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European Journal of Cancer 40 (2004) 2287–2292

European Journal of Cancer

www.ejconline.com

Methotrexate inhibits the glyoxalase system *in vivo* in children with acute lymphoid leukaemia

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Received 4 February 2004; received in revised form 17 May 2004; accepted 29 June 2004
Available online 10 August 2004

Abstract

The inhibition of glyoxalase I leads to antitumour activity through the accumulation of methylglyoxal. Our earlier observations suggested that methotrexate (MTX) may affect the glyoxalase system. This prompted a serial study of the drug on this metabolic pathway. Ten children with acute lymphoid leukaemia (ALL), admitted to our department between January 2002 and July 2003, were enrolled. Plasma D-lactate was assayed before, 24 and 72 h after the start of four consecutive MTX infusions (5 g/m^2/24 h) in each patient. Inhibition of glyoxalase I was tested *in vitro*, using human erythrocyte lysates and yeast enzyme. The elevated initial plasma D-lactate levels ($P < 0.02$) fell significantly ($P < 0.001$) in response to 24 h MTX infusions. *In vitro*, MTX, folic and folinic acids inhibited the activity of glyoxalase I. Thus, MTX seems to affect the α-oxoaldehyde metabolism *in vivo*, as a likely consequence of glyoxalase I inhibition. This action probably contributes to the anticancer activity and toxicity of the drug.

Keywords: Diabetes; Folic acid; Folinic acid; Glyoxalase; Leukaemia; Methotrexate; Methylglyoxal; D-Lactate; Thiamine

1. Introduction

Methotrexate (MTX) is one of the most widely applied chemotherapeutic drugs and is used in the treatment of different types of cancer, leukaemia, and inflammatory disorders such as psoriasis and rheumatoid arthritis. It is a folic acid analogue that affects the enzyme dihydrofolate reductase, blocking the conversion of dihydrofolate to tetrahydrofolate, thereby disrupting the synthesis of DNA and RNA components. It also inhibits thymidylate synthase, preventing the production of deoxythymidylate. The inhibition of DNA synthesis and proliferation by the drug also affects normal cells, leading to side-effects, such as acute gastrointestinal toxicity. In a previous study, we tested the plasma D-lactate level as a possible indicator of injuries to the intestinal mucosa, and that study included some patients undergoing high-dose MTX treatment.

D-Lactate is a product of the α-oxoaldehyde metabolism. The α-oxoaldehydes glyoxal and methylglyoxal are formed by lipid peroxidation, glycation and degradation of glycolytic intermediates [1]. They are electrophilic compounds that bind readily to nucleic acids and proteins and form stable adducts inducing mutagenesis, apoptosis, protein degradation and the formation of advanced glycation end-products. Several enzymes (e.g., aldose reductase, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenases) are capable of metabolising α-oxoaldehydes, but the highly active glyoxalase system is considered the main route of methylglyoxal detoxification [2]. The system consists of two enzymes, glyoxalase I and II, and a catalytic amount of glutathione. The metabolic flux through this pathway results in the production of D-lactate (Fig. 1).

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doi:10.1016/j.ejca.2004.06.024

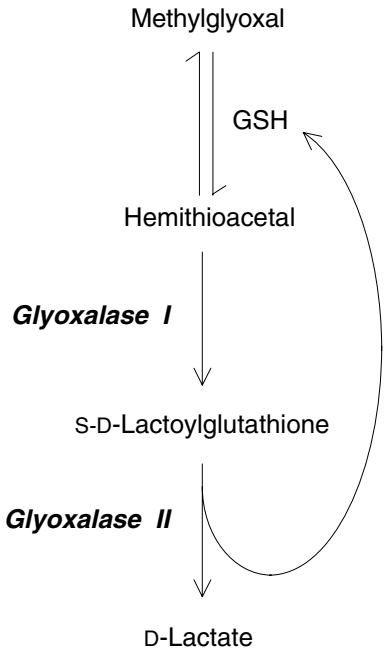

Fig. 1. The metabolic pathway of the glyoxalase system. The non-enzymatic reaction of methylglyoxal with reduced glutathione (GSH) produces the hemithioacetal. Glyoxalase I catalyses the intramolecular redox reaction of the hemithioacetal forming the thioester, S–D-lactoylglutathione. In the last step, glyoxalase II hydrolyses the ester to D-lactate and GSH.

