Dipeptidyl Peptidase IV as a Potential Target for Selective Prodrug Activation and Chemotherapeutic Action in Cancers

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ABSTRACT: The efficacy of chemotherapeutic drugs is often offset by severe side effects attributable to poor selectivity and toxicity to normal cells. Recently, the enzyme dipeptidyl peptidase IV (DPPIV) was considered as a potential target for the delivery of chemotherapeutic drugs. The purpose of this study was to investigate the feasibility of targeting chemotherapeutic drugs to DPPIV as a strategy to enhance their specificity. The expression profile of DPPIV was obtained for seven cancer cell lines using DNA microarray data from the DTP database, and was validated by RT-PCR. A prodrug was then synthesized by linking the cytotoxic drug melphalan to a proline-glycine dipeptide moiety, followed by hydrolysis studies in the seven cell lines with a standard substrate, as well as the glycyl-prolyl-melphalan (GP-Mel). Lastly, cell proliferation studies were carried out to demonstrate enzyme-dependent activation of the candidate prodrug. The relative RT-PCR expression levels of DPPIV in the cancer cell lines exhibited linear correlation with U95Av2 Affymetrix data \( r^2 = 0.94 \), and with specific activity of a standard substrate, glycine-proline-p-nitroanilide \( r^2 = 0.96 \). The significantly higher antiproliferative activity of GP-Mel in Caco-2 cells \( (G_{50} = 261 \mu M) \) compared to that in SK-MEL-5 cells \( (G_{50} = 807 \mu M) \) was consistent with the 9-fold higher specific activity of the prodrug in Caco-2 cells \( (5.14 \text{ pmol/min/\mu g protein}) \) compared to SK-MEL-5 cells \( (0.68 \text{ pmol/min/\mu g protein}) \) and with DPPIV expression levels in these cells. Our results demonstrate the great potential to exploit DPPIV as a prodrug activating enzyme for efficient chemotherapeutic drug targeting.

KEYWORDS: drug targeting, dipeptidyl peptidase IV (DPPIV), enzyme-targeted delivery, enzyme-dependent prodrug activation, selective cytotoxic action

1. INTRODUCTION

Chemotherapeutic drugs, alone or as an adjuvant therapy to surgery and radiation, are a vital part in cancer treatment. However, their effectiveness is often offset by the severe side effects caused by poor selectivity and toxicity to normal cells. In recent years, the rapid advance in the fields of bioinformatics and genomics facilitated the identification of numerous target molecules that are uniquely or overly expressed in cancer cells, including certain receptors and enzymes. Thus, a DPPIV-cleavable prodrug containing a proline dipeptide conjugated to a cytotoxic chemotherapeutic agent may limit nonselective cytotoxic effects attributable to poor selectivity and toxicity to normal cells. In recent years, the rapid advance in the fields of bioinformatics and genomics facilitated the identification of numerous target molecules that are uniquely or overly expressed in cancer cells, including certain receptors and enzymes. Thus, a DPPIV-cleavable prodrug containing a proline dipeptide conjugated to a cytotoxic chemotherapeutic agent may limit nonselective cytotoxic effects.

The enzyme dipeptidyl peptidase IV (DPPIV) (EC 3.4.14.5) is also known as CD26 (T-cell surface activation antigen) or adenosine deaminase (ADA), which was first identified in 1966. DPPIV is a member of the prolyl oligopeptidase (POP) family of serine proteases and specifically hydrolyzes the peptide bond C-terminal to proline or alanine in the penultimate P1 position. It is normally present on the apical membrane surface of several cell types, including T cells and B cells, and natural killer (NK) cells, epithelial cells of kidney, intestine brush border membranes, plasma, and endothelial cells of blood vessels, and is involved in many diverse physiological processes. In addition, it was recently discovered that DPPIV is overly expressed in human renal cell carcinoma tissues, and it is thought to play a role in the pathogenesis of other various human cancers as well. Moreover, proline-containing substrates are hydrolyzed exclusively by proline-specific enzymes and are less susceptible to nonselective peptidases and proteases. Thus, a DPPIV-cleavable prodrug containing a proline dipeptide conjugated to a cytotoxic chemotherapeutic agent may limit nonselective activation.

