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Decreased cytotoxicity and increased antimitotic activity of a proline analogue of chlorambucil as a prodrug susceptible to the action of fibroblast's prolidase

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We synthesized an proline analogue of chlorambucil (CH-pro) as a prodrug susceptible to the action of ubiquitously distributed, cytosolic imidopeptidase – prolidase [E.C.3.4.13.9]. A conjugation of chlorambucil (CH) with proline through an imido-bond resulted in the formation of a good substrate for prolidase. We have compared several aspects of biological actions of CH and its prodrug in cultured normal human skin fibroblasts. The prodrug was found to be more effectively transported into the cells than the free drug. Moreover, in opposition to CH, CH-pro had no inhibitory effect on fibroblast's prolidase activity against the endogenous substrate, glycyl-L-proline. Lower cytotoxicity and a higher antimitotic activity of the prodrug, compared to the free drug, was observed. CH and CH-pro at concentrations of 25 μM led to a 30% and 10%, decrease in cell viability in confluent human skin fibroblasts. IC_{50} values of CH and CH-pro for DNA synthesis was found to be 30 μM and 7 μM, suggesting higher antimitotic potency of the pro-drug compared to the free drug. CH-pro also evoked lower ability to inhibit collagen biosynthesis in cultured fibroblasts than the free drug. IC_{50} values of CH and CH-pro for collagen biosynthesis were found at about 15 μM and 30 μM, respectively. Targeting of prolidase as a prodrug-converting enzyme may serve as a novel strategy in pharmacotherapy of various diseases, leading to the increase in therapeutic efficacy and reduction in untoward side effects of antineoplastic agents.

1. Introduction

The use of all classes of antimitotic compounds is accompanied by a wide variety of untoward side effects [1]. In order to minimize their side effects, efforts were taken to construct pro-drugs. For instance, daunorubicin (DNR) was conjugated to serum albumin by an amide bond [2]. The conjugate was found to be internalized into the tumour cells (of mice bearing L-1210) and hydrolyzed by intracellular cathepsin, releasing DNR. High tumour-targeting efficiency and decreased toxicity of the conjugate were observed. Some other conjugates showed similar effects [3]. It seems that the formulation of pro-drugs and targeting of intracellular hydrolases as prodrug-converting enzymes may serve as an effective strategy leading to an increase in specificity, therapeutic efficacy and to reduction in untoward side effects of antineoplastic agents.

Chlorambucil (CH) belongs to the class of antitumour agents with an alkylating and cross-linking action on guanine and possibly other bases of deoxyribonucleic acid, which results in arresting cell division [1]. It has a wide spectrum of antineoplastic activity [1]. Preliminary results have shown that a proline analogue of chlorambucil (CH-pro), conjugated through an imido-bond may serve as a substrate for prolidase.

Prolidase [E.C.3.4.13.9] is a cytosolic exopeptidase that cleaves imidodipeptides with C-terminal proline [4, 5]. The primary 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 [6]. The presence of prolidase in cytoplasm allows suspecting that it may be targeted as a pro-drug converting enzyme.

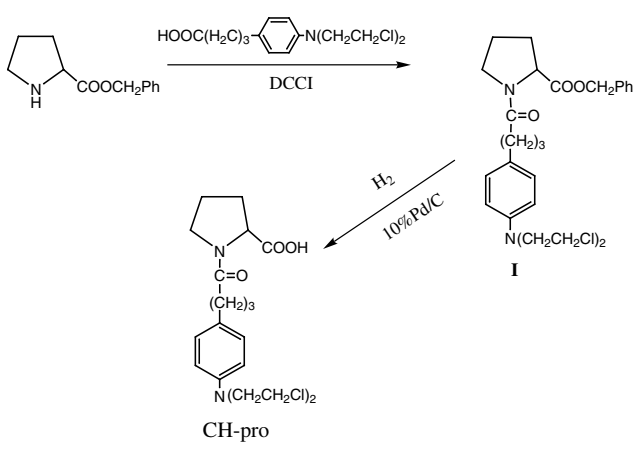
The specific objective of the studies was to examine the susceptibility of CH-pro to the action of prolidase, its ability to penetrate fibroblast's cell membrane, evaluate the pro-drug cytotoxicity, its effect on DNA and collagen biosynthesis and prolidase activity in the cells.

