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Structure–Activity Relationships of Carboxymethylpullulan-Peptide-Doxorubicin Conjugates—Systematic Modification of Peptide Spacers

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Abstract—A series of carboxymethylpullulan (CMPul)-doxorubicin (DXR) conjugates bound by peptide spacers of different compositions and lengths were prepared and evaluated for their in vivo antitumor effects. Systematic study of the peptide spacers indicated that CMPul-DXR conjugates bound via appropriate dipeptide spacers were more potent than DXR. © 2000 Elsevier Science Ltd. All rights reserved.

In trying to increase the therapeutic index of a parent drug, it is often chemically conjugated with macromolecules, because such conjugates can alter its pharmacokinetic properties depending on the characteristics of the carrier macromolecules. Drug release can be controlled by the selection of a drug-carrier linkage offering more stability in the bloodstream with degradation in or near cancer cells. Most macromolecules are captured by pinocytic internalization, followed by transfer into the endosomal compartment of the cell. They are then directed into a secondary lysosomal compartment which contains a variety of lysosomal enzymes.[1] Consequently, peptide spacers enzymatically cleavable by lysosomal enzyme are often used to link a drug with a carrier. Several examples of such conjugates have been proposed for not only antitumor drugs[2,3] but also antibacterial[4] and antiviral drugs.[5]

The structural requirements for efficient drug release from macromolecular prodrugs have been intensively investigated in the presence of rat liver lysosomal enzymes or individual cysteine proteases such as cathepsine B and L.[2] However, little information is available about how the systematic replacement of the length and sequence of the peptide spacer is related to the efficacy of animal models[6,7] and tissue distribution. Our previous report described CMPul-Gly-Gly-Phe-Gly-DXR to be the most potent conjugate against Walker 256 carcinosarcoma (Walker 256) among CMPul-DXR conjugates bound via three types of tetrapeptide spacers.[8] In order to examine the effect of the length and composition of the peptide spacer on the in vivo antitumor effect, we describe the antitumor activity against rats bearing Walker 256 of CMPul-DXR conjugates bound via peptide spacers differing by systematic replacement. We also examined the amount of DXR released from the CMPul-DXR conjugates in the presence of lysosomal enzymes and cathepsine B and determined the tissue distribution of the five CMPul-DXR conjugates selected.

Synthesis

Scheme 1 presents the synthetic route for CMPul-peptide-DXR conjugates. Condensation of *N*-triphenylmethyl (trityl) peptides (**1**) with DXR in the presence of dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HOSu) gave *N*-trityl aminoacyl derivatives. The *N*-trityl groups were smoothly removed by treatment with 75% AcOH for 1 h at 25 °C. The acetate

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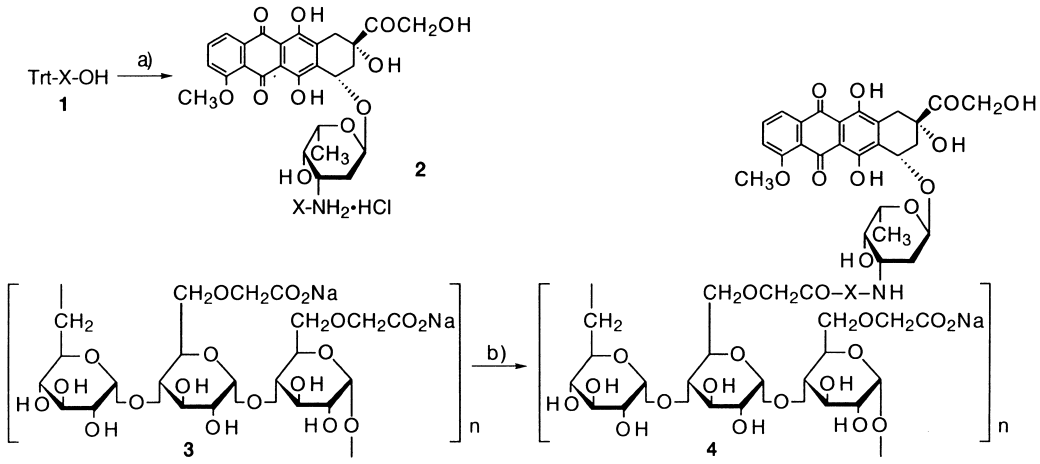

