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Bioconjugate Chem., **2008**, 19 (11), 2260-2269 • Publication Date (Web): 30 October 2008

Design, Synthesis, and *In Vitro* Activity of Novel Drug Delivery Systems Containing Tuftsin Derivatives and Methotrexate

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During the past decade, biodegradable polymers or oligopeptides recognized by cell-surface receptors have been shown to increase drug specificity, lowering systemic drug toxicity in contrast to small-size fast-acting drugs. The goal of the present study was to develop anticancer bioconjugates based on chemotactic drug targeting (CDT). These constructs are composed of methotrexate (Mtx) attached to a tuftsin-like peptide carrier through an enzyme-labile pentapeptide spacer (GFLGC) and several copies of a chemotactic targeting moiety (H-TKPR, For-TKPR, H-TKPKG, and Ac-TKPKG). Carriers with targeting moieties in the branches were prepared by solid-phase synthesis using mixed Boc and Fmoc strategies. The drug molecule connected to an enzyme-labile spacer was attached to the branched oligopeptide in solution. *In vitro* chemotaxis, cellular uptake, and cytotoxicity assays were carried out on the MonoMac6 cell line. The most effective conjugates with H-TKPR or Ac-TKPKG targeting moieties in the branches, which have the most advantageous chemotactic properties, can be internalized rapidly, and these conjugates trigger higher toxic effect than the free drug (Mtx). The results suggest that our tuftsin-based drug delivery systems might be potential candidates for targeting cancer chemotherapy.

INTRODUCTION

Numerous drug delivery systems (DDS) have been developed over the years to establish novel antitumor therapeutics. The main goal of these developments is the investigation of various approaches toward targeting toxic drugs to tumor cells using their conjugated forms with carriers and/or targeting moieties. In this way, a controlled location and higher concentration of toxic drugs can be reached at the site of action; furthermore, side effects and therapeutical doses can be reduced. The application of drug delivery systems for targeting chemotherapy might result in increased selectivity and efficiency in the cancer treatment (*1, 2*).

Peptide-based biodegradable conjugates represent one group of DDS. These conjugates consist of a drug molecule, an enzyme-labile or pH-sensitive spacer sequence, a biodegradable peptide carrier, and targeting moieties (e.g., peptide hormones, lectins, sugars, or antibodies) (*3*), which have been shown to bind to cell-surface receptors and can be internalized by receptor-mediated endocytosis. Chemotactic drug targeting (CDT), a special form of drug targeting, was investigated in our laboratory (*4*). Previously, we reported on the synthesis of chemotactic drug delivery systems for drug targeting. We used the well-known chemotactic peptides (For-Met-Leu-Phe and For-Nle-Leu-Phe) as targeting moieties, capable to bind to specific chemotactic receptors on phagocytic cells (*5*). In this study, we have further developed this system by modifying the chemotactic targeting moieties. Conjugates composed of chemotactically active ligands (chemotactic peptides), drug, and carrier induce chemotactic activity of target cells. In this way, responder cells accumulate in the high-concentration region of tissues, and internalization of the drug is also increased. To achieve conjugates suitable for CDT, chemotactic peptides as targeting moieties were built up as branches in the carrier.

In this study, a sequential oligopeptide, a tetratuftsin derivative, was applied as carrier. Human tuftsin, a naturally occurring tetrapeptide (H-TKPR-OH) was described by Najjar and Nishioka in 1970 (*6*). Tuftsin is the 289−292 sequence in the CH2 domain of the Fc fragment of the heavy chain of IgG (*7*). It is an immunopotentiating peptide, which stimulates phagocytosis and cell migration (*8*) and possesses antitumor activity (*9, 10*). In our present study, a tuftsin analogue (H-TKPK-OH) derived from canin and having human tuftsin-like activities, elongated with Gly at the *C*-terminal (H-TKPKG-OH), was used. The carrier in multifunctional conjugates was the H-$[TKPKG]_4$-NH_2 tetramer (OT20) (*11*). Inasmuch as the human tuftsin and its analogue have a chemotactic effect on monocytes (*12, 13*), these peptides and their formylated or acetylated derivatives at the *N*-terminal were attached to the carrier and applied as targeting moieties (H-TKPR, For-TKPR, H-TKPKG, Ac-TKPKG) in the branches.

Methotrexate, (*S*)-2-(4-(((2,4-diaminopteridin-6-yl)methyl)-methylamino)benzamido)pentanedioic acid (Mtx), an antimetabolite and antifolate molecule was incorporated as an anticancer drug. Mtx is used for chemotherapy in cancers and autoimmune diseases (*14*−*16*). It is mainly applied for the treatment of acute lymphoblastic leukemia (*17*), but it is also used against leishmania (*18, 19*) or in the treatment of rheumatoid arthritis (RA) (*20*). However, methotrexate could cause severe toxicity; the most frequent reactions include low white blood cell counts and toxicity of the liver and bone marrow resulting in immunosuppression.

To decrease the toxic side effects, the release of free drug molecules in target cells is important. The release of the Mtx from the conjugates can be influenced by the type of linkage applied between the drug and the carrier. The use of specific

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10.1021/bc800115w CCC: $40.75
Published on Web 10/30/2008

oligopeptide sequences recognized by intracellular enzymes is a promising approach for increasing the site-specific release of the drug; therefore, Mtx was coupled to the carrier *via* a cathepsin B-cleavable tetrapeptide spacer sequence (GFLG). This linker is frequently used in drug conjugates and could be cleaved in the lysosomes (*21, 22*). For the present study, the spacer sequence was elongated at the *C*-terminal with a cysteine to introduce a thiol group for conjugation through the thioether bond to the carrier.

In this paper, the synthesis and characterization as well as the chemotactic and cytotoxic properties of the new bioconjugates and their components (carrier, carrier with targeting moieties in the branches, Mtx, and Mtx-spacer) are reported. To ensure the release of the drug from the conjugates, enzymatic digestions were investigated using Cathepsin B. The chemotaxis, cellular uptake, and apoptosis assays were carried out on human MonoMac6 cell line, representing monocytic cells with a closely related pattern of surface receptors, and they display phenotypic and functional features of mature human monocytes and macrophages (*23*). Inasmuch as monocytes and macrophages express tuftsin receptors (*24, 25*), MonoMac6 cells might express these receptors as well. In this way, we can ensure that our conjugates might be internalized by receptor-mediated endocytosis.

Chemotaxis assays were carried out with the Mtx-conjugates and with their components. Each of the conjugates could elicit significant chemotactic effect on MonoMac6 cells. Cellular uptake of the CF-labeled analogues of the conjugates was studied by flow cytometry. Rapid internalization of each CF-labeled conjugate was observed. We investigated the cytotoxicity of Mtx-conjugates and Mtx *via* apoptosis assay on the MonoMac6 cell line. Mtx-conjugates and the Mtx itself induced significant cell death at 1 μM concentration after 24 or 48 h. Our data show that MonoMac6 cells are highly susceptible to the toxic effects of the conjugates through enhanced cell death by necrosis.

Our results indicated that these kind of drug delivery systems are rather effective on the MonoMac6 cell line. Some of our conjugates were attractants for the cells, could be taken up considerably, and could elicit a toxic effect inside the cells.

