Identification of the Bromosulfophthalein-sensitive Efflux Route for Methotrexate as the Site of Action of Vincristine in the Vincristine-dependent Enhancement of Methotrexate Uptake in L1210 Cells

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ABSTRACT

The mechanism by which vincristine enhances the uptake of methotrexate in leukemic L1210 mouse cells has been investigated. Methotrexate uptake after 30 min at 37°C increased 44% relative to untreated controls in cells suspended in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/bicarbonate-buffered saline medium containing 20 μM vincristine. This stimulation was half-maximal at a vincristine concentration of 4 μM. An enhancement of methotrexate uptake by vincristine was also observed in the presence of glucose but a reversal could be achieved by prior treatment of the cells with taxol, a drug which prevents microtubule disassembly by vincristine. When the effect of vincristine was determined on the individual influx and efflux components for methotrexate, the increased uptake of methotrexate correlated with the inhibition of the unidirectional efflux route for methotrexate that is sensitive to bromosulfophthalein. Inhibition of this route was half-maximal at 3 μM vincristine and it exceeded 90% at high concentrations of the inhibitor. Transport via the bidirectional exchange carrier for methotrexate was not affected by vincristine, while the unidirectional efflux route sensitive to probenecid was inhibited by vincristine but only at concentrations 10-fold higher than required to inhibit the bromosulfophthalein-sensitive route. Vincristine did not increase methotrexate uptake in CCRF-CEM lymphoblasts, a human cell line which contains much lower levels of bromosulfophthalein-sensitive efflux route for methotrexate. Concentrations of vincristine which inhibited the bromosulfophthalein-sensitive efflux route of L1210 cells by 80–90% had little or no effect on the intracellular pH or on intracellular levels of ATP, GTP, cyclic AMP, K⁺, or oxidized glutathione. A significant increase was observed in the cellular uptake of tetrathenylphosphonium ions, suggesting that vincristine causes a hyperpolarization of the plasma membrane.

INTRODUCTION

Methotrexate has been employed for several decades in the chemotherapeutic treatment of various types of cancerous cells. Recent approaches, however, have typically rejected regimens involving methotrexate or other drugs used alone in favor of combination drug therapies. The result has been an increased interest in identifying complementary or synergistic drug combinations. Studies by Zager et al. (1) and Fyfe and Goldman (2) in vitro showed that methotrexate uptake could be increased by the addition of vincristine to L1210 or Ehrlich ascites tumor cells. In addition, combinations of methotrexate and vincristine, when given at specified time intervals, led to an increased mean survival of mice implanted with L1210 cells (1, 3, 4). Other studies (5), however, indicated that vincristine could increase cellular uptake of methotrexate in L1210 cells in vivo but that combinations of these drugs did not exhibit therapeutic synergism.

Revised 5/20/88; accepted 7/20/88.

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1 This work was supported by Grants CH-229 from the American Cancer Society and Grant CA-23970 from the National Cancer Institute. This is Research Institute manuscript 5181-BCR.

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Mechanism studies have shown that vincristine enhances the uptake of methotrexate in Ehrlich ascites (2) and probably also L1210 (3) cells by selectively inhibiting methotrexate efflux. The efflux of methotrexate in L1210 cells is a complex process which is kinetically distinct from influx (6–10), exhibits a strong dependence on the metabolic state of the cell (6, 9), and is comprised of three components which have different sensitivities to specific inhibitors (10, 11). The present study was initiated to identify the component or components of methotrexate efflux that are inhibited by vincristine and to probe possible biochemical mechanisms by which vincristine could exert an effect on the efflux of methotrexate. The results indicate that vincristine is a potent and specific inhibitor of the efflux route for methotrexate which is sensitive to bromosulfophthalein. In addition, the mechanism of inhibition by vincristine appears to differ from that of various other compounds which also block the bromosulfophthalein-sensitive efflux route of methotrexate. Comparative studies showed further that human CCRF-CEM lymphoblastoid cells, which contain relatively low levels of a bromosulfophthalein-sensitive efflux route for methotrexate (12), do not exhibit an increased uptake of methotrexate in the presence of vincristine.

