Bioorganic & Medicinal Chemistry 17 (2009) 4647–4656

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Novel hyaluronic acid–methotrexate conjugates for osteoarthritis treatment

Akie Homma [a,*], Haruhiko Sato [a], Akira Okamachi [a], Takashi Emura [a], Takenori Ishizawa [a], Tatsuya Kato [a], Tetsu Matsuura [a], Shigeo Sato [a], Tatsuya Tamura [a], Yoshinobu Higuchi [a], Tomoyuki Watanabe [a], Hidetomo Kitamura [a], Kentaro Asanuma [a], Tadao Yamazaki [a], Masahisa Ikemi [b], Hironoshin Kitagawa [b], Tadashi Morikawa [b], Hitoshi Ikeya [b], Kazuaki Maeda [b], Koichi Takahashi [b], Kenji Nohmi [b], Noriyuki Izutani [b], Makoto Kanda [b], Ryochi Suzuki [b]

[a] Research Division, Chugai Pharmaceutical Co., Ltd, 1-135, Komakado, Gotemba, Shizuoka 412-8513, Japan
[b] Research Center, Denki Kagaku Kogyo K. K, 3-5-1 Asahimachi, Machida, Tokyo 194-8560, Japan

ARTICLE INFO

*Article history:*
Received 5 March 2009
Revised 27 April 2009
Accepted 28 April 2009
Available online 3 May 2009

*Keywords:*
Hyaluronic acid
Methotrexate
Osteoarthritis
Targeting drug delivery system

ABSTRACT

Hyaluronic acid (HA) provides synovial fluid viscoelasticity and has a lubricating effect. Injections of HA preparations into the knee joint are widely used as osteoarthritis therapy. The current HA products reduce pain but do not fully control inflammation. Oral methotrexate (MTX) has anti-inflammatory efficacy but is associated with severe adverse events. Based on the rationale that a conjugation of HA and MTX would combine the efficacy of the two clinically evaluated agents and avoid the risks of MTX alone, we designed HA–MTX conjugates in which the MTX connects with the HA through peptides susceptible to cleavage by lysosomal enzymes. Intra-articular injection of our HA–MTX conjugate (conjugate **4**) produced a significant reduction of the knee swelling in antigen-induced arthritis rat, whereas free MTX, HA or a mixture of HA and MTX showed no or marginal effects on the model. The efficacy of conjugate **4** was almost the same as that of MTX oral treatment. Conjugate **4** has potential as a compound for the treatment of osteoarthritis.

© 2009 Elsevier Ltd. All rights reserved.

## 1. Introduction

Osteoarthritis (OA) is characterized by degeneration of articular cartilage[1–4] and is commonly described as a noninflammatory disease. However, there is growing evidence suggesting that synovial inflammation is reflected in many of the signs and symptoms of the disease.[5,6] It is therefore anticipated that inhibition of the inflammatory component of OA may provide effective therapeutics for the treatment of OA.[7,8]

Oral methotrexate (MTX, Fig. 1) provides a very effective treatment to control the synovial inflammation of rheumatoid arthritis (RA).[9,10] Several lines of evidence have suggested that some pathological changes observed in the synovial inflammation of RA are also observed in that of OA.[6,11] It seems likely that MTX would be effective in altering at least some aspects of the OA pathogenic process. However, MTX is frequently associated with adverse events such as pneumonitis, liver fibrosis and myelosuppression that limit the application of the drug to the other arthritic diseases such as OA.[12,13] Intra-articular injection of MTX is a possible alternative route to reduce adverse effects while maintaining the antiproliferative and anti-inflammatory effects of the drug. Therefore, an effective carrier that would deliver MTX to the synovium and lower the systemic side effects is desirable.

Hyaluronic acid (HA) is an endogenous polysaccharide providing synovial fluid viscoelasticity and a lubricant effect.[14–16] Intra-articular HA is widely used as a symptom-modifying treatment for OA of the knee.[1,4,7,17–24] Intra-articularly injected HA is distributed in the synovium, and synovial cells have a mechanism for incorporating HA through the cell surface receptors such as CD44[22,25,26] for HA. It has been reported that HA can be used as a carrier when coupled with various agents.[27–39] Those conjugates are internalized into the cell through cell surface receptors, followed by intracellular release of the active drug.[40] In this study, we designed HA–MTX conjugates based on the rationale that a conjugation of HA and MTX would combine the efficacy of two clinically evaluated agents and avoid the risks of MTX. Current HA products reduce pain but do not fully control inflammation. We anticipate that HA–MTX conjugates would be

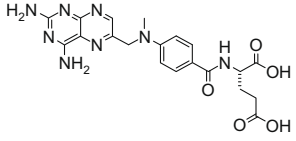


**Figure 1.** Methotrexate (MTX).

* Corresponding author. Tel.: +81 550 87 8643.
*E-mail address:* honmaake@chugai-pharm.co.jp (A. Homma).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.04.063

a safe and more efficacious intra-articular injection alternative to current HA products.

Herein we report the design of new HA–MTX conjugates. The conjugates demonstrated antiproliferative and anti-inflammatory efficacy in vitro and in vivo suggesting their capability to be a prototype for a future OA drug.

## 2. Design of conjugates

The template structure of the conjugates is shown in Figure 2. Several studies have suggested that conjugates of MTX coupled to drug carriers appear to increase therapeutic efficacy and reduce the side effects of MTX.[12,13,41–48] However, use of HA as a drug carrier to target MTX to the joints of OA patients has not been reported. Due to the properties of HA, HA–MTX conjugates can preferably accumulate in the synovium and be internalized into synovial cells through cell surface HA receptors. MTX acts inside the cell, thus the release of a pharmacologically active form of MTX from HA is important. We therefore introduced a lysosomal enzyme-sensitive peptide chain into the HA–MTX conjugate. We also introduced linkers as well as peptides to the conjugates to avoid a potential steric barrier to the proteases by the HA backbone. In the present study, we selected 4,7,10-trioxa-1,13-tridecanediamine (PEG13) as the linker, which is considered to be of sufficient length so as not to be a steric hindrance. To prove the concept, we synthesized three different types of HA–MTX conjugates. Type 1 (conjugates **1** and **2**) has no peptide. Type 2 (conjugate **3**, Gly-Phe-Leu-Gly[49]) and type 3 (conjugate **4**, Asn-Phe-Phe[50]) have peptide chains known to be susceptible to cleavage by lysosomal enzymes.

## 3. Synthesis

The synthesis of conjugates **1**–**4** is described in Schemes 1–3. To avoid epimerization of amino acids, the peptide was elongated toward the N-terminal.[51] In the case of **1** and **2** without a peptide, the control of the α- or γ- connection was almost perfect; however, **3** contained a mixture of α/γ of 3/1. Compound **18** was obtained as a mixture of α/γ (also 3/1) because of the equilibrium of the migration. The isomers were detectable and separable by HPLC. However, pure compound **18** was not obtained because of re-migration while removing the solvents. The equilibrium may have occurred during the coupling reaction between **18** and HA, and so the ratio of isomers was determined again by NMR analysis after conjugation. Similar equilibrium of migration was observed in the case of conjugate **4**. The pteridine part of **23** shown in Scheme 3 was pre-synthesized because of ease of use. The MTX binding ratio was calculated by the dividing the concentration of the MTX by the concentration of the disaccharide unit (GlcU-GlcNAC). These concentrations were obtained by gel permeation chromatography (GPC) analysis of the conjugates. GPC analysis determines both the binding ratio and the molecular weight (MW) of the conjugates at the same time and thus we demonstrated that the HA–MTX conjugates prepared have a MW as high as HA (~2000 kDa). Actually, we conducted a $^1$H NMR analysis and identified the acetyl of HA and the 7-position proton of the pteridine ring; however, the integration ratio was not robust because the HA peaks tended to be broad, and the respective pteridine 7-position proton peaks of the free MTX and the HA–MTX conjugate did not separate well. For the reaction of sodium hyaluronate with compounds **10a**, **10b**, **18** and **25**, tris[2-(2-methoxyethoxy)ethyl]-amine was used to carry out the reaction under non-acidic conditions. This amine was also expected to contribute to the homogenization in the reaction as a phase transfer catalyst.[52] As a matter of fact, the reaction mixture of the water–THF solution appeared homogeneous and elastic gel formed was easy to stir; the desired conjugates were obtained by basic hydrolysis of the methyl ester of the Glu of the MTX. When equivalents of 0.05–0.1 of compound **25** were used, the MTX binding ratio of **4** became 0.5–3%. Conjugates **1**, **2** and **3** were synthesized the same as **4**. During the coupling step, the MW of the conjugates tended to decrease. HA with a high MW (~2000 kDa) is known to be the preferred analgesic for knee joints of OA patients.[1,7,17–22] Therefore, it is critical that the HA–MTX conjugate maintains a MW as high as that of HA. To meet the requirement, the conjugation was optimized by using 1:1 water–THF solvent and 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOOBt); the reactions were carried out at 5 °C for 20 h. The MTX binding ratios for conjugates **1**, **2**, **3** and **4** were, respectively, 3.1%, 2.7%, 1.3% and 1.2% with MWs of 1610, 1610, 1850 and 1780 kDa.

## 4. Antiproliferative effect on human synovial fibroblasts stimulated by TNF-α

Effects of the HA–MTX conjugates with or without peptide chains on cell proliferation were examined using human

