New pemetrexed-peptide conjugates: synthesis, characterization and *in vitro* cytostatic effect on non-small cell lung carcinoma (NCI-H358) and human leukemia (HL-60) cells

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Pemetrexed (Pem) is a novel antimetabolite type of anticancer drug that demonstrated promising clinical activity in a wide variety of solid tumors, including non-small cell lung carcinoma and malignant pleural mesothelioma. It inhibits enzymes involved in the folate pathway, for which the presence of its free carboxylic groups is necessary. The heteroaromatic ring system of Pem has a modifiable amino group, which opens a possibility to apply a new strategy to conjugate Pem to carrier molecules. Considering this as well as the necessity of untouched carboxylic groups of Pem in the new conjugates, we developed a new synthesis strategy. Here, we describe the synthesis and the characterization of new Pem-peptide conjugates in which cell-penetrating octaarginine or/and lung-targeting H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$ peptide is attached to the drug by thioether bond. The conjugates characterized by RP-HPLC and MS exhibited cytostatic effect *in vitro* on non-small cell lung carcinoma as well as on human leukemia cell lines. The IC$_{50}$ values of the conjugates were similar, but the conjugates with H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$ sequence were slightly more effective. Our data show that the *in vitro* cytostatic effect of the free Pem was essentially maintained after conjugation with cell-penetrating or cell-targeting peptides. Thus, the conjugation strategy reported could lead to the development of a new generation of active Pem conjugates. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: synthesis of pemetrexed-oligoarginine conjugates; pemetrexed; IELLQAR lung-targeting peptide; *in vitro* cytostatic effect of conjugates; non-small cell lung carcinoma; human leukemia

Introduction

Perturbation of nucleotide metabolism for cancer therapy has primarily focused on the utilization of nucleoside and nucleic acid base analogues, which compete with their physiological counterparts for incorporation into DNA and RNA, as well as antifolate agents. One of the first antifolate agents was methotrexate, and still it is in clinical use [1]. Much is known about its mode of action and the mechanisms by which tumors exhibit inherent or acquired resistance to this drug [2]. During the preparation of new analogues, a promising agent, pemetrexed (Pem, LY231514 or Alimta, Figure 1.), has been developed by Eli Lilly and Company (Indianapolis, IN, USA).

Pem is an antimetabolite inhibiting at least three enzymes (thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyltransferase) involved in the folate pathway [3,4].

After being transported into the cell via the reduced folate carrier, Pem binds to folate receptor-α with a very high affinity [5]. In the cells, Pem is polyglutamated to the active pentaglutamide by a reaction catalyzed by folylpolyglutamate synthase. Pem proved to be a better substrate for folylpolyglutamate synthase as compared with methotrexate [6]. The polyglutamate derivatives (the pentaglutamate is the predominant intracellular form) are potent inhibitors of thymidylate synthase and are also weaker inhibitors of glycinamide ribonucleotide formyltransferase. Pem and its polyglutamate derivatives also inhibit dihydrofolate reductase but with less potency than methotrexate. Compared with methotrexate, Pem polyglutamates have an increased intracellular half-life, resulting in prolonged drug action in malignant cells [7].

Pem has demonstrated promising clinical activity in a wide variety of solid tumors, including non-small cell lung (NSCLC), breast, colorectal, pancreatic, gastric, bladder and malignant pleural mesothelioma [8].

NSCLC is the most common form of lung cancer, which is the leading cause of cancer mortality in men worldwide. First-line therapy of NSCLC is based on platinum agents, and docetaxel is the standard second-line treatment option. Pem was compared

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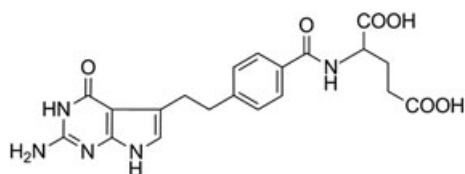‡*These authors contributed equally to this work.*

Figure 1. Chemical structure of Pem.

with docetaxel in a randomized trial involving 571 patients with locally advanced metastatic NSCLC after prior chemotherapy. Pem did not show superiority over docetaxel on the primary endpoint of survival, and there were no statistically significant differences between Pem disodium and docetaxel with respect to the secondary endpoints such as objective response rate. However, Pem disodium had a more favorable safety profile than docetaxel; for example, it caused significantly less neutropenia and febrile neutropenia [9].

Pem is also a substrate for multidrug resistance protein transporters; therefore, resistance can rise against it [3]. Like other antineoplastic agents, Pem has side effects, e.g. decreasing the number of neutrophils, nausea, vomiting and stomatitis.

