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Interaction of *N*-hydroxy(sulfo)succinimide active esters with the reduced folate/methotrexate transport system from human leukemic CCRF-CEM cells

Gerrit Jansen [1], G. Robbin Westerhof [1], Gert Rijksen [2] and Jan H. Schornagel [1]

[1] *Department of Internal Medicine, Oncology Unit and* [2] *Laboratory of Medical Enzymology, Department of Hematology, University Hospital Utrecht, Utrecht (The Netherlands)*

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The membrane impermeant protein cross-linker 3,3′-dithiobissulfosuccinimidyl propionate (DTSSP) is a well-known inhibitor of human erythrocyte band 3-mediated inorganic anion transport. We observed that DTSSP is also a potent inhibitor of reduced folate/methotrexate transport in human CCRF-CEM leukemia cells. An interaction of DTSSP with the reduced folate/MTX is substantiated by findings that: (a) like MTX transport itself, the concentration of DTSSP required for half-maximal inhibition of [^{3}H]methotrexate transport varied substantially with the anionic composition of the external medium. In a saline buffer and an anion-deficient buffer the I_{50} values were 7 and 1 μM, respectively; (b) saturation of the carrier with 1–5 μM methotrexate completely protected the transport system from interaction by DTSSP; (c) methotrexate transport activity in DTSSP-treated cells could be restored after cleavage of the disulfide bond in DTSSP under mild reducing conditions; and (d) pretreatment of cells with DTSSP reduced the incorporation of [^{3}H]methotrexate after labeling with an *N*-hydroxysuccinimide ester of [^{3}H]methotrexate (NHS-MTX), another potent inhibitor of methotrexate transport. Comparison of DTSSP- and NHS-MTX-induced inhibition of methotrexate transport showed that DTSSP inhibition, in contrast to NHS-MTX inhibition, was (a) less potent, (b) dependent on buffer conditions, (c) reversible by reducing agents, and (d) required only a very low molar ratio of methotrexate over DTSSP to afford maximal protection.

Introduction

Membrane transport of the cytotoxic folate antagonist methotrexate (MTX) has been extensively studied in a great number of tumor cells and cell lines [1–5]. These studies have shown that in many tumor cells, including human CCRF-CEM leukemia cells, 5-substituted reduced folates required for cell growth as well as MTX are accumulated via the same carrier-mediated process. One of the most intruiging features of the reduced folate/MTX transport system is that the transport activity can be influenced by a wide variety of structurally unrelated anions [2,6–8], suggesting that the driving force in the uptake of MTX in carrier-mediated transport is provided by anion gradients in which anions can act as exchange substrates for MTX [2,6,9].

The identification and isolation of the reduced folate/MTX carrier have been impeded by the low cellular levels of the carrier protein [10]. Several reagents have been used for labeling of the putative carrier. They can be divided into three groups, based upon the site of interaction with the transport protein. The sulfhydryl reagents *p*-chloromercuriphenylsulfonate (pCMPS) [11] or *N*-ethylmaleimide [11,12] are rather non-specific inhibitors of MTX transport. A second group of inhibitors employs the affinity of the transport system for large inorganic anions. Effective labeling reagents include 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) [7] and -8-azido AMP [13]. The most potent inhibitors of MTX transport consisted of chemically or photodynamically activated (anti)folate compounds. I_{50} values of 20 and 8 nM, respectively, for an *N*-hydroxysuccinimide ester of MTX [10,14] and a

Abbreviations: MTX, methotrexate; NHS-MTX, *N*-hydroxysuccinimide ester of MTX; DTSSP, 3,3′-dithiobissulfosuccinimidyl propionate; MHS (buffer B), magnesium/Hepes/sucrose; HBSS (buffer A), Hepes-buffered saline solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Correspondence: G. Jansen, Department of Internal Medicine, Oncology Unit, University Hospital Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands.

photoaffinity analogue of MTX [15], were substantially lower than for reagents in the first two categories.

One major disadvantage of all these labeling procedures is that they accomplish an irreversible inactivation of the transport system with complete loss of transport activity. This study reports that the cleavable membrane-impermeant protein cross-linker 3,3'-dithiobissulfosuccinimidyl propionate (DTSSP), a well-known inhibitor of erythrocyte lactate and band-3 mediated anion transport [16–19], is also a potent inhibitor of MTX transport in human leukemia cells. In addition, we observed that transport activity is restored after cleavage of the disulfide bond in DTSSP under reducing conditions. The characteristics of inhibition are compared with those of an *N*-hydroxysuccinimide ester of MTX, another potent inhibitor of MTX transport [10,14].

