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Proline-linked nitrosoureas as prolidase-convertible prodrugs in human breast cancer cells

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Abstract:
A number of novel proline-linked nitrosoureas (**1**–**4**) were synthesized and examined for cytotoxicity and influence on DNA and collagen biosynthesis in MDA-MB-231 and MCF-7 human breast cancer cells. Evaluation of the cytotoxicity of these compounds employing a MTT assay and inhibition of [^{3}H]thymidine incorporation into DNA in both MDA-MB-231 and MCF-7 breast cancer cells demonstrated that compound **2**, the most active of the series, proved to be only slightly less potent than carmustine. It has also been found that carmustine did not inhibit MCF-7 cells prolidase activity, while compounds **1**–**4** significantly increased its activity, when used at 50–250 μM concentrations. Proline-linked nitrosoureas (**1**–**4**) also had lower ability to inhibit collagen biosynthesis in MCF-7 cells, compared to carmustine. The expression of β_1-integrin receptor and phosphorylated MAPK, ERK_1 and ERK_2 was significantly decreased in MCF-7 cells incubated for 24 h with 60 μM of compounds **2** and **4** compared to the control, untreated cells, whereas under the same conditions carmustine did not evoke any changes in expression of all these signaling proteins, as shown by Western immunoblot analysis. These results indicate the proline-linked nitrosoureas (**1**–**4**), represent multifunctional inhibitors of breast cancer cell growth and metabolism.

Key words:
nitrosoureas, breast cancer cells, prolidase, collagen biosynthesis, β_1-integrin

Abbreviations: DMEM – Dulbecco's minimal essential medium, ERK_1 and ERK_2 – extracellular-signal-regulated kinase 1 and kinase 2, FAK – non-receptor focal adhesion kinase $pp125^{FAK}$, FBS – fetal bovine serum, IGF-I – insulin-like growth factor I, MAPK –mitogen activated protein kinases, MTT – 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, PAGE – polyacrylamide gel electrophoresis, SDS – sodium dodecyl sulfate

Introduction

Cytotoxic agents that act by covalent modification of DNA were the first modern anticancer chemotherapeutics and remain major components of combination chemotherapy regimens [15]. In combination with drugs that act by other mechanisms, alkylating antitumor drugs have produced impressive and even curative responses in the treatment of some cancers. 2-Chloroethylnitrosoureas belong to the alkylating antitumor agents and some of them have been applied for the treatment of human cancers, mainly lymphomas, gliomas, a few solid tumors and melanomas [9, 48]. More recently, they have been incorporated into multiagent high-dose chemotherapy regimens with stem cell support in patients with breast cancer, neuroblastoma, glioma, melanoma and sarcomas [37]. Despite their

broad antitumor activity, the clinical usefulness of nitrosoureas has been limited by delayed onset, cumulative myelosuppression and pulmonary toxicity [9, 48]. Therefore, although the most widely used compound in this class, carmustine (Fig. 1), was approved for clinical use in the mid 1960s, efforts to develop new analogues with a better therapeutic index have continued to the present day [9, 48].

In the present study, we report on the synthesis and biological evaluations of four new alkylating agents utilizing L-proline derivatives as carriers of the cytotoxic nitrosoureas (Fig. 1). We hypothesized that coupling of L-proline through imido-bond to anticancer drugs might create prodrugs which would be locally activated by tumor-associated prolidase and consequently would be less toxic to normal cells that have lower prolidase activity. This strategy should be of benefit particularly in case of antineoplastic prodrugs, since at least some tumor tissues possess increased prolidase activity compared to normal tissues [2, 32, 37]. In such a case the release of the drug from the prodrug would be more efficient in neoplastic tissues than in normal tissues [46].

Prolidase [EC 3.4.13.9] is a cytosolic exopeptidase that cleaves imidodipeptides with C-terminal proline [14, 25, 34]. The primary biological function of the enzyme involves the metabolism of proline-containing protein degradation products and the recycling of proline from imidodipeptides for resynthesis of proline-containing proteins, mainly collagen [10]. Prolidase from various sources hydrolyzes dipeptides in which the C-terminal amino acid proline or hydroxyproline is linked through its tertiary nitrogen to the carbonyl of an amino acid residue bearing a free α-amino group [25]. However, good substrates can be produced, when methionyl group or haloacetylprolines replaces the amino group in imidodipeptides, suggesting that α-amino group is not an absolute specificity requirement for prolidase [25, 33].

Collagen biosynthesis and prolidase activity are coordinately regulated in MCF-7 and MDA-MB-231 cells [49]. Prolidase [EC 3.4.13.9] catalyzes the hydrolysis of imidodipeptides [35], releasing proline, which is used for collagen resynthesis [51] and cell growth [13]. Prolidase activity is regulated by the signal mediated by activated β_1-integrin receptors [42]. Stimulated β_1-integrin receptors induce autophosphorylation of non-receptor focal adhesion kinase $pp125^{FAK}$ (FAK) [20], which is then capable of interacting with adaptor-proteins, such as Grb2, through Src and Shc proteins. This interaction allows for activation of further signaling cascade through son of sevenless protein (Sos-1), Ras and Raf proteins [24] and subsequently, two mitogen activated protein kinases (MAPK), extracellular-signal-regulated kinase 1 (ERK_1) and kinase 2 (ERK_2) [45]. This results in induction of transcription factors and stimulation of the expression of genes for integrins, metalloproteinases, proteases and many other proteins involved in the regulation of cell growth and differentiation [26].

Collagen biosynthesis and cell growth is stimulated by insulin-like growth factor I (IGF-I) [17]. IGF-I, acting predominantly through the IGF-I receptor [28], has been demonstrated to stimulate proliferation, promote survival, enhance metastatic potential of breast cancer cells [19] and prevent apoptosis [1]. The MAP-kinase (ERK_1 and ERK_2) pathway induced by activated IGF-I receptor is considered to play a central role in carcinogenesis and tumor progression. IGF-I receptor signaling involves the same proteins and kinases as the β_1-integrin transduction pathway, except for the participation of FAK kinase and Src protein [6].

