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Cytotoxicity and effect on collagen biosynthesis of proline analogue of melphalan as a prolidase-convertible prodrug in cultured human skin fibroblasts

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Abstract

Proline analogue of melphalan (MEL-PRO) was synthesised 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 an imido-bond resulted in formation of a good substrate for prolidase. The susceptibility of MEL-PRO to the action of prolidase was found to be similar, compared to glycyl-proline—the most abundant, endogenous substrate for prolidase and about 6-fold higher compared to its substrate—glycyl-hydroxyproline. We have compared the transport of MEL and its prodrug through cell membrane, their antimitotic activity, cytotoxicity and effect on collagen biosynthesis in cultured, normal human skin fibroblasts. The prodrug was found to be more effectively transported into the cells than the free drug. Moreover, a lower cytotoxicity, antimitotic activity and inhibitory effect on collagen biosynthesis of the prodrug, compared to the free drug were observed after 24 h of incubation. MEL and MEL-PRO at concentrations of 12 μM led to the decrease in cell viability in confluent human skin fibroblasts by about 40 and 20%, respectively, during 24 h of incubation. IC_{50} of MEL for DNA synthesis (measured by thymidine incorporation assay) was found at about 7 μM, while MEL-PRO used at this concentration produced about 35% reduction in thymidine incorporation. Similarly, MEL and MEL-PRO used at 7 μM concentrations inhibited collagen biosynthesis in fibroblasts cultured for 24 h to about 30 and 80% of control values, respectively. However, when the cells were cultured with the drugs for 72 h, similar effects of both drugs on DNA and collagen biosynthesis were observed. The data suggest that MEL-PRO may serve as a prolidase-convertible prodrug that evokes lower cytotoxicity, antimitotic activity, and lower inhibitory effect on collagen biosynthesis in fibroblast cultures, compared to the free drug.

Keywords: Prodrug; Proline analogue of melphalan; Prolidase; Collagen; Human skin fibroblasts

1. Introduction

Melphalan (MEL) belongs to the class of antitumour 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 minimise these side effects, efforts were undertaken to construct prodrugs [2–4]. Our previous results have shown that the proline analogue of melphalan (MEL-PRO) (Fig. 1), conjugated through imido-bond may serve as a substrate for purified pig kidney prolidase [5].

Prolidase [E.C.3.4.13.9] is a ubiquitously distributed cytosolic exopeptidase that cleaves imidodipeptides with C-terminal proline [6,7]. 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 [8]. The presence of prolidase in cytoplasm allows us to suspect that it may be targeted as a MEL-PRO-converting enzyme. The

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specific objective of present studies was to examine the susceptibility of MEL-PRO to the action of fibroblast's prolidase, the ability of MEL and MEL-PRO to penetrate cell membrane, their cytotoxicity, effect on DNA and collagen biosynthesis in normal cultured human skin fibroblasts.

2. Experimental

2.1. Material

Glycyl-proline (GLY-PRO), bacterial collagenase (type VII), trypsin, bovine serum albumin (BSA), pig kidney prolidase, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and MEL 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).

2.2. Fibroblast cultures

Normal human skin fibroblasts, obtained by punch biopsy from an 11 year-old male donor, were maintained in DMEM supplemented with 10% FBS, 2 mmol/l glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37 °C in 5% CO_2 in an incubator. The cells were used between the 12th and 14th passages. The fibroblasts were subcultivated by trypsinisation. Subconfluent cells from Costar Flasks were detached with 0.05% trypsin and 0.02% ethylenodiaminetetraacetic acid (EDTA) in the calcium-free phosphate buffered saline (PBS). For prolidase assay the cells were cultured in six-well plates (Costar). For these experiments, cells were counted in haemocytometers and cultured at 1×10^5 cells per well in 2 ml of growth medium. Cells reached confluency at day 6 after inoculation and in most cases such cells were used for the experiments.

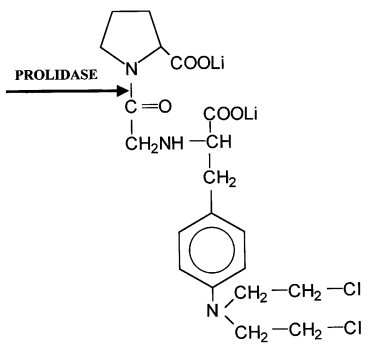

Fig. 1. The chemical structure of MEL-PRO. An arrow indicates the imido-bond susceptible to the action of prolidase.

