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Methotrexate and epirubicin conjugates as potential antitumor drugs

Koniugaty metotrexatu i epirubicyny jako potencjalne leki przeciwnowotworowe

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Summary

Introduction: The use of hybrid molecules has become one of the most significant approaches in new cytotoxic drug design. This study describes synthesis and characterization of conjugates consisting of two well-known and characterized chemotherapeutic agents: methotrexate (MTX) and epirubicin (EPR). The synthesized conjugates combine two significant anticancer strategies: combinatory therapy and targeted therapy. These two drugs were chosen because they have different mechanisms of action, which can increase the anticancer effect of the obtained conjugates. MTX, which is a folic acid analog, has high cytotoxic properties and can serve as a targeting moiety that can reach folate receptors (FRs) overexpresing tumor cells. Combination of nonselective drugs such as EPR with MTX can increase the selectivity of the obtained conjugates, while maintaining the high cytotoxic properties.

Materials and methods: Conjugates were purified by RP-HPLC and the structure was investigated by MS and MS/MS methods. The effect of the conjugates on proliferation of LoVo, LoVo/Dx, MCF-7 and MV-4-11 human cancer cell lines was determined by SRB or MTT assay.

Results: The conjugation reaction results in the formation of monosubstituted (α, γ) and disubstituted MTX derivatives. *In vitro* proliferation data demonstrate that the conjugates synthesized in our study show lower cytotoxic properties than both chemotherapeutics used alone.

Discussion: Epirubicin cytotoxicity was not observed in obtained conjugates. Effective drugs release after internalization needs further investigation.

Keywords: **Methotrexate • Epirubicin • Combinatory therapy • Targeted therapy • Bioconjugates**

* Both authors contributed equally to this work.

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Abbreviations: **AICAR** – Aminoimidazolecarboxamide ribonucleotide, **DCC** – *N,N'*-dicyclohexylcarbodiimide, **DHFR** – Dihydrofolate reductase, **DMF** – *N,N*-Dimethylformamide, **ESI** – Electrospray ionization, **EPR** – Epirubicin, **FA** – Folic acid, **FR** – Folate receptor, **GAR** – glycineamide ribonucleotide, **MTT** – *N'*-anilino-*N*-[(4,5-dimethyl-1,3-thiazol-2yl)imino]benzenecarboximidamide, **MTX** – Methotrexate, **NHS** – 1-Hydroxy-2,5-pyrrolidinedione, **PCFT** – protoncoupled folate transporter, **RFC** – reduced folate carrier, **RPC** – Reversed phase chromatography, **SRB** – 2-(3-diethylamino-6-diethylazaniumylidene-xanthen-9-yl)-5-sulfo-benzenesulfonate, **TEA** – *N,N*-Diethylethanamine, **TOF** – Time of flight, **TS** – Thymidylate synthase.

