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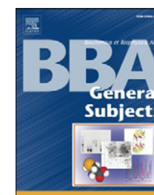

The antileukemic activity of modified fibrinogen–methotrexate conjugate

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ABSTRACT

Background: The search for new, innovative methods to treat all types of diseases, especially cancer-related ones, is a challenge taken by pharmaceutical companies and academic institutions. The use of conjugates containing widely-known and widely-used bioactive substances is one of the ways to solve this problem. Research into drug binding with macromolecular carrier systems has joined the search for new therapeutic strategies.
Methods: The main goal of this paper is the potential offered by the use of fibrinogen derivatives as an antileukemic drug carrier. Physicochemical properties of the obtained conjugate were analyzed, characterizing alterations in relation to the starting carrier and analyzing biological implications. The intraperitoneally (*i.p.*) inoculated P388 mouse leukemia model for *in vivo* studies was used.
Results and conclusions: Conjugates consisting of a fibrinogen derivative with a covalently bound anticancer drug were developed. Carrier preparation and a conjugate synthesis in aqueous solution were formulated, as well as purification of the conjugate was performed. The study showed that the survival of leukemia mice treated with FH–MTX conjugate was indeed significantly longer than survival in both untreated animals (control) and mice treated with unbound MTX. A significant increase in the antileukemic activity of MTX conjugated with hydrolysed fibrinogen was observed as compared with the unconjugated drug. Reported data suggest that hydrolysed fibrinogen can serve as a carrier molecule for the MTX drug with the aim of enhancing its antileukemic activity.
General significance: Conjugates consisting of a fibrinogen derivative with a covalently bound anticancer drug seem to be a promising anticancer drug.

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1. Introduction

The majority of currently used anticancer drugs represent low molecular weight small compounds [1,2]. This explains their traditional disadvantages, such as fast metabolism and excretion from an organism, as well as unfavorable biodistribution and low specificity [3]. Conjugation of drugs with macromolecular carriers has been recommended as a potential way to correct these problems. Such conjugates were designed with the aim of enhancing delivery and to improve the selectivity and pharmacological properties of both conventional and innovative drugs [4,5].

Methotrexate (MTX) is one of the oldest antifolate drugs widely used in the treatment of cancers, rheumatoid arthritis and other diseases [6–8]. Despite its strong drug efficacy, it has a relatively low plasma half-life time. We have recently studied different conjugated forms of MTX based on dextrans [9], albumin [10], fibrinogen [11] and glycated protein [12] carriers. These studies revealed fibrinogen to be the most promising carrier for MTX. Many prerequisites, such as accumulation in tumors, determined that this type of protein was selected. Fibrinogen–MTX conjugates were characterized by considerably higher antileukemic efficacy in comparison to unconjugated drugs. One of the proposed mechanisms of this phenomenon assumes retention of fibrinogen/fibrin and its conjugates in the peritoneum of animals with ascite tumors and a gradual release of the active drug from the conjugates as a result of its enzymatic degradation.

In this paper, the synthesis and determination of the *in vitro* and *in vivo* antileukemic properties of MTX conjugated with fibrinogen subjected to limited hydrolysis were described.

The intraperitoneally inoculated P388 mouse leukemia model for *in vivo* studies was used. A significant increase in the antileukemic activity of MTX conjugated with hydrolysed fibrinogen as compared with the unconjugated one was observed.

In addition, the observation was made that the conjugate was well tolerated by experimental mice with no apparent differences in initial weight drop as compared to the free drug, suggesting that the

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conjugation process preferentially increased the activity of MTX towards leukemic cells without increasing the overall toxicity of the treatment in animals.

2. Material and methods

2.1. Carrier preparation (FH)

Fibrinogen from bovine plasma (F) including ≥70% protein and containing sodium chloride and sodium citrate (Fluka, Germany) was dialyzed with MWCO 12 kDa dialyzing tubing (Serva, Germany) for 48 h against ultrapure water to remove salts. After dialysis the solution was lyophilized and dissolved in 85% formic acid (POCH, Poland) reaching final fibrinogen concentration of 20 mg/ml. The mixture was heated to 98 °C at reflux for 2 h, afterwards cooled in an ice-bath to room temperature and extensively dialyzed against ultrapure water for 2 h and then lyophilized. Fibrinogen concentration was determined in 100 mM sodium bicarbonate at 280 nm by absorption coefficient $5.12\times10^5 M^{-1}\ cm^{-1}$.

