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Il Farmaco 55 (2000) 736–741

IL FARMACO

Short Communication

Prolidase-activated prodrug for cancer chemotherapy Cytotoxic activity of proline analogue of chlorambucil in breast cancer MCF-7 cells

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Received 20 March 2000; accepted 12 September 2000

Abstract

Although prolidase [EC 3.4.13.9] is found in normal cells, substantially increased levels are found in some neoplastic tissues. Because prolidase possesses the ability to hydrolyse imido bonds of various low molecular weight compounds coupled to L-proline, we hypothesized that coupling of L-proline through an imido bond to anticancer drugs might create prodrugs which would be locally activated by tumour-associated prolidase and consequently would be less toxic to normal cells that evoke lower prolidase activity. To test this concept we have synthesized a conjugate of chlorambucil–proline (CH–pro) as a possible prodrug. Treatment of this prodrug with prolidase generated the L-proline and the free drug, demonstrating its substrate susceptibility to prolidase. We have compared several aspects of biological actions of chlorambucil (CH) and its prodrug in breast cancer MCF-7 cells. IC_{50} values for chlorambucil and for CH–pro in DNA synthesis were found to be 54 and 16 μM, respectively. CH–pro also exhibited a lesser ability to inhibit collagen biosynthesis in breast cancer MCF-7 cells compared to the free drug. The IC_{50} values for chlorambucil and for CH–pro in collagen biosynthesis were found to be about 32 and 80 μM, respectively. This suggests that the targeting of prolidase may serve as a potential strategy for converting antineoplastic prodrugs.

Keywords: Prolidase; Chlorambucil; Proline analogue of chlorambucil

1. Introduction

A major limitation of chemotherapy for cancer is that anticancer drugs do not distinguish between neoplastic and normal cells. Their toxicity to non-target tissues limits the amount of drug that can be administered, and therefore the chemotherapy may be less effective. One approach to overcome the toxicity of anticancer drugs to normal tissue is to construct a prodrug with lower hydrofobicity and cytotoxicity but preferentially activated in cancer cells.

Prolidase [EC 3.4.13.9] is a cytosolic exopeptidase that cleaves imidodipeptides with C-terminal proline [1,2]. 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 resyn-

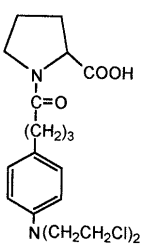

Fig. 1. The chemical structure of proline analogue of chlorambucil (CH–pro).

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0014-827X/00/$ - see front matter
PII: S0014-827X(00)00095-1

thesis, mainly collagen [3]. Prolidase from various sources hydrolyze 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 [4]. However, when a methionyl group or haloacetylprolines replace the amino group in imidodipeptides, good substrates result, suggesting that the α-amino group is not an absolute specificity requirement for prolidase [4]. We hypothesized that conjugation of L-proline through an imido bond to chlorambucil (CH) might create prodrugs which would be locally activated by tumour-associated prolidase and consequently would be less toxic to normal cells. The specific objective of the studies was to examine the susceptibility of CH–pro (Fig. 1) to the action of prolidase and evaluate the prodrug cytotoxicity, its effect on DNA, and collagen biosynthesis and prolidase activity in breast cancer MCF-7 cells.

2. Experimental

2.1. 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). L-5-[^{3}H]proline (28 Ci/mM) and the ECL-western detection system were received from Amersham (UK). [^{3}H]Thymidine (6.7 Ci/mM) was the product of NEN (USA).

2.2. Chemistry

The synthesis of the proline analogue of chlorambucil (CH–pro) was reported previously [5]. Chlorambucil was condensed with L-proline benzyl ester by using the carbodiimide coupling method. The coupling reaction was carried out in chloroform in the presence of a dicyclohexylcarbodiimide. The protecting benzyl group was removed by catalytic hydrogenation at room temperature and atmospheric pressure. The structure of CH–pro was confirmed by elemental and spectral analyses [5].