MATERIALS AND METHODS

Chemicals. [3',5',9-3H]Methotrexate (20 Ci/mmol) was obtained from Moravek Biochemicals, diluted with unlabeled methotrexate to a specific activity of 60,000 cpm/nmol, and purified by thin-layer chromatography on Baker-flex cellulose sheets (J. T. Baker) using 100 mM Na-HEPES, pH 7.5, as the solvent. The zone containing the [3H]methotrexate was scraped from the plate and eluted with 2% ethanol. Stock solutions of [3H]methotrexate were used immediately or stored at −20°C for up to 30 days. α-[7-14C]Phthalic acid (5 mCi/mmol, ICN Pharmaceuticals); [2-14C]-5,5-dimethylxazolidine-2,4-dione (60 mCi/mmol, New England Nuclear); and (phenyl-1-14C)tetrathenylphosphonium bromide (19.2 mCi/mmol, New England Nuclear) were employed directly from the supplier. Methotrexate, vincristine, bromosulfophthalein (sulfobromophthalein), carbonylcyanide m-chlorophenyldrazone, valinomycin, antimycin A, protein kinase, glutathione reductase, N-hydroxysuccinimide, and 1-ethyl(3,3-dimethylaminopropyl)carbodiimide were obtained from Sigma Chemical Co. Taxol was obtained from the Natural Products Branch of the National Cancer Institute. Stock solutions of Taxol were prepared in dimethyl sulfoxide (10 mM final concentration) and stored up to 7 days at −20°C.

Preparation of NHS-Methotrexate. NHS-methotrexate (11, 13) was prepared by dissolving methotrexate, free acid (2.2 mg), 1-ethyl-3(3,3-dimethylaminopropyl)carbodiimide (7.8 mg), and N-hydroxysuccinimide (5.8 mg) in 2.0 ml of anhydrous dimethyl sulfoxide and allowing the mixture to stand for 60 min at 23°C. The concentration of activated compound was determined from the extinction coefficient of methotrexate of 18.9 at 302 nm and pH 7.

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Cells. L1210 mouse cells were grown as described previously (13) in RPMI 1640 medium containing 2.5% fetal bovine serum, washed with

[14C]Na-HEPES, pH 7.5, as the solvent. The zone containing the [3H]methotrexate was scraped from the plate and eluted with 2% ethanol. Stock solutions of [3H]methotrexate were used immediately or stored at −20°C for up to 30 days. α-[7-14C]Phthalic acid (5 mCi/mmol, ICN Pharmaceuticals); [2-14C]-5,5-dimethylxazolidine-2,4-dione (60 mCi/mmol, New England Nuclear); and (phenyl-1-14C)tetrathenylphosphonium bromide (19.2 mCi/mmol, New England Nuclear) were employed directly from the supplier. Methotrexate, vincristine, bromosulfophthalein (sulfobromophthalein), carbonylcyanide m-chlorophenyldrazone, valinomycin, antimycin A, protein kinase, glutathione reductase, N-hydroxysuccinimide, and 1-ethyl(3,3-dimethylaminopropyl)carbodiimide were obtained from Sigma Chemical Co. Taxol was obtained from the Natural Products Branch of the National Cancer Institute. Stock solutions of Taxol were prepared in dimethyl sulfoxide (10 mM final concentration) and stored up to 7 days at −20°C.

