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ORIGINAL ARTICLE

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The antiproliferative action of a melphalan hexapeptide with collagenase-cleavable site

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Abstract *Purpose*: The objective of the present study was to examine the relevance of collagenase in the antitumor action of a melphalan peptide (MHP) with a collagenase-cleavable sequence. The question was addressed as to whether collagenase may act as an activator or a target in the antiproliferative mechanism of MHP. *Methods*: Melphalan was inserted into peptides representing the sequence Pro-Gln-Gly-Ile-Ala.Gly of the collagenase-cleavable site in collagens. Changes in growth and collagenase IV activities of HT-1080, HT-29, HT-168, and MCF-7 cell cultures were investigated. *Results*: The present investigations provide data indicating that Pro-Gln-Gly-Ile-Mel-Gly (melphalan hexapeptide, MHP) is a substrate for both bacterial and 72-kDa type IV collagenases and that in this way it can generate Ile-Mel-Gly (melphalan tripeptide, MTP) of higher cytotoxic potency. Indeed, the formation of MTP was detected in the conditioned medium of HT-1080, a collagenase IV-producing human fibrosarcoma. In a comparison of equimolar concentrations of melphalan and its two peptide derivatives (MHP and MTP), superior antiproliferative action of MTP was seen in HT-29, HT-1080, and HT-168 tumor cell cultures. However, the relatively modest cytostatic actions of MHP were increased when bacterial collagenase was added to the cell cultures. After melphalan treatment, reduced levels of both 92 and 72-kDa type IV collagenases were seen in the HT-1080 cell cultures. However, the reduction of collagenase activity and the cell counts did not run parallel in the MTP- or MHP-treated cultures; indeed, collagenase activity related to cell numbers showed an elevated level. *Conclusions*: As the conversion of MHP to the more toxic MTP was detected in the presence of collagenases, it is possible that collagenase-directed activation of prodrugs may be a promising approach for the development of more selective cytostatic drugs against malignant tumors with high collagenase activities.

Key words Prodrugs · Collagenase · Melphalan · HT-1080

Abbreviations *Z* Benzyloxycarbonyl · *Boc* terc.butyloxycarbonyl · *Su* succinimide · *OMe* methyl ester · *OEt* ethyl ester · *ONp* *p*-nitrophenyl ester · *Mel* melphalan · *MTP* isoleucyl-melphalyl-glycine · *MHP* prolyl-glutaminyl-glycyl-isoleucyl-melphalyl-glycine · *MMP* matrix metalloproteinase

Introduction

As important elements of the extracellular matrix, collagens are subject to significant alterations in various pathological lesions, including infiltrative tumor growth and tumor metastasis. The degradation of collagens is governed by a closely related family of enzymes having a similar site of action on the polypeptide chain but showing different specificity for the various types of collagen. Of the collagenases in eukaryotic cells that are designated as matrix metalloproteases, gelatinase A (MMP-2) and gelatinase B (MMP-9), or 72- and 92-kDa type IV collagenase, respectively, show high specificity for collagen IV [2, 3, 5, 7, 13, 25, 26]. Indeed, both MMP-2 and MMP-9 activities have been reported to be remarkably higher in several cancer tissues relative to normal counterparts [19] and are related to the malignant phenotype because degradation of basement membrane components, such as type IV collagen, is necessary for cancer cells to metastasize [13, 17]. MMPs participating in malignant progression are produced either in the tumor cells or in the surrounding mesenchymal cells, being induced by tumor-cell-associated factors [12, 21]. Consequently, collagenase may be

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utilized in chemotherapeutic drug design either as a potential target or as an activator for agents in the inactive (latent) form [10, 14].

In the present study the question was addressed as to whether linkage of melphalan to a collagenase-cleavable peptide fragment would change its cytotoxic and collagenase-inhibitory action.

Since all vertebrate collagenases cleave uniformly the Gly-Ile peptide bond in collagens, in this report the terms collagenases and matrix metalloproteinases are equally used. Nevertheless, in evaluation of the cleavage of the Gly-Ile peptide bond it is more relevant to refer to collagenase activity, whereas in the gelatin zymography technique the specific type of collagenase IV (i.e., MMP-2 or MMP-9) has to be named.

