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Proline analogue of melphalan as a prodrug susceptible to the action of prolidase in breast cancer MDA-MB 231 cells

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Abstract

Proline analogue of melphalan (Mel-pro) was synthesized as a prodrug susceptible to the action of ubiquitously distributed, cytosolic imidodipeptidase–prolidase [E.C.3.4.13.9]. Conjugation of melphalan (Mel) with proline (Pro) through imido-bond resulted in formation of a good substrate for prolidase. Cytosolic location of prolidase in neoplastic cell suggests that proline analogue of melphalan (Mel-pro) may serve as a prolidase convertible prodrug. We have compared several aspects of pharmacologic actions of Mel and Mel-pro in estrogen-independent breast cancer MDA-MB 231 cells. It has been found that Mel-pro is more effectively transported into the MDA-MB 231 cells, evokes higher cytotoxicity, similar inhibitory effect on DNA synthesis, lower inhibitory effect on collagen biosynthesis and reduces IGF-I receptor and MAPkinase expression in MDA-MB 231 cells, compared to Mel. The results suggest that targeting of prolidase as a Mel-pro-converting enzyme may serve as a potential strategy in pharmacotherapy of breast cancer.

Keywords: Prodrug; Proline analogue of melphalan; Prolidase; Breast cancer cells; Collagen

1. Introduction

Melphalan (Mel) belongs to the class of antitumor agents with an alkylating and cross-linking action on guanine and possibly other bases of deoxyribonucleic acid that result in arresting cell division [1]. The use of alkylating agents in pharmacotherapy of neoplastic diseases is accompanied by a wide variety of untoward side effects [1]. In order to minimize their side effects, efforts were undertaken to construct prodrugs [2–4]. Our previous results [5,6] have shown that proline analogue of melphalan, *N*-[[[[(S)-carboxy]pyrrolidin-1yl]carbonyl]methyl]-4-[bis(2-chloroethyl)amino]-2 phenylalanine dilithium salt (Mel-pro) may serve as a substrate for purified pig kidney prolidase (Fig. 1).

Prolidase [E.C.3.4.13.9] is a ubiquitously distributed cytosolic exopeptidase that cleaves imidodipeptides with C-terminal proline [7,8]. The biological function of the enzyme involves the metabolism of proline-containing protein degradation products and the recycling of proline from imidodipeptides for proline-containing protein resynthesis, mainly collagen [9]. The presence of prolidase in cytoplasm allows to suspect that it may be targeted as a Mel-pro-converting enzyme. This strategy should be of benefit particularly in case of antineoplastic prodrugs, since at least some tumour tissues evoke increased prolidase activity compared to normal tissues [10]. In such a case the release of the drug from the prodrug would be more efficient in neoplastic tissues than in normal tissues. The specific objective of present studies was to examine the susceptibility of Mel-pro to the action of prolidase in estrogen-independent breast cancer MDA-MB 231 cells, the ability of Mel and Mel-pro to penetrate cell membrane, their cytotoxicity, effect on DNA and collagen biosynthesis, expression of IGF-I receptor and MAP kinase in MDA-MB 231 cells.

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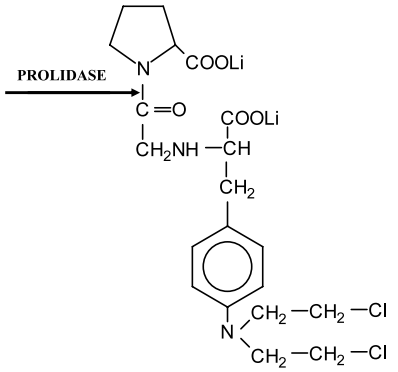

Fig. 1. The chemical structure of proline analogue of melphalan (Melpro). An arrow indicates imido-bond susceptible to the action of prolidase.

