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Prolidase, a Potential Enzyme Target for Melanoma: Design of Proline-Containing Dipeptide-like Prodrugs

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Abstract: Bioinformatics tools such as Perl, Visual Basic, Cluster, and TreeView were used to analyze public gene expression databases in order to identify potential enzyme targets for prodrug strategies. The analyses indicated 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. RT-PCR expression of prolidase and hydrolytic activity against *N*-glycyl-L-proline (GLY-PRO), a standard substrate of prolidase, determined in tumor cell lines, exhibited a high correlation ($r^2 = 0.95$). These results suggest the possibility of targeting prolidase with prodrugs of anticancer agents for enhanced selectivity. The feasibility of such a scenario was tested by (a) synthesizing prodrugs of melphalan that comprised linkage of the carboxy terminus of the L-phenylalanine moiety of melphalan to the N-terminus of L and D stereoisomers of proline and (b) determining their bioconversion and antiproliferative activities in SK-MEL-5 cells, a melanoma cancer cell line with high expression levels of prolidase. The results of hydrolysis studies of the L- and D-proline prodrugs of melphalan, designated as prophalan-L and prophalan-D, respectively, indicated a ~7-fold higher rate of activation of prophalan-L compared to prophalan-D in SK-MEL-5 cell homogenates. Prophalan-L exhibited cytotoxicity ($GI_{50} = 74.8\ \mu M$) comparable to that of melphalan ($GI_{50} = 57.0\ \mu M$) in SK-MEL-5 cells while prophalan-D was ineffective, suggesting that prolidase-specific activation to the parent drug may be essential for cytotoxic action. Thus, melphalan prodrugs such as prophalan-L that are cleavable by prolidase offer the potential for enhanced selectivity by facilitating cytotoxic activity only in cells overexpressing prolidase.

Keywords: Bioinformatics; prolidase; enzyme targeting; melanoma; prodrugs; melphalan

Introduction

The completion of the human genome project[1] and the subsequent development of a variety of bioinformatics tools have facilitated detailed studies of the genetic basis of multifactorial diseases. Such studies have revealed the role and importance of genes that are differentially expressed and can be used as markers of the disease state or as possible targets to achieve highly selective delivery of appropriate medicinal agents.[2] Enzymes that are differentially expressed

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in disease states are possible targets since enzymes comprise 28% of all drug targets.[3] Of these, hydrolases (E.C. 3) such as esterases (E.C. 3.1) and peptidases (E.C. 3.4) are of special importance since prodrugs containing ester or amide linkages are quite common. Based on the IUBMB (International Union of Biochemistry and Molecular Biology) classification, currently 342 enzymes present in humans have been classified as hydrolases. The majority of hydrolases are either esterases (96, 28.07%), or peptidases (115, 33.63%). Moreover, hydrolases comprise 4% (1227 proteins) of the total gene products[1] with most of the hydrolases being proteases.

The number of public genomic databases and data analysis tools has increased exponentially in recent years,[4−6] and several analysis tools such as Perl (programming language) and Visual Basic have been widely used to retrieve/sort the microarray data from these databases to identify possible drug targets. Further, several cell lines derived from human tumors and organs have been characterized by the NCI (NCI 60) and are extensively used as experimental models of neoplastic disease.[7] DNA microarrays and the U95Av2 gene chip database from Novartis were used to survey the variation in expression of around 8000 distinct human transcripts in these 60 cell lines using various bioinformatics tools. It is thus possible to examine published gene expression profiles of various cancers and of cancer cell lines derived from human tumors and identify specific enzymes that are differentially overexpressed in certain cancer tissues/cells. In this report we describe the utilization of bioinformatics tools in facilitating identification of activating enzymes as potential targets for anticancer prodrugs. Of several possible enzymes that were so identified, prolidase was found to be overexpressed in melanoma cancer cell lines and was selected as the most desirable enzyme target. Prolidase (E.C. 3.4.13.9), or Xaa-Pro dipeptidase, is a 493 amino acid cytosolic dipeptidase that hydrolyzes dipeptides with proline or hydroxyproline at the carboxy terminus and has been reported to be overexpressed in certain pancreatic tumors.[8] In this report, we also describe the characterization of prolidase activity in various cancer cell lines using a standard substrate and validate such prolidase-specific activation by ascertaining their correlation with prolidase expression levels determined with RT-PCR experiments. We also describe the synthesis of proline prodrugs of an established anticancer agent, melphalan, and demonstrate the feasibility of prolidase-specific targeting with cell proliferation studies in a melanoma cell line.

Experimental Section

Materials. The cancer cell lines SK-MEL-5 (melanoma), K-562 (leukemia), MCF-7 (breast), NCI-H522 (lung), U-251 (CNS), Caco-2 (colon), and HepG2 (liver) were obtained from National Cancer Institute (Bethesda, MD) or ATCC (Rockville, MD). RPMI-1640 media, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), fetal calf serum (FCS), and trypsin-EDTA were obtained from GIBCO BRL (Grand Island, NY). 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, glycylproline (GLY-PRO), prolidase from porcine kidney (134 units/mg solid), melphalan (*p*-di(2-chloroethyl)amino-L-phenylalanine), 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 Co. (St. Louis, MO). Phenylalanylproline (D-PHE-L-PRO, L-PHE-D-PRO, and L-PHE-L-PRO) and L-proline benzyl ester hydrochloride were obtained from Bachem (Torrance, CA). Palladium 10%, di-*tert*-butyl dicarbonate, and dicyclohexylcarbodiimide 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 units/μL), AMV reverse transcriptase (5 units/μL), 25 mM magnesium sulfate, and AMV/*Tfl* 5X reaction buffer were obtained from Promega (Madison, WI). The sense and antisense primers were designed using Primer Select and custom-ordered from Invitrogen (Carlsbad, CA) along with the 4−20% TBE gels. All other chemicals and reagents used were of analytical or HPLC grade.