Earlier in our group, peptide conjugates of methotrexate have been developed and *in vitro* cytostatic activity was evaluated [10,11]. In these conjugates, the carboxyl groups of methotrexate have been used for conjugation, which are required for the polyglutamation of the drug inside the cells. Pem has a different heteroaromatic ring system with an amino group, which could be modified. It opens a possibility to apply a new conjugation strategy.

Peptide H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$ was identified from a phage peptide library by Fukuda *et al.* [12,13] to mimic the carbohydrate ligands of E-selectin, sialyl Lewis X. E-selectin and P-selectin are expressed on activated vascular endothelial cells, whereas L-selectin is present on lymphocytes. Their ligands, such as sialyl Lewis X, are cell surface carbohydrates, which are characteristic of various stages of differentiation and are expressed in a tissue-specific and cell-specific manner. They play a role in cell–cell interaction and can also be found on the surface of tumor cells (sialyl Lewis X: breast, colon and lung carcinoma; sialyl Lewis A: colorectal and pancreas adenocarcinoma) related to the formation of selectin-mediated tumor metastasis [14]. It was found that the phage harboring the H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$ sequence and also the synthetic peptide bind to E-selectin, P-selectin and L-selectin in a calcium-dependent manner. *In vitro* investigations proved that E-selectin-mediated adhesion of certain tumor cells enriched with sialyl Lewis X oligosaccharides (HL-60 human leukemia, B16-FTIII·M melanoma) can be inhibited by this phage or peptide. *In vivo*, the peptide inhibited sialyl Lewis X-dependent lung colonization of tumor cells (B16-FTIII·M melanoma, HAL-8Luc human lung cells) and thus prevented the formation of tumor metastasis. The targeting potential of peptide H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$ *in vivo* by injecting a biotinylated derivative to wild-type and E/P selectin-deficient mutant mice was also demonstrated [15]. Targeting by peptide H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$ was first reported by Hildebrandt *et al.* [16] using functionalized dextran-coated superparamagnetic iron oxide nanoparticles with negatively charge connecting to peptide H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$ derivatives. However, in this study, electrostatic interactions were utilized for the development of a new target-specific imaging probe in clinical magnetic resonance imaging.

Oligoarginines (Arg$_n$, $n = 5$–15) are well-known cell-penetrating peptides with the capability to deliver covalently attached cargo molecules [17]. However, the mechanism of their translocation through the cell membrane is not fully understood [18–21]. It is known that the guanidine head group of Arg plays an important role in the uptake mechanism, and an optimum number of arginine residue is required for effective internalization [22]. The mechanism and efficacy of cellular uptake depends also on the physicochemical properties of the cargo molecules and on the cell line studied [23]. Different types of cargo molecules were coupled covalently to oligoarginine peptides for delivery purposes (e.g. peptides, proteins and liposomes) [24,25]. Delivery of small organic molecules/drugs by cell-penetrating peptides is also increasing. For example, transmembrane delivery of taxol [26], adenosine derivatives [27], ferrocene derivatives [28] or daunomicin [29] was carried out successfully. Amide, ester, thioether, oxime or hydrazone linkage was applied for the synthesis of these conjugates, and their effect was investigated *in vitro* and/or *in vivo*.

In this paper, synthesis and characterization of new Pem-peptide conjugates are described. In these compounds, octaarginine and/or peptide IELLQAR is attached to the drug by thioether linkage (Figure 2).

Our aim was to create Pem conjugates in which free carboxylic groups are present because they are required for the activity of folic acid antagonists. Therefore, we have synthesized chloroacetyl-pemetrexed-dimethylester [ClAc-Pem(OMe)$_2$] and also modified the peptides by adding Gly-Gly-Cys sequence at the C-terminal for the synthesis of conjugates. After conjugation, which resulted in thioether bond between the partners, we removed the methyl ester protecting groups from the carboxylic groups of Pem. The conjugates were purified and analyzed by RP-HPLC and were identified by ESI-MS. *In vitro* cytostatic effect of the new conjugates was determined on HL-60 human leukemia and NCI-H358 NSCLC cell lines. We found that the cytostatic effect of the free Pem was essentially preserved after covalent attachment of Pem with cell-penetrating or cell-targeting peptides and also with their 'hybrid' combined structure.