2.2. Conjugate preparation (FH–MTX)

Dry product (FH) was dissolved in 100 mM $NaHCO_3$ solution and reacted with MTX anhydride according to the method previously described [13]. MTX was purchased from EBEWE Pharma (Austria). FH conjugate was dialyzed for 96 h against 100 mM $NaHCO_3$ (POCH, Poland) to remove free MTX.

The analysis of total MTX in preparations was based on absorption spectrophotometry in 100 mM sodium bicarbonate at 372 nm by absorption coefficient 8571 $M^{-1}\ cm^{-1}$. Determination of unbound MTX was based on size exclusion chromatography with UV–VIS detection at a wavelength of 302 nm. A Superdex® Peptide column (150 × 4.6 mm) and mobile phase 100 mM sodium bicarbonate with a flow rate of 400 µl/min were applied.

The total protein concentration in the conjugate was calculated using the BCA protein assay (Sigma-Aldrich) and a standard curve with a known concentration of bovine fibrinogen.

2.3. SDS-PAGE

Polyacrylamide gel electrophoresis of fibrinogen, FH and FH–MTX was carried out according to Laemmli [14] in 10% gel under reducing and non-reducing conditions. Protein detection in gels was carried out by silver staining [15]. A Carestream Imaging System S4000MM PRO was used to scan and store images of the gels. For creation of density profile plots, ImageJ software [16] was employed.

2.4. DLS measurement

Fibrinogen, FH and FH–MTX were characterized by dynamic light scattering (DLS) to obtain hydrodynamic parameters and polydispersity information. This technique measures time-dependent fluctuations in the intensity of scattered light. Analysis of these intensity fluctuations enables the determination of the diffusion coefficients of particles, which are converted into a size distribution. The sample solution was illuminated by a 633 nm laser, and the intensity of light scattered at an angle of 173° was measured. The experimental result consists of the overall average size, size distributions by intensity, and the overall polydispersity index — PDI. Compounds were prepared in 50 mM $NaHCO_3$ solutions. All samples were measured using a Zetasizer Nano ZS (Malvern Instruments) in a low volume quartz cuvette (12 µl). All solutions were clear without evidence of insoluble material. The following parameters were used: protein refractive index (1.450), solvent viscosity (8.979×10^{-4} Pa × s), temperature (25 °C), fibrinogen concentration (1 mg/ml), FH, and FH–MTX concentration (10 mg/ml of total proteins).

2.5. Cell line

P388 (murine leukemia) were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained in culture or were frozen in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Twenty-four hours before addition of the tested preparations, the cells were plated in 96-well plates (Sarstedt, Germany) at a density of 0.5×10^4 cells per well and were cultured in a mixture of RPMI 1640 and Opti-MEM (1:1) medium, supplemented with 2 mM glutamine (Sigma-Aldrich Chemie GmbH, Germany), 100 mg/ml streptomycin (Polfa, Tarchomin, Poland), 100 U/ml penicillin (Polfa), and 5% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Germany). The cells were cultured at 37 °C in a humid atmosphere saturated with 5% CO_2. Passages of P388 leukemia cells in BDF mice were carried out according to the NIH/NCI standard screening protocols for *in vivo* assessment [17,18].