The specificity of the amino acids around the scissile bond has been studied by Van Wart et al. [18, 24] with the aid of peptide substrates. It has been reported that the minimal chain length required by the enzymes is a hexapeptide, and substrate-specificity studies with type IV collagenases reveal that the amino acid in the P'_2 position (i.e., alanine) can be modified relatively freely without decreasing the enzymatic activity [17]. Since it was especially favorable to replace alanine with phenylalanine, in our laboratory, melphalan (i.e., phenylalanine mustard) was inserted into the P'_2 position of the hexapeptide collagen sequence.

In our previous investigations with melphalan-containing peptides we observed that the longer the peptide carrier, the less toxic the congener; thus, we assumed that a hexapeptide-Mel-conjugate (MHP) would show less cytotoxicity and would be activated in the presence of collagenase [11, 23]. Conceivably, the activation involving the release of the more toxic tripeptide-Mel conjugate (MTP) would make the action of MHP more selective against tumors with high collagenase activity (Fig. 1).

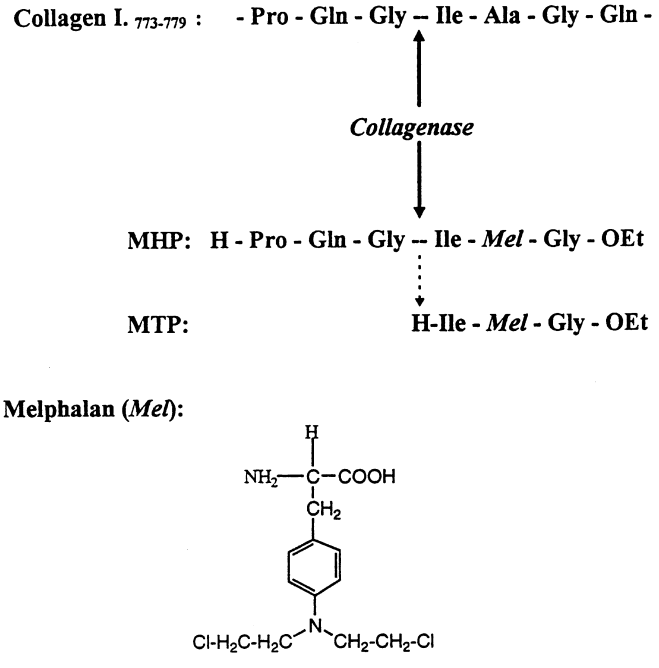


Fig. 1 The prodrug concept of MHP

Materials and methods

Bacterial collagenase type IV from *Clostridium hystolyticum* was purchased from Sigma Chemical Co. Recombinant human 72-kDa type IV collagenase was a generous gift from Dr. U. Bergmann, Oulu University, Finland. Merck Kieselgel-precoated sheets number 5553 were used for *thin-layer chromatography* (TLC), and Merck Kieselgel 60 sheets number 10832 were used for column chromatography. High-performance liquid chromatography (HPLC) was performed on a Knauer instrument.

For, MTP and MHP were synthesized in solution according to conventional peptide-synthesis procedures [1] except that precautions owing to the presence of melphalan in the sequence were taken into account as described elsewhere [22]. Purifications of the end products were carried out by silica-gel column chromatography. The steps of the synthesis are illustrated in Fig. 2.

Chemical characteristics of MTP and MHP

H-Ile-Mel-Gly-OEt x 2HCl

R_f:0.42 (chloroform:methanol = 10:1). Analytical calculations were done for $C_{23}H_{38}N_4O_4Cl_4$ (576.39): C 47.92, H 6.64, N 9.72, and Cl 24.60%; we found C 47.73, H 6.74, N 9.34, and Cl 23.97%.