EXPERIMENTAL PROCEDURES

Materials. Amino acid derivatives were purchased from Reanal (Budapest, Hungary) or NovaBiochem (Läufelfingen, Switzerland), MBHA and Rink-Amide MBHA resins were NovaBiochem products. Reagents for synthesis [*N,N*′-dicyclohexylcabodiimide (DCC), *N,N*′-diisopropylcarbodiimide (DIC), *N*-diisopropyl-ethylamine (DIEA), 1-hydroxybenzotriazole (HOBt), 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), piperidine, trifluoroacetic acid (TFA), hydrogen fluoride (HF), *p*-cresol, *p*-thiocresol, thioanisole, phenol, 1,2-ethanedithiol (EDT), 1,4-DL-dithiothreitol (DTT), and 5(6)-carboxyfluorescein (CF) were purchased from Fluka (Buchs, Switzerland). Acetic anhydride (Ac_2O), dimethylsulfoxide (DMSO), and solvents for the synthesis were obtained from Reanal. HPLC-grade acetonitrile and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were ordered from Sigma-Aldrich Kft. (Budapest, Hungary). Methotrexate was purchased from Lederle Laboratories (Gosport, UK). Chloroacetic acid pentachlorophenyl ester (ClAc-OPcp) and formic acid trichlorophenyl ester (For-OTcp) were prepared in our laboratory from the appropriate acids and phenol derivatives (Fluka products) with DCC according to the method described by Martinezet al. (*26*).

Synthesis of the Components: H-TKPR-NH_2 (1), H-TKPKG-NH_2 (2), Ac-[TKPKG]$_4$-NH_2 (OT20) (3). The peptides were built up on MBHA resin (1.04 mmol/g capacity) by stepwise synthesis using standard Boc chemistry. The synthetic protocol was as follows: (i) washing of the resin with DCM (3 × 0.5 min), (ii) removal of Boc protecting groups with 33% TFA in DCM (2 + 20 min), (iii) DCM washing (5 × 0.5 min), (iv) neutralization with 10% DIEA in DCM (4 × 1 min), (v) DCM washing (4 × 0.5 min), (vi) coupling of 3 equiv Boc-amino acid derivative-DCC-HOBt (60 min), (vii) DMF washing (3 × 1 min), (viii) DCM washing (2 × 0.5 min), and (ix) ninhydrin or isatin assay (*27, 28*). The following amino acid derivatives were applied: Boc-Thr(Bzl)-OH, Boc-Lys(ClZ)-OH, Boc-Pro-OH, Boc-Gly-OH, and Boc-Arg(Tos)-OH. After the synthesis, the components, which were used as control peptides in the bioassays, were cleaved from the resin simultaneously with the removal of the protecting groups in the presence of *p*-cresol, using HF (10 mL HF/1 g *p*-cresol). The cleavage was carried out at 0 °C for 90 min. The crude peptides were purified by reverse-phase high-performance liquid chomatography (RP-HPLC), and the purified components were characterized by analytical RP-HPLC and electrospray ionization mass spectrometry (ESI-MS) (Table 1).

Table 1. Characteristics of Compounds

code	R_t[a] (min)	[M][b] calculated	[M][b] found
1	7.8	499.3	499.5
2	7.6	528.4	529.7
3	12.5	2103.4	2103.2
4	11.4	4036.1	4035.7
5	14.6	4148.1	4147.8
6	10.8	4152.4	4152.0
7	13.9	4320.4	4320.1
8	11.5	4275.0	4274.9
9	14.6	4387.0	4386.8
10	10.8	4391.1	4391.1
11	14.1	4559.1	4559.4
12	24.5	930.7	930.3
13	22.3	6063.4	6063.4
14	23.0	6175.4	6175.5
15	23.2	6182.5	6182.3
16	23.4	6348.5	6348.8
17	31.3,32.1	851.9	852.3
18	26.0	5909.0	5909.2
19	26.6	6020.2	6020.9
20	26.7	6024.5	6024.2
21	26.9	6192.2	6192.3

[a] RP-HPLC column: Phenomenex Synergy C_{12} (4 μm, 80 Å, 250 × 4.6 mm); eluents: 0.1% TFA/water (A eluent), 0.1% TFA/acetonitrile-water 80:20 v/v (B eluent); flow rate 1 mL/min; gradient: 0 min 0% B, 5 min 0% B, 50 min 90% B. [b] ESI-MS was performed on a Bruker Esquire 3000+ ion trap mass spectrometer. Standard resolution mode resolving 2+ ions @ 13 000 u/s. Maximum resolution mode resolving 3+ and 4+ ions @ 1650 u/s. Extended mass range 200−6 000 *m/z* @ 27 000 u/s. Unsurpassed sensitivity in the low pg range.

Synthesis of the Branched Chain Oligopeptides: Ac-[TKPK(H-TKPR)G]$_4$-NH_2 (4), Ac-[TKPK(For-TKPR)G]$_4$-NH_2 (5), Ac-[TKPK(H-TKPKG)G]$_4$-NH_2 (6), Ac-[TKPK-(Ac-TKPKG)G]$_4$-NH_2 (7). The carrier backbone was built up on MBHA resin using the Boc strategy as described earlier (*29*). The following amino acid derivatives were applied: Boc-Thr(Bzl)-OH, Boc-Pro-OH, Boc-Gly-OH, Boc-Lys(ClZ)-OH in position 2, and Boc-Lys(Fmoc)-OH in position 4 in the repeat units. Prior to the synthesis of the branches, the *N*-terminus of the backbone was acetylated. Fmoc groups of the lysine side chains were removed selectively and the appropriate branches were built up in every second lysine residue using the mixed Fmoc/Bzl strategy. For this, the following amino acid derivatives were used: Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Lys(ClZ)-OH, Fmoc-Pro-OH, Fmoc-Thr(Bzl)-OH, and Z-Thr-OH.

In the case of peptides H-TKPR and H-TKPKG in the branches (peptides **4** and **6**), Z-Thr-OH was used for the incorporation of the *N*-terminal amino acid. In this way, a free amino terminus was formed in the branches after removal of the protecting groups with HF. In the case of For-TKPR and

Ac-TKPKG in the branches (branched peptides **5** and **7**), Fmoc-Thr(Bzl)-OH was applied. After the removal of the last Fmoc group from the *N*-terminus of the branches, a formyl (compound **5**) or acetyl group (compound **7**) was attached to the free α-amino groups using either For-OTcp (20 equiv in DMF for 3 h) or Ac_2O (Ac_2O/DIEA/DMF = 1:1:3 mL for 0.5 h).

The branched peptides were cleaved with HF, were purified and characterized in the same way as described above. Characteristics of the purified compounds are listed in Table 1.

Synthesis of the Branched Peptides with Chloroacetylated *N*-Terminal Lysine in the Backbone: ClAc-K(ClAc)-[TKPK(H-TKPR)G]$_4$-NH$_2$ (8), ClAc-K(ClAc)-[TKPK(For-TKPR)G]$_4$-NH$_2$ (9), ClAc-K(ClAc)-[TKPK(H-TKPKG)G]$_4$-NH$_2$(10),ClAc-K(ClAc)-[TKPK(Ac-TKPKG)-G]$_4$-NH$_2$ (11). The carrier backbone was built up on MBHA resin using the Boc strategy as described above, and a Boc-Lys(Boc)-OH was attached to the *N*-terminus using DIC/HOBt activation. Fmoc groups were removed and the branches were built up to form the chemotactic targeting sequences similarly to peptide **4**−**7**. Boc groups from *N*-terminal Lys were removed, and chloroacetylation was performed using 10 equiv of ClAc-OPcp in DMF. The chloroacetylated peptides were cleaved and worked up similarly to the previously described derivatives, but 10 mL HF/0.5 g *p*-cresol and 0.5 g *p*-thiocresol was used as the cleavage mixture that is suggested to haloacetylated peptides (*30*). The reaction time was 90 min at 0 °C. The crude peptides were purified by semipreparative RP-HPLC, and the purified components were characterized by analytical RP-HPLC and ESI-MS. Analytical data of chloroacetylated peptides are presented in Table 1.

