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Neopterin-inhibitor of dihydrofolate reductase and hypothetical action in the human body

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ABSTRACT

Concentration of neopterin in the organism increases during many diseases and therefore it is used as a marker to assess the severity of these disease, applied treatment severity, oxidative stress and the cellular immune response. In this paper we attempt to determine the new properties of neopterin and its hypothetical role in DHFR activity in the human body.

During the study we worked with neopterin by SIGMA, purity ≥ 98% (HPLC), and a set of reagents to determine the activity of dihydrofolate reductase. The study was performed using a spectrophotometer at a wavelength of

λ = 340 nm.

The obtained results have confirmed that neopterin inhibits the activity of DHFR, both when it is fully dissolved in 1 N HCl as a suspension in a buffer (pH = 7.5) and as a supernatant.

DHFR inhibition depends on the neopterin concentration in the reaction mixture.

The results of our research and our discovery of new properties of neopterin as a DHFR inhibitor allow us to determine the hypothetical importance of neopterin in the body's defence system.

KEY WOEDS:- neopterin; inhibition of DHFR; folate metabolism; defence mechanisms

ACADEMIC DISCIP AND SUB-DISCIPLINES:-

medical sciences; medical biology; biochemistry in medicine

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TYPE (METHOD/APPROACH):- experimental in vitro

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INTRODUCTION

Neopterin is a derivative of pteridine, a molecule of molecular weight 253 Da, an aromatic compound, which has a tricarbon side chain at carbon 6 [1], 6-d-erythro-trihydroxypropyl-pterin. What is more, among its discovered properties, e.g. the ability to fluoresce, which has been used in numerous studies [2-4], sensitivity to sunlight, or a possible ability to occur in the form of stereoisomers. Neopterin has been also identified as a biochemical indicator of cell-mediated immune responses [5] that is released from monocytes/macrophages stimulated with interferon γ (INF-γ) [6] as a result of the activation of T helper cells [7, 8]. Monitoring levels of neopterin in blood and urine is used in clinical practice to assess the course of disease and effectiveness of the treatment [9], in transplantology, to assess the graft acceptance by the body [10, 11], and in transfusiology, to assess the degree of infection risk [12].The course of neopterin synthesis was established under physiological conditions and during cellular immune responses with increased immune-induced oxidative stress [7].

Despite numerous research into physicochemical properties of neopterin [1-12], so far it has been impossible to define its role in the human body.

It is difficult to agree that neopterin is redundant in the body and that it is there only to be used as a marker to assess the effectiveness of treatment or monitor a disease of different a etiology [12].

MATERIAL AND METHODS

For studies there was selected 6-D-erythro-neopterin (D-(+)-Neopterin by SIGMA), purity ≥ 98% (HPLC), which is the most abundant in the biological material. A set of reagents from Sigma (Dihydrofolate Reductase Assay Kit) CS0340 was used to determine the activity of DHFR. The preparation of neopterin for studies was as follows: 10 mg of neopterin was dissolved in 0.5 ml 1N HCl, and 10 mg (a suspension) in 2.2 ml of buffer of pH 7.5 (SIGMA).

The evaluation of the neopterin impact on DHFR enzyme inhibition was performed using a set of reagents from SIGMA kit (Dihydrofolate Reductase Assay Kit), which is based on an enzyme derived from human tissue. The kit makes it possible to perform conventional studies of the enzyme (DHFR), kinetics of the reaction, and effects of various compounds on the activity of dihydrofolate reductase. Dihydrofolate reductase (DHFR) catalyses the reduction process of 7.8 dihydrofolate (DHF) to 5,6,7,8 tetrahydrofolate (THF) using NADPH as a cofactor.

The DHFR activity test was implemented using a UV-7504 spectrophotometer by Hangzhou Ausheng Instruments Co. at the wavelength λ = 340 nm, and the decrease of absorption over the time of the process duration was measured. The DHFR activity was measured for 4 to 5 minutes, measuring the absorption every 0.5 or 1 minute. In the reference sample, where the decrease in absorption was measured, a mixture was used without dihydrofolic acid – the substrate of the reaction.