H-Pro-Gln-Gly-Ile-Mel-Gly-OEt x 2HCl

R_f:0.64 (ethyl acetate: pyridine: acetic acid: water = 30:20:6:11). Analytical calculations were done for $C_{35}H_{56}N_8O_8Cl_4$ (858.69) and Cl 6.51; we found Cl 17.26. Amino acid analysis (after 24 h of hydrolysis) at 105 °C, 6 *N* HCl revealed Pro 1.03, Glu 1.08, Gly 2.12, and Ile 0.85.

Decomposition studies

Hydrolysis of the mustard group of peptides containing melphalan, MTP, and MHP

Solvolysis was estimated upon incubation of MTP at 3 mg/ml or of MHP at 1.5 mg/ml in borate buffer (0.05 *M* boric acid, 0.2 *M* NaCl, 0.005 *M* $CaCl_2$, pH 7.6 adjusted with NaOH solution) at 37 °C. At intervals, 20-µg samples were taken and analyzed by reversed-phase HPLC (Beckmann Ultrasphere ODS 5 µm,

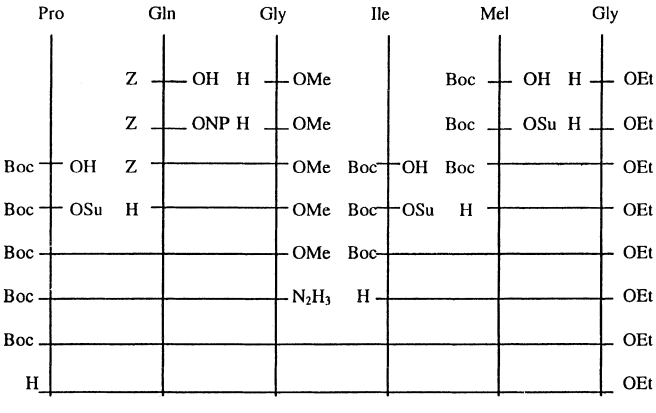


Fig. 2 The synthesis scheme of the peptides containing melphalan (MTP, MHP)

4.6 × 150 mm column) using acetonitrile gradient (0 to 60% over 25 min) in 0.1% trifluoroacetic acid. The amounts of the substrates measured at 220 nm were related to the area under the peak of the zero-time sample.

Conversion of MHP in the presence of the C. histolyticum enzyme

200-µl solution of MHP (3 mg in 1 ml buffer) was incubated at 37 °C with 1.5 µl enzyme solution (1 mg in 1 ml buffer) and samples were analyzed as described above.

Conversion of MHP in the presence of the 72-kDa type IV collagenase

In all, 100 µl 0.1 µ*M* enzyme solution was activated by a 1-h incubation at 37 °C with 100 µl 1 m*M* phenylmercuric acetate solution in borate buffer. Then, 200 µl substrate solution was added to the activated enzyme solution, the incubation was continued, and samples were taken and analyzed as described above.

Conversion of MHP in the conditioned medium of HT-1080 cell culture

MHP at a concentration of 10 µg/ml was incubated in the conditioned medium of HT-1080 cell culture in the absence or presence of 1 m*M* phenanthroline for 24 h at 37 °C. After 24 h, 100-µl samples were taken for HPLC analysis using acetonitrile gradient in 0.1% trifluoroacetic acid. The peaks related to MHP or MTP were identified with the aid of appropriate internal controls, i.e., MHP or MTP incubated in buffer A solution for 24 h at 37 °C.

Measurements of antiproliferative activity

In the present experiments, cell cultures with various MMP-producing capacity were applied. HT-1080 produced both MMP-9 and MMP-2, but only MMP-2 occurred in the conditioned medium of HT-168/M1 cell culture, whereas much less was found in the HT-29 culture and none was detected in the MCF-7 culture (Fig. 3).