The purpose of this study was to investigate the feasibility of exploiting DPPIV as a prodrug activating enzyme, to allow specific targeting of chemotherapeutic agents to cancer cells. To
that end, the expression profiles of DPPIV in 60 cancer cell lines (NCI 60) were obtained, and 7 cell lines, which represent the spectrum of DPPIV expression, were selected. A candidate prodrug was then synthesized by linking the cytotoxic drug melphalan (Figure 1A) to a proline dipeptide, creating glycyl-prolyl-melphalan prodrug (GP-Mel; Figure 1B) with expected DPPIV affinity. Functional activity studies of DPPIV with the prodrug, in the absence vs presence of inhibitor, confirmed that the prodrug is a specific DPPIV substrate. Finally, hydrolysis and cell proliferation studies were performed in high and low DPPIV expression cancer cell lines. Overall, this work indicates that DPPIV may be exploited as a prodrug activating enzyme for efficient chemotherapeutic drug targeting.

2. MATERIALS AND METHODS

2.1. Materials. Melphalan, porcine kidney dipeptidyl peptidase (porcine DPPIV), glycyl-prolyl-p-nitroanilide (GP-pNA), glycyl-phenylalanyl-p-nitroanilide (GP-pNA), glycyl-arginyl-p-nitroanilide (GR-pNA), p-nitroaniline (pNA), diprotin A (Ile-Pro-Ile), XTT, PMS (N-methylidibenzopyrazine methyl sulfate), and ladder SYBR green were purchased from Sigma Chemical Co. (St. Louis, MO). N,N-Dimethylformamide (DMF), piperidine, triisopropylsilane (TIS), triethanolamine (TEA), and trifluoroacetic acid (TFA) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Boc-L-Gly-L-Pro, 1-hydroxybenzotriazole anhydrous (HOBT), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), triisopropylsilane (TIS), N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu), and Wang resin (100−200 mesh) were obtained from Calbiochem-Novabiochem (San Diego, CA). Access RT-PCR kit, DNA blue/orange loading dye, 1 kb ladder and 100 bp ladder were obtained from Promega (Madison, WI). 4−20% TBE gels, TRIzol reagent, and custom-ordered DPPIV sense and antisense primers were from Invitrogen Life Technologies (Carlsbad, CA). The cancer cell lines IGROVI (ovarian), PC-3 (prostate), 786-O (renal), SK-MEL-5 (melanoma), SK-OV-3 (ovarian), Caco-2 (colon), and HepG2 (liver) were obtained from National Cancer Institute or ATCC. Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640, fetal bovine serum
(FBS), fetal calf serum (FCS), phosphate buffered saline (PBS), and trypsin-EDTA were purchased from GIBCO BRL (Grand Island, NY). Minimal essential medium (MEM) was obtained from ATCC. All HPLC grade solvents (acetonitrile, DMF, ethyl acetate, and piperidine) used for peptide synthesis or HPLC analysis were obtained from EM Sciences (Gibbstown, NJ). Trifluoroacetic acid for HPLC analysis was obtained from Pierce (Rockville, IL). All other chemicals and reagents used were of analytical or HPLC grade.

2.2. Synthesis of L-Glycyl-L-prolyl-melphalan Prodrug.

The solid-phase synthesis of the L-glycyl-L-prolyl prodrug of melphalan was carried out in a stepwise fashion with Wang resin as described below and is summarized in the schematic shown in Figure 2.

Step 1. Fmoc-L-melphalan synthesis: Fmoc-OSu (300 mg, 0.89 mmol) was added to an ice-cold solution of melphalan, 1 (200 mg, 0.82 mmol), in a mixture of acetone (20 mL), distilled water (10 mL), and NaHCO₃ (300 mg). The mixture was stirred for 1 h at 0 °C and then reacted at room temperature for 16 h. After the reaction was complete, the mixture was concentrated. 15 mL of ethyl acetate and 15 mL of distilled water were added. 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 MgSO₄. The filtrate was concentrated under vacuum to yield Fmoc-L-melphalan, 2 (330 mg, yield 98%).