2.6. Antiproliferative assays in vitro

The *in vitro* cytotoxic effect was studied after 72-h exposure of the cultured cells to different concentrations of the test preparations (based on the total contents of the MTX), using the SRB assay as described by Skehan et al. [19]. Briefly, the cells were attached to the bottom of plastic wells by fixing them with cold 80% trichloroacetic acid (TCA; Sigma-Aldrich Chemie GmbH, Germany). The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with culture medium, but without the cells. The cellular material fixed with TCA was then stained for 30 min with 0.4% sulforhodamine B (Sigma-Aldrich Chemie Gmbh, Germany) dissolved in 1% acetic acid (POCH, Poland). Unbound dye was removed by rinsing (4×) with 1% acetic acid. The protein-bound dye was extracted with 10 mM TRIS base (POCH) for determination of optical density (at 540 nm) in a 96-well microtiter plate reader (Multiskan RC photometer, Labsystems, Helsinki, Finland).

2.7. Experimental animals

Male B6D2F1 mice, aged 16–20 weeks were used. The mice were supplied from the Animal Breeding Centre of the Medical Academy, Wroclaw, Poland, and were maintained in standard laboratory conditions. Experiments were performed according to the Guide for the Care and Use of Laboratory Animals (National Academy of Science, National Academy Press, Washington, D.C.) and were approved by the First Local Ethical Committee for the use of Laboratory Animals, Wroclaw, Poland. Mice were injected with 10^6 leukemia (P388) cells *i.p.* (day 0), and 24 h later (day 1) each mouse was injected once *i.p.* with the appropriate agent. All doses (80 µmol/kg) were based on the contents of the MTX in the conjugate. Body weight and survival data were collected on a daily basis throughout the duration of the experiment.

2.8. Data handling

The *in vitro* results were presented in terms of IC_{50} values. The IC_{50} is the concentration of a tested agent which inhibits the proliferation of 50% of the cancer cell population. Average IC_{50} values for each preparation were calculated using data from three independent experiments. The antileukemic effect *in vivo* was evaluated as the increase in lifespan (ILS) of treated mice over the control, calculated from the following formula: $(MSTT/MSTC)\times100-100$, where MSTT is the median survival time of treated animals and MSTC is the median survival time of untreated control mice.