Introduction

Most cytostatic agents exhibit serious side effects due to their lack of selectivity for cancer cells. Current efforts are focused on more specific drug design. An intensively exploited technique in new anticancer drug discovery is a hybrid molecule formation where two or more active molecules are linked together [7,20]. The conjugation of a cytotoxic drug with the leading molecule can help to overcome the limitation of non-selectivity. The obtained conjugated drug should show higher selectivity for cancer cells resulting in lower toxicity for normal cells and tissues [14,27]. Tumor cells intensive divisions have a high demand for nucleotides that are essential during DNA replication. For this reason, a significant number of cancer cells show overexpression of folate receptors (FR-positive cancers), which makes these receptors an attractive target in selective drug delivery [9]. Methotrexate (MTX) can serve as a targeting moiety that not only has the ability to reach FR-positive tumor cells but also shows high cytostatic properties [12,31]. Another critical issue in effective drug design is cancer cells cytotoxic resistance. To overcome this problem, combinatory therapy utilizing agents targeting several different, often unrelated pathways, was applied. A hybrid molecule containing two or more drugs is advantageous because it provides a way for synergistic therapeutic action. This strategy involves the development of multifunctional drugs with more than one molecular target [25]. Methotrexate is one of the most widely used cytotoxic drugs in the treatment of several types of cancer such as leukemia, lymphoma and breast cancer [2]. This drug is a folic acid (FA) antimetabolite. Folic acid derivatives (folates) are involved in the transport of carbon groups that are required during purines and pyrimidines synthesis. MTX belongs to dihydrofolate reductase (DHFR) inhibitors and possesses greater affinity to DHFR than FA [25,36]. Interaction between MTX and DHFR induces a deficiency of reduced folate cofactors in the cell, which inhibit cofactor-dependent enzymes like glycineamide ribonucleotide (GAR) transformylase, aminoimidazolecarboxamide ribonucleotide (AICAR) transformylase and thymidylate synthase (TS). Inhibition of these enzymes results in DNA synthesis retardation [10,28]. We distinguish three groups of MTX transporters in mammalian cells: reduced folate carrier (RFC), proton-coupled folate transporter (PCFT) and folate receptors (FRs). RFC belongs to antiporters and utilizes the organic phosphate gradient to transport folates through the cell membrane whereas PCFT exploits the proton gradient to that purpose. Folate receptors (FRs) are expressed at the cell surface as glycosylphosphatidylinositol-anchored glycoproteins. These receptors have the ability to bind MTX and transport this molecule into the cell through receptor-mediated endocytosis [2,32,35]. Most efficient MTX and folates transport occurs through reduced folate carrier (RFC), whereas FA shows low affinity to these transporters [35]. All of these molecules can undergo cellular uptake exploiting folate receptors [2,32]. MTX, as well as FA, after internalization can undergo polyglutamination, which extends the residence time of these molecules inside the cell [25,32]. Both carboxylic groups of MTX are utilized in conjugate design since MTX with a modified α or γ carboxylic group is able to be transported through FR. However, free α-carboxylic group is preferable [9,17]. Epirubicin (EPR) is a semisynthetic, anthracycline antibiotic, derivative of doxorubicin. EPR consists of hydrophobic aglycone attached by β-glycosidic bond to an aminosugar ring [3,16]. Best known mechanisms of EPR action are:

- intercalation into DNA, which interferes with replication and transcription of DNA;
- generation of free radicals, which leads to biomolecules and DNA damage and lipid peroxidation;

• inhibition of topoisomerase II, which causes DNA damage and induction of apoptosis [5,18,24,29].

Epirubicin is characterized by high cytotoxicity, but a non-specific drug delivery results in various side effects [34]. The aminosugar is intensively exploited to produce epirubicin conjugates and modified antibiotic molecules retain cytotoxic properties *in vitro*. Drug derivatives created by substituting C3' amino group possess a similar cytotoxic activity as non-modified drugs [4,8,23]. Here, we report a methotrexate-epirubicin conjugate strategy in which two different drugs and two different types of therapies are integrated. The formation of an amide bond between the MTX carboxylic group and the amine group of EPR resulted in hybrid MTX-EPR molecules. Synthetized conjugates combine combinatory therapy and targeted therapy, both of which are considered to be meaningful anticancer approaches. We expected that a combination of these two strategies possess a huge potential and can be highly beneficial in new anticancer drugs design. Molecular targets of exploited molecules are located in different cell compartments. MTX is an inhibitor of cytosolic enzyme (DHFR), whereas EPR's primary target is DNA located in the nucleus. Methotrexate serves a dual role in synthesized molecules. Apart from the high cytotoxicity it possesses, it has also targeting molecule properties, enabling selective cancer cell targeting through receptor-mediated endocytosis. EPR as free drug is transported into the cell through membrane diffusion, resulting in high cytotoxicity. A leading molecule like MTX can enhance EPR's selectivity, thereby limiting the side effects of applied therapy [18,34]. The primary objective of our study was to investigate cytotoxic properties of the obtained MTX-EPR hybrid molecules. The antiproliferative activity of synthetized conjugates was evaluated in human tumor cell lines (LoVo, LoVo/Dx, MCF-7, MV-4-11) *in vitro.*