2.3. Collagen production

Incorporation of a radioactive precursor into proteins was measured after labelling confluent cells in serum-free medium with varying concentrations of MEL or MEL-PRO for 24 h with the 5-[^{3}H]proline (5 μCi/ml, 28 Ci/mmol) as described previously [9]. The incorporation into collagen was determined by digesting proteins with purified *Clostridium histolyticum* collagenase according to the method of Peterkofsky et al. [10]. 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. [11], which is based on the measurement of proline by Chinard's reagent [12]. 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 six 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. [13]. Enzyme activity was calculated as nanomoles of released proline per minute per milligram of supernatant protein.

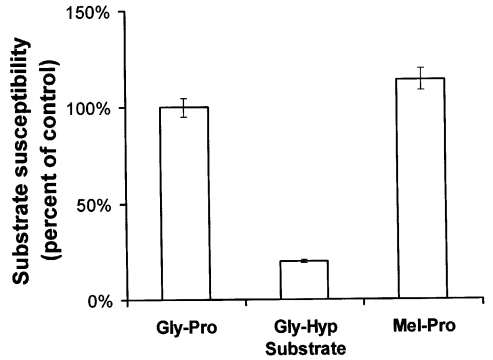

Fig. 2. Susceptibility of MEL-PRO, GLY-PRO and GLY-HYP to the action of prolidase. The susceptibility of GLY-PRO to the action of prolidase was considered as 100%. Mean values ± SD from six assays are presented.

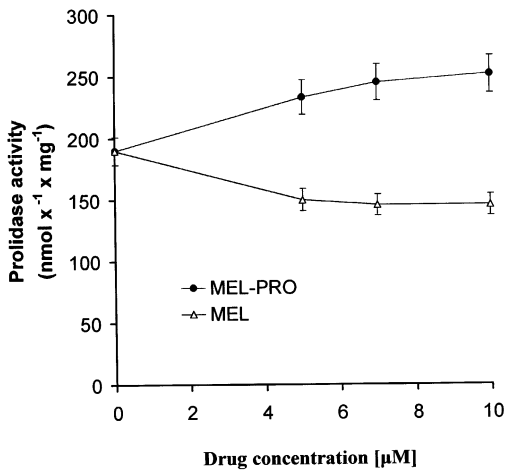

Fig. 3. Prolidase activity in confluent human skin fibroblasts cultured for 24 h in the presence of different concentrations of MEL or MEL-PRO. Mean values ± SD from three independent experiments done in duplicates are presented.

2.5. Cell viability assay

The assay was performed according to the method of Carmichael [14] using MTT. Confluent cells, cultured for the indicated period of time with various concentrations of studies drugs in six-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 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 fibroblasts cultured 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 fibroblast 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 ($1.8 \pm 0.1 \times 10^5$ cells/well) plates were incubated with varying concentrations of MEL or MEL-PRO and 0.5 μCi of [^{3}H]thymidine for the indicated period of time at 37 °C. Cells were rinsed three times with PBS, solubilised 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 scintilation counter.

2.7. Drug accumulation in the cells

Growth medium was removed from confluent human skin fibroblasts and the monolayer was washed three times with 1 ml of medium. For accumulation studies, 50 μl of the drug (0.2 mmol/l) was added in dimethyl sulfoxide (1%, final concentration) to 1 ml of fresh medium and the cells were incubated for the indicated time at 37 °C in CO_2 incubator. 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 F_{254} (0.2 mm) in methanol. The chromatograms were analysed at UV (254 nm).

2.8. Statistical analysis

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

3. Results

Preparation of MEL-PRO was satisfactorily achieved by standard chemical transformations according to the method described previously [5].

As can be seen from Fig. 2, MEL-PRO shows slightly higher susceptibility to the action of fibroblast's prolidase, compared to standard prolidase substrate—glycyl-L-proline (GLY-PRO) and about 6-fold higher susceptibility, compared to its substrate—glycyl-L-hydroxyproline (GLY-HYP). It suggests that MEL-PRO may represent a prolidase-convertible prodrug. Moreover, we have found that MEL-PRO by itself does not significantly affect fibroblast's prolidase activity. The experiment presented in Fig. 3 shows the effect of MEL and MEL-PRO on prolidase activity in confluent human skin fibroblasts cultured for 24 h. It has been found that MEL significantly (by about 20%) inhibited the fibroblast's prolidase activity, while MEL-PRO up-