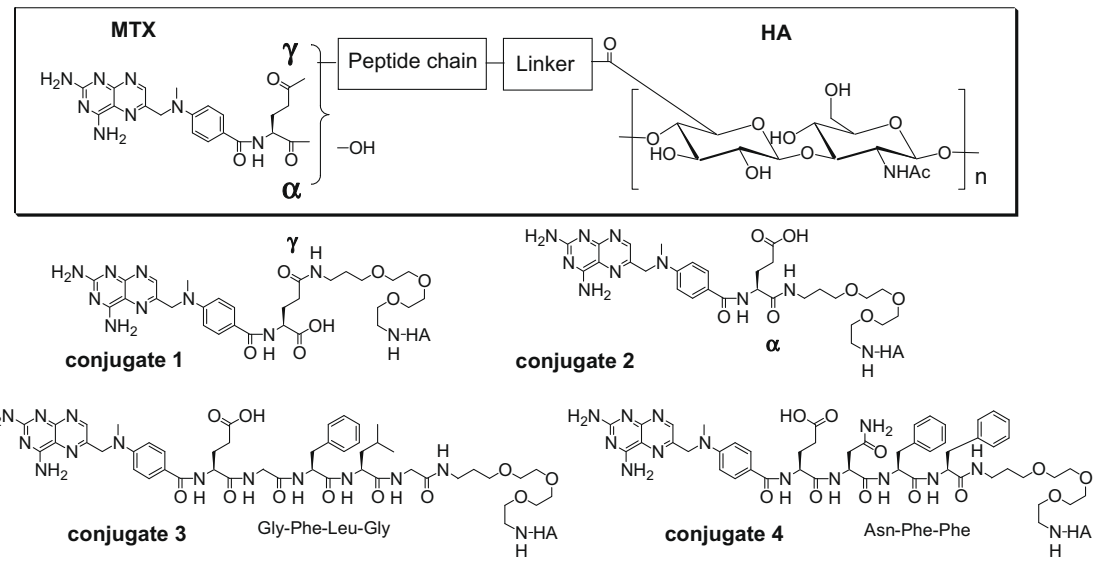
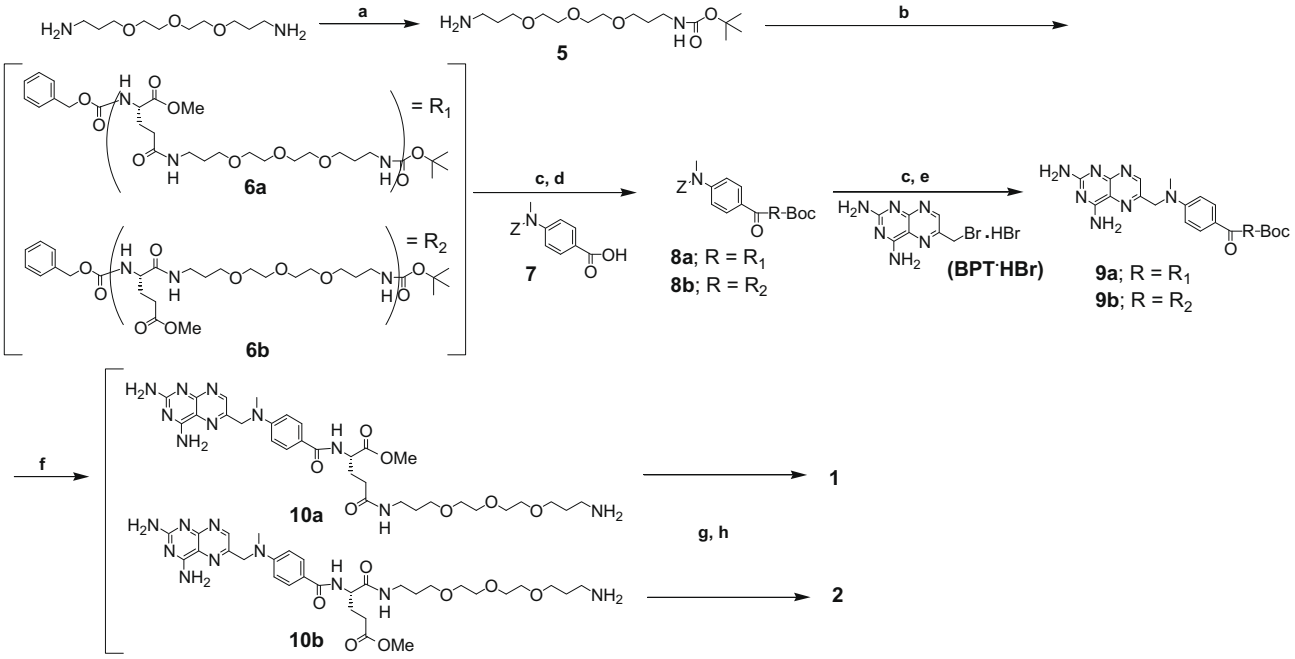
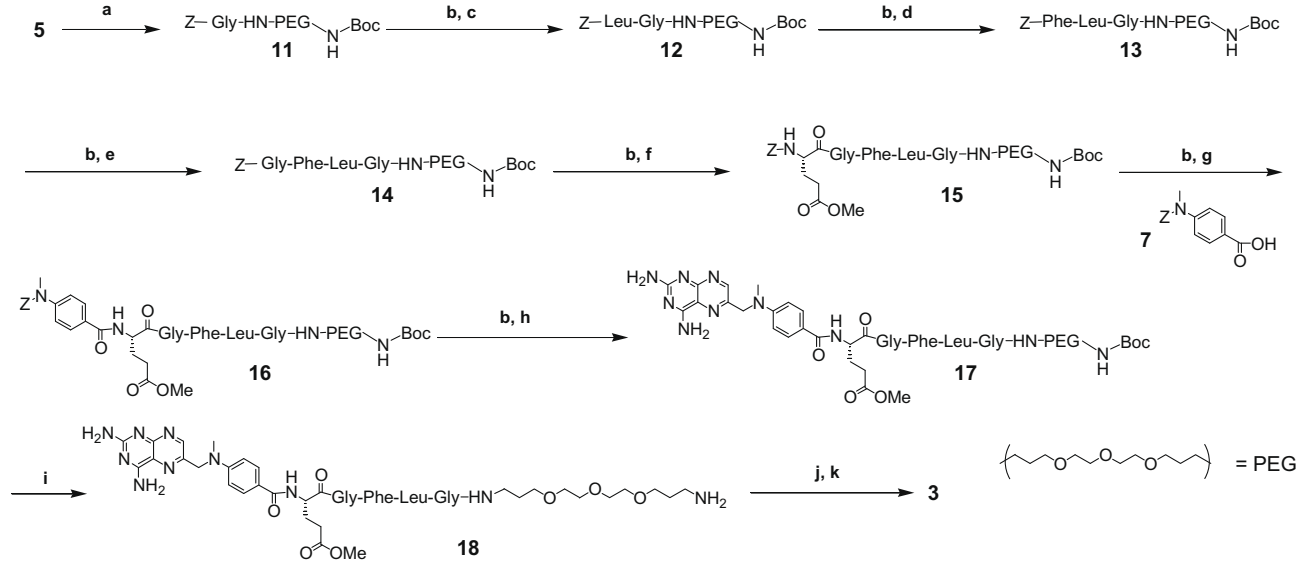



**Figure 2.** Drug design and structures of conjugates **1**–**4**.

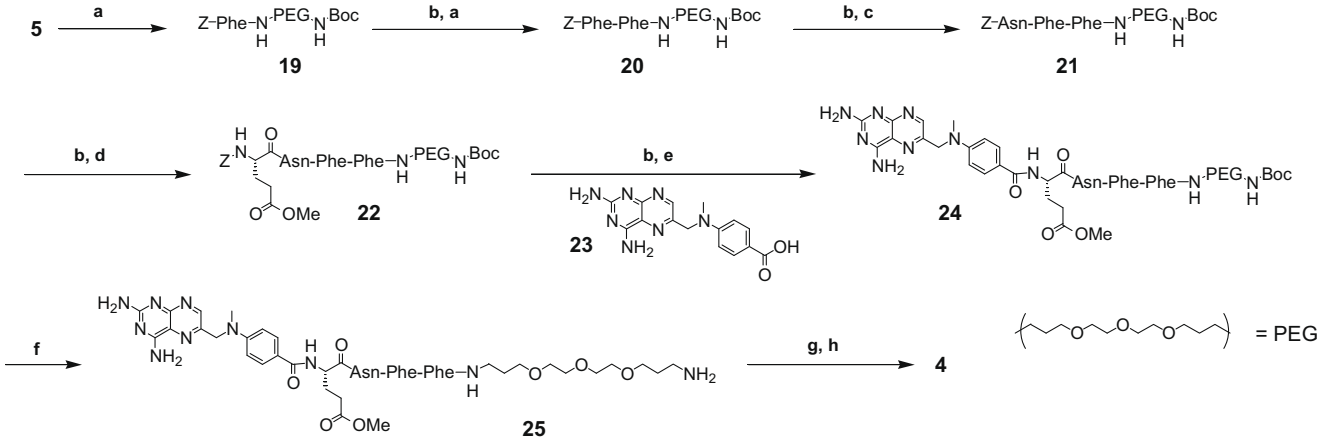


**Scheme 1.** Reagents and conditions: (a) $Boc_2O$, THF, −5 °C; (b) Z-Glu(OMe)-OH or Z-Glu(OH)-OMe, EDC–HOBt, DMF, 0 °C to rt; (c) $H_2$, Pd/C, methanol, rt; (d) (4-benzyloxycarbonylmethylamino)benzoic acid (**7**), EDC–HOBt, DMF, 0 °C to rt; (e) BPT·HBr, DMF, 65 °C; (f) TFA, 0 °C; (g) sodium hyaluronate, tris[2-(2-methoxyethoxy)ethyl]amine, EDC–HOOBt, THF–$H_2O$, 5 °C; (h) NaOH aq, 5 °C.



**Scheme 2.** Reagents and conditions: (a) Z-Gly-OH, EDC–HOSu, DMF, 0 °C to rt; (b) $H_2$, Pd/C, methanol, rt; (c) Z-Leu-ONp, $Et_3N$, DMF, rt; (d) Z-Phe-OH, EDC–HOBt, DMF, 0 °C to rt; (e) Z-Gly-OH, EDC–HOBt, DMF, 0 °C to rt; (f) Z-Glu(OMe)-OH, EDC–HOBt, DMF, 0 °C to rt; (g) (4-benzyloxycarbonylmethylamino)benzoic acid (**7**), EDC–HOBt, DMF, 0 °C to rt; (h) BPT·HBr, DMF, 65 °C; (i) TFA, 0 °C; (j) sodium hyaluronate, tris[2-(2-methoxyethoxy)ethyl]amine, EDC–HOOBt, THF–$H_2O$, 5 °C; (k) NaOH aq, 5 °C.

fibroblast-like synoviocytes (HFLS) stimulated by TNF-α. As shown in Table 1, conjugates lacking a peptide chain (**1** and **2**) showed no suppressive effects. In contrast, conjugates **3** (Gly-Phe-Leu-Gly) and **4** (Asn-Phe-Phe) clearly inhibited proliferation of HFLS, indicating that the peptide chains are required for the inhibitory effect of HA–MTX conjugates on HFLS proliferation. As mentioned above, synovial cells have a mechanism for incorporating HA through cell surface receptors for HA such as CD44. HA–MTX conjugates can also be incorporated into the synovial cells in a similar manner. It is therefore likely that the HA–MTX conjugates were cleaved by lysosomal enzymes such as cathepsins and the MTX released as a biologically active form inside the cells. We have not established a method for analyzing the metabolism of HA–MTX conjugates so we prepared MTX analogs with the peptides of conjugates **3** and **4**, MTX-Gly-Phe-Leu-Gly and MTX-Asn-Phe-Phe, and examined whether they were cleaved by cathepsins. MTX-monopeptides are known to have activity[42–46,53] or be further cleaved to MTX by a peptidase such as a carboxypeptidase and, in our preliminary studies, both Gly-Phe-Leu-Gly and Asn-Phe-Phe were cleaved by cathepsins B, D and L to produce MTX, MTX-Gly or MTX-Asn. These results suggest that conjugates **3** and **4** are likely to be cleaved by cathepsins and release biologically




**Scheme 3.** Reagents and conditions: (a) Z-Phe-OH, EDC–HOBt, DMF, 0 °C to rt; (b) $H_2$, Pd/C, methanol, rt; (c) Z-Asn-OH, EDC–HOBt, DMF, 0 °C to rt; (d) Z-Glu(OMe)-OH, EDC–HOBt, DMF, 0 °C to rt; (e) **23**, EDC–HOBt, DMF, rt; (f) TFA, 0 °C; (g) sodium hyaluronate, tris[2-(2-methoxyethoxy)ethyl]amine, EDC–HOOBt, THF–$H_2O$, 5 °C; (h) NaOH aq, 5 °C.

**Table 1**
Effects of the peptide chain of HA–MTX conjugates on inhibition of human synovial fibroblasts

| Compounds | Inhibition of cell proliferation $IC_{50}$, μmol/L [a] | |
|---|---|---|
| HA | — | (>1.0) |
| **1** | >77 | (1.0) |
| **2** | >67 | (1.0) |
| **3** | 0.30 | (0.01) |
| **4** | 0.35 | (0.01) |
| MTX | 0.05 | — |

Data represent $IC_{50}$ values of the compounds converted to equivalent concentrations of MTX.
[a] Equivalent to the concentration of HA (mg/mL).

active MTX analogs inside the cell. Further studies are required to clarify the mechanism of conjugates **3** and **4** in synovial cells.