Materials and Methods

Chemicals

[^{3}H]Methotrexate (spec. act. 10–20 Ci/mmol) was obtained from Moravek Biochemicals, Brea, CA and purified prior to use by TLC as described before [2,20]. Radiochemical purity was >99% after rechromatography. [^{35}S]Sulfate (carrier free) was from New England Nuclear and diluted with unlabeled sulfate to a specific activity of 3.5 mCi/mmol. DTSSP was purchased from Pierce Chemical Co. *N*-Hydroxysuccinimide (NHS), 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDAC) and (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Sigma Chemical Co. Unlabeled methotrexate was a generous gift by Pharmachemie, Haarlem, The Netherlands.

Cells

Human leukemic CCRF-CEM cells were grown as a suspension culture in RPMI-1640 medium supplemented with 5% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml) and streptomycine (100 μg/ml), at 37°C in a humidified atmosphere. Cells were harvested during the logarithmic phase of growth, washed with the buffer of choice and resuspended to a density of $1.5 \cdot 10^7$ cells per ml. Buffers used as suspending media for cells were: (a) Hepes-buffered saline solution (buffer A) containing 107 mM NaCl, 20 mM Hepes, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 7.0 mM D-glucose, (pH 7.4) with NaOH; (b) Mg-Hepes sucrose (buffer B) containing 20 mM Hepes, 225 mM sucrose (pH 7.4) with MgO; and (c) K-Hepes containing 160 mM Hepes, 2 mM $MgCl_2$ (pH 7.4) with KOH [7].

Treatment of cells with DTSSP and NHS-MTX

Freshly harvested cells ($1.5 \cdot 10^7$ per ml in buffer A or B) were exposed to appropriate concentrations of DTSSP or NHS-MTX/NHS-[^{3}H]MTX, synthesized as described by Henderson et al. [10,14]. DTSSP stock solutions were prepared immediately before use. Incubations for 5 min at 25°C were followed by two successive washes at 25°C in the respective buffer for [^{3}H]MTX or [^{35}S]sulfate uptake (see below). In other experiments, CCRF-CEM cells ($1.5 \cdot 10^7$/ml in buffer B) were incubated for 5 min at 25°C with 2.5 μM DTSSP. After washing with buffer B, cells were incubated for 5 min at 37°C in buffer B supplemented with 5–10 mM dithiotreitol (DTT). At this time cells were washed twice with MHS buffer and analyzed for [^{3}H]MTX and [^{35}S]sulfate uptake, and NHS-[^{3}H]MTX labeling.

NHS-[^{3}H]MTX labeling

CCRF-CEM cells were washed with buffer B (pH 7.0) and resuspended to a density of $2 \cdot 10^7$ per ml. NHS-[^{3}H]MTX was then added to a final concentration of 50 nM in the presence or absence of 1 mM unlabeled MTX incubated for 5 min at 25°C. Next, cells were washed twice in buffer A and resuspended in 0.5 ml of buffer A containing 2% CHAPS. After incubation for 1 h at 4°C, the extract was centrifuged in an Eppendorf microfuge (1 min, $13000 \times g$). An equal volume of methanol was added to the supernatant and proteins were allowed to precipitate for 1 h, −20°C. The precipitated protein was recovered by centrifugation, solubilized with 2% sodium dodecyl sulfate and analyzed for radioactivity by liquid scintillation counting.

[^{3}H]MTX/[^{35}S]sulfate uptake

[^{3}H]MTX influx was determined in 1 ml incubation mixtures containing 2 μM [^{3}H]MTX (spec. act. 0.5 Ci/mmol) and $1.5 \cdot 10^7$ cells in the buffer of choice. After 5 min of incubation at 37°C, uptake was terminated by the addition of 9 vol. of ice-cold transport buffer. The cells were centrifuged (5 min, $800 \times g$) and washed with 10 ml ice-cold buffer. The final pellet was suspended in 0.5 ml water and analyzed for radioactivity.