2. Experimental

2.1. Materials

Glycyl-proline (Gly-pro), bacterial collagenase (type VII), trypsin, bovine serum albumin, pig kidney prolidase, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), polyclonal anti-human insulin-like growth factor-I (IGF-IsR), monoclonal anti-MAP kinase and melphalan were purchased from Sigma Chemical Co. (USA). Dulbecco's minimal essential medium (DMEM) and foetal bovine serum (FBS) used in cell culture were products of Gibco (USA). Glutamine, penicillin and streptomycin were obtained from Quality Biological Inc. (USA). L-5[^{3}H]-proline (28 Ci/mmol) was received from Amersham (UK). [^{3}H]-thymidine (6.7 Ci/mmol) was the product of NEN (USA). Nitrocellulose membrane (0.2 μm) and sodium dodecylsulphate-polyacrylamide gel eletrophoresis (SDS-PAGE) were received from Bio-Rad Laboratories (USA). Alkaline phosphatase-conjugated antibody against rabbits Fc IgG and alkaline phosphatase-conjugated antibody against mouse Fc IgG were obtained from Promega Corp. (USA).

2.2. MDA-MB 231 cell culture

Breast cancer MDA-MB 231 cells were maintained in DMEM supplemented with 10% 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 confluency at day 3 and in most cases such cells were used for the assays.

2.3. Collagen production

Incorporation of radioactive precursor into proteins was measured after labelling subconfluent cells in serum-free medium with varying concentrations of melphalan or its proline analogue for 24 h with the 5-[^{3}H]-proline (5 μCi/ml, 28 Ci/mmol) as described previously [11]. The incorporation into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase according to the method of Peterkofsky et al. [12]. The results are shown as combined values for cell plus medium fractions.

2.4. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al. [13], which is based on the measurement of proline by Chinard's reagent [14]. Briefly, the monolayer was washed three times with 0.15 mol/l NaCl. Cells were collected by scraping and suspended in 0.15 mol/l NaCl, centrifuged at low speed ($200 \times g$) and the supernatant was discarded. The cell pellet (from 6 wells) was suspended in 0.3 ml of 0.05 mol/l Tris–HCl, pH 7.8, and sonicated three times for 10 s at 0 °C. The samples were then centrifuged ($18\,000 \times g$, 30 min) at 4 °C. Supernatant was used for protein determination and then prolidase activity assay. The activation of prolidase requires preincubation with manganese, therefore 0.1 ml of the supernatant was incubated with 0.1 ml of 0.05 mol/l Tris–HCl, pH 7.8 containing 2 mmol/l $MnCl_2$ for 2 h at 37 °C. After the preincubation, the prolidase reaction was initiated by adding 0.1 ml of the preincubated mixture to 0.1 ml of 94 mmol/l Gly-Pro to a final concentration of 47 mmol/l. After the additional incubation for 1 h at 37 °C, the reaction was terminated with 1 ml of 0.45 mol/l 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 mol/l 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 by using the proline standards. Protein concentration was measured by the method of Lowry et al. [15]. Enzyme activity was calculated as nanomoles of released proline per minute per milligram of supernatant protein.

2.5. Cell viability assay

The assay was performed according to the method of Carmichael [16] using MTT. Subconfluent cells, cultured for indicated period of time with various concen-

trations of studies drugs 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. The medium was removed and 1 ml of 0.1 mol/l HCl in absolute isopropanol was added to attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm. Cell viability of MDA-MB 231 cells in the presence of drugs was calculated as a per cent of control cells.

2.6. Mitogenic assay

To examine the effect of studied drugs on MDA-MB 231 cells proliferation, the cells were seeded in 24-well tissue culture dishes at 1×10^5 cells/well with 1 ml of growth medium. After 48 h ($1.8 \perp 0.1 \times 10^5$ cells/well) plates were incubated with varying concentrations of melphalan or its proline analogue and 0.5 μCi of [^{3}H]thymidine for indicated period of time at 37 °C. Cells were rinsed three times with PBS, solubilized with 1 ml of 0.1 mol/l sodium hydroxide containing 1% SDS, scintilation fluid (9 ml) was added and radioactivity incorporation into DNA was measured in scintillation counter.