The novel proline-linked nitrosoureas (**1**–**4**) have been synthesized (Fig. 1) and their cytotoxicity has been tested in both MCF-7 and MDA-MB-231 breast cancer cells. In this study, the effects of these compounds on collagen and DNA biosynthesis, β_1-integrin receptor, IGF-I receptor, and phosphorylated MAP-kinases (ERK_1 and ERK_2) expression in human breast cancer cells were compared to those evoked by carmustine.

Materials and Methods

Chemistry

Melting points were determined on Büchi 535 melting-point apparatus and were uncorrected. ^1H and ^{13}C NMR spectra were recorded on Brucker AC 200F apparatus using tetramethylsilane as an internal standard. Chemical shifts are expressed in δ values (ppm). Multiplicity of resonance peaks are indicated as singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Elemental analysis of C, H, and N was performed on a Perkin Elmer 240 analyzer and satisfactory results within ± 0.4% of calculated values were

obtained. 2-Chloroethyl isocyanate, 2-bromoethyl isocyanate, 3-chloropropyl isocyanate, 4-bromophenyl isocyanate, L-proline, sodium nitrate(III) and diethyl ether were purchased from Sigma Chemical Co. (USA).

General method of synthesis for compounds 1–4

To a solution of 16 mmol of L-proline and 16 mmol of NaOH in 17 ml of water, 16 mmol of isocyanate in 11 ml of toluene were added dropwise with stirring and ice-cooling. After 2 h both phases were separated. To the aqueous phase, 32.5 mmol of sodium nitrate(III) was added followed by 12 ml of 36% hydrochloric acid which was added in several portions so that temperature did not rise above 5°C. Stirring for 45 min was followed by extraction with 3 × 20 ml of diethyl ether. The diethyl ether phase was washed with water, dried over $MgSO_4$ and the solvent was removed by rotary evaporation. The remaining residue was recrystallized from methanol.

N-[N'-(2-chloroethyl)-N'-nitrosocarbamoyl]-L--proline **(1)**

Yield = 43%, m.p. = 147°C. ^{1}H-NMR (DMSO-d_6) δ: 1.92 (m, 2H, γ-CH_2 proline), 2.16 (m, 2H, β-CH_2 proline), 3.24 (m, 2H, δ-CH proline), 3.54 (t, 2H, -CH_2-Cl, *J* = 6.2 Hz), 4.07 (t, 1H, α-CH proline, *J* = 8.24), 4.20 (t, 2H, N-CH_2-, *J* = 6.2 Hz), 8.21 (br, 1H, COOH). ^{13}C-NMR (DMSO-d_6) δ: 26.8 (γ-C proline), 27.2 (β-C proline), 38.6 (-CH_2-), 42.1 (CH_2-Cl), 46.0 (δ-C proline), 58.2 (α-C proline), 154.0 (CO), 173.9 (COOH). *Anal.* Calcd. for $C_9H_{14}ClN_3O_4$ (263.8): C, 41.00; H, 5.35; N, 15.94; Cl, 13.45. Found: C, 40.94; H, 5.32; N, 15.43; Cl, 13.59%.

N-[N'-(2-bromoethyl)-N'-nitrosocarbamoyl]-L--proline **(2)**

Yield = 34%, m.p. = 80°C. ^{1}H-NMR (DMSO-d_6) δ: 1.92 (m, 2H, γ-CH_2 proline), 2.16 (m, 2H, β-CH_2 proline), 3.24 (m, 2H, δ-CH proline), 3.66 (t, 2H, N-CH_2-, *J* = 6.2 Hz), 3.72 (t, 2H, -CH_2-Br, *J* = 6.2 Hz), 4.07 (t, 1H, α-CH proline, *J* = 8.24), 8.21 (br, 1H, COOH). ^{13}C-NMR (DMSO-d_6) δ: 25.7 (CH_2-Br), 26.8 (γ-C proline), 27.2 (β-C proline), 48.6 (-CH_2-), 46.0 (δ-C proline), 58.2 (α-C proline), 154.0 (CO), 173.9 (COOH). *Anal.* Calcd. for $C_8H_{12}BrN_3O_4$ (294.1): C, 32.67; H, 4.11; N, 14.29. Found: C, 32.84; H, 4.22; N, 14.23%.

N-[N'-(3-chloropropyl)-N'-nitrosocarbamoyl]-L--proline **(3)**

Yield = 51%, yellow oil. ^{1}H-NMR (DMSO-d_6) δ: 1.92 (m, 2H, γ-CH_2 proline), 2.16 (m, 2H, β-CH_2 proline), 3.24 (m, 2H, δ-CH proline), 3.40–3.58 (m, 6H, -$(CH_2)_3$-), 4.07 (t, 1H, α-CH proline, *J* = 8.24), 6.11 (br, 1H, COOH). ^{13}C-NMR (DMSO-d_6) δ: 26.8 (γ-C proline), 27.2 (β-C proline), 30.6 (-CH_2-), 36.7 (CH_2-Cl), 46.0 (δ-C proline), 45.3 (N-CH_2), 58.2 (α-C proline), 154.0 (CO), 173.9 (COOH). *Anal.* Calcd. for $C_9H_{14}ClN_3O_4$ (263.7): C, 41.00; H, 5.35; N, 15.94; Cl, 13.45. Found: C, 40.94; H, 5.32; N, 15.43; Cl, 13.59%.