[^{35}S]Sulfate influx was measured as described by Henderson and Zeveley [7] in 1 ml mixtures containing 0.5 mM [^{35}S]sulfate (3.5 mCi/mmol) and $1.5 \cdot 10^7$ cells in K-Hepes (pH 7.4). After incubation for 3 min at 37°C, cells were diluted with 9 vol. ice-cold 150 mM KCl. After centrifugation (5 min, $800 \times g$, 4°C), the cells were washed with 10 ml ice-cold 150 mM KCl and analyzed for radioactivity in an Isocap-300 scintillation counter (Chicago Nuclear). Counting efficiencies for [^{3}H] and [^{35}S] were 53 and 86%, respectively.

Results

Effects of DTSSP and NHS-MTX on [^{3}H]MTX and [^{35}S]sulfate transport in CCRF-CEM cells

DTSSP is a potent inhibitor of band 3-mediated anion transport in human erythrocytes [17,18]. Like-

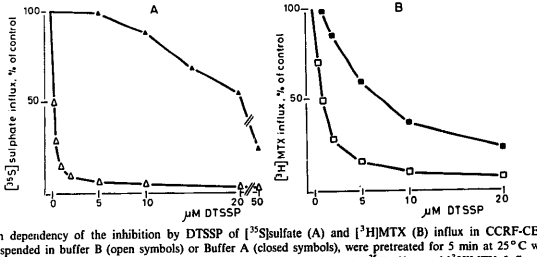

Fig. 1. Concentration dependency of the inhibition by DTSSP of [^{35}S]sulfate (A) and [^{3}H]MTX (B) influx in CCRF-CEM cells. $1.5 \cdot 10^7$ CCRF-CEM cells, suspended in buffer B (open symbols) or Buffer A (closed symbols), were pretreated for 5 min at 25 °C with the indicated concentrations of DTTSP, washed twice with the indicated buffer, and analyzed for influx of [^{35}S]sulfate and [^{3}H]MTX. Influx of [^{3}H]MTX was measured at 2 μM extracellular concentration in buffer B, and influx of [^{35}S]sulfate at 0.5 mM extracellular concentration in K-Hepes (pH 7.4) as described in Materials and Methods. The 100% values represent an influx of 925 pmol [^{35}S]sulfate per min per 10^7 cells and 4.1 pmol [^{3}H]MTX per min per 10^7 cells, respectively.

wise, DTSSP was found to be an effective inhibitor of anion transport in human CCRF-CEM leukemia cells (Fig. 1A). Utilizing [^{35}S]sulfate as a test substrate [7], the effective DTSSP concentration for half-maximal influx inhibition (I_{50}) is, however, highly dependent on the choice of buffer conditions. In a physiological saline buffer (buffer A) the I_{50} value for [^{35}S]sulfate transport inhibition is 27 μM, in an anion-deficient buffer (buffer B) this value is 100-fold lower (0.25 μM). Fig. 1B shows that DTSSP is also a potent inhibitor of [^{3}H]MTX transport in CCRF-CEM cells. Although the influence of buffer composition is of lesser importance than for [^{35}S]sulfate transport, there is a 7-fold difference in I_{50} value, 1 and 7 μM in buffers A and B, respectively. Interestingly, the K_i values for MTX transport in Buffer B ($K_i = 0.7$ μM) and buffer A ($K_i = 5$ μM) [2] differ to the same extent.

NHS-MTX is one of the most potent irreversible inhibitors of [^{3}H]MTX transport in CCRF-CEM cells [2]. The I_{50} for inhibition is 45 nM, irrespective the use of buffer A or B (not shown). NHS-MTX in concentrations up to 500 nM, however, has no inhibitory effect on [^{35}S]sulfate transport in CCRF-CEM cells.