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Cells. L1210 mouse cells were grown as described previously (13) in RPMI 1640 medium containing 2.5% fetal bovine serum, washed with

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100 volumes of buffer, and resuspended to the desired density. CCRF-CEM human cells were grown similarly (12) except that the amount of fetal bovine serum was increased to 5%. Unless otherwise stated, the buffer employed as the suspending medium was HEPES/bicarbonate-buffered saline (HBBS), 20 mM HEPES, 5 mM sodium bicarbonate, 135 mM NaCl, 5 mM KCl, 2 mM MgCl₂, pH 7.4 with NaOH. For pretreatment with taxol, L1210 cells were collected by centrifugation, washed once with fresh RPMI 1640 medium (without added serum), resuspended in the same medium to 2 × 10^7/ml, and incubated for 2 h at 37°C with 100 μM taxol. Cultures of control and taxol-treated cells contained 1% dimethyl sulfoxide.

Uptake Measurements. Methotrexate uptake was measured (11) in duplicate samples containing cells (2 × 10⁷), the desired additions, [³H]methotrexate (5.0 μM), and HBBS, pH 7.4, in a final volume of 1.0 ml. After incubation for the desired times, the cells were diluted with 4 ml of ice-cold saline (1 mM Na-phosphate and 160 mM NaCl, pH 7.4), recovered by centrifugation for 5 min at 4°C (1000 × g), washed with 4 ml of saline, resuspended in 0.5 ml saline, and analyzed for radioactivity in 10 ml of Safety-solv (Research Products International). Uptake at 0°C served as the control. Protein concentrations were determined by the biuret method (14) using bovine serum albumin as the standard. Results are expressed in picomoles of methotrexate transported out of cells/min/mg protein. The concentration of [³H]methotrexate employed in uptake measurements (5.0 μM) approximates the Kᵢ for half-maximal methotrexate influx in this buffer system.⁴

Efflux Measurements. Methotrexate efflux was measured (11) in cells suspended to 5 × 10⁷/ml in HBBS incubated for 20 min at 37°C with 10.0 μM [³H]methotrexate and the desired concentration of vincristine, washed (at 4°C) to remove extracellular substrate, treated (if desired) with 10 μM NHS-methotrexate for 5 min at 23°C (11), and resuspended in HBBS. The extracellular concentration of [³H]methotrexate that was employed during these loading procedures (10.0 μM) was higher than was employed for influx measurements (5.0 μM), but this concentration was necessary to achieve intracellular substrate levels which were at least 5-fold higher than intracellular level of dihydrofolate reductase (15). When treatment with NHS-methotrexate was performed, control samples without NHS-methotrexate were also incubated at 23°C so that a comparable cell load was maintained in both cases; during this short incubation, less than 5% of the label exited the cells. Efflux mixtures were prepared in duplicate and consisted of cells (2 × 10⁷/ml), the desired additions of vincristine (at the same concentration employed initially), bromosulfophthalein (200 μM), or probenecid (1.0 mM), and HBBS in a final volume of 1.0 ml. For time-course determinations, the cells were incubated for the indicated times at 37°C, diluted with 7 ml of ice-cold isotonic saline, and recovered by centrifugation for 5 min at 4°C (1000 × g). Cell pellets were suspended in 0.5 ml of saline and analyzed for radioactivity in 10 ml of Safety-solv. Samples were then served as the control and assayed for ATP recovery by centrifugation (20 min at 20,000 × g), and fractionated on a C18 After Ultrasphere ODS column using a tetrabutylammonium phosphate, ammonium phosphate, and methanol solvent system described previously (16). Methotrexate elutes under these conditions at 38 min (76 mL), whereas polyglutamates of methotrexate elute at longer time intervals (16).

Nucleotide Triphosphate Determinations. For measurements of ATP, washed cells were suspended in HBBS buffer to 2 × 10⁷/ml, exposed to 0.5-ml portions to the indicated concentrations of vincristine or antimycin A for 30 min at 37°C, recovered by centrifugation, and eluted with 0.5 M 0.3 M NaOH. After 5 min at 37°C, the samples were neutralized by the addition of 0.5 ml of 0.6 M NaOH and 4 ml of 20 mM K-phosphate, pH 7, and assayed by the procedure of Cheer et al. (17). For GTP determinations, cell samples were treated similarly except that cell lysis was achieved by heating for 1 min at 100°C. After recovering the supernatant fraction by centrifugation, the coupled enzyme assay of Karl (18) was employed to measure GTP levels.