The sources of plasma D-lactate are the endogenous α-oxoaldehyde metabolism, the enteric microorganisms and fermented foodstuffs. Elevated plasma levels of the metabolite have been reported in diabetes [3] and triose phosphate isomerase deficiency [4], due to the enhanced production of endogenous methylglyoxal; in necrotising enterocolitis [5] and short bowel syndrome [6], due to bacterial overgrowth; in carbohydrate malabsorption or the ingestion of large amounts of carbohydrate, due to the increased delivery of nutrients to the colon [7]; in diminished colon motility, due to the increased fermentation of nutrients [7]; in bowel ischaemia, due to mucosal injury [8]; and after the ingestion of fermented milk products [9].

In our previous study, the changes (a marked fall in plasma D-lactate; data not shown) observed in the patients receiving MTX therapy did not seem to indicate the condition of the intestinal mucosa, but rather raised the possibility that MTX inhibits the endogenous α-oxoaldehyde metabolism. The fall in plasma D-lactate following MTX treatment seemed to be of special interest, as impairment of the methylglyoxal metabolism is cytotoxic and glyoxalase I inhibitors exhibit antitumour activity.

These data prompted a systematic study of the influence of high-dose MTX therapy on the level of plasma D-lactate in patients with acute lymphoid leukaemia (ALL). The effects of MTX, folic acid and folinic acid (leucovorin) on the activity of glyoxalase I were tested *in vitro*, using human erythrocyte lysates and yeast enzyme.

2. Patients and methods

2.1. Patients

Ten children (5 girls/5 boys; age: 7.6 ± 5.4 years, mean ± standard deviation (SD)) with newly-diagnosed and pathologically-confirmed ALL, who were admitted to the Department of Paediatrics, University of Szeged between January 2000 and July 2002, were enrolled. All these children were treated according to the ALL-BFM Berlin-Frankfurt-Münster 95 protocol. As consolidation therapy, they received high-dose MTX infusions (5 g/m^2/24 h every every (q) 14 days, 4×) supplemented with intrathecal MTX (12 mg/m^2 in a single dose q 14 days, 4×) and followed by folinic acid rescue (15 mg/m^2 intravenously (i.v.) at 42, 48 and 54 h after the start of the infusion). 6-Mercaptopurine was started 1 week before the first MTX infusion and was administered in an oral dose of 25 mg/m^2/day every evening from day 1 to day 56. The patients did not receive any other therapy for 2 weeks before the initiation of and during the consolidation therapy. In each patient, 4 consecutive MTX cycles were followed. Blood was collected immediately before (0 h), and 24 and 72 h after the start of the MTX infusion. The patients were on a normal diet, but without any fermented milk product, from the day before and during the 72 h observation period. Blood was always collected in a fasting state. Altogether 36 treatment cycles could be evaluated; data of 4 cycles were omitted because of blood transfusions administered during the observation period. Normal values of D-lactate were determined in 14 healthy children admitted for selective surgery (7 girls and 7 boys; age: 7.8 ± 3.7 years, mean ± SD). Blood for the *in vitro* assay of glyoxalase I activity was obtained from 4 healthy adult volunteers.

The study was approved by the Human Investigation Review Board of the University of Szeged and informed parental consent was obtained before the collection of blood samples.

2.2. Materials

(+)-Amethopterin, folic acid, (+/−)-folinic acid Ca salt, glyoxalase I (EC 4.4.1.5), D-(−)-lactic acid, D-lactic dehydrogenase (EC 1.1.1.28), methylglyoxal, oxidised nicotinamide adenine dinucleotide and reduced glutathione (GSH) for the assays were purchased from the Sigma Chemical Co. (St. Louis, MO). The MTX radioimmunoassay kit was from the Institute of Isotope Chemistry (Budapest, Hungary). All other chemicals were commercial products of reagent grade.

2.3. D-Lactate

Venous blood anticoagulated with ethylene diamine tetra acetic acid (EDTA) was immediately separated by centrifugation (4 °C, 1500*g*, 10 min). Plasma was added to vials containing 4 M perchloric acid and samples were stored at −72 °C for up to one month before analysis. The concentration of D-lactate was measured by end-point enzymatic assay with D-lactic dehydrogenase and fluorometric detection of reduced nicotinamide adenine dinucleotide (NADH) [10]. (The within-run and inter-day coefficients of variation (CVs) were 3.2% and 5.1%, respectively.)

