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β-Cyclodextrin/epoxysuccinyl Peptide Conjugates: a New Drug Targeting System for Tumor Cells

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Abstract—β-Cyclodextrin is known to form inclusion complexes with hydrophobic drugs. Several tumor cell lines are known to secrete and/or contain membrane-associated cathepsin B which is possibly involved in invasion and metastasis. Based on these informations, our recently developed *endo*-epoxysuccinyl peptide inhibitor MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH for cathepsin B was conjugated with β-cyclodextrin to obtain a site-directed drug carrier system. Furthermore, the conjugate was shown to form an inclusion complex with the cytotoxic drug methotrexate.

Several tumor cell lines are known to secrete cathepsin B and/or to contain membrane-associated cathepsin B which is thought to be involved in invasion and metastasis.[1,2] Thus, extracellular cathepsin B could be an interesting target for selective tumor therapy. Panchal et al.[3] have demonstrated that prodrug forms of pore-forming toxins, which were designed for specific activation by cathepsin B, are able to permeabilize and thus to kill tumor cells. An alternative approach could be a suitable carrier molecule equipped with an appropriate address sequence for cathepsin B loaded with a cytotoxic drug. In this respect, cyclodextrins are widely used due to their ability to form inclusion complexes with a wide variety of pharmaceutical active compounds.[4] Since cyclodextrins lack information for site specific delivery in the organism, they have been functionalized with signal molecules such as opioid peptides,[5,6] gastrin peptides,[7] and mono- and oligosaccharides[8] as specific ligands of cell-surface receptors. Recently, we have studied the accessibility of the tripeptide aldehyde Ac-Leu-Leu-Nle-H as inhibitor of cysteine proteases when linked covalently to β-cyclodextrin.[9] The observed K_i value for the inhibition of cathepsin B by this conjugate was only slightly increased compared to the parent inhibitor indicating that the recognition process between inhibitor and enzyme is not impaired. These results stimulated us to synthesize a β-cyclodextrin/protease inhibitor conjugate with a cathepsin B-selective inhibitor and to analyse its potential ability as carrier system to address tumor cell cathepsin B.

Based on the carboxydipeptidase activity of cathepsin B and on the *retro*-binding mode of its propeptide in the zymogen form we recently constructed a new type of *endo*-epoxysuccinyl-peptide, i.e. MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-OH (**1**).[10] With this design strategy we achieved (i) to selectively inhibit cathepsin B among the cysteine proteinases by the specific salt bridge interaction of the C-terminal carboxylate function with histidine 110 and 111 of the occluding loop, and (ii) to span the whole substrate binding groove at the S subsite with the propeptide portion (46–48). In fact, docking experiments of the inhibitor into the active site cleft of cathepsin B (data not shown) using the X-ray structure[11] of cathepsin B clearly show that the terminal glycine residue is exposed to the surface of the protein, thus allowing for chemical manipulation at this inhibitor terminus (Fig. 1). Using ϵ-aminohexanoic acid as additional fully flexible 6 C spacer for the attachment to mono-(6-deoxy-6-amino)-β-cyclodextrin steric clashes of the bulky cyclodextrin moiety with the enzyme should be avoided.