2. Investigations and results

Preparation of the proline analogue of chlorambucil (CH-pro) was satisfactorily achieved by standard chemical transformations according to the reaction sequence shown in the Scheme. Condensation of L-proline benzyl ester with chlorambucil (CH) in dry chloroform in the presence *N,N′*-dicyclohexylcarbodiimide gave the benzyl ester of the proline analogue of chlorambucil (I). The C-terminal benzyl protecting group was removed by catalytic hydrogenation at room temperature and at atmospheric pressure to yield the desired CH-pro confirmed by NMR and elemental analysis.

CH-pro shows susceptibility to the action of fibroblast's prolidase in a range similar to that observed in the case of glycyl-L-hydroxyproline (Fig. 1).

Scheme



Confluent human skin fibroblasts were used to test the effect of CH and CH-pro on prolidase activity and collagen biosynthesis. The rationale for the use of confluent cells in the experiments was that prolidase activity [7] and

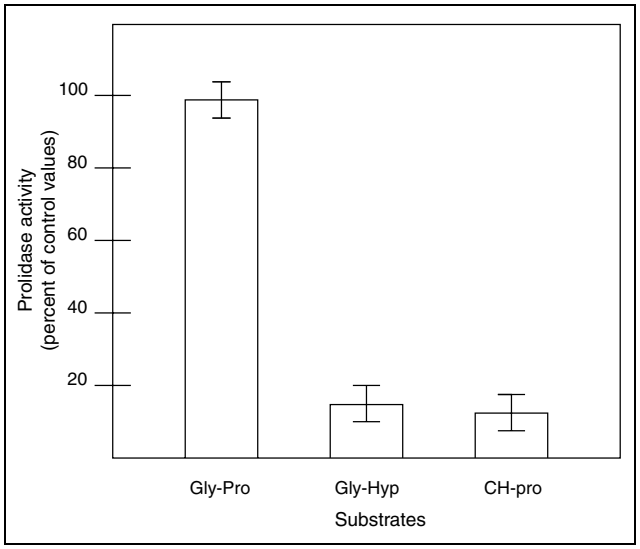


Fig. 1: Susceptibility of proline analogue of chlorambucil (CH-pro), glycyl-L-proline (Gly-Pro) and glycyl-L-hydroxyproline (Gly-Pro) to the action of prolidase. The susceptibility of Gly-Pro to the action prolidase was considered as 100%. Mean values ± SD from 6 assays are presented

collagen biosynthesis [8] are dependent on cell density and rise when cell density increases. Prolidase activity and collagen biosynthesis were measured in fibroblasts treated for 24 h with different concentrations of studied drugs. As it can be seen from Fig. 2, CH at concentration of 50 μM induced a significant decrease in fibroblasts' prolidase activity against gly-L-pro, as a substrate while its proline analogue (CH-pro) had no significant effect on the activity. However, the decrease in prolidase activity due to the treatment of confluent cells with CH was not accompanied by any differences in the amount of the enzyme protein recovered from these cells as shown by western immunoblot analysis (Fig. 3). It may suggest that the inhibitory effect of CH on prolidase activity is a posttranslational event.

Both of the studied drugs inhibited collagen biosynthesis in fibroblasts, in a dose dependent manner, but with different potency (Fig. 4). IC_{50} of CH and CH-pro for collagen biosynthesis were found at about 15 μM and 30 μM, respectively. In both experiments IC_{50} values were calculated on the basis of drug concentrations in the medium of cultured cells.

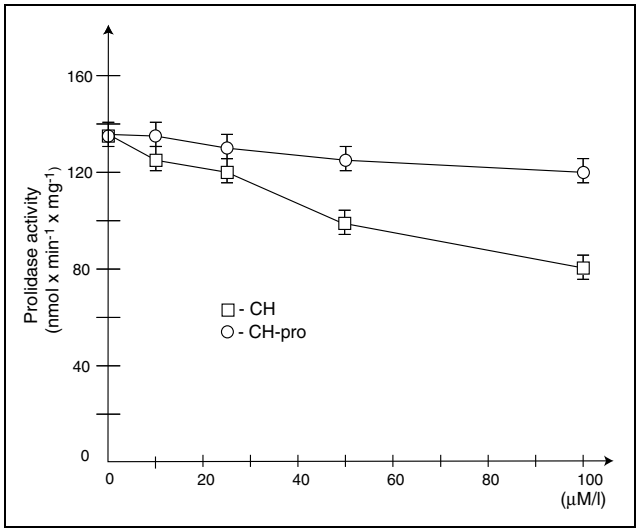


Fig. 2: Prolidase activity in confluent human skin fibroblasts cultured for 24 hours in the presence of different concentration of CH or CH-pro. Mean values from 3 independent experiments done in duplicates are presented