Scheme 1. Reagents and conditions: (a) (i) DCC-HOSu, DMF, 4 °C; (ii) DXR, DMF, 4 °C; (iii) 75% AcOH; (iv) AG® 2-X8 resin (Cl^-); (b) **2**, EEDQ, DMF:H_2O (1:1).

salts of peptidyl DXR were converted to their hydrochloride salts (**2**) by eluting the substrate through AG® 2-X8 ion exchange resin (Cl^- form, Bio Rad).[9] CMPul (**3**), which was prepared by treatment of pullulan (molecular weight, 150 kDa) with chloroacetic acid in alkaline solution, was conjugated with peptidyl-DXR·HCl (**2**) in the presence of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in a solution of DMF:H_2O (1:1) to afford the resulting conjugates (**4**). The DXR contents of CMPul-DXR conjugates, determined by measuring the absorbance at 480 nm, were 5.6–6.9% as shown in Table 1.

Antitumor Effects In Vivo

Walker 256 cells were inoculated intramuscularly to the inguinal region of 6-weeks-old female Wistar rats at 1×10^7 cells per rat. CMPul-DXR conjugates or DXR·HCl were dissolved in saline and administered once intravenously at 3 days after the tumor inoculation. Five animals were used in at least four groups. The tumor was excised and weighed at 4 days after its administration for rats bearing Walker 256 tumor. The tumor weight of the treated group (*T*) was compared with that of the control group (*C*). The ED_{50} was calculated from *T*/*C* (%) by Probit analysis.

In Vitro Digestion by Lysosomal Enzymes

A mixture of rat liver lysosomes was isolated by differential centrifugation according to the method of Trouet.[10] CMPul-DXR conjugates (DXR = 18 μM) were incubated at 37 °C in 0.2 M citrate buffer (pH 5.5) (1 mL), containing EDTA (1 mM), 0.2% Triton X-100, reduced glutathione (5 mM) and enzyme solution (1.0 mg/mL). At 24 h after the incubation, 0.1 mL of the incubation mixture was placed in a propylene tube and MeOH solution (0.1 mL) containing daunorubicin (DNR) (1.9 μM) was added as an internal standard. One mL of H_2O, 1 M Na_2CO_3/$NaHCO_3$ (pH 9.8) (0.1 mL) and $CHCl_3$:MeOH (3:1) (2.5 mL) were added and mixed. The organic layer was evaporated and free DXR was determined by HPLC using the method described previously.[11] In the case of cathepsine B, 1 unit/mL solution was used instead of lysosomal enzymes.

Table 1. In vivo antitumor effects and in vitro drug release of CMPul-DXR conjugates

	Spacer	DXR content[a] (%)	Relative antitumor effect[b]	Lysosome[c] (%)	Cathepsine B[c] (%)
4a	Gly-Gly-Gly-Phe-Gly	6.1	0.39	60.5	17.5
4b	Gly-Gly-Phe-Gly	6.5	0.42	48.1	12.3
4c	Gly-Phe-Gly	6.2	0.40	46.2	12.9
4d	Phe-Gly	6.5	0.36	21.9	2.9
4e	Gly-Gly	6.9	>6.1	0.0	0.6
4f	Gly-Phe	6.3	>6.1	0.0	0.0
4g	Phe-Phe	5.6	1.77	0.9	0.0
4h	D-Phe-Gly	6.2	>2.0	0.7	0.0
4i	Tyr-Gly	5.6	0.34	27.8	2.4
4j	Leu-Gly	5.6	0.38	25.4	2.9
4k	Ala-Gly	6.8	1.8	0.3	0.3
4l	Ser-Gly	6.8	>2.0	0.1	0.1
4m	Gly-Leu-Phe-Gly	6.2	0.40	89.0	41.0