## 5. Anti-inflammatory effect in rat antigen-induced arthritis

The in vivo anti-inflammatory effects of the HA–MTX conjugates were evaluated in antigen-induced arthritis rats. Arthritis was induced in one knee joint by intra-articular injection of methylated bovine serum albumin (mBSA).[54–56] Conjugates **3**, **4**, HA (at a dose of 0.3 mg as HA) and vehicle (saline) were intra-articularly injected once a week from 7 days before inducing arthritis. MTX, at the dose of 0.5 mg/kg and vehicle (PBS) were administered orally 5 times a week from 7 days before inducing arthritis. Conjugate **4** showed significant reduction of knee swelling but HA did not (Fig. 3A). Conjugate **3** having a similar MTX binding ratio and MW of HA showed only tendency to inhibit knee swelling, suggesting that cleavage of the conjugate in vivo depends on the peptide composition. In quantifying the AUC (in this case, joint swelling over time), the efficacy of conjugate **4** was almost the same as the oral treatment of MTX (Fig. 3C). The weekly dosage of MTX administered was ~10 nmol for conjugate **4** and 1500 nmol for oral MTX.

In the comparison of the efficacy of intra-articular injection of HA–MTX conjugate **4** with free MTX, HA, and a mixture of HA and MTX in antigen-induced arthritis rats, conjugate **4** showed a significant reduction in knee swelling, whereas free MTX did not (Fig. 4). Free HA and HA + MTX showed only marginal suppression of joint swelling. These results are consistent with our theory that intra-articularly-injected free MTX or a mixture of HA and MTX disappear from the joint cavity, but HA–MTX conjugates remain much longer.

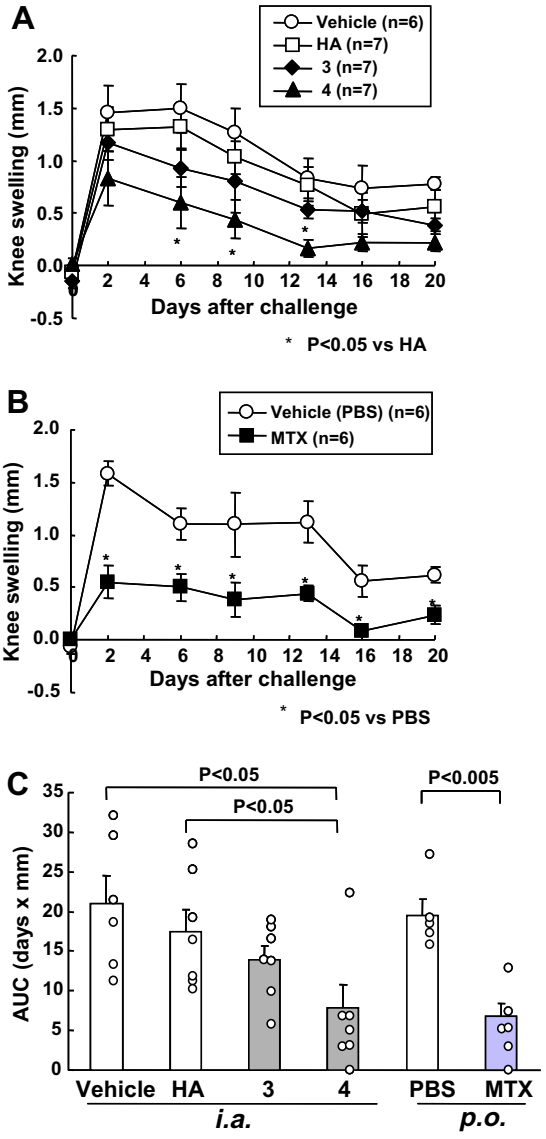



**Figure 3.** Effects of HA, conjugates **3**, **4** and MTX on knee swelling in rats with antigen-induced arthritis. Knee swelling, the difference in width between the right and left knee was measured from days 0 to 20 (A and B) and the AUC for knee swelling (C) calculated. Results are expressed as mean ± SE or mean + SE.

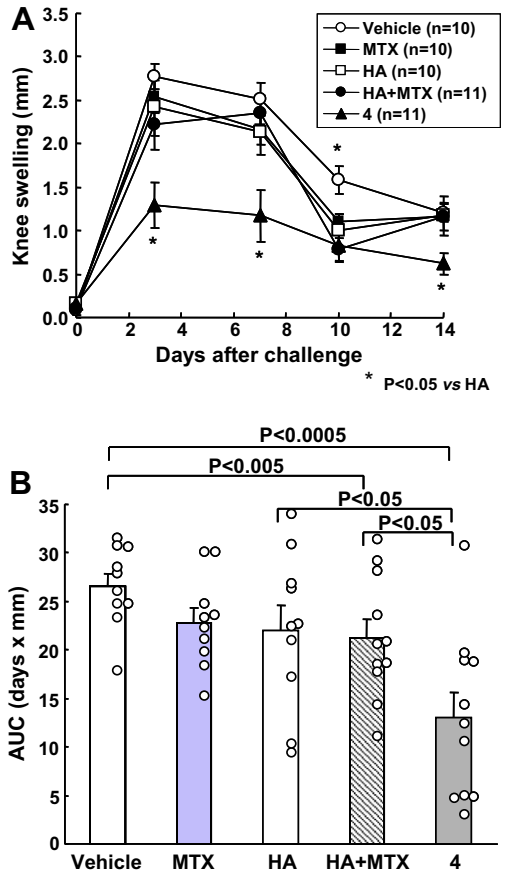



**Figure 4.** Effects of HA, MTX, HA + MTX and conjugate **4** on knee swelling in rats with antigen-induced arthritis. Knee swelling (the difference in width between the right and left knee) was measured from days 0 to 14 (A), and the AUC for knee swelling was calculated (B). Results are expressed as mean ± SE or mean + SE.

## 6. Conclusion

We synthesized novel HA–MTX conjugates with a peptide chain and linker and examined the potential of these compounds have as anti-arthritic drugs using both in vitro and in vivo assay systems. One HA–MTX conjugate (**4**) was found to have both antiproliferative and anti-inflammatory effects. The antiproliferative effect of the conjugates on HFLS in comparison with non-peptide conjugates indicates that enzymatic cleavage of the peptide is requisite for exerting activity. In antigen-induced arthritis rats, intra-articularly-injected conjugate **4** suppressed knee swelling as potently as does oral MTX. However, intra-articularly injected free MTX and the mixture of HA and MTX showed little or no effect in the antigen-induced rats, probably due to rapid clearance from the joint cavity. The dose of the MTX of conjugate **4** injected was approximately one hundred fiftieth the amount of an oral dose of MTX. Taken together, these results suggest that conjugate **4** is a promising lead compound for the development of safe and effective future OA drugs.

## 7. Experimental

### 7.1. Chemistry

All amino acid derivatives, 1-hydroxybenzotriazole monohydrate (HOBt·$H_2O$) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) were purchased from Watanabe Chemical Industries, Ltd. Hyaluronic acid was supplied by Denki Kagaku Kogyo, K. K. All solvents were purchased from Kanto Chemical Co., Inc. 4-Methylaminobenzoic acid was purchased from Tokyo Chemical Industry Co., Ltd. 6-Bromomethyl-pteridine-2,4-diamine trihydrobromide (BPT·HBr) was purchased from Ube Industries, Ltd. All other compounds were of the best available commercial grade. $^1$H NMR spectra were recorded on a JEOL ECP 270 or 500 MHz or a Mercury 300 MHz instrument in $CDCl_3$, DMSO-$d_6$ or deuterium oxide solutions. Low-resolution mass spectra were determined on LC/PDA/MS (HP 1100/TSP UV 6000/LCQ Classic, LC–MS) system using Cadenza CD-$C_{18}$ (3.0 mm I.D. × 20 mm) column at 35 °C. λ was 190–400 nm total area with retention times evaluated in minutes. The solvents ran as follows: (A) 0.05% TFA, $H_2O$, (B) 0.05% TFA, MeCN, gradient (A/B): 95/5–0/100 (9.5 min), 0/100 (2.5 min), flow rate: 1.0 mL/min. The binding ratio of MTX and the MW of HA–MTX conjugates were determined by GPC on an LC/PDA/RI (Waters Alliance 2695/2996/2414) system using Shodex OHpak SB-806 HQ (8.0 mm I.D. × 300 mm) column at 40 °C. RI was analyzed at 35 °C. λ was detected at 259 nm. The solvent ran as follows: 50 mM sodium phosphate (pH 6.0), Flow rate: 0.3 mL/min, injection volume: 100 μL.

#### 7.1.1. 4-[(2,4-Diaminopteridin-6-ylmethyl)-methylamino]benzoic acid (23)

**7.1.1.1. 4-Methylaminobenzoic acid methyl ester.**[57] A dry methanol (500 mL) solution of 10.28 g (68.0 mmol) of 4-methylaminobenzoic acid was cooled by ice bath. Then 7.4 ml (102.0 mmol) of $SOCl_2$ was added. After 5 min in an ice bath, the reaction mixture was refluxed for 3 h. The reaction mixture was then quenched with saturated $NaHCO_3$ at 0 °C and extracted with ethyl acetate. The organic layer was washed with brine, dried with anhydrous sodium sulfate, filtered and evaporated. The product was purified by silica gel column chromatography (eluent; chloroform/methanol, 100/5) to give the titled ester as a white solid (10.96 g, 98%). $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 2.88 (d, 3H, $J$ = 4.9 Hz), 3.85 (s, 3H), 4.16 (s, 1H), 6.55 (d, 2H, $J$ = 8.9 Hz), 7.87 (d, 2H, $J$ = 8.9 Hz).

**7.1.1.2. 4-[(2,4-Diaminopteridin-6-ylmethyl)-methylamino]benzoic acid methyl ester.**[58–60] 4-Methylaminobenzoic acid methyl ester (10.96 g, 66.3 mmol) and BPT·HBr (22.29 g, 66.3 mmol) were dissolved in 200 mL of *N*,*N*-dimethylacetamide (DMA). After being kept at 60–70 °C for 1 day, the reaction mixture was cooled to 0 °C, and ethyl acetate and saturated $NaHCO_3$ were added. The precipitate was filtered and dried at 30 °C for 1 day. The titled ester (21.54 g, 63.5 mmol, 96%) was obtained as a yellow solid. $^1$H NMR (270 MHz, DMSO-$d_6$) $\delta$: 3.25 (s, 3H), 3.74 (s, 3H), 4.84 (s, 2H), 6.85 (d, 2H, $J$ = 8.9 Hz), 7.30 (br, 2H), 7.75 (d, 2H, $J$ = 8.9 Hz), 8.21 (br, 1H), 8.43 (br, 1H), 8.66 (s, 1H). LC–MS $m/z$: 340.1 $(M+H)^+$.

**7.1.1.3. 4-[(2,4-Diaminopteridin-6-ylmethyl)-methylamino]benzoic acid (23).**[61] 16.54 g (48.7 mmol) of 4-[(2,4-diaminopteridin-6-ylmethyl)methylamino]-benzoic acid methyl ester was dissolved in 300 mL of DMSO and aqueous NaOH (5.85 g, 164.2 mmol of NaOH dissolved in 70 mL of water) was added to the solution. After stirring for 3.5 h, 400 mL of water was added to the reaction mixture. The solution was cooled to 0 °C and neutralized to pH 5 with a 10% acetic acid solution. The precipitate was filtered and dried at 30 °C. The titled compound (12.1 g, 37.2 mmol, 76%) was obtained as a yellow solid. $^1$H NMR (270 MHz, DMSO-$d_6$) $\delta$: 3.22 (s, 3H), 4.78 (s, 2H), 6.82 (d, 2H, $J$ = 8.6 Hz), 7.43 (br, 1H), 7.70 (br, 1H), 7.73 (d, 2H, $J$ = 8.6 Hz), 8.58 (s, 1H). LC–MS $m/z$: 326.1 $(M+H)^+$.

