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Synthesis and characterization of methotrexate containing peptide bioconjugates

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INTRODUCTION

Leishmaniasis, the disease caused by unicellular parasites belonging to *Leishmania* is a very serious problem in the tropics and in the Mediterranean region.[1] Nonetheless, the availability of anti-*Leishmania* drugs remained limited. The increased resistance continuously maintain the claim for develop new chemotherapeutics. In the visceral form of the disease, parasites infect the cells of the reticulo-endothelial system, predominantly macrophages. During my doctoral work I was working with methotrexate (MTX) polypeptide conjugates, as potential compounds for the treatment of leishmaniasis. The use of MTX is limited by serious side effects including bone marrow damage, hepato-, neuro- and nephrotoxicity and respiratory complications. In our research group we are concerned since several years to target infected macrophages by the synthesis and characterization of MTX conjugates. During my work I have used polylysine backbone based branched polypeptides containing hydrophobic amino acid or arginine, as a targeting unit.

AIMS

The design and synthesis of antiparasitic MTX polypeptide conjugates against *Leishmania donovani*, characterization of functional properties and the establishment of structure-effect relationships.

To synthesize the poly[Lys(X_i-DL-Ala_m)], where X: Ile, Nle, Leu, Val, Arg (IAK, *Nle*AK, LAK, VAK, RAK), furthermore poly[Lys(X_i)], where X: Leu (L_iK) with triphosgene reagent.

During the synthesis of the arginine-containing polymer (RAK), a new method was developed for the selective cleavage of the guanidino-NO_2 protecting group in the presence of benzyl-type protecting groups with sodium borohydride.

To demonstrate the structure-activity relationship, the effect of pH and ionic strength on the polypeptides structure in solution was studied, and oligopeptide conjugates were designed to model the structure of the macromolecules basic monomeric unit.

[1] *World Health Organization (2010) Control of Leishmaniases. WHO Technical Report Series no. 949. Report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22–26 March 2010*

As the active compound has to be transported into infected macrophage cells to reach the parasites, my aim was to investigate the cytotoxicity and cellular uptake of polypeptides and their MTX conjugates in macrophage cells and to study the *in vitro* anti-*Leishmania* effects on *L. donovani* promastigota and *L Pifanoi* amastigota cells.

METHODS

Synthesis of poly[Lys(X_i-DL-Ala$_m$)], where X: Leu, Ile, Nle, Val, Arg (LAK, IAK, NleAK, VAK, RAK) and poly[Lys(X_i)], where X: Leu (L_iK) polymers

I have optimized the synthesis of L-Lys(Z)-NCA[2]. In the modified synthesis: Triphosgene was dissolved in tetrahydrofuran and the solution was held at 60°C for 1 hour. The finely powdered H-L-Lys(Z)-OH was added to the solution in 5 portions. After stirring for another two hours at 60°C, the total amount of H-L-Lys(Z)-OH was dissolved. The clear solution was evaporated using a rotary vacuum evaporator. The treatment of leaving phosgene was resolved by appropriate trapping. The resulting oil was purified by crystallization. The composition of the polypeptides was determined by amino acid analysis.

Selective removal of Z-Arg(NO_2)-OH NH-NO_2 protecting group with $NaBH_4$

In connection with the synthesis of RAK, a new reduction procedure was developed to selectively remove the NH-NO_2 protecting group. Z-Arg(NO_2)-OH and copper acetylacetonate catalyst in abs. ethanol was stirred and sodium borohydride was added. The reaction was stopped by the addition of concentrated acetic acid and RP-HPLC analysis was performed to determine the conversion. The formation of the product was confirmed by mass spectrometry.

Analysis the conformation of branched-chain polypeptides with ECD spectroscopy

The polypeptide samples were dissolved in 0.5 to 1.0 mg/mL in 0.02, 0.2 or 2.0 M NaCl and the pH was adjusted with 1.0 M HCl or 1.0 M NaOH. The [θ] MR values were determined based on the lysine unit of the main chain, including the full side chain. Interpretation of the ECD spectra of polypeptides at various pH (pH 3, pH 7.4, pH 12) and ionic strength (0.02M, 0.2M, 2.0M) based on the data from the literature.[3]

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[2] *Katchalski E.; Grossfeld I.; Frankel, M. Poly-condensation of alpha-amino acid derivatives; poly-lysine J. Am. Chem. Soc. (1948) 70(6): 2094–2101.*