Synthesis of Mtx$^{\gamma}$-GFLGC-NH$_2$ (12) and CF-GFLGC-NH$_2$ (17). The synthesis and isomer separation of Mtx-GFLGC-NH_2 peptide were published earlier (*4*). The Mtx-containing GFLGC peptide was built up on Rink-Amide MBHA resin using standard Fmoc chemistry. The synthetic protocol was as follows: (i) washing of the resin with DMF (3 × 1 min), (ii) removal of Fmoc protecting groups with 2% piperidine and 2% DBU in DMF (2 + 2 + 5 + 20 min), (iii) DMF washing (8 × 1 min), (iv) coupling of 3 equiv Fmoc-amino acid derivative-DIC-HOBt (60 min), (v) DMF washing (3 × 1 min), (vi) DCM washing (2 × 0.5 min), and (vii) ninhydrin or isatin assay (*27, 28*). A trityl group was used for the side chain protection of Cys. The peptide was cleaved from the resin with 10 mL TFA using scavengers (phenol/thioanisole/EDT/water = 0.75 g/0.5 mL/0.25 mL/0.5 mL). The crude products were purified, and the isomers were separated by semipreparative RP-HPLC. CF-GFLGC-NH_2 was prepared in the same way, but the isomers of 5(6)-carboxyfluoresceine were not separated. The pure compounds were characterized by analytical HPLC and ESI-MS (Table 1).

Conjugation of the Chloroacetylated Peptides with Mtx$^{\gamma}$-GFLGC-NH$_2$ or CF-GFLGC-NH$_2$ (13−16 and 18−21). The chloroacetylated peptides were dissolved in 0.1 M Tris buffer (pH 8.2) at 10 mg/mL peptide concentration and Mtx$^{\gamma}$-GFLGC-NH_2 or CF-GFLGC-NH_2 was added to the solution in solid form at regular intervals. Under these conditions, thioether bonds can be formed between the chloroacetyl group of the branched peptides and the thiol group of the Cys in the spacer sequence resulting in compounds **13**−**16** and **18**−**21**. The conjugation reaction was monitored by analytical HPLC. At the end of the reaction, the mixtures were acidified and purified by RP-HPLC directly. The yields of the conjugations were 50−60% calculated for the carrier. The pure compounds were characterized by analytical RP-HPLC and ESI-MS (Table 1).

Reverse-Phase High-Performance Liquid Chomatography (RP-HPLC). Analytical RP-HPLC was performed on a Knauer (H. Knauer, Bad Homburg, Germany) HPLC system using a Phenomenex Synergy C_{12} column (4 μm, 80 Å, 250 × 4.6 mm I.D.) (Torrance, CA) as the stationary phase. Linear gradient elution (0 min 0% B, 5 min 0% B, 50 min 90% B) was used with the following eluents: 0.1% TFA/water (A eluent), 0.1% TFA/acetonitrile−water 80:20 v/v (B eluent). The flow rate was 1 mL/min at ambient temperature, and the peaks were detected at λ = 220 nm.

The crude peptides and conjugates were purified on a semipreparative Phenomenex Jupiter C_{18} column (10 μm, 300 Å, 250 × 10 mm I.D.) (Torrance, CA). The applied flow rate was 4 mL/min at ambient temperature, and the peaks were detected at λ = 220 nm. The same eluents as described above with an appropriate linear gradient were applied.

Electrosray Ionization Mass Spectromerty (ESI-MS). ESI-MS was performed on a Bruker Esquire 3000+ Ion trap mass spectrometer (Bremen, Germany), operating in continuous sample injection mode at 4 μL/min flow rate. Samples were dissolved in 50% acetonitrile/50% water containing 0.01% acetic acid. Mass spectra were recorded in positive ion mode in the *m*/*z* 200−1500 range.

Enzymatic Digestion by Cathepsin B. Conditions for digestion (enzyme concentration, pH, temperature) were chosen to approximate the lysosomal medium as a model for intracellular drug release. Compounds **12** and **15** were dissolved in a solution of 5 mM cysteine/2 mM EDTA (pH = 5). For the digestions, 200 μL aliquots of the solutions were used, and 0.1 unit of human liver cathepsin B (Merck Kft., Budapest, Hungary) was added to the solutions. The mixtures—giving the following initial concentrations: [cathepsin B]:[substrate] = 1:50—were incubated at 37 °C for 15 min and 1 h. Stabilities of the compounds were also measured without cathepsin B at pH 5 under the same conditions. After the incubation, the enzyme-containing solutions as well as the control samples were measured using tandem mass spectrometry, and the fragments were identified.

Cell Culture. Human monocytic MonoMac6 cells were grown in suspension in RPMI-1640 (Sigma Ltd. St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Cergy-Pontoise, France) L-glutamine (2 mM) and gentamicin (35 μM). Cells were routinely maintained on plastic tissue culture dishes at 37 °C in an incubator with a humidified atmosphere containing 5% CO_2/95% air.

Chemotaxis Assay. The chemotactic ability of MonoMac6 cells was evaluated using a 96-well modified Boyden chamber (NeuroProbe, Gaithersburg, MD) (*31*). Polycarbonate filters with a pore size of 8 μm were used. Due to the requirements of cell-physiological experiments, the peptides were dissolved in an identical substance to culturing free of the ligand to be studied. For this purpose, RPMI-1640 supplemented with 10% heat-inactivated FCS, L-glutamine (2 mM/mL), and gentamicin (35 μM) was used. The concentration dependence of the chemotactic response was determined to be in the 0.0001−1 μM range. The 96-well plate was placed into the chamber, 395 μL of peptide solution was added to the bottom wells for each experiment, and the chamber was locked. The cells in medium (100 μL) at density of 10^5 cell/mL were applied to each of the top wells. The incubation time was 3 h at 37 °C in a humidified atmosphere containing 5% CO_2/95% air, allowing migration across the membrane. After the incubation, nonmigrated cells were removed from the upper surface of the membrane, and the number of cells, which were migrated into the 96-well plate through a filter, was determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (*32, 33*). For this, 20 μL of 12 μM MTT solution was added to wells. Cells were incubated with MTT at 37 °C for 24 h, then the plates were centrifuged at 2000 rpm for 5 min. Sample supernatants were removed, formazan crystals were solubilized in DMSO,

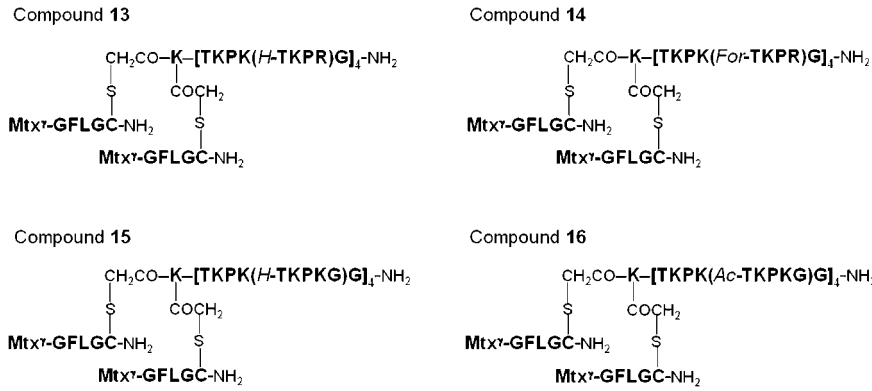


Figure 1. Schematic structure of the Mtx conjugates.

and the colorimetric measurement was carried out by an ELISA reader (Labsystems Multiscan MS-Reader, Helsinki, Finland) at $\lambda = 540$ and $\lambda = 620$ nm. Each data point represents the average of fifteen parallel measurements.