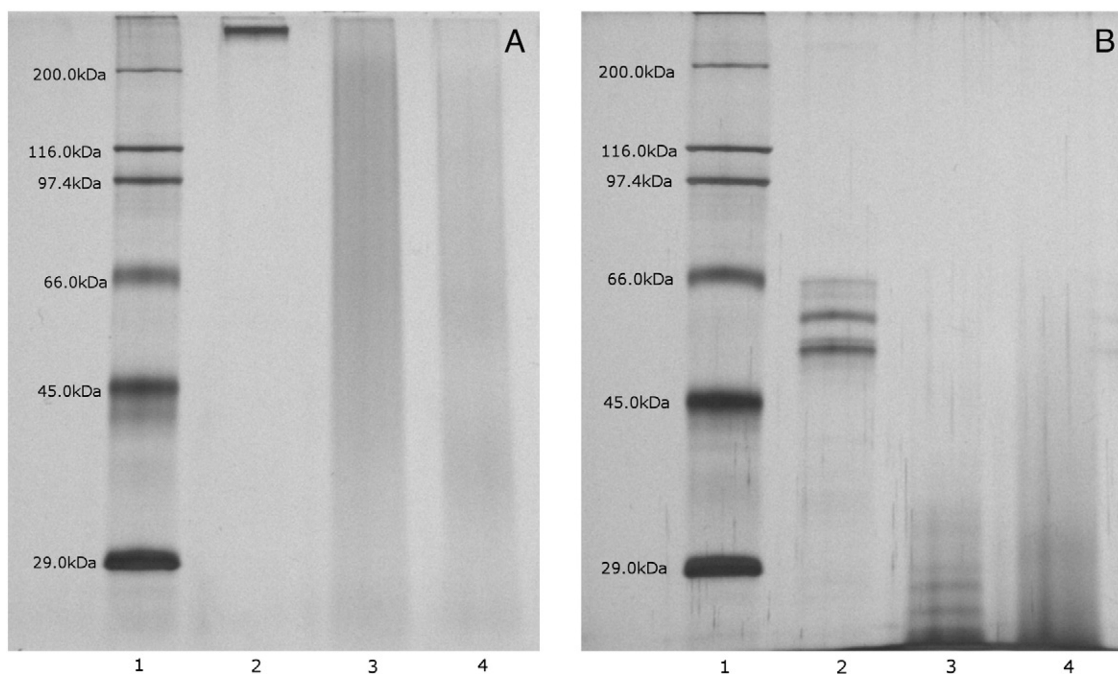

Fig. 1. SDS PAGE in 10% gel in the absence of 2-mercaptoethanol (A) and in the presence of 2% 2-mercaptoethanol (B). Lane 1: molecular weight standard; lane 2: bovine fibrinogen (carrier precursor); lane 3: FH — bovine fibrinogen subjected to limited hydrolysis; lane 4: FH–MTX — conjugate.

2.9. Statistical evaluation

Survival data in experimental *in vivo* groups were compared using Cox's F test with the Bonferroni correction for multiple comparisons (p adjusted = p counted × N, where N = number of pairwise comparisons). p values less than 0.05 were considered significant.

3. Results and discussion

The time of the acid hydrolysis of bovine fibrinogen was optimized to obtain a statistically heterogeneous mixture of proteins/peptides. The resulting mixture was used as a carrier for MTX. MTX anhydride is expected to react mainly with lysine residues and with N-terminal

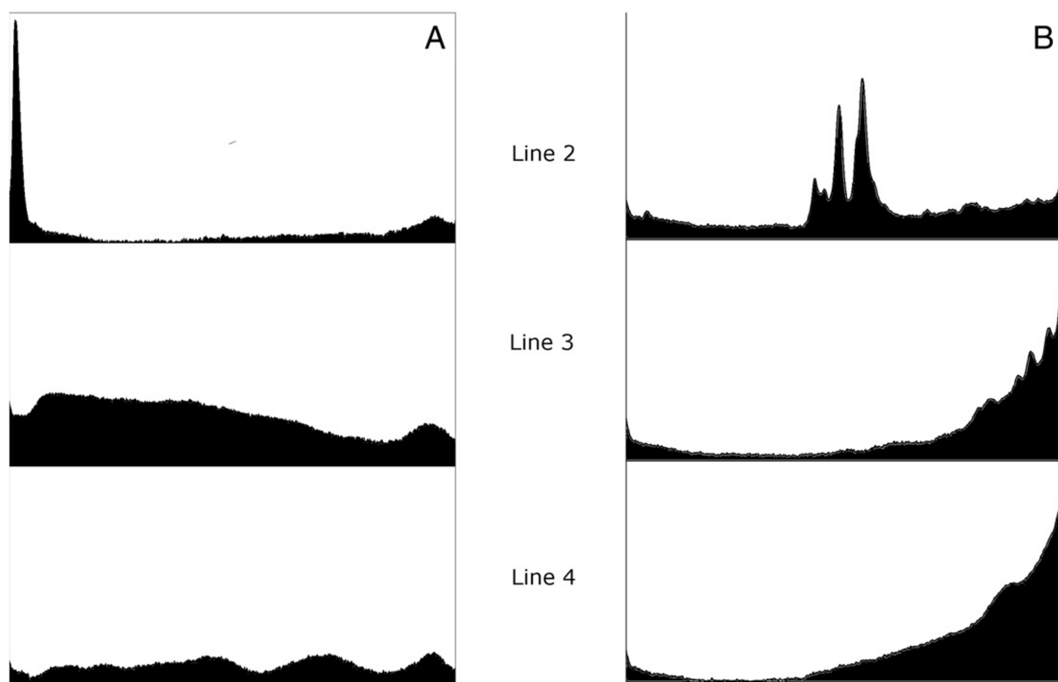

Fig. 2. Densitometric analysis of gels presented in Fig. 1. (A) Electrophoresis in the absence of 2-mercaptoethanol, and (B) electrophoresis in the presence of 2% 2-mercaptoethanol. Line 2: bovine fibrinogen (carrier precursor); line 3: FH — bovine fibrinogen subjected to limited hydrolysis; line 4: FH–MTX — conjugate.