[3] *Votavovàa, H.; Hudecz, F.; Kajtár, J.; Szekerke, M.; Sponar, J.; Bláha K. Conformation of branched polypeptides based on poly(L-lysine): Circular dicroism study. Coll. Czech. Chem. Commun. (1979) 45: 942-949.*

Synthesis of MTX conjugates

The conjugation of MTX (0.2 equivalents calculated for the monomer unit) was synthesized by using benzotriazol-1-yloxypyrrolidone-phosphonium hexafluorophosphate (PyBOP) coupling agent in dimethylformamide in the presence of *N,N*-diisopropylethylamine (DIEA) base. The conjugates were purified by dialysis against distilled water and the product was isolated by freeze-drying. The MTX content was determined by amino acid analysis.

Fluorescence labelling of branched-chain polymer polypeptides and MTX conjugates with 5(6)-carboxyfluorescein (Cf), purification and characterization

For the fluorescence labelling of the polymers, 5(6)-carboxyfluorescein N-hydroxysuccinimide ester (Cf-OSu, 0.1 equivalent calculated for the monomer unit) was used in the presence of DIEA base. The conjugates were purified by dialysis against distilled water and the product was isolated by freeze-drying. Fluorescein content was determined by UV-VIS spectroscopy.

Synthesis of fluorophore containing MTX model oligopeptides

In the case of Boc strategy, synthesis was performed on MBHA resin. The Boc protecting group was cleaved with 33% TFA/DCM (V/V) and Fmoc protecting group with 2% piperidine + 2% DBU/DMF (V/V/V) solution. For the coupling of amino acids, MTX and Cf a mixture of DIC/HOBt coupling agents was used. The duration of the coupling reaction was 60 minutes. The success of the coupling was justified by ninhydrin method developed by Kaiser. Several methods were used to cleave the peptides from the resin. The cleavage was performed on the one hand with TMSOTf, in the presence of 50% thioanisole, 25% ethanedithiol (EDT), 25% *p*-cresol (V/V/V) and in the presence of TFA, on the other hand with HF in the presence of *p*-cresol or anisole scavengers.

In the Fmoc strategy, I used Rink-Amide MBHA resin, the cleavage of the protective groups was consistent with the Boc strategy. The cleavage mixture used to cleave peptides from the resin was 95% TFA, 2.5% H_2O, 2.5% TIS (V/V/V) solution.

The crude product was dissolved on the glass filter in 10% acetic acid solution and filtered off from the resin and was isolated by freeze-drying from the solution. The peptides were purified by semi-preparative HPLC, identified by mass spectrometry and purified by analytical HPLC.

Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC was used to purify the synthesized oligopeptides. The purity of the crude product was confirmed by a Knauer analytical HPLC (Knauer GmbH, Berlin, Germany) using Phenomenex Synergi C12, 4 μ, 120 A, 4.6 x 300 mm column. The eluents were: Eluent A 0.1% TFA/H_2O (V/V), eluent B 0.1% TFA / H_2O-ACN (20:80 V/V). The separation is carried out at room temperature at a flow rate of 1 ml/min. For the separation I applied linear gradient (20-80% B (5-20 minutes)), detection was done by UV photometer, λ = 220 nm.

Separation of the MTX oligopeptide isomers was carried out with 10-100% B (5-55 min) linear gradient elution under the same conditions.

Mass spectrometry

The molecular mass of the compounds was performed using a Bruker Esqiure 3000+ ion trap mass spectrometer (Bruker, Karlsruhe, Germany) using ESI ionization. The device can also record MS^n (fragment selection, further fragmentation). Samples were dissolved in 0.1% acetic acid ACN/H_2O 1:1 (V/V) solvent mixture. The sample was injected with a syringe pump at 10 μl/min flow rate. The spectra were taken with 4.0 kV surge voltage and 40.0 V orifice voltage. I used the device in positive and negative mode at 50-3000 m/Z, with a sampling rate of 13,000 m/Z/sec.