Cyclic AMP Determinations. Cyclic AMP levels were measured in cells that had been washed with HBBS and then resuspended in a modified HEPES-buffered saline (5 mM HEPES-150 mM NaCl-5 mM KCl-2 mM MgCl₂, pH 7.4 with NaOH) with a reduced buffering capacity. Assay samples contained cells (2.5 × 10⁷) and the indicated concentrations of vincristine in a final volume of 1.0 ml and were incubated for 30 min at 37°C. The cell mixtures were then heated for 1 min at 100°C and centrifuged for 5 min at 30,000 × g. The supernatant fraction was retained and assayed for cyclic AMP by the radioisotopic dilution assay of Gilman (19) using a cyclic AMP binding protein (protein kinase) from bovine heart.

Uptake of Tetraphenylphosphonium Ion (TPP⁺). The membrane potential of L1210 cells was estimated by the equilibrium uptake of [¹⁴C]TPPP⁺ across the cell membrane as described previously (20). Uptake was measured in cells (1 × 10⁷) that had been washed with HBBS, suspended in 0.45 ml of the same buffer containing the desired concentration of vincristine or antimycin A, and incubated for 5 min at 37°C. [¹⁴C]TPPP⁺ (0.05 μM, 40 μM) was then added and the incubation was continued for 25 min at 37°C. Samples were then diluted with 9 ml of ice-cold saline, pelleted by centrifugation, and analyzed for associated radioactivity. Cells treated similarly but preincubated for 5 min at 37°C with 20 μM carbonylcyanide-m-chlorophenylhydrazide and 5 μM valinomycin prior to the addition of the [¹⁴C]TPPP⁺ served as the control.

Measurements of Intracellular pH. Intracellular pH was determined (20) by the distribution of the weak acid, 5,5-dimethylxazolidine-2,4-dione (DMO⁺), across the cell membrane. Cells (2 × 10⁷) were suspended in 0.48 ml of the desired buffer containing the indicated concentration of vincristine and were preincubated for 30 min at 37°C. [¹⁴C]DMO⁺ (0.02 ml, 50 μM) was then added (2.0 μM final concentration) and the incubation was allowed to proceed for an additional 1 min at 37°C. Samples were then chilled to 0°C, and the cells were recovered by centrifugation at 1000 × g (5 min, 4°C). Residual radioactivity was removed from around the pellet with a cotton swab. The cells were suspended in 0.5 ml of saline and analyzed for radioactivity. Uptake of [¹⁴C]phthalate at 0°C (conditions under which internalization of this anion is minimal) served as the control for the extracellular water space. Calculations of intracellular pH employed a cell volume of 5 × 10⁻¹⁰ ml (7).

K⁺ Determinations. Intracellular concentrations of K⁺ were determined (20) employing cells (10⁷) that had been suspended in 5 ml of buffer containing the desired concentration of vincristine and incubated for 30 min at 37°C. The cells were recovered by centrifugation, washed twice with 5 ml of ice-cold isotonic saline, and lysed by the addition of 1.0 ml of 2 mM sodium HEPES-100 mM NaCl, pH 7.5, containing 1% Triton X-100. Samples were then diluted with 9 ml of the HEPES-NaCl solution and analyzed for K⁺ employing an Orion 93-19 K⁺-specific electrode.