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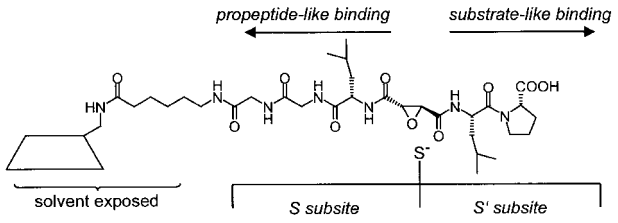
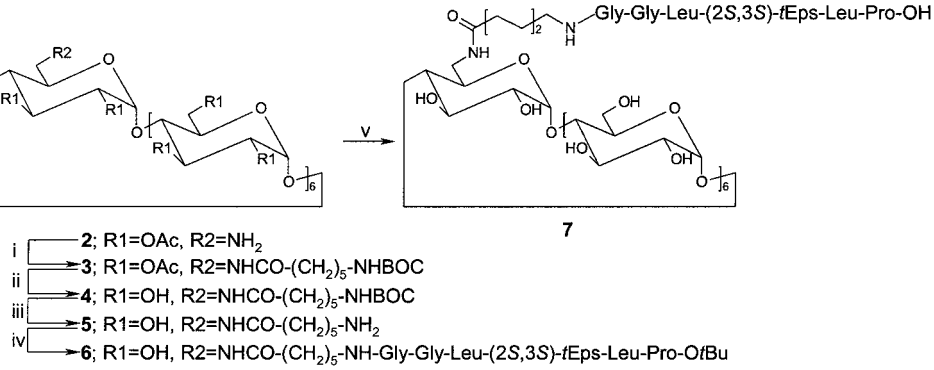


Figure 1. Proposed interaction of the *endo*-epoxysuccinyl peptide/β-cyclodextrin conjugate **7** with cathepsin B.

Starting point of the synthesis was mono-(2,3-di-*O*-acetyl-6-deoxy-6-amino)-hexakis-(2,3,6-tri-*O*-acetyl)-β-cyclodextrin (**2**)[9] which was reacted with BOC-protected ϵ-aminohexanoic acid using EDC/HOBt (Scheme 1). After two deprotection steps, the spacer-functionalized β-cyclodextrin (**4**) was coupled with HO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu (obtained by saponification of MeO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu)[10] using PyBOP.[12] Finally, the conjugate **7**[13] was obtained by acidolytic cleavage of the *tert*-butyl ester function with 90% TFA.

The β-cyclodextrin/epoxysuccinyl peptide conjugate **7** was analysed by 2D NMR-spectroscopy. TOCSY and NOESY-spectra recorded in H_2O:D_2O (9:1) allowed the full assignment of all signals of the peptide portion as well as of the substituted sugar unit. Furthermore, no evidence could be derived from the NOESY-spectrum for an interaction of the peptide portion with the β-cyclodextrin cavity in terms of host-guest complexation.

Methotrexate (*N*-{4-[(2,4-di-aminopteridin-6-yl methyl) methylamino]benzoyl}-L-(+)-glutamic acid) a cytotoxic drug which is known to form a 1:1-inclusion complex with β-cyclodextrin[14] was chosen to investigate the ability of the conjugate **7** to form an inclusion complex. Both the observation of a signal at m/z = 1127.6 in the ESI-MS[15] (Fig. 2) which can be attributed to the double charged 1:1-complex of the conjugate **7**



Scheme 1. Synthesis of a β-cyclodextrin/epoxysuccinyl peptide conjugate **7**. Reaction conditions: (i) BOC-ϵAhx-OH/EDC/HOBt/$CHCl_3$ (91%); (ii) KOH/MeOH/H_2O, then Amberlyst 15 (88%); (iii) 90% TFA (90%); (iv) HO-Gly-Gly-Leu-(2*S*,3*S*)-*t*Eps-Leu-Pro-O*t*Bu/PyBOP/NEt_3/DMF (57%); (v) 90% TFA (74%).

