Comparative Studies on the Transport of Aminopterin, Methotrexate, and Methasquin by the L1210 Leukemia Cell

Francis M. Sirotnak and Ruth C. Donsbach

Division of Drug Resistance, Sloan-Kettering Institute for Cancer Research, New York, New York 10021

SUMMARY

Comparison was made of the uptake in L1210 leukemia cells of the agents aminopterin, methotrexate, and the quinazoline antifolate methasquin. Intracellular drug content was measured by titration with a bacterial dihydrofolate reductase. All three agents gain entry into the cell by carrier-mediated transport, since uptake is temperature dependent, saturable, and concentrative. Of the three drugs, aminopterin is transported most efficiently. At the same rate-limiting external concentration, the transport of methotrexate is one-fourth as efficient as that of aminopterin and the transport of methasquin is one-twentieth as efficient. Differences in the ability of the three drugs to be transported, as indicated by the respective Michaelis constants for initial uptake velocity, seem related to a difference in the affinity of a carrier component for each drug. All three drugs appear to be transported by the same mechanism, since competition for the same carrier component among the three drugs, as well as between them and the normal analogs, folate and folinate, was demonstrated with the expected quantitative relationships. The rate of efflux of aminopterin and methotrexate from the L1210 cell is far greater than the rate for methasquin. The extent of concentrative uptake during influx (uptake) of aminopterin is somewhat higher than that for methotrexate or methasquin at a rate-limiting concentration for influx.

INTRODUCTION

The manner by which antifolates penetrate tumor cells has been of some interest to a number of workers (3-5, 7-10, 15, 18-20, 27, 28). In most of the studies reported, the uptake of antifolate by the cell appears to occur mainly by a carrier-mediated active transport process. Moreover, the absence of appreciable transport capacity in tumor cells has been associated with instances of poor chemotherapeutic response (10, 15, 18-20) and also with the development of resistance during therapy (3, 5, 20, 27, 28). The uptake of the antifolates, notably methotrexate, has been studied in greatest detail in the L1210 leukemia cell (7, 8, 18, 27, 28). In those cells, most of the uptake is clearly the result of a transport phenomenon, since it occurs against a concentration gradient, is temperature dependent, requires a readily available source of energy, and exhibits Michaelis-Menten saturation kinetics. This study is a comparison in detail of the uptake in L1210 leukemic cells of aminopterin, methotrexate, and methasquin. Differences in the ability of the three drugs to be transported, as indicated by the respective Michaelis constants for initial uptake velocity, seem related to a difference in the affinity of a carrier component for each drug. All three drugs appear to be transported by the same mechanism, since competition for the same carrier component among the three drugs, as well as between them and the normal analogs, folate and folinate, was demonstrated with the expected quantitative relationships. The rate of efflux of aminopterin and methotrexate from the L1210 cell is far greater than the rate for methasquin. The extent of concentrative uptake during influx (uptake) of aminopterin is somewhat higher than that for methotrexate or methasquin at a rate-limiting concentration for influx.

MATERIALS AND METHODS

The in vivo maintenance and transplantation of the ascitic L1210 line (V) have been described (13). Methotrexate and aminopterin were supplied by Lederle Laboratories, Pearl River, N. Y. Methasquin was provided by Parke Davis and Co., Detroit, Mich. Methotrexate-3H was purchased from Amer-Sham/Searle Corp., Des Plaines, Ill. The aminopterin and both the labeled and unlabeled samples of methotrexate were purified by chromatography (24). The final purity of all drug samples was checked bioautographically (6). The specific activity of the radioactive methotrexate used was 0.5 mCi/mg. H2-folate was synthesized by the method of Blakley (2).
by heat extraction, and the cellular debris was discarded after centrifugation.

Enzyme Assay for Antifolate. The content of nonradioactive drug in the cell extracts was determined by titration (30) with a partially purified (29) dihydrofolate reductase from a high-level recombinant strain of Diplococcus pneumoniae (26).

The standard tube assay devised measures the inhibition by drug of the reduction of H\textsubscript{2}-folate to H\textsubscript{4}-folate by the enzyme (23). Known amounts of drug or varying amounts of sample, diluted in 0.05 M potassium phosphate buffer (pH 7.3) containing 12.8 mM 2-mercaptoethanol, were added to test tubes held in ice. Five-tenths ml of 0.1 mM solution of TPNH in buffer and 0.1 ml of the enzyme preparation diluted 1/500 in buffer were then added. The mixture was brought to a volume of 1 ml and was allowed to stand at room temperature for 4 min. After the tubes were placed on ice, 0.5 ml of H\textsubscript{2}-folate (0.1 mM) was added, and the tubes were reincubated at 37° for 12 min. After the tubes again were placed on ice, 1 ml of distilled H\textsubscript{2}O containing 100 ng methasquin was added to stop the reaction. The spectrophotometric absorbance was determined at 340 nm against blank mixtures containing no enzyme. A change in absorbance of 0.15 to 0.2 with linear reaction kinetics for at least 15 min is obtained with the amount of enzyme selected.