Quantitation of Oxidized Glutathione. Intracellular concentrations of oxidized glutathione were determined by the procedure of Srivastava et al. (21) employing assay mixtures containing 8 × 10⁷ cells in 3 ml of HBBS. After incubation for 30 min at 37°C with the desired concentration of vincristine, the samples were treated with N-ethylmalimide and then trichloroacetic acid, clarified by centrifugation, extracted with ether, and then assayed enzymatically with glutathione reductase for the presence of oxidized glutathione. Samples containing buffer alone or buffer containing a known amount of oxidized glutathione were employed as controls for interference and substrate recovery, respectively.

RESULTS

Effects of Vincristine on the Kinetics of Methotrexate Uptake. The effect of vincristine on the time-dependent uptake of meth-
METHOTREXATE TRANSPORT IN L1210 CELLS

The disruption of microtubules by Vinca alkaloids (23, 24), had little effect on the uptake of methotrexate relative to an untreated control (Table 1). However, these treated cells no longer exhibited the ability to increase intracellular concentrations of methotrexate in the presence of vincristine.

Effects of Vincristine on Methotrexate Efflux. The observation that vincristine increases the total uptake of methotrexate

Fig. 1. Comparative effect of vincristine and antimycin A on the uptake of methotrexate by (A) L1210 cells and (B) CCRF-CEM cells. Uptake was measured at 37°C in HBBS, pH 7.4, containing 5.0 μM [3H]methotrexate and buffer alone (no addition), 20 μM vincristine (VCR), or 2 μM antimycin A (Anti A). See "Materials and Methods" for further details.

Table 1 Reversal by taxol of the vincristine-dependent enhancement of methotrexate uptake by L1210 cells

Pretreatment with taxol was performed in freshly-harvested cells that had been washed, resuspended to 4 x 10^7/ml in RPMI 1640 medium (without serum), and incubated with gentle shaking for 2 h at 37°C in the presence or absence of 100 μM taxol. Both control and taxol-treated cultures contained dimethylsulfoxide at a final concentration of 1%. Cells were collected by centrifugation, washed with HBBS, and examined for methotrexate uptake by incubation for 30 min at 37°C in HBBS containing 5.0 μM [3H]methotrexate and (where indicated) 20 μM vincristine.

<table>
<thead>
<tr>
<th>Pretreatment addition</th>
<th>Assay addition</th>
<th>Methotrexate uptake (pmol/mg protein)</th>
<th>Relative uptake (% change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>64.3</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>Vincristine</td>
<td>92.5</td>
<td>+44</td>
</tr>
<tr>
<td>Taxol</td>
<td>None</td>
<td>65.3</td>
<td>+2</td>
</tr>
<tr>
<td>Taxol</td>
<td>Vincristine</td>
<td>66.6</td>
<td>+4</td>
</tr>
</tbody>
</table>

Pretreatment of L1210 cells for 2 h at 37°C with 100 μM taxol, a drug which has been shown in other cells to prevent the disruption of microtubules by Vinca alkaloids (23, 24), had no effect on the uptake of methotrexate relative to an untreated control (Table 1). However, these treated cells no longer exhibited the ability to increase intracellular concentrations of methotrexate in the presence of vincristine.
without affecting influx (see Fig. 1A) supports the previous finding in Ehrlich ascites cells (2) that efflux was being preferentially reduced by vincristine. To confirm this conclusion for L1210 cells, the effect of vincristine on methotrexate efflux was determined (Fig. 3). In these measurements, it was necessary to add vincristine both during the loading procedure and during efflux determinations since the time required for vincristine to act upon the efflux process (10–20 min) was substantially longer than the half-time (3–4 min) for methotrexate efflux. As a consequence, vincristine-treated cells became loaded with labeled substrate to slightly higher levels than control cells. The internalized label, however, remained essentially unmetabolized methotrexate. When cells were incubated with 10.0 μM [3H]methotrexate for 30 min at 37°C in the absence and presence of 40 μM vincristine, intact [3H]methotrexate accounted for 93 and 95%, respectively, of the total labeled material as determined by high performance liquid chromatography analysis (see “Materials and Methods” for further details). The remaining 5 to 7% of the label fractionated as a broad peak whose position (85 ml) is consistent with the formation of a polyglutamate of methotrexate. From values for the t1/2 of efflux (no addition curves, Fig. 3), vincristine (Fig. 3B) was found to reduce total methotrexate efflux by 52% relative to the untreated control (Fig. 3A).