Identification of Target Enzymes in NCI 60 Cancer Cell Lines. The microarray data (web_hooks_gc_txt.gz) and the U95Av2 Affymetrix data on the DTP database (http://dtp.nci.nih.gov/mtargets/download.html) were downloaded as a text file. The programming language Perl was used to sift through more than 8000 genes to find all enzymes with names that end with the suffix "ase". The enzyme text file was then used to separate the enzymes into different classes, such as hydrolases, peptidases, and esterases. The text files were then converted to an excel sheet. Visual Basic was then used to arrange the data retrieved so that the expression of each enzyme gene in the 60 cancer cell lines could be easily visualized using visual tools such as Cluster (version 2.11)

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and TreeView (version 1.5) programs.[9−11] The sorted expression data for the 60 cancer cell lines was clustered using hierarchical clustering, and a file was created (.cdt) such that the expression of the genes in 60 cancer cell lines could be visualized using TreeView. The expression levels of the genes in the cancer cell lines are color coded as follows: green (expression lower than the control), black (expression the same as that of the control), and red (expression higher than that of the control or overexpressed). The potential enzyme targets were selected on the basis of the differential expression in various cell lines or tissues with emphasis on high expression in a particular tissue as compared to other tissues and high substrate specificity of the enzyme.

Selection of Cancer Cell Lines and Cell Culture. Candidate cancer cell lines for hydrolysis studies with GLY-PRO 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, MCF-7, and K-562, represent cells with high, medium, and low expression of prolidase, reasonably short doubling times, and standard growth requirements. In addition, Caco-2 as well as HepG2 cell lines were also used in the studies.

Cells were cultured at 37 °C in 5% CO_2 and 90% relative humidity. SK-MEL-5, K-562, 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. 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 according to the directions of the manufacturer. The 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′-CTGCAGGGCGGGGAGGAGAC-3′) and antisense (22-mer, 5′-CGCCCCGGGAGTAGCAGTAGTG-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 were measured using Metamorph software.

Hydrolysis of GLY-PRO by Porcine Kidney Prolidase with and without Specific Inhibitor Cbz-PRO. The commercial availability of prolidase from porcine kidney and its close alignment with the human prolidase (97% identity; BLASTP2.2.6) dictated its use in hydrolysis studies. The hydrolysis of a standard substrate, GLY-PRO, and that of several other substrates by porcine kidney prolidase were examined. Briefly, prolidase solution was prepared by suspending the lyophilized solid (0.8 mg solid, 0.551 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 protocol (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 (3.45 mg/mL). Reagent A was incubated at 40 °C for 30 min to activate the enzyme. Reagent B consisted of 6.3 mM GLY-PRO (2.53 mM for L-PHE-L-PRO, L-PHE-D-PRO, and D-PHE-L-PRO) and 200 mM manganese chloride. The activated reagent A was added to reagent B for a final GLY-PRO concentration of 5 mM (2 mM for L-PHE-L-PRO, L-PHE-D-PRO, and D-PHE-L-PRO) and incubated for 10 min at 40 °C. A final concentration of 2 mM for L-PHE-L-PRO, L-PHE-D-PRO, and D-PHE-L-PRO in the reaction mixture was chosen in order to afford comparisons at concentrations close to the K_m for Gly-PRO. Solutions of reagent A without the enzyme mixed with reagent B as described above served as controls. The competitive inhibition of GLY-PRO 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 GLY-PRO. The enzymatic reaction was quenched by adding 10% ice-cold trifluoroacetic acid. The mixtures were then centrifuged at 1500 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 were determined in all hydrolysis experiments by assaying the amounts of proline released.

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.[12] using Chinard's reagent (25 g of ninhydrin in 600 mL of 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 was then pipetted into a flat-bottom 96-well plate and the absorbance read at 495 nm using a precision microplate reader (Emax, Molecular Devices). The amount

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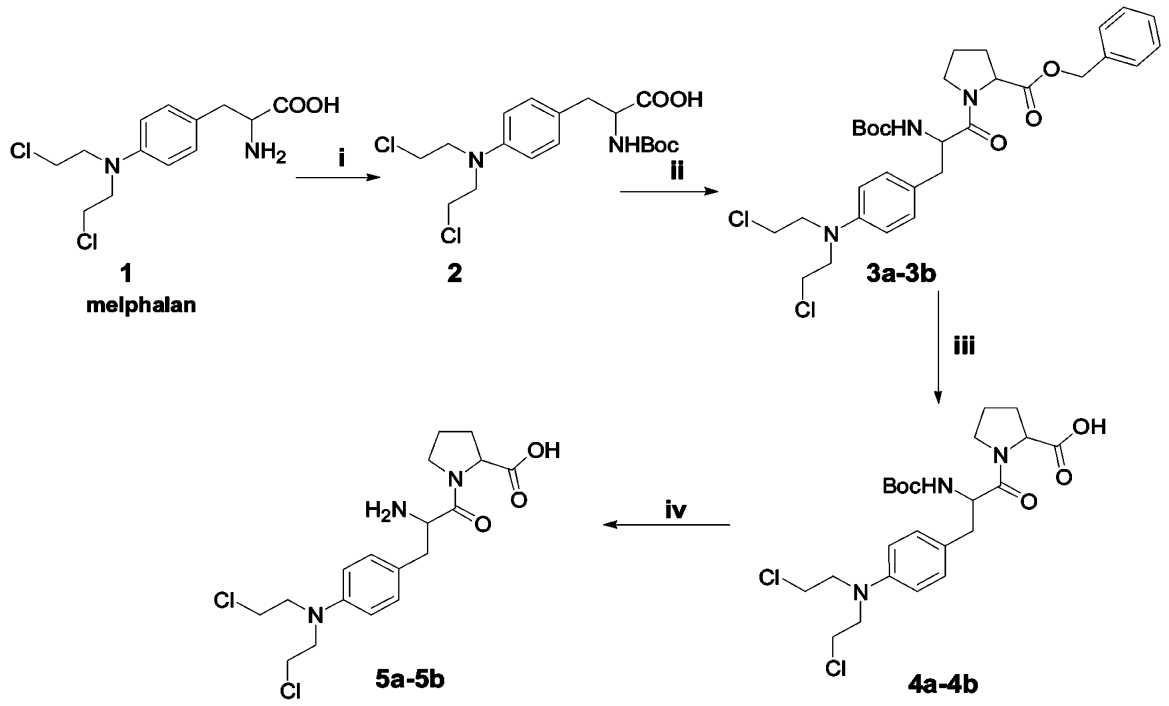

Figure 1. Synthesis and structures of proline analogues of melphalan. Reagents: (i) di-*tert*-butyl dicarbonate, NaOH, dioxane, H_2O; (ii) DCC, TEA, L-proline benzyl ester or D-proline benzyl ester; (iii) 10% Pd/C, EtOH; (iv) HCl(g)/dioxane.

of released proline was then calculated using standard curves generated using proline. The standard curves generated showed excellent linearity over the proline concentration range, 30 μM to 2 mM, examined, and the limit of detection was around 10 μM.