2.4. Glyoxalase I

The activity of this enzyme was assayed by measuring the initial rate of formation of S-D-lactoylglutathione from the hemithioacetal in the presence of diluted erythrocyte lysate or yeast glyoxalase I [11]. The reaction mixture contained 2 mM GSH and 2 mM methylglyoxal in 100 mM sodium phosphate buffer, pH 6.6, preincubated at 37 °C for 10 min, and 30 μl/ml 1:40 haemolysate, or yeast enzyme (43 mU/ml). The reaction was monitored spectrophotometrically at 240 nm. MTX (10–300 μM), folic acid (5–250 μM), or folinic acid (50–450 μM) was added to the assay mixture at the start of preincubation. (The within-run and inter-day CVs were 4.5% and 6.4%, respectively.)

2.5. Methotrexate

At 24, 42 and 72 h after the start of the MTX infusion, plasma MTX levels were measured by radioimmunoassay, using [I^{125}]-MTX. (The within-run and inter-day CVs at 1×10^{-7} M were 2.1% and 3.4%, respectively.)

2.6. Methotrexate toxicity

Patients were monitored during the 72 h observation period for acute toxic symptoms, including stomatitis, diarrhoea, vomiting or elevated aminotransferase/alanine aminotransferase (AST/ALT) levels. The role of glyoxalase I inhibition in the toxicity of MTX was assessed by comparing the changes in D-lactate levels during those treatment cycles which were followed by acute side-effects with those in the cycles without toxic manifestations.

2.7. Statistical analysis

One-way analysis of variance followed by the Tukey test was used to determine significant differences over time within the plasma D-lactate levels. The differences between the patient and control groups and between the patients with and without toxic symptoms were assessed by the Student *t*-test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. D-Lactate

The plasma level of D-lactate was elevated ($P < 0.02$) in the ALL patients before therapy compared with the healthy controls (Fig. 2(a)). The initial D-lactate values were similar and followed the same pattern of changes during the 4 consecutive MTX infusions in each patient. MTX induced a significant ($P < 0.001$) fall in the D-lactate level, but at 72 h its concentration was again close to the initial value (Fig. 2(b)).

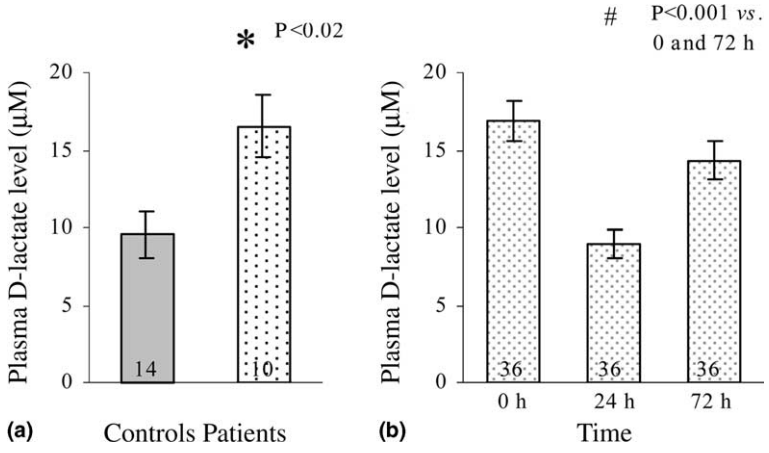

Fig. 2. (a) Patients with acute lymphoid leukaemia exhibit an elevated *in vivo* plasma D-lactate level (mean ± Standard error of the mean (SEM)). (b) Methotrexate induces a significant, transient fall in plasma D-lactate concentrations, as demonstrated by the data obtained from 36 treatment cycles (mean ± SEM).

3.2. Glyoxalase I inhibition

The glyoxalase I activity of human red blood cell lysates, and also the activity of yeast glyoxalase I were inhibited by all three folates (Table 1).