#### 7.1.2. *N-tert*-butoxycarbonyl-4,7,10-trioxa-1,13-tridecandiamine (Boc-NH-PEG-$NH_2$, 5)[62]

To a solution of 50 g (0.227 mol) of 4,7,10-trioxa-1,13-tridecandiamine in dry THF (80 mL) under an nitrogen atmosphere at a

temperature of −5 °C was added dropwise di-*tert*-butyl dicarbonate ($Boc_2O$, 14.6 g, 0.07 mol, 0.3 equiv) in dry THF (80 mL). After 1 h, THF was evaporated in vacuo and to the resulting mixture was added 1 N HCl (to pH 4) at a temperature of 0 °C and extracted with ethyl acetate (500 mL) to remove the diprotected byproduct. To the aqueous layer was added a $Na_2CO_3$ solution (to pH 10) and brine and stirred for an hour. Then the solution was extracted with ethyl acetate and the organic layer was dried over sodium sulfate and evaporated. The product was purified by silica gel column chromatography (eluent; chloroform/methanol/17% $NH_3$aq, 100/10/1), obtaining 5.84 g (0.018 mol, 8.0%) of desired compound **5** as an oil. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$: 1.44 (s, 9H), 1.71–1.80 (m, 4H), 2.06 (br, 2H), 2.83 (t, 2H, $J$ = 6.6 Hz), 3.22 (q, 2H, $J$ = 6.0 Hz), 3.52–3.67 (m, 12H), 5.09 (br, 1H). LC–MS $m/z$: 321.0 $(M+H)^+$.

### 7.1.3. Z-Glu(OMe)-NH-PEG-NH-Boc (6b)[63,64]

To a solution of 500 mg (1.56 mmol) of **5** in 5 mL of dry *N*,*N*-dimethylformamide (DMF) was added *N*-α-carbobenzoxy-L-glutamic acid γ-methyl ester (Z-Glu(OMe)-OH, 461 mg, 1.56 mmol, 1.00 equiv), HOBt·$H_2O$ (119 mg, 0.78 mmol, 0.50 equiv) and EDC·HCl (329 mg, 1.72 mmol, 1.10 equiv) at 0 °C. After being kept at 0 °C for 2 h, the reaction mixture was warmed to room temperature. After 3 h ethyl acetate was added. The organic layer was washed with saturated $NaHCO_3$ and brine, dried over anhydrous sodium sulfate and evaporated. The product was purified by silica gel column chromatography (eluent; dichloromethane/methanol, 100/3) to afford 892 mg (1.49 mmol, 95.7%) of the desired compound **6b** as colorless oil. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.43 (s, 9H), 1.72–1.76 (m, 4H), 1.89–2.22 (m, 2H), 2.38–2.57 (m, 2H), 3.18–3.21 (m, 2H), 3.35–3.37 (2H, m), 3.49–3.64 (m, 12H), 3.66 (s, 3H), 4.21 (br, 1H), 5.02 (br, 1H), 5.10 (s, 2H), 5.79 (br, 1H), 6.90 (br, 1H), 7.26–7.34 (m, 5H). LC–MS $m/z$: 598.3 $(M+H)^+$.

### 7.1.4. Z-Glu-OMe(NH-PEG-NH-Boc) (6a)

Substitution of *N*-α-carbobenzoxy-L-glutamic acid α-methyl ester (Z-Glu(OH)-OMe) for Z-Glu(OMe)-OH, γ-compound (931 mg, 1.56 mmol, 99.8%) was obtained as a colorless oil. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.43 (s, 9H), 1.69–1.78 (m, 4H), 1.96–2.23 (m, 4H), 3.19–3.13 (m, 2H), 3.34–3.38 (m, 2H), 3.49–3.61 (m, 12H), 3.74 (s, 3H), 4.34 (br, 1H), 5.00 (br, 1H), 5.01 (s, 2H), 5.94 (br, 1H), 6.50 (br, 1H), 7.26–7.34 (m, 5H). LC–MS $m/z$: 598.3 $(M+H)^+$.

### 7.1.5. 4-(Benzyloxycarbonylmethylamino)benzoic acid (7)[65]

To a solution of 4.0 g (26.5 mmol) of 4-methylaminobenzoic acid in100 mL of ether was added $NaHCO_3$ solution (10 g, 119 mmol in 65 mL of water) and dropwise of benzyl chloroformate (6.7 g, 39.3 mmol, 1.5 equiv) at the temperature of 0 °C. The reaction mixture was stirred at 0 °C for 1.5 h and at room temperature for 2.5 h. Then the solution was cooled to 0 °C and 50 mL of 1 N HCl solution added. The precipitate which formed was filtered and dried at reduced pressure. The filtrate was dissolved with ethyl acetate and the organic layer was washed with brine, dried over sodium sulfate and evaporated. The precipitate and the residue were suspended in ethyl acetate and hexane. The desired product (6.37 g, 22.3 mmol, 84%) was obtained by filtration. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 3.38 (s, 3H), 5.20 (s, 2H), 7.34 (m, 5H), 7.39 (d, 2H, $J$ = 8.4 Hz), 8.07 (d, 2H, $J$ = 8.6 Hz). LC–MS $m/z$: 285.9 $(M+H)^+$.

### 7.1.6. 4-Z-N(Me)$C_6H_4$-C(=O)-Glu(OMe)-NH-PEG-NH-Boc (8b)

889 mg (1.49 mmol) of compound **6b** was dissolved in 10 mL of methanol. To the mixture, 30 mg of 10% palladium on carbon (Pd–C) was added. After stirring under hydrogen atmosphere for 80 min, the reaction mixture was filtered and evaporated to dryness at reduced pressure, and the residue was dissolved in 10 mL of dry DMF. To the solution 4-(benzyloxycarbonylmethylamino)benzoic acid (**7**, 509 mg, 1.78 mmol, 1.20 equiv), HOBt·$H_2O$ (228 mg, 1.49 mmol, 1.0 equiv) and EDC·HCl (341 mg, 1.78 mmol, 1.20 equiv) were added at 0 °C. The reaction mixture was warmed to room temperature, stirred for 18 h, and ethyl acetate added. The organic layer was washed with saturated $NaHCO_3$ and brine, dried over sodium sulfate and evaporated. The desired compound **8b** was purified by silica gel column chromatography (eluent; dichloromethane/methanol, 100/5) to afford 1.06 g (1.45 mmol, 97%) as colorless oil. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.43 (s, 9H), 1.73–1.82 (m, 4H), 2.06–2.39 (m, 2H), 2.45–2.68 (m, 2H), 3.17–3.31 (m, 2H), 3.35 (s, 3H), 3.36–3.49 (m, 2H), 3.56–3.63 (m, 12H), 3.65 (s, 3H), 4.61 (br, 1H), 5.10 (br, 1H), 5.18 (s, 2H), 7.00 (br, 1H), 7.30–7.36 (m, 8H), 7.81 (d, 2H, $J$ = 8.4 Hz). LC–MS $m/z$: 731.3 $(M+H)^+$.

### 7.1.7. 4-Z-N(Me)$C_6H_4$-C(=O)-Glu-OMe(NH-PEG-NH-Boc) (8a)

Substitution of a γ-derived compound **6a** (928 mg, 1.55 mmol) for α-derived one and the use of **7** (532 mg, 1.86 mmol, 1.20 equiv), HOBt·$H_2O$ (237 mg, 1.66 mmol, 1.0 equiv) and EDC·HCl (357 mg, 1.86 mmol, 1.20 equiv) provided γ-compound **8a** (931 mg, 1.56 mmol, 99.8%) as a colorless oil. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.43 (s, 9H), 1.71–1.76 (m, 4H), 2.19–2.40 (m, 4H), 3.16–3.33 (m, 2H), 3.33–3.40 (m, 2H), 3.35 (s, 3H), 3.48–3.60 (m, 12H), 3.76 (s, 3H), 4.65 (br, 1H), 5.00 (br, 1H), 5.18 (s, 2H), 6.72 (br, 1H), 7.30–7.36 (m, 7H), 7.88 (d, 2H, $J$ = 8.4 Hz), 8.10 (br, 1H). LC–MS $m/z$: 731.3 $(M+H)^+$.

### 7.1.8. MTX(OMe)-α-NH-PEG-NH-Boc (9b)[59]

1.06 g (1.45 mmol) of compound **8b** was dissolved in 10 mL of methanol. To the mixture, 30 mg of 10% Pd–C was added. After stirring under hydrogen atmosphere for 1 h, the reaction mixture was filtered and evaporated, and the residue was dissolved in 7 mL of dry DMA. To the solution, BPT·HBr (536 mg, 1.60 mmol, 1.10 equiv) was added and stirred at 65 °C for 9 h. The reaction mixture was diluted dichloromethane and purified by column chromatography (aminosilica gel, eluent; dichloromethane/methanol, 100/3–100/10, 100/2–100/3, 2 times). The desired compound **9b** was obtained 611 mg (0.79 mmol, 55%) as a yellow amorphous substance. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.42 (s, 9H), 1.73–1.80 (m, 4H), 2.05–2.70 (m, 4H), 3.16–3.25 (m, 2H), 3.20 (s, 3H), 3.36–3.40 (m, 2H), 3.51–3.59 (m, 12H), 3.64 (s, 3H), 4.62 (br, 1H), 4.76 (s, 2H), 5.15 (br, 3H), 6.76 (d, 2H, $J$ = 8.6 Hz), 6.50–7.20 (br, 4H), 7.72 (d, 2H, $J$ = 8.6 Hz), 8.66 (s, 1H). LC–MS $m/z$: 771.4 $(M+H)^+$.

### 7.1.9. MTX(OMe)-γ-NH-PEG-NH-Boc (9a)

Substitution of a γ-derived compound (**8a**, 1.00 g, 1.37 mmol) for an α-derived one and the use of BPT·HBr (483 mg, 1.44 mmol, 1.05 equiv) in 10 mL of DMA provided γ-compound (**9a**, 622 mg, 0.81 mmol, 59%) as a yellow amorphous substance. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.43 (s, 9H), 1.69–1.76 (m, 4H), 2.09–2.42 (m, 4H), 3.20 (s, 3H), 3.20–3.40 (m, 4H), 3.47–3.60 (m, 12H), 3.75 (s, 3H), 4.67 (br, 1H), 4.76 (s, 2H), 5.16 (br, 3H), 6.77 (d, 2H, $J$ = 8.9 Hz), 6.60–7.60 (br, 4H), 7.76 (d, 2H, $J$ = 8.9 Hz), 8.66 (s, 1H). LC–MS $m/z$: 771.4 $(M+H)^+$.

### 7.1.10. MTX(OMe)-α-NH-PEG-$NH_2$ (10b)

285 mg (0.37 mmol) of compound **9b** was dissolved in 3 mL of trifluoroacetic acid (TFA) at a temperature of 0 °C. After the reaction mixture was kept at 0 °C for 0.5 h, dichloromethane was added and evaporated to remove TFA and solvent. The residue was purified by column chromatography (aminosilica gel, eluent; dichloromethane/methanol, 100/5–100/8). The titled compound **10b** (211 mg, 0.31 mmol, 85%) was obtained as yellow oil. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.68–1.75 (m, 4H), 2.17–2.41 (m, 4H), 2.77 (t, 2H, $J$ = 6.6 Hz), 3.16 (s, 3H), 3.28–3.40 (m, 2H), 3.46–3.60 (m, 12H), 3.74 (s, 3H), 4.66 (br, 3H), 5.51 (s, 2H), 6.00–6.73 (br, 2H),

6.73 (d, 2H, $J$ = 8.9 Hz), 7.11 (br, 1H), 7.65–7.76 (m, 3H), 8.62 (s, 1H). LC–MS $m/z$: 671.4 $(M+H)^+$.