Cytotoxicity of polypeptides and MTX conjugates to mouse bone marrow derived macrophages

The experiments were carried out in cooperation with Dr. Rita Szabó. Macrophage cells were divided into 96-well tissue culture plates with 10^4/well starting numbers in R10 M-CSF medium. The cells were treated with polypeptides and conjugates at concentrations ranging from 0.8 to 100 μg/ml using a 5x runner dilution in serum-free RPMI 1640 medium for 1 and 24 hours at 37°C. As controls, untreated cells were supplied to the same volume of media as the solution of the active ingredient. The amount of viable cells was determined by MTT assay. The absorbance was measured by ELISA reader (Labsystems iEMS Reader) at λ = 540 nm and λ = 620 nm reference wavelength.

Cytotoxicity was determined by $Cytotoxicity[\%] = (1-A_{Manage}/A_{Control})\times 100$ formula. The cytotoxicity % values plotted as a function of concentration, from which the 50% inhibition concentration (IC_{50}) was determined.

Determination of Cf-labeled polypeptides and conjugate cellular uptake into mouse bone marrow derived macrophages

Experiments were carried out in cooperation with Dr. Rita Szabó. Cells were divided 24 hours prior to treatment into 24 well suspending plates at 10^5 cells/well starting cell count. Incubated with Cf-labeled compounds in serum-free medium at 37°C for 1 hour at 0.16; 0.8; 4; 20 and 100 μg/ml. Cell uptake was measured by flow cytometry at BD LSRII (Franklin Lakes, NJ, United States) measuring 5-10000 cells (λ_{ex} = 488 nm, λ_{em} = 519 nm). The median of the fluorescence intensity of the obtained histograms was depicted by subtracting the control value depending on the treatment concentration. The statistical analysis was performed by a two-sample t-test (p = 0.05).

Investigation of cell uptake by fluorescence microscopy

The experiments were carried out in cooperation with Dr. Rita Szabó. Cells were divided 24 hours prior to the treatment into 24 well cell culture plates with 10^5 cells/well starting cell count. The cells were incubated for 1 hour at 37°C with Cf polypeptide and Cf conjugate compounds in a serum free medium at 100 μg/ml. The location of the labeled compounds was monitored following CKX41 fluorescence microscopy (λ_{ex} = 488 nm). Pictures were recorded with the Olympus E250 camera attached to the microscope and edited with ImageJ.

The antiparasitic effect of MTX conjugates in vitro on L. donovani promastigota and L. pifanoi amastigota cells

The experiments were carried out by me after training in cooperation with the Research Group of Dr. Luís Rivas (Eucaryotic Antibiotic Peptides Laboratory, Centro de Investigaciones Biologicas (CIB), Spanish National Research Council (CSIC), Madrid) based on the methods developed by them[4] and they made it available to me.

Determination of the viability of parasite cells after short time treatment:

To a sterile 96-well plate 60 μl/well parasitic suspension (20×10^6 cells/ml HBSS) was divided. 60 μl/well of the 100 μg/ml solution of the compound was added, and incubated for 4 hours at 27°C for promastigote cells and 32°C for amastigote cells. For statistical significance, three parallel measurements were performed at each point. For the measurements, positive control (untreated parasites) was used as a reference. I added 50 μl of MTT solution (final MTT

[4] *Luque-Ortega, J.R.; Rivas, L. Characterization of the leishmanicidal activity of antimicrobial peptides. Methods Mol Biol. (2010) 618:393-420.*

concentration 0.5 μg/ml) to each well and then incubated at 27°C or 32°C, until the holes of the positive control (untreated parasites) showed a visible color (about 30 minutes). Then, 50 μl of 10% (w/v) SDS solution was added to each well, including the blank, to stop the reaction and then measure the absorbance. The MTT method was used to determine the number of living cells. I read the absorbance at a wavelength of λ=595 nm with a 600 nm filter microplate reader. Negative control served as a medium without cell. Cytotoxicity was determined by *Cytotoxicity [%] = (1-*$A_{Managed}$ */* $A_{Control}$*)×100* formula.

Determination of the viability of parasite cells after long time treatment:
After 4 hours incubation, 20 μl of cell suspension was pipetted onto a new sterile 96 well plate, which was containing 180 μl/well phenol red free, 10% HIFCS containing RPMI 1640 medium for promastigote cells or amastigote media for amastigote cells. The cells were incubated for a further 3 days at 27°C or 32°C. Then, 50 μl MTT solution (final MTT concentration 0.5 μg/ml) was added to each well and then incubated at 27°C or 32°C, until the positive control wells, which were containing untreated parasites were clearly visible (about 30 minutes). Then, 50 μl of 10 % (w/v) SDS solution was added to each well to stop the reaction, and the absorbance was measured analogously to the short-term treatment.