3.3. Methotrexate

The plasma MTX levels displayed wide variations; the medians and ranges at 24, 48 and 72 h were as follows: 216 (100–384), 1.2 (0.6–36) and 0.11 (0–4.1) μM, respectively.

3.4. MTX toxicities

The manifestation of acute toxic symptoms (stomatitis, diarrhoea, vomiting or elevated AST/ALT, Table 2) varied from treatment cycle to cycle in each patient. However, those MTX infusions which were followed by acute side-effects also resulted in more pronounced changes in plasma D-lactate values (at 24 h: $P < 0.05$; at 72 h: $P < 0.001$) (Fig. 3).

Table 1
The IC_{50} values of glyoxalase I inhibition by folic acid and its analogues (mean ± SD of four determinations)

	Methotrexate (μM)	Folic acid (μM)	Folinic acid (μM)
Haemolysate	125 ± 4.6	112 ± 5.8	221 ± 9.6
Yeast glyoxalase	206 ± 6.5	154 ± 2.2	310 ± 12.6

IC_{50}, concentration causing 50% inhibition, SD, standard deviation.

Table 2
Selected toxicities of high dose MTX therapy in the children with acute lymphoid leukaemia (ALL) ($n = 36$ treatment cycles)

	Grade 1	Grade 2	Grade 3	Grade 4
Vomiting	16 (44%)	8 (22%)	1 (3%)	–
Stomatitis	6 (17%)	1 (3%)	1 (3%)	–
Diarrhoea	1 (3%)	1 (3%)	–	–
AST[a]/ALT[b] elevation	4 (11%)	3 (8%)	3 (8%)	–

[a] AST, aspartate aminotransferase.
[b] ALT, alanine aminotransferase. All toxicities were graded using the National Cancer Institute Common Toxicity Criteria, version 2.0.

4. Discussion

The present data indicate that MTX inhibits the metabolism of α-oxoaldehydes *in vivo* in leukaemic children, as a likely consequence of glyoxalase I inhibition. This observation is in accordance with the findings of an earlier *in vitro* study, which compared the antiglyoxalase activities of some well-known anticancer drugs and demonstrated that MTX was as potent as the standard inhibitor S-octylglutathione [12].

Methylglyoxal is an α-oxoaldehyde formed by glycation and degradation of glycolytic intermediates. Tumour cells have an increased flux through glycolysis with a concomitant high rate of methylglyoxal formation [13]. In the present study, we found elevated concentrations of D-lactate *in vivo* in the plasma of the leukaemic patients, indicating an increased rate of formation and degradation of methylglyoxal. It would be of interest to follow plasma D-lactate during the course of leukaemia in order to determine its value as a possible indicator of disease activity.

Inhibition of the glyoxalase I in tumour cells elevates the intracellular methylglyoxal level and induces apoptosis [13]. The mechanism of the latter process is not fully understood, but a marked increase in the production of reactive oxygen species seems to be involved [14]. Since the 1970s, several glyoxalase I inhibitors have been tested and proved to bear antitumour activity, both *in vitro* and *in vivo* [15,16]. These data suggest that, besides the inhibition of dihydrofolate reductase and

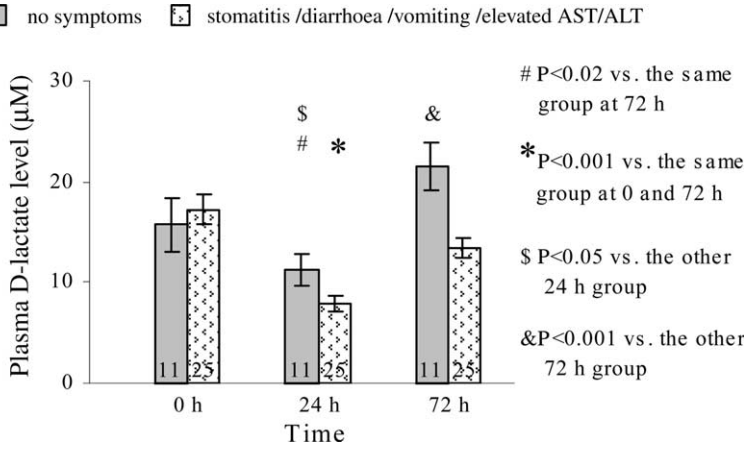

Fig. 3. The fall in plasma D-lactate level proved to be more pronounced when the methotrexate infusions were followed by the manifestation of acute toxic symptoms (mean ± SEM). AST, aspartate aminotransferase; ALT, alanine aminotransferase.

thymidylate synthase, the glyoxalase I inhibitory property of MTX may potentially contribute to the anticancer and toxic actions of the drug.