Materials and Methods

Materials

Pem disodium salt was obtained from Leo Chemical (Hong Kong); Fmoc-Rink Amide MBHA resin (0.72 mmol/g) was purchased from Novabiochem (Laufelfingen, Switzerland). All amino acid derivative were from Bachem (Bubendorf, Switzerland) or Reanal (Budapest, Hungary). Scavengers (thioanisole, ethanedithiol, phenol), coupling agents (DIC, HOBt, DIEA), cleavage reagents
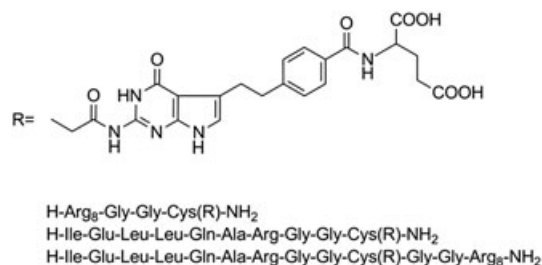

H-Arg$_8$-Gly-Gly-Cys(R)-NH$_2$
H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys(R)-NH$_2$
H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys(R)-Gly-Gly-Arg$_8$-NH$_2$

Figure 2. Chemical structure of Pem-peptide .

(piperidine, DBU, TFA) and tris(hydroxymethyl)-aminomethane (Tris) were Fluka (Buchs, Switzerland) products. All solvents, acetic acid (AcOH), DMF, DCM, diethyl ether, acetonitrile (ACN) and TFA, were from Reanal (Budapest, Hungary). Chloroacetic anhydride, 3 - (4,5 - dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI-1640 medium and fetal calf serum (FCS) were obtained from Sigma-Aldrich (Budapest, Hungary). HPMI (D-glucose, $NaHCO_3$, NaCl, HEPES, KCl, $MgCl_2$, $CaCl_2$, $Na_2HPO_4 \times 2H_2O$) was prepared in our laboratory with ingredients from Sigma-Aldrich (Budapest, Hungary).

Synthesis and Purification

Pem(OMe)₂

$Pem(OMe)_2$ was synthesized by methylation of Pem by $SOCl_2$ in methanol. Briefly, 100 ml MeOH was cooled to $-10\,^\circ$C, then 11 ml (150 mmol) $SOCl_2$ was slowly added. The mixture was kept in $-10\,^\circ$C for about 20 min, and 1.0 g (2.1 mmol) Pem disodium salt was added. The mixture was heated to room temperature, and it was refluxed for 3 h. NaCl formed was filtered off, and methanol was removed in vacuum. The remaining $SOCl_2$ was removed from the crude product by repeated solving in hot methanol. After the removal of the solvent, the product was crystallized from ethanol. The crystals were filtered off, washed with diethyl ether and dried in desiccating pistol. The yield was 90%. (light green crystals, unstable above $217\,^\circ$C; Anal. Calcd. for $Pem(OMe)_2 \times HCl$ $C_{22}H_{26}N_5O_6Cl$: C, 53.65%; H, 5.28%; N, 14.22%; Cl, 7.22%. Found: C, 52.24%; H, 5.15%; N, 14.45%; Cl, 6.43%.).

ClAc-Pem(OMe)₂

$ClAc\text{-}Pem(OMe)_2$ was synthesized by chloroacetylation of $Pem(OMe)_2$ by chloroacetic anhydride in DMF. $Pem(OMe)_2$ (290 mg, 0.60 mmol) was dissolved in 4 ml DMF. DIEA (110 µL, 0.65 mmol) and 1.5 g (8.77 mmol) chloroacetic anhydride was added, and the mixture was stirred at $40\,^\circ$C for 5 h. The reaction was followed by analytical RP-HPLC; monochloroacetylized and dichloroacetylized products were formed. The volume of 4 ml of ACN : distilled water (80 : 20, v/v%) mixture containing 0.1% TFA and 2 ml distilled water were added to the mixture and was left for 15 h at room temperature. After this, the mixture contained mostly monochloroacetyl Pem, which was precipitated by addition of distilled water and filtered off. The crude product was crystallized from ethanol, filtered, washed with diethyl ether and dried in desiccating pistol. The yield was 70% (light brown crystals, unstable above $185\,^\circ$C; Anal. Calcd. for $ClAc\text{-}Pem(OMe)_2$ $C_{24}H_{26}ClN_5O_7$: C, 54.19%; H 4.93%; N, 13.17%; Cl, 6.68%. Found: C, 53.20%; H, 4.71%; N, 12.60%; Cl, 6.69%.).

