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Design, synthesis of methotrexate-diosgenin conjugates and biological evaluation of their effect on methotrexate transport-resistant cells

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ABSTRACT

A series of methotrexate-diosgenin conjugates was designed and synthesized to enhance the passive internalization of methotrexate (MTX) into transport-resistant cells. The inhibitory effects of these conjugates on dihydrofolate reductase (DHFR), and their anti-proliferation behaviors against a transport-resistant breast cancer cell line, MDA-MB-231, were investigated. All of the synthesized conjugates retained an ability to inhibit DHFR after the diosgenin substitution. The MTX conjugates were much more potent against methotrexate-resistant MDA-MB-231 cells than MTX. Conjugate **18**, containing a disulfide bond, exhibited the most potent anti-proliferative and DHFR inhibitory effects (IC_{50} = 4.1 μM and 17.21 nM, respectively). Anti-proliferative activity was higher in the conjugate with a longer space linker (conjugate **21**) than those with shorter linkers (conjugates **19** and **20**). These results suggest that diosgenin conjugation of MTX may be an effective way to overcome its transport resistance in cancer cells.

1. Introduction

Methotrexate (MTX, compound **2**, Fig. 1) is a major chemotherapeutic agent used in the treatment of various types of cancers such as malignant lymphoma, osteosarcoma, and breast cancer [1,2]. However, therapy with MTX has several limitations such as myelosuppression, nephrotoxicity, and gastrointestinal mucositis [3]. Cellular factors that impede its clinical efficacy include diminished glutamate polymerization, decreased dihydrofolate reductase (DHFR) activity, elevated DHFR expression, and lack of expression of a reduced folate carrier (RFC) that is a main transporter for folate analogs [3–5]. A deficiency in RFC-mediated transport is the primary and frequent mechanism of resistance to MTX, and the main reason for discontinuation of treatment with this drug [6]. Extensive efforts have been made to overcome folate transporter-related resistance of MTX [1]. Lipophilic modification of MTX at the γ-carboxylic group of glutamate shows considerable potential to overcome these limitations [7–9]. However, the therapeutic efficacy of the resulting conjugates is always limited by their decreased solubility, reduced DHFR affinity resulting from the lipophilic substitutions of MTX, and inefficient release of MTX.

Considering the limitations described above, we have assumed that naturally occurring hydrophobic molecules through the hydrophobic modification of MTX would probably overcome the RFC-mediated transport resistance. Diosgenin (compound **1**, Fig. 1), a naturally occurring steroidal saponin, is mainly isolated from legumes and yams, and exhibits high biocompatibility and various pharmacological activities such as anti-inflammation [7]. immune-protection [8], and anticancer [9] including that against colon carcinoma, HCT-116 cells [10], and breast cancer cells [11]. DG is a biocompatible, biodegradable, and non-toxic compound based on a previous toxicological and pharmacokinetic study [12]. Besides, DG is a strong hydrophobic molecule ($\log P$ = 5.7) [13] with cholesterol-like properties. Furthermore, DG was employed recently to modify various therapeutic agents [14–16]. Herein, we report the synthesis and biological evaluation of new series of MTX-DG conjugates via various polyamine linkers (Fig. 2).

2. Experimental section

2.1. Materials and methods

Methotrexate (MTX, purity > 98%), anhydrous *N*-methylmorpholine (NMM), triphosgene, O-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), anhydrous dichloromethane, and *N*-carboethoxyphthalimide were obtained from

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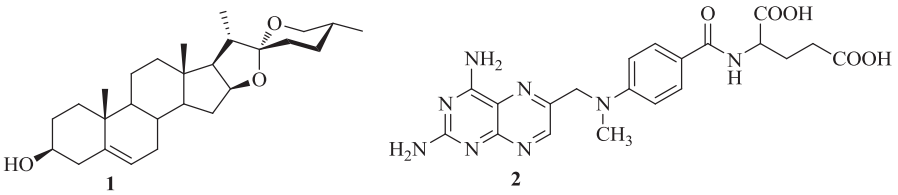

Fig. 1. Structures of diosgenin (**1**) and methotrexate (**2**).

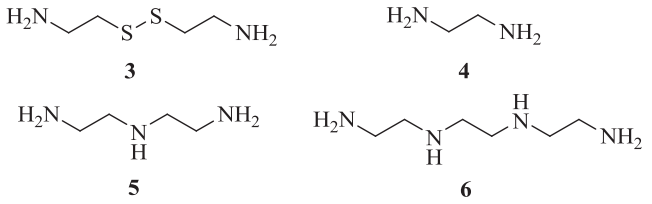

Fig. 2. The polyamines used as linkers.

Tokyo Chemical Industry (Tokyo, Japan). Cystamine dihydrochloride, phthalic anhydride, anhydrous pyridine, di-tert-butyl dicarbonate, and anhydrous *N,N*-dimethylformide (DMF) were ordered from Alfa Aesar (Haverhill, Massachusetts, USA). Trifluoroacetic acid (TFA), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dihydrofolate reductase assay kit, triethylenetetramine (TETA), diethylenetriamine (DETA), diosgenin (DG), and ethylenediamine (EDA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, and an antibiotic-antimycotic (100×) solution were obtained from Life Technologies (Carlsbad, CA, USA). All solvents and reagents were chemical grade unless otherwise stated.