Conditions of the enzymatic reaction:

In a sample with a volume of 1000 µl there could be observed 0.0015 (1.5×10^{-3}) of DHFR enzymatic activity unit, 6µl NADPH (10 mM), 5µl of dihydrofolic acid and various amounts of inhibitor solution, as stated in the description under the drawings. The enzymatic reaction was carried out in a buffer of pH=7.5.

In this study, the following designations of mixtures were introduced for which absorption measurements were made:

A: DHFR (enzyme without the inhibitor for the first measurement in Figure 1)

B: DHFR + 5µl methotrexate - solution (pattern 100µl)

C: DHFR + 5µl neopterin - suspension (10 mg/2,2 ml buffer o pH=7,5)

D: DHFR + 5 µl neopterin - solution (10 mg/0,5 ml 1 N HCl)

E: DHFR + 2.5 µl neopterin - solution (10 mg/0,5 ml 1 N HCl)

F: DHFR + 55 µl supernatant neopterin - after centrifugation of the suspension (10 mg/2,2 ml buforu o pH=7,5)

G: DHFR (enzyme without the inhibitor for the second measurement in Figure 2)

RESULTS

Figure 1 and Figure 2 present the time dependencies of absorbance for all mixtures. Moreover, Table 1 includes the measurement results as average values.

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Figure 1

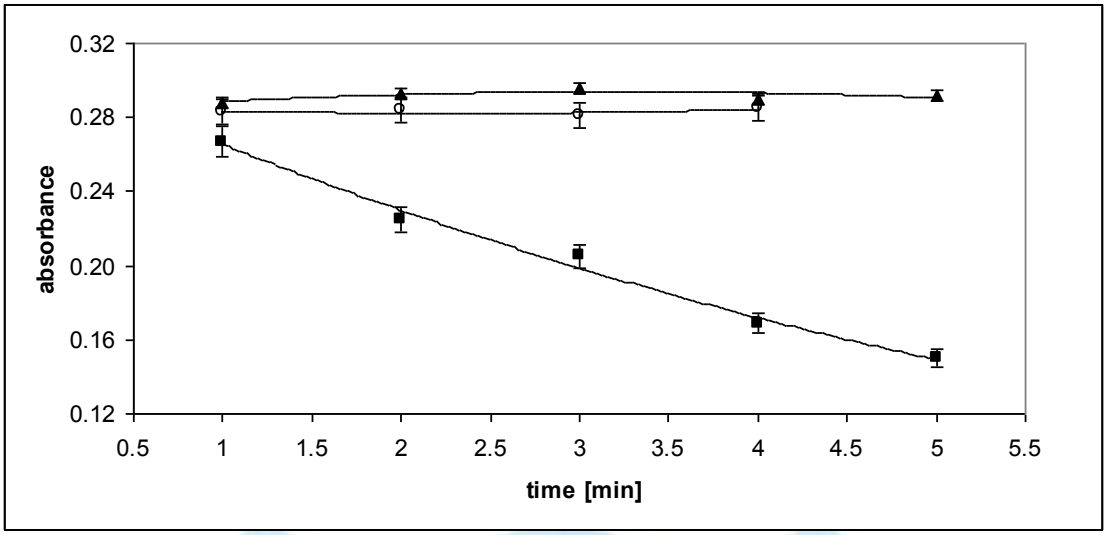

Figure 1 Dependency of absorbance on time for A (■), B (o) and C (▲). The curves show mean values of absorption from three measurements

Figure 1 shows the plots of absorption for mixtures A, B and C versus time. The numerical values of the absorption curves for A, B and C decrease respectively and show almost no change in the function of time. In addition, the absorption for B and C has similar values for the same measurement time. This result indicates that neopterin (in suspension) with the same concentration as methotrexate in the mixture with DHFR results in complete inhibition of this enzyme.

