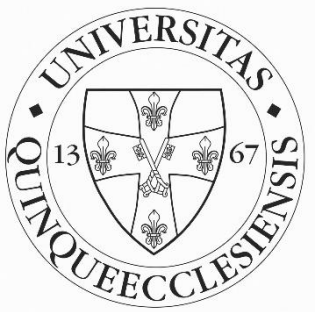
Secondary interactions and antioxidant properties of methotrexate and some of its analogones

PhD thesis



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1. Introduction

Methotrexate (MTX) has been a widely used antirheumatic and anti-tumor drug for decades. It is a photosensitive compound, it is degraded by ultraviolet (UV) light, but only a limited amount of data is available on its photodegradation products. Its secondary interactions with human serum albumin (HSA) have been studied, but it is not known how MTX or its photodegradation products affect the stability of this protein, so one of the aims of our research was to describe this.

MTX can cause a number of side effects that are currently only partially preventable in clinical practice. One of the most important goals of modern drug therapy is that the active substances delivered to the body have a targeted effect only on the organs or tissues to be treated, thus reducing the number and severity of side effects. Two promising methods have been examined that can potentially help to achieve this goal in the case of MTX: photopharmacological transformation and host-guest complexation.

Photopharmacology is a new field of science that aims to create compounds that can undergo a conformational change upon light exposure, thus altering their function. A photopharmacological derivative of MTX (Phototrexate, PHX) was developed a few years ago. Various properties of the latter compound were compared with the parent molecule in this work.

Resorcinarene-based cavitands are cup-shaped, cyclic host molecules. The molecular moieties attached to the backbone can significantly affect the molecules that a given cavitand can complex and the strength of the secondary interaction. Therefore, cavitands may be suitable for the design of drug delivery systems. No information has been available on their interactions with MTX or PHX, so related research has been conducted.

Because cardiovascular diseases can also be considered as chronic systemic inflammations, research of the cardioprotective effects of anti-inflammatory drugs has become increasingly important over the past decade. These studies may be warranted in the case of both MTX and PHX, so these compounds have been compared and antioxidant activity of PHX has been described for the first time.

2. Aims

MTX is a molecule known for decades, however, relatively little data are available on its secondary interactions with proteins and host molecules. In addition, the physicochemical properties and interactions of its photodegradation products (DFP and ABGA) and its photopharmacological derivative (PHX) are almost completely undiscovered. Therefore, in the course of my work I set the following goals:

- Thermodynamic characterization of the interactions of HSA with MTX and with photodegradation products of MTX and the description of the effect of these interactions on protein stability.
- Thermodynamic characterization of the interaction of MTX and PHX with cavitand derivatives.
- Investigation and comparison of the antioxidant properties of MTX and PHX using several different methods.

3. Methods

Fluorimetric measurements were performed with a Fluorolog τ3 spectrofluorimeter (Jobin-Yvon / Spex, Longjumeau, France). The bandwidths on the excitation and emission sides of the monochromators were set to 5.25-5.25 nm, and a perpendicular excitation-observation arrangement was applied. Lockable quartz cuvettes with an optical path length of 1 cm were used for the measurements.

The preparation of the photodegradation products of MTX (DFP, ABGA) and the time monitoring of the photodegradation process were performed with spectrofluorimeter (λex = 302 nm) at room temperature. The concentration of MTX was 1 mM in pH 7.4 phosphate buffered saline (PBS).

The interactions of HSA with MTX and with the photodegradation products of MTX were investigated with spectrofluorimeter (λ_{ex}= 371 nm) in PBS (pH 7.4). The concentration of HSA in the samples was constant (1 μM) and that of the other reagents was varying (0–450 μM), and the emissions of each sample were measured at 288 K, 293 K, 298 K, 303 K, and 308 K. Binding constants and thermodynamic parameters of the interactions were determined using the Benesi-Hildebrand and the van ’t Hoff equations.

The kinetics of the thermal denaturation of HSA were measured by differential scanning calorimetry (μSC, Setaram, Lyon, France) using different heating rates with the Kissinger equation. The effect of MTX and its photodegradation products on the thermal denaturation of HAS was investigated. Data were evaluated using Calisto Thermal Analysis Software (Setaram Instrumentation, Lyon, France).

Isomerization of *trans*-PHX was performed by UV irradiation (λ = 366 nm) using a Fluotest UV lamp (Original Hanau, Hanau, Germany). To monitor the isomerization process, the absorbance spectra of the samples were recorded every minute during illumination until the isomerization was complete. UV-Vis absorption spectra were recorded with a Specord Plus 210 (Analytik Jena, Jena, Germany) spectrophotometer, except for the ABTS assays, for which a Specord 40 (Analytik Jena, Jena, Germany) spectrophotometer was used.