N-[N'-(4-bromophenyl)-N'-nitrosocarbamoyl]-L--proline **(4)**

Yield = 76%, mp = 196°C. ^{1}H-NMR (DMSO-d_6) δ: 1.92 (m, 2H, γ-CH_2 proline), 2.16 (m, 2H, β-CH_2 proline), 3.24 (m, 2H, δ-CH proline), 4.09 (t, 1H, α-CH proline, *J* = 8.24), 7.41 (d, 2H, Ar-H, *J* = 6.97), 7.47 (d, 2H, Ar-H, *J* = 6.99 Hz), 8.46 (br, 1H, COOH). ^{13}C-NMR (DMSO-d_6) δ: 26.2 (γ-C proline), 28.3 (β-C proline), 46.1 (δ-C proline), 58.2 (α-C proline), 113.2 (Ar), 121.3 (Ar), 131.0 (Ar), 139.8 (Ar), 153.5 (CO), 173.9 (COOH). *Anal.* Calcd. for $C_{12}H_{12}BrN_3O_4$ (342.2): C, 42.08; H, 3.51; N, 12.27. Found: C, 42.04; H, 3.52; N, 12.23%.

Pharmacology

Materials

Stock cultures of breast cancer cells MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection, Rockville, MD (USA). Dulbecco's minimal essential medium (DMEM) and fetal bovine serum (FBS) used in cell culture were products of Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biologicals Inc. (USA). [^{3}H]thymidine (6.7 Ci/mmol) was the product of NEN (USA). Control antiVEGF monoclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-FAK antibody, anti-Human IGF-I receptor antibody, anti-goat immunoglobulin antibody, anti-mouse immunoglobulin antibody, aprotinin, bacterial collagenase, 5-bromo-

4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate reagent (BCIP/NBT), leupeptin, glycyl-L-proline, L-proline, glycyl-L-hydroksyproline, monoclonal anti-posphorylated MAPK (ERK_1 and ERK_2) antibody, porcine kidney prolidase, Nonidet P-40, phenylmethylsulfonyl fluoride and Protein A-Sepharose were provided by Sigma Chemical Co. (USA), as were most other chemicals and buffers used. Nitrocellulose membrane (0.2 μm), sodium dodecylsulfate (SDS), polyacrylamide, molecular weight standards and Coomassie Brilliant Blue R-250 were received from Bio-Rad Laboratories (USA). 5-[^{3}H]Proline (28 Ci/mmol) was purchased from Amersham (UK). A goat polyclonal anti-β_1-integrin antibody (N-20) and a rabbit polyclonal anti-α_2-integrin antibody (H-293) were obtained from Santa Cruz Biotechnology (USA). Polyclonal (rabbit) anti-human prolidase antibody was a gift from Dr. James Phang (NCI-Frederick Cancer Research and Development Center, Frederick, MD, USA). Anti-Rabbit immunoglobulin was obtained from Promega Corp. (USA). [^{3}H]thymidine (6.7 Ci/mmol) was purchased from ICN Biomedicals, USA and Scintillation Coctail "Ultima Gold XR" from Packard (USA).

Cell culture

Human breast cancer MDA-MB-231 and MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin and 0.02% EDTA in calcium-free phosphate buffered saline, counted in hemocytometers and plated at 5×10^5 cells per well of 6-well plates (Nunc) in 2 ml of growth medium (DMEM without phenol red with 10% CPSR1). Cells reached about 80% of confluence on day 3 and in most cases such cells were used for the assays.

DNA synthesis assay

MCF-7 and MDA-MB-231 cells were seeded in 6-well plates and were incubated with varying concentrations of carmustine or compounds **1**–**4** and 0.5 μCi of [^{3}H]thymidine for 24 h at 37°C. The cells were then harvested by trypsinization, washed with cold phosphate-buffered saline and centrifuged for 10 min at 1500 × g several times (4–5) until the dpm in the washes were similar to the reagent control. The recovered radioactivity was determined by liquid scintillation counting and the values were expressed as dpm/well.

Cell viability assay

The assay was performed according to the method of Carmichael using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [8]. Confluent cells, cultured for 24 h with various concentrations of studied compounds in 6-well plates were washed three times with PBS and then incubated for 4 h in 1 ml of MTT solution (0.5 mg/ml of PBS) at 37°C in 5% CO_2 in an incubator. The medium was removed and 1 ml of 0.1 mol/l HCI in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Viability of breast cancer cells cultured in the presence of the studied compounds was calculated as a per cent of control cells. The experiments were performed in triplicates. After treatment of the cells with drug, the ratio of viable to dead cells in tested and control (untreated) cells was calculated for each drug concentration. Viable cell number was plotted versus drug concentration, and IC_{50} values were calculated from dose-response curves as the concentration of drugs that reduce the number of viable cells to 50% of control using an Origin 7.5 software (OriginLab, USA).

Collagen production

Incorporation of a radioactive precursor into proteins was measured after labeling of the cells in growth medium, containing varying concentrations of carmustine and compounds **1**–**4** for 24 h, with 5-[^{3}H]proline (5 μCi/ml, 28 Ci/mmol) as described previously [38]. Incorporation of the tracer into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase, according to the method of Peterkofsky [43]. Results are shown as combined values for cell plus medium fractions.

Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al. [34] which is based on the measurement of proline by Chinard's reagent. Briefly, the monolayer was washed three times with 0.15 M NaCl. Cells were collected by scraping and suspended

in 0.15 M NaCl, centrifuged at low speed (200 × g) and the supernatant was discarded. The cell pellet (from 1 well) was suspended in 0.3 ml of 0.05 M Tris-HCl, pH 7.8, and sonicated 3 times for 10 s at 0°C. Samples were then centrifuged (16,000 × g, 30 min) at 4°C. Supernatant was used for protein determination and then prolidase activity assay. Activation of prolidase requires preincubation with manganese, therefore, 0.1 ml of supernatant was incubated with 0.1 ml of 0.05 M Tris-HCl, pH 7.8 containing 2 mM $MnCl_2$ for 2 h at 37°C. After preincubation, the prolidase reaction was initiated by adding 0.1 ml of the preincubated mixture to 0.1 ml of 0.094 M gly-L-pro to a final concentration of 47 mM. After additional incubation for 1 h at 37°C, the reaction was terminated with 1 ml of 0.45 M trichloroacetic acid. In the parallel tubes, reaction was terminated at time "zero" (without incubation). The released proline was determined by adding 0.5 ml of the trichloroacetic acid supernatant to 2 ml of a 1:1 mixture of glacial acetic acid: Chinard's reagent (25 g of ninhydrin dissolved at 70°C in 600 ml of glacial acetic acid and 400 ml of 6 M orthophosphoric acid) and incubated for 10 min at 90°C. The amount of proline released was determined colorimetrically by reading an absorbance at 515 nm and calculated from calibration curve for proline standard. Protein concentration was measured by the method of Lowry [29]. Enzyme activity was calculated as nanomoles of released proline per minute per milligram of supernatant protein.

Hydrolysis of Gly-Pro, Gly-Hyp and compounds 1–4 by porcine kidney prolidase with and without specific inhibitor Z-Pro

Prolidase solution was prepared by suspending the lyophilized solid enzyme (0.8 mg of enzyme, 0.551 mg of 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 $MnCl_2$, 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 or Gly-Hyp and 200 mM $MnCl_2$. The activated reagent A was added to reagent B to obtain a final Gly-Pro or Gly-Hyp concentration of 5 mM and incubated for 10 min at 40°C. 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 Z-Pro was determined by supplementing Z-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 for 20 min, and the supernatant was withdrawn for colorimetric assay of the released proline content as described above. The extent of hydrolysis of the compounds **1**–**4** by porcine kidney prolidase and the effect of the competitive inhibitor Z-Pro were determined using a slight modification of the procedure described above for Gly-Pro and Gly-Hyp. Thus, the studies were performed as described for Gly-Pro except that (a) reagent B contained 2.53 mM concentrations of compounds **1**–**4** and (b) the hydrolysis was carried out for 30 min at 40°C. In inhibition studies, the concentration of Z-Pro used was also 2.53 mM. The extent of hydrolysis was determined by assaying released proline content by colorimetric analysis as described above.

Immunoprecipitation

Subconfluent cells in 6-well plates were rinsed with phosphate-buffered saline, scraped out of the wells and centrifuged at 1000 × g for 3 min. Then the cells (from 6 wells) were solubilized with lysis buffer containing 10 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, at 4°C for 10 min. The insoluble material was removed by centrifugation at 10,000 × g for 5 min at 4°C. Supernatant containing 100 μg of protein was added to 100 μg of Protein A-Sepharose that had been linked to primary antibody in the following manner. Protein A-Sepharose was washed 3 times with lysis buffer and 100 μl of suspension containing about 100 μg of beads was incubated for 1 h at 4°C with 20 μl of primary antibody. Then, the conjugate was incubated for 1 h at 4°C with shaking. Immunoprecipitate was washed four times with lysis buffer. Proteins were released from the beads by boiling in SDS sample buffer and loaded onto a 10% SDS-polyacrylamide gel. The immunoprecipitates were analyzed by Western immunoblot method.

SDS-PAGE

Slab SDS/PAGE was performed according to the method of Laemmli [27].

Western immunoblot analysis

After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris-HCl, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transferred to 0.2-μm pore-sized nitrocellulose at 100 mA for 1 h by using a LKB 2117 Multiphor II electrophoresis unit. The membrane was blocked with 5% dried milk in TBS-T for 1 h at room temperature, with slow shaking. The nitrocellulose was incubated with polyclonal antibody against human prolidase at concentration 1:3000, monoclonal antibody against IGF-I receptor at concentration 1:500, monoclonal antibody against β_1-integrin subunit at concentration 1:1000 and phosphorylated MAPK at concentration 1:5000 in 5% dried milk in Tris-buffered saline with Tween 20 (TBS-T) (20 mmol/l Tris-HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween 20) for 1 h. In order to analyze prolidase, the second antibody-alkaline phosphatase conjugated anti-rabbit immunoglobulin (whole molecule) was added to reach a final dilution of 1:5000; in order to analyze the β_1-integrin subunit, and phosphorylated MAP-kinases second antibody-alkaline phosphatase conjugated anti-mouse immunoglobulin (whole molecule) was added as 1:7500 dilution; and in order to analyze IGF-I receptor, the second antibody-alkaline phosphatase conjugated anti-goat immunoglobulin (whole molecule) was added as 1:5000 dilution. All antibodies were diluted in TBS-T and incubated for 60 min with gentle shaking. Then nitrocellulose was washed with TBS-T (5 × 5 min) and submitted to Sigma-Fast BCIP/NBT reagent.

Statistical analysis

In all experiments, the mean values for three assays ± standard deviations (SD) were calculated. The results were submitted to statistical analysis using the Student's *t*-test. Differences were considered significant at $p < 0.05$. Mean values, the standard deviations and the number of measurements in the group (n) are presented in the Figures. These statistical analyses were carried out using Origin 7.5 software (OriginLab, USA).