[a]The amount of DXR was estimated spectrophotometrically at 480 nm in PBS.
[b](ED_{50} of CMPul-DXR conjugate/ED_{50} of DXR)×100. ED_{50} was determined for at least four data points. ED_{50} of DXR was 1.17 mg/kg. *T*/*C* of **4e** and **4f** at 8 mg/kg were 99 and 95%, and that of **4h** and **4l** at 2.4 mg/kg were 93 and 74%, respectively.
[c]DXR release from CMPul-DXR conjugates after 24 h in the presence of enzyme at 37 °C.

Evaluation of Tissue Distribution In Vivo

Walker 256 cells were inoculated intramuscularly to the inguinal region of 6-weeks-old female Wistar rats at 1×10^7 cells per rat. After 5 days, CMPul-DXR conjugates or DXR·HCl in saline was administered intravenously at a dose of 2.5 mg/kg as DXR. Blood samples were collected and the rats were sacrificed under ether anesthesia at 6 h after the administration. The heart, liver, spleen, and tumor were excised and weighed. Blood was centrifuged at 3000 rpm for 5 min to obtain plasma samples. Plasma samples were diluted with 4 volumes of phosphate-buffered saline (PBS) (pH 7.4), and the tissues were homogenized with 5 volumes of PBS and subjected to the HPLC assay described previously.[12]

Results and Discussion

The therapeutic effects of CMPul-DXR conjugates and DXR were determined by the ED_{50} as shown in Table 1. Initially, in order to examine the optimal length of the peptide spacer of CMPul-DXR conjugates on the in vivo antitumor effect, we synthesized these conjugates bound by Phe-Gly with elongation in the N-terminal direction by Gly from zero to three (**4a–d**). We also compared the in vivo antitumor effect of these conjugates with that of the CMPul-DXR conjugate bound by Gly-Leu-Phe-Gly (**4m**), which is known as spacer of *N*-(2-hydroxypropyl)-methacrylamide (HPMA)-DXR conjugate highly cleavable by lysosomal enzymes.[7] Since all CMPul-DXR conjugates (**4a–d, 4m**) showed the same potency and a greater antitumor effect than DXR, CMPul-Phe-Gly-DXR (**4d**) was selected as our prototype to be subjected to further transformation. We next compared the in vivo antitumor effects of four CMPul-DXR conjugates bound by dipeptide spacers with the combination of Phe and Gly. Great differences in antitumor effects were noted among the four conjugates (**4d–g**). Although CMPul-Phe-Gly-DXR (**4d**) showed a higher antitumor effect than DXR, CMPul-Phe-Phe-DXR (**4g**) exhibited a lesser antitumor effect than DXR. CMPul-Gly-Gly-DXR (**4e**) and CMPul-Gly-Phe-DXR (**4f**) showed no antitumor effect at the high dose of 8 mg/kg. Next, we explored the N-terminal amino acid of the dipeptide spacer with Gly at the C-terminal amino acid kept constant. Hydrophobic amino acid such as Tyr (**4i**) or Leu (**4j**) led to the same potency as Phe (**4d**). However, this trend was not observed when D-Phe (**4h**) was used. CMPul-Ala-Gly-DXR (**4k**) or CMPul-Ser-Gly-DXR (**4l**) showed less antitumor activity than DXR.