The inhibition of dihydrofolate reductase activity (Chart 1) by all 3 drugs is linear with an increase in concentration to about 80% inhibition. The amount of drug detectable in this assay is between 0.05 to 0.5 ng. The range can be varied by the use of greater or lesser amounts of enzyme and by a decrease or an increase in the corresponding incubation period.

Two types of controls are essential for an accurate determination of drug content in samples, particularly at low dilution. Spectrophotometer readings must be corrected for the contribution made by the cell extract to the total absorbance at 340 nm and for any effect on the enzyme activity of the samples obtained from untreated cells.

Dihydrofolate Reductase Content of L1210 Leukemic Cells. The amount of dihydrofolate reductase in leukemic cells was determined by titration (30) with methasquin. Washed cells were disrupted sonically (for 45 sec) with a probe-type sonicator (Measuring and Scientific Equipment Co., Ltd., London, England) or with a Potter-Elvehjem homogenizer. The amount of breakage obtained by each method was determined by a comparison of the protein values (22) of both sonic extracts and homogenates with the values obtained for a preparation that was completely lysed by 1% sodium lauryl sulfonate. The amount of dihydrofolate reductase found in this subline of the L1210 leukemia was 3.7 ± 0.6 nmoles/g, dry weight.

RESULTS

The Uptake of Antifolate with Time. From studies of methotrexate uptake reported previously (8, 18, 20, 27, 28), it is apparent that this antifolate gains entry into L1210 cells in 2 ways: by nonfacilitated passive diffusion and by active transport. Diffusion accounts for only a minor component of the total drug uptake at therapeutic levels and can be readily distinguished from transport by the fact that it is a temperature-independent process. The relative contribution to uptake made by both transport and diffusion processes can be determined by comparing the uptake at 37° with the uptake at 0°.

As in earlier experiments from this laboratory (27, 28) and elsewhere (7, 8), the uptake (influx) of methotrexate at 37° occurs at a constant rate, initially. When the internal concentration of drug is above the dihydrofolate reductase level, the rate diminishes gradually until a steady-state level (equilibrium) is reached. The uptake of methotrexate with time is shown in Chart 2. At an external concentration of 0.45 \( \mu \)M in this experiment, the initial rate of uptake was 0.23 n mole/min/g, dry weight. A steady-state level is reached in about 40 min. At the same external concentration, the uptake of aminopterin occurs in a similar manner, but about 4 times (0.81 n mole/min/g, dry weight) more rapidly, reaching a somewhat higher steady-state level in about the same time. Methasquin, on the other hand, appears to exhibit a more complex mode of uptake than is exhibited by the other 2 drugs. At an external concentration of 0.45 \( \mu \)M, the association of this drug with cells is very rapid within the 1st 1 or 2 min, followed by a much slower but constant rate of accumulation during the remainder of the incubation period. That only the slowly associating fraction of drug is a result of transport will become apparent as additional data are presented. Thus the rate of methasquin uptake by transport in this experiment was only 0.039 n mole/min/g, dry weight. The
results of the association of drug with cells at 0° (Chart 2) also provide a useful comparison of the 3 antifolates. The uptake (at an external concentration of 0.45 μM) of both aminopterin and methotrexate at 0° is rapid but is quantitatively negligible. Uptake at 37°, then, represents transport almost entirely. A much larger quantity of methasquin (10- to 12-fold) rapidly associates with L1210 cells at 0°. This result is quantitatively identical to that seen at 37°, except for the absence of the 2nd phase of gradual uptake seen at the higher temperature. The nature of this rapidly associating fraction of methasquin is not immediately apparent. It could represent greater nonfacilitated uptake (passive diffusion) of this drug; however, it also might result merely from more tenacious adsorption of drug on the cell surface. More extensive washing of cells following methasquin treatment did not reduce the amount of this fraction of drug that remained in association with the cells.