RESULTS AND CONCLUSIONS

1.

Using the more safe triphosgene by modifying the method described in the literature, I developed a novel process for the synthesis of L-Lys(Z)-NCA, as starting material for the polylysine "backbone".

2.

I have produced several new and previously described branched-chain polymers: poly[Lys(X_i-DL-Ala$_m$)], where X: Leu, Ile, Nle, Val, Arg (LAK, IAK, *Nle*AK, VAK, RAK) and poly[Lys(Leu_i)], where X: Leu (L_iK).

3.

During the design of the synthesis of RAK polymer, I developed a method for cleaving the guanidino-nitro protecting group, leaving the Z protecting group intact, thus providing orthogonality. Examining the Z-Arg(NO_2)-OH sample compound, it was found that with

sodium borohydride in the presence of the copper(II)-acetyl acetate catalyst the NO_2 protecting group can be removed with no effect on the protecting group Z. Such a selective procedure has not been known in the literature so far.

4.

Solution conformation of the polypeptides was studied by ECD spectroscopy. It has been found that the presence of both the alkyl amino acid side chain (Leu, Ile, Nle, Val) and the positively charged amino acid side chain (Arg) at physiological conditions (pH~7.4, 0.2 M NaCl) could form of an ordered structure. During the study of the effect of pH and ionic strength, it has been found that the degree of ordered structure increases with increasing pH and ionic strength.

5.

I have prepared and characterized novel conjugates in which the polypeptide of the branched chain [(poly[Lys(X_i-DL-Ala$_m$)], where X: Leu, Ile, Nle, Val, Arg (LAK, IAK, NleAK, VAK, RAK) or poly[Lys(Leu$_i$)] (L_iK)] methotrexate and/or 5(6)-carboxyfluorescein. The syntheses were carried out using an active ester or PyBOP coupling agent.

6.

I have prepared oligopeptides and oligopeptide-MTX conjugates representing the "monomeric unit" of the poly[Lys-(MTX-DL-Ala$_m$)], (MTX-AK) conjugate, the K(MTX-AaAa)-NH_2 (oligo)peptide and its fluorescently labeled derivative of the N α-amino group (Cf-K(MTX-AaAa)-NH_2] and its shorter derivatives. Despite the simple structure, during the peptide synthesis I have identified a number of problems/side reactions, the most surprising was the formation of incomplete sequences that suggest that the lactone carboxyl group of the fluorescein can acylate under certain conditions. My experiences suggest that anisole-type scavengers in the highly acidic medium are alkylating methotrexate and I also have demonstrated that the MTX coupling with DIC/HOBt creates not only two constitutive isomers for the two carboxyl groups, but also cause racemization of the glutamic acid unit.

7.

The most significant challenge in the chemotherapy of leishmaniasis is that the antiparasitic drug have to enter into the macrophages to reach the parasites. Therefore, it was important to clarify whether the polymers and their MTX conjugates enter the macrophage cells, and whether the presence of MTX influences the extent of polymer uptake. To clarify, fluorescein-

labeled conjugates were studied by fluorescence microscopy and in flow cytometric assays. It was found that, the cellular uptake of both the MTX-*Nle*AK conjugate and the *Nle*AK without the active agent was the highest.

Studying the cytotoxicity of MTX conjugates, I found that most of the polypeptides did not exhibit toxicity under the experimental conditions, but arginine-containing conjugates (RAK) were outstanding, whereas the L_iK polypeptide was a few tenths lower, but toxic to the macrophages. Only MTX-RAK and MTX-L_iK conjugates are toxic, to about the same extent.

8.

The antiparasitic activity of the compounds produced was investigated *in vitro* by free *L. donovani* promastigote and *L. pifanoi* amastigote parasite cells. Furthermore, I studied the cellular uptake and localization of compounds with *L. pifanoi* amastigota parasite infected mouse abdominal origin macrophage cells.

Surprisingly, for promastigote cells, the investigated polypeptides were much more toxic than their MTX conjugates or than the free MTX drug. I found that L_iK, IAK and *Nle*AK polypeptides, MTX-L_iK, MTX-IAK and MTX-*Nle*AK conjugates were the most cytotoxic. Confocal microscopic assays confirmed that the conjugates enter the parasite cells.