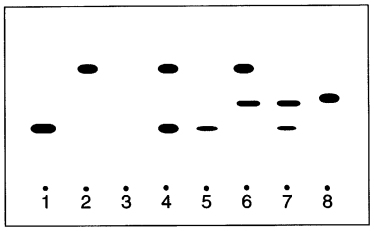

Fig. 4. The comparison between MEL and MEL-PRO accumulation in human skin fibroblasts during 24 h incubation with 10 μM concentrations of the drugs: (1) MEL standard, (2) growth medium, (3) methanol extract of control cells, (4) growth medium from cells treated with MEL, (5) methanol extract of cells treated with MEL, (6) growth medium from cells treated with MEL-PRO, (7) methanol extract of cells treated with MEL-PRO, and (8) MEL-PRO standard.

regulated the activity (by about 20%), when both were used at 5–10 μM concentrations.

We have compared the transport of MEL and its prodrug through the fibroblast's cell membrane. The cells were cultured for 24 h in the presence of 10 μM 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. 4). In the case of MEL, a slight amount of the drug was found in the cells (Fig. 4, line 5), while most of it was present in the medium of the cells (Fig. 4, line 4). MEL-PRO however, was similarly distributed between the medium (Fig. 4, line 6) and the cells (Fig. 4, line 7). In addition, the presence of MEL was found in the cells treated with MEL-PRO (Fig. 4, line 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.

The comparison between effects of the drugs on antimitotic activity and cytotoxicity in cultured, normal human skin fibroblasts was performed. Significantly lower cytotoxicity of the prodrug, compared to the free drug was observed during 24 h of incubation (Table 1). Both compounds at concentrations of 12 μM led to the decrease in cell viability in confluent human skin fibroblasts, however MEL produced about 40% and MEL-PRO only about 20% reduction in cell viability, compared to control (Table 1). When the cells were incubated with the drugs for 48 and 72 h, a similar decrease in the cell viability was found. At 12 μM concentration both drugs produced about 70 and 85% reduction in cell viability after 48 and 72 h of incubation, respectively (Table 1). It suggests that during the prolonged time of incubation MEL-PRO was hydrolysed by prolidase, releasing free MEL that evoked toxic effect on the cells.

Similarly, antimitotic activity of MEL-PRO was significantly lower, compared to MEL (Fig. 5A). IC_{50} of MEL for DNA synthesis (measured by thymidine incorporation assay) was found at about 7 μM, while MEL-PRO used at this concentration produced only about 35% reduction in thymidine incorporation, suggesting lower antimitotic potency of the prodrug compared to the free drug during 24 h incubation. However, when the cells were incubated with the drugs for 48 and 72 h, a similar decrease in DNA synthesis was observed (Fig. 5B).

Cellular hydrolysis of MEL-PRO also contributes to an increase in proline concentration in cytoplasm. Since imido-bound proline can be reused for collagen synthesis [8], we have compared the effect of MEL and MEL-PRO on the synthesis of this protein in fibroblasts cultured for 24, 48 and 72 h. 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 cells incubated with MEL. After 72 h of incubation both drugs inhibited collagen biosynthesis to the same extent.

Table 1
Time course experiment for viability of confluent human skin fibroblasts incubated with different concentrations of MEL or 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
2	97 ± 2	58 ± 4	42 ± 6	98 ± 2	61 ± 6	44 ± 5
5	83 ± 2	45 ± 3	36 ± 5	93 ± 2	50 ± 6	38 ± 4
7	66 ± 2	34 ± 5	22 ± 3	90 ± 2	40 ± 5	25 ± 3
10	60 ± 3	33 ± 6	20 ± 4	86 ± 1	36 ± 4	22 ± 6
12	59 ± 2	30 ± 5	18 ± 2	82 ± 1	32 ± 4	19 ± 4