Hydrolysis of GLY-PRO in Cancer Cells and Inhibition by Cbz-PRO. Prolidase activity in the selected cancer cell lines was determined using the standard substrate, GLY-PRO. 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 3000 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 18000*g* for 30 min at 4 °C. The supernatant was then used in GLY-PRO hydrolysis studies and to determine protein content. The protein assay was carried out using the method by Lowry et al.[13] 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 (2 h incubation time for enzyme activation adopted from previous studies[14,15]). The hydrolysis (prolidase) reaction was initiated by adding 0.1 mL of the preincubated mixture to a 0.1 mL solution of 0.05 M Tris-HCl, pH 7.8 buffer containing 4 mM GLY-PRO. In competitive inhibition studies, 0.1 mL of the preincubated mixture was added to 0.1 mL solutions of 0.05 M Tris-HCl, pH 7.8 buffer containing 4 mM GLY-PRO and 4 mM Cbz-PRO. Mixtures of 0.1 mL solutions of 0.05 M Tris-HCl, pH 7.8 buffer containing 4 mM GLY-PRO 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 15 min. After 15 min the reaction was quenched by withdrawing 150 μL of the reaction mixture and adding it to 150 μL of cold 10% trifluoroacetic acid solution. The quenched mixture was centrifuged at 1500 rpm for 20 min at 4 °C, and 100 μL of the supernatant waswere withdrawn for colorimetric assays of proline content.

Synthesis of Proline Prodrugs of Melphalan. The synthesis of the proline prodrugs of melphalan, prophalan-L and prophalan-D, were carried out in a stepwise fashion as described below and summarized in the schematic shown in Figure 1.

***t*-Boc Protected L-Melphalan, 2.** Di-*tert*-butyl dicarbonate (196 mg, 0.89 mmol) was added to an ice-cold solution of melphalan (250 mg, 0.82 mmol) in a mixture of dioxane (2 mL), distilled water (1 mL), and 1 N NaOH (1 mL). The mixture was stirred for 1 h at 0 °C and then for 16 h at room temperature. After the reaction was complete, the

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mixture was concentrated and ethyl acetate and distilled water were added. The pH of the mixture was adjusted to 2 with hydrochloric acid, and the aqueous phase was then extracted with ethyl acetate (3 × 15 mL). The combined organic phases were washed with distilled water and brine and dried over $MgSO_4$, and the filtrate was concentrated under vacuum to yield compound **2** (330 mg, yield 98%).

4-[Bis(2-chloroethyl)butyloxycarbonyl]-L-phenylalanyl-L-proline Benzyl Ester, 3a. L-Proline benzyl ester hydrochloride (197 mg, 0.82 mmol) was dissolved in chloroform (15 mL), and to the stirred solution was added triethylamine (0.14 mL). The solution was cooled to 0 °C before addition of compound **2** (330 mg, 0.82 mmol). Dicyclohexylcarbodiimide (DCC, 165 mg, 0.82 mmol) was then added to the mixture. The reaction mixture was stirred for 3 h at 0 °C and then allowed to warm to room temperature, and stirring was continued for another 24 h. The reaction mixture was filtered and chloroform removed under reduced pressure. The residue was extracted with ethyl acetate and washed with distilled water and brine. The organic layer was dried over $MgSO_4$ and concentrated under vacuum. The residue was subjected to column chromatography to yield compound **3a** (545 mg, yield 75%).

^{1}H NMR ($CDCl_3$): δ 1.38 (s, 9H, $(CH_3)_3$), 1.93−2.30 (m, 4H), 3.05−3.64 (m, 12H), 5.03 (m, 1H), 4.41 (m, 1H), 4.55−4.64 (m, 2H, $PhCH_2$), 6.60 (m, 2H, Ar), 7.05 (m, 2H, Ar), 7.18−7.25 (m, 5H, Ar).

4-[Bis(2-chloroethyl)butyloxycarbonyl]-L-phenylalanyl-D-proline Benzyl Ester, 3b. Compound **3b** was synthesized using the procedure described for **3a** except for the starting material. Thus, D-proline benzyl ester was used instead of L-proline benzyl ester. The yield of **3b** was 70%.

^{1}H NMR ($CDCl_3$): δ 1.40 (s, 9H, $(CH_3)_3$), 1.91−2.25 (m, 4H), 3.05−3.66 (m, 12H), 4.42 (m, 1H), 5.02 (m, 1H), 4.56−4.67 (m, 2H, $PhCH_2$), 6.61 (m, 2H, Ar), 7.06 (m, 2H, Ar), 7.18−7.25 (m, 5H, Ar).

4-[Bis(2-chloroethyl)butyloxycarbonyl]-L-phenylalanyl-L-proline, 4a. Compound **3a** (520 mg, 0.88 mmol) was dissolved in 15 mL of anhydrous ethanol, and 80 mg of 10% Pd/C was added. The mixture was vigorously stirred under hydrogen at room temperature for 12 h, at which time TLC analysis indicated complete removal of the benzyl group. The catalyst was filtered through a bed of Celite and washed with ethanol. The filtrate was concentrated under vacuum, and the residue was applied on a silica gel column and eluted using a graded series of methylene chloride/methanol mixtures (ratios graded from 10:1 to 1:1) as the eluant. The desired fractions were combined and concentrated to yield **4a** (312 mg, yield 71%).

^{1}H NMR ($CDCl_3$): δ 1.39 (s, 9H, $(CH_3)_3$), 1.89−2.25 (m, 4H), 3.03−3.62 (m, 12H), 4.41 (m, 1H), 5.03 (m, 1H), 6.61 (m, 2H, Ar), 7.07 (m, 2H, Ar).