The binding constants and thermodynamic parameters of the MTX-tetrakis(androst-4-en-3-one-17-ethinyl)-cavitand (TAC), *trans*-PHX-TAC, *trans*-PHX-tetrakis(3,5-dicarboxylatophenoxy)-cavitand (TDC), *cis*-PHX-TAC and *cis*-PHX-TDC interactions were also determined by fluorimetric method, applying the Benesi Hildebrand and the van ‘t Hoff equations. The solvent was dimethyl sulfoxide (DMSO) and λ_{ex} was 390 nm for MTX and 366 nm for PHX. In all cases, the concentrations of MTX (10 μM) and PHX (50 μM) were constant and those of the cavitand derivatives were varying (0-80 μM for MTX and 0-450 μM for PHX). Emission spectra were recorded at several temperatures.

Xanthine oxidase (XO) assay with hypoxanthine (HX) substrate was used to compare the superoxide radical scavenging properties of MTX and PHX. Electron paramagnetic resonance (EPR) spectroscopic measurements were performed with a MiniScope MS 200 device (Magnettech, Berlin, Germany) and MiniScopeCtrl software was used to evaluate the data. Krebs-HEPES EPR buffer was used as solvent and the spin trap was 1-hydroxy-2,2,6,6-tetramethyl-piperidin-4-ol (N-hydroxy-TEMPOL). Samples contained 10 mU/ml XO, 20 μM HX, 100 μM N-hydroxy-TEMPOL, and varying concentrations of MTX (0-5 mM) and *trans*- or *cis*-PHX (0 -2 mM). Samples containing only N-hydroxy-TEMPOL and only XO and HX were also measured as controls. The solutions were sampled hourly while keeping them at 37 °C. From the change in the amplitudes of the EPR signals over time, the reaction rate constants of radical production were determined.

Trolox equivalent antioxidant capacity (TEAC) values of MTX and PHX were compared by ABTS assay. The concentration of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$) in the stock solution was 2.45 mM. This was diluted with

the solvent (PBS) until its absorbance was 0.70 at 734 nm at 37 °C. MTX, trans-PHX, cis-PHX, and Trolox solutions were added, and the concentrations of these substances were varied in the samples (2.5 μM, 7.5 μM, 10 μM, 12.5 μM). Prior to the measurements, samples were incubated for 6 minutes at 37 °C.

The thermodynamic parameters of the complex formation of cavitands with MTX and PHX were also determined using HyperChem 8.0 (Hypercube Inc., Gainesville, FL, USA) software. Because this modeling is intended to be informative, equilibrium conformations and vibrational-rotational analysis (that was needed to calculate entropy) were determined using the semiempirical AM1 method.

Statistical evaluations were performed by one-way analysis of variance (ANOVA) using OriginLab 8.1 software (OriginLab Corporation, Northampton, MA, USA; $p < 0.05$).

4. Results and Discussion

4.1. The interaction of HSA with MTX and with its photodegradation products

Photodegradation products of MTX (DFP, ABGA) were prepared by illuminating MTX solution (1 mM) with UV light (λ_{ex} = 302 nm). As a result of the illumination, the photodegradation products were present in increasing concentrations, and a broad emission peak with a maximum at 458 nm appeared, which was used to monitor the reaction. When the intensity of the emission peak did not increase further (after about 4000 min of illumination), it was concluded that MTX was degraded and only DFP and ABGA were present in the solution.

The interactions of HSA with MTX and with the photodegradation products of MTX were investigated by fluorimetry (λ_{ex}=371 nm) using the Benesi-Hildebrand equation and the van 't Hoff equation. Taking into account the emission peak at 455 nm, the binding constants of the interactions were calculated and their thermodynamic parameters were determined. The calculated binding constants indicate that both MTX and its photodegradation products are stably bound by HSA in the temperature range of the measurements, and the strength of the bonds shows only a slight temperature dependence. This means that the strength of the interactions at the studied temperatures varies negligibly, which is advantageous for the calorimetric measurements, because the dissociation of small molecules does not affect the results even at high temperatures. In

this temperature range, at least 99,99% of the reagents are present in HSA-bound form. According to our calculations, HSA develops a stronger secondary interaction with MTX than with its photodegradation products.