The procedure described previously for separating methotrexate efflux in L1210 cells into three distinct routes by their sensitivity to specific inhibitors (10, 11) was employed to examine the effect of vincristine on each of the individual efflux routes for methotrexate. In this procedure, efflux proceeding via the methotrexate influx carrier is quantitated by its inhibition by NHS-methotrexate, a specific and highly reactive stoichiometric inhibitor of this transport system (13). The other remaining efflux components function unidirectionally outward and can be inhibited by bromosulfophthalein and probenecid, respectively. When the effect of treatment with NHS-methotrexate was determined in control cells (Fig. 3A) and in cells exposed to vincristine (Fig. 3B), the amount of initial efflux (in pmol/min/mg protein) which could be inhibited by NHS-methotrexate was the same in both cases. Vincristine thus exerted little or no effect on efflux via the methotrexate influx carrier.

Further analysis revealed that vincristine is a potent inhibitor of the portion of methotrexate efflux which is sensitive to bromosulfophthalein (Fig. 4). In control cells treated with NHS-methotrexate alone (Fig. 4A), efflux was more rapid and bromosulfophthalein reduced this efflux by 72%, whereas the slow efflux observed after treatment with vincristine (Fig. 4B) was reduced only 20% by bromosulfophthalein. The bromosulfophthalein-sensitive efflux in both control (Fig. 4A) and vincristine-treated (Fig. 4B) cells was approximately the same (t1/2 = 20 min) and corresponded to efflux via a route sensitive to probenecid (data not shown).

The sensitivity of the bromosulfophthalein-sensitive efflux route to varying concentrations of vincristine is shown in Fig. 5A. Efflux via this route was reduced by 50% at a vincristine concentration of 3 μM, and a maximum inhibition of 90% was obtained at higher concentrations of the inhibitor. The probenecid-sensitive route (Fig. 5B) was also reduced progressively by increasing concentrations of vincristine but the midpoint for inhibition (43 μM) was more than 10-fold higher than for the bromosulfophthalein-sensitive route. Efflux via the methotrexate influx carrier was not affected by vincristine concentrations up to 40 μM (data not shown).

Effect of Vincristine on Cellular Metabolism. Since antimycin A and other metabolic inhibitors have been shown to block the bromosulfophthalein-sensitive efflux route for methotrexate (11), the effects of vincristine on parameters relating to cellular energetics were measured. ATP levels, even though highly sensitive to low concentrations of antimycin A (Fig. 6A), were not affected by vincristine (Fig. 6A; Table 2) even at a concentration (40 μM) which was 10-fold higher than required for half-maximal inhibition of bromosulfophthalein-sensitive methotrexate efflux. Similarly, intracellular concentrations of cyclic AMP were not affected by vincristine, whereas a slight decline was noted in intracellular concentrations of GTP (Table 2). These latter compounds were examined since the structure of microtubules is regulated by GTP hydrolysis (25, 26) and by
This increase was half-maximal at a vincristine concentration of 7 nM (Fig. 6B). Conversely, exposure of the cells to antimycin A led to a decrease in TPP⁺ uptake of 21% (Fig. 6B).

Measurements were also performed to determine the intracellular concentration of oxidized glutathione since the latter had been shown to increase in H-35 rat hepatoma cells exposed to vincristine (29). Oxidized glutathione levels, however, did not vary between control cells and cells incubated with 20 μM vincristine for 30 min at 37°C (Table 2).