4-[Bis(2-chloroethyl)butyloxycarbonyl]-L-phenylalanyl-D-proline, 4b. Compound **4b** was synthesized using the procedure described for **4a** except for the starting material. Thus, compound **3b** was used instead of compound **3a**. The yield of compound **4b** was 65%.

^{1}H NMR ($CDCl_3$): δ 1.37 (s, 9H, $(CH_3)_3$), 1.89−2.30 (m, 4H), 3.03−3.62 (m, 12H), 4.40 (m, 1H), 5.01 (m, 1H), 6.62 (m, 2H, Ar), 7.09 (m, 2H, Ar).

4-[Bis(2-chloroethyl)]-L-phenylalanyl-L-proline, 5a. A solution of **4a** (300 mg, 0.6 mmol) in 5 mL of hydrogen chloride saturated dioxane was stirred for 25 min at 20 °C. The mixture was concentrated under vacuum, and the residue was washed with pentane, to yield **5a** as the hydrochloride salt (210 mg, yield 80%).

^{1}H NMR ($CDCl_3$): δ 1.48−1.73 (m, 6H), 2.60−2.75 (m, 2H), 3.17−3.70 (m, 10H), 4.00 (m, 1H), 6.65 (m, 2H, Ar), 7.09 (m, 2H, Ar). ESI-MS: 402.0 $(M + H)^+$.

4-[Bis(2-chloroethyl)]-L-phenylalanyl-D-proline, 5b. Compound **5b** was synthesized using the procedure described for **5a** except for the starting material. Thus, compound **4b** was used instead of compound **4a**. The yield of compound **5b** was 81%.

^{1}H NMR ($CDCl_3$): δ 1.45−1.70 (m, 6H), 2.60−2.73 (m, 2H), 3.17−3.72 (m, 10H), 4.01 (m, 1H), 6.67 (m, 2H, Ar), 7.08 (m, 2H, Ar). ESI-MS: 402.0 $(M + H)^+$.

Hydrolysis of Melphalan Prodrugs by Porcine Kidney Prolidase. The extent of hydrolysis of the melphalan prodrugs (activation) by porcine kidney prolidase and the effect of the competitive inhibitor Cbz-PRO were determined using a slight modification of the procedure described above for GLY-PRO and other substrates. Thus, the studies were performed as described for GLY-PRO except that (a) reagent B contained 2.53 mM concentrations of prophalan-L or prophalan-D and (b) the hydrolysis reaction was carried out for 30 min at 40 °C. In inhibition studies, the concentration of Cbz-PRO used was also 2.53 mM. The extent of hydrolysis was determined by assaying released proline content by colorimetric analysis as described above.

Hydrolysis of Melphalan Prodrugs by SK-MEL-5 Cell Homogenates. The extent of hydrolysis of melphalan prodrugs in SK-MEL-5 cell homogenates was determined in a manner similar to that described for GLY-PRO hydrolysis in cell homogenates. However, the concentrations of prophalan-L and prophalan-D used were 2 mM each instead of 4 mM for GLY-PRO and the hydrolysis reaction was performed over 60 min instead of 15 min for GLY-PRO. SK-MEL-5 cell homogenates were prepared as described above.

Stability of Melphalan Prodrugs in Cell Proliferation Growth Media. The stability of the two melphalan prodrugs, prophalan-L and prophalan-D, in growth media under conditions identical to those in cell proliferation studies was determined. Thus, 1 mM prophalan-L or 1 mM prophalan-D was prepared in phenol-free RPMI-1640 medium supplemented with FBS and incubated at 37 °C. The stability studies were carried out over 48 h, and samples were withdrawn periodically and assayed for released proline as described above. The RPMI-1640 medium supplemented with FBS but without the prodrugs served as control to eliminate any interference from endogenous proline in the medium.