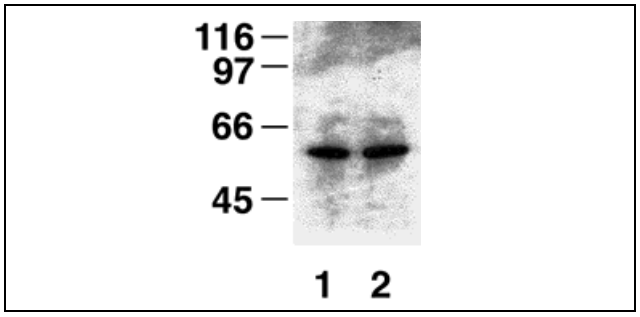


Fig. 3: Western immunoblot analysis of prolidase from cytosol of confluent human skin fibroblasts cultured for 24 hours in the presence of CH (lane 1) and CH-pro (lane 2). The same amount of supernatant protein (20 μg) was run in each lane

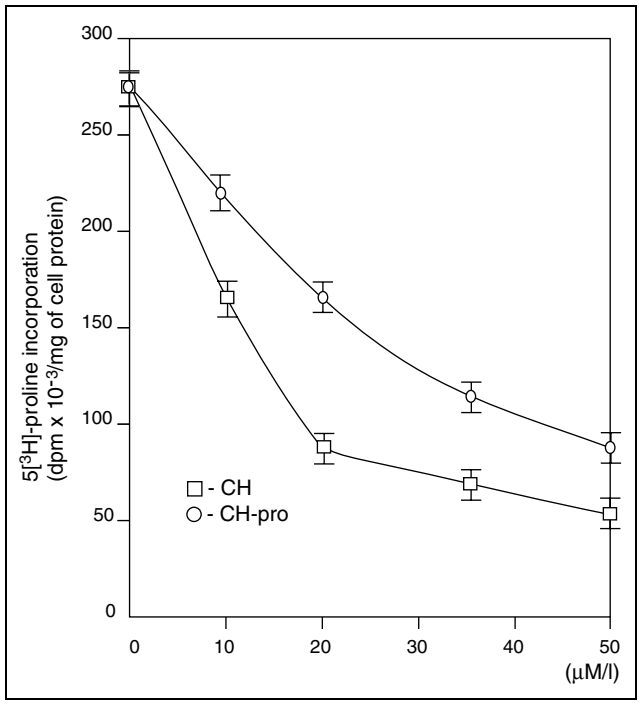


Fig. 4: 5[^{3}H]-Proline incorporation into protein susceptible to the action of bacterial collagenase in confluent human skin fibroblasts cultured for 24 hours in the presence of different concentration of CH or CH-pro. Mean values from 3 independent experiments done in duplicates are presented

Since CH is known as a highly cytotoxic agent, we have compared the viability of fibroblasts cultured with different concentrations of CH and CH-pro. Cell viability was measured by the method of Carmichael et al. [9] using tetrazolinum salt. The viability of cells incubated for 24 h

Table: Viability of confluent human skin fibroblasts treated for 24 h with different concentrations of chlorambucil (CH) or proline analogue of chlorambucil (CH-pro)

Concentration (μM/l)	Viability of cells (% of control) CH	Viability of cells (% of control) CH-pro
0	100	100
10	77 ± 2	95 ± 2
25	70 ± 3	89 ± 2
50	61 ± 2	79 ± 2
75	57 ± 1	75 ± 2
100	48 ± 1	70 ± 2