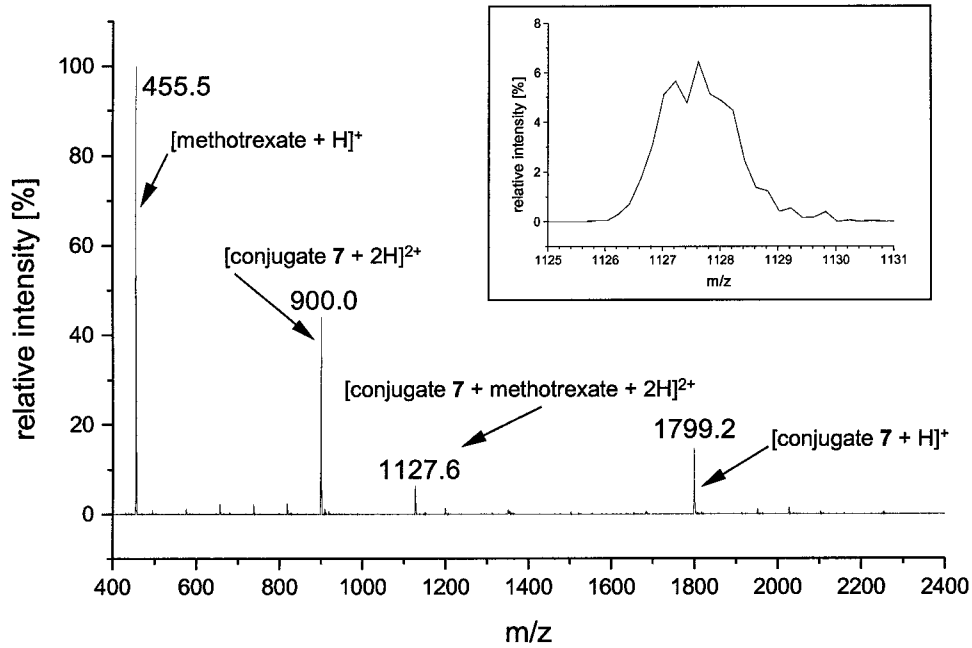


Figure 2. ESI-MS of methotrexate in presence of an equimolar concentration of conjugate **7**. The insert shows the mass signal of the double charged 1:1-complex of conjugate **7** with methotrexate.

with methotrexate and the formation of an isosbestic point at 333 nm in the UV-vis spectrum[16] of methotrexate in the presence of increasing concentration of the conjugate **7** (Fig. 3) show that the β-cyclodextrin portion in the conjugate **7** is capable to form an inclusion complex.

A comparison of the second order rate constants[17] (Table 1) of inhibition of cathepsin B by the β-cyclodextrin/inhibitor conjugate **7** and the parent inhibitor **1** clearly confirmed that conjugation exerts marginal effects on the inhibitory potency. The k_2/K_i value is reduced only by a factor of 0.7. These findings validate our design strategy. Conversely, the selectivity of the conjugate **7** for cathepsin B versus cathepsin L is increased by a factor of 2. This result is surprising since the selectivity ratio between cathepsin B and papain is not affected by the conjugation. A comparison of the X-ray structures of cathepsin L and papain revealed a high degree of similarity,[18] however, the S2 pocket of cathepsin L is much narrower and smaller than that of cathepsin B. Therefore, it seems that the β-cyclodextrin portion of the conjugate **7** influences indirectly the optimal interaction of the P2 Leu with the corresponding subsite of cathepsin L resulting in a stronger reduction of affinity compared with cathepsin B.

The membrane permeability of inhibitor **1** and of its conjugate **7** was assessed with MCF-7 breast cancer cells known to contain membrane-associated cathepsin B (Fig. 4) as well as with fibroblasts and HaCaT-cells (data not shown).[19] As control for a membrane-permeable compound the prodrug epoxysuccinyl peptide E64d was used. Both the inhibitor **1** and its cyclodextrin conjugate **7** inhibit quantitatively the endogenous

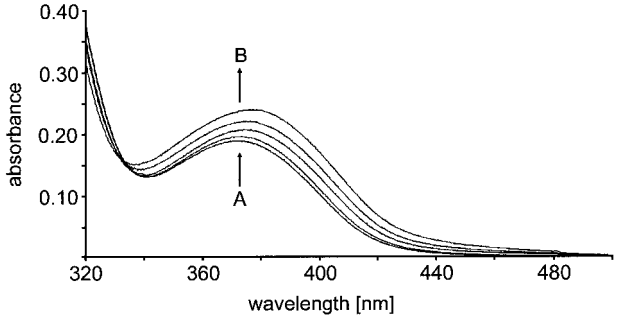


Figure 3. UV/vis-spectrum of methotrexate at increasing concentrations of conjugate **7**: *c* (methotrexate) = 10 μM in 50 mM phosphate buffer (pH = 8.0), *c* (conjugate **7**) = 0, 0.1, 0.5, 1.0, and 2.0 mM, read from A to B.