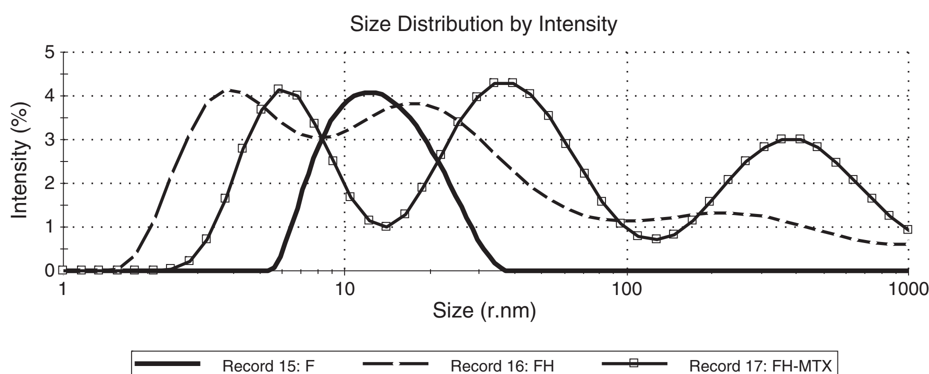

Fig. 3. Characterization of fibrinogen, FH and FH–MTX by dynamic light scattering technique. Size distributions are shown according to intensity.

Table 1
Hydrodynamic parameters of fibrinogen (F), FH and FH–MTX.

Compound	Hydrodynamic radius — r [nm]	Polydispersity index (PDI)
F	12.66 ± 5.499	0.189
FH	12.09 ± 9.318	0.594
FH–MTX	24.60 ± 20.60	0.701

Table 2
Antiproliferative activity *in vitro* of FH–MTX conjugate in comparison with free MTX. All concentrations were based on the total contents of the MTX.

IC_{50} (SD) μM	
Compound	Cell line — P388
MTX	0.156 ± 0.00742
FH–MTX	1.98 ± 0.364

amino groups during the conjugation reactions. The resulting conjugate FH–MTX contained 159 mol MTX covalently bound to 1 mol of proteins (BCA total protein) and 0.49% of unbound MTX. The obtained samples were analyzed by SDS-PAGE (Fig. 1 and 2).

The hydrodynamic parameters of fibrinogen for 50 mM $NaHCO_3$ conditions were 12.66 nm (hydrodynamic radius) and 0.189 PDI. These were typical values for large proteins and had previously been reported in the literature [20]. Other data presented more than one peak in the size distribution (Fig. 3). PDI was indicative of the overall breadth of the size distribution and clearly revealed the heterogeneous nature of samples FH and FH–MTX (Table 1).

After characterization of the conjugate preparation its cytotoxic activity to unbound MTX in the *in vitro* assay using the P388 mouse leukemia cell line was compared. The results of cytotoxic activity *in vitro* were expressed as IC_{50}. As shown in Table 2, the FH–MTX conjugate revealed approximately a one-order-of-magnitude weaker cytotoxic activity *in vitro* than MTX alone. FH itself did not reveal any cytotoxic activity *in vitro*.

Based on previous evidence that the poor performance of conjugates *in vitro* does not necessarily predict diminished activity *in vivo*, a hypothesis was proposed that the FH–MTX conjugate could still show enhanced antileukemic activity *in vivo* as compared to unbound MTX due to the slow release of the active drug. To investigate this, B6D2F1 mice were injected with P388 mouse leukemia and the animals were treated in experimental groups with 80 μmol/kg of either unbound MTX or FH–MTX conjugate injected intraperitoneally. The study showed that the survival of leukemia mice treated with FH–MTX conjugate was indeed significantly longer than survival in both untreated animals (control) and mice treated with unbound MTX with $p = 0.008$ and 0.038, respectively (Fig. 4). No mice died due to the therapy in any of the experimental groups and the data on body weight changes suggested that overall toxicities of both unbound MTX and FH–MTX conjugate were comparable as illustrated by similar decreases in weight during the first few days after therapy administration (Fig. 5).

Gathered data demonstrate that a conjugate of MTX with hydrolysed fibrinogen has a higher antileukemic activity in the *in vivo* mouse model of leukemia as compared to the unconjugated one.