### DISCUSSION

Vincristine has been shown previously to mediate the increased uptake of methotrexate in Ehrlich ascites (2) and L1210 tumor cells (1, 3). These results were explained by the ability of vincristine to mediate a preferential reduction in methotrexate efflux relative to influx (2). A decrease in efflux would perturb the balance between influx and efflux and thereby cause total uptake to increase to a new steady-state reflecting the extent to which efflux had been inhibited. This induced increase in methotrexate uptake by tumor cells in vitro also led to the suggestion that the efficacy of methotrexate in killing tumor cells in vivo might be improved by its use in combination with vincristine (1, 2). Studies with mice showed subsequently that combinations of vincristine and methotrexate can exhibit synergistic effects in the reduction of growth of L1210 cells, although this effect was highly dependent upon the administration interval, and an increase in animal survival was not always observed (3–5). A favorable response to methotrexate/vincristine combination therapy might also be possible for acute human myelogenous leukemia cells since the latter exhibit a vincristine-induced increase in the uptake of methotrexate (30). However, this therapy would have no apparent advantage in killing leukemia CCRF-CEM human lymphoblasts since vincristine has little or no effect on methotrexate uptake by these cells (Fig. 1B) (22).

Vincristine was shown in the present study to increase the uptake of methotrexate in L1210 cells by a preferential reduction in methotrexate efflux relative to influx and to mediate this effect via reduction in one of the three efflux routes for methotrexate (see Figs. 3–5). Inhibition of efflux was associated with the route previously identified by its sensitivity to bromosulfophthalein (11). This route, which is the major component of methotrexate efflux in energized L1210 cells (11), was inhibited to a maximum of 90% at concentrations of vincristine above 20 μM, and it was reduced half-maximally at a vincristine concentration of 3 μM (Fig. 5). A similar concentration of vincristine (4 μM) produced a half-maximal increase in methotrexate uptake (Fig. 2), indicating that the increase in methotrexate uptake by vincristine correlates directly with inhibition of this specific efflux route. CCRF-CEM human lymphoblasts, which contain much lower levels of a bromosulfophthalein-sensitive efflux component for methotrexate but share with...
L1210 cells the other two efflux routes (12, 20), failed to accumulate elevated levels of methotrexate in response to vincristine (Fig. 1B), further implicating the bromosulfophthalein-sensitive route as the site of action of vincristine. Analysis of the other efflux routes revealed that efflux via the methotrexate influx carrier was unaffected by vincristine, while the probenecid-sensitive route was inhibited by vincristine but only at concentrations 10-fold higher than required to inhibit the bromosulfophthalein-sensitive route (Fig. 5). The lack of an effect by vincristine on efflux via the influx carrier was also consistent with the findings that vincristine was unable to increase total uptake of methotrexate after exposure of L1210 cells to antimycin A. Efflux under the latter conditions proceeds almost exclusively via the methotrexate influx carrier system (11).

Previous studies have shown that compounds which reduce intracellular ATP inhibit the bromosulfophthalein-sensitive efflux of methotrexate (11, 20). Some efflux inhibitors, however, do not exhibit this property. One example is prostaglandin A₁, which has no effect on intracellular levels of ATP but (at pH 6.2) inhibits methotrexate efflux half-maximally at the relatively low concentration of 0.5 μM (20). Results of the present study indicate that vincristine represents another inhibitor of this same efflux route which has no effect on intracellular concentrations of ATP (Fig. 6A; Table 2). Vincristine retains a high capacity for enhancing methotrexate uptake in the presence of glucose (Fig. 2), indicating that the ATP produced from glucose metabolism cannot reverse the vincristine-dependent inhibition of methotrexate efflux. Various other parameters relating to cellular metabolism were also relatively unaffected by vincristine. These include the intracellular pH and the intracellular concentrations of GTP, cyclic AMP, K⁺, and oxidized glutathione. A vincristine-dependent rise was noted in the uptake of TPP⁺, a lipid-soluble cation whose uptake responds to changes in the membrane potential (28), and, moreover, the concentration of vincristine (7 μM) required for a half-maximal rise in TPP⁺ uptake (Fig. 6) was similar to the concentration of vincristine (3 μM) required for half-maximal inhibition of bromosulfophthalein-sensitive methotrexate efflux (Fig. 5). However, the importance of this apparent enhancement in membrane potential to the efflux mechanism remains unclear since other efflux inhibitors such as prostaglandin A₁ produce a reduction in TPP⁺ uptake (20). Various compounds with distinctly different effects on cellular metabolism thus appear to act as inhibitors of the bromosulfophthalein-sensitive efflux of methotrexate in L1210 cells.