Step 2. Fmoc-L-melphalan (330 mg) and Wang resin (0.1 mmol) were added into a 100 mL round reaction bottle with 20 mL of DMF. HOBT (0.3 mmol), HBTU (0.3 mmol), and TEA (0.3 mmol) were then added to the resin mixture and stirred overnight at room temperature. The Wang resin, 3, was washed 3 times with 10 mL of DMF after filtration and then washed 3 times for 10 min each in 10 mL of 20% piperidine in DMF.

Step 3. Boc-L-glycyl-L-proline (0.3 mmol) and Wang resin 3 were added to 20 mL of DMF in a 100 mL round reaction bottle. HOBT (0.3 mmol), HBTU (0.3 mmol), and TEA (0.3 mmol) were then added and stirred overnight at room temperature. The resultant Wang resin, 4, was washed 3 times with 10 mL of DMF after filtration and then washed 3 times for 10 min each in 10 mL of 20% piperidine in DMF. Wang resin 4 was then added to 10 mL of cleavage buffer (TFA:TIS:H₂O, 95:2.5:2.5) and stirred for 1 h at room temperature. After filtration, the solution was concentrated and cold ether (10 mL) was added to precipitate out the prodrg 5. After removing ether, the residues were reconstituted with 5 mL of distilled water and lyophilized. The TFA salt of the prodrg was obtained as a white powder.

The purity of the melphalan prodrg was determined by HPLC to be 95%. Electroospray ionization mass spectra (ESI-MS) were obtained on a Thermoquest LCQ electrospary ionization mass spectrometer. The observed molecular weight of the prodrg was found to be consistent with that required by its structure. The spectral identity of the prodrg was then confirmed using proton nuclear magnetic resonance spectra (¹H NMR). ¹H NMR spectra were obtained with a 500 mHz Bruker NMR spectrometer.

1-L-Glycyl-L-prolyl-melphalan: yield 13%; percent purity 95%; ¹H NMR (DMSO-d₄) δ 1.95–2.20 (m, 4H, CH₂ on proline), 3.05–3.35 (m, 12H, CH₂), 4.44–4.80 (m, 5H, αCH₂, γCH₂), 6.50–6.90 (m, 4H, phenol H); ESI-MS 459.4 (M + H)⁺.

2.3. Identification of Target Enzyme in NCI 60 Cancer Cell Lines.

The microarray data and the U95Ava II Affymetrix data were downloaded from the DTP database (https://wiki.nci.nih.gov/display/NCIDTPdata/Molecular+Target+Data). The programming language Perl was used to sift through more than 26,000 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 as described earlier. Briefly, 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 and TreeView programs. The sorted data from peptidases was normalized using a pool of 12 cell lines (NCI-H226, COLO 205, MCF-7, HS 578T, OVCA-3, OVCA-4, K-562, HL-60 (TB), CAKI-1, LOX IMVI, PC-3, SNB-19) as reference and clustered according to the origin of the cell lines. 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 potential enzyme target, DPPIV, was 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.

2.4. Selection of Cancer Cell Lines and Cell Culture.

Candidate cancer cell lines were selected from the NCI 60 cell lines based on the expression levels of DPPIV, doubling time, and growth requirements. Thus, the cancer cell lines selected, IGROV1, PC-3, 786-0, SK-OV-3, and SK-MEL-5, represent cells with high, medium, and low expression of DPPIV, reasonably short doubling times, and standard growth requirements. In addition, Caco-2 as well as HepG2 cell lines were also used in the studies.