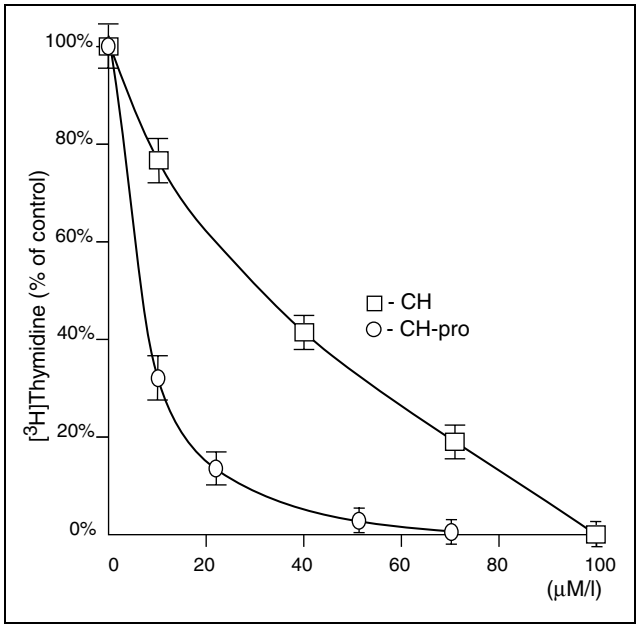


Fig. 5: DNA synthesis in confluent human skin fibroblasts cultured for 24 hours with different concentrations of CH or CH-pro. Mean values from 3 independent experiments done in duplicates are presented

with indicated concentrations of CH and CH-pro is presented in the Table. CH at the concentration of 25 μM (at which collagen synthesis was decreased by over 3-fold) produced only about 30% reduction of cell viability in confluent human skin fibroblasts. CH-pro had significantly lower cell toxicity. About 10% reduction in cell viability was found at 25 μM concentration of the drug.

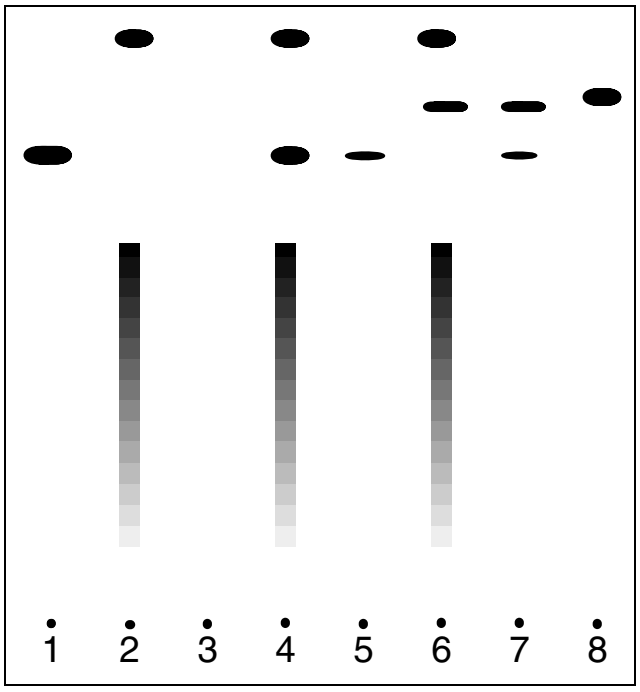


Fig. 6: The comparison between CH and CH-pro accumulation in human skin fibroblasts
1. CH standard
2. serum – free medium
3. methanol extract of control cells
4. serum – free medium from cells treated with CH
5. methanol extract of cells trated with CH
6. serum – free medium from cells trated with CH-pro
7. methanol extract of cells treated with CH-pro
8. CH-pro standard

The effect of CH and CH-pro on DNA synthesis was measured in confluent fibroblasts, treated with the drugs for 24 h. As it can be seen from Fig. 5, IC_{50} of CH and CH-pro for DNA synthesis was found to be about 30 μM and 7 μM, respectively, suggesting higher antimitotic potency of the pro-drug compared to the free drug.