Vincristine is toxic to cells since it binds to tubulin and causes a disassembly of the microtubule matrix which is required for mitosis and cell division. In a reciprocal fashion, taxol stabilizes microtubules and can prevent microtubule disassembly in the presence of vincristine (23, 24). Since taxol also reverses the ability of vincristine to mediate an increase in methotrexate uptake (Table 1), microtubule disassembly appears to be the initial event which is required for and leads to the inhibition by vincristine of the bromosulfophthalein-sensitive efflux of methotrexate. These findings suggest that the carrier protein which mediates the bromosulfophthalein-sensitive efflux of methotrexate may be directly linked to or closely associated with the cellular cytoskeleton. The latter is primarily composed of microfilaments and microtubules and has been shown to be physically associated with the plasma membrane (31–33) and to control various membrane-associated functions (34).

Studies of the physiological function of the bromosulfophthalein-sensitive efflux system for methotrexate have indicated that this efflux route has various common features with the mediated efflux of cyclic AMP (35–38). Methotrexate and cyclic AMP efflux each proceed unidirectionally, are blocked by metabolic inhibitors, increase upon the addition of glucose, and share a common inhibitory response to various compounds including prostaglandin A₁ and probenecid (20). Cyclic AMP efflux has also been shown to be inhibited by Vinca alkaloids (35), a feature shown by the present results to be also shared with the bromosulfophthalein-sensitive efflux of methotrexate. It is thus possible that methotrexate acts as a fortuitous substrate for an efflux system whose primary function is to extrude cyclic AMP or adenine nucleotides. A possible physiological function for this system might then be to mediate extracellular signalling processes (39). However, similarities also exist between the efflux of methotrexate and a unidirectional and ATP-dependent efflux of oxidized glutathione in human erythrocytes (40–42) and also the efflux of anionic dyes via a probenecid-sensitive route in human macrophages (43). These latter correlations are suggestive of an alternative possibility that the bromosulfophthalein-sensitive efflux system in L1210 cells may have a broad substrate specificity and that its physiological function may be to detoxify cells by extruding unwanted anions or zwitterions.

The bromosulfophthalein-sensitive efflux of methotrexate in L1210 cells also has common features with the unidirectional extrusion of vincristine and other drugs from multiply resistant tumor cells. Drug efflux from these cells (44–48) appears to be unidirectional for the outward movement of substrates, is blocked by metabolic inhibitors, and is enhanced by glucose, characteristics which are shared with the efflux of methotrexate and cyclic AMP. This efflux system, however, appears to function only with neutral and cationic drugs and hence would not be expected to accommodate anions such as methotrexate and cyclic AMP.

REFERENCES


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on “The Effects of Radiation on Aqueous Solutions,” which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is “Physical Measurements for Radiobiology” and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray’s lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, “The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration,” November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O₂ consumption, it is seen that the amount of glucose “cleavage products” exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

0.65 (+0.27) + 0.35 (−0.16) = +0.12

a figure identical to the observed +0.12 for normal leukocytes.
Identification of the Bromosulfophthalein-sensitive Efflux Route for Methotrexate as the Site of Action of Vincristine in the Vincristine-dependent Enhancement of Methotrexate Uptake in L1210 Cells

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