2.5. Isolation of mRNA and RT-PCR Experiment.

Total RNA was lysed and purified from each cancer cell line with TRizol reagent according to the manufacturer’s protocol. Isolated RNA (0.5 µg) was reverse transcribed to cDNA with AMV reverse transcriptase, Oligo (dT), and Access RT-PCR kit following the standard protocol. Each of the 20 cycles of amplification consisted of denaturation (94 °C for 25 s), annealing (50 °C for 25 s), and extension (68 °C for 40 s). The primer set used for DPPIV amplification was the sense primer (5′-CCCTCTACTCTGATGACTC-GC-3′) and the antisense primer (5′-GTGCGCCTAAGGATCCTTCATCTTC-3′). PCR product was identified by electrophoresis in 4–20% TBE gels followed by SYBR green staining. The gel was then visualized with UV light, and the relative intensities of the RT-PCR product bands were measured using Metamorph software.

2.6. Hydrolysis of Glycyl-prolyl-p-nitroanilide (GP-pNA) by Porcine DPPIV with and without Specific Inhibitor Diprotin A.

Porcine kidney DPPIV was used in the studies due to its commercial availability and its close alignment with human DPPIV (88% homology). Stock solutions of porcine DPPIV were prepared by reconstituting lyophilized powder (0.87 unit, 23 units/mg) in 1 mL of 0.1 M Tris-HCl (pH 8.0) and stored at −80 °C. Solutions of the various substrates glycyl-prolyl-p-nitroanilide (GP-pNA), glycyl-phenylalaninlyl-p-nitroanilide (GF-pNA), or glycyl-argininyl-
2.7. Hydrolysis of GP-pNA in Cancer Cells and Inhibition by Diprotin A. DPPIV activity in the selected cancer cells was determined using the standard substrate GP-pNA. Briefly, cells were grown to 90% confluence in 150 mm culture plates, washed carefully 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 phosphate buffered saline (PBS, pH 7.4), and sonicated for 5 s two times on ice. The sonicated cell suspension was centrifuged at 1800g for 30 min at 4 °C. The supernatant was then used in hydrolysis studies, and to determine protein content (Bio-Rad DC protein assay). The protein content was adjusted to approximately 1000 μg/mL by appropriate dilutions before use in hydrolysis studies.

Hydrolysis studies were conducted in a 96-well microplate containing the cell homogenate suspensions at 37 °C. The reactions were initiated by the addition of GP-pNA (final concentration 1 mM) to the suspensions. The absorbance was monitored every minute for a total of 30–60 min using a precision microplate reader (Emax, Molecular Devices) at 405 nm. The competitive inhibition of hydrolysis of the substrates by porcine kidney DPPIV in the presence of the inhibitor diprotin A was determined by including 0.4 mM diprotin A in the diluted DPPIV mixture prior to incubation. Initial hydrolysis rates were determined in all hydrolysis experiments by assaying the amounts of pNA released.

2.8. Hydrolysis of GP-Mel by Porcine Kidney DPPIV. Hydrolysis of the melphalan prodrug by porcine kidney DPPIV was determined by assaying the DPPIV mixture prior to incubation. Initial hydrolysis rates were determined in all hydrolysis experiments by assaying the amounts of melphalan released per minute.

2.9. Hydrolysis of GP-Mel by Caco-2 and SK-MEL-5 Cell Homogenates. The extent of hydrolysis of GP-Mel in Caco-2 and SK-MEL-5 cell homogenates was determined as follows. Caco-2 and SK-MEL-5 cells and cell homogenates were prepared as described earlier. The hydrolysis reactions were carried out in 96-well plates (Corning, Corning, NY). 230 μL of the cell suspensions (1000 μg/mL protein) were placed in triplicate wells and the reactions initiated by the addition of GP-Mel (final concentration 1 mM in mixture) and incubated at 37 °C. At predetermined time points 40 μL aliquots were removed and added to two volumes of 10% ice-cold TFA to quench the reaction and precipitate protein. In the inhibition studies, diprotin A and GP-Mel were both added (1 mM final concentration) to the cell suspensions, incubated at 37 °C, and sampled as described above. The reactions were monitored for 30–60 min. The quenched precipitated samples were then filtered through a 0.45 μm filter plate and centrifuged at 1800g and 4 °C for 20 min. The recovered filtrate was analyzed by HPLC as described below. DPPIV activity was expressed as the amount (picomoles) of melphalan released per minute, normalized to the amount of protein.