Figure 2

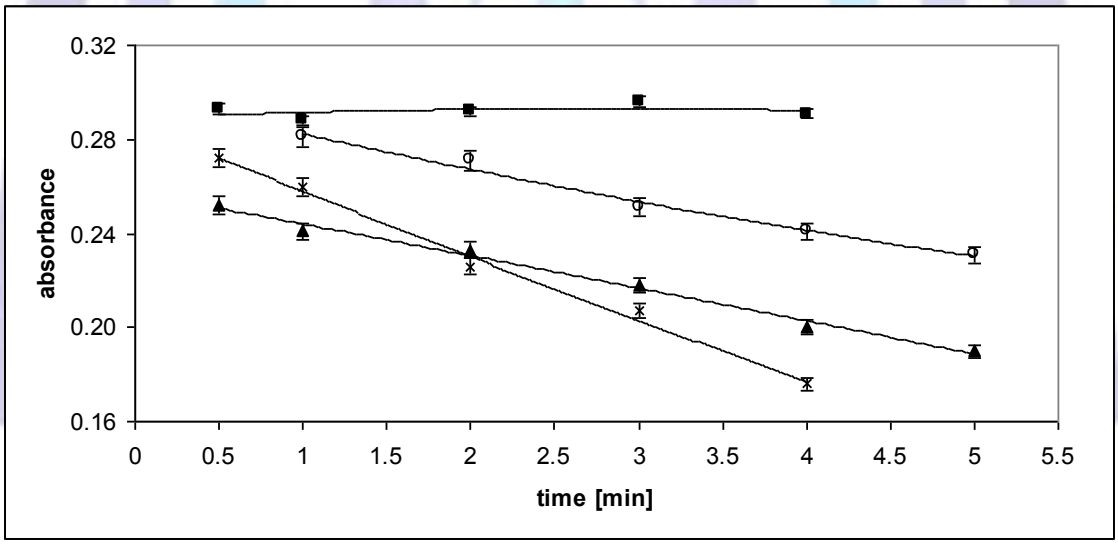

Figure 2 Dependency of absorbance on time for D (■), E (o), F (▲), G (x). The curves show mean values from three measurements

Figure 2 shows time dependencies of absorption for mixtures D, E, F, and G. The curves show the effect of neopterin concentration on DHFR activity and differ in terms of inclination towards the time axis. The greatest inclination, and thus a significantly higher change in absorption in a function of time when compared to other courses, is shown by the G curve. The total inhibition of DHFR activity is evident for the D curve, the absorption of which is almost constant and does not depend on time. Other curves for E and F, where absorption changes over time, show a lower degree of DHFR inhibition compared to the D curve.

The result of the study on the effect of neopterin dissolution on an environment with the selected pH, acidic (10 mg/0.5 ml 1 N HCl) and pH = 7.5 confirms its varied solubility whilst maintaining the capability of neopterin to inhibit DHFR, both when it has been fully dissolved (D and E in Figure 2) in a form of a suspension (C in Figure1), or a supernatant (F in Figure 2). The degree of DHFR inhibition by neopterin depends on its concentration in the environment (C in Figure 1 and D, E, and F in Figure 2).

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Table 1

Table 1 Time dependencies of mean values of absorbance for mixtures A, B, C, D, E, F and G

absorbance for mixtures	time [min]					
	0,5 min.	**1** min.	**2** min.	**3** min.	**4** min.	**5** min.
A	--------	**0,268**	**0,226**	**0,205**	**0,169**	**0,150**
B	--------	**0,283**	**0, 285**	**0,281**	**0,285**	--------
C	--------	**0,289**	**0,293**	**0,297**	**0,290**	**0,292**
D	**0,293**	**0,288**	**0,293**	**0,296**	**0,292**	--------
E	--------	**0,281**	**0,271**	**0,252**	**0,241**	**0,231**
F	**0,252**	**0,241**	**0,234**	**0,218**	**0,200**	**0,190**
G	**0,272**	**0,261**	**0,226**	**0,207**	**0,177**	--------

DISCUSSION

In our studies, neopterin that had been dissolved in 1N HCl and added to the tested sample in an amount of 100 µg (5 µl), which equaled the concentration of 400 µM of neopterin in the reaction mixture, completely inhibited the activity of DHFR (D in Figure 2). On the other hand, however, when its amount equaled

50 µg (2,5 µl), which was the concentration of 200 µM of neopterin in the reaction mixture, it only partially inhibited DHFR activity (E in Figure 2). Testing of neopterin dissolved in an acidic environment has proved that neopterin does not lose its DHFR inhibiting properties and that – depending on the concentration in the reaction mixture – it may partially or completely decelerate the activity of this enzyme. Neopterin, which is less soluble in a buffer of pH = 7.5 and forms a suspension in it, as well exhibits the DHFR inhibiting properties.