Protection studies

The inhibitory effect of DTSSP on [^{3}H]MTX uptake could be prevented by coincubation of cells with unlabeled MTX (Fig. 2). Substantial protection is already observed at 1 μM MTX and protection is almost complete at 5 μM MTX, a 2.5-fold excess over the utilized DTSSP concentration. MTX (5 μM) had no protective effect on DTSSP inhibition of [^{35}S]sulfate transport (not shown). In order to exclude the possibility that the protective effect of MTX on DTSSP inhibition of [^{3}H]MTX transport is caused by direct interaction of DTSSP with MTX, other compounds known as

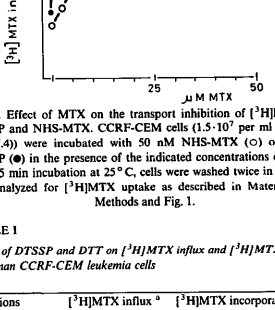

Fig. 2. Effect of MTX on the transport inhibition of [^{3}H]MTX by DTSSP and NHS-MTX. CCRF-CEM cells ($1.5 \cdot 10^7$ per ml in MHS (pH 7.4)) were incubated with 50 nM NHS-MTX (○) or 2 μM DTSSP (●) in the presence of the indicated concentrations of MTX. After 5 min incubation at 25 °C, cells were washed twice in buffer B and analyzed for [^{3}H]MTX uptake as described in Materials and Methods and Fig. 1.

TABLE 1

Effect of DTSSP and DTT on [^{3}H]MTX influx and [^{3}H]MTX binding of human CCRF-CEM leukemia cells

Additions		[^{3}H]MTX influx [a]	[^{3}H]MTX incorporation [b]
DTSSP (μM)	DTT (mM)	(% of control) ($n = 3$)	(% of control) ($n = 2$)
0	10	104 ± 10	106
2.5	0	29 ± 5	26
2.5	5	71 ± 10	n.d.
2.5	10	85 ± 9	82

[a] Influx buffer B, extracellular [^{3}H]MTX concentration 2 μM, mean ± S.D.

[b] Labeling with 50 nM NHS-[^{3}H]MTX. Specific incorporation of [^{3}H]MTX in control cells was 0.24 pmol per 10^7 cells.

n, number of determinations, n.d., not determined.

quenchers of DTSSP (Tris and glycine) [16–18], were analyzed for their protective effect. Incubation of cells with 5 μM Tris or glycine, in contrast to MTX, did not offer protection against DTSSP inhibition of [^{3}H]MTX transport (not shown). As shown further in Fig. 2, NHS-MTX inhibition requires a much higher excess of MTX to accomplish a protective effect. At 50 μM MTX, a 1000-fold molar excess over NHS-MTX, there is only a partial (75%) protection against inhibition.

Effect of dithiotreitol on DTSSP-treated cells

Cleavage of the disulfide bond in DTSSP can be accomplished under reducing conditions [17]. With respect to [^{3}H]MTX transport, the effect of dithiotreitol (DTT) on DTSSP-treated cells is shown in Table I. Exposure of DTSSP-treated CCRF-CEM cells to buffer B supplemented with 5–10 mM DTT relieved the inhibition of [^{3}H]MTX transport to 70–85% of untreated controls. On the other hand, [^{35}S]sulfate uptake inhibition of DTSSP (Fig. 1A) was not restored under these conditions (not shown).

Sites of interaction of DTSSP and NHS-MTX on the reduced folate / MTX carrier protein

To examine whether DTSSP and NHS-MTX share common or separated interaction sites on the transport protein, CCRF-CEM cells were labeled with NHS-[^{3}H]MTX after treatment with DTSSP. Pretreatment of CCRF-CEM cells with 2.5 μM DTSSP substantially reduced the incorporation of [^{3}H]MTX after labeling with NHS-[^{3}H]MTX (Table I). However, in CCRF-CEM cells treated with DTSSP, and subsequently with 10 mM dithiotreitol, the incorporation of [^{3}H]MTX was reestablished to 82% of the level of untreated controls.

Discussion

It is generally accepted that in most tumors the transport of MTX is mediated by a high-affinity/low-capacity transport system which normally deals with the uptake of natural reduce dfolates required for cell growth. The importance of the carrier system is illustrated by the fact that quantitative and/or qualitative defects of this transport system have been associated with tumor-cell resistance to MTX, either in vitro or in vivo [3–5,20–22]. From a therapeutical point of view, knowledge as to properties of the 'MTX-carrier', are most relevant to the problem of drug resistance.