Results

Nitrosourea derivatives **1**–**4** (Fig. 1) were prepared by reaction of L-proline with 2-chloroethyl isocyanate (**1**), 2-bromoethylisocyanate (**2**), 3-chloropropyl isocyanate

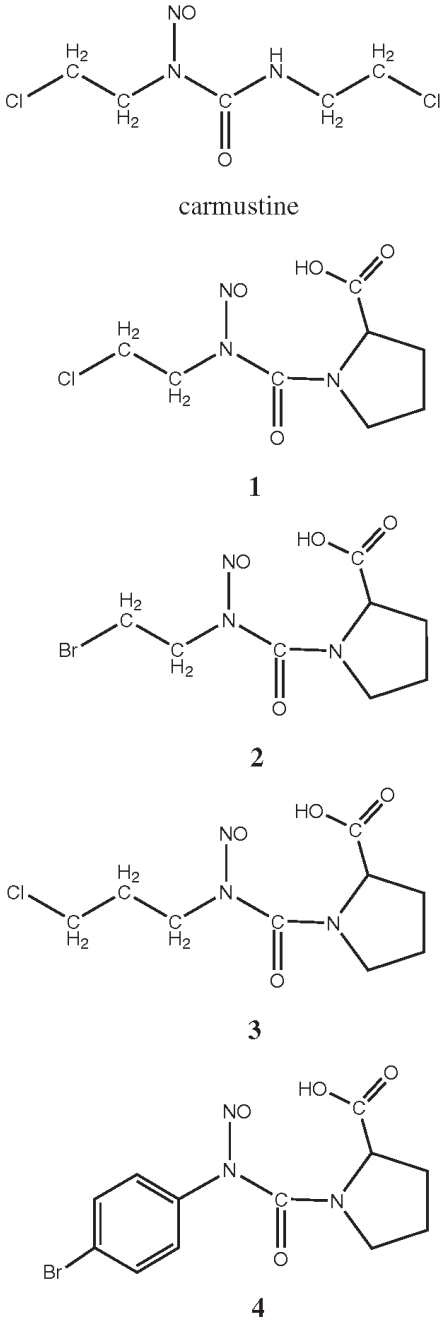

Fig. 1. The chemical structure of carmustine and compounds **1**–**4**

(**3**) or 4-bromophenyl isocyanate (**4**) followed by subsequent nitrosation of the resulting ureas [12, 47]. The chemical structures of **1**–**4** were proved by ^{1}H and ^{13}C NMR and elemental analysis.

In order to compare cytotoxicity of **1**–**4** and carmustine, viability of breast cancer cells was measured by the method of Carmichael et al. [8] using tetrazolium salt (Fig. 2). We have found that compounds **1**–**4** decreased the number of viable cells in both estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) breast cancer cells. Although the cytotoxicity was concentration-dependent in both cell lines, it was more pronounced in MDA-MB-231 than in MCF-7 (Fig. 2). In terms of reduction in cell viability, the compounds rank in both MCF-7 and MDA-MB-231 cells in the following order: carmustine > **2** > **4** > **1** > **3**. The values of IC_{50} were relatively higher for **2** and **4** which possess a bromoethyl and 4-bromophenyl function, respectively. Among the derivatives, compound **2** in both MDA-MB-231 and MCF-7 proved to be only slightly less potent than carmustine, with IC_{50} values of 88 ± 2