Cellular Uptake. MonoMac6 cells were cultured in RPMI-1640 supplemented with 10% FCS, L-glutamine (2 mM), and gentamicin (35 μM). Cell culture was maintained at 37 °C in a humidified atmosphere with 5% CO_2. To study the cellular uptake of fluorescent-labeled analogues of the conjugates, 10^5 cells per well were plated on 24-well plates. After 24 h incubation at 37 °C, cells were treated with the conjugates dissolved in serum-free RPMI-1640 for 1.5 h. The compounds were used in the 0.1−100 μM concentration range. Cells treated with serum-free medium for 1.5 h were used as control. After incubation, cells were washed with HPMI (glucose, $NaHCO_3$, NaCl, HEPES, KCl, $MgCl_2$, $CaCl_2$, $Na_2HPO_4 \times 2H_2O$) and were treated with trypsin for 10 min. The trypsinization was stopped by HPMI supplemented with 10% fetal calf serum, and cells were moved from the plate to FACS tubes. After washing, cells were resuspended in HPMI. The increase of the fluorescence of MonoMac6 cells after this preparation was monitored by flow cytometry (BD LSR II, BD Bioscience, San Jose, CA). Data were analyzed with *FACSDiVa* software. Fluorescence mean values of the untreated control were subtracted from the mean fluorescence of treated samples. Statistical analysis of data was performed using the Student's *t* test at the 95% confidence level.

Annexin V Apoptosis Assay. Mtx and Mtx-conjugates were dissolved in RPMI-1640 supplemented with 10% heat-inactivated FCS, L-glutamine (2 mM), and gentamicin (35 μM). Flow cytometry and cell staining was applied with fluorescein isothiocyanate- (FITC) labeled annexin-V (green fluorescence) (*34*−*37*), simultaneously with propidium iodide (PI) stain (red fluorescence) (*38*), to discriminate intact cells from apoptotic cells and necrotic cells after treatment with the conjugates and Mtx. The apoptotic/necrotic effect of Mtx and of conjugates was determined in the 0.01−1 μM range. Briefly, after cultured cells were pretreated with Mtx or conjugates for 3, 24, and 48 h at 37 °C in a humidified atmosphere containing 5% CO_2, cells were harvested and washed with phosphate-buffered saline (PBS). Cells were then resuspended in 100 μL of Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4), incubated with 5 μL of Annexin-V-FITC at room temperature in the dark for 15 min followed by adding 400 μL of Annexin-V binding buffer to the cells. The cell suspensions were vortexed and analyzed immediately by flow cytometry using a Becton Dickinson FACS-Calibur flow cytometer with *Cell Quest* software (Becton Dickinson, Mountain View, CA). Finally, 5 μL PI was added to each tube, and the tubes were vortexed and analyzed again by flow cytometry. Untreated cells displayed 0% annexin+/PI− (early apoptosis) and 0% annexin−/PI+ (necrosis) after 3, 24, and 48 h incubation.

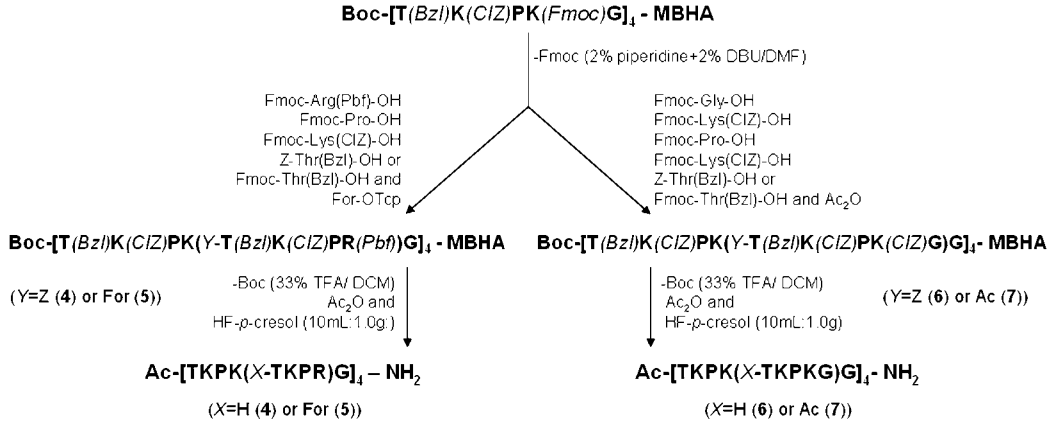
Statistical Evaluation of Data. Data shown in the figures demonstrate the averages ± SE values. The statistical analysis was done by the application of the *t* test of *Origin 7.0*.

RESULTS AND DISCUSSION

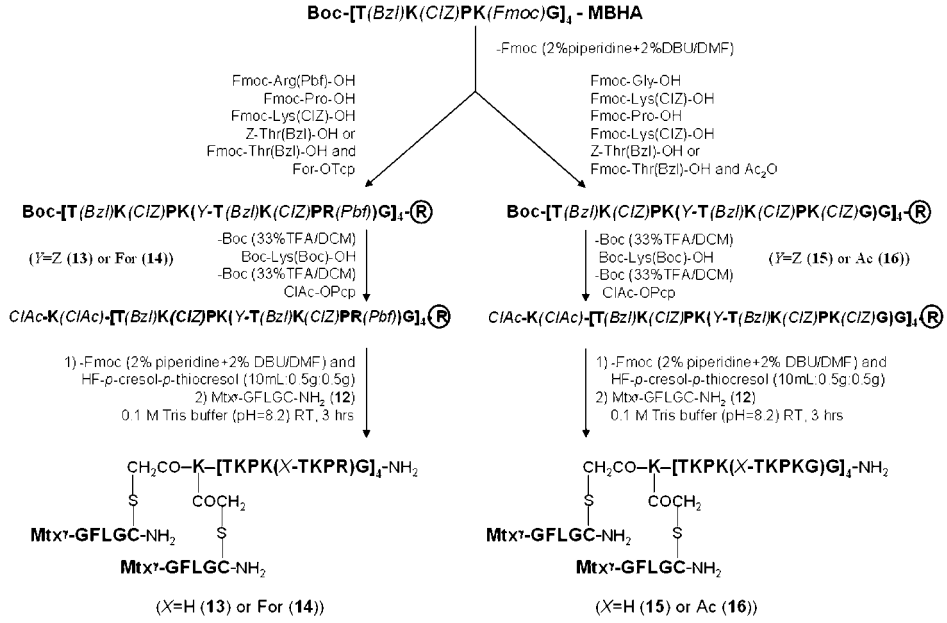
New anticancer drug delivery systems based on chemotactic drug targeting (CDT) were developed consisting of Mtx as the anticancer drug attached to an immunomodulatory carrier molecule through an enzyme-labile peptide spacer and targeting sequences with chemotactic activities. Four novel Mtx-conjugates (Figure 1), their Mtx-CF exchanged analogues, and their components were synthesized, purified, and characterized. These peptide conjugates were tested in various relevant biological assays: chemotaxis, cellular uptake, and cytotoxicity on the MonoMac6 cell line.