In order to find out if metabolic actions of the studied drugs are due to the differences in their transport into the cells, the experiment presented in Fig. 6 was performed according to the protocol described in the experimental section. It is apparent that the influx of CH-pro (Fig. 6, line 7) into the fibroblasts is greater than that of free drug (Fig. 6, line 5). In the case of cells treated with CH, most of the drug was recovered from medium (Fig. 6, line 4) and only its small portion from the cells (Fig. 6, line 5) after 24 h of incubation. In opposition, slight portion of added CH-pro was found in the medium (Fig. 6, line 6) whereas most of it was recovered from the cells in a form of CH and CH-pro (Fig. 6, line 7). It suggests that during the time of incubation, CH-pro was hydrolyzed by prolidase, yielding the free drug.

3. Discussion

The N-acylproline linkage is unique in peptides in that it involves a tertiary amide. Most proteases cannot cleave that bond except a specific, cytosolic imidodipeptidase, prolidase [5]. Cytosolic location of this imidodipeptidase suggests that it may serve as a prodrug-converting enzyme. Conjugation of chlorambucil with proline through an imido-bond resulted in the formation of a good substrate for prolidase, however, with weak susceptibility. Its susceptibility was comparable to the well known endogenous prolidase substrate, glycyl-L-hydroxyproline [5].

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 CH occurs by a passive transport mechanism [10, 11]. Moreover, in opposition to CH, CH-pro had no inhibitory effect on prolidase activity against the endogenous substrate, gly-L-pro in cultured human skin fibroblasts. This feature is of importance since the prodrug susceptibility to the action of prolidase is rather low. The low ratio of hydrolysis of the prodrug could be of some benefit in view of toxicity and time of action. In fact, we have found that the prodrug was much less cytotoxic than the free drug. On the other hand, CH-pro evoked higher antimitotic potency, compared to the free drug. The lower cytotoxicity and higher antimitotic activity of the prodrug is rather an unusual phenomenon. In general, CH as well as other nitrogen mustards produce DNA crosslinks and monoadducts in a variety of cells [1, 12]. The formation of DNA crosslinks is known to inhibit replication, while DNA monoadducts contribute to the cytotoxic effects of the drugs by inhibiting transcription. Whether CH-pro evokes higher ability to form DNA crosslinks than the free drug remains to be explored.

The low ratio of hydrolysis of the prodrug may also be of benefit since it has been found that some neoplastic tissues evoke much higher prolidase activity than normal tissues [13], suggesting that the prodrug would be preferentially targeted into neoplastic tissues.

CH-pro also evoked a lower inhibitory effect on collagen biosynthesis in the cultured fibroblasts than the free drug. This phenomenon may be due to its lower cytotoxicity,

enhanced delivery of proline into the cells and lack of its effect on prolidase activity inhibition, compared to the free drug.

Lower ability of CH-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. It may explain the differences in the rate of collagen biosynthesis inhibition by the studied drugs.

The presented data postulate that targeting of prolidase as a prodrug-converting enzyme may serve as an effective strategy in pharmacotherapy of neoplastic diseases. We suggest that CH-pro represents such a prodrug that evokes higher antimitotic activity and lower cytotoxicity on cultured human skin fibroblasts than the free drug. Whether this prodrug evokes a similar activity in neoplastic cells remains to be explored.

4. Experimental

4.1. Materials

Chlorambucil, glycyl-proline (Gly-Pro), bacterial collagenase (type VII), trypsin, bovine serum albumin (BSA) and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (USA) as were most other chemicals used. 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 Biologicals Inc. (USA). Polyclonal anti-human prolidase antibody was the gift of Dr. C. R. Scriver (Montreal Children's Hospital, Montreal, Quebec, Canada). Nitrocellulose membrane (0.2 μm) and sodium dodecylsulphate – polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were received from Bio-Rad Laboratories (USA). Horseradish peroxidase labelled anti rabbit immunoglobulin G antibody was purchased from Promega Corp. (USA). L-5[^{3}H]proline (28 Ci/mmol) and ECL-western detection system were received from Amersham (UK). [^{3}H]Thymidine (6.7 Ci/mmol) was the product of NEN (USA).

4.2. Apparatus

Melting points were determined on Buchi 535 apparatus and were uncorrected. ^{1}H NMR (200 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on a Bruker AC 200F spectrometer, using TMS as an internal standard. TLC was performed on Silica Gel 60 F_{254} (Merck) and visualized with UV or with iodine vapour. Chromatographic purification on silica gel (Merck, grade 60, 240–400 mesh) was carried out by flash or gravity methods.