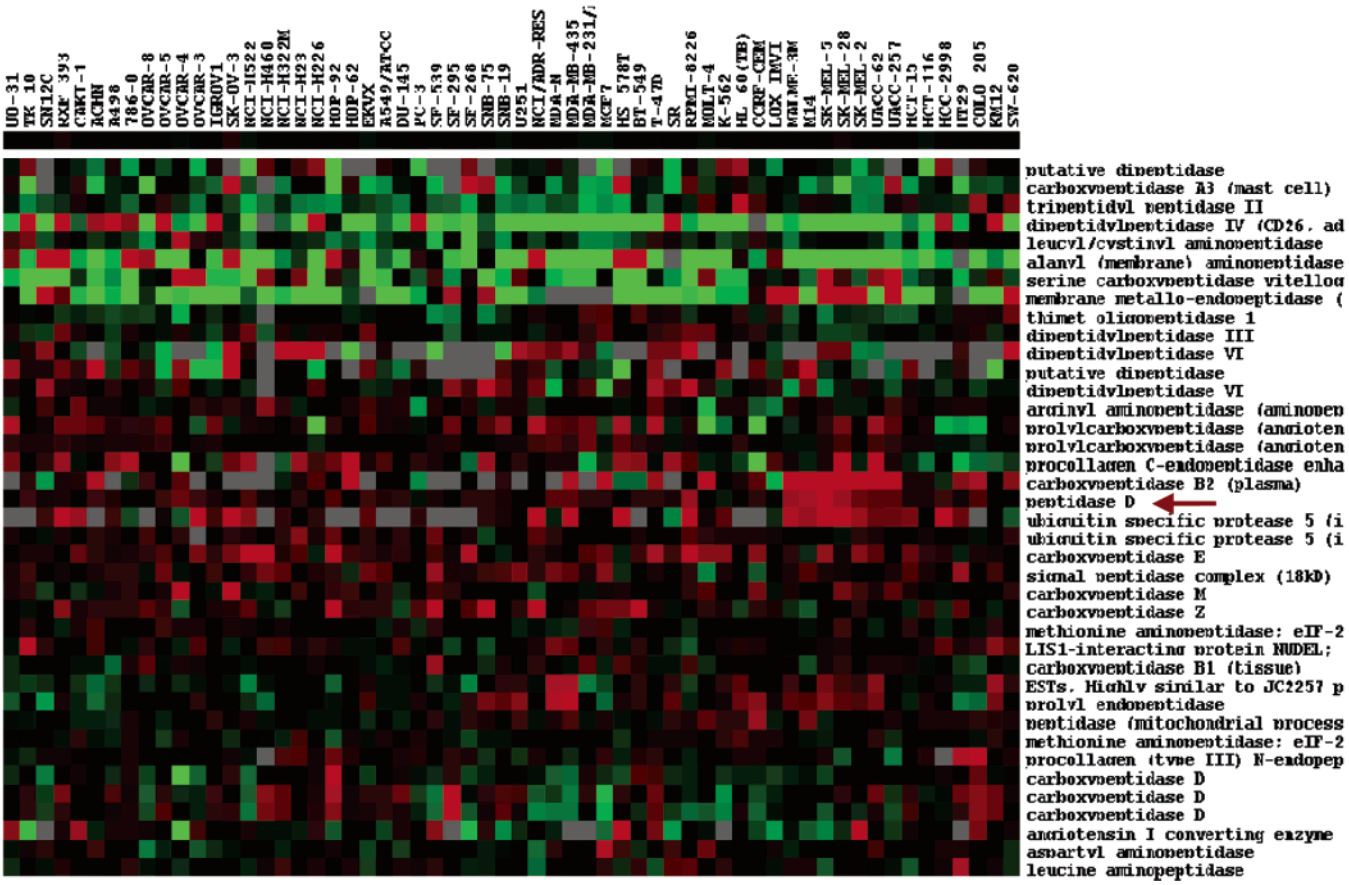

Figure 2. The peptidase TreeView showing the expression of genes sorted as peptidases in the NCI 60 cancer cell lines. The red dots indicate higher expression than control, green dots indicate lower expression than control, black dots indicate expression equal to control, and the gray dots indicate missing data. Peptidase D or prolidase shows high expression in all the melanoma cell lines while the expression in the cell lines from other tissues was similar to control.

Cell Proliferation Assays. Cell proliferation assays were conducted to determine the cytotoxic activities of the prodrugs and the parent drug. Cell proliferation assays were carried out with SK-MEL-5 cells since the expression of prolidase was found to be highest in these cells based on both RT-PCR results and microarray expression data. SK-MEL-5 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 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 medium in the 96-well plate was aspirated and replaced with drug solutions in the phenol-free medium. Growth medium 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 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/prodrug concentration to compute the GI_{50} values for melphalan, prophalan-L, and prophalan-D.

Results

Identification of Target Enzyme: Prolidase. After the genes had been sorted as hydrolases, peptidases, and esterases, they were clustered and their expression in the 60 cancer cell lines was visualized using TreeView as shown in Figure 2, a TreeView for peptidases. An examination of the TreeView suggested that enzymes such as peptidase D (prolidase) may be a potential target for prodrug strategy since they appear to be differentially overexpressed in melanoma compared to cell lines derived from other tissues and control cells. It is evident from Figure 2 that prolidase expression is relatively higher in SK-MEL-5, a melanoma cell line, compared to its expression in MCF-7 breast adenocarcinoma and K-562 leukemia cell lines. Thus, the U95Av2 Affymetrix gene chip expression of prolidase in the five cell lines SK-MEL-5, U-251, NCI-H522, MCF-7, and K-562 was 325.3, 171.4, 154.8, 114.1, and 41.5,

Table 1. Specific Activity of GLY-PRO in the Presence and Absence of Cbz-PRO in Various Cancer Cell Lines (Expressed as pmol/min/μg of Protein, Mean $\pm$ SD, $n = 3$) and Prolidase Expression Determined with RT-PCR and with U95Av2 Affymetrix GeneChip

cell Line	sp act. w/o Cbz-PRO	sp act. w/ Cbz-PRO	% inhibn	rel RT-PCR expression	GeneChip expression[a]
SK-MEL-5	228.1 $\pm$ 6.3	51.1 $\pm$ 1.4	77.6	1.29	325.3
Caco-2	234.6 $\pm$ 13.1	24.1 $\pm$ 5.2	89.8	1.25	
NCI-H522	134.2 $\pm$ 9.0	nd[b]	100.0	1.21	154.8
HepG2	103.4 $\pm$ 4.0	19.7 $\pm$ 13.9	80.7	1.19	
U-251	108.6 $\pm$ 2.4	17.9 $\pm$ 2.9	83.6	1.18	171.4
MCF-7	50.7 $\pm$ 2.5	4.9 $\pm$ 1.8	90.2	1.09	114.1
K-562	37.9 $\pm$ 2.9	10.9 $\pm$ 1.4	71.1	1.00	41.5

[a] Novartis U95Av2 Affymetrix data on the DTP database (http://dtp.nci.nih.gov/mtargets/download.html). [b] Not detected.

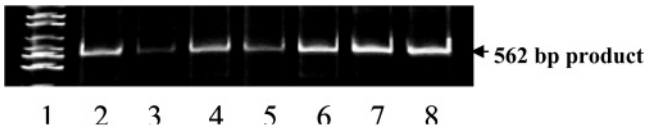

Figure 3. Expression profiles of prolidase (562 bp product DNA) in cancer cell lines determined using semiquantitative RT-PCR: 100 base pair DNA ladder (lane 1), HepG2 cells (lane 2), K-562 (lane 3), U-251 (lane 4), MCF-7 (lane 5), NCI-H522 (lane 6), Caco-2 (lane 7), and SK-MEL-5 (lane 8).

respectively (Table 1). These cell lines were chosen for further characterization for prolidase activity with standard substrates such as GLY-PRO as well as for validation of prolidase expression with RT-PCR determinations.

Expression of Prolidase in Cancer Cell Lines: RT-PCR and Microarray. The expression of prolidase in the cancer cell lines selected using microarray data, as well as in Caco-2 and HepG2 cells, was determined using RT-PCR. Figure 3 shows the expression profiles of prolidase obtained with the seven cancer lines. The results of the semiquantitative RT-PCR analysis indicated that prolidase expression was highest with SK-MEL-5 cells (melanoma) and lowest with K-562 (leukemia) cells. Thus, the relative expression of prolidase determined with RT-PCR in the seven cancer cell lines examined listed in Table 1 was in the order SK-MEL-5 (1.28), Caco-2 (1.25), NCI-H522 (1.21), HepG2 (1.19), U-251 (1.18), MCF-7 (1.09), K-562 (1.00) and is consistent with the gene chip expression data.