Peptides (H-Arg₈-Gly-Gly-Cys-NH₂, H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-NH₂, H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-Gly-Gly-Arg₈-NH₂)

Cysteine containing peptides ($H\text{-}Arg_8\text{-}Gly\text{-}Gly\text{-}Cys\text{-}NH_2$, $H\text{-}Ile\text{-}Glu\text{-}Leu\text{-}Leu\text{-}Gln\text{-}Ala\text{-}Arg\text{-}Gly\text{-}Gly\text{-}Cys\text{-}NH_2$, $H\text{-}Ile\text{-}Glu\text{-}Leu\text{-}Leu\text{-}Gln\text{-}Ala\text{-}Arg\text{-}Gly\text{-}Gly\text{-}Cys\text{-}Gly\text{-}Gly\text{-}Arg_8\text{-}NH_2$) were synthesized on solid phase using Fmoc strategy on Fmoc-Rink Amide MBHA resin (0.72 mmol/g). All amino acids were coupled as N^α-Fmoc derivatives: Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Cys(Trt)-OH, Fmoc-Ile-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Leu-OH, Fmoc-Ala-OH. Coupling was performed using DIC/HOBt *in situ* active ester methodology in DMF; coupling time was 60–90 min. The success of the coupling was monitored by ninhydrin reaction. The peptide was cleaved from the resin with TFA in the presence of scavengers (TFA : water : thioanisole : ethanedithiol : crystalline phenol = 10 ml : 0.5 ml : 0.5 ml : 0.25 ml : 0.75 g). The crude product was purified by a semipreparative RP-HPLC instrument (Knauer, Bad Homburg, Germany) on a Phenomenex Jupiter (C18, 300 Å, 10 µm, 250 × 10 mm, Phenomenex, Torrance, CA, USA) column. Linear gradient elution was developed from 1% to 40% eluent B from 5 to 44 min. Eluent A was 0.1% TFA in water, whereas eluent B contained 0.1% TFA in methanol–water (90 : 10 v/v%). Experiments were carried out at a flow rate of 4 ml/min at room temperature. Sample was applied in eluent A or in the mixture of eluents A and B, the sample concentration was 5 mg/ml, and 2 ml of solution was injected. Peaks were detected at $\lambda = 220$ nm. The purified peptides were dissolved in distilled water or AcOH : distilled water (10 : 90, v/v%) and freeze dried.

Conjugation of Pem dimethylester with peptide [Arg₈-Gly- Gly-Cys - Pem(OMe)₂, Ile -Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-Pem(OMe)₂, Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-(Pem(OMe)₂)-Gly-Gly-Arg₈]

Reaction was performed between $ClAc\text{-}Pem(OMe)_2$ and cysteine-containing peptide in 0.1 M Tris-buffer (pH = 8.0) : DMF (45 : 55, v/v %) mixed solvent. Chloroacetyl group of Pem and thiol group of cysteine reacted forming thioether bond. $ClAc\text{-}Pem(OMe)_2$ (15 mg, 0.03 mmol) was dissolved in 11 ml DMF, then 9 ml 0.1 M Tris-buffer (pH = 8) was added to the solution. The peptide was slowly added in small portion to the mixture. The reaction was followed by analytical RP-HPLC; the product was formed in 1 day. The solvent was removed in vacuum, the crude product was dissolved in eluents A and B mixed solvent and was purified by semipreparative RP-HPLC as it was described previously (Paragraph Peptides).

Pem-peptide conjugates [Arg₈-Gly-Gly-Cys-Pem, Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-Pem, Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-(Pem)-Gly-Gly-Arg₈]

Peptide conjugate of Pem was prepared by removing the methyl ester group from the conjugate of $Pem(OMe)_2$. The dimethylester conjugate was dissolved in 0.1 M NaOH : acetone (50 : 50, v/v%) mixed solvent. NaOH was used in about 2.5 times excess regarding the methylester groups. Dimethylester conjugates (10–15 mg) were dissolved in 400–450 µL solvent, and the mixture was kept at $5\,^\circ$C. The reaction was followed by RP-HPLC; the product was formed in 60–90 min. The remaining NaOH was neutralized by adding 200 µL 0.1 M HCl solution to the reaction mixture. The crude conjugate was dissolved in eluents A and B mixed solvent and was purified by semipreparative RP-HPLC as described previously (Paragraph Peptides).

Characterization of Compounds

Analytical RP-HPLC

All peptides and conjugates were analyzed by RP-HPLC using a Knauer (Bad Homburg, Germany) instrument. RP-HPLC measurements were performed using Supleco Suplecosil™ LC-18-DB (C18, 120 Å, 5 µm, 4.6 × 250 mm; Bellefonte, PA, USA) column. A linear gradient elution was developed from 5% to 95% eluent B from 5 to 50 min. Eluent A was 0.1% TFA in water, whereas eluent B contained 0.1% TFA in ACN : water (80 : 20, v/v%). Experiments were carried out at a flow rate of 1 ml/min at room temperature. Samples were applied in eluent A or in the mixture of eluents A

and B, and 20 μL of solution was injected. Peaks were detected at $\lambda = 220$ nm.