The thermal denaturation of HSA in samples containing only HSA, HSA and MTX, and HSA and photodegradation products of MTX was examined at different heating rates (0.5 K/min, 0.8 K/min, 1 K/min, 1.2 K/min) by differential scanning calorimetry. The thermogramms showed two peaks in each case, the first of which was taken into account during the evaluation of data because it provides information on the denaturation of the MTX-binding domain of HSA. When plotting the Kissinger functions, the lines fitted well to the data points ($R^2 > 0.900$), so it was concluded that (since denaturation of HSA occurs according to first-order kinetics), the Kissinger method is suitable for the determination of activation energy (E_a).

Based on our calculations, MTX significantly increases E_a, however, the photodegradation products of MTX more than doubled the value of this studied parameter. From this, it was concluded that these compounds increase the thermal stability of HSA. Denatured proteins act as mediators of inflammation, so that compounds that have a protein stabilizing effect often have an anti-inflammatory effect, for example, the protein stabilizing effect of non-steroidal anti-inflammatory drugs has been described. MTX is also an anti-inflammatory (anti-rheumatic) agent, so we hypothesize that in addition to the many mechanisms that have been elucidated so far, protein stabilization may play a role in exerting its effects.

4.2. The interactions of MTX and PHX with cavitand derivatives

The interaction of MTX with a cavitand derivative was investigated for the first time in the literature. The fluorimetric measurements required to determine the binding constants of the MTX-TAC complexes were recorded using an excitation wavelength of 390 nm. Emission spectra were observed with constant MTX (10 μM) and varying TAC concentrations (0–80 μM) at 293.15, 296.48, 299.82, 303.15, 306.48, 309.82 and 313.15 K. The applied solvent was DMSO.

The intensity values for the peak of the emission spectra at 540 nm were used for the calculations with the Benesi-Hildebrand equation to obtain the binding constants for different temperatures. Based on the results, TAC forms stable complexes with MTX. After determining the binding constants, we plotted the van 't Hoff function of the

interaction, which consists of two lines instead of one, which means that the mechanism of complex formation may differ depending on the temperature within the studied temperature range. Complex formation was also investigated using computer modeling by HyperChem software. Based on the results of the modeling, two types of complexes (open and closed) can be formed. At lower temperatures, the increase in entropy is accompanied by an increase in enthalpy, while at higher temperatures there is a decrease in entropy and enthalpy. This is because in the lower temperature region (293.00–299.67 K) of the studied temperature range, the TAC is in the closed conformation, so that MTX interacts primarily with the arms of the host molecule with an ethisterone group. In contrast, in the higher temperature region (303.00-313.00 K), the TAC can be found in the open conformation, with the ethisterone arms spaced apart, so MTX interacts with the rigid, aromatic rings (with the cavity of the host molecule).

The increase in entropy during the formation of complexes in the closed conformation is due to the loss of the solvation shell of MTX and TAC, but this is an energy-requiring process covered by enthalpy. For this reason, we experience a positive enthalpy change in the gross process. However, in the case of the open conformation, the stiffening of the cavitand backbone and the MTX molecule results in a significant decrease in entropy, and the significant decrease in enthalpy is also due to the strong interaction of the well-matched reactants.

PHX is a relatively new molecule, so its interaction with macrocyclic host molecules was described for the first time in the literature. The interactions of both isomers (*trans*- and *cis*-PHX) with two types of cavitand derivatives (TAC and TDC) were investigated by fluorimetric measurements.

In order to study both isomers of PHX, we first had to make sure that the more stable *trans* isomer could be completely converted to the less stable (but pharmacologically more effective) *cis* isomer. For this purpose, the sample was illuminated with a UV lamp at a wavelength of 366 nm. Absorption spectra were recorded every minute during illumination. Based on our results, approx. 15 minutes of UV exposure was required for complete isomerization.

The isomers of PHX show an absorption peak around 370 nm (a slight hypochromic shift is observed during the *trans* → *cis* conversion). The absorbance values of the cavitand derivatives are much less around the same wavelength, so an excitation wavelength of 366 nm was used during the fluorimetric measurements. Fluorimetric emission spectra were registered applying constant *trans*- and *cis*-PHX concentrations

(50 μM) and varying TAC and TDC concentrations (0–450 μM) at 293 K, 298 K, 303 K, and 308 K. To determine the binding constants of the different complexes, the Benesi-Hildebrand equation was used, taking into account the intensity values of the emission peak at 490 nm.

After calculating the binding constants, the thermodynamic parameters of the formation of the complexes were determined using the van 't Hoff equation. We also plotted the van 't Hoff functions of the interactions, from which we concluded that the two types of cavitand derivatives form complexes with the isomers of PHX with different mechanisms.