Materials and methods

Methotrexate used in this research was purchased from EBEWE Pharma Ges.m.b.H.Nfg.KG. Epirubicin hydrochloride was a kind gift from Laboratory of Lipids and Liposomes, Faculty of Biotechnology, University of Wroclaw. DCC and NHS were obtained from Fluka. All other reagents and solvents were of analytical or HPLC grade and were used without further purification. Human MV-4-11 (leukemia, FR-positive [15]), MCF-7 (breast cancer, FR-negative [6], MTX resistant cell line [30]), LoVo (colon cancer, FR-positive [19]) cell lines were obtained from American Type Culture Collection (Rockville, Maryland, U.S.A.). LoVo/Dx (colon cancer, doxorubicin resistant LoVo subline, FR-positive as LoVo) was a kind gift from prof. E. Borowski, Medical University of Gdańsk. All cell lines were maintained at the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. MV-4-11 cells were cultured in RPMI 1640 medium (Gibco, Scotland, UK) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 1.0 mM sodium pyruvate, 10% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). MCF-7 cells in Eagle medium (IIET, Wroclaw, Poland), supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, 10% fetal bovine serum and 0.8 mg/L of insulin (all from Sigma Aldrich Chemie GmbH, Steinheim, Germany). LoVo and LoVo/Dx cells were cultured in a mixture of Opti-MEM and RPMI 1640 medium (1:1, both from Gibco, Scotland, UK) supplemented with 1.0 mM sodium pyruvate and 5% fetal bovine serum. LoVo/Dx medium was supplemented with doxorubicin (100 ng doxorubicin per 1 ml medium). All culture media were supplemented with 100 units/ml penicillin, and 100 µg/ml streptomycin (both from PolfaTarchomin S.A., Warsaw, Poland). Cell lines were grown at 37 °C with 5% CO_2 humidified atmosphere. The synthesis reaction was performed in DMF. The carboxylic group of MTX was activated by use of DCC and NHS. The molar ratio of reagents was 1:1:1.1:4:1.1 (MTX:EPR:DCC:NHS:TEA). TEA was necessary as the amino group of EPR was in the form of a hydrochloride and reaction requires basic conditions. The mixture was incubated for 24 hours at 4 °C. The conjugates were purified by use of HPLC (Dionex ultimate 300) on a reverse phase column (Waters Symmetry® C18, 3.5 µm, 4.6×75 mm). To detect conjugates UV-VIS methods were used, MTX absorption maximum is at 302 nm and EPR at 480 nm. A linear gradient of water and acetonitrile, both containing 0.1 % formic acid, from 5 to 95 % organic phase, was used as an eluent in 50-minute runs. Runs were performed at room temperature at a 0.9 mL/minute flow rate. To confirm the structures MS and MS/MS techniques were used (Brucker micrOTOFQII). The molecules were ionized by ESI, precursor ions $[M+H]^+$ were selected by quadrupole and submitted fragmentation by collision with argon atoms in collision cell. The ions were detected in TOF detector. For cytotoxicity assays, cells were placed in 96-well flat-bottom plates (Sarstedt, Inc. Newton, NC,USA) at a density of 1×10^4 cells per well, 24 h before the addition of the tested compounds. Cells were exposed for 72 h to various concentrations (0.01, 0.1, 1, 10, µg/ml) of EPR, MTX or MTX-EPR conjugates. The MTT (MV-4-11) or SRB (MCF-7, LoVo, LoVo/Dx) assay was performed in order to evaluate the cytotoxic effect of the tested compounds as described previously [33]. The percent of proliferation inhibition was calculated according to the formula: % of proliferation inhibition =|[(ApAm)/(AcAm)×100(%)]-100(%)|, where: Ap absorbance of treated cells; Am absorbance of culture medium; Ac absorbance of control cells. The results were also calculated as the IC_{50} (inhibitory concentration 50%), i.e. the dose of tested compound which inhibits the proliferation of cancer cells by 50%. IC_{50} values were calculated for each experiment separately [21] and the mean value ± SD are presented in Table 1. Each compound at a given concentration was tested in triplicates in a given experiment; each experiment was repeated 3-7 times. Statistical analysis was performed using STATISTICA version 10 (StatSoft Inc., USA). The data were analyzed by Kruskal-Wallis ANOVA. Multiple Comparisons p values (2-tailed) and Mann-Whitney test were performed for further analysis. P-values less than 0.05 were considered significant.

