Pharmaceutical Research, Vol. 24, No. 7, July 2007 (© 2007)
DOI: 10.1007/s11095-007-9249-9

Research Paper

Proline Prodrug of Melphalan Targeted to Prolidase, a Prodrug Activating Enzyme Overexpressed in Melanoma

Sachin Mittal,[1] Xueqin Song,[1] Balvinder S. Vig,[1,2] and Gordon L. Amidon[1,3,4]

Received November 14, 2006; accepted January 22, 2007; published online March 22, 2007

Purpose. To determine the bioactivation and uptake of prolidase-targeted proline prodrugs of melphalan in six cancer cell lines with variable prolidase expression and to evaluate prolidase-dependence of prodrug cytotoxicity in the cell lines compared to that of the parent drug, melphalan.
Materials and Methods. Hydrolysis, cell uptake, and cell proliferation studies of melphalan and the L- and D-proline prodrugs of melphalan, prophalan-L and prophalan-D, respectively, were conducted in the cancer cell lines using established procedures.
Results. The bioactivation of prophalan-L in the cancer cell lines exhibited high correlation with their prolidase expression levels ($r^2 = 0.86$). There were no significant differences in uptake of melphalan and its prodrugs. The cytotoxicity of prophalan-L (GI_{50}) in cancer cells also showed high correlation with prolidase expression ($r^2 = 0.88$), while prophalan-D was ineffective at comparable concentrations. A prolidase targeting index (ratio of melphalan to prophalan-L cytotoxicity normalized to their uptake) was computed and showed high correlation with prolidase expression ($r^2 = 0.82$).
Conclusions. The data corroborates the specificity of prophalan-L activation by prolidase as well as prolidase-targeted cytotoxicity of prophalan-L in cancer cell lines. Hence, prophalan-L, a stable prodrug of melphalan, exhibits potential for efficiently targeting melanoma with reduced systemic toxicity.

KEY WORDS: melanoma; melphalan prodrugs; prolidase; prophalan-L; targeting.

INTRODUCTION

Adverse effects associated with chemotherapy include unacceptable damage to normal cells and organs, a narrow therapeutic index, a relatively poor selectivity for neoplastic cells, and multidrug resistance upon prolonged treatment due to up-regulation of efflux pumps, increased glutathione *S*-transferase expression, and enhanced DNA repair (1–3). The selectivity of anticancer agents can be improved by appropriate design of prodrugs of anticancer agents targeted to enzymes or transporters that are overexpressed in cancers. Information on the expression and substrate specificity of such molecular targets would facilitate design of prodrugs for enzyme-prodrug targeting strategies. These strategies had been handicapped due to incomplete knowledge of desirable molecular targets (4); however, recent advances in bioinformatics tools and mapping of the human genome have allowed the identification of such molecular targets for incorporation into enzyme-targeted prodrug strategies.

We previously reported an analysis of public gene expression databases that revealed that prolidase might be a desirable enzyme target based on its differential expression in melanoma cancer cell lines and its high substrate specificity for dipeptides containing proline at the carboxy terminus (5). Thus, the activation of a standard substrate of prolidase, Gly-Pro, determined in NCI 60 cancer cell lines, exhibited good correlation with prolidase expression in these cells. Melanoma, one of the top five cancers afflicting both men and women, is curable if detected early, but becomes fatal in patients with deep primary tumors or tumors that metastasize to regional lymph nodes. Dacarbazine, the first drug approved by FDA for the treatment of melanoma, is used alone or in combination but has a response rate of only 10–20% (6,7). Melphalan, phenylalanine mustard (L-PAM), originally approved for the treatment of multiple myeloma, has recently been used in the treatment of a variety of cancers such as ovarian cancer, breast cancer, colorectal cancer and melanoma (8). However, due to severe toxic side-effects such as bone marrow suppression, leukopenia and thrombocyto-

[1] Department of Pharmaceutical Sciences, College of Pharmacy, University of Michigan, 428 Church Street, Ann Arbor, Michigan 48109, USA.
[2] Exploratory Biopharmaceutics & Stability, Drug Delivery Enablement, Pharmaceutical Research Institute, Bristol-Myers Squibb Company, New Brunswick, New Jersey 08502, USA.
[3] College of Pharmacy, The University of Michigan, 428 Church Street, Ann Arbor, Michigan 48109-1065, USA.
[4] To whom correspondence should be addressed. (e-mail: glamidon@umich.edu)

ABBREVIATIONS: Caco-2, colon adenocarcinoma; Cbz-PRO, *N*-benzyloxycarbonyl L-proline; HepG2, hepatocellular carcinoma; HFF, human foreskin fibroblast; MCF-7, breast adenocarcinoma; NCI-H522, lung adenocarcinoma; PMS, *N*-methyl dibenzopyrazine methyl sulfate; Prophalan-D, *p*-di(2-chloroethyl)amino-L-phenylalanyl-D-proline; Prophalan-L, *p*-di(2-chloroethyl)amino-L-phenylalanyl-L-proline; SK-MEL-5, melanoma; U-251, CNS glioblastoma; XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate.

penia, novel alternative treatment approaches have become a necessity. Thus, establishment of prolidase as a potential enzyme target for melphalan prodrugs could be significant in achieving desirable melanoma therapeutics.

In the previous study, we had also reported the synthesis of proline prodrugs of melphalan and their cytotoxic activity in SK-MEL-5 melanoma cell lines (5). It was found that the L-proline prodrug of melphalan (Prophalan-L) was a significantly better substrate for prolidase than the D-proline prodrug (Prophalan-D). Further, the observation that prophalan-L was cyctotoxic in SK-MEL-5 cell proliferation assays while prophalan-D was inherently ineffective suggested selective cytotoxic action in SK-MEL-5 cells that was dependent on bioactivation of the L-proline prodrug by prolidase (5). These preliminary studies suggested that such selective activation of melphalan prodrugs by prolidase in melanoma cancer cells lines could provide enhanced therapeutic efficacy and reduce systemic toxicity associated with melphalan.

In this report, we describe the results of bioactivation, uptake, and cytotoxic activity of the proline prodrugs in six NCI-60 cancer cell lines with variable prolidase expression levels to determine the viability of prolidase as an enzyme target for melanoma, and prophalan-L as a novel melphalan prodrug for targeting melanoma.