Synthesis. Linear peptides representing the components of the designed chemotactic branched oligopeptides and the carrier backbone (tetratuftsin derivative) were synthesized by standard Boc chemistry. The branches on carrier backbone were built up using Fmoc/Bzl strategy. In the case of the branched peptides without Mtx, the *N*-terminus of the backbone was acetylated (**4**−**7**), as outlined in Scheme 1. In the case of the drug or CF-labeled oligopeptide conjugates, an additional Lys residue was coupled to the *N*-terminus of the carrier backbone, and then both α- and ε-amino groups were chloroacetylated to form two conjugation sites (Scheme 2). Peptides were cleaved from the resin with liquid HF in the presence of appropriate scavengers. In addition, Mtx or CF containing spacer peptides (GFLG) elongated with a Cys residue at the *C*-terminus (Mtx$^{\gamma}$-GFLGC-NH_2 (**12**), CF-GFLGC-NH_2 (**17**)) were synthesized by Fmoc chemistry. The peptides were purified by RP-HPLC. The isomers (α, α-D, and γ) of Mtx containing peptide could be separated (*4*), but in case of the 5- and 6-carboxyfluoresceine derivatives, the separation was not successful. In order to form conjugates, the purified chloroacetylated branched oligopeptides were dissolved in 0.1 M Tris buffer (pH 8.2), and Mtx$^{\gamma}$-GFLGC-NH_2 or CF-GFLGC-NH_2 was added to the solution in solid form at regular intervals over 3 h at RT. Under these conditions, a thioether bond was formed between the chloroacetyl group of the branched oligopeptides and the thiol group of the cysteine in the spacer peptide resulting in compounds **13**−**16** (Scheme 2) and **18**−**21**. All compounds were purified by RP-HPLC and characterized by analytical HPLC and ESI-MS (Table 1). The chemoselective ligation followed by RP-HLPC purification resulted in conjugates (Figure 1) with 50−60% yields and 93−97% purity in all cases. While the two isomers of CF-

Scheme 1. Synthesis of Acetylated Branched Peptides



Scheme 2. Synthesis of Mtx-Conjugates



GFLGC-NH_2 were separated on the applied analytical HPLC column, the CF labeled conjugates (**18**−**21**) gave only single peaks.

Enzymatic Digestion by Cathepsin B. To determine the intracellular drug release enzymatic digestions with cathepsin B, a lysosomal enzyme, were carried out with the drug-spacer Mtx^{γ}-GFLGC-NH_2 (**12**) and conjugate **15** (Figure 1). In both cases, the main fragment split by cathepsin B was Mtx^{γ}-Gly-OH ($[M + H^+] = 512.3$), but Mtx^{γ}-Gly-Phe-OH ($[M + H^+] = 659.4$) and Mtx^{γ}-Gly-Phe-Leu-Gly-OH ($[M + H^+] = 829.5$) fragments were also identified by mass spectrometry. However, no significant enzymatic cleavage between Leu and Gly was observed (Figure 2). In the case of the conjugate (compound **15**), the multiple charged peaks of $[M + H^+] = 4560.0$ fragment (381.4 (12+); 415.4 (11+); 457.0 (10+); 506.3 (9+); 570.9 (8+); 652.5 (7+)) were also detected, which do not include the two Mtx^{γ}-GFLG sequences on the carrier backbone (Figure 3). No significant split in the oligotuftsin backbone could be detected, and the cleavage pattern did not change when the incubation time was increased from 15 min to 1 h. Our results suggested that the main cleavage site is between the Gly and Phe amino acid residues in the spacer sequence; thus, the drug can be released from the conjugates in the lysosomes. It is worth noting that the cleavage of the Ala-Cys bond in the lysosomal preparations was observed in the case of a conjugate of H-LLEDPVGTVAC-NH_2 (HSV epitope peptide) attached to the OT20 carrier through a thioether bond (*39*). On the basis of this similarity, we can hypothesize that peptides with Cys-amide at the *C*-terminus might provide a cleavage site for cathepsin B, which cannot be prevented by conjugation of Cys through thioether bond. This type of conjugation with an appropriate spacer sequence might provide some benefits to an effective drug release from drug delivery systems.

Chemotactic Activity of Compounds 1, 2, and 3. Chemotactic activity of H-TKPR-NH_2 (**1**), H-TKPKG-NH_2 (**2**), and Ac-[TKPKG]$_4$-NH_2 (OT20) (**3**) was examined. Compound **1** triggered a significant chemoattractant effect at 1 μM (337.9 ± 7.6%) and 10^{-3} μM (203.0 ± 5.0%), but at 10^{-1} μM, it was repellent (68.8 ± 18.2%), and at the other concentrations, it was neutral. In contrast, compound **2** was significantly repellent at 10^{-1} μM (55.6 ± 18.2%) and 10^{-4} μM (75.0 ± 7.8%), and neutral at the other concentrations. The tetratuftsin derivative carrier (**3**) also had chemorepellent behavior at 1 μM (56.5 ± 20.3%). However, no significant chemoattractant or chemorepellent effect was observed at the other concentrations on MonoMac6. These data indicated that the oligomerization of H-TKPKG-NH_2 (**2**) produced a carrier molecule (**3**) without a significant change of chemotactic activity (Supporting Information S2).

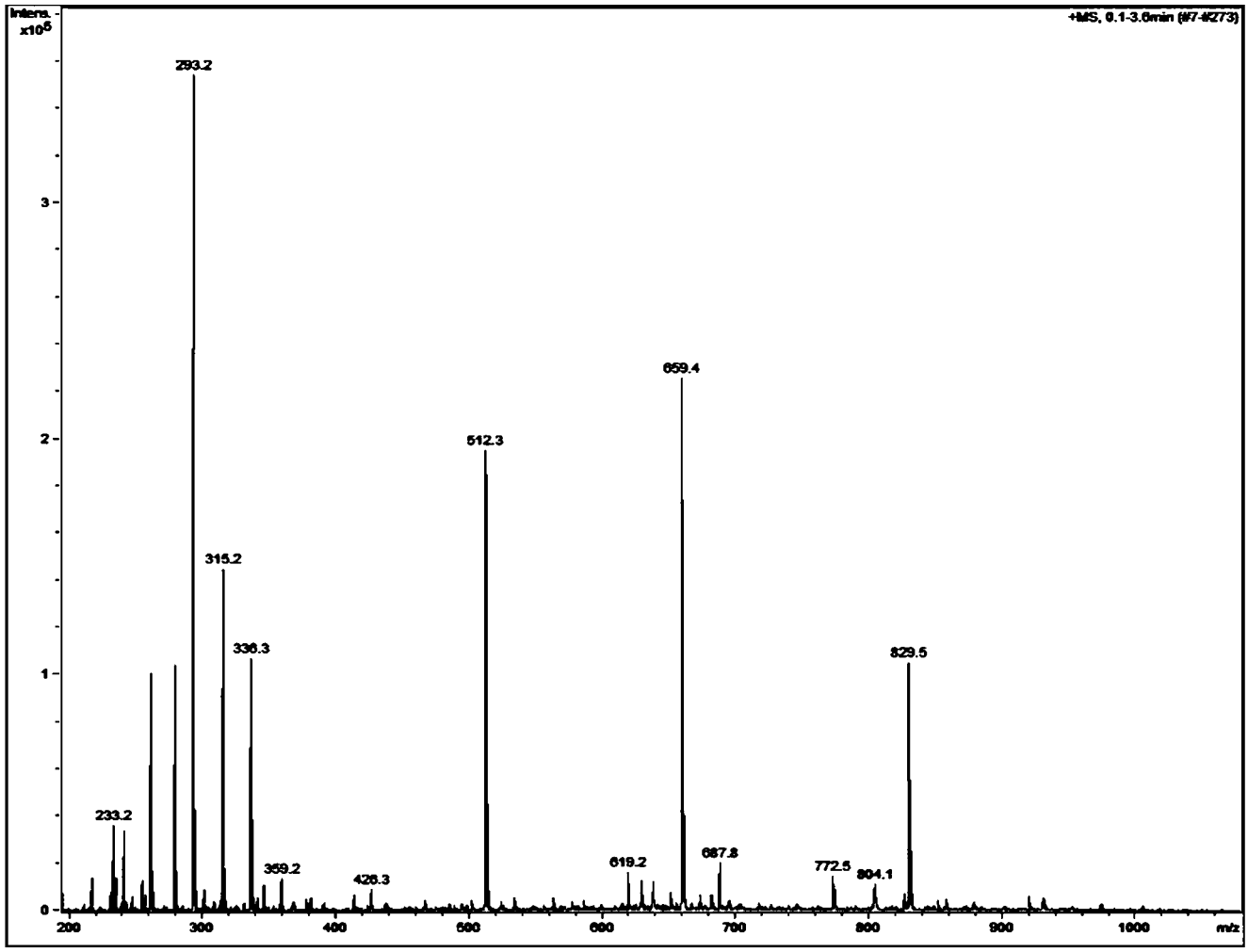


Figure 2. Mass spectrum of Mtx$^{\gamma}$-GFLGC-NH_2 (**12**) after digestion with cathepsin B.