Results

MTX-EPR conjugates synthesis method is summarized in Figure 1. First step of synthesis was MTX-NHS active esters formation that has been achieved by the presence of DCC and NHS in the reaction mixture. The resultant MTX-NHS esters can easily react with primary amine group of EPR aminosugar ring resulting in MTX-EPR hybrid molecules. MTX possesses two carboxylic groups: α and γ, both of which can be activated using DCC and NHS activation method. Therefore, a set of three target molecules can be created during the synthesis: mono-substituted derivatives (α, γ) and a disubstituted derivative.

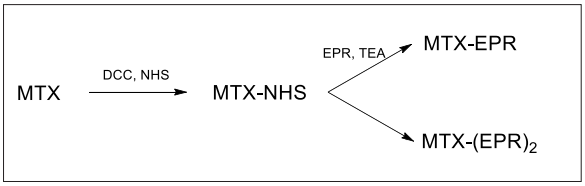

Fig. 1. MTX forms NHS active esters that are able to react with primary amine group of EPR resulting in MTX and EPR conjugates formation

Both derivatives (mono – and disubstituted) were purified using reversed phase chromatography (RPC) and the HPLC profile of obtained compounds is demonstrated in Figure 2.

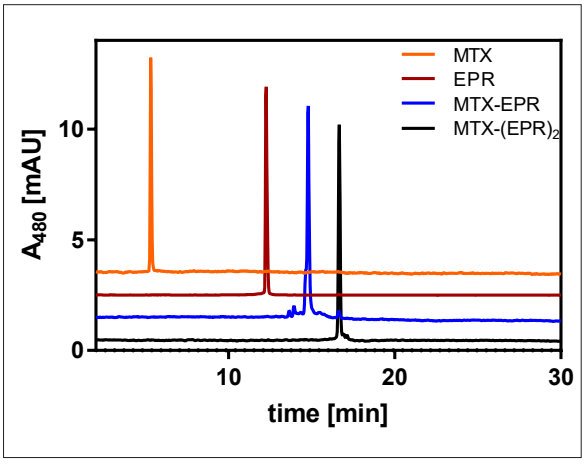

Fig. 2. HPLC analysis of synthesized compounds investigated by 480 nm

The structures of purified conjugates were confirmed by means of MS and MS/MS methods (Figure 3). m/z= 980.3435 corresponds to monosubstituted MTX derivative and peak with value m/z= 1505.5045 indicates disubstituted MTX derivative formation. Conjugates fragmentation data (mono – and disubstituted derivatives) show peaks characteristic for MTX and EPR molecules. We did not identify whether the α or γ carboxyl group was modified in the monosubstituted derivative.

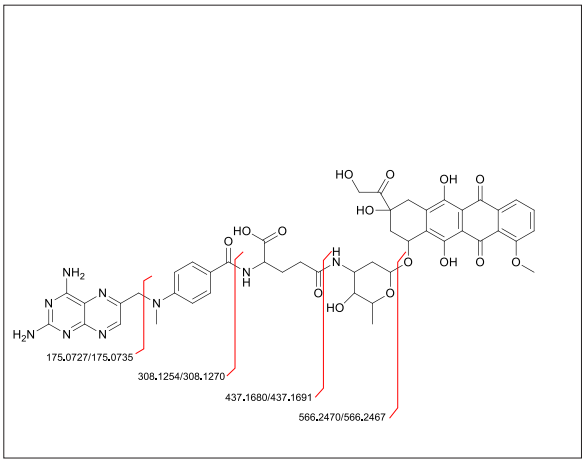

Fig. 3. ESI-MS CID fragmentation diagram for γ-monosubstituted derivative (m/z = 980.3435), calcd/found (m/z) values for fragmentation ions are shown in this figure