2.7. Drug accumulation in the cells

Growth medium was removed from breast cancer MDA-MB 231 cells and the monolayer was washed three times with 1 ml of medium. For accumulation studies, 50 μl of the drug (40 mmol/l) was added in dimethyl sulfoxide (1%, final concentration) to 1 ml of fresh medium and the cells were incubated for 4 h. After that, the medium and cells were separated by centrifugation ($200 \times g$, 10 min). The medium was evaporated to dryness in a vacuum and the residue was dissolved in 0.5 ml of methanol. The cells were washed three times with fresh medium, suspended in 0.5 ml of methanol, sonicated and centrifuged at $16\,000 \times g$ for 10 min. The respective samples were submitted to thin layer chromatography on DC-Alufolien Kieselgel 60 F254 (0.2 mm) in methanol. The chromatograms were analysed at UV (254 nm).

2.8. SDS-PAGE

Slab SDS-PAGE was used, according to the method of Laemmli [17]. Samples of cell supernatants (20–50 μg of protein) were incubated for 5 min. at 100 °C in 62.5 mmol/l Tris–HCl, pH 6.8, containing 2.0% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.001% Bromophenol blue. Samples were electrophoresed on a 0.1% SDS-polyacrylamide slab gel (composed of 4% stacking gel and a 7.5% separating gel) at 50 Vper gel for 1.5 h at room temperature in a pH 8.3 running buffer containing 25 mmol/l Tris, 192 mmol/l glycine and 0.1% SDS.

2.9. Western immunoblot analysis

After SDS-PAGE, the gels were allowed to equilibrate for 5 min in 25 mmol/l Tris, 0.2 mol/l glycine in 20% (v/v) methanol. The protein was transfered to 0.2 μm pore-sized nitrocellulose at 100 mA for 1 h by using a LKB 2117 Multiphor II electrophoresis unit according to the method described in the manual accompanying the unit. Nitrocellulose, containing molecular weight standards was stained for 1 min. with 0.2% Panceau S, positions od standards were marked with S&S NC marker (Schleicher and Schuell) and destained by TBS-T solution (20 mmol/l Tris–HCl buffer, pH 7.4, containing 150 mmol/l NaCl and 0.05% Tween 20). Nitrocellulose was blocked with 5% dried milk in TBS-T for 1 h in room temperature, slowly shaking. Then the nitrocellulose was incubated with polyclonal anti-human insulin-like growth factor-I (IGF-IsR) at concentration 1:1000 or monoclonal anti-MAP kinase at concentration 1:5000 in 5% dried milk in TBS-T for 1 h as previously. After the incubation, nitrocellulose was washed with TBS-T (1×15 and 2×10 min) intensively shaking. In order to analyze IGF-IsR, alkaline phosphatase-conjugated antibody against rabbits Fc IgG was added at concentration 1:5000 and in order to analyze MAP kinase, alkaline phosphatase-conjugated antibody against mouse Fc IgG (whole molecule) was added at concentration 1:5000 in TBS-T and incubated for 30 min, slowly shaking. Nitrocellulose was washed with TBS-T (five times for 5 min) and submitted to Sigma-Fast BCIP/NBT reagent.

2.10. Statistical analysis

In all experiments, the mean values for six independent experiments ± standard deviation (SD) were calculated, unless otherwise indicated.

3. Results

Preparation of proline analogue of melphalan, *N*-[[[[(S)-carboxy]pyrrolidin-1yl]carbonyl]methyl]-4-[bis(2-chloroethyl)amino]-2 phenylalanine dilithium salt (Mel-pro) was satisfactorily archieved by standard chemical transformations according to the method described previously [5]. As can be seen from Fig. 2 Mel-pro shows higher (by about 20%) susceptibility to the action of breast cancer MDA-MB 231 cells prolidase, compared to standard prolidase substrate – glycyl-L-proline (Gly-pro) and about sixfold higher susceptibility, compared to another its substrate – glycyl-L-hydroxyproline (Gly-hyp). In the presence of prolidase inhibitor, 5 mM acetylsalicylic acid [18], cell homogenate looses ability to