Results described in this paper appear interesting when considered together with other reported data showing that fibrin may facilitate the persistence and progression of malignancy [21–25]. Since

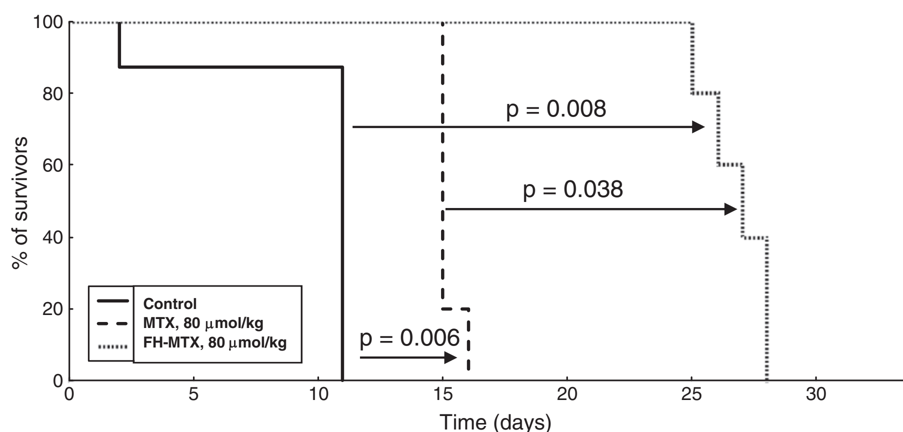

Fig. 4. Survival data of leukemia-bearing mice in control group (untreated, n = 8), and mice treated with either free MTX (n = 5) or FH–MTX (n = 5). The increase in lifespan over the control group (ILS) was 36% for the group treated with free MTX and 145% for the group treated with FH–MTX conjugate. All doses were based on the total contents of the MTX.

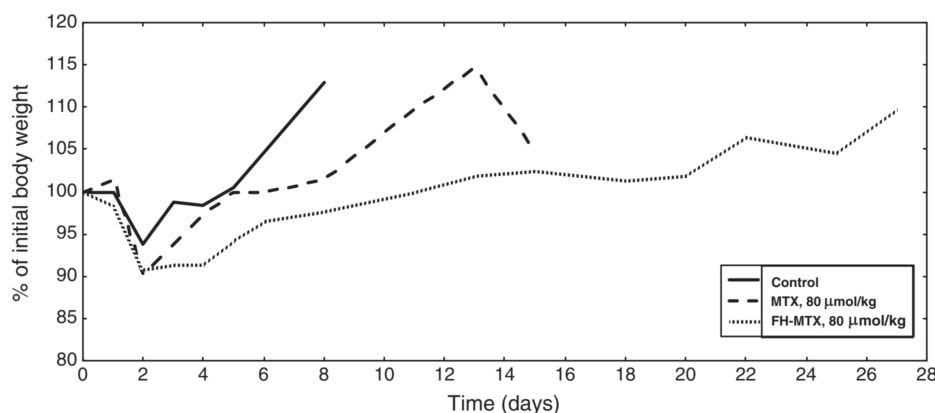

Fig. 5. Dynamics of body weight in untreated leukemia-bearing mice or those treated with 80 μmol/kg of either MTX or FH–MTX conjugate. All doses were based on the total contents of the MTX.

fibrinogen is more commonly found in the connective tissue of a variety of human malignancies than fibrin, attention should be directed towards the potential contribution of this molecule (as well as of fibrin) to tumor progression and invasion [25]. The proteolytic degradation of the fibrinogen leads to the formation of a heterogeneous mixture of derived proteins and peptides. This mixture – FH – seems to be a promising anticancer drug carrier.

MTX covalently bound to macromolecules and the proteolysis process of such a system might be the cause of the prolonged persistence of drugs in the body [26].

Data reported here suggest that hydrolysed fibrinogen can serve as a carrier molecule for the MTX drug with the aim of enhancing its antileukemic activity.

Acknowledgements

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