Antiproliferative potential of synthesized compounds was preliminarily evaluated by *in vitro* assays in human cancer cell lines. The activity of tested MTX-EPR conjugates was compared to the activity of methotrexate (MTX) and epirubicin (EPR) used alone, as a reference agents. IC_{50} values of investigated molecules were determined and are summarized in Table 1. MTX revealed antiproliferative activity against MV-4-11, LoVo and LoVo/Dx cell lines with nanomolar IC_{50} values. As we expected, antifolate was not active towards the FR-negative MCF-7 cell line (calculated IC_{50} value is above 22 μM). The synthetized conjugates were also not active against MTX resistant MCF-7 cancer cell line regardless of high antiproliferative activity of EPR alone (IC_{50} = 0.55 μM). All conjugates revealed activity towards MTX sensitive, FR-positive cell lines (MV-4-11, LoVo, LoVo/Dx) and the lowest IC_{50} value for both of synthetized compounds was observed on the MV-4-11 cell line. Besides LoVo/Dx, all other tested cell lines are EPR sensitive but low antiproliferative effect of conjugates was observed only in two cell lines (MV-4-11, LoVo). Both monosubstituted as well as disubstituted derivatives revealed lower antiproliferative activity against cancer cell lines used.

Discussion

MTX and EPR are two well characterized and widely used chemotherapeutics in anticancer therapy [11,22]. The study indicates that it is possible to synthetize new drugs by using already applied molecules. Combination of two different agents in one molecule creates an opportunity to overcome cytotoxic resistance; this approach is widely exploited in combinatory therapies. One of the main objectives of conjugates synthesized in this study was to enhance conjugate selectivity, which can be achieved by exploiting MTX as a leading molecule. We were hoping that after the loss of carboxylic groups, which are required for reduced folate carrier (RFC) internalization [35], the main drug delivery mechanism will be FR-mediated endocytosis. The con-

Table 1. The in vitro antiproliferative activity of MTX-EPR conjugates in comparison with free MTX and EPR

Compound	Cancer cell line, IC50±SD [μM]			
	MV-4-11	LoVo	LoVo/Dx	MCF-7
EPR	0.028±0.002	0.316±0.036	6.241±2.208	0.550±0.072
MTX	<0.022	0.057±0.024	0.051±0.025	>22.005
MTX-EPR	3.449±1.174	22.369±2.082	20.675±3.490	>32.655
MTX-$(EPR)_2$	2.106±0.810	9.818±4.298	>21.256	>21.256

jugates revealed a cytotoxic effect on FR-positive MV-4-11, LoVo and LoVo/Dx cell lines. However, the IC_{50} values of the conjugates in *in vitro* assays were higher than values obtained for free drugs (MTX, EPR), which indicates lower cytotoxic activity. These data can be caused by several different factors. Conjugation through the γ-carboxylic group of MTX can prevent polyglutamination [26] and conjugate residence time inside the cell can be reduced. Modification through the α-carboxylic group can perturb interaction between MTX and DHFR [17] and reduce the capacity of MTX binding to FRs [12,31]. EPR is transported into the cell through cell membrane diffusion [18]; this transport can be disturbed after conjugation to MTX and the internalization of conjugate can be achieved only by receptor-mediated endocytosis. After internalization, the conjugates can be strongly bound to DHFR because of MTX high affinity to this enzyme and the drug can be arrested in the cytoplasm, thereby preventing penetration into the nucleus. Low conjugates activity in MCF-7 cell line suggests the lack of EPR activity in the synthesized molecules. The monosubstituted derivative shows similar IC_{50} values for EPR sensitive LoVo cell line and EPR resistant LoVo/Dx subline, also indicating the absence of EPR activity after internalization. However, disubstituted derivative shows better antiproliferative activity than the monosubstituted derivative to LoVo cell line. The presence of two EPR molecules can enable conjugate transportation to the nucleus and cytotoxicity exerting. Synthesis of MTX-EPR conjugates possessing a cleavable linker between MTX and EPR should be considered as the next step. Various cleavable linkers (e.g. valine-citrulline) are exploited in new anticancer drugs design [1]. Effective free drug release after internalization can enhance cytotoxic effect of MTX-EPR hybrid molecules. Synergistic drugs action combined with MTX leading molecule properties can be highly beneficial in the production of new cytotoxic compounds [13]. Forasmuch as MTX-EPR conjugates possess anticancer activity, further studies could enable the creation of a new anticancer agent, which could find clinical application.

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