Mass spectrometry

The identification of conjugates and peptides was achieved by ESI-MS on a Bruker Daltonics Esquire 3000 Plus (Bremen, Germany) ion trap mass spectrometer, operating in continuous sample injection at 4 μL/min flow rate. Samples were dissolved in ACN : water (50 : 50, v/v%) mixture containing 0.1 v/v% AcOH. Mass spectra were recorded in positive ion mode in the *m/z* 200–2000 range.

In vitro cytostatic effect

HL-60 human leukemia cells [ATCC no. CCL-240 (American Type Culture Collection, Manassas, VA, USA)] were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine and 160 μg/ml gentamycin, whereas NCI-H358 human NSCLC cells (ATCC no. CRL-5807) were cultured in RPMI-1640 medium supplemented with 10% FCS and 2 mM L-glutamine. Cell culture was maintained at 37 °C in a humidified atmosphere with 5% CO_2. To study the cytostatic effect of the conjugates, MTT-assay was carried out [30]. Cells of 5×10^3 per well were plated on 96-well plates. After 24 h incubation at 37 °C, the cells were treated for 3 h with the conjugates dissolved in serum-free RPMI-1640 medium. The compounds were tested in the $2.6 \times 10^{-4} - 10^2$ μM concentration range. Cells treated with serum-free medium for 3 h were used for control. After treatment and incubation, the cells were washed twice with serum-free medium and cultured for 72 h in complete medium. On the fourth day, MTT-assay was carried out. An MTT-solution of 45 μL (2 mg/ml) was added to each well (final concentration is 367 μg/ml). After 3.5 h incubation, purple crystals were formed by mitochondrial dehydrogenase enzyme of live cells. The cells were centrifuged for 5 min at 863 g, and supernatant was removed. Crystals were dissolved in DMSO, and the optical density (OD) of the samples was measured at $\lambda = 540$ and 620 nm using ELISA reader (Labsystems MS reader, Finland). OD_{620} was substracted from OD_{540}. The percent of cytostasis was calculated using the following equation:

$$\text{Cytostasis}\% = \left[1 - \frac{OD_{treated}}{OD_{control}}\right] \times 100$$

Where $OD_{treated}$ and $OD_{control}$ correspond to the optical densities of treated and control cells, respectively. Cytostasis% was plotted as a function of concentration, fitted to a sigmoidal curve, and the IC_{50} value was determined by right of this curve and illustrated in a bar chart.

Results and Discussion

In this study, we report on the synthesis and characterization of novel Pem-peptide conjugates containing cell-penetrating octaarginine and/or lung-targeting H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH₂ peptide with thioether bond between the two moieties. The conjugates were characterized by RP-HPLC and ESI-MS. The cytostatic effect of conjugates as well as free Pem derivatives was studied on NCI-H358 NSCLC and on HL-60 human leukemia cell lines.

Synthesis and Purification

Preparation of methyl ester protected Pem compound [Pem(OMe)₂]

For the temporary protection of carboxylic groups, we have synthesized Pem dimethylester by the reaction between Pem disodium salt and thionyl chloride in methanol. The outline of the synthesis is shown in Figure 3. The reaction went smoothly. Apart from the dimethylester product, NaCl side product was formed, which had to be filtered off from the methanol solution. Pure Pem(OMe)₂ was obtained by crystallization from ethanol; light green crystals were precipitated in 1 day. The compound was characterized by elemental analysis and ESI-MS and retention times (t_R) were obtained by analytical RP-HPLC. The characteristics of the compound are summarized in Table 1.

Preparation of ClAc-Pem(OMe)₂

For the preparation of the conjugates, we have synthesized ClAc-Pem(OMe)₂ by the reaction of chloroacetic acid anhydride and Pem(OMe)₂ in the presence of DIEA in DMF. The outline of the synthesis is shown in Figure 3.

The amino group of Pem is hardly reactive; therefore, we have used 15 times molar excess even of the very reactive agent, chloroacetic acid anhydride at elevated temperature (40 °C). In this reaction, monochloroacetyl and dichloroacetyl derivatives could be formed. However, under conditions we developed, only the dichloroacetyl-Pem compound was obtained. After crystallization from ethanol, pure, light brown crystals were collected. The compound was characterized by elemental analysis and MS. Retention time value (t_R) was also obtained by analytical RP-HPLC (Figure 4). The characteristics of the compound are summarized in Table 1.