Previous studies [3,5,11,12,14,15] have shown that the transport system is susceptible to modifying interactions, leading to (ir)reversible inhibition of MTX transport. In particular, physiological anions and a wide variety of structurally unrelated anions are competitive inhibitors of MTX transport. Likewise, MTX transport is irreversibly inhibited by the large inorganic anion DIDS [7], which is also a potent inhibitor of band 3-mediated anion transport in human erythrocytes [16–18]. In light of these observations, the hypothesis has been put forward that an anion-exchange mechanism is involved in the uptake of MTX by exchanging an extracellular MTX (divalent anion) for an intracellular anion [6,9,10].

In this study we compared the effect on MTX transport in human leukemic CCRF-CEM cells of two compounds. First, an *N*-hydroxysuccinimide ester of MTX (NHS-MTX), in which the nucleophilic *N*-hydroxysuccinimide group is esterified to the α- and/or γ-carboxylic group of the glutamate side-chain of the MTX molecule [14]. The second compound is a membrane-impermeable thiol-cleavable cross-linking agent; an *N*-hydroxysulfosuccinimide active ester of the dicarboxylic acid dithiobispropionate (DTSSP).

DTSSP is known as a potent inhibitor of anion transport in human erythrocytes by cross-linking subunits of the band 3 protein to covalent dimers at the extracytoplasmic membrane face [17,18]. As shown in Fig. 1A, DTSSP is not only an effective inhibitor of anion transport in human erythrocytes, but also in human CCRF-CEM leukemia cells. The concentration of DTSSP required for half-maximal inhibition of [^{35}S]sulfate transport are highly dependent on the presence of anions in the buffer systems employed. In a non-physiological (anion-deficient) buffer system, DTSSP has a 100-fold higher inhibiting potency as compared to physiological buffer systems. This suggests that anions can protect, probably by competitive inhibition, the site of interaction of DTSSP at the anion transporter.

Apart from inhibiting anion transport, DTSSP was found to be a potent inhibitor of MTX transport in CCRF-CEM cells. Although DTSSP was not as potent an inhibitor as NHS-MTX, several lines of evidence support the interaction of DTSSP with the MTX influx carrier. These include: (a) saturation of the influx carrier with MTX protected from inhibition of MTX influx by DTSSP (Fig. 2); (b) DTSSP interaction with the carrier reduced the incorporation of [^{3}H]MTX in the carrier protein after NHS-[^{3}H]MTX incubation (Table I); and (c) cleavage of the disulfide bond in DTSSP, restored [^{3}H]MTX transport activity. A number of these characteristics differed from NHS-MTX induced inhibition of MTX transport. Inhibition by NHS-MTX was accomplished at much lower concentrations than by DTSSP, showed no dependence on anionic composition of buffer systems, but required a very high molar excess of MTX to afford protection from inhibition. These latter observations are in agreement with Henderson et al. [10,14]. As DTSSP is an *N*-hydroxysulfosuccinimide ester of a dicarboxylic anion, it probably interacts with that site of the transport protein which is involved in the competitive blocking of MTX transport by anions [2,6,8,9]. This hypothesis is supported by the effects of

(anionic) buffer compositions (Fig. 1B) and protection by MTX (Fig. 2). It is not clear from these experiments whether the formation of an intra- or intermolecular cross-link accounted for the inhibition of MTX transport by DTSSP. Polyacrylamide gel electrophoresis for the identification of an intermolecular cross-link (like for erythrocyte band 3 subunits) [17,18], was not feasible due to the low cellular levels of the carrier protein. Moreover, the protective effect of MTX for inhibition by DTSSP (Fig. 2), and the lack of evidence for the presence of multiple subunits of the reduced folate/MTX carrier, suggest an intramolecular rather than an intermolecular interaction of DTSSP with the carrier although this should not necessarily be a cross-link. One end of the DTSSP could react with a reactive amino group, and the other end might not have access to a reactive group. Consequently, a bulky, negatively charged sulfosuccinimido group will be attached to the protein. The DTT, in cleaving the disulfide, would release this charged group, leaving the remainder of the protein-DTSSP residue much smaller and uncharged. A relief of inhibition of transport could therefore result from DTT treatment even if DTSSP had not originally formed a cross-link. Irrespective of the exact mechanism of inhibition of MTX transport, radiolabeled DTSSP might be an interesting new probe for labeling of the reduced folate/MTX carrier. Unlike other labeling reagents, DTSSP interaction does not irreversibly modify the carrier and transport activity may be preserved.

Acknowledgements

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