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

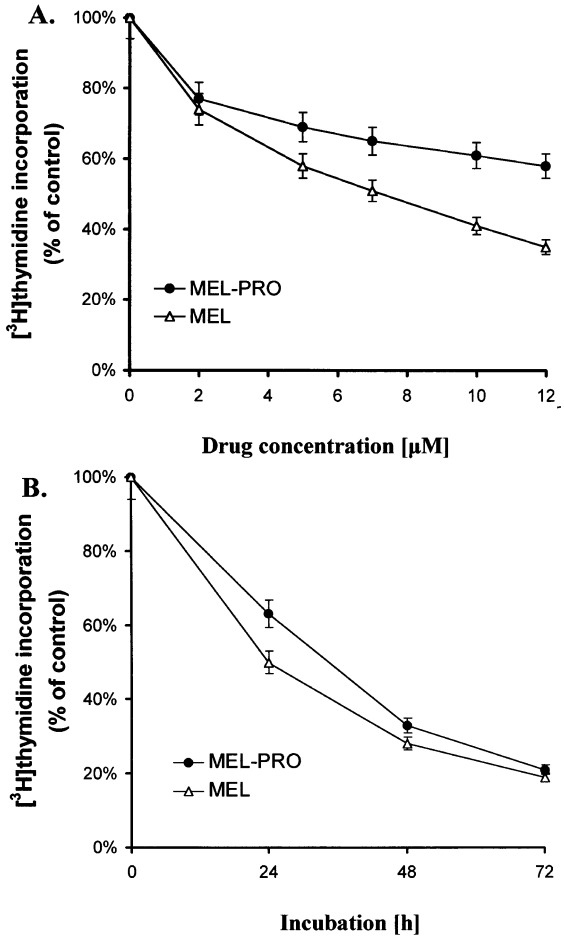

Fig. 5. Concentration-dependent effect (A) and time course experiment (B) for DNA synthesis (measured by [^{3}H]thymidine incorporation assay) in semiconfluent human skin fibroblasts cultured with MEL or MEL-PRO. (A) The cells were incubated for 24 h with different concentrations of the drugs. (B) The cells were incubated for the indicated time with 7 μM concentrations of the drugs. The concentrations represent IC_{50} of MEL for DNA synthesis (see panel A). Mean values ± SD from six assays are presented.

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 [7]. Cytosolic location of this imidodipeptidase suggests that it may serve as a prodrug-converting enzyme. In fact, conjugation of MEL 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 normal cultured human skin fibroblasts. 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 [15,16]. Moreover, in contrast to MEL, MEL-PRO had no inhibitory effect on prolidase activity against endogenous substrate, GLY-PRO, in cultured human skin fibroblasts. 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. It seems that the prodrug is not cytotoxic for fibroblasts. The observed cytotoxicity of the prodrug was probably due to MEL that was released during hydrolysis of the prodrug by prolidase. In fact, we have found that the prodrug was much less cytotoxic than the free drug. The same phenomenon was observed with respect to DNA and collagen biosynthesis. MEL-PRO was also found to evoke a lower inhibitory effect on collagen biosynthesis in the cultured fibroblasts, compared to the free drug. This phenomenon may be due to its lower cytotoxicity, enhanced delivery of MEL-PRO-derived proline into the cells and lack of its effect on prolidase activity inhibition, compared to the free drug. Lower ability of MEL-PRO to inhibit collagen biosynthesis may result (at least in part) from the delivery of proline into the cells, the process that provides main substrate for collagen biosynthesis [8]. 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 DNA and collagen biosynthesis were observed, compared to MEL.

The data presented postulate that targeting of prolidase as a prodrug-converting enzyme may serve as a potential strategy in pharmacotherapy of various diseases. The finding that MEL-PRO evokes susceptibility

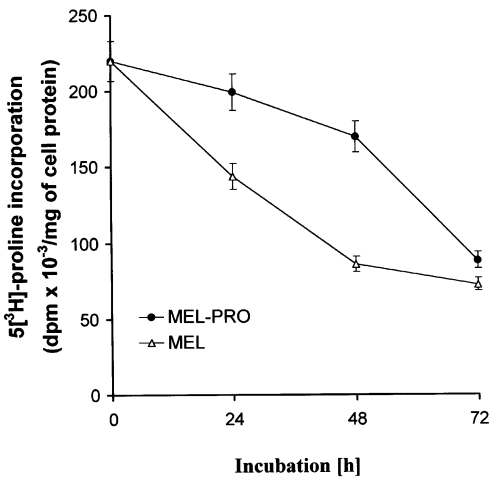

Fig. 6. Time course experiment for collagen biosynthesis (measured by [^{3}H]proline incorporation into protein susceptible to the action of bacterial collagenase) in confluent human skin fibroblasts cultured in the presence of 7 μM concentration of MEL or MEL-PRO. Mean values ± SD from three independent experiments done in duplicates are presented.

to the action of prolidase creates a 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 [17]. Simultaneously, the neoplastic tissues evoke higher collagenolytic activity and decreased collagen biosynthesis [18] which are known to promote metastasis. In such a case the lower ability of MEL-PRO to inhibit collagen biosynthesis, compared to MEL, would be of benefit. Whether this prodrug evokes a similar activity in neoplastic cells remains to be explored.

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