4.3. Synthesis

4.3.1. N-[4-[4-(N,N-bis(2-Chloroethyl)amino)phenyl]butyryl]-L-proline benzyl ester (**I**)

L-Proline benzyl ester hydrochloride (1.5 g, 6.2 mmol) was dissolved in 20 ml of chloroform. Triethylamine (0.86 ml, 6.2 mmol) was added to this stirred solution. The solution was cooled to 0 °C before adding chlorambucil (CH) (1.89 g, 6.2 mmol). This provided a clear solution into which *N,N*'-dicyclohexylcarbodiimide (DCCI) (1.28 g, 6.2 mmol) was added all at once. It was stirred at 0 °C for 2 h, and then warmed to room temperature where it was kept for 24 h. The precipitate of dicyclohexylurea was removed by filtration. Concentration in vacuo gave a colourless solid, which crystallized from methanol to give **I** (2.1 g, 76%).

M.p. 85 °C; ^{1}H NMR ($CDCl_3$): δ 7.23 (m, 5 H, Ph), 7.06 (m, 2 H, Ar), 6.61 (m, 2 H, Ar), 5.22 (m, 2 H, $PhCH_2$), 4.38 (1 H, α-CH proline), 3.48 (2 H, δ-CH proline), 3.64 (t, 4 H, NCH_2), 3.56 (t, 4 H, CH_2Cl), 2.97 (m, 2 H, CH_2CO), 2.64 (t, 2 H, CH_2), 2.21 (q, 2 H, CH_2), 1.82–1.68 (m, 4 H, β- and γ-CH_2 proline); ^{13}C NMR ($CDCl_3$) δ 172.0, 169.0, 139.6, 139.2, 136.9, 136.7, 131.1, 130.7, 128.6, 126.1, 67.3, 50.4, 48.6, 47.1, 42.4, 34.9, 28.9, 27.8, 27.4, 23.5.

4.3.2. N-[4-[4-(N,N-bis(2-Chloroethyl)amino)phenyl]butyryl]-L-proline (CH-pro)

Compound **I** (1.7 g, 3.8 mmol) was dissolved in 21 ml of anh. ethanol, and 0.1 g of 10% Pd/C was added under a blanket of N_2. The mixture was stirred vigorously at room temperature for 6 h, at which time TLC analysis indicated complete removal of the benzyl group. The catalyst was filtered through a bed of Celite and washed with ethanol (4 × 15 ml). Concentration in vacuo gave an oil, which was chromatographed on a silica gel column using methanol/methylene chloride (8 : 2) as the eluant. The desired fractions were combined and concentrated to give CH-pro (1.1 g, 74%).

M.p. 138 °C; ^{1}H NMR ($CDCl_3$) δ 9.58 (s, 1 H, COOH), 7.12 (m, 2 H, Ar), 6.65 (m, 2 H, Ar), 4.36 (m, 1 H, α-CH proline), 3.48 (m, 2 H, δ-CH proline), 3.64 (t, 4 H, NCH_2), 3.56 (t, 4 H, CH_2Cl), 2.92 (m, 2 H, CH_2), 2.64 (t, 2 H, CH_2), 2.21 (q, 2 H, CH_2), 1.82–1.69 (m, 4 H, β- and γ-CH_2 proline); ^{13}C NMR ($CDCl_3$) δ 176.6, 172.0, 136.9, 136.3, 129.3, 128.9, 50.4, 48.7, 47.2, 41.9, 35.0, 28.9, 27.7, 27.4, 23.4.

$C_{19}H_{25}N_2O_3Cl_2$ (401.3)	C%	H%	N%
Calculated:	56.82	6.48	6.98
Found:	56.93	6.29	6.85

4.4. Biochemical studies

4.4.1. 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 μg/ml penicillin, 50 μg/ml streptomycin at 37 °C in a 5% CO_2 in an incubator. The cells were used between the 12th to 14th passages. The fibroblasts were subcultivated by trypsinization. Subconfluent cells from Costar flasks were detached with 0.05% trypsin, 0.02% ethylenediaminetetraacetic acid (EDTA) in the calcium-free phosphate buffered saline (PBS). For the prolidase assay cells were cultured in 6 well plates (Costar). For these experiments cells were counted in hemocytometers and inoculated at 1×10^5 cells per well in 2 ml of growth medium. The cells reached confluency on 6th day after the inoculation and in most cases such cells were used for the experiments. Confluent cells were treated for 24 h with the studied drug in growth medium.