Hydrolysis of Standard Substrates by Porcine Kidney Prolidase. The specific activity of a standard substrate GLY-PRO for porcine kidney prolidase, determined using assays of released proline, was 17.5 $\pm$ 0.6 nmol/min/μg of prolidase. In the presence of the competitive inhibitor Cbz-PRO, GLY-PRO specific activity decreased substantially to 3.3 $\pm$ 1.3 nmol/min/μg of prolidase. The significant inhibition ($\sim$81%) in the presence of Cbz-PRO suggests that determinations of GLY-PRO activity in cell homogenates may be accurate indicators of prolidase activity in the cells.

Other substrates of prolidase, L-PHE-L-PRO, L-PHE-D-PRO, and D-PHE-L-PRO, were also examined with porcine kidney prolidase, and their activities relative to GLY-PRO

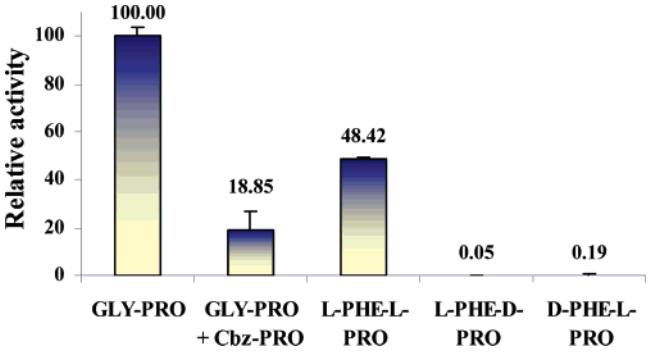

Figure 4. Relative activities of L-PHE-L-PRO, L-PHE-D-PRO, and D-PHE-L-PRO with porcine kidney prolidase compared to that of standard substrate GLY-PRO (specific activity of GLY-PRO = 17.5 $\pm$ 0.6 pmol/min/μg of prolidase, mean $\pm$ SD, $n = 3$).

were found to be 48.42%, 0.05%, and 0.19%, respectively (Figure 4). The results clearly reflect the requirement of an L configuration for both amino acids in the dipeptide for candidate prolidase substrates.

Hydrolysis of GLY-PRO in Cancer Cells and Inhibition by Cbz-PRO. Prolidase activity in various cancer cell homogenates was determined using the standard prolidase substrate GLY-PRO and is presented in Table 1. The results indicate that prolidase activity was in the order SK-MEL-5 $\approx$ Caco-2 $\gg$ NCI-H522 $>$ HepG2 $\approx$ U-251 $>$ MCF-7 $>$ K-562. GLY-PRO hydrolysis in the presence of the competitive inhibitor Cbz-PRO was inhibited substantially in all cancer cell systems tested. Thus, the average inhibition in the seven cell lines was $\sim$85% with individual values ranging from 71% to 100% (Table 1). The extent of inhibition observed in cell homogenates with human prolidase compares favorably with the extent of inhibition obtained using porcine kidney prolidase.

Correlation between Prolidase Activity and Expression. The specific activity in the cancer cell lines determined using GLY-PRO was correlated with the relative RT-PCR expression in the cell lines. A good linear correlation ($r^2 = 0.81$) was observed between RT-PCR expression of prolidase in the cancer cell lines and the specific activity of GLY-PRO. An exponential fit, however, appears to be more appropriate exhibiting an excellent correlation coefficient ($r^2 = 0.95$). A good linear correlation ($r^2 = 0.93$) was also observed between the specific activity of GLY-PRO and U95Av2 Affymetrix gene chip prolidase expression from the NCI database.

Hydrolysis (Activation) of Melphalan Prodrugs by Porcine Kidney Prolidase, in SK-MEL-5 Cell Homogenates and in RPMI-1640 Growth Medium. The specific activity of porcine kidney prolidase for prophalan-L was 366.7 $\pm$ 14.4 pmol/min/μg of prolidase. In the presence of Cbz-PRO, porcine kidney prolidase activity for prophalan-L was inhibited by $\sim$64% to 132.5 $\pm$ 6.3 pmol/min/μg of prolidase. The specific activity of porcine kidney prolidase for the D analogue was roughly 100-fold lower compared to the L-proline analogue (3.2 $\pm$ 1.4 pmol/min/μg of prolidase),

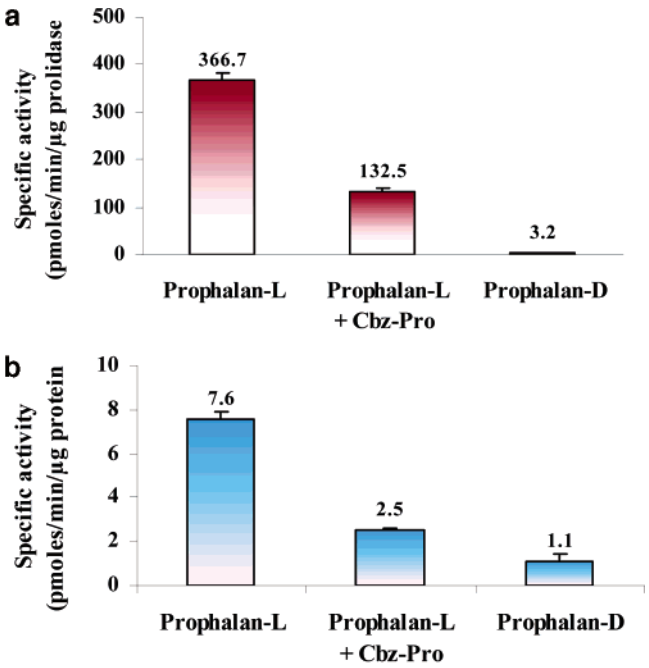

Figure 5. (a) Specific activity of melphalan prodrugs for porcine kidney prolidase in the presence and absence of Cbz-Pro (mean ± SD, $n = 3$). (b) Specific activity of melphalan prodrugs in SK-MEL-5 cell homogenates in the presence and absence of Cbz-Pro (mean ± SD, $n = 3$).

suggesting substrate specificity of prolidase. These results are plotted in Figure 5a.