MATERIALS AND METHODS

Materials

SK-MEL-5 (melanoma), MCF-7 (Breast), NCI-H522 (Lung), U-251 (CNS), Caco-2 (colon), and HepG2 (liver) cancer cell lines were obtained from National Cancer Institute (Bethesda, MD) or ATCC. RPMI-1640 medium, Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), fetal calf serum (FCS), and trypsin-EDTA were obtained from GIBCO BRL (Grand Island, NY) while minimal essential medium (MEM) was obtained from ATCC. Cell culture supplies were purchased from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). L-proline, D-proline, glutathione (reduced form), glacial acetic acid, *o*-phosphoric acid, ninhydrin, prolidase from porcine kidney (134 units/mg solid), melphalan (4-[Bis(2-chloroethyl)amino]-L-phenylalanine), chlorambucil (4-[Bis(2-chloroethyl)amino]benzebutyric acid), XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate), PMS (*N*-methyl dibenzopyrazine methyl sulfate), and manganese chloride tetrahydrate were purchased from Sigma Chemical Company (St. Louis, MO). The L-proline benzyl ester hydrochloride was obtained from Bachem (Torrance, CA). Palladium 10%, di-tert-butyl dicarbonate, and dicyclohexyl-

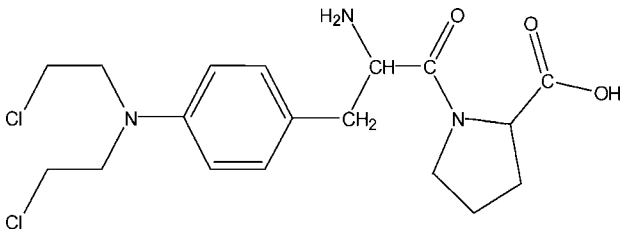

Fig. 1. Structure of prophalan, the proline prodrug of melphalan.

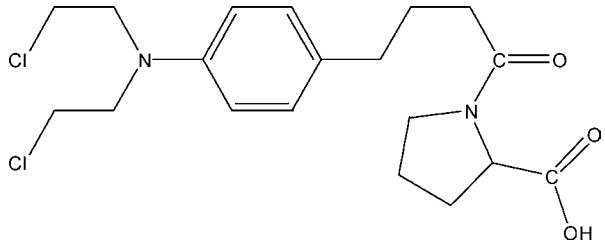

Fig. 2. Structure of the proline prodrug of chlorambucil.

carbodiimide were purchased from Aldrich (Milwaukee, WI). *N*-benzyloxycarbonyl L-proline (Cbz-PRO) was purchased from Novabiochem (San Diego, CA). Access RT-PCR kit consisting of nuclease free water, dNTP mix, *Tfl* DNA polymerase (5 U/μl), AMV reverse transcriptase (5 U/μl), 25 mM magnesium sulfate, and AMV/*Tfl* 5X reaction buffer was obtained from Promega (Madison, WI). The sense and anti-sense primers were designed using Primer select2® and ordered from Invitrogen (Carlsbad, CA) along with the 4–20% TBE gels. All other chemicals and reagents used were of analytical or HPLC grade.

Synthesis of Proline Prodrugs of Melphalan

The synthesis of the proline prodrugs of melphalan, prophalan-L and prophalan-D, was carried out in a step-wise fashion as described earlier (5). Briefly, melphalan was condensed with L-proline benzyl ester (or D-proline benzyl ester) by using the carbodiimide coupling method. The coupling reaction was carried out in chloroform in the presence of dicyclohexylcarbodiimide. The protecting benzyl group was removed by catalytic dehydrogenation at room temperature and atmospheric pressure. ESI-MS and ^{1}H NMR spectral analyses data that confirmed the structural identities of the proline prodrugs (Fig. 1) were also reported earlier (5).

Synthesis of Proline Prodrug of Chlorambucil

Chlorambucil is a nitrogen-mustard with a high degree of structural similarity to melphalan. The proline prodrug of chlorambucil (chlorambucil-L-proline) was synthesized in a step-wise fashion using chlorambucil and L-proline benzyl ester as described earlier (9). The structure of the prodrug (Fig. 2) was confirmed by elemental and spectral analyses.

^{1}H-NMR (DMSO): δ 7.03 (d, 2H, J = 8.79 Hz), 6.66 (d, 2H, J = 9 Hz), 4.25–4.53 (m, 1H), 3.44–3.69 (m, 10H), 2.21–2.46 (m, 6H), 1.69–1.74 (m, 4H); ESI-MS: 400 $(M–H)^+$

Hydrolysis of Chlorambucil and Melphalan Prodrugs by Porcine Kidney Prolidase

The hydrolysis of prophalan-L, prophalan-D, and proline prodrug of chlorambucil by porcine kidney prolidase was examined. Porcine kidney prolidase was used in hydrolysis studies since it was commercially available and more importantly because of its close alignment with human prolidase (97% identity; BLASTP2.2.6). Briefly, prolidase solution was prepared by suspending the lyophilized solid (1.2 mg solid, 0.7133 mg protein) in 50 mM cold Tris-HCl buffer (pH 8.0 at 40°C) to yield a 5 mg/ml solution. The enzyme activation and assay were carried out according to the manufacturer's pro-

tocol (Sigma) by preparing two sets of reagents, Reagent A and Reagent B. Reagent A consisted of 50 mM pH 7.8 Tris HCl buffer (pH 8.0 at 40°C), 200 mM manganese chloride, 30 mM glutathione, and porcine kidney prolidase solution (2.97 mg/ml). Reagent A was incubated at 40°C for 30 min to activate the enzyme. Reagent B consisted of 2.53 mM prophalan-L or prophalan-D or chlorambucil prodrug and 200 mM manganese chloride. The activated Reagent A (a final enzyme concentration of 28.18 μg/ml in the reaction mixture) was added to Reagent B for a final prodrug concentration of 2 mM and incubated for 2 min (prophalan-L) and 30 min (prophalan-D and chlorambucil prodrug) at 40°C. Solutions of Reagent A without the enzyme mixed with Reagent B as described above served as controls. The competitive inhibition of prodrug hydrolysis by porcine kidney prolidase in the presence of the inhibitor Cbz-PRO was determined by including Cbz-PRO in Reagent B at the same concentration as the prodrug. The enzymatic reaction was quenched by adding 10% ice-cold TFA (trifluoroacetic acid). The mixtures were then centrifuged at 1,500 rpm (221 g) for 20 min and the supernatant was withdrawn for colorimetric assay of the released proline content as described below. Thus, initial hydrolysis rates (<10% of substrate hydrolysis) were calculated for the three compounds.

