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Research paper

Cell-penetrating conjugates of pentaglutamylated methotrexate as potential anticancer drugs against resistant tumor cells

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ABSTRACT

The emerging resistance of tumor cells against methotrexate (MTX) is one of the major limitations of the MTX treatment of tumorous diseases. The disturbance in the polyglutamation which is a main step in the mechanism of methotrexate action is often the reason of the resistance. Delivery of polyglutamylated MTX into cells may evade the mechanisms that are responsible for drug resistance. In this study conjugates of methotrexate and its pentaglutamylated derivatives with cell-penetrating peptides – penetratin and octaarginine – were investigated. The cellular-uptake and *in vitro* cytostatic activity of conjugates were examined on breast cancer cell cultures (MDA-MB-231 as resistant and MCF-7 as sensitive cell culture). These cell cultures showed very different behaviour towards the conjugates. Although the presence of pentaglutamyl moiety significantly decreased the internalisation of conjugates, some of them were significantly active *in vitro*. All of the conjugates were able to penetrate in some extent into both cell types, but only the conjugates of penetratin showed *in vitro* cytostatic activity. The most effective conjugates were the MTX-Glu$_5$-Penetratin(desMet) and MTX-Glu$_5$-GFLG-Penetratin(desMet). The latter was effective on both cell cultures while the former was active only on the resistant tumor cells. Our results suggest that the translocation of polyglutamylated MTX may be a new way to treat sensitive and more importantly resistant tumors. While both penetratin and octaarginine peptides were successfully used to deliver several kinds of cargos earlier in our case the activity of penetratin conjugates was more pronounced.

1. Introduction

Methotrexate (4-amino-10-methylfolic acid, MTX) was the first antimetabolite used in the treatment of tumorous diseases from the 1950s. As an effective anticancer drug it is clinically used to treat childhood acute lymphoblastic leukemia and a number of other haematologic malignancies [1]. Methotrexate, belonging to the antifolate antimetabolite family, can be internalised into cells by specific transporters - the membrane-bound reduced folate carrier (RFC-1) and/or folate-binding proteins (folate receptors Frα, FRβ and FRγ) [2]. Inside the cell sequential addition of Glu moiety/moieties occurs by the enzyme folylpolyglutamate synthetase (FPGS) [3–5]. Polyglutamylated MTX exhibiting higher affinity to folate-metabolizing enzymes play crucial role in the metabolism of folic acid and disturb the biosynthesis of thymidine and purine, inhibit the synthesis of DNA and could lead to cell death.

During the polyglutamation 5–8 glutamic acid residues are coupled to the MTX *via* amide linkage forming γ-peptide bonds. The MTX is a poor substrate of the FPGS which catalyses the glutamic acid attachment and its binding competes with intracellular folates [6]. The polyglutamylated forms of MTX can inhibit the dihydrofolate reductase and timidylate synthase enzymes. The presence of the polyglutamyl moiety essentially prevents the efflux of MTX from the cell because derivatives containing more than three glutamic acid residues are not substrates of multidrug resistance protein (MRP 3) [7] and of the folate transporter systems [8]. Thus the polyglutamation is a very important process in the action of MTX. The concentration of polyglutamylated MTX in the cytosol is modulated by the activity of FPGS and of the enzyme γ-glutamyl hydrolase (GGH), catalysing the removal of Glu residues [9,10]. Therefore, the presence of polyglutamylated MTX derivatives depends on the balance of glutamic acid attachment and removal.

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Although several second and third generation antifolates have been developed for clinical use, the main drawback is the development of the evolving drug resistance. There are various processes which are responsible for the drug resistance: i) decreased cellular uptake caused by the malfunction of folate transporters [11]; ii) increased efflux related to the overexpression of multidrug resistance transporters [12]; and/or iii) decreased level of polyglutamation due to inactivation or decreased activation of respective enzymes that take part in these processes [13]. The drug resistance can be intrinsic or acquired. Some studies showed that the defect in the polyglutamation can result in the intrinsic drug resistance [14,15]. Therefore the intracellular delivery of polyglutamylated MTX may provide an effective antitumour drug against resistant tumor cells as well.

Polyglutamylated MTX derivatives are very hydrophilic, and they are not substrates of folate transporter systems (RFC-1 and/or FRs) delivering folates into the cells. Consequently, these compounds are not able to enter through the cell membrane. This disadvantage may be overcome by using cell-penetrating peptides (CPPs). CPPs are short oligopeptides (natural and/or of synthetic origin) with cell internalisation capability. Among the first peptides discovered, derivatives from proteins: HIV-1 Tat [16,17] and Antennapedia protein of *Drosophila* could be mentioned [18]. These oligopeptides are able to penetrate across the cell membrane even in the presence of various, covalently attached cargos (e.g. peptides, peptide-nucleic acids, oligonucleotides) [19].

For targeted delivery into sensitive and/or resistant tumor cells MTX or pemetrexed [20] has been conjugated to protein [21], peptides including CPP [22–25], polymeric polypeptides [26], dendrimers [27,28] and oligosaccharides [29].

