, despite higher uptakes of the two prodrugs compared to melphalan.

The *in vivo* studies in the murine melanoma model were performed to validate the *in vitro* observations and required evaluation of (a) the therapeutic efficacy of prophalan-L compared to prophalan-D and (b) the therapeutic efficacy and systemic toxicity of prophalan-L compared to melphalan. The activity in isolated tumor homogenates was also compared to the hydrolytic activity in B16-F10 cell homogenates to determine any *in vivo* regulation of prolidase activity. Tumor growth inhibition by prophalan-L and melphalan was similar, suggesting efficient activation of prophalan-L to melphalan in the tumor. The significantly lower toxicity associated with prophalan-L compared to melphalan suggests that prophalan-L bioactivation to melphalan in systemic circulation is quite low (as indicated by its stability in mouse plasma) thereby reducing systemic toxic effects. The minimal therapeutic effect and systemic toxicity of prophalan-D, on the other hand, is consistent with its lower bioactivation to melphalan by prolidase as well as its stability in plasma. The average tumor sizes on day 14 for the three treatment groups – melphalan, prophalan-L, and prophalan-D, exhibited an excellent linear correlation with their respective GI_{50} values obtained *in vitro* in B16-F10 cells ($r^2 = 0.95$; plot not shown). This *in vivo–in vitro* correlation not only validates the *in vitro* results obtained with B16-F10 cells but also suggests the possibility of a higher therapeutic index of prophalan-L in a nude mouse model with a human melanoma xenograft since human melanoma cells exhibited ~7-fold higher activity compared to the mouse melanoma cells in this study [20].

Treatment with higher doses of melphalan (12.2 μg/g) and prophalan-L (equimolar concentration) resulted in a greater reduction of tumor growth suggesting that a greater therapeutic index might be possible with human melanoma cells which exhibit higher hydrolysis and cytotoxicity for prophalan-L [20] compared to murine melanoma cells. Further, at high doses, a dramatic increase in toxicity was evidenced by a mortality of 100% of the melphalan-treated animals by day 15. In comparison, only 16% of the animals treated with high-dose prophalan-L showed comparable toxicity at day 15. Further, studies with isolated tumor homogenates demonstrated an approximately 1.7-fold higher hydrolytic activity of prophalan-L compared to B16-F10 cell homogenates indicating a slight upregulation of activity *in vivo*. However, high-dose prophalan-L treatment did not increase survival of mice compared to that of the control group at day 16.

The results of the preliminary animal studies described in this report are consistent with previous *in vitro* studies and validate the potential of prophalan-L to selectively target prolidase overexpression in melanomas. The similarity of therapeutic effects between melphalan and prophalan-L coupled with the significantly lower systemic toxicity of the prodrug compared to melphalan suggests that the therapeutic window with prophalan-L may be significantly enhanced over that of melphalan.

Acknowledgments

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