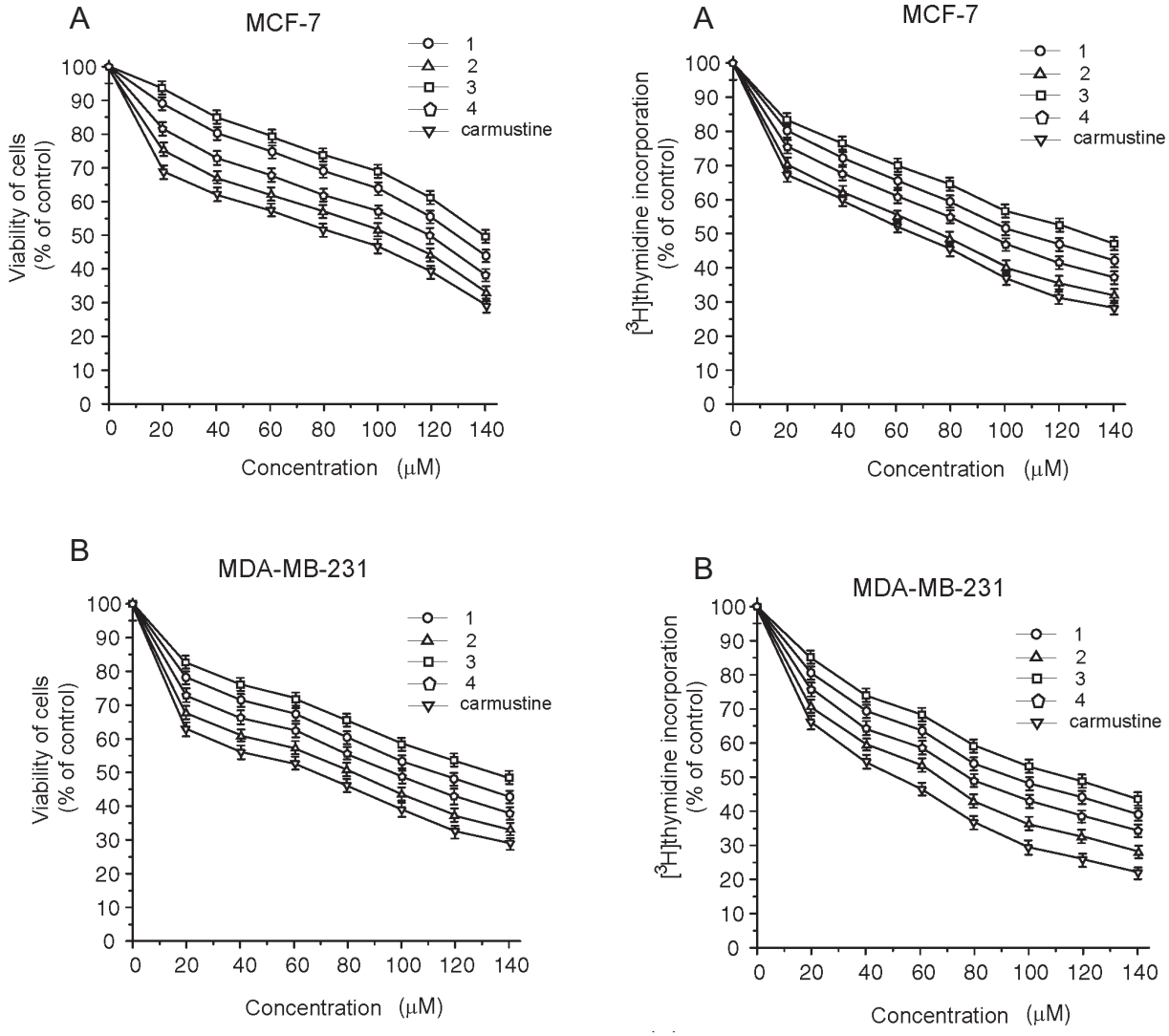

Fig. 2. Viability of MCF-7 **(A)** and MDA-MB-231 **(B)** cells treated for 24 h with different concentrations of compounds **1**–**4** and carmustine. Mean values ± SD of 3 independent experiments (n = 4) done in duplicates are presented

Fig. 3. Antiproliferative effects of compounds **1**–**4** and carmustine in cultured breast cancer MCF-7 **(A)** and MDA-MB-231 **(B)** cells as measured by inhibition of [^{3}H]thymidine incorporation into DNA. Mean values ± SD of 3 independent experiments (n = 4) done in duplicates are presented

and 102 ± 2 μM, respectively, compared to 72 ± 2 and 87 ± 2 μM for carmustine.

To analyze if the inhibition in cell viability was due to decreased cell proliferation, we measured DNA synthesis in the presence of compounds **1**–**4** and carmustine (Fig. 3). All tested compounds showed concentration-dependent activity, yet with different potency. Furthermore, the obtained profiles of DNA synthesis were similar in MCF-7 and MDA-MB-231 (Fig. 3). The concentrations of compounds **1**, **2**, **3** and **4** needed to inhibit [^{3}H]thymidine incorporation into DNA by 50% (IC_{50}) in MCF-7 were found to be 108 ± 4 μM, 78 ± 4 μM, 130 ± 4 μM, and 92 ± 4 μM, respectively, suggesting lower cytotoxic potency of these compounds compared to carmustine (IC_{50} = 71 ± 4 μM). The concentrations of compounds **1**, **2**, **3**, **4** and carmustine required for 50% inhibition of [^{3}H]thymidine incorporation into DNA in breast cancer MDA-MB-231 cells (IC_{50}) was found to be 107 ± 4 μM, 69 ± 4 μM, 92 ± 4 μM, 83 ± 4 μM and 57 ± 4 μM, respectively.

The activity of prolidase was determined according to the method of Myara et al. [34] which is based on the measurement of proline by Chinard's reagent [11]. The compounds **1**–**4** were insoluble in aqueous solutions but were solubilized in the presence of 10% DMSO. It has been found that in the presence of 1% DMSO, the purified prolidase has similar catalytic activity toward standard prolidase substrate – glycyl-L-proline as in the absence of DMSO [3]. The compounds **1**–**4** were found to be good substrates for prolidase, however, with weak susceptibility. Compounds **1**–**4** show susceptibility to cleavage by prolidase in a concentration range similar to that of the well-known endogenous prolidase substrate, glycyl-L-hydroxyproline (Fig. 4). The prolidase inhibitor Z-Pro inhibited the specific activity of Gly-Pro, Gly-Hyd and compounds **1**–**4** by roughly 80% (Fig. 4).

Experiment presented in Figure 5 shows the effect of carmustine and compounds **1**–**4** on prolidase activity in breast cancer cells incubated with the drugs for 24 h. It is evident from Figure 5 that prolidase expression is 3-fold higher in MDA-MB-231 cells compared to MCF-7 breast cancer cells. It has been found that carmustine did not inhibit breast cancer cell prolidase activity, while compounds **1**–**4** increased the activity by 80 to 110% in MCF-7 cells and by 20 to 40% in

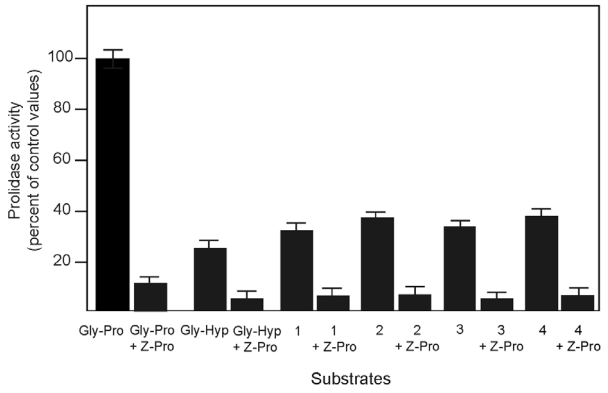

Fig. 4. Susceptibility of compounds **1**–**4**, glycyl-L-proline (Gly-Pro) and glycyl-L-hydroxyproline (Gly-Hyp) to the action of prolidase in the presence and absence of Z-Pro. The susceptibility of glycyl-L-proline to the action of prolidase was considered as 100%. Mean values ± SD of 6 assays are presented

