Cancer Chemother Pharmacol (1988) 21: 313–318

Cancer Chemotherapy and Pharmacology

Effect of methotrexate on homocysteine and other sulfur compounds in tissues of rats fed a normal or a defined, choline-deficient diet*

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Summary. Methotrexate (MTX) affects homocysteine (Hcy) metabolism in both cultured cells and patients, and this may be explained by a lack of the 5-methyltetrahydrofolate required for salvage of Hcy to methionine. We here report the effect of MTX on Hcy in serum and Hcy, *S*-adenosylhomocysteine (AdoHcy), *S*-adenosylmethionine (AdoMet) and reduced glutathione (GSH) in tissues of rats fed either a normal or a defined, choline-deficient (CD) diet. The CD diet alone did not affect the amounts of Hcy in serum and tissues, but decreased the amount of AdoMet in most tissues and increased the GSH content in the liver. MTX increased the amount of Hcy about 2-fold in serum, liver and kidney, and decreased the amount of AdoMet in liver and kidney, whereas the AdoHcy content in these tissues was essentially unaffected. Accordingly, both choline deficiency and MTX treatment reduced the AdoMet to AdoHcy ratio. The increased GSH in the liver induced by CD diet seemed to be abolished by MTX. In the spleen MTX had only a marginal effect on the Hcy and AdoMet content and decreased the GSH content. It is concluded that the increase in serum Hcy during MTX exposure probably reflects a disturbance of the Hcy metabolism in some tissues, and especially in the liver. Altered metabolism of other sulfur-containing metabolites may only partly be related to the inhibition of Hcy salvage, and some metabolic effects of MTX may be modulated by tissue-specific metabolic pathways as well as by the diet.

Introduction

Methotrexate (MTX) inhibits the enzyme dihydrofolate reductase, and thereby blocks the regeneration of tetrahydrofolate (THF) from dihydrofolate (DHF). This is expected to cause cellular depletion of reduced folates [16]. Determination of folates in tissues has met with considerable difficulty, and it was not until recently that the effect of MTX on the various species of reduced folates was evaluated. Notably, in two independent reports it is shown that one of these compounds, 5-methyl-THF, is selectively and rapidly depleted during MTX exposure [1, 17].

Homocysteine (Hcy) is formed from the endogenous transmethylase inhibitor *S*-adenosylhomocysteine (AdoHcy) and is metabolized either to cystathionine or to methionine [19]. Cystathionine is further metabolized to cysteine, which is a precursor of the important thiol, glutathione (GSH). In the methionine synthase reaction, 5-methyl-THF serves as a methyl donor. This metabolic relationship suggests that MTX inhibits the 5-methyl-THF-dependent salvage of Hcy to methionine. Data obtained in cultured cells [32] or in patients receiving high-dose MTX [23] accord with this possibility. The Hcy egress from cultured cells was greatly enhanced in the presence of MTX, and plasma and urinary Hcy increased during MTX treatment [23, 32].

Long-term treatment of patients [21] or laboratory rats [9] with MTX induces hepatic injury characterized by fatty infiltration and fibrosis. Notably, exogenous choline antagonizes the hepatotoxic effects of MTX in the rat [12, 29]. Experimental data suggest that the hepatotoxicity is caused by lack of 5-methyl-THF necessary for the endogenous synthesis of methionine from Hcy [5]. This in turn may cause increased consumption of choline, to supply betaine for the betaine-Hcy-transmethylase reaction. In addition, lack of methionine may slow down the AdoMet-dependent steps [34] in choline biosynthesis. Notably, there is some indication that MTX may produce the choline-deficient state only when the diet lacks vitamin B_{12} [3].

A diagram showing the metabolic relationship between Hcy, GSH, choline and reduced folates is shown in Fig. 1.