The obtained results presented in Figure 1 and Table 1 show that neopterin added to the mixture in an enzymatic reaction in a form of suspension with the same concentration as the methotrexate formula (curve for B) completely inhibits the activity of DHFR (curve for C). The environmental pH = 7.5, in which neopterin formed a suspension, did not result in losing its DHFR inhibiting properties. The plots for B and C in Figure 1, and for D in Figure 2 clearly show that high concentrations of neopterin inhibited the activity of DHFR.

It is different when it comes to the activity of supernatant obtained after centrifugation of the suspension. The supernatant, in contact with DHFR, shows inhibition which is much weaker (F in Figure 2) than the activity of neopterin in the suspension (C in Figure 1). The plot for F in Figure 2 clearly indicates that the activity of neopterin in the supernatant affecting inhibition of the enzyme in question is the weakest one in comparison to the plots for B and C in Figure 1, and for D and E in Figure 2. The dissimilarities in inhibition visible between the curves in Figure 1 and Figure 2 result from the difference in the concentration of neopterin, the amount of which is the lowest in supernatant.

The obtained results in Table 1, Figure 1 and Figure 2 show that for neopterin dissolved in the solution, suspension and in supernatant its ability to inhibit DHFR does not depend on the pH of the environment in which it is dissolved (acidic or in a buffer close to neutral), but it does depend on the concentration of neopterin in the reaction mixture. Thus, in the conditions of changing environmental pH, e.g. in a sick organism, the effect of neopterin's DHFR inhibiting activity depends primarily on its concentration. Our study has been the first one to perform the assessment of the effect of environmental pH and research the ability of neopterin as a DHFR inhibitor. No data on this topic can be found throughout the available literature. Therefore, in discussions it is difficult to refer to any similar research. Neopterin is synthesized in the body and under physiological conditions its concentration in serum is less than 10 nmol/l by norm. The concentration of neopterin grows in different disease entities. It seems, however, that it does not reach the level of neopterin that could completely inhibit the activity of DHFR in the body. In our studies the neopterin concentration of 400 µM in the reaction mixture is equivalent to 100 µg (5µl), while 200 µM corresponds to 50µg (2,5µl). Accordingly, the amount of neopterin is much higher than the concentrations estimated in a variety of disease conditions. For example, in patients with hepatitis C, its level in plasma reaches slightly more than 36 nmol/l [13]; in patients with the severe acute respiratory syndrome

(SARS), prior to treatment, the mean neopterin concentrations are in range of 34 nmol/l [14]; in patients with different cancers neopterin concentrations are in the range of 23-30 nmol/l [15, 16] and in the case of RA – 11-12 nmol/l [17]. Nevertheless, these examples do not indicate that neopterin in the body cannot occur locally at much higher concentrations, depending on the disease and its severity, causing a slight increase in the concentration above the normal range in the whole body. The varied levels of neopterin concentration in the same and different diseases can depend on many factors related to the efficiency of the body's defense mechanisms, location of the outbreak of inflammation, the number of pathogens or current disease stage.

The results of our research and our discovery of the new properties of neopterin as a DHFR inhibitor, as well as the available literature, allow us to determine the importance of neopterin in organism. The increase in neopterin levels is observed after non-specific immune response in which Th1 cells release IFN-γ which activates monocytes/macrophages to synthesize neopterin [5-8].

The release of large amounts of neopterin outside monocytes/macrophages in the disease focus or in the generalized form of the disease throughout the body increases the concentration of neopterin [18, 19]. According to our research results, which confirmed the correlation between neopterin concentration and the degree of DHFR inhibition within the specified time, it can be assumed that the higher the concentration of neopterin in the body, the more intensive the DHFR inhibition. DHFR is among the most important, if not the most important one, enzymes in the conversion of folic acid and folates. Reduction of the enzyme activity will result in the inhibition or attenuation of these changes in pathogens, redundant cells in the body and in normal cells. This means that the conversion of folic acid and folates required by pathogens and any cell in the body to exist will be (depending on the concentrations of neopterin) restricted or interrupted, causing damage to the cells, inhibition of their metabolism, lack of suitable life conditions and death [20].