Determination of Kinetic Parameters

The kinetic parameters of porcine kidney prolidase for the hydrolysis of prophalan-L were determined as follows. Kinetic measurements were carried out in 50 mM pH 7.8 Tris HCl buffer (pH 8.0 at 40°C) supplemented with 200 mM manganese chloride to which the activated Reagent A containing prolidase (28.18 μg/ml prolidase concentration in reaction mixture) was added as described in the previous section. Kinetic parameters were calculated from the initial velocity data (<10% substrate hydrolysis) at prophalan-L concentrations ranging from 0.05 to 4 mM. The Michaelis-Menten equation was fitted to the data by the nonlinear least square regression analysis (one-site saturation kinetics) using Sigma Plot 8.0 (SPSS Inc. Chicago, IL).

Selection of Cancer Cell Lines and Cell Culture

Candidate cancer cell lines for hydrolysis and cytotoxicity studies were selected from the NCI 60 cell lines based on the expression levels of prolidase, doubling time, and growth requirements. Thus, the cancer cell lines selected, SK-MEL-5, NCI-H522, U-251, and MCF-7, represent cells with high, medium and low expression of prolidase, reasonably short doubling times, and standard growth requirements. In addition, Caco-2 and HepG2 cell lines were also used in the studies. HFF cells were included to elicit comparisons between the tumor cell activity (SK-MEL-5) and non-tumor cell activity (HFF) from the same tissue.

Cells were cultured at 37°C in 5% CO_2 and 90% relative humidity. SK-MEL-5 and U-251 cell lines were cultured in RPMI-1640 supplemented with 10% FBS. NCI-H522 and MCF-7 cell lines were cultured in RPMI-1640 supplemented with 10% FCS. Caco-2 cells were cultured in DMEM supplemented with 10% FBS and 1% nonessential amino acids. HFF and HepG2 cells were cultured in MEM supplemented with 10% FBS.

Reverse Transcription-PCR of Prolidase mRNA in Cancer Cell Lines

Total cellular RNA was isolated from each cancer cell line using Trizol® reagent as per the directions of the manufacturer. The isolated RNA (0.5 μg) was reverse transcribed for 45 min at 48°C using AMV reverse transcriptase, oligo (dT) as the downstream primer, and Access RT-PCR kit (Promega). The primer pair, sense (20-mer, 5′-CTGCAGG GCGGGGAGGAGAC-3′), and anti-sense (22-mer, 5′-CGC CCCGGGAGTAGCAGTAGTG-3′), were used for the PCR amplification. The PCR conditions were as follows: initial 2 min denaturation at 94°C, followed by 20 PCR amplification cycles (30 s at 94°C, 30 s at 56°C, 1 min at 68°C) and a final 7 min extension at 68°C. The RT-PCR product was separated on 4–20% TBE gel. The gel was run at 200 V in TBE buffer (1X) and then the DNA separated on the gel was stained with SYBR green. The gel was then visualized in UV light and the relative intensities of the RT-PCR product bands measured using Metamorph® software. RT-PCR measurements were conducted three times with each cell line.

Hydrolysis of Proline Prodrugs of Melphalan in Cancer Cells and Inhibition by Cbz-PRO

The hydrolysis of the substrate prodrug, prophalan-L, and the negative control prophalan-D was determined in the selected cancer cell lines to assess the extent of prolidase activity in the cells. Briefly, cancer 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 resuspended in 0.15 M NaCl and then centrifuged at 3,000 rpm for 5 min. The cell pellet was resuspended 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.* (10). The protein content was adjusted to approximately 1,000 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. In competitive inhibition studies, 0.1 ml of the pre-incubated mixture was added to 0.1 ml solutions of 0.05 M Tris-HCl, pH 7.8 buffer containing 2 mM prophalan-L and 2 mM Cbz-PRO. 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 1,500 rpm for 20 min at 4°C and 100 μl of the supernatant were withdrawn for colorimetric assays of proline content. The initial hydrolysis rates were then calculated for the two prodrugs.

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.* (11) utilizing Chinard's reagent (25 g ninhydrin in 600 ml glacial acetic acid and 400 ml of 6 M o-phosphoric acid). Briefly, 200 μl each of Chinard's reagent and glacial acetic acid were added to 100 μl of the test sample, and the mixture was incubated at 90°C for 10 min. Two hundred microliters of the mixture were then pipeted into a flat-bottom 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 from standard curves generated using pure proline solutions. The standard curves generated showed excellent linearity over the proline concentration range of 30 μM–2 mM examined, and the limit of detection being approximately 10 μM.

Cell Proliferation Assay in Cancer Cells

Cell proliferation assays were conducted to determine and compare the cytotoxic activities of the prodrugs, prophalan-L and prophalan-D, and the parent drug melphalan. Cell proliferation assays were carried out with the selected cancer cell lines as well as with HFF cells. The cancer cells were plated overnight in a 96-well cell culture plate at a density of 5,000 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 medium supplemented with FBS. Stock solutions were serially diluted to obtain a total of six drug concentrations, 1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM and 0.03125 mM, for cell proliferation studies. After 24 h, the media in the 96-well plate was aspirated and replaced with drug solutions in the phenol-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 medium 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 4 h 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 or prodrug concentration to compute the GI_{50} values for melphalan, prophalan-L and prophalan-D in all the cell lines studied.

Uptake of Melphalan, Prophalan-L, and Prophalan-D in Cancer Cell Lines

Uptake studies were conducted with the cancer cell lines by seeding around 300,000 cells per well in a 6-well plate. After the cells were confluent, the media was 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 ml of 0.5 mM drug or prodrug solution prepared in uptake buffer for 20 min on a shaker at room temperature. After 20 min, the drug or prodrug solution was removed and the cells were washed three times with 1 ml ice-cold PBS (phosphate buffered saline, pH 7.4). The PBS was aspirated and 1 ml 0.1% SDS solution was added to cells and shaken vigorously for 1 hr to detach and lyse the cells. The cell suspension was then transferred to an eppendorf tube and TFA (final concentration of 5% v/v) was added to precipitate protein. The mixture was then briefly sonicated and the tube was centrifuged at 9,720 g for 5 min after which the supernatant was filtered (0.45 μm, Whatman GF membrane filter) and analyzed by HPLC. The cell pellet was dissolved in 1% SDS and assayed for protein content.

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.