Preparation of peptides oligoarginine and H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH₂ with C-terminal cysteine (H-Arg₈-Gly-Gly-Cys-NH₂, H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-NH₂, H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-Gly-Gly-Arg₈-NH₂)

For the preparation of the conjugates, first we have synthesized C-terminally modified octaarginine and H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH₂ peptides as well as H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-Gly-Gly-Arg₈-NH₂ peptide on solid phase by Fmoc strategy using *in situ* active ester coupling strategy. Cysteine was built into the sequence for the purpose to create a thioether bond with the chloroacetyl group of Pem. In our laboratory, it was proved that this type of reaction proceeds better if the cysteine residue is present at the C-terminal end [31]. We have incorporated Gly-Gly dipeptide as spacer between the two moieties. The peptides were purified by semipreparative RP-HPLC, characterized by ESI-MS, and retention time value (t_R) was obtained by analytical RP-HPLC. The characteristics of the peptides are summarized in Table 1.

Conjugation of Pem(OMe)₂ with peptide [Arg₈-Gly-Gly-Cys-Pem(OMe)₂, Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-Pem(OMe)₂, Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-(Pem(OMe)₂)-Gly-Gly-Arg₈]

Peptide conjugates of Pem(OMe)₂ were prepared by the reaction between chloroacetyl group of Pem(OMe)₂ and thiol group of cysteine, present in the peptide sequence, under alkaline circumstances (pH = 8.0). Thioether bond was formed, which is stable in acidic or basic circumstances. We observed the presence of peptide dimer linked by thioether bond in the crude product.
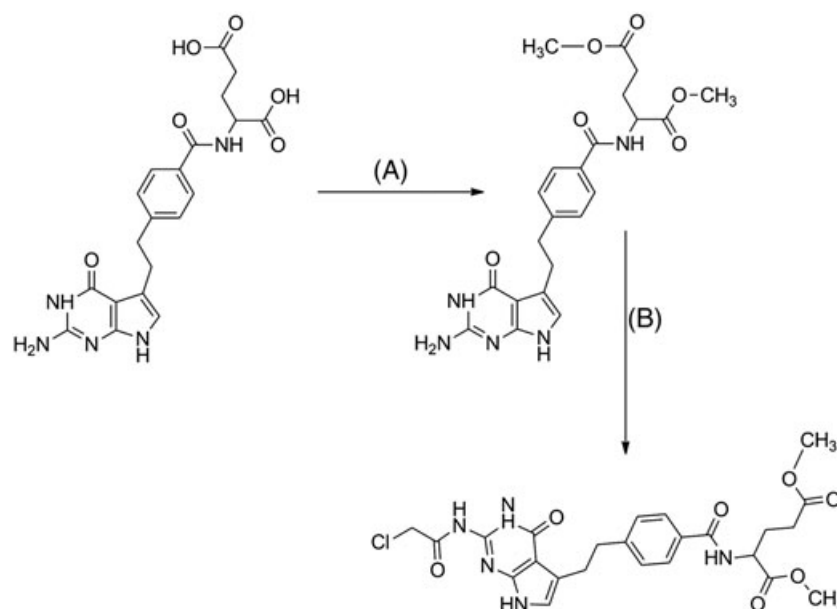
Figure 3. Outline of the synthesis of ClAc-Pem(OMe)$_2$ from Pem [(A) SOCl2 in MeOH, reflux 4.5 h; (B) chloroacetyl anhydride, DIEA in DMF, 40 °C, slightly acidic conditions] see details in Materials and Methods.

The conjugates were purified by semipreparative RP-HPLC, characterized by ESI-MS, and retention time value (t_R) was obtained by analytical RP-HPLC. The characteristics of these conjugates are also presented in Table 1.

Preparation of Pem-peptide conjugates [Arg$_8$-Gly-Gly-Cys-Pem, Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-Pem, Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-Cys-(Pem)-Gly-Gly-Arg$_8$]

Peptide conjugates with Pem were prepared by removing the methylester protecting groups from the carboxylic groups of Pem. The removal of the protecting groups is indispensable because the free carboxylic groups are essential for the biological activity of Pem [4,5]. In alkaline circumstances – e.g. by treatment

with NaOH solution – the methyl ester groups can be removed from the carboxylic groups. However, under alkaline conditions, there is a chance of the cleavage of the acetyl group from the amino group of Pem. In addition, the first amino acid in the peptide sequence could be racemized too. Therefore, the molar excess of NaOH applied for cleavage, the temperature and also the reaction time had to be carefully set for each reaction. We found that 2–3 times molar excess of 0.1 M NaOH solution and the same volume of acetone was the most appropriate as cleavage mixture for 60–90 min at 5 °C. We have monitored the reaction by analytical RP-HPLC and have terminated the reaction in time to avoid undesired cleavages by adding 0.1 M HCl solution to the mixture. The conjugates were purified by semipreparative