2.3. MCF-7 cultures

Breast cancer MCF-7 cells were maintained in DMEM supplemented with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Cells were cultured in Costar flasks and subconfluent cells were detached with 0.05% trypsin, 0.02% EDTA in calcium-free phosphate-buffered saline, counted in hemocytometers and cultured at 5×10^5 cells per well of six-well plates (Nunc, Wiesbaden, Germany) in 2 ml of growth medium. Cells reached about 80% of confluence at day 3 after and in most cases such cells were used for the assays.

2.4. Collagen production

The incorporation of a 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/mM) as described previously [6]. Incorporation into collagen was determined by digesting proteins with purified *C. histolyticum* collagenase according to the method of Peterkofsky et al. [7]. Results are shown as combined values for cell plus medium fractions. IC_{50} values, indicating the ligand concentration reducing 5-[^{3}H]proline incorporation by 50%, were obtained by a nonlinear regression analysis of the data points using one phase exponential decay method.

2.5. Determination of prolidase activity

The activity of prolidase was determined according to the method of Myara et al. [8], which is based on the measurement of proline by Chinard's reagent [9]. 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 ($200 \times g$) 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 three times for 10 s at 0°C. Samples were then centrifuged ($16\,000 \times g$, 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 pre-incubation, the prolidase reaction was initiated by adding 0.1 ml of the pre-incubated 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

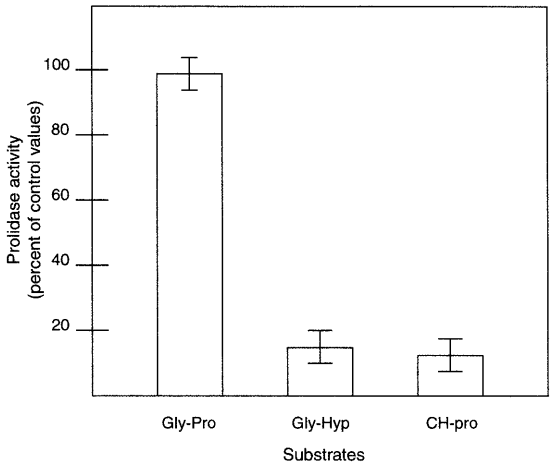

Fig. 2. Susceptibility of proline analogue of chlorambucil (CH–pro), glycyl-L-proline (Gly-Pro) and glycyl-L-hydroxyproline (Gly-Hyp) to the action of prolidase. The susceptibility of Gly-Pro to the action prolidase was considered as 100%. Mean values ± SD from six assays are presented.

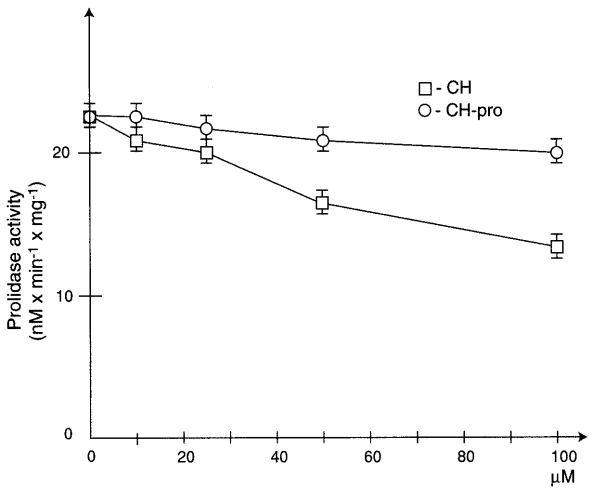

Fig. 3. Prolidase activity in breast cancer MCF-7 cells cultured for 24 h in the presence of different concentration of CH or CH–pro. Mean values from three independent experiments done in duplicates are presented.

reading an absorbance at 515 nm and calculated from calibration curve for proline standards. Protein concentration was measured by the method of Lowry et al. [10]. Enzyme activity was calculated as nanomoles of released proline per minute per milligram of supernatant protein.

2.6. Cell viability assay

The assay was performed according to the method of Carmichael [11] 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 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 a 5% CO_2 incubator. The medium was removed and 1 ml of 0.1 M HCl in absolute isopropanol was added to 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.