Human fibrosarcoma cells (HT-1080), melanoma cells (HT-168/M1), colon adenocarcinoma cells (HT-29), and mammary adenocarcinoma cells (MCF-7) were cultured in RPMI 1640 medium containing 10% fetal calf serum and gentamycin at 50 µg/ml. The cultures were incubated in a humidified atmosphere containing 5% CO_2. Test compounds were dissolved in ethanol containing 1% hydrochloric acid and were diluted with the culture medium to the molar concentrations indicated below in Results. The solvents at the highest concentration (0.00074% HCl and 0.094% ethanol) used for the test compounds at 10^{-4} *M* concentration showed less than 10% growth inhibition, but none was seen at in the concentrations used for 10^{-5} and 10^{-6} *M* concentrations of the test compounds. The cell cultures grown in 96-well plates were treated with the test compounds or with only diluted solvents at 24 h after seeding for 24, 48, or 72 h. For assessment of cell proliferation, both the tetrazolium dye (MTT) test and the sulforhodamine B (SRB) test were used as reported elsewhere [15].

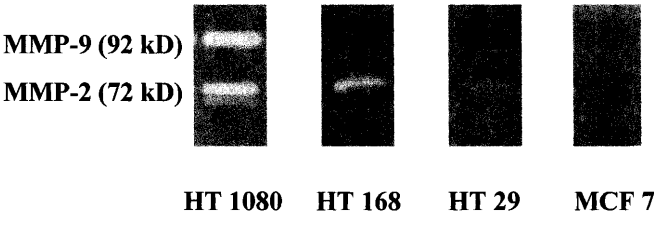


Fig. 3 MMP activities in the medium of HT-1080, HT-168/M1, HT-29, and MCF-7 cell cultures. Enzyme activities were measured as described in Materials and methods

Assay for collagenase activity

HT-1080 cell cultures were treated with the test compounds when grown in serum-containing medium for 18 h, then the medium was replaced and fresh medium without serum was added. At the end of the subsequent 24-h incubation period the activity and the molecular weight of the collagenases were determined using gelatin zymography [9, 27]. In brief, the conditioned medium (20 µl/lane) was separated by electrophoresis at 25 mA for 5 h in an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel containing gelatin at 0.3 mg/ml. After electrophoresis the gel was washed in 2.5% Triton-X-100 for 30 min to remove the SDS and was incubated for 20 h at 37 °C in 50 m*M* TRIS-HCl/5 m*M* $CaCl_2$/1 *M* $ZnCl_2$ (pH 7.6) buffer containing 0.2% NaN_3. Areas of gelatinolytic activity were visualized by staining of the gel with 0.1% Coomassie Brilliant Blue in 50% methanol followed by destaining in 10% methanol + 10% acetic acid. Gelatinolytic enzymes were detected as transparent bands on the blue background of the Coomassie-blue-stained slab gel. The collagenase activity was quantitated by densitometric measurement of these bands using bacterial collagenase as the standard.

Statistical analysis

The dose and effect relationship was determined according to the computer program described by Chou and Chou [6].

Results

Decomposition of the peptides containing melphalan

Peptides containing melphalan decompose in buffer solution through solvolytic (hydrolytic) reactions characteristic of alkylating agents with a mustard group. A similar decomposition rate (60–70% after 3 h) was found in the case of both MHP and MTP in borate buffer at 37 °C as monitored by HPLC analysis (Fig. 4).

To obtain more relevant results for the interpretation of the cytotoxic effect of MHP and MTP, we also measured the alkylating capacity using the γ-(4-nitrobenzyl)-pyridine assay, which is used for determining the alkylating ability of cytotoxic agents [8]. The results of these investigations (data not shown) are in good correlation with the above-mentioned HPLC data, indicating no difference in the chemical reactivity of melphalan and its peptide derivatives.