Trouet et al. showed that succinyalbumin-DNR conjugate bound through Leu-Ala-Leu or Ala-Leu-Ala-Leu was much more active against L 1210 leukemia in mice than DNR, while the conjugate bound by Ala-Leu was similar to DNR at low doses.[6] Subr et al. reported that the HPMA-DXR conjugate bound by biodegradable tri- or tetrapeptide was more active than DXR against mice bearing L 1210.[7] However, our finding is the first example of the composition of amino acids of dipeptide spacers significantly affecting the in vivo antitumor activity and of the in vivo effectiveness of an appropriate dipeptide spacer between the carrier and the drug.

The release of DXR from the conjugates in the presence of rat liver lysosomal enzyme or cathepsine B over 24 h is shown in Table 1. The drug release from four conjugates (**4a–d**) having peptide spacer of different lengths increased with the peptide spacer size. Furthermore, the largest drug release was observed using Gly-Leu-Phe-Gly (**4m**) as the spacer. The amounts of drug release from CMPul-Phe-Gly-DXR (**4d**), CMPul-Tyr-Gly-DXR (**4i**) and CMPul-Leu-Gly-DXR (**4j**) were very similar. On the other hand, other conjugates (**4e–g, 4h, 4l**), which showed less or no activity than DXR, released less than 1% of DXR in the presence of both enzymes. Recently, Dubowchik et al. demonstrated that cathepsine B-mediated drug release was enhanced when self-immolative *p*-aminobenzylcarbonyl spacer was inserted between DXR and lysosomal protease-sensitive peptides. They showed that dipeptides composed of a basic or strongly hydrogen bonding amino acid (Lys, Arg or Cit) at P_1 and a hydrophobic amino acid (i.e. Phe or Val) at P_2 are substrates for cathepsine B.[13] However, synthesis of these compounds were complicated and conjugation of the derivatives with macromolecules including monoclonal antibody would be also difficult because of the presence of a hydrogen bonding amino acid at P_1. We showed that dipeptides composed of a hydrophilic amino acid Gly instead of a hydrogen bonding amino acid at C-terminal amino acid and a hydrophobic amino acid (Phe, Tyr or Leu) at N-terminal amino acid are good substrates for lysosomal enzyme and cathepsine B.

Tissue distribution of the five CMPul-DXR conjugates selected and DXR was studied in rat bearing Walker 256 and compared with that of DXR. The results are shown in Table 2. We separately determined the concentration of free DXR, which was released from CMPul-DXR conjugate, and aglycone, which was obtained by acid hydrolysis of both CMPul-DXR conjugate and free DXR, as total DXR by the HPLC method.[12] All conjugates have similar concentrations of total DXR in each tissue. In particular, the tumor concentration of each conjugate was over 5 times higher than that of DXR. All conjugates were also highly distributed in the reticuloendothelial organs, such as the liver and spleen. On the other hand, each conjugate has different tissue concentrations of free DXR. When CMPul-D-Phe-Gly-DXR (**4h**), which showed no antitumor effect, was administered intravenously, no free DXR was detected in each tissue. A small amount of free DXR was detected in each tissue in the case of CMPul-Ala-Gly-DXR (**4k**), which was less effective than DXR. On the other hand, CMPul-Gly-Gly-Phe-Gly-DXR (**4b**), CMPul-Phe-Gly-DXR (**4d**) and CMPul-Leu-Gly-DXR (**4j**), which were more potent than DXR, showed the free DXR in each tissue. The tumor concentration of those three CMPul-DXR conjugates was above 3-fold higher than that of DXR. We have previously demonstrated that the in vivo antitumor effect of CMPul-DXR conjugates is correlated with the tumor

Table 2. Tissue distribution of total DXR (upper) and free DXR (under) of CMPul-DXR conjugates and DXR at 6 h after intravenous administration at a dose of 2.5 mg/kg as DXR for rat bearing Walker 256[a]