Chemotactic Activity of Mtx and Mtx$^{\gamma}$-GFLGC-NH_2. Chemotactic activity of Mtx and Mtx$^{\gamma}$-GFLGC-NH_2 (**12**) molecules was also tested on MonoMac6. The Mtx itself was significantly repellent at the lower concentration range (10^{-2} μM, 47.3 ± 14.6%, 10^{-3} μM, 78.6 ± 19.6%; 10^{-4} μM, 66.9 ± 20.7%) and neutral at the higher concentrations. In contrast to Mtx, compound **12**, the drug containing enzyme sensible spacer sequence of the total CDT bioconjugate, was found to be significantly attractant at the lower concentration range (10^{-2} μM: 145.5 ± 4.8%, 10^{-3} μM: 153.5 ± 4.6%, 10^{-4} μM: 138.5 ± 6.5%), but it was repellent at the highest concentration (1 μM: 63.5 ± 16.1%). The effect of GFLGC on the repellent moiety of Mtx seems to be very advantageous with respect to the biological/clinical activity of the CDT bioconjugate (Supporting Information S3).

Chemotactic Activity of the Branched Peptides and the Mtx-Containing Bioconjugates. Four different targeting moieties in branches (H-TKPR, For-TKPR, H-TKPKG, Ac-TKPKG) were built up on the tetratuftsin carrier, resulting in the *N*-terminal acetylated branched peptides (**4**, **5**, **6**, **7**). In the case of the Mtx conjugates, chloroacetylation of both amino groups of Lys inserted in the *N*-terminus of the carrier was performed and these chloroacetylated branched peptides were conjugated with the Mtx$^{\gamma}$-GFLGC-NH_2 in solution (Scheme 2). This reaction resulted in four Mtx-containing conjugates (**13**, **14**, **15**, **16**). The four drug−oligopeptide conjugates as well as the corresponding branched oligopeptides were tested in chemotaxis assay on MonoMac6 cells. In the case of H-TKPR branches, the branched peptide (**4**) had a chemoattractant effect at 1 μM (115.1 ± 3.5%), and it was slightly repellent at the lower concentration range (10^{-2} μM, 82.6 ± 6.9%; 10^{-3} μM, 85.0 ± 6.1%) (Supporting Information S4). When Mtx$^{\gamma}$-GFLGC-NH_2 was coupled to the peptide resulting in compound **13**, significant chemoattractant behavior appeared over the whole concentration range (10^{-1} μM, 174.7 ± 4.3%; 10^{-2} μM, 306.9 ± 2.3%; 10^{-3} μM, 321.8 ± 2.3%; 10^{-4} μM, 289.6 ± 3.2%) (Figure 4A). In this case, the coupling of the Mtx$^{\gamma}$-GFLGC-NH_2 considerably amended the biological properties of the branched peptide.

In the case of the formylated branches, the branched peptide (**5**) had a chemoattractant effect at 1 μM (135.2 ± 7.4%), but it was repellent at the lower concentration range (10^{-2} μM, 57.5 ± 17.6%; 10^{-4} μM, 68.0 ± 16.7%) (Supporting Information S4). The conjugation with Mtx$^{\gamma}$-GFLGC-NH_2 does not modulate the chemotactic behavior of the molecule (**14**), the conjugate was still significantly attractant at 1 μM (157.6 ± 7.6%), and in the lower concentration range, it could elicit a chemorepellent effect (10^{-2} μM, 70.5 ± 14.1%; 10^{-4} μM, 73.9 ± 12.3%) (Figure 4B).

In case of H-TKPKG branches, the acetylated branched peptide (**6**) showed a biphasic effect; it could trigger a chemorepellent effect at the higher concentration range (1 μM, 53.3 ± 16.2%; 10^{-1} μM, 30.9 ± 20.3%), but it exhibited significant attractant chemotactic activity at the lower concentration range (10^{-2} μM, 388.9 ± 1.9%; 10^{-3} μM, 396.0 ± 2.1%; 10^{-4} μM, 251.4 ± 4.0%) (Supporting Information S4). The coupling of the Mtx$^{\gamma}$-GFLGC-NH_2 to the branched peptide resulted in adverse and less significant effects; the conjugate (**15**) was attractant at 10^{-1} μM (160.0 ± 1.9%), but it could

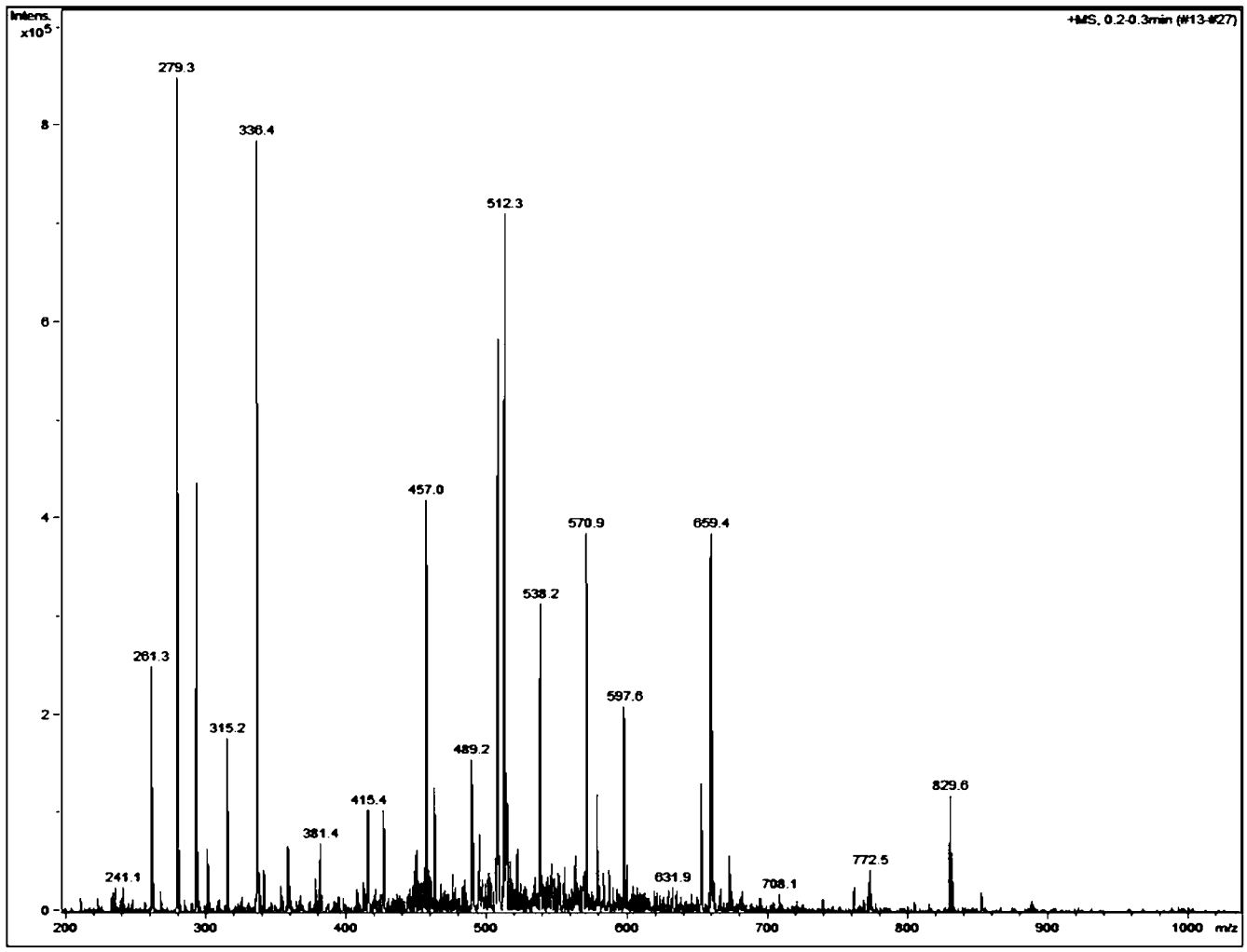


Figure 3. Mass spectrum of conjugate **15** after digestion with cathepsin B.