The enzymatic cleavage of MHP was examined using *Clostridium hystolyticum* bacterial collagenase or 72-kDa type IV collagenase, and the cleavage products were identified by HPLC analysis. In the presence of the 72-kDa enzyme the Gly-Ile bond was hydrolyzed and MTP was identified, as the conclusions of previous studies had predicted [4, 17]. Although the reported substrate specificity of the bacterial enzyme [20] would have suggested the cleavage before glycine, in our case the same cleavage products were formed in the presence of both the bacterial and the vertebrate collagenases. This finding in agreement with our previous results, when we observed that under certain conditions the bacterial enzyme hydrolyzes the peptide bond not before but after glycine [4]. The decompositions of MHP in the

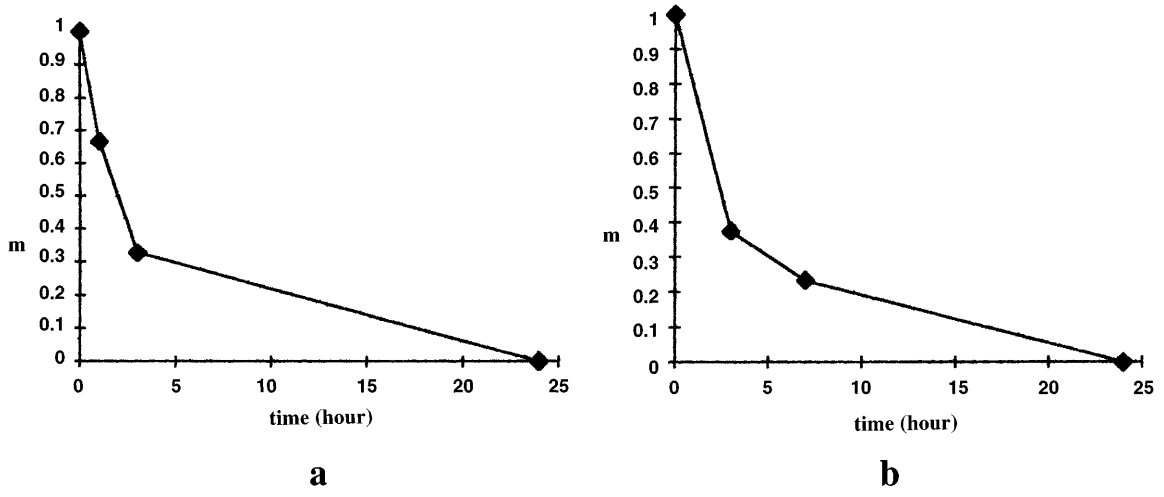


Fig. 4a,b Hydrolysis of the bis(2-chloroethyl)amino group of **a** Pro-Gln-Gly-Ile-Mel-Gly (MHP) and **b** Ile-Mel-Gly (MTP) in borate buffer at 37 °C, monitored by determination of the undecomposed melphalan-peptide conjugate as described in Materials and methods

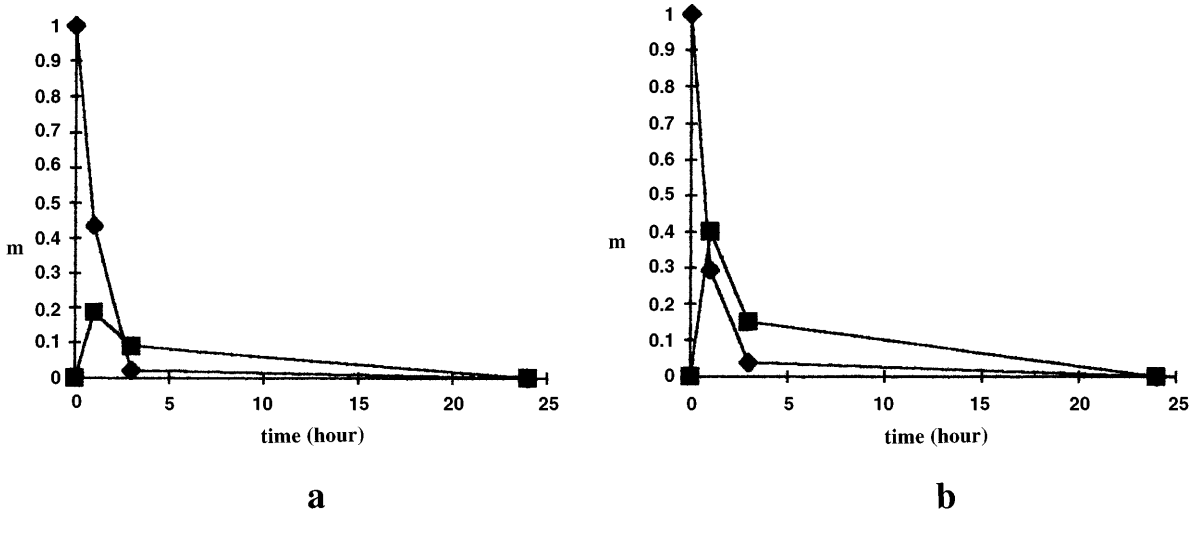
presence of enzymes are shown in Fig. 5, where the data on the formation and decomposition of MTP are also illustrated. This graph shows the overall decomposition derived from the enzymatic cleavage of the peptide bond and the hydrolysis of the mustard group as well.