RESULTS

Hydrolysis of Melphalan and Chlorambucil Prodrugs by Porcine Kidney Prolidase

The specific activity of porcine kidney prolidase for prophalan-L, determined using assay of released proline, was 591.4 ± 47.3 pmoles/min/μg of prolidase. In the presence of the competitive inhibitor Cbz-PRO, prophalan-L specific activity decreased substantially to 197.2 ± 36.1 pmoles/min/μg prolidase. Prophalan-D and the proline prodrug of chlorambucil were also examined with porcine kidney prolidase and their activities relative to prophalan-L were observed to be 0.86% and 0%, respectively (Fig. 3). The kinetic parameters, K_m and V_{max}, for prophalan-L hydrolysis by prolidase as determined from a V_0 versus [S] plot (Fig. 4), were 0.97 ± 0.16 mM and $1{,}193.0 \pm 70.2$ pmoles/min/μg of prolidase, respectively.

Hydrolysis of Proline Prodrugs of Melphalan in Cancer Cells and Inhibition by Cbz-PRO

The specific activity of prolidase for the proline prodrugs of melphalan in cell homogenates of the various cancer cell

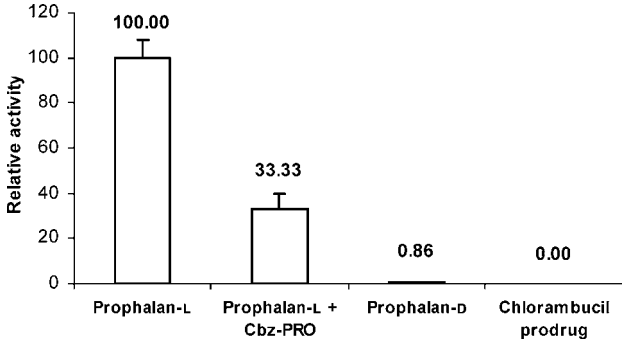

Fig. 3. Relative activities of prophalan-L (in the presence and absence of Cbz-PRO), prophalan-D, and proline prodrug of chlorambucil, with porcine kidney prolidase (mean ± SD, $n = 3$). Activity of prophalan-L set to 100.0

lines and in HFF control cells in the presence and absence of the specific inhibitor CbzPro are listed in Table I. Table I also shows the percent inhibition of prolidase activity in the cell homogenates. The results indicate that prolidase activity was in the order: SK-MEL-5 ≫ NCI-H522 > Caco-2 ≈ HepG2 > HFF > U-251 > MCF-7 for prophalan-L. Prophalan-L hydrolysis in the presence of the competitive inhibitor Cbz-PRO was significantly inhibited in all cancer cell systems tested. Thus, the average inhibition in the seven cell lines was ~70% with individual values ranging from 60–83% (Table I). The melanoma cell line SK-MEL-5 demonstrated approximately 5.5-fold higher hydrolysis of the prodrug compared to the non-cancerous HFF cells. The specific activity of prophalan-L in the seven systems in the presence and absence of Cbz-PRO are plotted in Fig. 5.

Expression of Prolidase in Cancer Cell Lines using RT-PCR

The expression of prolidase in the selected cancer cell lines excepting HFF control cells, determined using semi-

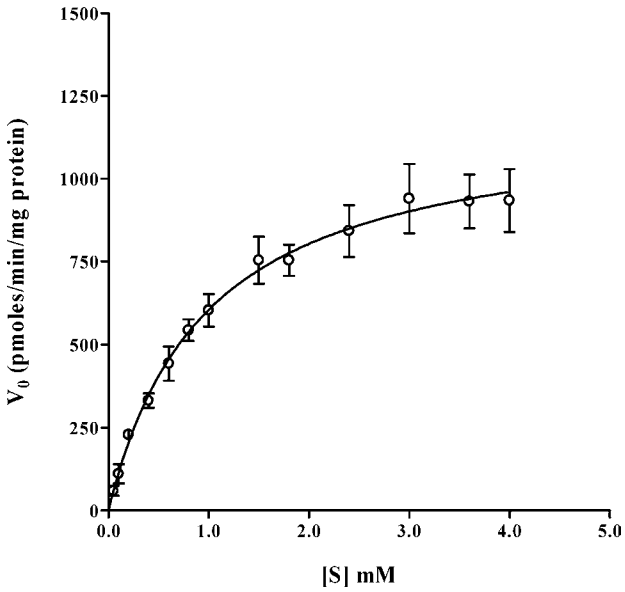

Fig. 4. Prophalan-L kinetic profile with porcine kidney prolidase (mean ± SD, $n = 3$).

Table I. Specific Activity of Prophalan-L in the Presence and Absence of Cbz-PRO in Various Cancer Cell Lines (expressed as pmoles/min/μg protein, mean ± SD, $n = 3$) and Prolidase Expression Determined with RT-PCR

Cell Lines	Specific Activity w/o Cbz-PRO	Specific Activity w/Cbz-PRO	Percent Inhibition	Relative RT-PCR Expression
SK-MEL-5	7.61 ± 0.27	2.49 ± 0.08	67.3	1.29
Caco-2	2.22 ± 0.08	0.49 ± 0.20	78.0	1.25
NCI-H522	2.40 ± 0.08	0.82 ± 0.42	66.0	1.21
HepG2	2.14 ± 0.13	0.36 ± 0.15	83.3	1.19
U-251	1.33 ± 0.35	0.53 ± 0.13	60.0	1.18
MCF-7	0.71 ± 0.15	0.22 ± 0.15	69.0	1.09
HFF[a]	1.35 ± 0.29	0.42 ± 0.15	68.8	1.21
Plasma	0.22 ± 0.01	–		–

[a] Human foreskin fibroblasts, non-cancerous cell line

quantitative RT-PCR analysis, was reported earlier (5). The relative expression of prolidase determined with RT-PCR in the six cancer cell lines examined was in the order: SK-MEL-5 (1.28); Caco-2 (1.25); NCI-H522 (1.21); HFF (1.21); HepG2 (1.19); U-251 (1.18); and MCF-7 (1.09) (Table I). The RT-PCR expression of prolidase in the cancer cell lines and HFF cells was normalized with RT-PCR prolidase expression in K-562 leukemia cells that was set to unity (5).