The formation of these complexes was also investigated by computer modeling using HyperChem software. The thermodynamic parameters of the formation of the complexes were also determined with the help of theoretical modeling. The calculated data supported our hypothesis based on the fluorimetric measurements that the different cavitand derivatives form complexes with the isomers of PHX with different mechanisms. This is due to the difference in the structure of the cavitands.

In the case of TAC, an energy investment (enthalpy increase) is required to "open" the ethisterone arms, only then can the guest molecule interact with the aromatic cavity. The increase in entropy is caused by the loss of the PHX and TAC solvation shell (and thus by the "released" solvent molecules). However, TDC has significantly smaller functional groups (1,3-dicarboxylato-5-phenoxy), whose movement requires much less energy, and the loss of their solvate shell results in less entropy increase. As a result of complex formation, host and guest molecules that become stiffer cause a net entropy decrease in this case.

4.3. Xanthine-oxidase (XO) assay with hypoxanthine (HX) substrate

The superoxide radical scavenging properties of MTX, *trans*-PHX and *cis*-PHX were compared by EPR spectroscopy using an XO assay. The averages of the amplitudes of the EPR signals as a function of time were plotted, and the reaction rate constants (k) were determined using the curves fitted to the data points to give information about the rate of superoxide radical production in each sample.

The rate of free radical production is important data because when this rate reaches the limit of performance of biological radical scavenging systems (e.g., superoxide

dismutase, catalase, glutathione peroxidase) in organisms, cells are exposed to oxidative stress.

Based on our results, it is characteristic of all three tested substances that the higher the concentration present in the samples, the greater the rate of radical production. Of the compounds tested, *trans*-PHX reduced the rate of radical production to a greater extent than *cis*-PHX or MTX at all investigated concentrations. This result is particularly interesting considering that *cis*-PHX is the active isomer in terms of cytotoxicity, yet *trans*-PHX was found to be more effective in this study (in terms of radical scavenging effect). At the lowest concentration used, *cis*-PHX reduced the rate of radical production to a greater extent than MTX, but at other concentrations the effect of MTX was more pronounced.

Control measurements were also performed to demonstrate that if only the spin trap is present in the sample, it alone does not give an EPR signal, but its presence is required for signal formation. For this purpose, samples containing only N-hydroxy-TEMPOL and only XO and HX were also measured. Controls did not give an evaluable EPR signal.

4.4. ABTS assay

The TEAC values of the previously tested compounds (MTX, *trans*-PHX and *cis*-PHX) were determined using ABTS assay with spectrophotometric method. The absorbance values of samples containing the same concentration of $ABTS^{\bullet+}$ and different concentrations of Trolox, MTX, *trans*-PHX and *cis*-PHX were measured at 734 nm.

Significantly higher TEAC values were obtained for both isomers of PHX than for MTX, however, there was no significant difference between *trans*- and *cis*-PHX.

5. Conclusions

Based on our results, MTX and its photodegradation products (DFP, ABGA) have a protein stabilizing effect. This effect may even have therapeutic significance in the future.

Examining the interactions of MTX and PHX with cavitand derivatives, it was found that these complexes may have different conformations, which can be characterized by different thermodynamic parameters. These studies may contribute to a more targeted MTX therapy in the future, which may result in a significant increase in drug tolerability.

Comparing the antioxidant properties of MTX and PHX, we concluded that *trans*-PHX is a better antioxidant than MTX, although the cytotoxic effect of MTX is not

characteristic of *trans*-PHX. This result may warrant studies to investigate the potential antirheumatic and cardioprotective effects of trans-PHX.

The main limitation of *in vitro* methods is that in the model systems that we create, the molecules are not in the same medium as in the living organism, so of course their behavior is not exactly the same as that observed in *in vivo* studies. Nevertheless, *in vitro* research is important, as its results may form the basis of future *in vivo* studies.

6. New findings

1. The photodegradation products of MTX (DFP, ABGA) interact with HSA. The binding constants of the interaction were determined.

2. MTX and its photodegradation products increase the activation energy of heat denaturation of HSA, thus stabilizing the protein. This effect is more pronounced for photodegradation products.

3. MTX forms stable complexes with TAC. Based on the determination of the binding constants of the complexes at different temperatures, complex formation occurs by different mechanisms depending on the temperature.

4. Both isomers of PHX form stable complexes with TAC and TDC cavitand derivatives. Based on the determination of the binding constants of the complexes at different temperatures, different cavitand derivatives form complexes with PHX by different mechanisms.