In spite of the potency of polyglutamylated MTX as effective drug against resistant tumors only few studies were reported on conjugates with this molecule. An early paper described conjugates in which MTX with different length of polyglutamyl chains were attached to polymeric polylysine (average M = 27,000 and 52,000) [30]. In these conjugates the polyglutamylated MTX was coupled *via* amide bond between the α/γ-carboxylic group of glutamic acids and ε-amino group of lysine side chains. Since the unprotected form was used in the conjugation reaction the exact structure of the conjugates (the carboxylic group involved in the formation of the amide bond) is unknown. The number of MTX attached to the polymer was between 2 and 11. The conjugates showed inhibitory activity of sensitive (H35) and MTX transport resistant (H35R) strains of hepatoma cells. In a recent study the polyglutamylated forms of MTX were conjugated with sequence-defined oligo (ethanamino)amide [31]. These compounds and the free MTX exhibited similar activity against human cervix carcinoma KB cells *in vitro*.

Based on our promising results on cell penetrating peptide - antitumour drug (daunomycin, vinblastine [32,33]) or enzyme activator/substrate peptide [34,35] conjugates our aim was to study the cell-penetrating peptide concept to deliver pentaglutamylated methotrexate into tumor cells. Here we describe the synthesis and characterization of a novel set of conjugates in which pentaglutamylated MTX is covalently attached to the N-terminal of cell-penetrating peptides *via* peptide bond. Two conjugate families are reported here with two different types of CPPs (octaarginine or modified penetratin) possessing different mechanisms of uptake. The cytostatic effect as well as cellular uptake properties of conjugates were studied on sensitive and resistant breast tumor cell lines *in vitro*. Data collected were comparatively analysed with those obtained with MTX-conjugates without pentaglutamyl moiety as well. We observed that the presence of penetratin (desMet) highly influenced the activity of pentaglutamylated MTX on resistant breast cancer cells even in comparison with the MTX-conjugate, but not on MCF-7 cells. Interestingly, no *in vitro* cytotoxic effect was documented with conjugate containing Arg_8 as CPP. Our results show that the insertion of an enzyme labile spacer (GFLG) between pentaglutamylated MTX and penetratin (desMet) could maintain (MDA-MB-231) or increase (MCF-7 cell) the activity of the conjugate as well.

2. Materials and methods

All amino acid derivatives, *N,N'*-diisopropylcarbodiimide (DIC) and Rink-amide MBHA resin were purchased from IRIS Biotech GmbH (Marktredwitz, Germany). *N,N*-diisopropylethylamine (DIEA), 1.8-diazabicyclo [5.4.0]undec-7-ene (DBU), thioanisole, 1,2-ethandithiol (EDT) were FLUKA (Buchs, Switzerland), while 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA) and phenol were Sigma Aldrich (Budapest, Hungary) products. Solvents for synthesis and purification were obtained from Molar Chemicals Ltd (Budapest, Hungary). Carboxyfluorescein (cf) and all other chemicals used in biological experiments were purchased from Sigma Aldrich (Hungary). Buffers were prepared with distilled water.

2.1. Synthesis of peptides and peptide-conjugates

The conjugates were built up by solid-phase peptide synthesis on Rink amide MBHA resin using Fmoc/^{t}Bu strategy. The side chain of amino acids were protected by 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf, in Arg), by trityl (Trt, in Asn, Gln), by *tert*-butyl group (^{t}Bu, in Glu) and by *tert*-butyloxycarbonyl group (Boc, in Lys). The N-terminal Fmoc protecting group was removed with 2% piperidine in the presence of 2% 1.8-diazabicyclo [5.4.0]undec-7-ene (DBU) in DMF (2 + 2+5 + 10 min). This reagent was removed by washing with DMF (8 × 0.5 min). In the coupling reaction, amino acid derivatives, which were used in 3 M excess to the resin capacity, were activated by *N,N'*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) dissolved in DMF. The reaction proceeded at RT for 60 min. Then, the resin was washed with DMF (2 × 0.5 min) and dichloromethane (DCM) (3 × 0.5 min). Ninhydrin assay was used to monitor the efficiency of the coupling reaction [36]. After the removal of the terminal N^{α}-Fmoc group, methotrexate (MTX) or 5 (6)-carboxyfluorescein (cf) were attached to the peptide using the HOBt-DIC coupling reagents. The free compounds were obtained by cleavage from the resin with 10 mL TFA containing 0.75 g phenol, 0.5 mL distilled water, 0.5 mL thioanisole and 0.25 mL 1,2-ethandithiol (EDT) as scavengers. Crude products were precipitated by dry diethyl-ether, dissolved in 10% acetic acid and freeze-dried. The crude peptide was purified by RP-HPLC as described below. The purified compounds were characterized by analytical RP-HPLC and ESI-MS (Table. 1). The purity of the compounds was higher than 95%.

2.2. RP-HPLC

Analytical RP-HPLC was performed on a Knauer (Herbert Knauer GmbH, Berlin, Germany) HPLC system using a Phenomenex Jupiter C18 column (250 × 4.6 mm I.D.) with 5 μm silica (300 Å pore size) (Torrance, CA USA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile-water (80:20, V/V)) was used at a flow rate of 1 mL/min at ambient temperature. Peaks were detected at λ = 220 nm. The samples were dissolved in eluent B. In the other cases Exformma (Exformma Technology (ASIA) Co., Ltd, Hong Kong, China) HPLC system was used. The column was Agilent Zorbax SB-C18 4.6 mm×150 mm, 100 Å. The applied linear