In the present study, the basal flux of metabolites through the glyoxalase system, as indicated by the plasma D-lactate, was not different before therapy between the patients with or without acute toxicities. By the end of the MTX infusion, however, the difference had become significant, and it was even more pronounced at 72 h. The lower D-lactate levels indicating a more expressed glyoxalase I inhibition in the event of toxic symptoms support the role of an altered α-oxoaldehyde metabolism in the *in vivo* actions of the drug.

This observation also indicated that MTX should prove more toxic in metabolic disorders involving an enhanced methylglyoxal production. In such disorders, the accumulation of toxic metabolites is accelerated in the non-cancer cells too. Diabetes is a common disease in which the impaired glycolysis leads to enhanced formation of the α-oxoaldehyde, methylglyoxal [3]. Indeed, studies in which the MTX hepatotoxicity was evaluated in patients with psoriasis, rheumatoid arthritis or dermatomyositis revealed a significant risk related to diabetes [17–19]. Furthermore, diabetes proved to be the strongest predictor of MTX-induced lung injury in rheumatoid patients [20].

In a recent study, the capacity of MTX therapy to prolong the remission phase at the onset of type 1 diabetes was investigated. However, no benefit was found from this therapy; on the contrary, islet failure, determined by insulin requirements, occurred earlier for those receiving MTX [21]. Methylglyoxal has been shown to contribute to glucose toxicity in insulin-secreting cells [22], and an inhibition of glyoxalase I by MTX may therefore explain the above phenomenon.

The application of thiamine has been recommended for the prevention of diabetic complications [23]. This substance is a stimulator of transketolase activity, and thereby diverts excess metabolites toward the pentose phosphate pathway, decreasing the formation of methylglyoxal. In contrast, a thiamine deficiency leads to enhanced methylglyoxal production. Testing Ehrlich ascites carcinoma and sarcoma-180 malignant tumours in mice demonstrated that the combination of MTX with hydroxythiamine (a thiamine antagonist), immobilised on monocarboxycellulose, resulted in higher antitumour efficacy compared with the agents applied individually [24]. Furthermore, a close correlation was found between the inhibition of transketolase in the tumours and the antiblastoma property of the preparation. This finding seems to support the presumed role of glyoxalase I inhibition in the actions of MTX.

In vitro, glyoxalase I was inhibited by MTX, folic acid and folinic acid. The folinic acid preparation used in the present study was a racemic mixture, containing equal amounts of the D and L diastereoisomers. These isomers differ substantially in biological activity, the L isomer being the active form [25]. The MTX and folic acid preparations contained exclusively active substances. This explains why the dose of folinic acid needed to achieve inhibition similar in extent to those obtained with folic acid and MTX was approximately twice as high. All three folates proved more effective when tested on haemolysates compared with the isolated enzyme protein. This phenomenon may be related either to the species differences (human *vs*. yeast enzyme) or the metabolism of folates and the formation of more active inhibitory derivatives by the red blood cells.

The abnormal activity or expression of glyoxalase I has been reported in several types of human cancer, including colon, renal and prostate cancers, and also in leukaemic cells [26–28]. Furthermore, this phenomenon has been identified as one of the factors responsible for resistance to chemotherapy; accordingly, glyoxalase inhibitors have been found to be effective drug resistance-reversing compounds [27,28].

In the present study, patients were treated with low dose (up to 60 mg/m^2/day) folinic acid in the 48 h following the MTX infusion. However, the plasma D-lactate levels gradually increased up to the end of the observation period, indicating that the tissue folate concentrations achieved were not sufficient for a significant *in vivo* inhibition of glyoxalase I. Folinic acid is frequently used in combination with fluorouracil in the therapy of gastrointestinal cancer as it potentiates the inhibitory action of the latter drug on the enzyme thymidylate synthase [29]. When patients with advanced cancer were treated with 5-fluorouracil and folinic acid (from 250 mg/m^2/day up to a maximum of 1000 mg/m^2/day), the mean plasma steady-state concentrations of the parent compound and its principal metabolite, 5-methyltetrahydrofolate were 2.7 and 5.1 μM (lower dose) and 15.3 and 16.5 μM (higher dose), respectively [30]. These data and the glyoxalase I inhibitory action of folates provide a rationale for an investigation of folinic acid and folic acid in chemotherapy regimens with regard to their ability to alter resistance to antitumour agent-induced apoptosis.