elicit a repellent effect in the lower concentration range (10^{-2} μM, 59.5 ± 19.3%; 10^{-3} μM, 71.9 ± 17.5%) (Figure 4C).

When the Ac-TKPKG branched peptide was studied, a significant attractant behavior was detected at 10^{-1} μM (139.6 ± 3.9%), which was retained, but shifted to the lower concentration range (10^{-2} μM: 146.1 ± 5.2%) after conjugation with Mtx$^{\gamma}$-GFLGC-NH_2 resulting in compound **16** (Supporting Information S4; Figure 4D).

Cellular Uptake of the CF-Labeled Conjugates. For the cellular uptake studies, the synthesis of CF-labeled conjugates was carried out. For this, CF-GFLGC-NH_2 was synthesized—similarly to Mtx$^{\gamma}$-GFLGC-NH_2—and coupled to the branched peptide *via* a thioether bond. Four CF-labeled conjugates (**18**, **19**, **20**, **21**) were studied. The mean fluorescence intensity of cells was quantitated by flow cytometry. After treatment for 90 min, cells were analyzed. We observed the concentration-dependent accumulation of fluorescence signal within the cells (Figure 5), indicating rapid internalization of each CF-labeled conjugate. A high level of internalization of the CF-labeled conjugates with For-TKPR branches was observed, while the internalization of the CF-labeled conjugates with H-TKPR branches was less pronounced (Figure 5A,B). Consequently, the formylation of the branches increased the cellular uptake of the conjugates. In the case of H-TKPKG branches, a relatively high internalization was observed, whereas the CF-labeled conjugates with Ac-TKPKG branches were internalized considerably within 90 min. In fact, the CF-labeled conjugates with Ac-TKPKG branches showed higher levels of internalization at all studied concentrations (Figure 5C,D). Consequently, the acetylation of the branches also increased the cellular uptake of the conjugates. The cellular uptake of the conjugates with H-TKPR branches was higher compared to the H-TKPKG branches. By the same token, the internalization of the conjugates with For-TKPR branches was compared to the conjugates with Ac-TKPKG branches: the rate of the cellular uptake was almost the same. A very high increase of fluorescence intensity in the cells was observed when the concentration of the conjugates was elevated from 4 μM to 20 μM. We believe that at lower concentration the conjugates are taken up in a receptor-mediated way, while at a higher concentration, they enter into the cells by fluidic endocytosis or the conjugates behave as cell penetrating peptides because of their strong positively charged character. The mechanism of the cellular uptake of these conjugates requires further study.

Apoptosis Assay. Phosphatidylserine (PS) is asymmetrically located in the lipid bilayer of the plasma membrane in mammalian cells. In cells undergoing apoptosis, PS is transferred from the cytoplasmic surface of the cell membrane to the outer surface of the plasma membrane and therefore serves as an early marker for apoptosis. By simultaneous staining with Annexin-V-FITC and PI, we could distinguish among intact cells, early apoptosis, late apoptosis, and cell death. Assessment of MonoMac6 cells exposed to 10^{-2}, 10^{-1}, and 1 μM Mtx and Mtx conjugates for different periods of time (3, 24, and 48 h) showed that 2−12% cells were undergoing necrosis (Figure 6; Supporting Information S5−6). MonoMac6 cells showed an an-

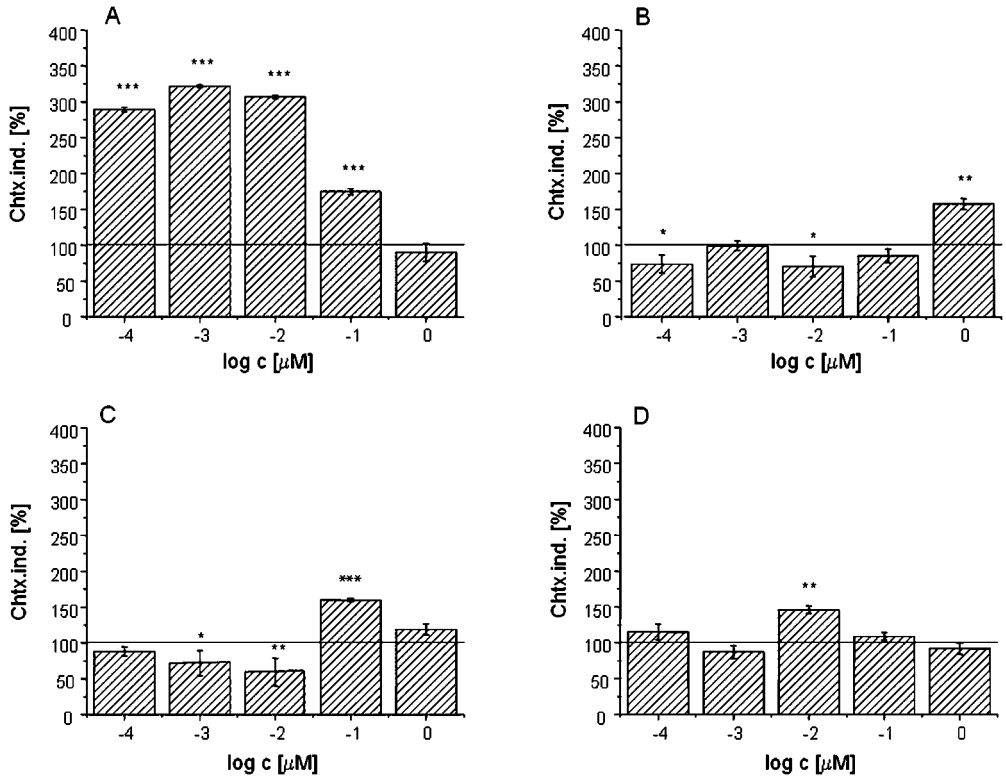


Figure 4. Chemotactic activity of Mtx conjugates with **A**, H-TKPR (**13**); **B**, For-TKPR (**14**); **C**, H-TKPKG (**15**); **D**: Ac-TKPKG (**16**) peptides in the branches. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