3. RESULTS

3.1. Identification of DPPIV and Selection of Cancer Cell Lines. 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 as described previously. The expression patterns of proline-specific peptidases such as prolidase/peptidase α, prolyl carboxypeptidase
Table 1. Specific Activity of GP-pNA in the Presence vs Absence of Diprotin A in Various Cancer Cell Lines (pmol/min/μg protein, Mean ± SD; n = 3) and DPPIV Expression Determined with RT-PCR and with U95Av2 Affymetrix GeneChip

| cell line | rel RT-PCR expression | GeneChip expression<sup>a</sup> | w/o diprotin A | with diprotin A | % inhibn |
|-----------|------------------------|-------------------------------|---------------|----------------|----------|
| Caco-2    | 13.49                  |                               | 18.40 ± 1.17  | 0.29 ± 0.38    | 98.4     |
| HepG2     | 12.71                  |                               | 20.16 ± 0.88  | 0.23 ± 0.49    | 98.9     |
| IGROVI    | 7.92                   | 171                           | 11.42 ± 0.12  | 0.25 ± 0.12    | 97.8     |
| PC-3      | 5.51                   | 138                           | 4.18 ± 0.11   | 0.30 ± 0.05    | 92.7     |
| 786-0     | 2.81                   | 88                            | 1.32 ± 0.10   | 0.08 ± 0.11    | 94.0     |
| SK-OV-3   | 1.88                   | 33                            | 1.50 ± 0.20   | nd<sup>b</sup> | 100.0    |
| SK-MEL-5 | 1.00                   | 6                             | 1.25 ± 0.02   | 0.19 ± 0.02    | 84.8     |

<sup>a</sup>Novartis U95Av2 Affymetrix data on the DTP database. <sup>b</sup>Not detected.

dase, prolyl oligopeptidase/prolyl endopeptidase, DPPIV/CD26, aminopeptidase P, and prolidase/peptidase D, were selected for further assessment. DPPIV was overexpressed in most renal carcinoma cell lines (UO-31, TK-10, SN12C, RXF 393, CAKI-1, ACHN, A498, and 786-0) compared to cancer cell lines derived from other tissues. The relatively higher DPPIV expression in 786-0 renal carcinoma cell line compared to its expression in SK-OV-3 ovarian carcinoma or SK-MEL-5 melanoma cell lines was consistent with Affymetrix expression data. Thus, in addition to the selection of IGROVI (ovarian cancer cell line), PC-3 (prostate cancer cell line), 786-0 (renal cancer cell line), SK-OV-3 (ovarian cancer cell line), and SK-MEL-5 (melanoma) based on the U95Av2 Affymetrix gene expression of DPPIV (Table 1), HepG2 and Caco-2 cells were also selected for characterization of DPPIV activity with standard substrates and RT-PCR determinations of DPPIV expression.

3.2. Expression of DPPIV in Human Cancer Cell Lines: RT-PCR. RT-PCR of extracellular mRNA in the seven selected human cancer cell lines was performed with primers specific for DPPIV. The expression profiles of DPPIV in the seven cancer cell lines evidenced by the band at 315 bp are shown in Figure 3. The results of semiquantitative RT-PCR analysis (Table 1) indicated that DPPIV was expressed at high levels in Caco-2 and HepG2 cells, and at very low levels in SK-MEL-5 cells. Thus, the relative RT-PCR expression (expression in SK-MEL-5 set at unity) in the seven cell lines listed in Table 1 were Caco-2 (13.49), HepG2 (12.71), IGROVI (7.92), PC-3 (5.51), 786-0 (2.81), SK-OV-3 (1.89), and SK-MEL-5 cells (1.0). The relative RT-PCR expression in the cancer cell lines exhibited an excellent linear correlation (r² = 0.94) with U95Av2 Affymetrix microarray data that was available for five of the seven cell lines (Figure 4).