Cell Proliferation Studies with Prophalan-L

The two melphalan prodrugs, prophalan-L and prophalan-D were evaluated for their anti-proliferative activity in the seven cell lines along with the parent drug melphalan. The GI_{50} values for prophalan-L and parent melphalan calculated from cell proliferation profiles are listed in Table II. Typical profiles for SKME-5 and U-251 cell lines, representing high and low prolidase expression levels, respectively, are depicted in Fig. 6. No apparent trends are evident with GI_{50} values for melphalan. The GI_{50} values for prophalan-L, however, appeared to be inversely related to prolidase expression in the cancer cells. Thus, prophalan-L GI_{50} values were in the order: SK-MEL-5 < Caco-2 < HFF ≈ NCI-H522 < U-251 < HepG2 < MCF-7. Prophalan-D, the D-analog, was relatively ineffective at comparable concentrations even in cell lines with high expression of prolidase such as SK-MEL-5. Further,

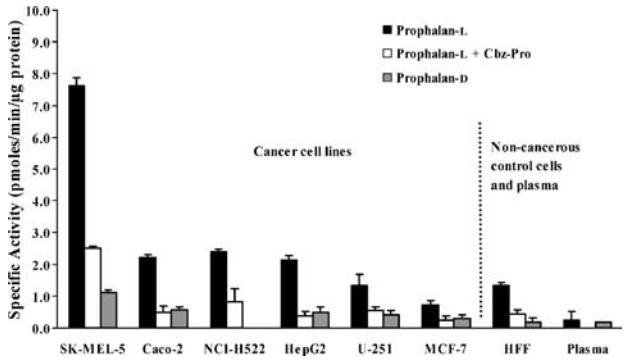

Fig. 5. Specific activity of prophalan-L in the presence and absence of Cbz-PRO and of prophalan-D in various cancer cell lines and in human plasma (expressed as pmoles/min/μg protein, mean ± SD, $n = 3$).

Table II. Melphalan and Prophalan-L Cytotoxicity (expressed as GI_{50}; $n = 3$) in Various Cell Lines and the Prolidase Targeting Index in the Cell Lines Calculated from the Melphalan and Prophalan-L GI_{50} Values and Normalized to Uptake

Cell lines	Melphalan GI_{50} (μM)	Prophalan-L GI_{50} (μM)	Prolidase Targeting Index[b]
SK-MEL-5	57.0	74.8	1.02
Caco-2	56.3	119.7	0.54
NCI-H522	60.0	149.3	0.33
HepG2	73.6	263.4	0.44
U-251	51.7	203.1	0.08
MCF-7	218.8	300.6	0.69
HFF[a]	24.9	146.6	–

[a] Human foreskin fibroblasts, non-cancerous cell line
[b] Prolidase Targeting Index = (Melphalan GI_{50}/Prophalan-L GI_{50})/(Prophalan-L uptake/Melphalan uptake)

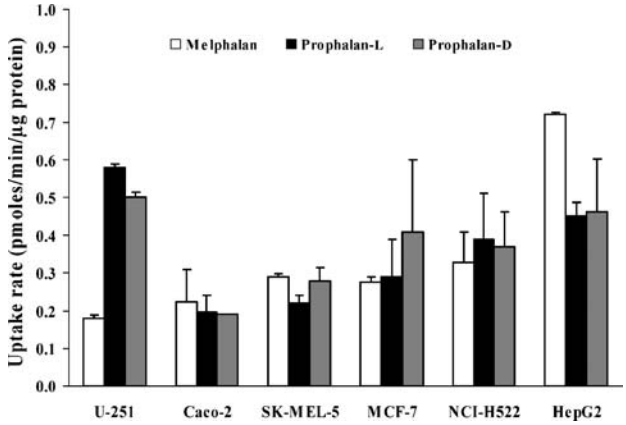

Fig. 7. Uptake rates in the cancer cell lines determined for melphalan, prophalan-L, and prophalan-D (expressed as pmoles/min/μg protein, mean ± SEM).

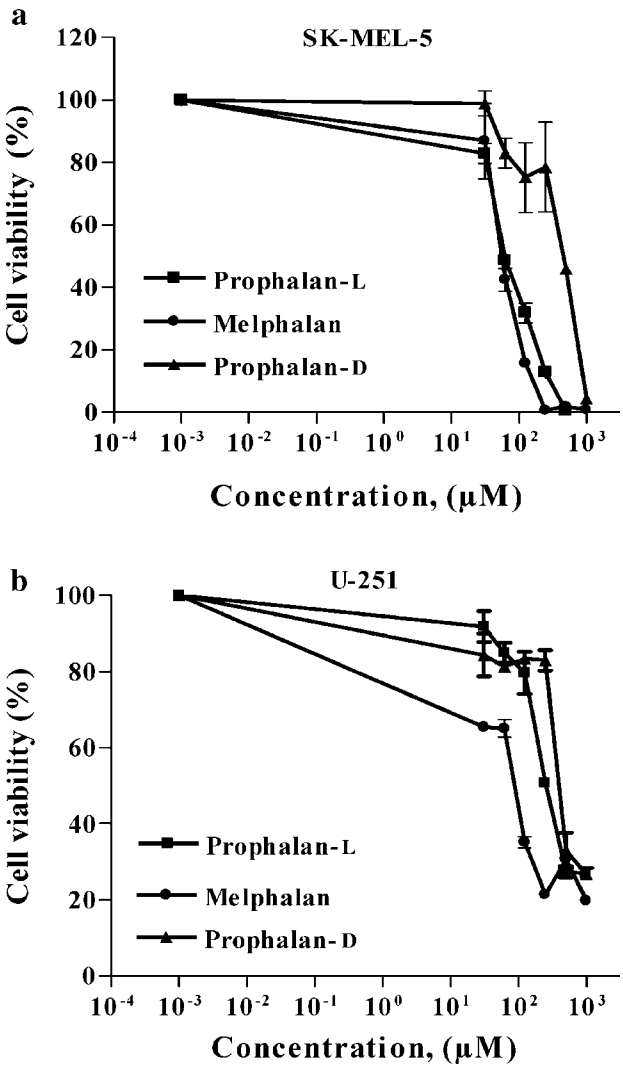

Fig. 6. Cell proliferation assays of melphalan, prophalan-L, and prophalan-D, after 48 h incubation with **a** SK-MEL-5 and **b** U-251 cell lines (expressed as mean ± SD, $n = 3$, for each treatment at each concentration).

prophalan-L was found to be less cytotoxic than melphalan under the conditions of the cell proliferation study, exhibiting a GI_{50} value of 74.8 μM compared to 57.0 μM for melphalan in the highest expression cell line, SK-MEL-5. More importantly, the cytotoxicity of prophalan-L was significantly lower ($GI_{50} = 147$ μM) than that of parent melphalan ($GI_{50} = 25$ μM) in the non-cancerous HFF control cells.