The activity profiles of the two melphalan proline analogue prodrugs in SK-MEL-5 cell homogenates exhibited differences that were qualitatively similar to that obtained with porcine kidney prolidase. Thus, the specific activities of prophalan-L and prophalan-D were 7.6 ± 0.3 and 1.1 ± 0.1 pmol/min/μg protein, respectively, suggesting a roughly 7-fold preference for the L analogue. Further, as observed with porcine kidney prolidase, Cbz-PRO inhibited the specific activity of prophalan-L by roughly 67% to 2.5 ± 0.1 pmol/min/μg protein. The activity profiles are plotted in Figure 5b.

The two proline prodrugs were found to exhibit high stability in phenol-free RPMI-1640 medium supplemented with FBS. Thus, the estimated half-lives were greater than 10 days for the two prodrugs.

Cell Proliferation Studies with Melphalan Prodrugs. The two melphalan prodrugs, prophalan-L and prophalan-D, were evaluated for their antiproliferative activity in SK-MEL-5 melanoma cells along with the parent drug melphalan. Figure 6 shows plots of percent cell viability as a function of prodrug or drug concentration for the three test compounds. The profiles suggest clearly that prophalan-L exhibits antiproliferative action similar to that of the parent drug whereas the D analogue, prophalan-D, was relatively ineffective at comparable concentrations. Thus, under the conditions of the cell proliferation study, prophalan-L exhibited a GI_{50} value of 74.8 μM compared to 57.0 μM for melphalan.

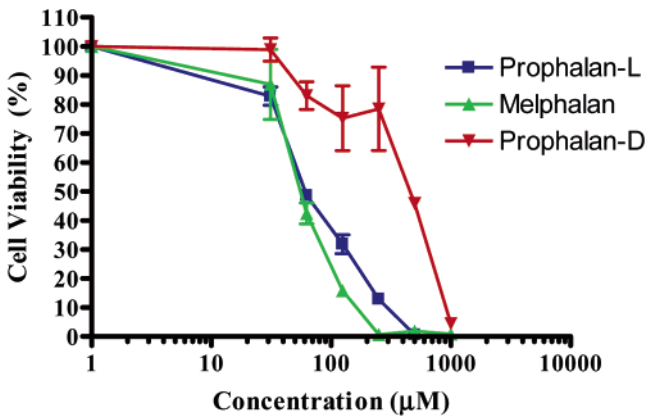

Figure 6. Cell proliferation assays of melphalan, prophalan-L, and prophalan-D after 48 h incubation with SK-MEL-5 cells. The data is expressed as mean ± SD ($n = 3$) for each treatment at each concentration. GI_{50} values for melphalan and prophalan-L were 57.0 μM and 74.8 μM, respectively, while prophalan-D was ineffective at comparable concentrations.

Discussion

In this study, we report the identification of prolidase, an enzyme with highly specific substrate requirements that is differentially overexpressed in melanomas, utilizing public gene expression databases and bioinformatics tools. Further, this report also describes the synthesis of prodrugs designed to target prolidase and demonstrates the feasibility of such targeting in cell proliferation studies with melanoma cells.

NCI 60 microarray/gene chip expression data revealed that the expression level of prolidase was high in all melanoma cell lines compared to a reference pool of 12 cancer cell lines**.** The consistently high expression of prolidase observed in all melanoma cell lines suggests that it might be a more suitable target compared to other enzymes that exhibit high expression in certain cell lines but not in others from the same tissue of origin. The consistent, high expression of prolidase in all melanoma cell lines also suggests that its occurrence in melanomas is highly probable and may ensure the selective antiproliferative action of prodrugs designed to target prolidase.

Prolidase is one of the very few proline-specific enzymes documented in the literature.[16,17] Some of the other proline-specific enzymes include prolinase, proline iminopeptidase, dipeptidyl peptidase IV and II, carboxypeptidase P, prolyl carboxypeptidase, prolyl oligopeptidase, aminopeptidase P, and HIV-proteinase. Prolidase is a 493 amino acid protein, and the subunit of this enzyme is a homodimer around 110 kDa.[18] Prolidase has unique substrate specificity since it acts

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on dipeptides with proline at the carboxy terminus, prefers dipeptides in the trans configuration,[19] and is a metalloenzyme requiring manganese (Mn^{2+}) as a cofactor for optimum catalytic activity.[20−22] The substrate requirements for prolidase action also include the presence of a free α-amino group and an L configuration of the proline moiety at the C-terminus.[21,23,24] It has also been observed that aminopeptidase P, the closest relative in substrate specificity to prolidase, hydrolyzes tripeptides or higher peptides but does not hydrolyze dipeptides. Thus, prolidase is one of the very specific peptidases for proline-containing dipeptides.[25]

The high substrate specificity of prolidase coupled with its high differential expression in melanomas suggested its suitability as an enzyme target. Thus, select melanoma cell lines along with other NCI 60 cancer cell lines with varying prolidase expression were chosen for further characterization. RT-PCR experiments were conducted with a total of seven cell lines to validate the NCI 60 microarray expression data. The RT-PCR results were consistent with the U95Av2 Affymetrix gene chip data exhibiting a high linear correlation ($r^2 = 0.86$), which suggests that oligonucleotide array microarray expression data may be reliably employed for selection of drug targets.

In order to assess prolidase activity in the cancer cell lines, it was first necessary to establish suitable assays with known substrates and specific inhibitors of the enzyme. The most commonly used substrates for studying prolidase activity include GLY-PRO, PHE-PRO, and glycyl-thiazolidinecarboxylate.[20,22,26] Although prolidase is a metalloproteinase, its activity has been shown to be inhibited by cysteine protease inhibitors such as *p*-chloromercuribenzoate[18] and *p*-hydroxymercuribenzoate.[22] Azetidine, pyrrolidine, and piperidine derivatives have also been designed for inhibition based on the postulated active site of prolidase, with *N*-benzyloxycarbonyl-L-proline (Cbz-PRO) inducing approximately 90% inhibition of activity[27] being the most potent. Thus, experiments were conducted with commercially available porcine kidney prolidase, GLY-PRO, as the standard substrate and Cbz-PRO as the inhibitor.