3.3. Hydrolysis of Standard Substrates by Porcine DPPIV. The specific activity of a standard substrate GP-pNA for porcine DPPIV was determined by the release of p-nitroanilide and the resultant effect on the clarity of the solution. The baseline activity was 14.02 ± 0.90 nmol/min/μg DPPIV. In the presence of the specific inhibitor diprotin A, it decreased substantially to 0.15 nmol/min/μg DPPIV. The kinetic parameters K<sub>m</sub> and V<sub>max</sub> obtained from a plot of V<sub>o</sub> versus [S] (Figure 5) were 231 ± 33 μM and 0.650 ± 0.037 μmol/min/mU DPPIV, respectively (fit estimate ± SD; n = 3). The K<sub>cat</sub> value determined from V<sub>max</sub> and DPPIV concentration (E<sub>0</sub>) was 36 s⁻¹. In contrast, GF-pNA or GR-pNA produced no detectable release of p-nitroanilide (negligible absorbance at 405 nm), indicating that these two molecules were resistant to cleavage by porcine DPPIV.

3.4. Hydrolysis of GP-pNA in Cancer Cell Homogenates and Inhibition by Diprotin A. The activity of DPPIV in various cancer cell homogenates was assessed using the standard substrate GP-pNA. The results shown in Figure 6 indicate that DPPIV activity was in the order HepG2 ≈ IGROVI ≫ PC-3 ≫ 786-0 ≥ SK-OV-3 ≥ SK-MEL-5 cells. Caco-2 and HepG2 cells exhibited high DPPIV activity (around 20 pmol/min/μg protein) whereas SK-MEL-5 cells showed the lowest activity (1.3 pmol/min/μg protein) among the cells tested (Figure 6). These results are in excellent correlation (r² = 0.96) with the relative RT-PCR expression of DPPIV in the cell lines (Figure 7). The hydrolysis of GP-pNA in the presence of diprotin A was significantly inhibited in all cancer cell
homogenates examined (Figure 6), with an average inhibition of 95% (range: 85−100%).

3.5. GP-Mel Model Prodrug Activation by Porcine Kidney DPPIV and Cell Homogenates. The specific activity of pure porcine enzyme against GP-Mel was 1.2 ± 0.1 nmol/min/μg protein. The activity was substantially lowered to 100 pmol/min/μg protein, a 93% inhibition, in the presence of diprotin A. DPPIV activity against the prodrug determined in Caco-2 and SK-MEL-5 homogenates was 5.14 ± 0.01 and 0.68 ± 0.03 pmol/min/μg protein, respectively, which was significantly inhibited by an average of 85% in the presence of diprotin A (Figure 8).

3.6. Cell Proliferation Studies. The antiproliferative activity of GP-Mel was determined in Caco-2 and SK-MEL-5 cancer cells and compared with that obtained with the parent drug melphalan. The percent cell viability profiles as a function of drug or prodrug concentration are shown in Figure 9. The profiles indicate that the antiproliferative action of melphalan was similar in Caco-2 and SK-MEL-5 cells with GI50 values of 34.9 μM and 56.6 μM, respectively. The GI50 value for GP-Mel in Caco-2 cells (261.3 μM), however, was significantly lower than that obtained with SK-MEL-5 cells (806.7 μM). The cytotoxic activity of GP-Mel in Caco-2 and SK-MEL-5 cells was consistent with the expression levels of DPPIV in these cells.