4.4.2. Collagen production

Incorporation of radioactive precursor into proteins was measured after labelling confluent cells in serum-free medium with varying concentrations of CH or CH-pro for 24 h with the 5[^{3}H]-proline (5 μCi/ml, 28 Ci/mmol) as described previously [13]. The incorporation into collagen was determined by digesting proteins with purified *C. histolyticum* collagenase according to the method of Peterkofsky et al. [14]. The results are shown as combined values for cell plus medium fractions.

4.4.3. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al. [15], which is based on the measurement of proline by Chinard's reagent [16]. 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 × 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 3 times for 10 s at 0 °C. The samples were then centrifuged (18,000 × 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 0.1ml of the supernatant was incubated with 0.1ml 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. [17]. Enzyme activity was calculated as nanomoles of released proline per minute per milligram of supernatant protein.

4.4.4. SDS-PAGE

Slab sodium dodecylsulphate – polyacrylamide gel electrophoresis (SDS-PAGE) was used, according to the method of Laemmli [18]. Samples of cell supernatants (50–100 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 containing 20 μg of protein were subjected to electrophoresis on a 0.1% SDS-polyacrylamide slab gel (composed of 4% stacking gel and a 7.5% separating gel) at 50 V per 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. The following Bio-Rad's unstained high molecular weight standards were used: galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa).

4.4.5. Western blot 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 transferred 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; the positions of standards were marked with S&S NC marker (Schleicher and Schuell, Germany) and destained in 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 at room temperature, with slow shaking. Then nitrocellulose was incubated with polyclonal antibody against human prolidase at a concentration of 1 : 3,000 in 5% dried milk in TBS-T for 1 h as previously. After the incubation, nitrocellulose was washed with TBS-T (1×15 min and 2×10 min) with slow shaking. In order to analyse prolidase, second antibody, horse radish peroxidase labelled antibody against rabbit's Fc IgG was added at a concentration of 1 : 5,000 in TBS-T and incubated for 30 min with slow shaking. Then, nitrocellulose was extensively washed with TBS-T (5×10 min) and submitted to ECL western blotting detection system (Amersham, UK) for 1 min. The nitrocellulose was covered with Saran Wrap and exposed to film for 30 s to 1 min.

4.4.6. Cell viability assay

The assay was performed according to the method of Carmichael [9] using 3-(4,5-di-methylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Confluent cells, cultured for 24 h with various concentrations of studied 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 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 the attached cells. Absorbance of converted dye in living cells was measured at a wavelength of 570 nm with background subtraction at 650 nm. Cell viability of fibroblasts cultured in the presence of drugs was calculated as a per cent of control cells.

4.4.7. 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) the plates were incubated with varying concentrations of CH or its CH-pro and 0.5 μCi of [^{3}H]thymidine for 24 h at 37 °C. Cells were rinsed 3 times with PBS, solubilized with 1 ml of 0.1 mol/l sodium hydroxide containing 1% SDS, scintillation fluid (9 ml) was added and radioactivity incorporation into DNA was measured with in scintillation counter.

4.4.8. Drug accumulation in the cells

Growth medium was removed from confluent human skin fibroblasts and the monolayer was washed 3 times with 1 ml of medium. For accumulation studies, 50 μl of the drug (2 mmol/l) was added in dimethyl sulfoxide (1%, final concentration) to 1 ml of fresh medium and the cells were incubated for 24 h at 37 °C in an CO_2 incubator. After that time 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 3 times with fresh medium, suspended in 0.5 ml of methanol, sonicated and centrifuged at $16{,}000 \times$ g for 10 min. Respective samples were submitted to TLC on DC-Alufolien Kieselgel 60 F_{254} (0.2 mm) in methanol. The chromatograms were analysed at UV (254 nm).

4.4.9. Statistical analysis

In all experiments, the mean values for six independent experiments ± standard deviations (SD) were calculated unless otherwise indicated. The results were subjected to statistical analysis using the Students t-test, accepting $p < 0.05$, as significant.

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