In the human body, high levels of neopterin as a DHFR inhibitor will be particularly dangerous for rapid cells division – and not only from a group of pathogens, but also for healthy cells in the body, such as bone marrow cells, gametes, cells of the mucous membrane sand hair follicles. It should be understood, however, that during a disease neopterin is synthesized not to destroy its own healthy cells, but to block (as soon as possible, even at the expense of the body's healthy cells) the metabolism and division of foreign "intruders" in order to prevent their rapid expansion and domination in the body. The faster and the more radically the defence mechanisms work, the faster the body is set free from unwanted "guests"; in this way healthy cells and tissues regeneration is faster and the return to homeostasis is quicker. The apogee of neopterin concentration starts towards the end of the non-specific immune response and begins to go down when the titre of forming antibodies begins to be detectable (seroconversion), namely until the beginning of the specific immune response [7]. Inhibition of DHFR by neopterin which leads to inhibition of pathogen metabolism may facilitate its damage and elimination by phagocytic cells, in particular macrophages in the process of phagocytosis. What is eliminated from the body by a non-specific immune response mechanism is removed by a specific immune response mechanisms with specific antibodies, which will target the pathogen and facilitate its elimination by macrophages on the process of phagocytosis. High concentrations of neopterin in the body and DHFR inhibition contribute to neopterin's becoming an antimetabolite of folic acid, folates, purines and pyrimidines in the cells, which is important in the destruction of cancer cells (rapidly dividing). It can also prevent intracellular bacteria from creating and using the required cellular metabolites, thus demonstrating a bactericidal effect. Neopterin can affect the inhibition of the synthesis of DNA and RNA as well as proteins; it can also lead to the inhibition of cell growth and their damage and apoptosis. These capabilities of neopterin action result from its DHFR inhibition capacity. Inhibition of DHFR activity prevents the creation of TH4-folate from DH2-folate, which is illustrated in the diagram of the part of folic acid conversion (Figure 3).