#### 7.1.11. MTX(OMe)-γ-NH-PEG-$NH_2$ (10a)

Substitution of a γ-derived compound (**9a**, 319 mg, 0.41 mmol) for an α-derived one provided γ-compound **10a** (238 mg, 0.35 mmol, 85%) as a yellow oil. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.67–1.80 (m, 4H), 2.06–2.31 (m, 2H), 2.41–2.60 (m, 2H), 2.79 (t, 2H, $J$ = 6.6 Hz), 3.17 (s, 3H), 3.34–3.42 (m, 2H), 3.50–3.61 (m, 12H), 3.62 (s, 3H), 4.65 (br, 1H), 4.73 (s, 2H), 5.50 (s, 2H), 6.00–6.73 (br, 2H), 6.73 (d, 2H, $J$ = 8.6 Hz), 7.22 (s, 1H), 7.55 (br, 1H), 7.71 (d, 2H, $J$ = 8.9 Hz), 8.63 (s, 1H). LC–MS $m/z$: 671.4 $(M+H)^+$.

#### 7.1.12. Z-Gly-NH-PEG-NH-Boc (11)

To a solution of 3.50 g (10.9 mmol) of **5** in 30 mL of dry DMF was added *N*-α-carbobenzoxy-glycine (Z-Gly-OH, 2.29 g, 10.9 mmol, 1.0 equiv), *N*-hydroxy succinimide (HOSu) (628 mg, 5.46 mmol, 0.5 equiv) and EDC·HCl (2.09 g, 10.9 mmol, 1.0 equiv) at the temperature of 0 °C. The reaction mixture was warmed to room temperature. After stirring for 17 h, ethyl acetate was added. The organic layer was washed with 10% citric acid, saturated $NaHCO_3$ and brine, dried over sodium sulfate and evaporated. The product was purified by silica gel column chromatography (eluent; dichloromethane/methanol, 100/3) to afford 4.76 g (9.30 mmol, 85%) of the desired compound **11** as a colorless oil. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 1.43 (s, 9H), 1.68–1.75 (m, 4H), 3.14–3.19 (m, 2H), 3.37–3.41 (m, 2H), 3.47–3.58 (m, 12H), 3.83–3.85 (m, 2H), 4.96 (br, 1H), 5.13 (s, 2H), 5.82 (br, 1H), 6.98 (br, 1H), 7.32–7.35 (m, 5H). LC–MS $m/z$: 512.3 $(M+H)^+$.

#### 7.1.13. Z-Leu-Gly-NH-PEG-NH-Boc (12)

4.76 g (9.30 mmol) of compound **11** was dissolved in 45 mL of methanol. To the mixture 250 mg of 10% Pd–C was added. After stirring under hydrogen atmosphere for 2 h, the reaction mixture was filtered and evaporated. The residue was dissolved in 30 mL of dry DMF. To the solution, *N*-carbobenzoxy-L-leucine-*p*-nitrophenyl ester (Z-Leu-ONp, 3.60 g, 9.30 mmol, 1.0 equiv) and triethylamine (1.29 mL, 9.30 mmol, 1.0 equiv) were added at a temperature of 0 °C. The reaction mixture was warmed to room temperature and ethyl acetate was added following stirring for 17 h. After following the same procedure as described for **11**, the residue was obtained. The desired compound **12** was purified by silica gel column chromatography (eluent; dichloromethane/methanol, 100/5) to afford 5.56 g (8.90 mmol, 96%) as a colorless oil. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 0.94 (d, 6H, $J$ = 5.9 Hz), 1.43 (s, 9H), 1.53–1.80 (m, 7H), 3.17–3.22 (m, 2H), 3.35–3.40 (m, 2H), 3.48–3.63 (m, 12H), 3.77–4.01 (m ,2H), 4.23 (br, 1H), 5.10 (br, 3H), 5.38 (br, 1H), 6.85 (br, 1H), 7.30–7.34 (m, 6H). LC–MS $m/z$: 625.6 $(M+H)^+$.

#### 7.1.14. Z-Phe-Leu-Gly-NH-PEG-NH-Boc (13)

5.07 g (8.11 mmol) of compound **12** was dissolved in 30 mL of methanol. To the mixture, 300 mg of 10% Pd–C was added. After stirring under hydrogen atmosphere for 2 h, the reaction mixture was filtered and evaporated. The residue was dissolved in 20 mL of dry DMF. To the solution, *N*-carbobenzoxy-L-phenylalanine (Z-Phe-OH, 2.67 g, 8.93 mmol, 1.10 equiv), HOBt·$H_2O$ (1.24 g, 8.11 mmol, 1.10 equiv) and EDC·HCl (1.71 g, 8.93 mmol, 1.10 equiv) were added at the temperature of 0 °C. The reaction mixture was warmed to room temperature and ethyl acetate was added following stirring for 17 h. After following the same procedure as described for **11**, the residue was obtained. The desired compound was suspended in ethyl acetate–hexane and the product (**13**, 5.87 g, 7.60 mmol, 94%) was obtained by filtration as a white powder. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 0.88 (d, 6H, $J$ = 4.3 Hz), 1.43 (s, 9H), 1.72–1.81 (m, 7H), 3.07–3.25 (m, 4H), 3.35–3.40 (m, 2H), 3.49–3.63 (m, 12H), 3.84 (br, 2H), 4.20 (br, 2H), 5.07 (s, 2H & br, 1H), 5.21 (br, 1H), 6.43 (br, 1H), 6.80 (br, 2H), 7.16–7.33 (m, 10H). LC–MS $m/z$: 772.5 $(M+H)^+$.

#### 7.1.15. Z-Gly-Phe-Leu-Gly-NH-PEG-NH-Boc (14)

5.35 g (6.93 mmol) of compound **13** was dissolved in 20 mL of methanol. To the mixture, 300 mg of 10% Pd–C was added. After stirring under hydrogen atmosphere for 2 h, the reaction mixture was filtered and evaporated. The residue was dissolved in 20 mL of dry DMF. To the solution, Z-Gly-OH (1.59 g, 7.62 mmol, 1.10 equiv), HOBt·$H_2O$ (1.06 g, 6.93 mmol, 1.00 equiv) and EDC·HCl (1.46 g, 7.62 mmol, 1.10 equiv) were added at the temperature of 0 °C. The reaction mixture was warmed to room temperature and ethyl acetate was added following stirring for 17 h. After following the same procedure as described for **11**, the residue was obtained. The desired compound **14** was purified by silica gel column chromatography (eluent; dichloromethane/methanol, 100/5) to afford 5.61 g (6.77 mmol, 98%) as an amorphous substance. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 0.89 (d, 6H, $J$ = 6.5 Hz), 1.43 (s, 9H), 1.67–1.87 (m, 7H), 3.10–3.12 (m, 4H), 3.30–3.32 (m, 2H), 3.47–3.59 (m, 12H), 3.79 (br, 2H), 3.87 (br, 2H), 4.42 (br, 1H), 4.65 (br, 1H), 5.02 (s, 2H & br, 1H), 6.19 (br, 1H), 6.90–7.34 (m, 14H). LC–MS $m/z$: 829.6 $(M+H)^+$, 852.5 $(M+Na)^+$.

#### 7.1.16. Z-Glu(OMe)-Gly-Phe-Leu-Gly-NH-PEG-NH-Boc (15)

1.93 g (2.33 mmol) of compound **14** was dissolved in 10 mL of methanol. To the mixture, 300 mg of 10% Pd–C was added. After stirring under hydrogen atmosphere for 3 h, the reaction mixture was filtered and evaporated. The residue was dissolved in 7 mL of dry DMF. To the solution Z-Glu(OMe)-OH (756 mg, 2.56 mmol, 1.10 equiv), HOBt·$H_2O$ (356 mg, 2.33 mmol, 1.00 equiv) and EDC·HCl (491 mg, 2.56 mmol, 1.10 equiv) were added at the temperature of 0 °C. The reaction mixture was warmed to room temperature and ethyl acetate was added following stirring for 17 h. After following the same procedure as described for **11**, the residue was obtained. The product **15** was purified by silica gel column chromatography (eluent; dichloromethane/methanol, 100/5) to afford 2.10 g (2.16 mmol, 93%) as an amorphous substance. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 0.89 (d, 6H, $J$ = 5.7 Hz), 1.43 (s, 9H), 1.44–1.82 (m, 7H), 1.90–2.21 (m, 2H), 2.40–2.48 (m, 2H), 3.00–3.38 (m, 6H), 3.47–3.63 (m, 12H), 3.64 (s, 3H), 3.65–4.02 (m, 4H,), 4.25 (br, 1H), 4.50–4.79 (br, 2H), 5.02–5.14 (m, 3H), 6.59 (br, 1H), 7.00 (br, 1H), 7.17–7.32 (m, 13H), 7.82 (br, 1H). LC–MS $m/z$: 972.9 $(M+H)^+$, 994.6 $(M+Na)^+$.

#### 7.1.17. 4-Z-N(Me)$C_6H_4$-C(=O)-Glu(OMe)-Gly-Phe-Leu-Gly-NH-PEG-NH-Boc (16)

2.1 g (2.16 mmol) of compound **15** was dissolved in 10 mL of methanol. To the mixture, 400 mg of 10% Pd–C was added. After stirring under hydrogen atmosphere for 9 h, to the reaction mixture, 100 mg of Pd–C was added again. After stirring for 4 h, the reaction mixture was filtered and evaporated. The residue was dissolved in 6 mL of dry DMF. To the solution, **7** (740 mg, 2.59 mmol, 1.20 equiv), HOBt·$H_2O$ (331 mg, 2.16 mmol, 1.0 equiv) and EDC·HCl (497 mg, 2.59 mmol, 1.20 equiv) were added at 0 °C. The reaction mixture was warmed to room temperature and stirred for 18 h, and ethyl acetate was added. The organic layer was washed with saturated $NaHCO_3$ and brine, dried over sodium sulfate then *n*-hexane was added. The precipitate which formed was filtered and dissolved in small-scale dichloromethane. The product, **16**, was purified by silica gel column chromatography (eluent; dichloromethane/methanol, 100/5–100/6) to afford 2.02 g (1.83 mmol, 85%) as a white solid. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 0.84–0.91 (m, 6H), 1.41 (s, 9H), 1.59–1.78 (m, 7H), 2.17–2.21 (m, 2H), 2.41–2.78 (m, 2H), 3.09–3.35 (m, 8H), 3.36 (s, 3H), 3.45–3.60 (m, 14H), 3.70 (s, 3H), 3.94–4.02 (m,

2H), 4.37–4.63 (br, 3H), 5.06 (br, 1H), 5.18 (s, 2H), 7.02 (br, 1H), 7.21–7.39 (m, 14H), 7.58 (br, 1H), 7.85 (d, 2H, $J$ = 8.1 Hz), 8.07 (br, 1H), 8.40 (br, 1H). LC–MS $m/z$: 1127.4 $(M+Na)^+$.