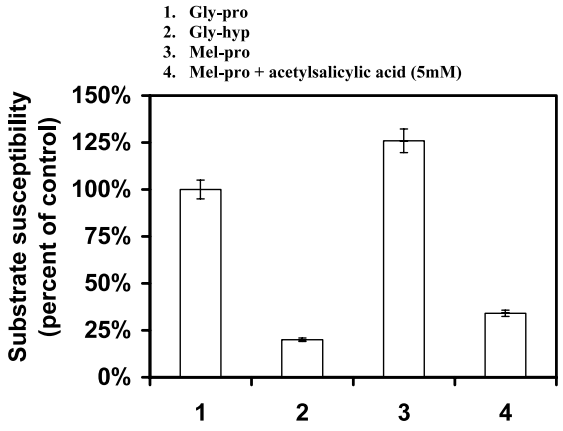

Fig. 2. Susceptibility of proline analogue of melphalan (Mel-Pro), glycyl-L-proline (Gly-Pro), glycyl-L-hydroxyproline (Gly-Hyp) and proline analogue of melphalan (Mel-pro) in the presence of 5 mM acetylsalicylic acid (prolidase inhibitor) to the action of prolidase from MDA-MB 231 cell homogenate. The susceptibility of Gly-Pro to the action of prolidase was considered as 100%. Mean values ± SD from six assays are presented.

convert Mel-pro (Fig. 2, lane 4). It suggests that Mel-pro may represent prolidase-convertable prodrug. Experiment presented on Fig. 3 shows the effect of Mel and Mel-pro on prolidase activity in estrogen-independent breast cancer MDA-MB 231 cells incubated with the drugs for 24 h. It has been found that Mel significantly (by about 30%) inhibited MDA-MB 231 cells prolidase activity, while Mel-pro up-regulated the activity (by about 25–30%), when both were used at 5–25 μM concentrations.

We have compared the transport of Mel and its prodrug through breast cancer MDA-MB 231 cells membrane. The cells were cultured for 4 h in the presence of 2 mM Mel or its proline analogue and after that time the presence of the drugs in the medium and the cells was analysed by thin layer chromatography

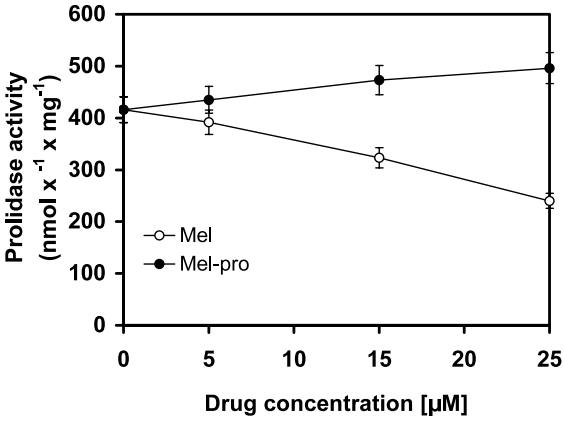

Fig. 3. Prolidase activity in breast cancer MDA-MB 231 cells incubated for 24 h in the presence of different concentration of melphalan (Mel) or proline analogue of melphalan (Mel-pro). Mean values ± SD from three independent experiments done in duplicates are presented.

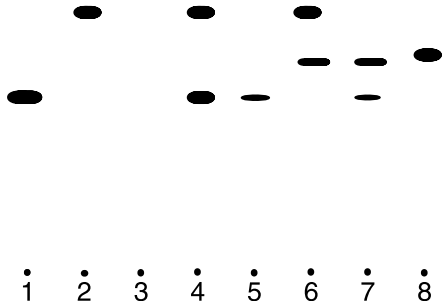

Fig. 4. The comparison between melphalan (Mel) and proline analogue of melphalan (Mel-pro) accumulation in breast cancer MDA-MB 231 cells during 4 h incubation with 2 mM concentrations of the drugs: (1) Mel standard, (2) growth medium, (3) methanol extract of control cells, (4) growth medium from cells treated with Mel, (5) methanol extract of cells treated with Mel, (6) growth medium from cells treated with Mel-pro, (7) methanol extract of cells treated with Mel-pro, (8) Mel-pro standard.