It has been postulated that Hcy in extracellular media may be a measure of the balance between Hcy production and utilization, and thereby the availability of 5-methyl-THF during antifolate treatment [23, 32, 33]. To evaluate this possibility it seems warranted to determine the alterations in the amount of Hcy and related compounds in whole tissues during drug exposure. Because of the importance of GSH in cellular detoxification processes [18], this thiol was also included in the study. The animals investigated received either a standard or a choline-deficient (CD) diet, since such a diet affects the amount of *S*-adenosylmethionine (AdoMet) and GSH in tissues [14, 26, 27] and therefore may modulate the metabolic effects of MTX.

Materials and methods

Chemicals and drugs. DL-Hcy, AdoHcy and reduced glutathione were purchased from Sigma Chemical Co. (St Louis, Mo), and AdoMet was obtained from Koch-Light lab-

* Supported by grants from the Norwegian Cancer Society and the Norwegian Society for Fighting Cancer

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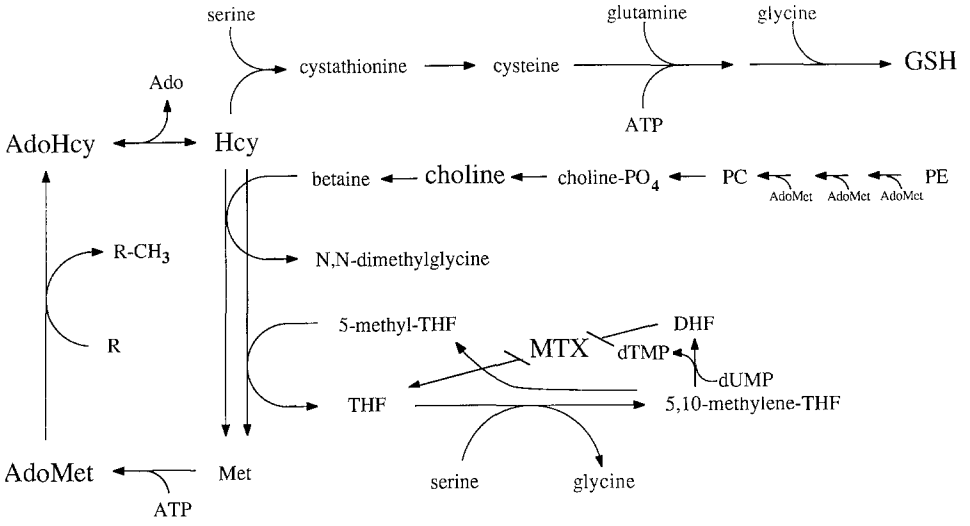

Fig. 1. The metabolism of sulfur compounds, choline and reduced folates, and its relation to the molecular target of MTX

oratories (Colnbrook, England). Monobromobimane was purchased from Calbiochem-Behring (La Jolla, Calif). MTX was from Nycomed (Oslo, Norway). Other chemicals were obtaind from commercial sources and were of reagent grade.

Animals and diets. Male Wistar rats weighing 180–220 g were used. They were housed individually in metal wire cages in a room with maintained 12-h light-dark cycles and a constant temperature of 20±3° C. The animals were acclimatized for at least 5 days under these conditions before the start of the experiments.

The animals were fed one of two diets.

Normal diet (ND) refers to commercial pelleted rat food containing 55% carbohydrate, 25% protein, 4% fat and all necessary minerals and vitamins, including vitamin B_{12} (24 μg/kg) and choline (600 mg/kg).

The choline-deficient (CD) diet was composed of 680 g sucrose, 200 g casein, 60 g soya oil, 40 g USP XIV salt mixture and 20 g vitamin mixture containing 25 mg thiamine hydrochloride, 22 mg riboflavine, 22 mg pyridoxine hydrochloride, 33 mg calcium pantothenate, 220 mg nicotinamide, 350 mg ascorbic acid, 5000 IU vitamin A, 600 IU vitamin D2, and 51 mg tocopherol in sufficient glucose to give 20 g.

The rats had free access to the diet and water.

Treatment of animals. The number of animals per group was chosen so that each metabolite in tissues of all animals could be assayed in a single run. This meant that between-day variations did not arise. The animals were divided into four groups, and each group contained four animals.