Antiproliferative actions

It can be seen in Fig. 6, comparing equimolar concentrations of melphalan and its two peptide conjugates in HT-1080 cell culture, that MTP has a remarkably higher and MHP, a much lower antiproliferative potency than does melphalan. These data indicate the following IC_{50} values: MTP 0.6 μM, melphalan 3.0 μM, and MHP 20.5 μM. Similarly, in the HT-168/M1 cell cultures, MTP again induced more extensive inhibition (IC_{50} 0.7 μM) of tumor cell proliferation than did MHP; indeed, the latter test compound yielded 50% inhibitory action only at concentrations above 100 μM.

In the next experiment the question was addressed as to whether the presence of collagen in the culture could modify the differences in the antiproliferative potency of the melphalan and its peptide derivatives. To this end, HT-29 cells were seeded in petri dishes coated with or without collagen. It is noteworthy that in this case, melphalan was much less active than MHP if the HT-29 cells were seeded on plastic. However, these cells grown on collagen were much more sensitive to melphalan and MTP, but not to MHP (Table 1). This suggests that melphalan and MTP may be more effective in cells grown on collagen relative to plastic, whereas collagen offered the cells some protection against MHP.

Fig. 5a,b Decomposition [enzymatic hydrolysis of the peptide bonds and solvolysis of the bis(2-chloroethyl)amino group] of Pro-Gln-Gly-Ile-Mel-Gly with the simultaneous formation and subsequent decomposition of Ile-Mel-Gly in borate buffer at 37 °C in the presence of **a** MMP-2 or **b** bacterial collagenase



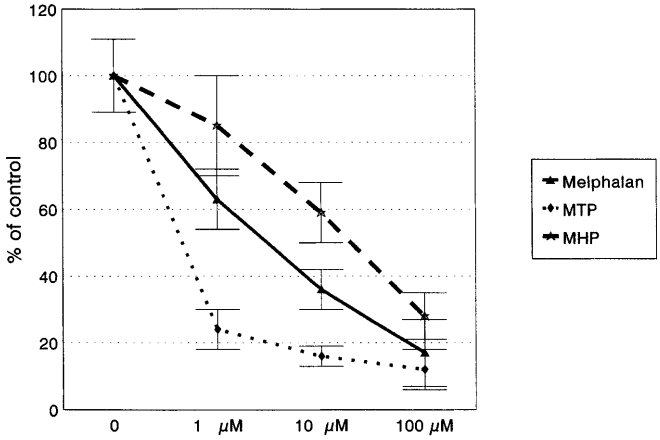


Fig. 6 Antiproliferative action of melphalan-peptide conjugates in HT-1080 cell cultures

Possible role of collagenases in MHP action

As MHP contains a collagenase-cleavable peptide, it was of interest to study whether there was some indication for a possible involvement of collagenases in the biological action of this compound. To this end, first the changes in collagenase activity after treatment with melphalan and its peptide conjugates were determined. Melphalan treatment resulted in a reduction in MMP-2 and MMP-9 activities, which more or less showed responses similar to the changes in cell growth. More interestingly, however, the reductions in collagenase activity and cell growth did not run parallel after treatment with the melphalan peptides (Table 2). Indeed, the collagenase activity in the cells was higher as compared with the untreated cells, especially after treatment with MHP.