For a study of the kinetics of methasquin uptake at 37° at an internal concentration above enzyme level, an external concentration of 2.25 μM was used. The initial association of this drug with cells was again very rapid (Chart 3). Following this, however, the rate of accumulation due to transport continued at a slower but constant rate to an internal concentration that was well above enzyme level by the end of the incubation period. By comparison, the mode of transport of methotrexate and aminopterin at this concentration (2.25 μM) is similar to that already shown in Chart 2, except that the initial rate of transport is greater and a higher steady-state level is eventually attained.

The uptake of the 3 antifolates at 37° was also measured at varying pH values within a physiological range. In earlier experiments with methotrexate (27), some difference in the rates of transport had been demonstrated in the range of 6.8 to 8.0, with maximum velocity at about 7.5. A similar result was attained with aminopterin and methasquin when the drugs were compared with methotrexate during these studies.

**Efflux of Intracellular Antifolate.** Other workers (8, 18) have shown a rapid temperature-dependent loss (efflux) of methotrexate following incubation of cells in drug-free medium. The efflux of aminopterin and methasquin was measured in this study and was compared with that of methotrexate. Leukemic cells were preloaded by incubation for 20 to 40 min at 37° with drug. The cells were then cooled, centrifuged, and resuspended in drug-free medium. Aliquots of each drug-treated suspension were incubated at 37 and 0° at varying times and were examined for drug content after being washed. The efflux of drug after the cells had been preloaded to 2 different intracellular levels of drug are shown in Chart 4. A rapid efflux of both aminopterin and methotrexate occurred at 37°, so that the internal level of both drugs reached the vicinity of the dihydrofolate reductase level in less than 20 min. After this level was reached, no further loss of drug could be detected. Methasquin, on the other hand, appeared to have a much slower rate of efflux. Enzyme level was reached only after a prolonged incubation period or when the cells were preloaded to a low level of drug (Chart 4). No loss of any of

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**Chart 2.** The uptake of antifolates by L1210 cells with time. One ml aliquots of cell suspension (2 × 10⁷ cells) were exposed to an external concentration (conc) of 0.45 μM for each drug and were incubated for varying times, as indicated. The aliquots were removed to an ice bath, diluted to 10 ml with cold, phosphate-buffered saline (see text), and washed 3 times prior to the drug assay. The data are average values obtained in 3 experiments.

**Chart 3.** The uptake of methasquin by L1210 cells with time. The experimental details are given in the text and in the legend for Chart 2. External concentration was 2.2 μM. The data are average values obtained in 3 experiments.
Antifolate Transport by L1210 Leukemia Cells

Chart 4. The efflux of antifolates from L1210 cells at 37°. A standard number of cells (2 x 10^7) were incubated with drug at 0.45 μM (A) or 2.2 μM (B) for 60 min at 37°. The cells were centrifuged, and resuspended in fresh medium without drug, and aliquots were removed at the times indicated. The data are average values obtained in 4 experiments. Additional details are given in the text and in Chart 2, legend.

In Chart 5 and Table 1. By an adjustment in the time of incubation, a measurement of initial uptake at each concentration of aminopterin and methotrexate was made before the amount accumulated internally reached the dihydrofolate reductase level, i.e., no free drug was present in the intracellular water. These measurements, since they were made in the absence of any appreciable efflux, should reflect the capacity for movement of drug in 1 direction across the membrane barrier. The importance of this precaution has already been stressed (8). A similar measurement of initial uptake velocity below target enzyme level was not possible for methasquin because of the considerably lower overall rate of uptake. However, an accurate measurement of initial uptake

the 3 drugs was observed when cells were preloaded below enzyme level or when they were preloaded and incubated at 0°.

In view of the substantial amount of methasquin, which might only be adsorbed onto the cell surface during the preloading step, it is interesting to note that the level of this drug during efflux (a value based on the total drug associated with the cells after washing), like the levels of aminopterin and methotrexate, eventually is nearly equivalent to the cellular dihydrofolate reductase content. Either one must assume that any methasquin adsorbed onto the cell surface also dissociates from the cell during the efflux incubation period at 37°, or else one is forced to conclude that the rapidly associating fraction represents drug that is present internally. In either case, the rate of methasquin dissociating from the cells during these experiments would appear to be an adequately valid measure of efflux since, at most, only 20% of the total methasquin associating with the cells during preloading would not be intracellular. (Compare the rapidly associating fraction of drug with the total uptake after 60 min as shown in Chart 3.)