The results of studies with the pure porcine enzyme clearly suggested the suitability of GLY-PRO as a substrate. The significant inhibition (∼81%) in the presence of Cbz-PRO suggests that determinations of GLY-PRO activity in human cancer cell homogenates may be accurate indicators of prolidase activity in the cells. The results of hydrolysis studies with other substrates such as L-PHE-L-PRO and L-PHE-D-PRO confirm previous studies stipulating the requirement of an L configuration for both amino acids in the dipeptide for candidate prolidase substrates, and suggest that D analogues may be useful as negative controls. The combined results suggest that GLY-PRO would be the standard substrate of choice for prolidase activity determinations in cancer cell homogenates.

The evaluation of human prolidase activity in cancer cell homogenates using GLY-PRO as the standard substrate appears to be justified on the basis of the observation that the average inhibition of activity by Cbz-PRO in the cells was comparable to that obtained with pure prolidase (porcine kidney). The observation that prolidase activity assessed using GLY-PRO hydrolysis profiles correlated well with both RT-PCR and U95Av2 gene chip prolidase expression data validated its use. Thus, the 4−6-fold higher prolidase activity in the SK-MEL-5 melanoma cell line compared to breast adenocarcinoma and leukemia cell lines was consistent with expression data. The combined prolidase expression and activity profiles indicated that proline-containing prodrugs of anticancer agents may be useful for targeted delivery to melanomas.

Prodrugs have been traditionally used to increase oral bioavailability, but recently prodrug strategies have also been employed to achieve drug targeting. The recent anticancer agents capecitabine (Xeloda),[28] and imatinib (Gleevec),[29,30] selectively target cancer cells by exploiting the differences between normal and cancerous cells. The successful clinical use of prodrugs such as capecitabine, tegafur,[31] temozo-

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lomide,[32] and irinotecan[32] clearly highlights the utility of prodrug strategies.

Proline prodrugs of melphalan, an L-phenylalanine analogue, have been previously synthesized and examined for their efficacy in breast carcinoma cell lines.[14,15,33,34] However, the proline moiety was attached via a linker to the amino terminal of melphalan. The proline prodrugs in this report were synthesized by direct coupling of the free carboxylic group of melphalan to the N-terminal of the proline imino acid in a manner similar to that reported for chlorambucil by Bielawska et al.,[35] and they are thus different from the previously reported proline prodrugs of melphalan. Although the rationale proposed for the synthesis of proline prodrugs of melphalan (and of neoplastic agents in general) involves the targeting of prolidase, the differences in linkage of the proline moiety to melphalan in our study and in the previous reports[14,15,33] may be crucial. One major difference concerns the end products of prolidase action on these prodrugs. Thus, the prodrugs described in our study would yield melphalan, whereas the prodrugs used in Chrzanowski et al.'s studies would generate N-substituted melphalan. The mode of linkage of the proline moiety to melphalan may also have an effect on their activation by prolidase.

The 100-fold preference by pure porcine kidney prolidase for the L-proline analogue of melphalan, prophalan-L, compared to the D analogue, prophalan-D, and the 7-fold higher activity in SK-MEL-5 cell homogenates of the L analogue are consistent with the dipeptide substrate requirements for prolidase action.

The antiproliferative activity profiles of melphalan, prophalan-L, and prophalan-D in SK-MEL-5 cells suggest strongly that the prodrugs themselves may be intrinsically inactive. Further, preliminary uptake studies in SK-MEL-5 cells indicated that the transport of melphalan and the two prodrugs into SK-MEL-5 cells was not significantly different from one another. Also, the roughly 6-fold lower antiproliferative activity of prophalan-D compared to prophalan-L is quite consistent with its 7-fold lower hydrolysis rate in SK-MEL-5 cell homogenates compared to prophalan-L. The relative GI_{50} values for melphalan, prophalan-L, and prophalan-D coupled with the high stability of the two prodrugs in growth medium ($t_{1/2}$ > 10 days) suggests that bioconversion of the prodrugs to the parent melphalan may determine their cytotoxic activity in the cells. The results of this study support the proposition that prolidase could serve as a target enzyme for the selective action of anticancer agents. However, in order to demonstrate selective delivery of the prodrugs to tumor (melanoma) tissues, it would be necessary to examine potential bioactivation of the prodrugs by prolidase expressed in normal tissues and in organs such as the liver and kidney. Further, the disposition of the prodrugs in systemic circulation would determine if the prodrugs possess sufficient stability characteristics to allow targeted delivery to melanoma tumor cells. The antitumor efficacy and toxicity profiles of prophalan-L and prophalan-D in mouse melanoma models relative to the parent drug melphalan will be investigated in future studies to resolve some of these issues.

Abbreviations Used

IUBMB, International Union of Biochemistry and Molecular Biology; E.C., enzyme classification; NCI, National Cancer Institute; DTP, Developmental Therapeutics Program; GLY-PRO, *N*-glycyl-L-proline; L-PHE-L-PRO, *N*-L-phenylalanyl-L-proline; L-PHE-D-PRO, *N*-L-phenylalanyl-D-proline; D-PHE-L-PRO, *N*-D-phenylalanyl-L-proline; Cbz-PRO, *N*-benzyloxycarbonyl-L-proline; prophalan-L, *p*-di(2-chloroethyl)amino-L-phenylalanyl-L-proline; prophalan-D, *p*-di(2-chloroethyl)amino-L-phenylalanyl-D-proline; SK-MEL-5, melanoma; Caco-2, colon adenocarcinoma; NCI-H522, lung adenocarcinoma; HepG2, hepatocellular carcinoma; U-251, CNS glioblastoma; MCF-7, breast adenocarcinoma; K-562, leukemia; XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate; PMS, *N*-methyl dibenzopyrazine methyl sulfate.

Acknowledgment. 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, MCF-7, and K-562 cell lines. We also thank Dr. Kyung-Dall Lee for use of the 96-well plate reader in colorimetric assays.

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