In the next experiments the modulation of MHP action by exogenous collagenase was investigated. To test this possibility, bacterial collagenase (which, according to our decomposition studies, cleaves the same peptide bond in MHP as MMP-2) was applied simultaneously with MHP to HT-29, MCF-7, or HT-168/M1 cell cultures. Since in HT-29 and MCF-7 cell culture, 10 μ*M* MHP showed no cell proliferation-inhibitory action and the HT-168/M1 cells were affected only to 25%, it is interesting that in the presence of bacterial collagenase the same MHP concentration became very effective. This may indicate that bacterial collagenase either generated the formation of the more cytotoxic MTP from MHP or, through another unclarified mechanism, enhanced the antiproliferative action of MHP, although by itself it did not reduce cell proliferation at concentrations below 25 μg/ml (Table 3).

To collect further data for the putative prodrug function of MHP we attempted to identify the presence of MTP in the conditioned medium of HT-1080 cell culture after a 24-h treatment with MHP. In this experiment, not only the formation of MTP but also the inhibition of the MHP conversion to MTP by phenanthroline, an inactivating agent of metalloproteases, was observed (Fig. 7).

Table 1 IC_{50} values of melphalan and related peptide derivatives in HT-29 cell cultures

	Plastic	Type I–III collagen
Melphalan	55.1 μ*M*	31.8 μ*M*
MTP	5.0 μ*M*	1.5 μ*M*
MHP	28.0 μ*M*	40.0 μ*M*

Table 3 Modulation of MHP action by collagenase in cell culture[a]

Cell lines	MHP 10 μ*M*	Collagenase[b] 5 μg/ml	MHP + collagenase
HT 168	74.2 ± 3.8	99.8 ± 11.9	4.8 ± 1.4
MCF 7	98.7 ± 11.1	109.8 ± 7.6	62.7 ± 8.1
HT 29	99.5 ± 8.0	112.5 ± 12.1	36.7 ± 3.2

[a] Growth of cell cultures was estimated 48 h after treatment using the SRB test as indicated in Materials and methods. Data are expressed as a percentage of control values
[b] Collagenase from *Clostridium histolyticum*

Discussion

To test the hypothesis that high levels of collagenase activity in malignant tumors could be utilized in an effort to achieve more selective antitumor action, melphalan was conjugated to a peptide representing the collagenase-cleavable amino acid sequence of collagens. To this end, Pro-Gln-Gly-Ile-Mel-Gly (MHP) was investigated as a potential prodrug of Ile-Mel-Gly (MTP). As it has previously been shown that the cytostatic action of melphalan peptides depends on fragment size [11, 22], it has been assumed that the conversion of

Table 2 Comparison of changes in cell proliferation and metalloprotease activities in HT-1080 cell cultures treated with melphalan or its peptide derivatives[a]

		Melphalan		MTP		MHP	
		1 μ*M*	10 μ*M*	1 μ*M*	10 μ*M*	1 μ*M*	10 μ*M*
Proliferation		65	38	25	14	85	60
MMP-2 activities	Cell	100	56	146	111	91	105
	Medium	65	50	55	43	135	65
MMP-9 activities	Cell	42	31	53	63	155	170
	Medium	60	42	51	35	128	102

[a] All data are expressed as a percentage of control values. Antiproliferative and MMP inhibitory actions were determined as described in Materials and methods