Kinetics of Antifolate Uptake. In studies with methotrexate (8, 27), the temperature-dependent uptake of drug was shown to exhibit Michaelis-Menten, or saturation, kinetics. The results of a similar analysis by the method of Lineweaver and Burk (21) of initial uptake velocity at varying concentrations of aminopterin, methotrexate, and methasquin are shown in Chart 5 and Table 1. By an adjustment in the time of incubation, a measurement of initial uptake at each concentration of aminopterin and methotrexate was made before the amount accumulated internally reached the dihydrofolate reductase level, i.e., no free drug was present in the intracellular water. These measurements, since they were made in the absence of any appreciable efflux, should reflect the capacity for movement of drug in 1 direction across the membrane barrier. The importance of this precaution has already been stressed (8). A similar measurement of initial uptake velocity below target enzyme level was not possible for methasquin because of the considerably lower overall rate of uptake. However, an accurate measurement of initial uptake velocity...
velocity for methasquin could still be made, since temperature-dependent uptake is linear with time to internal levels much higher than enzyme level (Chart 3). This difference in influx kinetics between methasquin and the other antifolates studied seems related to the low rate of efflux also demonstrated for this drug (Chart 4).

The results of the kinetic analysis (Chart 5) clearly demonstrate that the temperature-dependent uptake of all 3 drugs conforms to saturation kinetics. The Michaelis constant ($K_m$) for uptake varies widely (Table 1) among the 3 antifolates used. The respective values for methotrexate and methasquin are 3- and 20-fold greater than that for aminopterin, indicating a lower degree of saturability. The maximal levels of velocity of uptake achievable in this system with aminopterin and methotrexate, as indicated by the calculated $V_{max}$ values (Table 1), are approximately the same. The value obtained for methasquin was about one-half as much.

The Intracellular Concentration of Unbound Antifolate at Equilibrium. We have also compared the extent of carrier-mediated transport of aminopterin, methotrexate, and methasquin by measuring the concentrative uptake of each drug to a steady-state level (equilibrium). The L1210 cells were incubated with drug at $37^\circ$ for a period of time that was adequate to allow maximal uptake (usually 40 to 60 min). A determination of the free intracellular concentration of aminopterin and methotrexate (total intracellular drug less enzyme-bound drug) was made at external concentrations that were both equal to and below that level saturating the carrier system. As shown by the inhibition of uptake of radioactivity, labeled and unlabeled methotrexate compete about equally for the transport mechanism under these conditions. Both aminopterin and methasquin also exhibited an inhibitory effect on methotrexate-$^3$H uptake, indicating that all 3 drugs compete for the same carrier mechanism. Competition by aminopterin was greater than with unlabeled methotrexate, whereas methasquin competed poorly. These results agree closely with the data obtained in the other experiments showing the kinetics and relative rate of uptake for each drug.

### Table 1

**Kinetic properties of antifolate transport by L1210 leukemia cells**

<table>
<thead>
<tr>
<th>Drug</th>
<th>$K_m$ ($\times 10^{-6}$ M)</th>
<th>$V_{max}$ ($\times 10^{-9}$ M/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopterin</td>
<td>1.43</td>
<td>17.5</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>4.40</td>
<td>16.9</td>
</tr>
<tr>
<td>Methasquin</td>
<td>27.0</td>
<td>7.9</td>
</tr>
</tbody>
</table>

* Molar concentration.

### Table 2

**Total accumulation of antifolate in the L1210 leukemia cells at equilibrium following incubation at $37^\circ$**

<table>
<thead>
<tr>
<th>Drug</th>
<th>External concentration ($\mu$M)</th>
<th>Intracellular drug (Total (ng/g, dry wt))</th>
<th>Free (\muM)</th>
<th>Ratio, internal/external</th>
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</thead>
<tbody>
<tr>
<td>Aminopterin</td>
<td>0.45</td>
<td>7.5</td>
<td>2.30</td>
<td>5.1</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>2.70</td>
<td>24.0</td>
<td>12.50</td>
<td>4.6</td>
</tr>
<tr>
<td>Methasquin</td>
<td>4.50</td>
<td>21.6</td>
<td>11.10</td>
<td>2.4</td>
</tr>
<tr>
<td>2.70</td>
<td>20.0</td>
<td>10.30</td>
<td>3.80</td>
<td></td>
</tr>
</tbody>
</table>

* The calculation for the free intracellular drug concentration was based on a volume of 0.005 ml for the water content of the free intracellular space in $2 \times 10^7$ cells (27). The amount of drug bound to dihydrofolate reductase (3.75 ng/g, dry weight) was subtracted from the total amount accumulated intracellularly.