#### 7.1.18. MTX(OMe)-α-Gly-Phe-Leu-Gly-NH-PEG-NH-Boc (17)

2.02 g (1.83 mmol) of compound **16** was dissolved in 8 mL of methanol. To the mixture, 400 mg of 10% Pd–C was added. After stirring under hydrogen atmosphere for 2 h, the reaction mixture was filtered and evaporated. The residue was dissolved in 7 mL of dry DMA. To the solution, BPT·HBr (676 mg, 2.01 mmol, 110 equiv) was added and stirred at 65 °C for 19 h. The reaction mixture was diluted with dichloromethane and purified by column chromatography (aminosilica gel, eluent; dichloromethane/methanol, 100/4–100/6, 100/3–100/7, 2 times). The desired compound **17** was obtained 978 mg (0.85 mmol, 47%) as a yellow amorphous substance. $^1$H NMR (270 MHz, $CDCl_3$) $\delta$: 0.85–0.89 (m, 6H), 1.42 (s, 9H), 1.64–1.78 (m, 7H), 2.09–2.21 (m, 2H), 2.43–2.78 (m, 2H), 3.07–3.32 (m, 8H), 3.27 (s, 3H), 3.45–3.90 (m, 16H), 3.64 (s, 3H), 4.25 (br, 1H), 4.36 (br, 1H), 4.55–4.57 (br, 2H), 6.85 (d, 2H, $J$ = 8.9 Hz), 7.15–7.20 (m, 5H), 7.79 (d, 2H, $J$ = 8.9 Hz), 8.56 (s, 1H). LC–MS m/z: 1145.6 $(M+H)^+$, 1167.6 $(M+Na)^+$.

#### 7.1.19. MTX(OMe)-α/γ-Gly-Phe-Leu-Gly-NH-PEG-$NH_2$ (18)

841 mg (0.73 mmol) of compound **17** was dissolved in 7 mL of TFA at a temperature of 0 °C. After the reaction mixture was kept at 0 °C for 1 h, dichloromethane was added and evaporated to remove TFA and solvent. The residue was purified by column chromatography (aminosilica gel, eluent; dichloromethane/methanol, 100/10). The product **18** (723 mg, 0.69 mmol, 94%) was a mixture of α- and γ- isomers (3:1). The desired α-isomer was purified by separable HPLC but migrated again. The product was obtained as a free amine through column chromatography (aminosilica gel, eluent; dichloromethane/methanol, 100/10) as a yellow amorphous substance. $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$: 0.50–0.60 (m, 6H, Leu), 1.15–1.39 (m, 2H, Leu, 4H, PEG), 1.71–1.86 (m, 2H, Glu), 1.99–2.08 (br, 1H, Leu), 2.15 (t, 2H, $J$ = 7.2 Hz, Glu), 2.31–2.37 (m, 2H, PEG), 2.64–2.71 (m, 2H, Phe), 2.78–2.87 (m, 2H, PEG), 2.91 (s, 3H, NMe), 3.00–3.23 (m, 12H, PEG), 3.23 (s, 3H, Glu OMe), 3.28–3.48 (m, 4H, Gly), 3.88–3.95 (m, 1H, Hα Leu), 4.00–4.04 (m, 1H, Hα Glu), 4.14–4.23 (m, 1H, Hα Phe), 4.46 (s, 2H, $CH_2$ 6-pteridine), 6.22–6.30 (br, 2H, $NH_2$), 6.51 (d, 2H, $J$ = 9.6 Hz, 1,4-disubstituted benzene), 6.75–6.91 (m, 5H, Phe), 7.18–7.28 (m, 1H, NH PEG), 7.18–7.38 (br, 2H, $NH_2$), 7.44–7.53 (m, 2H, NH Gly), 7.51 (d, 2H, $J$ = 9.6 Hz, 1,4-disubstituted benzene), 7.64–7.66 (m, 1H, NH Leu), 7.66–7.71 (m, 1H, NH Phe), 8.14 (br, 1H, NH Glu), 8.12–8.30 (br, 2H, $NH_2$), 8.26 (s, 1H, CH 7-pteridine). The ratio was of α/γ was decided by HPLC (ODS, 25% MeCN–$H_2O$ iso., 30 min., 305–380 nm.). Retention times: 18.6 min. (α-isomer), 11.7 min. (γ-isomer). The ratio was 3/1. LC–MS $m/z$: 1045.7 $(M+H)^+$, 1067.7 $(M+Na)^+$.

For the analysis, the fraction of isomers of free carboxylic acid was obtained. Separated chemical shifts of α-isomer, $^1$H NMR (500 MHz, $D_2O$) $\delta$: 0.67–0.75 (m, 6H, side chain Leu), 1.17–1.28 (m, 1H, side chain Leu), 1.36–1.48 (m, 2H, side chain Leu), 2.00–2.06 (m, 2H, side chain Glu), 2.23–2.32 (m, 2H, side chain Glu), 2.83–2.91 (m, 2H, side chain Phe), 4.13–4.17 (m, 1H, Hα Leu), 4.20–4.24 (m, 1H, Hα Glu), 6.70 (d, 2H, $J$ = 9.1 Hz, 1,4-substituted benzene), 7.68 (d, 2H, $J$ = 9.1 Hz, 1,4-substituted benzene). LC–MS $m/z$: 1031.5 $(M+H)^+$. Retention time: 3.57 min.

Separated chemical shifts of γ-isomer, $^1$H NMR (500 MHz, $D_2O$) $\delta$: 0.62–0.71 (m, 6H, side chain Leu), 1.38–1.54 (m, 3H, side chain Leu), 1.86–1.93 (m, 1H, side chain Glu), 2.05–2.13 (m, 1H, side chain Glu), 2.13–2.21 (m, 1H, side chain Glu), 2.23–2.32 (m, 1H, side chain Glu), 2.70–2.81 (m, 1H, side chain Phe), 2.90–2.98 (m, 1H, side chain Phe), 4.09–4.13 (m, 1H, Hα Leu), 4.29–4.33 (m, 1H, Hα Glu), 6.73 (d, 2H, $J$ = 9.1 Hz, 1,4-disubstituted benzene), 7.72 (d, 2H, $J$ = 9.1 Hz, 1,4-disubstituted benzene). LC–MS $m/z$: 1031.6 $(M+H)^+$. Retention time: 3.46 min.

#### 7.1.20. Z-Phe-NH-PEG-NH-Boc (19)

To a solution of 5.0 g (15.6 mmol) of **5** in 150 mL of dry DMF was added Z-Phe-OH (4.9 g, 16.4 mmol, 1.05 equiv) and HOBt·$H_2O$ (2.51 g, 16.4 mmol, 1.05 equiv). The reaction mixture was stirred at 0 °C and EDC·HCl (3.29 g, 17.2 mmol, 1.10 equiv) was added. After keeping 0 °C for 1 h, the reaction mixture was warmed to room temperature. After 17 h ethyl acetate was added. The organic layer was washed with 10% citric acid, saturated $NaHCO_3$ and brine, dried over magnesium sulfate and evaporated. The product was purified by means of silica gel column chromatography (eluent; dichloromethane/methanol, 10/1) to afford 9.82 g (quant.) of the desired compound **19**. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$: 1.37 (s, 9H), 1.54–1.63 (m, 4H), 2.71–3.32 (m, 4H), 3.33–3.39 (m, 6H), 3.45–3.52 (m, 8H), 4.19 (m, 1H), 4.94 (s, 2H), 6.74 (br, 1H), 7.17–7.30 (m, 10H), 7.46 (d, 1H, $J$ = 8.4 Hz), 7.95 (br, 1H). LC–MS $m/z$: 624.2 $(M+Na)^+$.

#### 7.1.21. Z-Phe-Phe-NH-PEG-NH-Boc (20)

15.6 mmol of compound **19** was dissolved in 400 mL of methanol. To the mixture, 0.98 g of 10% Pd–C was added. After stirring under hydrogen atmosphere for 1.5 h, the reaction mixture was filtered and evaporated, and the residue was dissolved in 160 mL of dry DMF. To the solution, Z-Phe-OH (5.13 g, 17.1 mmol, 1.10 equiv), HOBt·$H_2O$ (2.62 g, 17.1 mmol, 1.10 equiv) were added. The reaction mixture was stirred at 0 °C and EDC·HCl (3.44 g, 17.9 mmol, 1.15 equiv) was added. After keeping 0 °C for 1 h, the reaction mixture was warmed to room temperature. After 14 h ethyl acetate was added. After following the same procedure as described for **19**, the desired compound **20** was obtained 12.1 g (quant.). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$: 1.37 (s, 9H), 1.54–1.63 (m, 4H), 2.67–3.13 (m, 6H), 3.33–3.39 (m, 6H), 3.45–3.50 (m, 8H), 4.24 (br 1H), 4.87 (br, 1H), 4.93 (s, 2H), 6.74 (br, 1H), 7.16–7.34 (m, 15H), 7.45 (d, 1H, $J$ = 8.7 Hz), 7.88 (br, 1H), 8.12 (d, 1H, $J$ = 8.1 Hz). LC–MS $m/z$: 771.3 $(M+Na)^+$.

#### 7.1.22. Z-Asn-Phe-Phe-NH-PEG-NH-Boc (21)

15.6 mmol of compound **20** was dissolved in 280 mL of methanol. To the mixture, 1.0 g of 10% Pd–C was added. After stirring under hydrogen atmosphere for 1.5 h, the reaction mixture was filtered and evaporated. The residue was dissolved in 160 mL of dry DMF. To the solution, carbobenzyloxy-L-asparagine (Z-Asn-OH, 4.51 g, 16.9 mmol, 1.08 equiv), HOBt·$H_2O$ (2.59 g, 16.9 mmol, 1.08 equiv) were added. The reaction mixture was stirred at 0 °C and was added EDC·HCl (3.40 g, 17.7 mmol, 1.13 equiv). After being kept at 0 °C for 1 h, the reaction mixture was warmed to room temperature. After 2 h, ethyl acetate was added. After using the same procedure as described for **19**, the desired compound **21** was obtained 14.8 g (quant.). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$: 1.37 (s, 9H), 1.54–1.63 (m, 4H), 2.27–2.57 (m, 2H), 2.74–3.08 (m, 6H), 3.28–3.36 (m, 6H), 3.45–3.51 (m, 8H), 4.29–4.43 (m, 3H), 5.00 (s, 2H), 6.74 (br, 1H), 6.67 (br, 1H), 7.10–7.37 (m, 15H), 7.45 (d, 1H, $J$ = 8.4 Hz), 7.64 (br, 2H), 8.05 (d, 1H, $J$ = 7.8 Hz), 8.18 (d, 1H, $J$ = 8.4 Hz). LC–MS $m/z$: 863.1 $(M+H)^+$, 885.4 $(M+Na)^+$.

#### 7.1.23. Z-Glu(OMe)-Asn-Phe-Phe-NH-PEG-NH-Boc (22)

2.62 g (3.04 mmol) of compound **21** was dissolved in 300 mL of methanol. To the mixture, 0.3 g of 10% Pd–C was added. After stirring under hydrogen atmosphere for 1.5 h, the reaction mixture was filtered and evaporated, and the residue was dissolved in 50 mL of dry DMF. To the solution, Z-Glu(OMe)-OH (941.3 mg, 3.20 mmol, 1.05 equiv) and HOBt·$H_2O$ (488.2 mg, 3.2 mmol, 1.08 equiv) were added. The reaction mixture was stirred at 0 °C and EDC·HCl (640 mg, 3.34 mmol, 1.10 equiv) was added. After being kept at 0 °C for 1 h, the reaction mixture was warmed to

room temperature. After 2 h, water was added. The product was obtained by filtration and washed with diluted HCl solution, $NaHCO_3$ solution and water. Then purified by silica gel column chromatography (eluent; dichloromethane/methanol, 10/1) to afford 2.50 g (2.48 mmol, 82%) of the desired product **22**. $^1$H NMR (300 MHz, DMSO-$d_6$); $\delta$: 1.36 (s, 9H), 1.54–1.63 (m, 4H), 1.67–1.81 (m, 2H), 2.26–2.60 (m, 2H), 2.68–3.01 (m, 8H), 3.26–3.36 (m, 6H), 3.45–3.51 (m, 8H), 3.57 (s, 3H), 4.02 (br, 1H), 4.32 (br, 2H), 4.53 (br, 1H), 5.01 (dd, 2H, $J$ = 12.3, 20.4 Hz), 6.75 (br, 1H), 7.06–7.49 (m, 19H), 8.16 (br, 2H), 8.25 (d, 1H, $J$ = 7.2 Hz). LC–MS $m/z$: 1028.5 $(M+Na)^+$.