Table 1. Characteristics of free peptides, ClAc-Pem(OMe)$_2$, and conjugates of Pem(OMe)$_2$

Compounds	MS (M)[a]		t_R (min)[b]
	Calculated	Measured	
IELLQARGGC-amide	1057.6	1057.4	25.6
IELLQARGGC[Pem(OMe)$_2$]-amide	1553.7	1553.5	32.3
IELLQARGGC[Pem]-amide	1525.7	1525.8	28.7
RRRRRRRRGGC-amide	1483.7	1483.5	19.1
RRRRRRRRGGC[Pem(OMe)$_2$]-amide	1979.2	1979.0	26.7
RRRRRRRRGGC[Pem]-amide	1951.2	1951.5	24.4
IELLQARGGCGGRRRRRRRR-amide	2421.8	2124.9	35.2
IELLQARGGC[Pem(OMe)$_2$]GGRRRRRRRR-amide	2917.3	2917.2	34.6
IELLQARGGC[Pem]GGRRRRRRRR-amide	2889.3	2890.3	33.3
Pem	427.1	427.3	32.2
Pem(OMe)$_2$	455.2	455.3	29.0
ClAc-Pem(OMe)$_2$	531.2	531.2	32.9

[a]ESI-MS.
[b]HPLC retention time, Supelcosil™ LC-18-DB (C18, 120 Å, 5 µm, 4.6 × 250 mm; Bellefonte, PA, USA) column, gradient: 0–5 min 5% B eluent, 5–50 min 95% B eluent, where eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in ACN : water (80 : 20, v/v%).
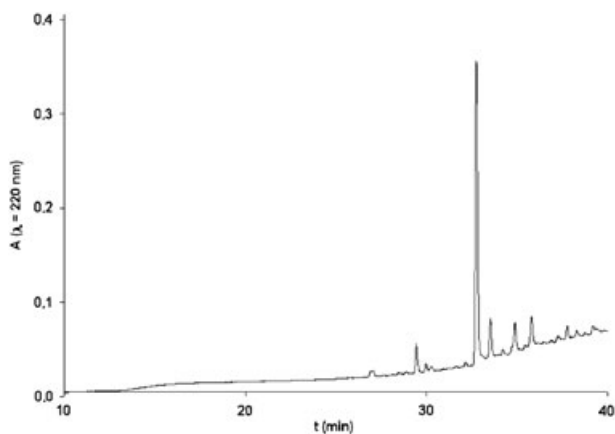
Figure 4. RP-HPLC chromatogram of ClAc-Pem(OMe)$_2$ after crystallization. Supelcosil™ LC-18-DB (C18, 120 Å, 5 μm, 4.6 × 250 mm; Bellefonte, PA, USA) column. Gradient: 0–5 min 5% B eluent, 5–50 min 95% B eluent, where eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in ACN–water (80:20 v/v%). Flow rate is 1.0 mL/min at room temperature. Peaks were detected at $\lambda = 220$ nm.
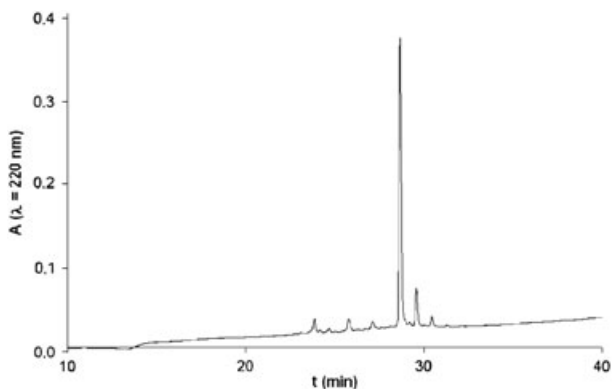