Table 1. Second order rate constants (k_2/K_i (M^{-1} s^{-1})) for the inhibition of papain, cathepsin B, and cathepsin L by inhibitor **1** and the cyclodextrin conjugate **7**

Inhibitor	Papain	Cathepsin B	Cathepsin L	Ratio CB/PA	Ratio CB/CL
1	14,800 ± 1077	1,520,000 ± 88,000	1,204 ± 29	103	1262
7	10,047 ± 794	1,050,000 ± 050	393 ± 28	105	2672

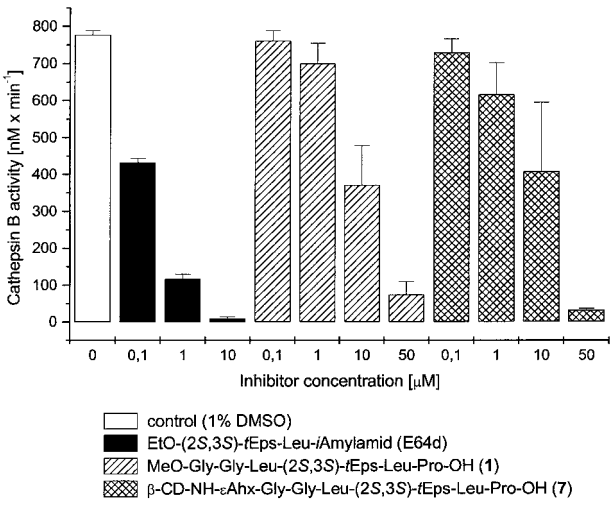


Figure 4. Membrane permeability of epoxysuccinyl-peptides into MCF-7 breast cancer cells. The columns represent mean values of three measurements with the standard deviation of the mean value.

cathepsin B activity of lysates of MCF-7 cells at nanomolar concentrations, identical to those determined for the isolated enzyme. On the contrary, with intact cells both the conjugate **7** and the parent inhibitor **1** block the endogenous cathepsin B activity only by 10–20% even at 1000-fold higher concentrations than those required for a complete inhibition of the enzyme in cell lysates. Therefore, both inhibitors are not cell-permeable at concentrations needed for full inhibition of lysosomal cathepsin B. Comparable results were obtained with fibroblasts and HaCaT-cells.

In conclusion, by the covalent attachment of our parent inhibitor **1** to the spacer-functionalized β-cyclodextrin a fully water-soluble conjugate **7** was obtained whose inhibitory potency in comparison to the parent inhibitor is only slightly reduced. The cyclodextrin cavity was proven to form an inclusion complex with the cytotoxic drug methotrexate. Furthermore, at the concentrations required for full inhibition of extracellular and/or membrane-bound cathepsin B the conjugate is not membrane-permeant. Therefore, the synthesized conjugate **7** exhibits all characteristics to act as a site-selective drug carrier system.