Thin layer chromatography with silica gel 60 F_{254} (Merck) was performed to monitor the progress of each reaction. Spots were visualized with ultraviolet light, a 5% sulfuric acid solution in ethanol, a ninhydrin solution, or iodine vapors. Flash chromatography was carried out on Merck silica gel 60 (70–230 mesh) with the eluent system presented below. Purification and purity determinations of the prepared conjugates were performed by a Skyam high-performance liquid chromatography (HPLC) system on a Luna reversed-phase C_{18} column (250 × 4.6 mm, 5 μm), using a gradient of 0.05% TFA aqueous solution (A) and acetonitrile or methanol (B) as the mobile phase at a constant flow rate of 1 mL/min. Detection was at 302 nm. The elution gradient started at 70% A and ended at 30% A during 45 min, then decreased to 20% eluent B during the next 15 min for acetonitrile system. For methanol system, the elution gradient (A) started at 90% and ended at 5% in within 10 min and then constant elution was used for next 45 min. Test samples were dissolved in 10% TFA in methanol or methanol alone and filtered through a 0.2 μm nylon filter before injection. Structural characterization of the synthetic products was done by a Varian Unity Plus 400 MHz spectrometer in $CDCl_3$ or DMSO-d_6. All chemical shifts (δ) are reported in parts per million (ppm) and multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, and m = multiplet). The molecular weight of the final conjugates was confirmed on a Shimadzu ultra-fast liquid chromatograph mass spectrometer (UFLC-MS) with the electron spray ionization (ESI) method.

2.2. Chemistry

2.2.1. Synthesis of compound **7**

To a solution of compound **5** (3.20 g, 31.0 mmol) in glacial acetic acid (200 mL) was added phthalic anhydride (11.0 g, 37.2 mmol) in solid form. The reaction mixture was refluxed at 80 °C for 2 h. The solvent was completely evaporated under reduced pressure and added 200 mL of hot ethanol with stirring. After stirring for 5 h, the precipitate was filtered, collected, and dried to give compound **7** in 95% yield. The spectrum pure compound **7** can be obtained by flash chromatography eluting with ethyl acetate. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.73 (m, 4H), 7.68 (m, 4H), 3.84–3.38 (t, *J* = 6.01, 4H), 3.30–2.97 (t, *J* = 6.01, 4H).

2.2.2. Synthesis of compound ***8***

To a solution of *N*-carboethoxyphthalimide (5.0 g, 22.8 mmol) and 6.35 mL of TEA in 50 mL of acetonitrile was added a solution of compound **6** (1.33 g, 11.4 mmol) in 10 mL of acetonitrile at 30 °C. The mixture was stirred for 2 h and then concentrated under vacuum to afford the bisphthalimide **8** in 65%. ^{1}H NMR (300 MHz, CD_3OD): δ = 7.89–7.84 (m, 4H), 7.82–7.79 (m, 4H), 3.90 (t, *J* = 5.68 Hz, 4H), 3.30–3.12 (t, *J* = 5.68 Hz, 4H), 3.01 (s, 4H).

2.2.3. Synthesis of compound ***9***

To a suspension mixture of compound 7 (8 g, 22 mmol) and K_2CO_3 (7.5 g, 55 mmol) in THF was added di-tert-butyl dicarbonate (5.7 g, 26.4 mmol) at ice cooled bath. The reaction mixture was warmed to room temperature and stirred for 10 h. The solvent was removed and the residue was dissolved in 200 mL of DCM. The organic layer was washed with H_2O (2 × 200 mL), dried with Na_2SO_4, filtered and concentrated. The crude product was recrystallized from a mixture of methanol/n-hexane or using flash chromatography eluting with 10% ethyl acetate in hexane to give **8** as a white power. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.83 (m, 4H), 7.68 (m, 4H), 3.84 (t, *J* = 6.01 Hz, 4H), 3.55 (t, *J* = 6.01 Hz, 4H), 1.06 (s, 9H).

2.2.4. Synthesis of compound ***10***

To a mixture suspension of compound **8** (6.0 g, 14.8 mmol) in 100 mL was added di-tert-butyl dicarbonate (7.1 g, 32.6 mmol) at ice cold bath. The reaction mixture was stirred for 10 h at room temperature. The solvent was removed and the residue was dissolved in 100 mL of DCM. The organic layer was washed with H_2O (2 × 200 mL) twice, dried with Na_2SO_4, and concentrated. The crude product was purified by flash chromatography with elution of ethyl acetate/methanol/$NH_3{\cdot}H_2O$ = 50/1/0.5 or recrystallized from a mixture of methanol and hexane to afford compound **10** in 90% yield as a white power. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.84 (m, 4H), 7.67 (m, 4H), 3.83–3.79 (t, *J* = 5.79, 4H), 3.51 (t, *J* = 5.79, 4H), 3.34–3.27 (t, *J* = 5.79, 4H), 1.23 (s, 18H).

2.2.5. Synthesis of compound ***11***

Compound **9** (8.5 g, 18.7 mmol) was suspended in 200 mL of ethanol and hydrazine monohydrate (18 mL, 20 equiv.) and stirred for 1 day at room temperature. After the reaction, the precipitate was filtered, and the filtrate was removed under reduced pressure and diluted with DCM (100 mL). The organic phase was washed with a small amount of saturated $NaHCO_3$ solution, dried over $MgSO_4$, filtered and concentrated to give **11** as light yellow oil in 76% yield, which was purified by flash chromatography with elution of 5–20% methanol in chloroform containing 0.5% aqueous ammonia. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.34 (br s, 4H), 2.93 (m, 4H), 1.46 (s, 9H).