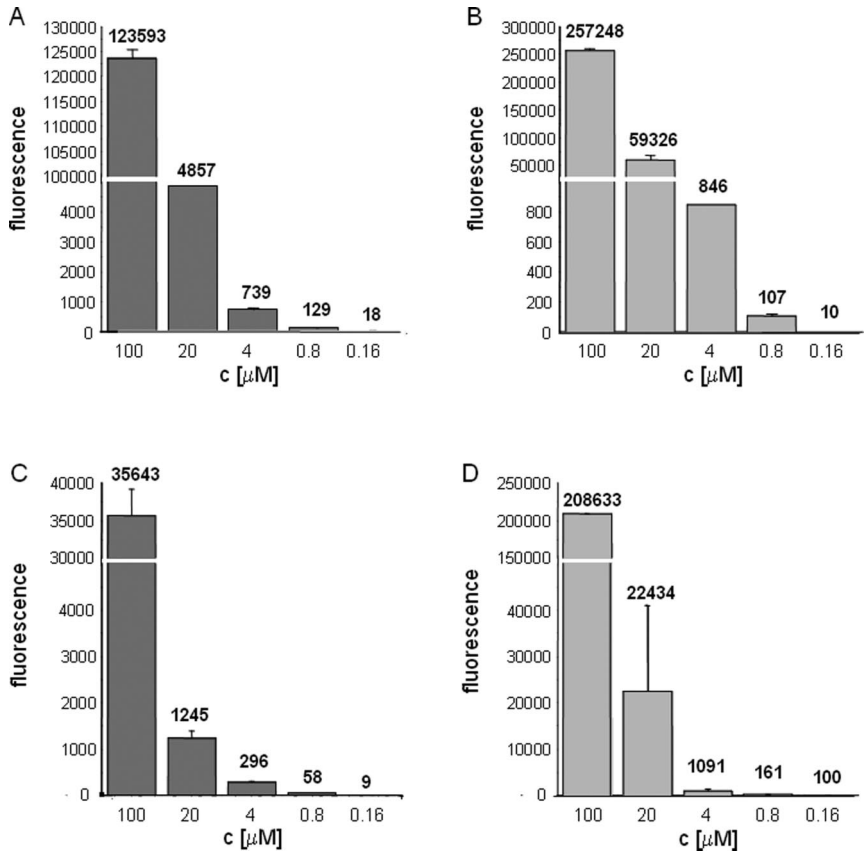


Figure 5. Cellular uptake of the CF-labeled conjugates with A, H-TKPR (**18**); B, For-TKPR (**19**); C, H-TKPKG (**20**); D, Ac-TKPKG (**21**) peptides in the branches.

nexin-/PI+ (indicating cell necrosis) pattern after 3, 24, and 48 h treatment with Mtx and Mtx conjugates in all concentrations. Only a few annexin+/PI− cells (indicating cell apoptosis) and annexin+/PI+ (indicating later stage of apoptotic cells) were observed. As it is shown in Figure 6 and Supporting Information S5−6, detection of MonoMac6 necrotic cells was dose- and

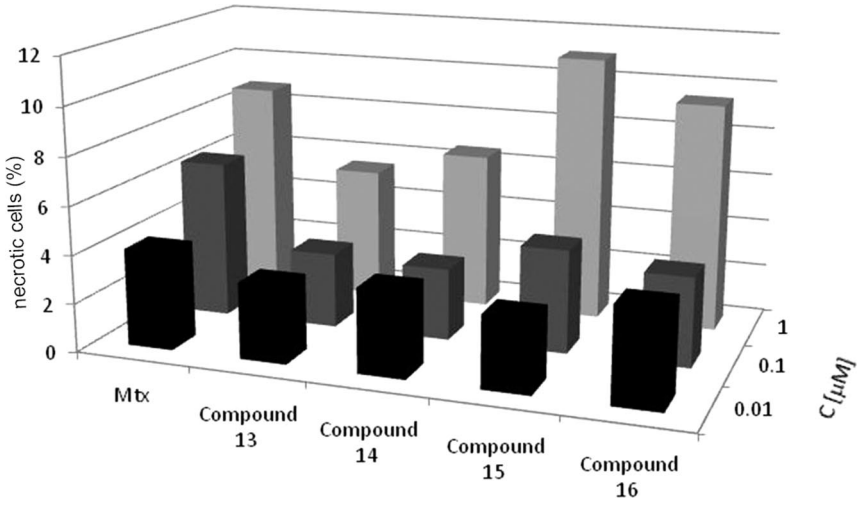


Figure 6. Effect of Mtx and Mtx-conjugates with H-TKPR (**13**), For-TKPR (**14**), H-TKPKG (**15**), and Ac-TKPKG (**16**) peptides in the branches in apoptotic assay on the MonoMac6 cell line after 24 h incubation.

time-dependent (maximum at 24 h, Figure 6). Mtx was more active than the conjugates at the concentrations of 0.01 and 0.1 μM, but at 1 μM dose, conjugates **15** and **16** showed higher toxity on MonoMac6 cells during the whole experiment.

CONCLUSION

In summary, new targeted peptide-based drug delivery systems were synthesized and biochemically characterized. These biodegradable conjugates consist of Mtx as the drug, an enzyme labile peptide spacer (GFLGC), a biodegradable peptide carrier (OT20), and four types of targeting peptide moieties (H-TKPR, For-TKPR, H-TKPKG, and Ac-TKPKG), which have been shown to bind to tuftsin receptors, and the conjugates can be internalized by receptor-mediated endocytosis. Inside the cells, in the lysosomes, the drug molecules could be released from the conjugates after enzymatic (cathepsin B) cleavage; therefore, our conjugates could elicit the proper anticancer effect. *In vitro* biological assays were performed with the prepared conjugates. Chemotaxis assays were carried out with the compounds and the branched peptides, as well as with the drug-conjugates. Each of the branched peptides and the drug conjugates could trigger a significant chemotactic effect on MonoMac6 cells, but the most effective conjugate was compound **13**, which was significantly attractant in a wide concentration range ($10^{-1}-10^{-4}$ μM). Rapid internalization of the CF-labeled conjugates was observed. Apoptosis assay was performed using simultaneous staining with Annexin-V-FITC and PI. MonoMac6 cells were undergoing necrosis after treatment with Mtx or with compunds **13**−**16**. Compounds **15** and **16** were the most effective ones; these conjugates triggered a higher toxic effect than the free drug (Mtx). Compound **13** induced necrosis in MonoMac6 cells. The effectiveness of compound **13** is comparable to that of the free drug at 3 or 48 h incubation (Supporting Information S5, S6). First and last, if we draw a parallel between Mtx and Mtx-conjugates (**13**, **14**, **15**, and **16**) we can conclude that the conjugates have many advantages in comparison with Mtx. The conjugates can attract the MonoMac6 cells in certain concentrations, especially compound **13**, which has the most advatageous chemotactic properties. Its significant chemoattractant behavior appears at a wide concentration range. Beside the chemotaxis, the conjugates can be internalized rapidly and at the same level as the Mtx, but the mechanism of internalization is different. Mtx can simply diffuse through the membrane without any specificity in contrast to the conjugates, which can be taken up by the cells through receptor-mediated endocytosis. By the same token, the conjugates are at least as cytotoxic as Mtx. To conclude the results of the biological assays, the most promising compounds are **13** and **16**. The results of the biological tests confirmed the feasibility of this new chemotactic drug targeting strategy for increasing the efficacy and specificity of chemotherapy especially in tumor treatments.

ACKNOWLEDGMENT

This work was supported by grants from the Hungarian National Science Fund (OTKA K 043576 and T 049814), ETT 202/2006, "Medichem 2" 1/A/005/2004, as well as GVOP-3.2.1.-2004-04-0005/3.0 and GVOP-3.2.1-2004-04-0352/3.0.

Supporting Information Available: (i) Chemotactic response of MonoMac6 cells induced by H-TKPR-NH_2, H-TKPKG-NH_2, the tetratuftsin derivative carrier (OT20), Mtx and Mtx$^{\gamma}$-GFLGC-NH_2; (ii) Chemotactic activity of the branched peptides withot drug molecule; (iii) Effect of Mtx and Mtx-conjugates with H-TKPR, For-TKPR, H-TKPKG, Ac-TKPKG peptides in the branches in apoptotic assay on MonoMac6 cell line after 3 and 48 h incubation. This material is available free of charge via the Internet at http://pubs.acs.org.

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BC800115W