(Fig. 4). In case of Mel, slight amount of the drug was found in the cells (Fig. 4, lane 5), while most of it was present in the medium of the cells (Fig. 4, lane 4). Mel-pro however, was similarly distributed between medium (Fig. 4, lane 6) and cells (Fig. 4, lane 7). In addition, the presence of Mel was found in the cells treated with Mel-pro (Fig. 4, lane 7), suggesting that during the time of incubation some amounts of the Mel-pro were converted into Mel. The experiment suggests that the prodrug was more effectively transported into the cells than the free drug.

Proline analogue of melphalan (Mel-pro) evokes higher cytotoxicity, compared to melphalan (Mel) during 24 h of incubation (Table 1). At 25 μM concentration Mel produced about 40% and Mel-pro about 55% reduction in cell viability, compared to control (Table 1). When the cells were incubated with the drugs for 48 and 72 h, similar decrease in the cell viability was found within wide range of concentrations.

Antimitotic activity of Mel-pro was slightly lower, compared to Mel (Fig. 5A). IC_{50} of Mel for DNA synthesis (measured by thymidine incorporation assay) was found at about 15 μM, while Mel-pro used at this concentration produced about 40% reduction in thymidine incorporation, suggesting lower antimitotic potency of the prodrug compared to the free drug during 24 h incubation. When the cells were incubated with the drugs for 48 and 72 h, similar decrease in DNA synthesis was observed (Fig. 5B).

Cellular hydrolysis of Mel-pro contributes also to increase in proline concentration in cytoplasm. Since imido-bound proline can be reused for collagen synthesis [9], we have compared the effect of Mel and Mel-pro on synthesis of this protein in breast cancer MDA-MB 231 cells. As can be seen from Fig. 6, both drugs inhibited collagen biosynthesis during the course of the experiment. However, the cells incubated with Mel-pro for 24 and 48 h produced much more collagen than the

Table 1
Time course experiment for viability of breast cancer MDA-MB 231 cells incubated with different concentrations of melphalan (Mel) or proline analogue of melphalan (Mel-pro)

Concentration [μM]	Cell viability (% of control)[a]					
	Mel			Mel-pro		
	24 h	48 h	72 h	24 h	48 h	72 h
0	100	100	100	100	100	100
5	76±2	52±4	32±6	60±2	37±6	23±5
10	70±2	46±3	30±5	55±2	33±6	20±4
15	68±2	37±5	23±3	50±2	29±5	18±3
20	67±3	30±6	19±4	47±1	26±4	14±6
25	58±2	26±5	14±2	45±1	21±4	10±4

[a] Mean values±SD from three independent experiments done in duplicates are presented.