1. One group was fed ND and received no drug. These are the control animals.
2. One group was fed the CD diet and received no drug.
3. One group was fed ND and received MTX by injection.
4. One group was fed CD diet and received MTX by injection. These animals are referred to as the CD+MTX group.

MTX was dissolved by adding sterile water to an ampulla containing sufficient sodium chloride to make an isotonic solution. The solution was injected i.p. once daily for 10 days, the amount corresponding to a dose of 0.350 mg/kg every 24 h. The control animals received injections of physiological saline.

At the end of the experiments, the animals were weighed, and then killed by decapitation and exsanguinated (for less than 30 s) to obtain blood samples; liver, kidney and spleen were rapidly removed and put in liquid nitrogen.

Determination of Hcy, AdoHcy and AdoMet in tissues. Frozen tissue specimens were homogenized in 0.8 *N* perchloric acid. Acid-soluble Hcy was determined by means of a radioenzymic assay [31], whereas AdoHcy and AdoMet were determined directly in the acid extract by using an HPLC method [32].

Determination of total Hcy in serum. Serum was prepared from whole blood, which was centrifuged at 1000 *g* for 10 min. Serum was then diluted in phosphate buffer, and the radioenzymic assay for Hcy was run in the presence of native proteins, to obtain total (i.e., free plus protein-bound) Hcy. Details have been published previously [22].

Determination of glutathione in tissues. Frozen tissue samples were rapidly homogenized (1:30, w:v) in 5% sulfosalicylic acid. The precipitated proteins were removed by centrifugation. Reduced GSH was determined in the acid extract by a slight modification of the method of Newton et al. [2], which involves derivatization of free sulfhydryl groups in the presence of the monobromobimane (Kosower's) reagent. The GSH-bimane derivative is then quantitated by chromatography on a 3-μm ODS Hypersil column, which is equilibrated and eluted with 14.2% methanol and 0.25% glacial acetic acid adjusted to pH 3.9 with sodium hydroxide. The column is washed by increasing the methanol concentration to 90%. The retention time of the GSH-bimane derivative was 3.2 min.

Statistical methods. The log-normal distribution of the values for Hcy in rat liver has been documented in a previous

report [28]. The number of experimental animals did not allow test for sample distribution, but the assumption is made that the values for Hcy, AdoHcy, AdoMet and GSH in rats fed a normal or a CD diet and/or receiving MTX also have a log-normal distribution.

Homogeneity of variance was tested for with Bartlett's test [24]. An analysis of variance (two-way) including a test for statistical interactions was used to compare values from different groups (14 out of 16) which showed identical variance. Different variance ($P < 0.025$) was found in two groups (Hcy in spleen of rats receiving CD diet plus MTX, and Hcy in serum from these rats). In these cases, Student's *t*-test was applied after logarithmic transformation of data, and the *P*-values were corrected using the Bonferroni correction. Significance values (*P*-values) were always expressed as two-tailed.

Results

Effects of diet

When the rats were given the defined CD diet for 10 days there was no change in the amount of Hcy in serum, no change in Hcy and AdoHcy content in liver or kidney, and a small increase in the amount of Hcy ($P < 0.005$) and AdoHcy ($P < 0.025$) in spleen.

The CD diet decreased AdoMet in the liver ($P < 0.005$) and the kidney ($P < 0.05$) but not in the spleen ($P > 0.25$). Calculation of the AdoMet-to-AdoHcy ratio showed that this parameter was decreased in the liver ($P < 0.005$) and spleen ($P < 0.025$), but not significantly in the kidney ($P > 0.25$).

The CD diet significantly increased the GSH level in the liver ($P < 0.05$), but not in the kidney or spleen (Table 1).