2.7. Cytotoxic assay

To examine the effect of studied drugs on fibroblast proliferation, the cells were seeded in twentyfour-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 CH or CH–pro and 0.5 μCi of [^{3}H]thymidine for 24 h at 37°C. Cells were rinsed three times with PBS, solubilized with 1 ml of 0.1 M sodium hydroxide containing 1% SDS, scintillation fluid (9 ml) was added and radioactivity incorporation into DNA was measured in scintillation counter. IC_{50} values, indicating the ligand concentration reducing [^{3}H]thymidine incorporation by 50%, were obtained by a nonlinear regression analysis of the data points using one phase exponential decay method.

2.8. Statistical analysis

In all experiments, the mean values for six independent experiments ± standard deviations (SD) were calculated, unless otherwise indicated. The results were submitted to statistical analysis using the Student's t-test, accepting $p < 0.05$, as significant.

3. Results

Conjugation of chlorambucil with proline through imido bond was described previously [5]. CH–pro was found as a good substrate for prolidase, however with weak susceptibility. Its susceptibility was comparable to the well known endogenous prolidase substrate, glycyl-L-hydroxyproline (Fig. 2).

Breast cancer MCF-7 cells were used to test the effect of CH and CH–pro on prolidase activity and collagen biosynthesis. Prolidase activity and collagen biosynthesis were measured in breast cancer MCF-7 cells treated for 24 h with different concentrations of studied drugs. As can be seen in Fig. 3, CH at a 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. Both studied drugs inhibited collagen biosynthesis in breast cancer MCF-7 cells in a dose

dependent manner, but with different potencies (Fig. 4). IC_{50} values for CH and CH–pro for collagen biosynthesis was found at about 32 and 80 μM, respectively. In both experiments IC_{50} values were calculated on the basis of drug concentrations in medium of MCF-7 cells.

Since CH is known as a highly cytotoxic agent [12], we have compared the viability of MCF-7 cells cultured with different concentrations of CH and CH–pro. Cell viability was measured by the method of Carmichael et al. [11] using tetrazolinum salt. Considering the possibility of the drugs interaction with tetrazolinum salt, the control test (non-specific reaction) was performed with both reagents incubated for 24 h in cell-free system. Values were corrected for non-specific reaction. The viability of cells incubated for 24 h with indicated concentrations of CH and CH–pro is presented in Table 1. As can be seen, CH at a concentration of 25 μM (at which collagen synthesis was decreased by about 40%) produced about 30% reduction of cells viability in breast cancer MCF-7 cells. CH–pro had lower toxicity on the cells. About 15% reduction in cell viability was found at 25 μM concentration of the drug.

The effect of CH and CH–pro on DNA synthesis was measured in breast cancer MCF-7 cells, treated with the drugs for 24 h. As can be seen in Fig. 5, the IC_{50} values for CH and for CH–pro in DNA synthesis were found to be about 54 and 16 μM, respectively.

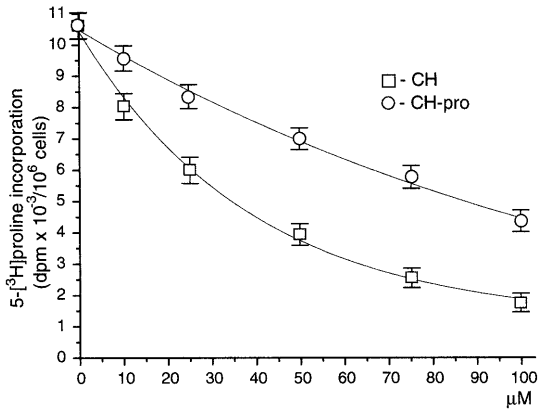

Fig. 4. 5-[^{3}H]proline incorporation into protein susceptible to the action of bacterial collagenase in breast cancer MCF-7 cells cultured for 24 h in the presence of different concentration of CH or CH–pro. Mean values from three independent experiments done in duplicates are presented.