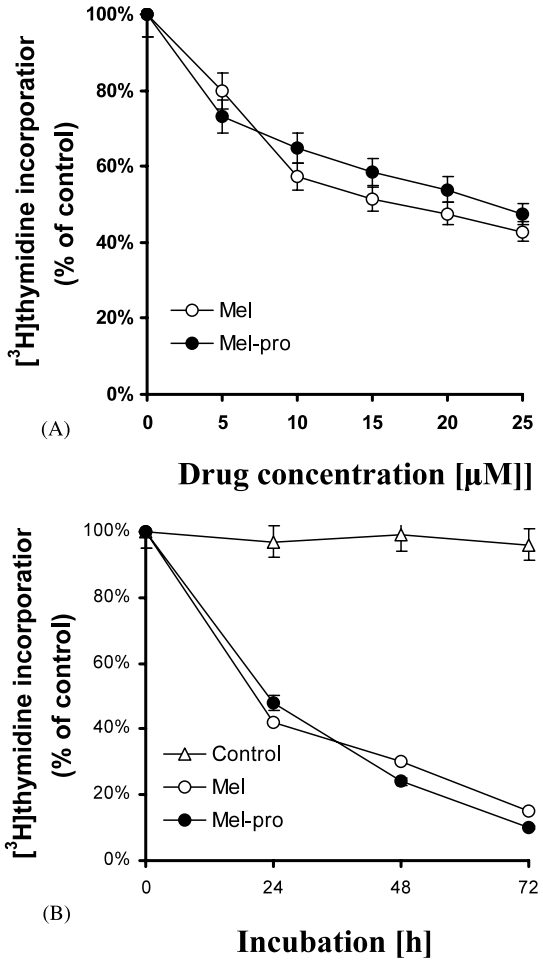

Fig. 5. Concentration-dependent effect (A) and time course experiment (B) for DNA synthesis (measured by [^{3}H]thymidine incorporation assay) in breast cancer MDA-MB 231 cells with melphalan (Mel) or its proline analogue (Mel-pro). (A) The cells were incubated for 24 h with different concentration of the drugs. (B) The cells were incubated for indicated time with 15 μM concentrations of the drugs. The concentrations represent IC_{50} of melphalan for DNA synthesis (see panel A). Mean values±SD from six assays are presented.

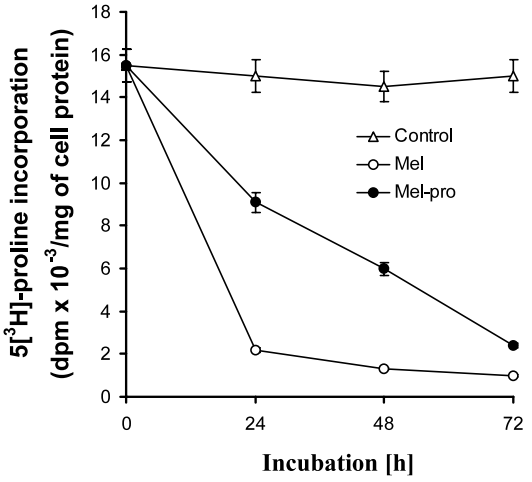

Fig. 6. Time course experiment for collagen biosynthesis (measured by [^{3}H]proline incorporation into protein susceptible to the action of bacterial collagenase) in breast cancer MDA-MB 231 cells in the presence of 15 μM concentration of Mel or Mel-pro. Mean values±SD from three independent experiments done in duplicates are presented.

cells incubated with Mel. After 72 h of incubation both drugs inhibited collagen biosynthesis to the same extent.

It is known that IGF-I is the main stimulator of collagen biosynthesis [11,19,20]. Its actions is regulated by IGF-I receptor expression that induces downstream signaling through MAP kinase pathway [21,22]. In order to evaluate whether Mel-pro and Mel affect the expression of IGF-I receptor and MAP kinase Western immunoblot analysis was performed. Interestingly, Mel did not affect the expression of both IGF-I receptor (Fig. 7A, lane 2) and MAP kinase (Fig. 7B, lane 2), while Mel-pro distinctly reduced the expression of both proteins (Fig. 7A, lane 3 and Fig. 7B, lane 3). It suggests that the inhibitory action of Mel and Mel-pro on collagen biosynthesis in MDA-MB 231 cells may undergo through different pathways.