Effect of MTX in rats fed a standard diet or a choline-deficient diet

Homocysteine in serum and tissues. MTX significantly increased the total amount of Hcy in serum from rats given a normal diet ($P < 0.005$), and seemed to increase the Hcy content in serum of rats fed a CD diet, but this could not be statistically verified because of unequal variance. The different spread was largely due to low Hcy in the serum of one rat exposed to choline deficiency plus MTX. Notably, this rat also showed relatively low Hcy in the spleen.

MTX increased Hcy about 2-fold in the liver ($P < 0.005$), and less, but significantly, in the kidney ($P < 0.025$), and slightly ($P < 0.01$) in the spleen. No interaction between MTX treatment and choline deficiency as they affect Hcy levels in liver and kidney was statistically verified ($P > 0.25$) (Table 1).

AdoHcy and AdoMet. Treatment had no significant effect on the amount of AdoHcy in liver ($P > 0.25$) and kidney ($P > 0.25$), but decreased it slightly in spleen ($P < 0.05$). In the liver the combination of CD diet plus MTX statistically showed a negative interaction ($P < 0.005$), which implies that the amount of AdoHcy in the liver of the treated rats receiving the CD diet was less than might be expected from the effect of each intervention, whereas in the spleen no interaction with diet was demonstrable ($P > 0.10$).

Administration of MTX decreased the AdoMet content in all tissues ($P < 0.005$).

It has been postulated that the ratio between AdoMet and AdoHcy may be critical for the overall transmethylation rate [30]. MTX treatment decreased the ratio in the liver ($P < 0.005$) and kidney ($P < 0.025$), but not in the spleen ($P > 0.25$). The negative interaction between CD

Table 1. Effect of methotrexate (MTX) on the amount of sulfur-containing metabolites in tissues of rats fed a normal or a choline-deficient (CD) diet[a]

Metabolite[b]	Control	MTX	CD diet	CD diet + MTX
Serum				
Hcy	9.2± 1.2	16.5± 1.9	9.0± 0.9	12.2± 4.8
Liver				
Hcy	3.7± 1.4	9.3± 1.6	3.5± 0.7	7.4± 1.7
AdoHcy	17.5± 2.9	22.0± 1.7	21.5± 2.0	16.7± 3.3
AdoMet	139.6±16.3	109.3± 6.6	95.4±12.1	68.6±10.0
[AdoMet]: [AdoHcy]	8.1± 1.1	5.0± 0.5	4.4± 0.3	4.2± 1.1
GSH	11692±3583	9764±679	18077±1489	10548±2490
Kidney				
Hcy	4.3± 1.0	6.2± 2.2	4.3± 0.4	5.4± 0
AdoHcy	11.3± 3.4	12.3± 4.4	10.9± 2.2	11.6± 1.0
AdoMet	81.0±12.0	54.5±10.2	60.9±12.2	50.5± 4.0
[AdoMet]: [AdoHcy]	7.6± 2.1	4.8± 1.5	5.7± 1.6	4.4± 0.3
GSH	3789±853	3678±769	4256±421	3975±521
Spleen				
Hcy	1.6± 0.1	2.1± 0.2	2.3± 0.2	2.3± 0.7
AdoHcy	1.0± 0.4	0.9± 0.1	1.7± 0.4	1.0± 0.2
AdoMet	76.5±14.9	54.2± 5.4	70.3± 6.6	57.2± 5.5
[AdoMet]: [AdoHcy]	87.0±46.9	64.1± 9.1	43.2± 8.4	59.3± 6.9
GSH	6367±909	4024±792	5779±1041	2711±1004

[a] The animal received MTX (0.350 mg/kg per day) i.p. for 10 days. The results are given as means of four determinations ± SD. The statistical evaluation of the data is given in the text

[b] The amounts of metabolites are given as nanomoles per milliliter of serum or nanomoles per gram of tissue. Hcy, homocysteine; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; GSH, glutathione

diet and MTX for the AdoHcy content in the liver was also found for the AdoMet-to-AdoHcy ratio ($P < 0.05$) (Table 1).