In conclusion, we have demonstrated that ALL patients display elevated plasma D-lactate levels *in vivo*, indicating an enhanced endogenous formation of methylglyoxal. MTX therapy inhibits the metabolism of this α-oxoaldehyde, as a likely consequence of glyoxalase I inhibition. This mechanism may be involved in the chemotherapeutic and toxic effects of the drug and may serve as a possible explanation for the increased cytotoxicity of MTX in diabetes mellitus. Both folic and folinic acids exert inhibitory action towards glyoxalase I, which suggests that it would be worthwhile to test these compounds as possible drug resistance-reversing agents.

Conflict of interest

None

Acknowledgements

The authors wish to thank Agota Fabian Nagy and Ilona Szecsi for their skillful and enthusiastic technical assistance. This study was supported by National Foundation for Scientific Research (OTKA) Grants T 34390 and T 35019.

References

1. Thornalley PJ. Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification – a role in pathogenesis and antiproliferative chemotherapy. *Gen Pharmacol* 1996, **27**, 565–573.
2. Vander Jagt DL, Hunsaker LA. Methylglyoxal metabolism and diabetic complications: roles of aldose reductase, glyoxalase I, betaine aldehyde dehydrogenase and 2-oxoaldehyde dehydrogenase. *Chem Biol Interact* 2003, **143–144**, 341–351.
3. Thornalley PJ, Hooper NI, Jennings PE, *et al*. The human red blood cell glyoxalase system in diabetes mellitus. *Diabetes Res Clin Pract* 1989, **7**, 115–120.
4. Karg E, Nemeth I, Horanyi M, Pinter S, Vecsei L, Hollan S. Diminished blood levels of reduced glutathione and α-tocopherol in two triosephosphate isomerase-deficient brothers. *Blood Cell Mol Dis* 2000, **29**, 91–100.
5. Garcia H, Smith FR, Cucinell SA. Urinary D-lactate excretion in infants with necrotizing enterocolitis. *J Pediatrics* 1984, **104**, 268–270.
6. Oh MS, Phelps KR, Traube M, Barbosa-Saldivar JL, Boxhill C, Carroll HJ. D-lactic acidosis in a man with the short-bowel syndrome. *N Engl J Med* 1979, **301**, 249–252.
7. Uribarri J, Oh MS, Carroll HJ. D-lactic acidosis. A review of clinical presentation, biochemical features, and pathophysiological mechanisms. *Medicine (Baltimore)* 1998, **77**, 73–82.
8. Sun XQ, Fu XB, Zhang R, *et al*. Relationship between plasma D(−)-lactate and intestinal damage after severe injuries in rats. *World J Gastroenterol* 2001, **7**, 555–558.
9. de Vrese M, Barth CA. Postprandial plasma D-lactate concentrations after yogurt ingestion. *Z Ernahrungswiss* 1991, **30**, 131–137.
10. McLellan AC, Phillips SA, Thornalley PJ. Fluorometric assay of D-lactate. *Anal Biochem* 1992, **206**, 12–16.
11. McLellan AC, Thornalley PJ. Sample storage conditions for the assay of glyoxalase activities in whole blood samples. *Ann Clin Biochem* 1992, **29**, 222–223.
12. Kraus JL, Pernice P, Ponce C. Inhibition of glyoxalase I by various clinically used anticancer drugs. *Res Commun Chem Pathol Pharmacol* 1988, **59**, 419–422.
13. Thornalley P. Advances in glyoxalase research. Glyoxalase expression in malignancy, antiproliferative effects of methylglyoxal, glyoxalase I inhibitor diesters and S–D-lactoylglutathione, and methylglyoxal-modified protein binding and endocytosis by the advanced glycation endproduct receptor. *Crit Rev Oncol Hematol* 1995, **20**, 99–128.