5. Based on the determination of the antioxidant properties of MTX, *trans*-PHX and *cis*-PHX, *trans*-PHX has a more pronounced antioxidant effect than MTX.

7. List of publications

7.1. Publications related to the present PhD thesis

Zsolt Preisz, Zoltán Nagymihály, Beáta Lemli, László Kollár, Sándor Kunsági-Máté. Weak Interaction of the Antimetabolite Drug Methotrexate with a Cavitand Derivative. *International Journal of Molecular Sciences* **2020,** 21, 4345. **[IF: 5,923; Q1]**

Zsolt Preisz, Sándor Kunsági-Máté. Effect of methotrexate and its photodegradation products on the temperature induced denaturation of human serum albumin. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **2021,** 245, 118905. **[IF: 4,098; Q2]**

Zsolt Preisz, Nóra Hartvig, Balázs Bognár, Tamás Kálai, Sándor Kunsági-Máté. Comparative EPR Study on the Scavenging Effect of Methotrexate with the Isomers of Its Photoswitchable Derivative. *Pharmaceuticals* **2021,** 14, 665. **[IF: 5,863; Q1]**

Zsolt Preisz, Zoltán Nagymihály, László Kollár, Tamás Kálai, Sándor Kunsági-Máté. Weak Interactions of the Isomers of Phototrexate and Two Cavitand Derivatives. *International Journal of Molecular Sciences* **2021,** 22, 10764. **[IF: 5,923; Q1]**

Cumulative impact factor related to the thesis: 21,807

7.2. Presentations and posters related to the present PhD thesis

Preisz Zsolt. A metotrexát és fotodegradációs termékeinek hatása a humán szérum albumin hődenaturációjára. **XXIV. Tavaszi Szél Konferencia** (Miskolc, Magyarország, 2021. 05. 28-29.; oral presentation).

Zsolt Preisz, Balázs Bognár, Tamás Kálai, László Kollár, Sándor Kunsági-Máté. Some Interactions of the Novel Photoswitchable Compound Phototrexate. **DDRS2021 Conference** (Budapest, Magyarország, 2021. 11. 15-17.; poster).

7.3. Other publications

Anita Bufa, Nelli Farkas, **Zsolt Preisz,** Viktória Poór, Csilla Páger, Sándor Szukits, Bálint Farkas, Péter Miklós Gőcze. Diagnostic relevance of urinary steroid profiles on ovarian

granulosa cell tumors: two case reports. *Journal of Medical Case Reports,* **2017,** 11, 166. **[IF: 0,885; Q3]**

Cumulative impact factor: 22,692

7.4. Other presentations and posters

Zsolt Preisz, Ferenc Kilár, Péter Miklós Gőcze, Nelli Farkas, Anita Bufa. Urinary steroid profiles of a woman with recurrent granulosa cell tumour of ovary. **MLDT 58. Nagygyűlése** (Szeged, Magyarország, 2016. 08. 25-27.; oral presentation).

Preisz Zsolt. Vizeletszteroid-profilok vizsgálata granulózasejtes petefészek daganatnál. **III. Cholnoky László Nemzetközi Szakkollégiumi Szimpózium** (Pécs, Magyarország, 2017. 05. 11-12.; oral presentation).

Zsolt Preisz, Ferenc Kilár, Péter Miklós Gőcze, Nelli Farkas, Anita Bufa. Urinary steroid profiles of women with granulosa cell tumour of ovary. **MLDT 59. Nagygyűlése** (Pécs, Magyarország, 2018. 08. 30-09. 01.; poster).

7.5. Editorial work

Luca Fanni Kajos, Cintia Bali, **Zsolt Preisz,** Petra Polgár. Adrienn Glázer-Kniesz, Ádám Tislér, Rebeka Szabó. 10. Jubileumi Interdiszciplináris Doktorandusz Konferencia 2021 Absztraktkötet; 10th Jubilee Interdisciplinary Doctoral Conference 2021 Book of Abstracts. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2021) 347 p. ISBN: 9789634298205.

Luca Fanni Kajos, Cintia Bali, **Zsolt Preisz,** Rebeka Szabó. X. Jubileumi Interdiszciplináris Doktorandusz Konferencia 2021 Tanulmánykötet; 10th Jubilee Interdisciplinary Doctoral Conference 2021 Conference Book. Pécs, Magyarország: Pécsi Tudományegyetem Doktorandusz Önkormányzat (2022) 872 p. ISBN: 9789634298199.

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