Glutathione. MTX decreased the GSH content in the liver ($P < 0.01$), especially in rats fed the CD diet. Thus, it seems that MTX counteracts the increased amount of GSH induced by a CD diet. However, the apparent differential effect of MTX on rats receiving normal versus CD diet was not sufficiently pronounced to document a statistically significant interaction ($P > 0.05$). MTX did not affect the GSH content in the kidney of rats fed either diet ($P > 0.10$).

In the spleen the response was somewhat different. The amount of GSH decreased following the administration of MTX ($P < 0.005$). No interaction between MTX and diet was demonstrable ($P > 0.10$) (Table 1).

Discussion

In the present work we investigated the effect of MTX on the amount of Hcy and other sulfur compounds in rats. The rats received either a standard diet or a defined CD diet. The standard diet is a commercially available pellet diet containing all vitamins and lipotropes; it is the one that has been fed to these animals throughout their lifespan and therefore supports their growth and normal function. Such a diet excludes any possibility that the MTX effects observed are limited to conditions of undefined nutritional deficiencies.

The CD diet is well-defined, lacking vitamin B_{12} and choline; it was given to these rats for 10 days. Vitamin B_{12} was excluded from the diet because it may antagonize some metabolic effects of MTX [3].

Animal studies have established that choline in the diet may modulate the hepatotoxic effects of MTX [5, 12, 29]. Therefore, we decided to evaluate the effect of MTX on sulfur compounds in tissues of rats receiving a normal diet, and of others receiving a CD diet. The CD diet alone did not affect Hcy in serum or tissues, but decreased AdoMet in most tissues and increased GSH in liver (Table 1). These effects of a diet devoid of choline on AdoMet and GSH are in accordance with data published by others [14, 26, 27].

MTX affects the Hcy metabolism both in cultured cells and in patients [23, 32]. This can be explained by rapid depletion of 5-methyl-THF, which in turn blocks the salvage of Hcy to methionine. Intracellular accumulation is prevented by the release of copious amounts of Hcy into the extracellular medium and by metabolism through alternate pathways [23, 32]. An increase in urinary and plasma Hcy may reflect cellular Hcy egress.

In the present paper this line is carried further by investigating the effect of MTX on Hcy and other sulfur compounds in tissues of the experimental rat. The effect of MTX on serum Hcy (Table 1) resembles the acute effect observed in patients [23]. Notably, diet did not affect the serum Hcy level (Table 1).

Alterations in serum Hcy probably reflect alterations of Hcy metabolism in tissues. The Hcy response in liver and kidney resembles the changes observed in serum. In contrast, the most striking effect in spleen was the increased amount of Hcy induced by choline deficiency, and only marginal effects, or none at all, of MTX exposure (Table 1). A similar response was observed in the heart (data not shown). These observations, together with the fact that the liver accounts for the bulk of transmethylation reactions producing Hcy [19], suggest that Hcy metabolism in the liver has a major impact on the serum Hcy level.

The differential response in liver and kidney versus spleen should be related to the special features of Hcy metabolism in liver and kidney. These tissues are rich in both betaine and 5-methyl-THF-homocysteine methyltransferase. In addition, the liver contains a high level of cystathionine β-synthase. In most other tissues, a single enzyme, 5-methyl-THF-homocysteine methyltransferase, is responsible for the intracellular handling of Hcy [19]. It is conceivable that the metabolic response to such events as choline deficiency and MTX exposure may be modulated by different metabolic pathways present in various tissues.

Addition of Hcy to various cells increases the AdoHcy content [30]. Therefore, the increase in intracellular Hcy following MTX exposure may induce AdoHcy accumulation through reversal or inhibition of the AdoHcy-hydrolase reaction. This possibility has previously been investigated in non-transformed and malignant fibroblasts, but no accumulation of cellular AdoHcy was demonstrated during MTX exposure [32]. A similar observation is made in whole tissue. There is no significant increase in AdoHcy in tissues following injection with MTX (Table 1). Hilton et al. [15] found a moderate increase in AdoHcy in liver of rats fed a normal diet during MTX exposure.