#### 7.1.24. MTX(OMe)-α-Asn-Phe-Phe-NH-PEG-NH-Boc (24)

302 mg (0.30 mmol) of compound **22** was dissolved in 2 mL of methanol and 6 mL of DMF. To the mixture, 120 mg of 10% Pd–C was added. After stirring under hydrogen atmosphere for 3 h, the reaction mixture was filtered and methanol was removed at reduced pressure. To the residue solution, compound **23** (117 mg, 0.36 mmol, 1.20 equiv), HOBt·$H_2O$ (46 mg, 0.30 mmol, 1.00 equiv) and EDC·HCl (69 mg, 0.36 mmol, 1.20 equiv) were added at the temperature of 0 °C. After stirring at room temperature for 19 h, brine was added to the solution. The yellow precipitate which formed was filtered and washed with water. The precipitate was diluted with DMF and recrystallized by the addition of methanol (2 times). The yellow solid was washed with methanol affording 180 mg (0.15 mmol, 51%) of product **20**. $^1$H NMR (270 MHz, DMSO-$d_6$) $\delta$: 1.36 (s, 9H), 1.54–1.61 (m, 4H), 1.89 (m, 2H), 2.31–2.43 (m, 2H), 2.71–3.38 (m, 19H), 3.45–3.51 (m, 6H), 3.55 (s, 3H), 4.30–4.35 (m, 3H), 4.49 (br, 1H), 4.79 (s, 2H), 6.60–6.83 (m, 5H), 7.03–7.24 (m, 11H), 7.48–7.72 (m, 4H), 7.73 (d, 2H, $J$ = 8.6 Hz), 8.07–8.23 (m, 4H), 8.57 (s, 1H). LC–MS $m/z$: 1179.4 $(M+H)^+$.

#### 7.1.25. MTX(OMe)-α-Asn-Phe-Phe-NH-PEG-$NH_2$ (25)

180 mg (0.15 mmol) of compound **24** was dissolved in 2 mL of TFA at a temperature of 0 °C. After the reaction mixture was kept at 0 °C for 0.5 h, dichloromethane was added and evaporated to remove TFA and solvent. The residue was purified by column chromatography (aminosilica gel, eluent; dichloromethane/methanol, 10/1) and suspended in methanol. The titled compound (**25**, 145 mg, 0.134 mmol, 88%) was obtained as a yellow solid by filtration and washed with methanol. $^1$H NMR (270 MHz, DMSO-$d_6$) $\delta$: 1.52–1.59 (m, 4H), 1.87–2.02 (m, 2H), 2.32–3.48 (m, 24H), 3.22 (s, 3H), 3.55 (s, 3H), 4.24–4.56 (m, 4H), 4.79 (s, 2H), 6.60 (br s, 2H), 6.81 (d, 2H, $J$ = 8.6 Hz), 7.04–7.75 (m, 17H), 8.07–8.26 (m, 4H), 8.56 (s, 1H). LC–MS $m/z$: 1079.5 $(M+H)^+$.

#### 7.1.26. General procedure of conjugation of amines (10a, 10b, 18 and 25) and HA

In what follows water meant extra-pure water and HA was treated under sterile condition.

A solution of 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOOBt) (0.125 mmol) and amine **10a**, **10b**, **18** or **25** (0.0625 mmol) in 20 mL of water–THF (1:1) solvent was added to a suspension of sodium hyaluronate (500 mg, MW: ca. 2300 kDa) in 10 mL of THF solution. To the mixture, tris[2-(2-methoxyethoxy)ethyl]amine (0.094 mmol) dissolved in 10 ml of water–THF (1:1) was added, followed by stirring at 5 °C. After 30 min, EDC·HCl (0.125 mmol) dissolved in water (10 mL) was added to the mixture, followed by stirring at 5 °C for 20 h. A 0.09 N NaOH aqueous solution (220 mL) was added to the reaction mixture, followed by stirring at 5 °C for 3.5 h. 1 N HCl solution (20 mL) was added to the reaction mixture for neutralization, sodium chloride solution (9 g in 45 mL of water) was added, followed by dropwise addition of ethanol (600 mL). The precipitate which formed was separated by centrifugation. The precipitate was dissolved in water (40 mL) to provide an aqueous solution of the HA–MTX conjugate. To the conjugate solution, sodium chloride (6 g) dissolved in 160 mL of water and 400 mL of ethanol were added. The precipitate which formed was separated by centrifugation and dissolved in 500 mL of water. To which sodium chloride (15 g) was added before filtration using a 0.45 μm filter (Stervex HV: Millipore), and then ethanol (1000 mL) was aseptically added dropwise. The precipitate was filtered and dried in vacuo. The conjugate was dissolved in 40 mL of phosphate buffer solution (2 mM sodium phosphate, 154 mM sodium chloride, pH 7.2) to provide a sterile aqueous solution of the HA–MTX conjugate. The MW was determined by GPC and the binding ratio of MTX was calculated by measuring the UV absorption (259 nm). The procedure for calculating the binding ratio of MTX was as follows: The concentration of HA in the conjugate was determined by the RI peak area relative to the standard HA solution. Next, the HA concentration was converted to an equivalent concentration of a disaccharide unit with one carboxyl group. The concentration of MTX in the conjugate was determined by the UV peak area relative to the standard MTX solution. The binding ratio of MTX was obtained by dividing the concentration of MTX by the concentration of the disaccharide unit.

**7.1.26.1. MTX-γ-NH-PEG-NH-HA (1).** Sodium hyaluronate (500 mg, MW: ca. 2300 kDa) was reacted with compound **10a** (0.0625 mmol) following the general procedure, providing an aqueous solution of **1**.

This aqueous solution was purified to provide a sterile aqueous solution of **1**. The MW and the binding ratio of MTX were 1610 kDa and 3.1%, respectively. $^1$H NMR (500 MHz, $D_2O$) $\delta$: 1.46 (m), 1.70 (br), 1.95 (br s), 2.21–2.34 (m), 2.87–3.01 (m), 3.20 (br s), 3.29 (br s), 3.36–3.49 (m), 3.63 (br s), 3.75 (br s), 4.25–4.61 (m), 6.90 (d, $J$ = 8.9 Hz), 7.66 (d, $J$ = 8.9 Hz), 8.62 (s).

**7.1.26.2. MTX-α-NH-PEG-NH-HA (2).** Sodium hyaluronate (500 mg, MW: ca. 2300 kDa) was reacted with compound **10b** (0.0625 mmol) following the general procedure, providing an aqueous solution of **2**.

This aqueous solution was purified to provide a sterile aqueous solution of **2**. The MW and the binding ratio of MTX were 1610 kDa and 2.7%, respectively. $^1$H NMR (500 MHz, $D_2O$) $\delta$: 1.72 (m), 1.95 (br s), 2.31 (m), 3.19 (br s), 3.27 (br s), 3.36–3.54 (m), 3.63 (br s), 3.75 (br s), 4.27–4.58 (m), 6.87 (d, $J$ = 9.0 Hz), 7.69 (d, $J$ = 9.0 Hz), 8.63 (s).

**7.1.26.3. MTX-αγ-GlyPheLeuGly-NH-PEG-NH-HA (3).** Sodium hyaluronate (500 mg, MW: ca. 2300 kDa) was reacted with compound **18** (0.0625 mmol) following the general procedure, providing an aqueous solution **3**.

This aqueous solution was purified to provide a sterile aqueous solution of **3**. The MW thereof and the binding ratio of MTX were 1850 kDa and 1.3%, respectively. $^1$H NMR (500 MHz, $D_2O$) $\delta$: 0.72 (d), 0.77 (d), *0.81* (d), 1.32 (m), 1.50 (m), 1.67–1.82 (m), 2.01 (br s), 2.23 (m), 2.33 (m), 2.75–3.03 (m), 3.51 (br s), 3.58 (br s), 3.71 (br s), 3.83 (br s), 4.16–4.28 (m), 4.46 (br s), 4.54 (br s), 6.85 (d), 6.92–7.06 (m), 7.75 (d), *7.78* (d), *8.63* (s), 8.65 (s).

The portions given in italics are minor signals. From the signals, **3** was deduced to be a mixture of α- and γ- isomers.

**7.1.26.4. MTX-α-AsnPhePhe-NH-PEG-NH-HA (4).** Sodium hyaluronate (500 mg, MW: ca. 2300 kDa) was reacted with compound **25** (0.0625 mmol) following the general procedure, providing an aqueous solution **4**.

This aqueous solution was purified to provide a sterile aqueous solution of **4**. The MW and the binding ratio of MTX were 1780 kDa and 1.2%, respectively. $^1$H NMR (500 MHz, $D_2O$) $\delta$: 1.60 (m), 1.80 (m), 2.02 (br s), 2.34 (m), 2.54 (m), 2.60–3.05 (m), 3.35 (br s), 3.52 (br s), 3.57 (br s), 3.64 (br s), 3.72 (br s), 3.83 (br s), 4.28

(m), 4.46 (br s), 4.55 (br s), 6.61 (d), *6.77* (t), 6.82–7.36 (m), *7.76* (d), 7.80 (d), 8.61 (s), *8.64* (s).

The portions given in italics are minor signals. From these signals, conjugate **4** was deduced to be a mixture of α- and γ-isomers.

### 7.2. Biology

#### 7.2.1. In vitro

HFLS (Cell Applications) was seeded at 5000 cells/well on a 96-well plate (Falcon) and cultured for 3 h in Iscove's modified Dulbecco's medium (IMDM) containing 5% FBS and 1X antibiotic–antimycotic (GIBCO BRL). After cellular attachment, TNF-α (recombinant human TNF-α, R & D Systems) (final concentration: 10 ng/mL) and each HA–MTX conjugate at each concentration was added, followed by cultivation for 5 days. Two days before the end of culture, 37 kBq/well of [$^3$H]-deoxyuridine was added to the cells (MORAVEK), followed by determining the uptake quantity (radioactivity) of [$^3$H]-deoxyuridine using a scintillation counter. Cells were recovered by unsticking them with 0.05% trypsin–0.2% EDTA.

Radioactivity was calculated as a relative value (% of control), using, as the control, radioactivity in the group of cells cultured without any added test substance. Since the concentration of a free carboxyl group is $2.49 \times 10^{-3}$ mol/L (1 g/401/L; 401 is the MW of *N*-acetylglucosamine + glucuronic acid) for each 1 mg/mL of hyaluronic acid, the MTX concentration in each HA–MTX was calculated by multiplying the value by the conjugation rate of MTX. (For 1 mg/mL of HA–MTX conjugate with a conjugation rate of MTX of 1%, the concentration of MTX was $2.49 \times 10^{-5}$ mol/L.) The value obtained was used to calculate the activity of cell proliferation inhibition (the $IC_{50}$ value) by a 4-parameter logistic method using analysis software (GraphPad Prism 3.02).