Uptake of Melphalan, Prophalan-L, and Prophalan-D in the Cancer Cell Lines

The uptake of melphalan, prophalan-L and prophalan-D was determined in the cancer cell lines. With a few exceptions, the uptake of melphalan, prophalan-L, and prophalan-D in a given cancer cell line was not significantly different (Fig. 7). Melphalan uptake in the cancer cell lines was in the order U-251 < Caco-2 < MCF-7 < SK-MEL-5 < NCI-H522 < HepG2, whereas prophalan-L uptake in the cancer cell lines increased in the order Caco-2 < SK-MEL-5 < MCF-7 < NCI-H522 < HepG2 < U-251. Prophalan-D uptake in the cancer cell lines was in the order Caco-2 < SK-MEL-5 < NCI-H522 < MCF-7 < HepG2 < U-251.

Correlation between Prophalan-L Activity and Prolidase Expression

The specific activity in the cancer cell lines determined using prophalan-L exhibited a linear correlation ($r^2 = 0.62$) with RT-PCR expression of prolidase in the cancer cell lines (Fig. 8). However, an exponential fit was found to provide a better correlation ($r^2 = 0.86$); inclusion of HFF cells lowered the correlation coefficient for an exponential fit to $r^2 = 0.79$. The antiproliferative activity of prophalan-L (GI_{50}) exhibited an excellent linear correlation with RT-PCR expression of prolidase in the cancer cell lines ($r^2 = 0.88$) (Fig. 9). Inclusion of HFF cells in the correlation lowered the correlation coefficient marginally to $r^2 = 0.87$. Melphalan GI_{50} values in cancer cells exhibited a poorer correlation with RT-PCR expression of prolidase in the cells ($r^2 = 0.66$); inclusion of HFF cells, however, further lowered the correlation coefficient ($r^2 = 0.60$).

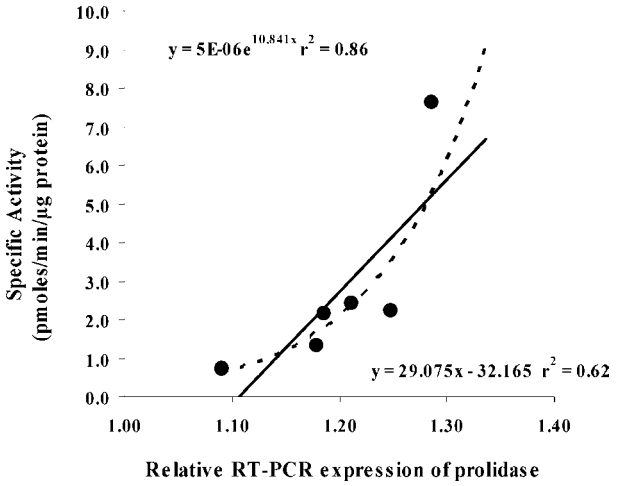

Fig. 8. Correlation of specific activity of prophalan-L in cancer cells with prolidase expression determined using RT-PCR.

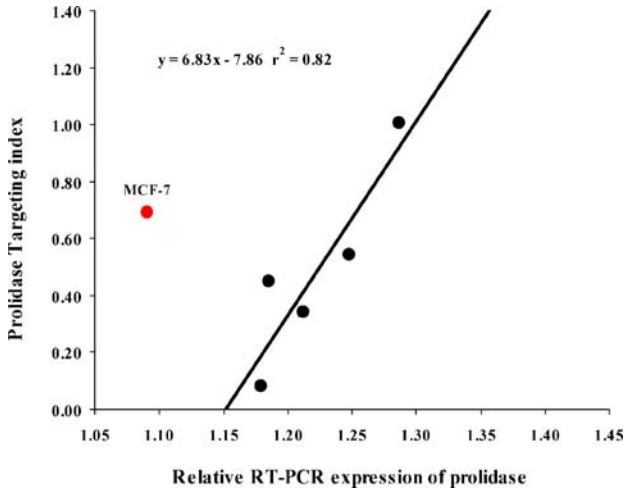

Fig. 10. Correlation of prolidase targeting index with prolidase expression determined using RT-PCR.

The relative cytotoxicity (GI_{50}) of melphalan and prophalan-L in each cell line normalized to their uptake, denoted as the prolidase targeting index, exhibited a good correlation ($r^2 = 0.82$) with prolidase expression in the cancer cell lines (Fig. 10).

DISCUSSION

Due to severe toxicity associated with most chemotherapeutic agents, a prodrug approach where the prodrug is selectively activated in cancerous tissues over-expressing the prodrug-activating enzyme(s) could reduce systemic toxicity while maintaining the therapeutic efficacy. In a previous report we had identified prolidase, an enzyme that is over-expressed in melanomas, as a suitable enzyme target for selective action of anticancer agents (5). In this study, we evaluate the targetability of prolidase using cancer cell lines with variable expression levels of prolidase. Thus, the activation of proline prodrugs of an anticancer agent melphalan as well their cytotoxic activity in these cancer cell lines was determined in order to validate our hypothesis. Further, this report also compares a chlorambucil prodrug to illustrate the influence of high specificity and differential expression on cytotoxicity and targetability of the enzyme. We also describe the results of studies aimed at elucidating the rate-determining step for cytotoxicity of the prodrugs which would provide a sound rationale for a successful enzyme-targeted prodrug strategy to be examined *in vivo*.

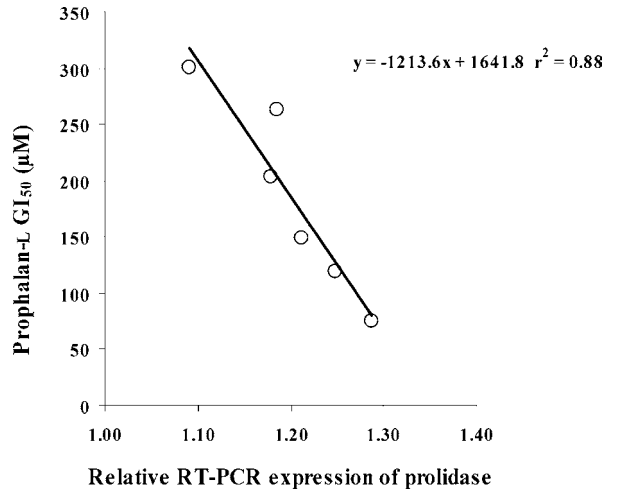

Fig. 9. Correlation of cytotoxicity of prophalan-L represented by its GI_{50} value in cancer cells with prolidase expression determined using RT-PCR.