Acknowledgement

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13. Compound **7** was isolated by preparative HPLC; yield: 74%; TLC (acetonitrile:water 7:3) $R_f = 0.35$; HPLC (Nucleosil 300/C18, linear gradient of acetonitrile/2% phosphoric acid from 5:95 to 80:20 in 30 min) $t_R = 12.8$ min; amino acid analysis of the acid hydrolysate (6 M HCl, 110 °C, 24 h): Pro 1.06 (1), Gly 2.00 (2), Leu 1.93 (2), peptide content: 88.4%; ^{1}H NMR (500 MHz; H_2O/D_2O 9:1): $\delta = 0.77$ (m, 3H, δ_2CH_3 Leu2), 0.81 (m, 3H, δ_1CH_3 Leu2), 0.82 (m, 3H, δ_2CH_3 Leu1), 0.84 (m, 3H, δ_1CH_3 Leu1), 1.15, 1.37, 1.44 (3 m, 6H, βCH_2 ϵAhx, γCH_2 ϵAhx, δCH_2 ϵAhx), 1.51, 1.60 (2 m, 3H, βCH_2 Leu1, γCH Leu1), 1.52 (2 m, 3H, βCH_2 Leu2, γCH Leu2), 1.89 (m, 2H, γCH_2 Pro), 1.94 (m, 1H, β_2CH_2 Pro), 2.12 (m, 2H, αCH_2 ϵAhx), 2.20 (m, 1H, β_1CH_2 Pro), 3.08 (m, 2H, ϵCH_2 ϵAhx), 3.19, 3.26 (2 m, 2H, H-6a,H-6b, substituted sugar unit), 3.42 (m, 1H, H-4, substituted sugar unit), 3.51 (m, 1H, H-2, substituted sugar unit), 3.52 (m, 1H, δ_2CH_2 Pro), 3.56, 3.59 (2 s, 2CH *t*Eps), 3.67 (m, 1H, H-5, substituted sugar unit), 3.75 (m, 1H, δ_1CH_2 Pro), 3.76 (m, 2H, αCH_2 Gly1), 3.79 (m, 1H, H-3, substituted sugar unit), 3.82 (m, 2H, αCH_2 Gly2), 4.26 (m, 1H, αCH Leu2), 4.30 (m, 1H, αCH Pro), 4.57 (m, 1H, αCH Leu1), 4.92 (m, 1H, H-1, substituted sugar unit), 7.64 (m, 1H, NH ϵAhx), 7.85 (m, 1H, NH substituted sugar unit), 8.09 (m, 1H, NH Gly2), 8.43 (m, 1H, NH Leu1), 8.45 (m, 1H, NH Gly1), 8.70 (m, 1H, NH Leu2); the numbering of the amino acids in compound **7** is indicated as follows: β-CD-NH-ϵAhx-Gly2-Gly1-Leu1-(2*S*,3*S*)-*t*Eps-Leu2-Pro-OH; MALDI-TOF-MS: $m/z = 1820.8$ $[M + Na]^+$; calcd. for: $C_{73}H_{119}N_7O_{44}$: 1797.7.

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15. The ESI-MS of an equimolar mixture of methotrexate and conjugate **7** (c (methotrexate) = c (conjugate **7**) = 0.1 mM) in 1% aqueous trifluoroacetic acid was recorded on a PE SCIEX API 165.
16. The spectra of methotrexate ($c = 10$ μM in 50 mM phosphate buffer, pH = 8.0) at increasing concentrations of the conjugate **7** ($c = 0$, 0.1, 0.5, 1.0, and 2.0 mM) were recorded on a Perkin–Elmer UV–vis spectrometer Lambda 19 using 1 cm cells at 25 °C.
17. The enzyme inhibition assay was performed as described previously.[10]

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19. Permeability assay: Briefly, MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with essential amino acids, 1 mM pyruvate, 1 μg/mL insulin, and 10% foetal calf serum. The culture was performed in 24-well plates where the cells were grown within a week to a confluent state (from initial 2×10^4 cells/well to app. 2×10^6 cells/well) in a humidified CO_2 incubator at 37 °C and 5% CO_2. After washing the cells three times with serum-free medium (SFM) at 37 °C, they were incubated at 37 °C with 300 μL of the inhibitor solution (SFM containing 1% DMSO) for 30 min. Then the cells were washed five times with PBS-buffer at room temperature followed by treatment with 200 μL lysis-buffer (0.5% Triton X-100, 50 mM sodium acetate pH 5.5, 2 mM EDTA) at room temperature for 30 min. The residual cathepsin B-activity was determined in the lysates with the fluorogenic substrate Z-Phe-Arg-NHMec and subsequent inhibition by inhibitor **1** (50 nM).