14. Amicarelli F, Colafarina S, Cattani F, *et al*. Scavenging system efficiency is crucial for cell resistance to ROS-mediated methylglyoxal injury. *Free Radic Biol Med* 2003, **35**, 856–871.
15. Vince R, Daluge S. Glyoxalase inhibitors. A possible approach to anticancer agents. *J Med Chem* 1971, **14**, 35–37.
16. Thornalley PJ, Edwards LG, Kang Y, *et al*. Antitumour activity of S-*p*-bromobenzoylglutathione cyclopentyl diester *in vitro* and *in vivo*. Inhibition of glyoxalase I and induction of apoptosis. *Biochem Pharmacol* 1996, **51**, 1365–1372.
17. Shergy WJ, Polisson RP, Caldwell DS, Rice JR, Pisetsky DS, Allen NB. Methotrexate-associated hepatotoxicity: retrospective analysis of 210 patients with rheumatoid arthritis. *Am J Med* 1988, **85**, 771–774.
18. Zieglschmid-Adams ME, Pandya AG, Cohen SB, Sontheimer RD. Treatment of dermatomyositis with methotrexate. *J Am Acad Dermatol* 1995, **32**(5 Pt 1), 754–757.
19. Malatjalian DA, Ross JB, Williams CN, Colwell SJ, Eastwood BJ. Methotrexate hepatotoxicity in psoriatics: report of 104 patients from Nova Scotia, with analysis of risks from obesity, diabetes and alcohol consumption during long-term follow-up. *Can J Gastroenterol* 1996, **10**, 369–375.
20. Alarcon GS, Kremer JM, Macaluso M, *et al*. Risk factors for methotrexate-induced lung injury in patients with rheumatoid arthritis. A multicenter, case-control study. Methotrexate-Lung Study Group. *Ann Intern Med* 1997, **127**, 356–364.
21. Buckingham BA, Sandborg CI. A randomized trial of methotrexate in newly-diagnosed patients with type 1 diabetes mellitus. *Clin Immunol* 2000, **96**, 86–90.
22. Sheader EA, Benson RS, Best L. Cytotoxic action of methylglyoxal on insulin secreting cells. *Biochem Pharmacol* 2001, **61**, 1381–1386.
23. Thornalley PJ, Jahan I, Ng R. Suppression of the accumulation of triosephosphates and increased formation of methylglyoxal in human red blood cells during hyperglycaemia by thiamine *in vitro*. *J Biochem* 2001, **129**, 543–549.
24. Zimatkina T, Yukshtovich T, Zimatkin S, Kaputsky F. Antitumor activity of hydroxythiamine and methotrexate immobilized on monocarboxycellulose. *Pol J Pharmacol* 1996, **48**, 163–169.
25. Lee PP, Schilsky RL. Inhibition of thymidylate synthase by the diastereoisomers of leucovorin. *Cancer Chemother Pharmacol* 1990, **26**, 273–277.
26. Ranganathan S, Tew KD. Analysis of glyoxalase-I from normal and tumor tissue from human colon. *Biochim Biophys Acta* 1993, **1182**, 311–316.
27. Sakamoto H, Mashima T, Kizaki A, *et al*. Glyoxalase I is involved in resistance of human leukemia cells to antitumour agent-induced apoptosis. *Blood* 2000, **95**, 3214–3218.
28. Sakamoto H, Mashima T, Sato S, Hashimoto Y, Yamori T, Tsuruo T. Selective activation of apoptosis program by S-*p*-bromobenzylglutathione cyclopentyl diester in glyoxalase I-overexpressing human lung cancer cells. *Clin Cancer Res* 2001, **7**, 2513–2518.
29. Petrellin N, Herrera L, Rustum Y, *et al*. A prospective randomized trial of 5-fluorouracil versus 5-fluorouracil and high dose leucovorin versus 5-fluorouracil and methotrexate in previously untreated patients with advanced colorectal carcinoma. *J Clin Oncol* 1987, **5**, 1559–1565.
30. Newman EM, Akman SA, Harrison JS, *et al*. Pharmakokinetics and toxicity of continuous infusion (6S)-folinic acid and bolus 5-fluorouracil in patients with advanced cancer. *Cancer Res* 1992, **52**, 2408–2412.