	Spacer	Tumor	Spleen	Liver	Heart	Plasma
4b	Gly-Gly-Phe-Gly	1.84 ± 0.11	2.34 ± 0.22	5.60 ± 0.24	0.35 ± 0.01	6.03 ± 0.32
		0.88 ± 0.35	1.74 ± 0.63	2.08 ± 0.78	0.09 ± 0.02	0
4d	Phe-Gly	2.38 ± 0.17	2.44 ± 0.18	3.78 ± 0.17	0.33 ± 0.03	4.43 ± 0.64
		1.16 ± 0.14	0.32 ± 0.03	0.53 ± 0.01	0.04 ± 0.01	0
4h	D-Phe-Gly	3.19 ± 0.39	4.42 ± 0.33	5.10 ± 0.15	0.30 ± 0.03	2.34 ± 0.22
		0	0	0	0	0
4j	Leu-Gly	4.13 ± 0.74	3.57 ± 0.17	4.47 ± 0.39	0.29 ± 0.02	2.67 ± 0.04
		1.75 ± 0.19	1.48 ± 0.27	1.58 ± 0.06	0.09 ± 0.00	0
4k	Ala-Gly	3.63 ± 0.24	5.36 ± 0.52	3.32 ± 0.22	0.35 ± 0.09	2.37 ± 0.13
		0.13 ± 0.00	0.11 ± 0.01	0.05 ± 0.01	0	0
DXR		0.35 ± 0.03	2.21 ± 0.22	0.54 ± 0.04	1.07 ± 0.11	0
		0.24 ± 0.03	1.01 ± 0.26	0.56 ± 0.06	1.13 ± 0.14	0

[a]% of dose/g tissue or mL. Data are mean ± S.E., n = three rats per point.

AUC of free DXR.[12] The present study also suggested that the in vivo antitumor effect of the conjugates was closely related with the amount of free DXR released from CMPul-DXR conjugate.

We found that CMPul-DXR conjugate bound to the tripeptide or larger peptides (**4a–c, 4m**) released several times more DXR than that from Phe-Gly (**4d**) over 24 h in the presence of lysosomal enzyme and cathepsine B. However, there was no preferential in vivo activity of the former conjugates against Walker 256. The results of the antitumor activity of CMPul-DXR conjugates compared well with that of in vivo tissue distribution. It is therefore likely that the CMPul-DXR conjugate was rapidly cleaved in vivo even with a low enzyme activity in vitro and this process contributed to loss of the preferential drug release in vivo of the conjugate with a high enzyme activity in vitro. Similar differences of enzyme activity in vitro and in vivo were observed with γ-glutamyl derivatives of sulfamethoxazole for kidney selective prodrug.[14]

Drug release from the macromolecular-drug conjugates are determined not only by the nature of the spacer between macromolecular carrier and drug but also the nature of the drug and the type of macromolecular carriers. For example, Rejmanova et al. investigated the degradability of HPMA-spacer-4-nitroanilide (NAP) copolymer by cathepsine B.[15] They found that HPMA-Gly-Phe-Leu-Gly-NAP released NAP first. On the other hand, Coessens et al. reported that antimicrobial drug norfloxacin was coupled with chloroformate-activated dextran via the same tetrapeptide spacer. The conjugate released Gly-norfloxacin in the presence of cathepsine B.[4] From the present results of in vitro digestion by lysosomal enzymes and cathepsine B, drug liberation from the conjugate paralleled each other in the two cases. Furthermore, hydrolysis of CMPul-DXR conjugates gave mostly DXR and little Gly-DXR was observed (data not shown). Therefore, we considered that CMPul-DXR conjugate releases DXR directly and cathepsine B plays an important role in the digestion of these conjugates.

In summary, systematic modification of peptide spacers showed that CMPul-DXR conjugates bound via appropriate dipeptide spacers such as Phe-Gly, Tyr-Gly or Leu-Gly were more potent than DXR. The CMPul-DXR conjugate was rapidly cleaved in vivo even with a low enzyme activity in vitro and this process contributed to loss of the preferential drug release in vivo of the conjugate with high enzyme activity in vitro.

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