Table 1
Viability of MCF-7 cells treated for 24 h with different concentrations of chlorambucil (CH) or proline analogue of chlorambucil (CH–pro)

Concentration (μM)	Viability of cells (% of control) CH	Viability of cells (% of control) CH–pro
0	100	100
10	82 ± 2	93 ± 2
25	73 ± 3	85 ± 2
50	65 ± 2	80 ± 2
75	56 ± 1	76 ± 2
100	52 ± 1	71 ± 2

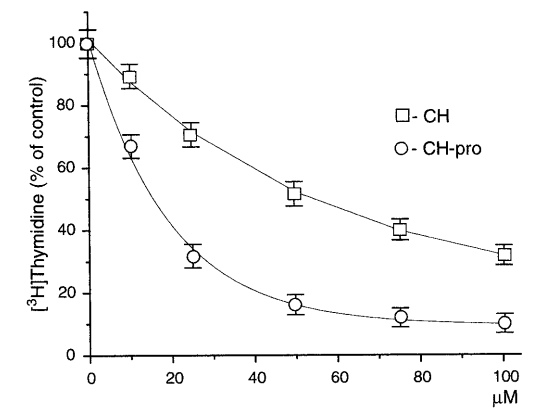

Fig. 5. DNA synthesis in breast cancer MCF-7 cultured for 24 h with different concentrations of CH or CH–pro. Mean values from three independent experiments done in duplicates are presented.

4. Discussion

Chlorambucil is a nitrogen mustard which is used in pharmacotherapy of chronic lymphatic leukemia, lymphomas, and advanced ovarian and breast carcinomas [13]. The clinical application of this anticancer drug is however, limited by its toxic side effects [12].

Proline analogue of chlorambucil (CH–pro) was found as a better therapeutic agent against breast cancer MCF-7 cells than CH. CH–pro in opposite to CH had no inhibitory effect on prolidase activity against endogenous substrate, gly-L-pro in breast cancer MCF-7 cells. 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 general, CH as well other nitrogen mustards produce DNA crosslinks and monoadducts in variety of cells [12,14]. The formation of DNA crosslinks is known to inhibit replication, while DNA monoadducts contribute to the cytotoxic effects of the drugs by inhibiting transcription [14]. Whether CH–pro evokes a greater ability to form DNA crosslinks than the free drug remains to be explored.

CH–pro also produced a lower inhibitory effect on collagen biosynthesis in breast cancer MCF-7 cells, compared to the free drug. This phenomenon may be due to the delivery of proline substrate for collagen biosynthesis into the cells and the lack of effect on prolidase activity inhibition, compared to the free drug.

The primary biological function of prolidase involves the metabolism of collagen degradation products and the recycling of proline from imidodipeptides for collagen synthesis [15–17]. 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 condition to lathyrism, can be attributed to this effect. On the other hand, enhanced liver prolidase activity was found during the fibrotic process [19]. 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 [20], during experimental aging of these cells [21], fibroblasts chemotaxis [22] and cell surface integrin receptor ligation [23]. Thus, as a result of CH-induced inhibition of prolidase activity, collagen biosynthesis may be decreased. The reduced 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 provide main substrate for collagen biosynthesis. It may explain the differences in the rate of collagen biosynthesis inhibition by the studied drugs.

It is known that tumour cells produce enhanced amounts of proteases that degrade collagen and modulate collagen interaction with integrin class of extracellular matrix (ECM) receptors [23]. Since this interaction has been shown to regulate cellular gene expression, differentiation and growth [24,25], and neoplastic transformation is accompanied by aberration of some ECM protein, mainly fibronectin and collagen [26,27], it is likely that disturbances in metabolism of neoplastic cells may in part be due to disregulation of tissue collagen synthesis and deposition. In view of these facts it seems that lower ability of CH–pro compared to CH to inhibit collagen biosynthesis in MCF-7 cells is of benefit.

The data presented postulate that targeting of prolidase as a prodrug-converting enzyme may serve as an effective strategy in pharmacotherapy of neoplastic diseases.

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