#### 7.2.2. In vivo

Six-week-old male LEW/Crj rats were purchased from Charles River Laboratories Japan, Inc. The rats were sensitized with 0.5 mL of an emulsion prepared from a 2 mg/mL mBSA (Calbiochem) aqueous solution and an equal amount of Freund's complete adjuvant (Difco) into the flank at 21 and 14 days before inducing arthritis. The arthritis was induced by administering 50 μL of a 2 mg/mL mBSA aqueous solution into the right knee joint. The left knee joint was untreated and served as the control. Knee joint swelling was assessed by measuring the width of each knee joint with calipers to define the left-right difference. The widths of all knee joints were measured twice a week from immediately before inducing arthritis to two weeks after to calculate the area under the curve (AUC) over time for joint swelling. In addition, the AUC was calculated using the value relative to the HA-treated control group (% of control). HA, free MTX, a mixture of HA and MTX and HA–MTX conjugates were administered into the right knee joint in an amounts of 50 μL before (7 and 1 day) and after (7 days) inducing arthritis.

At each measurement, the mean and standard deviation of AUC were calculated to perform an unpaired t-test between each test substance-treated group and the HA-treated group, and significant difference was judged to be present if the probability level was less than 5%. Statistical analysis was performed using SAS version 6.12 (SAS Institute Japan).

### Acknowledgments

The authors thank Professor Kunio Ogasawara for his helpful suggestions concerning this study. We also thank Ms. Frances Ford for proofreading the manuscript.

### References and notes

1. Wen, D. Y. *Am. Fam Physician* **2000**, *62*, 565.
2. Abramson, S. B.; Yazici, Y. *Adv. Drug Delivery Rev.* **2006**, *58*, 212.
3. Issa, S. N.; Sharma, L. *Curr. Rheumatol. Rep.* **2006**, *8*, 7.
4. Pinals, R. S. *Clin. Therap.* **1992**, *14*, 336.
5. Pelletier, J. P.; Martel-Pelletier, J. *Ann. Rheum. Dis.* **2003**, *62*, ii79.
6. Pelletier, J. P.; Martel-Pelletier, J.; Abramson, S. B. *Arthritis Rheum.* **2001**, *44*, 1237.
7. McCarty, M. F.; Russell, A. L.; Seed, M. P. *Med. Hypotheses* **2000**, *54*, 798.
8. de Isla, N. G.; Stoltz, J. F. *Biorheology* **2008**, *45*, 433.
9. Alarcon, G. S. *Immunopharmacology* **2000**, *47*, 259.
10. Swierkot, J.; Szechinski, J. *Pharmacol. Rep.* **2006**, *58*, 473.
11. Altman, R. D.; Gray, R. *Am. J. Med.* **1983**, *75*, 50.
12. Hamstra, D. A.; Page, M.; Maybaum, J.; Rehemtulla, A. *Cancer Res.* **2000**, *60*, 657.
13. Smal, M. A.; Dong, Z.; Cheung, H. T.; Asano, Y.; Escoffier, L.; Costello, M.; Tattersall, M. H. *Biochem. Pharmacol.* **1995**, *49*, 567.
14. Anadere, I.; Chmiel, H.; Laschner, W. *Biorheology* **1979**, *16*, 179.
15. Rwei, S.-P.; Chen, S.-W.; Mao, C.-F.; Fang, H.-W. *Biochem. Eng. J.* **2008**, *40*, 211.
16. Adam, N.; Ghosh, P. *Inflamm. Res.* **2001**, *50*, 294.
17. Adams, M. E.; Lussier, A. J.; Peyron, J. G. *Drug Safety* **2000**, *23*, 115.
18. Mihara, M.; Higo, S.; Uchiyama, Y.; Tanabe, K.; Saito, K. *Osteoarthritis Cartilage* **2007**, *15*, 543.
19. Pagnano, M.; Westrich, G. *Osteoarthritis Cartilage* **2005**, *13*, 751.
20. Hsieh, Y. S.; Yang, S. F.; Lue, K. H.; Chu, S. C.; Lu, K. H. *J. Orthopaed. Res.* **2008**, *26*, 475.
21. Kotevoglu, N.; Iyibozkurt, P. C.; Hiz, O.; Toktas, H.; Kuran, B. *Rheumatol. Int.* **2006**, *26*, 325.
22. Ghosh, P.; Guidolin, D. *Sem. Arthritis Rheum.* **2002**, *32*, 10.
23. Soltes, L.; Mendichi, R.; Kogan, G.; Mach, M. *Chem. Biodivers.* **2004**, *1*, 468.
24. Lavelle, E. D.; Lavelle, W.; Smith, H. S. *Anesthesiol. Clin.* **2007**, *25*, 841.
25. Entwistle, J.; Hall, C. L.; Turley, E. A. *J. Cell. Biochem.* **1996**, *61*, 569.
26. Knudson, W.; Chow, G.; Knudson, C. B. *Matrix Biol.* **2002**, *21*, 15.
27. Pek, Y. S.; Kurisawa, M.; Gao, S.; Chung, J. E.; Ying, J. Y. *Biomaterials* **2009**, *30*, 822.
28. Lee, F.; Chung, J. E.; Kurisawa, M. *J. Controlled Release* **2008**.
29. Kurisawa, M.; Chung, J. E.; Yang, Y. Y.; Gao, S. J.; Uyama, H. *Chem. Commun. (Camb)* **2005**, 4312.
30. Prestwich, G. D.; Kuo, J. W. *Curr. Pharm. Biotechnol.* **2008**, *9*, 242.
31. Prestwich, G. D.; Marecak, D. M.; Marecek, J. F.; Vercruysse, K. P.; Ziebell, M. R. *J. Controlled Release* **1998**, *53*, 93.
32. Lee, H.; Lee, K.; Park, T. G. *Bioconjugate Chem.* **2008**, *19*, 1319.
33. Yadav, A. K.; Mishra, P.; Jain, S.; Mishra, P.; Mishra, A. K.; Agrawal, G. P. *J. Drug Targeting* **2008**, *16*, 464.
34. Nogusa, H.; Hamana, H.; Yano, T.; Kajiki, M.; Yamamoto, K.; Okuno, S.; Sugawara, S.; Kashima, N.; Inoue, K. PTC Int. Appl. Patent WO 9419376, 1994.
35. Akima, K.; Iwata, Y.; Matsuo, K.; Watari, N. PTC Int. Appl. Patent WO 9206714, 1992.
36. Tanihara, M.; Kinoshita, H. PTC Int. Appl. Patent WO 9531223, 1995.
37. Tanihara, M.; Kinoshita, H. PTC Int. Appl. Patent JP 2000070356, 2000.
38. Ellwood, D. C. PTC Int. Appl. Patent JP2701865, 1998.
39. Li, H.; Liu, Y.; Shu, X. Z.; Gray, S. D.; Prestwich, G. D. *Biomacromolecules* **2004**, *5*, 895.
40. Jaracz, S.; Chen, J.; Kuznetsova, L. V.; Ojima, I. *Bioorg. Med. Chem.* **2005**, *13*, 5043.
41. Rosowsky, A.; Bader, H.; Freisheim, J. H. *J. Med. Chem.* **1991**, *34*, 574.
42. Rosowsky, A.; Wright, J. E.; Vaidya, C. M.; Forsch, R. A. *Pharmacol. Ther.* **2000**, *85*, 191.
43. Rosowsky, A.; Forsch, R. A.; Wright, J. E. *J. Med. Chem.* **2004**, *47*, 6958.
44. Smith, G. K.; Banks, S.; Blumenkopf, T. A.; Cory, M.; Humphreys, J.; Laethem, R. M.; Miller, J.; Moxham, C. P.; Mullin, R.; Ray, P. H.; Walton, L. M.; Wolfe, L. A., 3rd *J. Biol. Chem.* **1997**, *272*, 15804.
45. Szeto, D. W.; Cheng, Y. C.; Rosowsky, A.; Yu, C. S.; Modest, E. J.; Piper, J. R.; Temple, C., Jr.; Elliott, R. D.; Rose, J. D.; Montgomery, J. A. *Biochem. Pharmacol.* **1979**, *28*, 2633.
46. Huennekens, F. M. *Adv. Enzyme Regul.* **1997**, *37*, 77.
47. Chau, Y.; Tan, F. E.; Langer, R. *Bioconjugate Chem.* **2004**, *15*, 931.
48. Wunder, A.; Muller-Ladner, U.; Stelzer, E. H.; Funk, J.; Neumann, E.; Stehle, G.; Pap, T.; Sinn, H.; Gay, S.; Fiehn, C. *J. Immunol.* **2003**, *170*, 4793.
49. Rejmanova, P.; Kopecek, J.; Phol, J.; Baidys, M.; Kostka, V. *Die Makromol. Chem.* **1983**, *184*, 2009.
50. Fosang, A. J.; Last, K.; Fujii, Y.; Seiki, M.; Okada, Y. *FEBS Lett.* **1998**, *430*, 186.
51. Samy, R.; Kim, H. Y.; Brady, M.; Toogood, P. L. *J. Org. Chem.* **1999**, *64*, 2711.
52. McKillop, A.; Mills, L. S. *Synth. Commun.* **1987**, *17*, 647.
53. Piper, J. R.; Montgomery, J. A.; Sirotnak, F. M.; Chello, P. L. *J. Med. Chem.* **1982**, *25*, 182.
54. Mahmoodi, M.; Sahebjam, S.; Smookler, D.; Khokha, R.; Mort, J. S. *Am. J. Pathol.* **2005**, *166*, 1733.
55. Roth, A.; Mollenhauer, J.; Wagner, A.; Fuhrmann, R.; Straub, A.; Venbrocks, R. A.; Petrow, P.; Brauer, R.; Schubert, H.; Ozegowski, J.; Peschel, G.; Muller, P. J.; Kinne, R. W. *Arthritis Res. Ther.* **2005**, *7*, R677.
56. Griffiths, R. J. *Agents Actions* **1992**, *35*, 88.
57. Hosangadi, B. D.; Dave, R. H. *Tetrahedron Lett.* **1996**, *37*, 6375.
58. Kuefner, U.; Lohrmann, U.; Montejano, Y. D.; Vitols, K. S.; Huennekens, F. M. *Biochemistry* **1989**, *28*, 2288.
59. Piper, J. R.; Montgomery, J. A. *J. Org. Chem.* **1977**, *42*, 1977.
60. Montgomery, J. A.; Piper, J. R.; Elliotto, R. D.; Roberts, E. C.; Temple, C., Jr.; Shealy, Y. F. *J. Heterocycl. Chem.* **1979**, *16*, 537.
61. Elliotto, R. D.; Temple, C., Jr.; Montgomery, J. A. *J. Org. Chem.* **1970**, *35*, 1676.
62. Braun, M.; Hartnagel, U.; Ravanelli, E.; Schade, B.; Böttcher, C.; Vostrowsky, O.; Hirsch, A. *Eur. J. Org. Chem.* **2004**, 1983.
63. Albrecht, M.; Napp, M.; Schneider, M. *Synthesis* **2001**, *3*, 468.
64. Shi, Y.-J.; Cameron, M.; Dolling, U. H.; Lieberman, D. R.; Lynch, J. E.; Reamer, R. A.; Robbins, M. A.; Volante, R. P.; Reider, P. J. *Synlett* **2003**, 647.
65. Fu, S.-C. J.; Reiner, M.; Loo, T. L. *J. Org. Chem.* **1965**, *30*, 1277.