We had demonstrated in an earlier study that prophalan-L is a substrate of prolidase. Hydrolysis studies with porcine kidney prolidase revealed that prolidase was highly selective and specific for its substrates (5). The studies confirmed previously established substrate requirements for prolidase; L-proline at the C-terminus of the dipeptide and a free α-amino group in the dipeptide (12–14). Thus, prolidase is a very specific peptidase for proline-containing dipeptides (15). The significant inhibition (~67%) in the presence of Cbz-PRO, a specific inhibitor of prolidase (16), suggests that determination of prophalan-L activity in cell homogenates may be accurate indicators of prolidase activity in the cells.

The extent of inhibition in the presence of Cbz-Pro in cell homogenates with human prolidase compared favorably with the extent of Cbz-Pro inhibition observed using porcine kidney prolidase. Thus, the relative extent of hydrolysis of prophalan-L and prophalan-D, and the inhibition of prophalan-L hydrolysis by Cbz-PRO (~70%) in the cancer cell lines were similar to that observed with porcine kidney prolidase (prophalan-D hydrolysis being 4- to 7-fold lower), and validates the evaluation of prolidase activity in cancer cell homogenates. The hydrolytic activity of prophalan-L in the cell homogenates was comparable to carboxylesterase activity for irinotecan, an established prodrug of CPT-11 (17). The observation that prolidase hydrolytic activity for prophalan-L correlated well with prolidase RT-PCR expression data (Fig. 8) suggests that prolidase may be a viable target for melanoma therapy. The excellent linear correlations of prolidase activity and relative RT-PCR data for a limited number of cell lines with previously cited Affymetrix prolidase expression data (5) (r^2 values of 0.95 and 0.87, respectively;

plots not shown), provided validation for the RT-PCR data and its correlation with prolidase activity. Melanomas in general exhibit high expression levels of prolidase; thus, the approximately ten-fold higher prolidase activity in the SK-MEL-5 melanoma cell line compared to the breast adenocarcinoma cell line is consistent with their respective prolidase expression data.

Since the activation of the prodrug to the parent drug is essential for cytotoxic action, cytotoxicity profiles are expected to be dependent on the expression of prolidase, the prodrug activating enzyme. In this regard, prophalan-D, with approximately a 100-fold lower activity than prophalan-L with porcine kidney prolidase (Fig. 3), could serve as a negative control for cell proliferation studies in cell systems.

The cell proliferation studies demonstrated a four-fold higher cytotoxicity in the melanoma cells as compared to the breast adenocarcinoma cells, a trend similar to the hydrolysis profile. Prophalan-L cytotoxicity in the non-cancerous HFF cells, with relatively low prolidase activity and expression, was much lower than in the SK-MEL-5 cells even though the parent drug, melphalan, exhibited higher cytotoxicity in HFF cells ($GI_{50} = 25$ μM) compared to SK-MEL-5 cells ($GI_{50} = 57$ μM). Thus, prophalan-L was ~6-fold less cytotoxic than melphalan in the non-cancerous skin cells while exhibiting cytotoxicity similar to melphalan in melanoma cells with high prolidase expression.

The correlation between melphalan cytotoxicity (GI_{50}) and prolidase expression in the cancer cells was surprisingly decent ($r^2 = 0.66$). However, a closer examination indicated that melphalan's low cytotoxicity in MCF-7 cells dominated this correlation. Thus, exclusion of MCF-7 cells from this plot revealed the absence of any systematic correlation between cytotoxicity of melphalan and prolidase expression in the other five cancer cell lines—SK-MEL-5, NCI-H522, U-251, Caco-2 and HepG2 ($r^2 = 0.0004$). In contrast, the correlation between prophalan-L GI_{50} values and expression of prolidase was graded and only slightly lowered from $r^2 = 0.88$ (Fig. 8) to 0.83 upon exclusion of MCF-7 cells and suggests prolidase-dependent cytotoxic action of prophalan-L. Prophalan-D was inherently ineffective and the computed GI_{50} did not demonstrate any significant correlation with expression of prolidase.

Since cytotoxicity in a given cell line is a function of both uptake of prodrug as well as its bioactivation in the cell, it was necessary to examine uptake of the prodrugs and melphalan in order to ascertain the cytotoxicity governing step. The finding that prodrugs exhibited comparable or higher uptake than melphalan (a known substrate of LAT1 (L-type amino acid) transporter) in each cell line (18), suggested that mere differences in transport do not account for differences in GI_{50} or cytotoxic action. Thus, despite a 2.5-fold higher uptake of prophalan-L compared to melphalan in U-251 CNS cancer cells (Fig. 7), prophalan-L cytotoxicity was approximately 3-fold lower than that of melphalan in these cells (Table II). The lower cytoxicity of prophalan-L in U-251 cells, however, is quite consistent with the low prolidase activity and expression levels in these cells and underlines the relative insignificance of uptake compared to specific prolidase-targeted activation of the prodrug in determining cytotoxic action.

The role of specific transporters involved in prophalan-L uptake is speculative at this juncture. Melphalan uptake in the NCI 60 cell lines correlated highly ($r^2 = 0.96$) with the expression of LAT1 (SLC36A1) transporter in the cell lines (expression data from U95Av2 genechip from Novartis). Similarly, prophalan-L uptake in the NCI 60 cell lines exhibited high correlation ($r^2 = 0.97$) with the expression of monocarboxylic acid transporter (MCT; SLC16A1) and a fair correlation ($r^2 = 0.77$) with the organic anion transporters (OATs; SLC21A11) expression. However, no correlation was observed between prophalan-L uptake and amino acid (SLC36), oligopeptide (SLC15), organic cation (SLC22), sodium glucose co-transporter (SLC5), bile acid (SLC10), or nucleoside (SLC 28/29) transporters.