Figure 5. RP-HPLC chromatogram of the purified IELLQARGGC-Pem conjugate. Supelcosil™ LC-18-DB (C18, 120 Å, 5 μm, 4.6 × 250 mm; Bellefonte, PA, USA) column. Gradient: 0–5 min 5% B eluent, 5–50 min 95% B eluent, where eluent A: 0.1% TFA in water, eluent B: 0.1% TFA in ACN–water (80:20 v/v%). Flow rate is 1.0 mL/min at room temperature. Peaks were detected at $\lambda = 220$ nm.
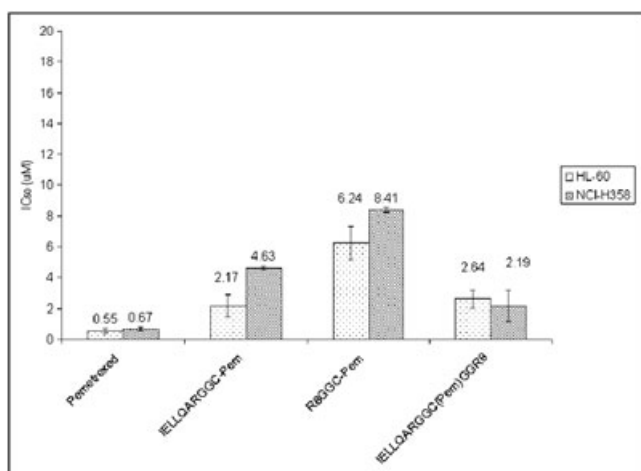

Figure 6. In vitro cytostatic effect of Pem and Pem-conjugates on HL-60 and NCI-H358 cells. Bars represent IC$_{50}$ ± SD values after treatment for 3 h.

RP-HPLC, characterized by MS, and retention time value (t_R) was obtained by analytical RP-HPLC (Figure 5). The characteristics of these conjugates are summarized in Table 1.

In Vitro Cytostatic Effect

In vitro cytostatic effect of Pem-peptide conjugates were studied and compared with that of the free drug (Pem) as well as Pem (OMe)$_2$ on NCI-H358 and HL-60 cell lines by MTT-assay. *In vitro* cytostatic effect of the compounds was characterized by their IC$_{50}$ values (Figure 6).

As expected, the free Pem was highly and similarly active on both cell lines. This could be documented by the similar IC$_{50}$ values on the studied cell lines (IC$_{50}$ = 0.67 μM for NCI-H358 and 0.55 μM for HL-60). It is interesting to note that Pem(OMe)$_2$ exhibited higher IC$_{50}$ values on both cell lines. However, this compound was more cytostatic on HL-60 (IC$_{50}$ = 9.44 μM) compared with NCI-H358 cells (IC$_{50}$ = 87.34 μM).

Conjugation of Pem with oligopeptides decreased its cytostatic effect. However, this was dependent on the peptide used and also on the cell line. Conjugates either with peptide H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-NH$_2$ or with peptide H-Arg$_8$-Gly-Gly-NH$_2$ were slightly more effective on HL-60 cells than on NCI-H358 cells. Also, the Pem-conjugate with peptide H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-NH$_2$ exhibited lower IC$_{50}$ values (IC$_{50}$ = 2.17 μM for HL-60 and IC$_{50}$ = 4.63 μM for NCI-H358) as compared with Pem-oligoarginine conjugate (IC$_{50}$ = 6.24 μM for HL-60 and IC$_{50}$ = 8.41 μM for NCI-H358).

Interestingly, Pem conjugated with the 'hybrid' peptide and comprising the H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$ sequence was the most active compound (IC$_{50}$ = 2.64 μM for HL-60 and IC$_{50}$ = 2.19 μM for NCI-H358). It is important to avoid the over interpretation of these data but is should be noted also that conjugates containing peptide H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-Gly-Gly-NH$_2$ were slightly more effective as compared with the only oligoarginine-containing conjugate.

Conclusion

Pem is a newly developed anticancer agent with promising effect against NSCLC and malignant pleural mesothelioma. Its long-time side effects and its effectiveness in curing other types of cancer are currently tested in clinical phase I, II or III. The modification of anticancer drugs by peptide could improve the *in vivo* activity or selectivity of the agents even circumvents multidrug resistance. Peptide conjugates could be well characterized, easily modified, and they are biocompatible, mostly water-soluble compounds.

The octaarginine peptide is well known about its ability to translocate through the cell membrane with different molecules attached to it. Peptide H-Ile-Glu-Leu-Leu-Gln-Ala-Arg-NH$_2$, which was identified by phage technology, could target the lung. With these two peptides presented in a single sequence, one could even target as well as translocate the drug at the same time.

Considering the necessity of untouched carboxylic groups of Pem in the new conjugates, we developed a new synthesis strategy, different from that applied during the synthesis of methotrexate-peptide conjugates. The differences between the heteroaromatic ring systems of the two drugs made this new

strategy to work. The applicability of this new conjugation strategy was proven by the IC_{50} values of the conjugates obtained in an *in vitro* cytostasis assay using two relevant cell lines. Pem conjugation with different targeting moieties and also further investigation related to the mechanism of action of the conjugates could lead to the development of a new generation of Pem conjugates with improved specificity.

Acknowledgements

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