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Figure 3

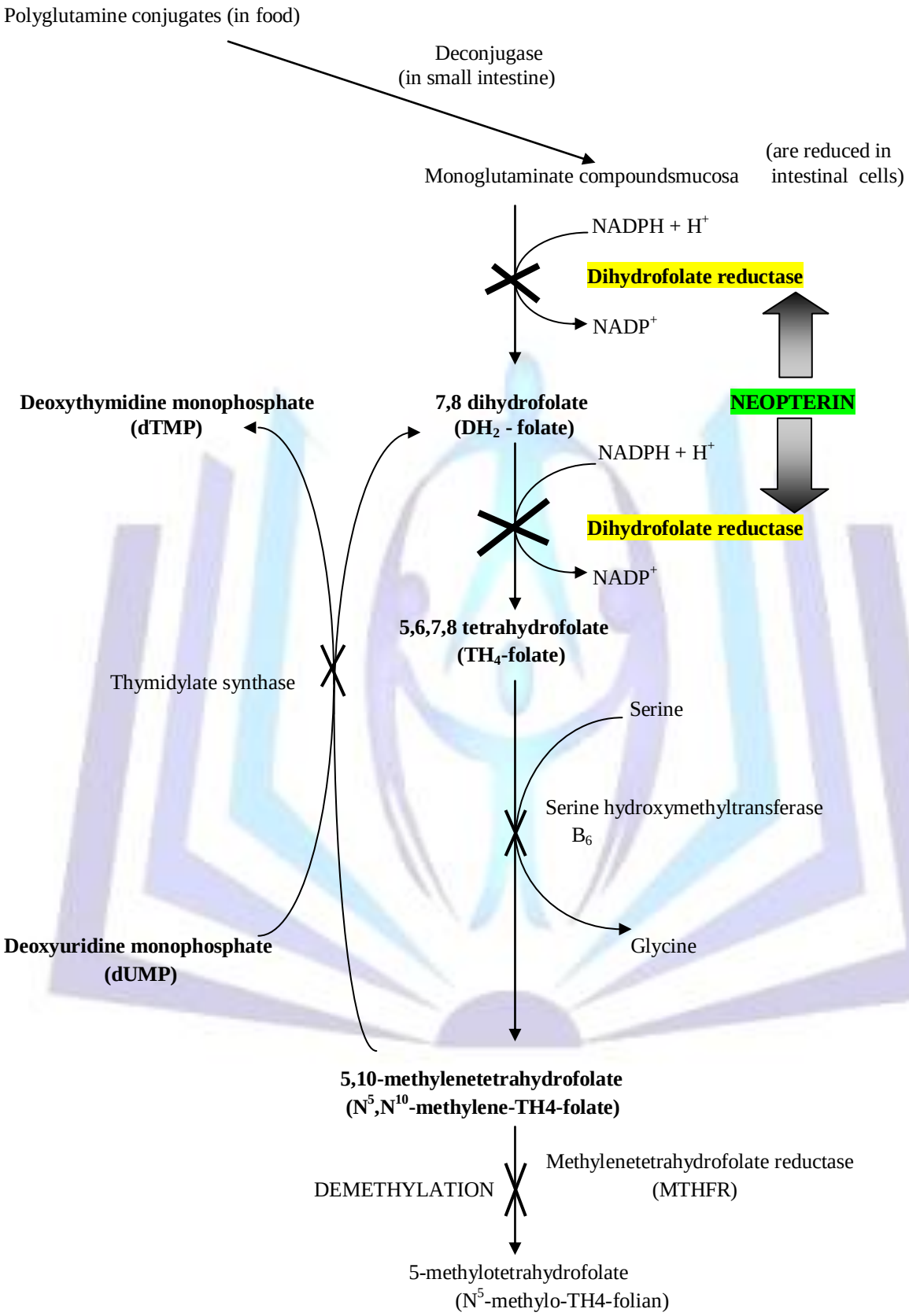

Figure 3 Blocking the activity of DHFR by neopterin in the selected fragment of folic acid and folate conversion

The consequence of that is termination of the transfer of active one-carbon groups, such as: methyl ($-CH_3$), methylene ($-CH_2-$), metenyl ($-CH_2=$), formyl (-CH=O), between the compounds involved in the metabolism of cells [20]. Inhibition of DHFR interrupts the possibility to create co-enzymes, which are necessary for the synthesis of the purines referred to above, as well as pyrimidines, and that are indispensible in the metabolism of homocysteine, methionine, serine, glycine or in catabolism of histidine to glutamate. As a result of DHFR inhibition by neopterin no 5,10-methylenetetrahydrofolate is formed in the conversion of folic acid, which is necessary to form deoxyuridine monophosphate from deoxythymidine monophosphate (dTMP), which is used as a thymidine by the cells for DNA synthesis [20].The selected part of conversion of folic acids and folates, accounting for the effect of high levels of neopterin on dihydrofolate reductase (DHFR), is shown in the diagram below (Figure 3). The consequence of DHFR inhibition in further metabolic conversion of folic acid is the absence of 5 methylotetrahydrofolate, which prevents the formation of methionine from homocysteine – an essential substrate for the synthesis of S-adenosylmethionine, which determines the DNA methylation [20]. On the basis of the above described dependencies, it can be concluded that the importance of neopterin as a DHFR inhibitor may have a certain function in the human body.

CONCLUSIONS

Neopterin is an inhibitor of DHFR and thus may perform such a physiological function in the human body. In the experiment DHFR inhibition depends on the neopterin concentration in the reaction mixture. The impact from the environmental pH determines the degree of solubility of neopterin, but it does not interfere with its properties as a DHFR inhibitor. As regards the body's defence mechanisms, neopterin may play an important role as an antimetabolite of pathogens and foreign cells and takes part in apoptosis. The results of our research and our discovery of new properties of neopterin as a DHFR inhibitor allow us to determine the hypothetical importance of neopterin in the body's defense system.

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