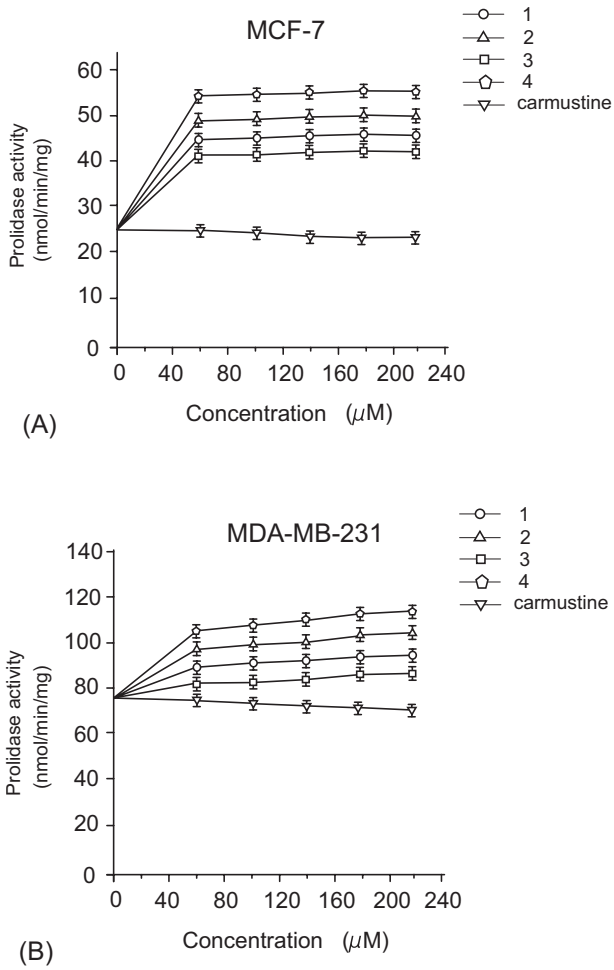

Fig. 5. Prolidase activity in breast cancer MCF-7 **(A)** and MDA-MB-231 **(B)** cells treated for 24 h different concentration of compounds **1**–**4** and carmustine. Mean values of three independent experiments done in duplicates ± standard deviation (SD) are presented

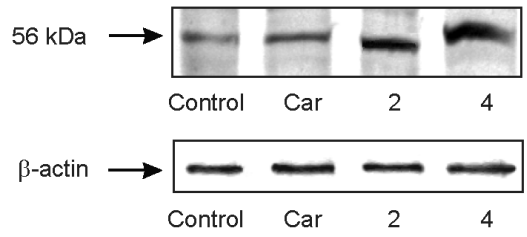

Fig. 6. Western immunoblot analysis for prolidase in control breast MCF-7 cells and the cells incubated for 24 h in the presence of 60 μM carmustine (Car), 60 μM compound **2** (2) or compound **4** (4). β-actin served as a loading control. This profile is representative of at least three different experiments. The samples contained 30 μg of protein from pooled cell extracts (n = 6). The arrows indicate the molecular mass of standards

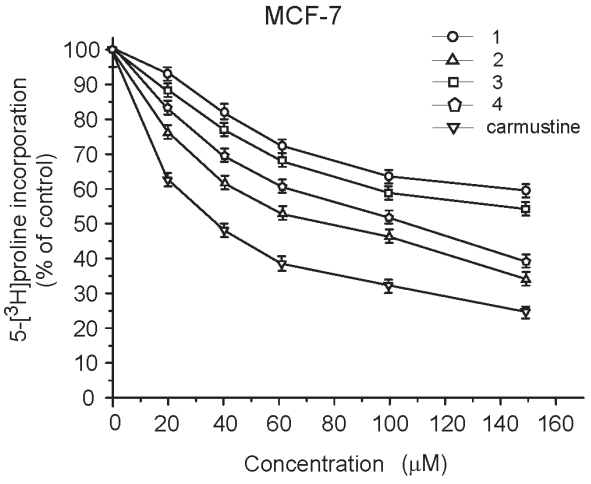

Fig. 7. Collagen synthesis, measured by 5-[^{3}H]proline incorporation into proteins susceptible to the action of bacterial collagenase, in breast cancer MCF-7 cells cultured for 24 h in the presence of different concentration of compounds **1**–**4** and carmustine. Mean values of three independent experiments done in duplicates ± standard deviation (SD) are presented

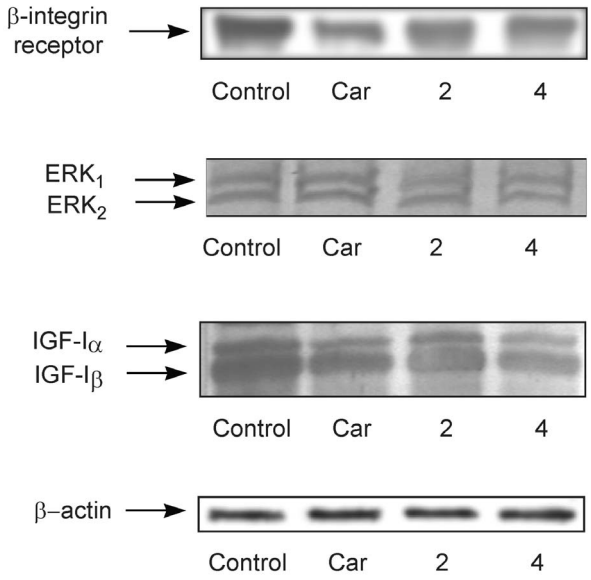

Fig. 8. Western immunoblot analysis for β_1-integrin receptor, phosphorylated MAP-kinases ERK_1 and ERK_2 and IGF-I receptor in control breast cancer MCF-7 cells and the cells incubated for 24 h in the presence of 60 μM carmustine (Car), 60 μM compound **2** (2) or compound **4** (4). β-actin served as a loading control. The results represent at least three different experiments. The samples contained 30 μg of protein from pooled cell extracts (n = 6)

MDA-MB-231 cells. The increase in prolidase activity induced by all compounds **1**–**4** in MCF-7 cell line was related to the changes in the enzyme expression as shown, for example, in Figure 6 for compounds **2** and **4** by Western immunoblot analysis.

Cellular hydrolysis of compounds **1**–**4** contributed to an increase in proline concentration in cytoplasm. Since imido-bound proline can be reused for collagen synthesis [10], we have compared the effect of carmustine and compounds **1**–**4** on synthesis of this protein in breast cancer cells. Collagen biosynthesis was measured in breast cancer MCF-7 cells treated for 24 h with different concentrations of the studied drugs. As shown in Figure 7, carmustine was found to be a more effective inhibitor of collagen biosynthesis than compounds **1**–**4**. The inhibitory effect was dose-dependent. IC_{50} for carmustine and compound **2** was 38 ± 4 μM and 82 ± 4 μM, respectively.