In the free amastigote cells, the L_iK and LAK polypeptides showed antiparasitic effect. Contrary to promastigotes, MTX-L_iK and MTX-RAK conjugate and MTX were toxic.

SUMMARY

In summary, I have produced new compounds during my research and made original observations. I find it an outstanding achievement that MTX-*Nle*AK conjugate enter the most effective into macrophages and infected macrophage cells and is toxic not only to promastigotes but also to amastigotes. Also perspective is the MTX-L_iK conjugate, which is moderately toxic to macrophage cells, but highly toxic and antiproliferative to both promastigote and amastigote cells. It is surprising that the drug-free L_iK polypeptide is itself highly effective against *Leishmania*, both for promastigote and amastigote cells.

PUBLICATIONS IN THE DOCTORAL RESEARCH TOPIC:

1. **Sebestyén, M**; Kóczán, G; Hudecz, F. Pitfalls in the synthesis of fluorescent methotrexate oligopeptide conjugates. *Amino Acids* (2016) 48: 2599 – 2604. DOI:10.1007/s00726-016-2285-1

2. **Sebestyén, M**; Kóczán, G; Csámpai, A; Hudecz, F. $NaBH_4$ – a novel method for the deprotection of N^{ω}-nitro-arginine. *Tetrahedron Letters* (2016) 57(5): 546-548. DOI: 10.1016/j.tetlet.2015.12.081

3. **Sebestyén, M** and Szabó, R; Kőhidai, L; Pállinger, É; Mező, G; Kóczán, Gy; Hudecz, F Synthesis, conformation and cytotoxicity of new, branched polymeric polypeptides containing hydrophobic amino acid or arginine moiety. *Struct Chem* (2017) 28: 527–535. DOI 10.1007/s11224-016-0901-z

4. Szabó, R; **Sebestyén, M**; Kóczán, Gy; Orosz, Á; Mező,G; Hudecz, F. Cellular Uptake Mechanism of Cationic Branched Polypeptides with Poly[L‑Lys] Backbone. *ACS Com. Sci.* (2017) 19: 246−254. DOI: 10.1021/acscombsci.6b00133

SCHOLARSHIP IN THE DOCTORAL RESEARCH TOPIC:

Campus Mundi Scholarship. Duration of the scholarship: 2016.07.26-08.23. Host institute: Eukaryotic Antibiotic Peptides Laboratory, Centro de Investigaciones Biologicas (CIB), Spanish National Research Council (CSIC) /Ramiro de Maeztu 9, 28040 Madrid/

PPRESENTATIONS IN THE DOCTORAL RESEARCH TOPIC:

1. Szabó, R; **Sebestyén, M**; Kóczán, G; Hudecz, F. Synthesis, cytotoxicity and cellular uptake of new, branched polymer conjugates containing hydrophobic amino acids or arginine and methotrexate *34th European Peptide Symposium*, Leipzig, Germany, 2016.09.04-2016.09.09.

2. **Sebestyén, M**; Kóczán, G; Hudecz F. Pitfalls in the synthesis of fluorescent methotrexate-containing oligopeptide conjugates. *14th International Congress on Amino Acids, Peptides and Proteins,* Vienna, Austria, 2015.08.03-2015.08.07.

3. **Sebestyén M**; Szabó R; Kóczán G; Hudecz F. Új, hidrofób aminosavat vagy arginint és metotrexátot tartalmazó elágazó láncú polimer konjugátumok szintézise, citotoxicitása és sejtfelvétele *MKE 2. Nemzeti Vegyészkonferencia (*Hajdúszoboszló, Hungary, 2015.08.31-2015.09.02.

4. Oláhné Szabó, R; **Sebestyén, M**; Kóczán, G; Kőhidai, L; Hudecz, F. Drug targeting strategy with polypeptide based methotrexate conjugates against leishmania infection. *17th International Congress of the Hungarian Society for Microbiology*, Budapest, Hungary,2015.07.8-2015.07.10.

5. **Sebestyén, M;** Kóczán, Gy; Hudecz, F.: $NaBH_4$ – a novel modest method for the deprotection of N^{ω}-nitro-arginine. *20th International Conference on Organic Synthesis*, Budapest, Hungary, 2014.06.29-2014.07.04.