MTX reduced the amount of AdoMet in all tissues investigated, whereas choline deficiency reduced the amount in liver and kidney (Table 1). Hilton et al. demonstrated a decreased AdoMet level in the liver of rats injected with MTX [15].

In general, low AdoMet may occur under conditions characterized either by a decrease in AdoMet synthesis and/or enhanced utilization.

Inhibition of the 5-methyl-THF-dependent salvage of Hcy to methionine by MTX may cause methionine deficiency, since the methionine-synthase reaction accounts for a significant portion of the total methionine supply in rats [11] and humans [19, 20]. This in turn may explain the reduction of the AdoMet content in tissues.

Reduction in AdoMet during choline deficiency may reflect either decreased methionine synthesis or increased metabolic demand for AdoMet. Lack of choline may reduce the amount of betaine [4] required for the betaine-homocysteine methyltransferase reaction, thereby inhibiting betaine-dependent methionine synthesis in liver and kidney. In addition, an increased demand for endogenous choline probably increases the amount of AdoMet consumed in the AdoMet-dependent steps [34] in choline biosynthesis (Fig. 1).

It has been suggested that low AdoMet in the liver favors fat deposition [10]. Whether the availability of AdoMet may affect the development of fatty metamorphosis induced in the liver by MTX exposure and choline deficiency should be considered.

GSH has been regarded as the most important nonprotein thiol [18]. AdoMet [10] or AdoHcy [7] seems to affect the amount of GSH in the liver [10] and L5178Y cells [7]. The amount of GSH in tissues may in turn modulate the cytostatic action of other chemotherapeutic agents [13, 25], some of which are administered together with MTX as cancer therapy. Therefore, it seemed warranted to investigate whether alteration of the metabolism of endogenous

sulfur compounds during choline-deficiency or MTX exposure may affect the amount of GSH.

Choline-deficiency induced a significant increase in the GSH content in liver, but the effect seemed to be counteracted by MTX treatment. No other effects of MTX or CD diet on GSH in liver or kidney were observed. Thus, in these organs the decreased amount of AdoMet following MTX exposure and CD diet was not paralleled by alterations in the GSH content (Table 1). Notably, GSH in the liver was related to the β-oxidation of fatty acids, which was enhanced by choline deficiency, and the increase was abolished by concurrent MTX administration (R. Berge, unpublished). The possible relationship between GSH and β-oxidation during MTX exposure of rats fed a normal or a CD diet is presently under study.

In contrast to liver and kidney, spleen showed decreasing GSH content in the order, normal diet $\geq$ choline deficiency $>$ normal diet plus MTX $>$ choline deficiency plus MTX. Thus, in this organ the GSH response resembled the alteration in AdoMet content. This observation may suggest a possible link between AdoMet and GSH in spleen.

The alterations in GSH content in the spleen contrast with the changes in the liver and kidney. In a previous paragraph we have described the metabolism of Hcy in liver and kidney versus spleen. In addition, hepatocytes [6], but not spleen lymphocytes [8], utilize methionine via the transsulfuration pathway for GSH synthesis. It is conceivable that the GSH response to interventions like MTX exposure and CD diet is modulated by metabolic features unique to particular tissues or cells.

Alteration in GSH content in some tissues following MTX exposure should be considered as a possible cause of interference with the action of other anticancer agents.

In conclusion, increased Hcy in extracellular media such as serum after MTX administration may result from the inhibition of 5-methyl-THF-dependent salvage of Hcy in tissues, especially in liver. There is a concurrent decrease in AdoMet and GSH in some tissues. This metabolic response is modulated by tissue-specific metabolic pathways and the composition of the diet. It is conceivable that altered metabolism of sulfur compounds may mediate some effects of MTX and modulate the cytostatic action of other anticancer drugs. These possibilities deserve further attention.

Acknowledgements: The technical assistance of A. Høylandskjær, G. Kvalheim and H. Bergesen is highly appreciated.

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Received June 7, 1987/Accepted December 8, 1987