4. DISCUSSION

In the past three decades, the science of molecular biology was revolutionized by the rapid advancement of the complementing fields of genomics and bioinformatics. This revolution had a profound effect on cancer research. Indeed, new molecular characteristics of cancer cells are discovered almost daily. Recently, a number of enzymes from the peptidase/protease class were found to be highly expressed in several types of tumors, and to play an important role in the pathophysiology of tumor cells. DPPIV has been previously associated with the onset and progression of several cancer types; Inamoto et al. demonstrated that the blockage of DDPIV reduced several cancer-related processes in the human renal carcinoma cell line Caki-2. It also reduced the tumor size and increased the survival of mice in a xenograft model. Furthermore, in patients with clear cell renal cell carcinoma (CCRCC), higher DPPIV activity was linked with a significant decrease in patients’ 5 year survival

Figure 5. GP-pNA hydrolysis kinetic profile with porcine DPPIV (mean ± SD, n = 3).

Figure 6. Specific activity of DPPIV against GP-pNA (pmol/min/μg protein) in 7 cell homogenates, in the absence vs presence of diprotin A (mean ± SD, n = 3).

Figure 7. Linear correlation of specific activity of DPPIV against GP-pNA (pmol/min/μg protein) in 7 cancer cell homogenates with DPPIV expression determined using RT-PCR.

Figure 8. Specific activity of DPPIV against GP-Mel (pmol/min/μg protein) in Caco-2 and SK-MEL-5 cell homogenates, in the absence vs presence of diprotin A (mean ± SD, n = 3).
DPP IV was also shown to be overexpressed in several human colon cancer tissues and in human colon cancer cell lines,33,42,43 and its inhibition reduced carcinogenesis in a rat model.44 In the prostate, DPP IV activity in cancerous versus benign prostatic hyperplasia was increased 2-fold.45,46 An elevation of DPP IV activity was also found in the prostatic secretions and the peripheral zone of the prostate, where most prostate cancers arise.45 Higher DPP IV levels in cancerous versus normal prostate tissue was correlated with PSA level,44,45 prostate cell line, combined with high substrate specificity, indicated that DPP IV may be a potential target molecule for the delivery of prostate cancers arising.45 Higher DPP IV levels in cancerous versus normal prostate tissue was correlated with PSA level,44,45 prostate cell line, combined with high substrate specificity, indicated that DPP IV may be a potential target molecule for the delivery of chemotherapeutic drugs, and sparked our interest in developing a DPP IV-degradable anticancer prodrug.

In conclusion, DPP IV was identified as a potential prodrug target due to its differential expression levels in tumor and normal tissues and relatively strict substrate specificity. A Gly-Pro dipeptide prodrug of melphalan, GP-Mel, was designed and synthesized, based on the highly specific substrate requirements of DPP IV. The finding that the activation and antiproliferative activity of GP-Mel in cells were highly dependent on DPP IV expression level in the cells; the cytotoxicity (represented by GI50) of GP-Mel in Caco-2 cells was 3-fold higher (260 μM) than that in SK-MEL-5 cells (800 μM) (Figure 9). In contrast, for the free parent drug melphalan, similar GI50 values were obtained in Caco-2 and in SK-MEL-5 cells (35 and 44 μM, respectively), demonstrating the nonspecific cytotoxic action of melphalan (Figure 9). The cytotoxic effect of GP-Mel on both cell lines was significantly lower in comparison to melphalan; the prodrug failed to show significant cytotoxic effect in concentrations equimolar to those required for maximal growth inhibition by melphalan. This result may indicate that the GP-Mel prodrug is not likely to be cytotoxic by itself, and further validates the key role of DPP IV in the activation of the GP-Mel prodrug. On the other hand, it may indicate insufficient activation, resulting in too low free drug levels. Overall, our results demonstrate the potential to exploit DPP IV as a prodrug activating enzyme for efficient chemotherapeutic drug targeting.

5. CONCLUSIONS
In conclusion, DPP IV was identified as a potential prodrug target due to its differential expression levels in tumor and normal tissues and relatively strict substrate specificity. A Gly-Pro dipeptide prodrug of melphalan, GP-Mel, was designed and synthesized, based on the highly specific substrate requirements of DPP IV. The finding that the activation and antiproliferative activity of GP-Mel in cells were highly dependent on DPP IV expression levels confirmed our hypothesis that DPP IV is a feasible functional prodrug target for effective and selective chemotherapeutic action.
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