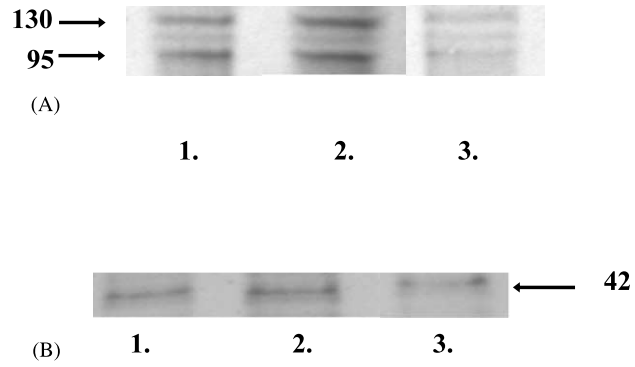

Fig. 7. Western immunoblot analysis of IGF-IsR (A) and MAP kinase (B) from cytosol of oestrogen-independent breast cancer MDA-MB 231 cells for 24 h in the presences of Control (lane 1), Mel 15 μM (lane 2) and Mel-pro 15 μM (lane 3). The same amount of supernatant protein (20 μg) was run in each lane.

4. Discussion

The *N*-acylproline linkage is unique in peptides in that it involves a tertiary amide. Most proteases cannot cleave that bond except specific, cytosolic imidodipeptidase, prolidase [8]. Cytosolic location of this imidodipeptidase suggests that it may serve as a prodrug-converting enzyme. In fact, conjugation of melphalan with proline through imido-bond resulted in the formation of a good substrate for prolidase. It suggests that Mel-pro may serve as a prolidase convertible prodrug.

We have studied several aspects of biological actions of the prodrug on oestrogen-independent breast cancer MDA-MB 231 cells. At first, we found that the prodrug was more effectively transported into the cells than the free drug. The nature of the transport is unknown at present. However, it is known that the uptake of some alkylating agents occur by a passive transport mechanism [23,24]. Moreover, in opposite to Mel, Mel-pro had no inhibitory effect on prolidase activity against its endogenous substrate Gly-pro, in MDA-MB 231 cells. The activity was even slightly increased by about 20% of control. This feature is of importance since the enzyme activity may determine the rate of the prodrug hydrolysis. Proline analogue of melphalan (Mel-pro) evoked slightly higher cytotoxicity for MDA-MB 231 cells, compared to melphalan (Mel). The observed cytotoxicity of the prodrug was probably due to melphalan that was released during hydrolysis of the prodrug by prolidase. The differences in cytotoxicity between the drug can be explained on the basis of higher ratio of Mel-pro transport into the cells, compared to Mel. However, Mel and Mel-pro evoked similar inhibition of DNA synthesis. Lower ability of Mel-pro to inhibit collagen biosynthesis may result (at least in part) from the delivery of proline into the cells (released from Mel-pro by prolidase) the process that provides main substrate for collagen biosynthesis [9]. It may explain the differences in the rate of collagen biosynthesis inhibition by the studied drugs. However, when the cells were submitted to prolonged incubation (72 h) with the prodrug, similar toxicity and effects on collagen biosynthesis were observed, compared to melphalan. Another mechanism of differential collagen biosynthesis regulation by studied drug may exist on the level of IGF-I receptor. This receptor is known to mediate signal that induce collagen gene expression [11,19,20] through MAP kinase pathway [21,22]. In opposite to Mel, Mel-pro induced decrease in expression of IGF-I receptor and MAP kinase. Although, the mechanism of this process in unknown it suggests that this feature of Mel-pro may be of benefit (from the point of its potential application in pharmacotherapy of neoplastic diseases) since IGF-I receptor is also involved in stimulation of cell division [11,19,20].

The presented data postulate that targeting of prolidase as a prodrug-converting enzyme may serve as a potential strategy in pharmacotherapy of neoplastic diseases. The finding that proline analogue of melphalan evokes susceptibility to the action of prolidase create possibility for its application in pharmacotherapy of neoplastic diseases. Previously it has been found that prolidase activity in lung adenocarcinoma is several fold higher, compared to normal lung tissue [10]. Simultaneously, the neoplastic tissues evoke decreased collagen biosynthesis and higher collagenolytic activity [25] which are known to promote metastasis. In such a case the lower ability of proline analogue of melphalan to inhibit collagen biosynthesis, compared to melphalan, would be of benefit. Whether this prodrug evokes a similar activity in other neoplastic cells remains to be explored.

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