Collagen biosynthesis and prolidase activity are regulated by signal generated by β_1-integrin receptor. The expression of the receptor in MCF cell lines was measured by Western immunoblot. As shown in Figure 8, treatment of the cells with 60 μM of carmustine evoked stronger inhibitory effect than 60 μM of compounds **2** and **4** on the expression of β_1-integrin receptor in MCF-7 cell lines. Expression of proteins involved in signaling cascade activated by β_1-integrin receptor was also evaluated. As shown in Figure 8, treatment of MCF-7 cell lines with 60 μM carmustine for 24 h did not affected the expression of phosphorylated MAP-kinases ERK_1 and ERK_2. In contrast, the compounds **2** and **4** inhibited expression of MAP-kinases in MCF-7 cell lines. Collagen biosynthesis is also known to be regulated by IGF-I receptor. Activation of this receptor plays an important role in cellular

transformation, mitogenesis and inhibition of apoptosis. The expression of the receptor in both cancer cell lines treated with 60 μM carmustine and 60 μM of compounds **2** and **4** for 24 h was inhibited by all compounds. Down-regulation of the receptor was more pronounced in the presence of compound **4** (Fig. 8).

Discussion

The present study was undertaken to extend our recent findings related to antineoplastic activity of new nitrosoureas. The compound **2**, which possesses a bromoethyl function, is the most cytotoxic proline analogue of nitrosoureas from among a series of derivatives we have synthesized to date. Our experimental studies have demonstrated that treatment with compounds **1**–**4** prevented the exponential growth and decreased the number of viable cells in both estrogen receptor-positive and estrogen receptor-negative breast cancer cells (Fig. 2). Because the antiproliferative effect of compounds **1**–**4** is independent of the estrogen receptor status of the breast cancer cells, these potent inhibitors are potential pharmacological agents for the treatment of both hormone responsive and nonresponsive breast cancer cells.

The N-acylproline linkage is unique in peptides in that it involves a tertiary amide. Most proteases cannot cleave that bond except for a specific, cytosolic imidodipeptidase, prolidase [35]. Cytosolic location of this imidodipeptidase suggests that it may serve as a prodrug-converting enzyme. Previously, it has been found that prolidase activity in several cancer lines is several-fold higher, compared to normal tissue [2, 32]. The finding that compounds **1**–**4** are susceptible to the action of prolidase creates a possibility for their application in pharmacotherapy of these neoplastic diseases.

The primary biological function of prolidase consists in the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen synthesis [10, 35]. It is evident that an absence of prolidase will severely impede the efficient recycling of collagen proline. The clinical symptoms related to collagen deficit, which led Goodman et al. [18] to liken the conditions to lathyrism, can be attributed to this effect. On the other hand, enhanced liver prolidase activity was found during the fibrotic process [36]. It suggests that prolidase, providing proline for collagen biosynthesis may regulate turnover of collagen and may be a rate-limiting factor in the regulation of collagen production. Recently, the link has been found between collagen production and prolidase activity in cultured human skin fibroblasts treated with anti-inflammatory drugs [31], during experimental aging of these cells [39, 40], fibroblast chemotaxis [41] and cell surface integrin receptor ligation [42]. Lower ability of compounds **1**–**4** to inhibit collagen biosynthesis may result (at least in part) from the delivery of proline into the cells, the process that provides the main substrate for collagen biosynthesis. It may explain the differences in the collagen biosynthesis inhibition rate by the studied drugs (Fig. 7).

It is known that tumor cells produce enhanced amounts of proteases that degrade collagen and modulate collagen interaction with integrin class of extracellular matrix (ECM) receptors [41]. Since this interaction has been shown to regulate cellular gene expression, differentiation and growth [4, 7] and neoplastic transformation is accompanied by aberration of some ECM protein, mainly fibronectin and collagen [22], it is likely that disturbances in metabolism of neoplastic cells may in part be due to dysregulation of tissue collagen synthesis and deposition. Simultaneously, the collagen biosynthesis is decreased in neoplastic tissues and collagenolytic activity is higher which is known to promote metastasis. Therefore, the lower ability of compounds **1**–**4** to inhibit collagen biosynthesis, compared to carmustine, would be of benefit.

Induction of these processes crucially depends on β_1-integrin receptor. Our findings show that incubation of MCF-7 cells in the presence of 60 μM of compounds **2** and **4** for 24 h caused a decrease in expression of β_1-integrin subunits (Fig. 8). This function of compounds **2** and **4** may suppress growth of cancer cells and induce apoptosis [5, 23]. Another important finding is that compounds **2** and **4** inhibit phospho-ERK activation. Up-regulation of those kinases was found in various breast cancers [44]. Blocking of those kinases was found to have proapoptotic and antiproliferative effect on MCF-7, which indicates a new target in the treatment of breast malignancies [16, 21].

Treatment of the cells with 60 μM of compounds **2** and **4** decreased expression of IGF-I receptor in MCF-7 lines (Fig. 8). IGF-I receptor is involved in cellular transformation, mitogenesis and inhibition of apoptosis [1]. Therefore, inhibition of the receptor

may represent an approach to the inhibition of tumor growth. Blockade of the receptor [30] or down-regulation of its expression [50] reduces cancer proliferation and induces apoptosis.

The presented data postulate that targeting of prolidase as a prodrug-converting enzyme may serve as a potential strategy in pharmacotherapy of neoplastic diseases. Although a more detailed analysis of the *in situ* coupling of these prodrugs to endogenous prolidase is warranted, we believe that the outlined prodrug strategy is an attractive approach that involves altering of the pharmacokinetic profile of established anticancer drugs in order to increase their therapeutic index.

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