A correlation plot of the ratio of prophalan-L to melphalan GI_{50} values normalized for uptake, the prolidase targeting index, with prolidase expression in cancer cell lines indicated that MCF-7 was once again an outlier ($r^2 = 0.10$). Exclusion of MCF-7 cells dramatically improved the correlation ($r^2 = 0.82$; Fig. 10). It is clear that the breakdown of targeting index correlations with prolidase expression is related to the rather low toxicity of melphalan obtained in this study in these cells. The much lower cytotoxicity in this study for MCF-7 cells is not consistent with values listed in the NCI DTP database (5 μM; (19)). It is to be noted that the relatively similar values for SK-MEL-5, U-251, and NCI-522 cell lines in this study (57.0, 52.0, and 60.0 μM) are qualitatively consistent with the similarity of values listed in the NCI database (20.0, 12.6, and 15.9 μM, respectively). Other authors have reported GI_{50} values ranging from 50–125 μM for melphalan in MCF-7 cells (20, 21). Despite this breakdown of the targeting index correlation in a cell line with relatively low prolidase expression levels, it is clear that the cytotoxic action of prophalan-L in the cell lines is significantly dependent on prolidase expression levels. This is quite clearly evidenced in the excellent linear correlation of prophalan-L GI_{50} with prolidase RT-PCR expression ($r^2 = 0.88$, Fig. 9). The results reported in this study support our hypothesis that prolidase-targeted activation and cytotoxicity with prophalan-L may be a viable therapeutic alternative to melphalan.

CONCLUSIONS

The results of the studies in this report suggest that prolidase is a viable enzyme target for selective activation of proline prodrugs of melphalan in melanoma cells. The correlations of specific activity of prolphalan-L as well as its cytotoxicity in various cancer lines with prolidase expression indicates that prophalan-L may exert its cytotoxic action following cytosolic bioactivation to the parent melphalan. The excellent correlation of the prolidase-targeting index with prolidase expression in the cells further underscores the suitability of the prodrug approach for selective cytotoxic action. Future studies in animal models such as the murine melanoma model should shed some light on the effect of this strategy on the therapeutic index of fschemotherapeutic agents.

ACKNOWLEDGMENTS

This work was supported by NIH Grant R01-GM37188 and by the College of Pharmacy, University of Michigan. We thank Dr. Gustavo Rosania for providing the SK-MEL-5, U-251, NCI-H522, and MCF-7 cell lines. We also thank Dr. Kyung-Dall Lee for use of the 96-well plate reader in colorimetric assays.

REFERENCES

1. A. A. Stavrovskaya. Cellular mechanisms of multidrug resistance of tumor cells. *Biochemistry (Mosc)* **65**:95–106 (2000).
2. D. Nielsen, C. Maare, and T. Skovsgaard. Cellular resistance to anthracyclines. *Gen. Pharmacol.* **27**:251–255 (1996).
3. R. M. Lowenthal and K. Eaton. Toxicity of chemotherapy. *Hematol. Oncol. Clin. North. Am.* **10**:967–990 (1996).
4. P. S. Huang and A. Oliff. Drug-targeting strategies in cancer therapy. *Curr. Opin. Genet. Dev.* **11**:104–110 (2001).
5. S. Mittal, X. Song, B. S. Vig, C. P. Landowski, I. Kim, J. M. Hilfinger, and G. L. Amidon. Prolidase, a potential enzyme target for melanoma: design of proline-containing dipeptide-like prodrugs. *Mol. Pharmacol.* **2**:37–46 (2005).
6. C. M. Anderson, A. C. Buzaid, and S. S. Legha. Systemic treatments for advanced cutaneous melanoma. *Oncology (Williston Park)* **9**:1149–1158 (1995).
7. S. S. Legha. Current therapy for malignant melanoma. *Semin. Oncol.* **16**:34–44 (1989).
8. C. Dollery. *Melphalan, Therapeutic Drugs, Vol. 2*, Churchill Livingstone, London, 1991, pp. 48–52.
9. A. Bielawska, K. Bielawski, and J. Palka. Prolidase as a prodrug converting enzyme I. Synthesis of proline analogue of chlorambucil and its susceptibility to the action of prolidase. *Rocz. Akad. Med. Bialymst.* **42**:148–155 (1997).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
11. I. Myara, C. Charpentier, and A. Lemonnier. Optimal conditions for prolidase assay by proline colorimetric determination: application to iminodipeptiduria. *Clin. Chim. Acta* **125**:193–205 (1982).
12. I. Myara, C. Cosson, N. Moatti, and A. Lemonnier. Human kidney prolidase-purification, preincubation properties and immunological reactivity. *Int. J. Biochem.* **26**:207–214 (1994).
13. J. P. Bai and G. L. Amidon. Structural specificity of mucosal-cell transport and metabolism of peptide drugs: implication for oral peptide drug delivery. *Pharm. Res.* **9**:969–978 (1992).
14. J. P. Bai, M. Hu, P. Subramanian, H. I. Mosberg, and G. L. Amidon. Utilization of peptide carrier system to improve intestinal absorption: targeting prolidase as a prodrug-converting enzyme. *J. Pharm. Sci.* **81**:113–116 (1992).
15. A. Yaron and F. Naider. Proline-dependent structural and biological properties of peptides and proteins. *Crit. Rev. Biochem. Mol. Biol.* **28**:31–81 (1993).
16. G. F. King, M. J. Crossley, and P. W. Kuchel. Inhibition and active-site modelling of prolidase. *Eur. J. Biochem.* **180**:377–384 (1989).
17. R. Humerickhouse, K. Lohrbach, L. Li, W. F. Bosron, and M. E. Dolan. Characterization of CPT-11 hydrolysis by human liver carboxylesterase isoforms hCE-1 and hCE-2. *Cancer Res.* **60**:1189–1192 (2000).
18. H. Uchino, Y. Kanai, D. K. Kim, M. F. Wempe, A. Chairoungdua, E. Morimoto, M. W. Anders, and H. Endou. Transport of amino acid-related compounds mediated by L-type amino acid transporter 1 (LAT1): insights into the mechanisms of substrate recognition. *Mol. Pharmacol.* **61**:729–737 (2002).
19. http://dtp.nci.nih.gov/dtpstandard/.
20. K. Chrzanowski and J. Palka. Proline analogue of melphalan as a prolidase-convertible pro-drug in breast cancer MCF-7 cells. *Folia Histochem. Cytobiol.* **39** Suppl 2, 209–211 (2001).
21. K. Bielawski, A. Bielawska, K. Sosnowska, W. Miltyk, K. Winnicka, and J. Palka. Novel amidine analogue of melphalan as a specific multifunctional inhibitor of growth and metabolism of human breast cancer cells. *Biochem. Pharmacol.* **72**:320–331 (2006).