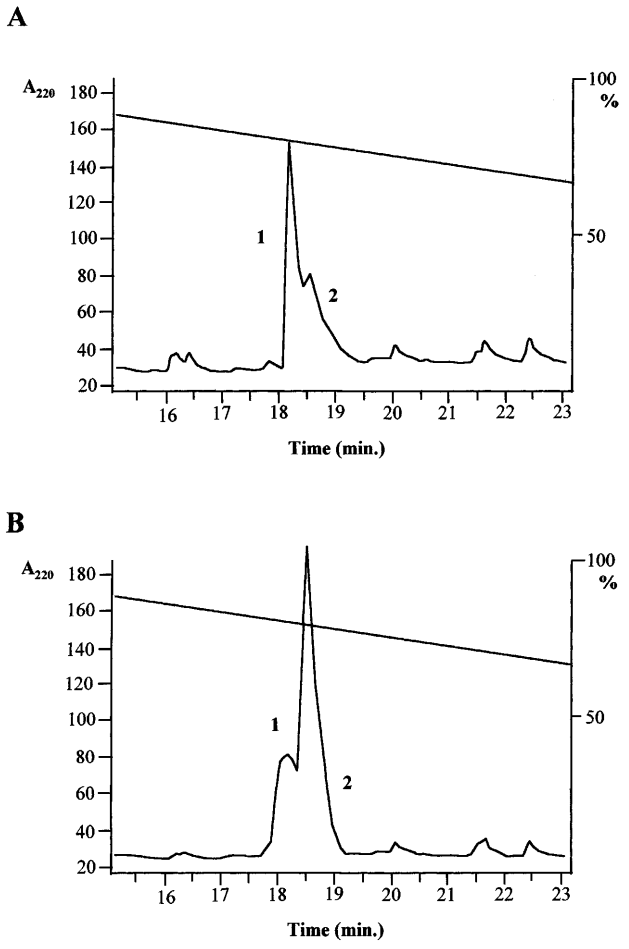


Fig. 7A,B Conversion of melphalan-hexapeptide conjugate (MHP) to melphalan tripeptide (MTP) in HT-1080 cell cultures. MHP was incubated at 10 μg/ml in the conditioned medium collected from HT-1080 cell culture for 24 h in the **A** absence or **B** presence of 1 m*M* phenanthroline as an inhibitor of collagenase activity. The perchloric acid extracts of the medium were analyzed by HPLC as described in Materials and methods. Peak *1* corresponds to MTP and peak *2*, to MHP, whereby the peptides contained the hydrolyzed mustard group after 24 h incubation as described in Materials and methods

MHP to the more toxic MTP directed by collagenases would offer higher therapeutic efficacy in animals bearing tumors with high collagenase activity. In the present study the question was addressed as to whether MHP would show any indication of collagenase-dependent antiproliferative action in tissue culture. In a comparison of the antiproliferative actions of melphalan, MHP, and MTP the superior action of MTP on an equimolar base was obvious in all tissue cultures used in this study. Since the alkylating-capacity values recorded for melphalan and its peptide derivatives, MHP and MTP, showed no difference, the possibility that any difference in their biological effectivity could be due to variations in chemical reactivities may be excluded.

In evaluations of the difference in the extent of cytotoxic action between MTP and MHP, several pharmacobiochemical factors should obviously be taken into account, such as cellular uptake, metabolism, damage to the target, and repair. Noteworthy, however, is that MHP generated MTP in the presence of either bacterial collagenase or MMP-2. The putative role of collagenase in the biological action of MHP gained some support in the experiment where MHP was tested after the addition of collagenase in cell cultures not producing collagenase. Since the very moderately acting MHP could be rendered more toxic in tissue culture by collagenase, it is conceivable that this compound may be regarded as a prodrug. This assumption gained further support in the experiment concluding that the formation of MTP from MHP could be abrogated in the presence of phenanthroline, an inhibitor of collagenase activity.

Since in the presence of collagen a greater production of collagenase has been reported [16], it may be of paramount interest that treatment with the two peptide derivatives of melphalan, MTP and, especially, MHP, resulted in an elevation of collagenase activity in the surviving cell populations. In this respect, these compounds showed differences from the biological activity of melphalan. The significant rise in MMP activities seen in cells treated with MHP may raise the possibility that this prodrug compound could induce activating enzyme for itself. If this notion can be proved in a forthcoming experiment, then the reason for the lack of correlation between MMP activity and susceptibility to MHP could be answered as well.

Consequently, although the present data clearly indicate that collagenases generate the highly cytotoxic MTP from MHP, it seems that MHP is a modest substrate for these enzymes because it is not as active as MTP, even in the presence of collagenase. This problem may be worked out in the future by the design of better collagenase substrates with cytotoxic potency.

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