![Chart 6](chart6.png)

**Chart 6.** The inhibition of methotrexate-$^3$H (met-$^3$H) uptake by varying amounts of unlabeled methotrexate, aminopterin, and methasquin. The external concentration of labeled methotrexate was 2.2 \muM. The data given are average values obtained in 2 experiments. See text and Chart 2, legend, for additional experimental details.
We have also measured the uptake at 37° of aminopterin, methotrexate, and methasquin in the presence of folic acid and folinic acid (5-formyltetrahydrofolate). The results (Table 3) agree with previous findings with methotrexate (8, 27). Whereas folinic acid is a good competitor of antifolate uptake, folic acid competes poorly. As would be expected on the basis of the results already described above, both analogs inhibit methasquin uptake at 37° far more than they do the uptake of the other 2 drugs.

**DISCUSSION**

It is apparent that the antifolates aminopterin, methotrexate, and methasquin are transported in the L1210 leukemia cell by the same carrier-mediated mechanism. Competition for the carrier component among the 3 antifolates and between the antifolates and the normal analogs, folic and folinic acid, was clearly demonstrated. Of the 3 drugs, aminopterin was transported most efficiently, with methotrexate being transported somewhat less efficiently. Methasquin transport was the least efficient by at least 2 orders of magnitude. Differences in the abilities of the 3 drugs to be transported seem related to a difference in the affinity of the carrier component for each drug. As indicated by the respective Michaelis constants for initial uptake velocity, more methotrexate and a great deal more methasquin were required for saturation of the system, compared with aminopterin. The relative extent of competition for uptake among the 3 drugs, and with folate and folinate as well, also appears to be a close quantitative reflection of the differences in affinity of the carrier component.

The data presented here do not entirely rule out the possibility that the antifolates share more than 1 carrier-mediated mechanism. However, in that case, each mechanism would be expected to exhibit approximately the same degree of saturability in order to account for the kinetic data.

The more rapid efflux of both aminopterin and methotrexate from L1210 cells, compared with methasquin, is of considerable interest. The marked temperature dependence of efflux that has been observed here for all 3 drugs agrees with the evidence presented earlier (8, 10) for their occurrence in L1210 leukemia cells has been obtained. In the current studies, L1210 cell enzyme extracts had no discernable effect on the inhibitory properties of any of the 3 antifolates.

An interesting difference between the 3 compounds studied was the relatively large amount of methasquin rapidly associating with the L1210 cells. We are now attempting to show whether a sizeable portion of this fraction of drug is actually inside the cell as a result of entry by some process other than active transport. A finding in favor of this type of entry would have possible therapeutic significance and perhaps would help to explain the potent antileukemic activity of this agent and the slower onset of resistance during in vivo therapy (11).

The ability of the L1210 cell to accumulate aminopterin and methotrexate rapidly (the former at a faster rate) to a concentration well above enzyme level at extremely low external concentrations explains to a great extent the therapeutic responsiveness of this neoplasm. Although methasquin influx is slower, once enzyme level is exceeded, the markedly slower rate of efflux would be crucial in maintaining a physiological effect when external levels of drug decreased. Since methasquin is a better inhibitor of dihydrofolate reductase in this leukemia (14), it is very likely that somewhat lower internal levels are required for total inhibition of this enzymatic activity.

**ACKNOWLEDGMENTS**

We acknowledge with gratitude the interest and advice of Dr. Dorris J. Hutchison and Dr. Frederick S. Phillips during the course of these studies. We also gratefully acknowledge the assistance of Dr. Franz A. Schmid in providing leukemic mice.

**REFERENCES**

1. Baker, B. R. Design of Active-site Directed Irreversible Enzyme

**Table 3**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>Methasquin</th>
<th>Aminopterin</th>
<th>Methotrexate</th>
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</thead>
<tbody>
<tr>
<td>Folic acid</td>
<td>60.0</td>
<td>71.0</td>
<td>97.5</td>
<td>90.0</td>
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<td></td>
<td>200.0</td>
<td>51.6</td>
<td>87.5</td>
<td>70.0</td>
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<tr>
<td>Folinic acid</td>
<td>2.2</td>
<td>54.3</td>
<td>61.6</td>
<td>65.7</td>
</tr>
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<td></td>
<td>6.6</td>
<td>10.2</td>
<td>42.4</td>
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<td></td>
<td>13.3</td>
<td>0</td>
<td>32.2</td>
<td>34.3</td>
</tr>
</tbody>
</table>

*Rate of uptake in the absence of drug = 100%.

*Concentration of drug was 2.2 µM.


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