Evidence for a Transport Carrier of Nitrogen Mustard in Nitrogen Mustard-sensitive and -resistant L5178Y Lymphoblasts

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SUMMARY

Uptake of nitrogen mustard (HN2)-\( ^{14} \)C by HN2-sensitive and -resistant L5178Y lymphoblasts followed simple Michaelis-Menten kinetics and demonstrated chemical specificity. Hydrolyzed HN2 and the monofunctional analog, dimethyl 2-chloroethylamine, inhibited uptake of HN2-\( ^{14} \)C, and the kinetics were those of competitive inhibition. Other structural analogs of HN2 such as chlorambucil, melphalan, and cyclophosphamide did not inhibit drug uptake. Transport proceeded against a concentration gradient as high as 35-fold and was almost completely inhibited at 4° and partially inhibited by ouabain and 2,4-dinitrophenol. These findings suggest that transport of HN2 is carrier-mediated and is an active process. The \( K_m \) for resistant cells was higher than that of sensitive cells, suggesting a decreased affinity of the transport carrier for drug. The \( V_{max} \) of resistant cells was lower than that of sensitive lymphoblasts, suggesting decreased transport capacity in resistant cells. The binding of HN2-\( ^{14} \)C to DNA, RNA, and protein of resistant lymphoblasts was significantly lower than that observed in each of the corresponding fractions of HN2-sensitive cells. The data suggest that resistance to HN2 is multifactorial, one factor being decreased uptake of HN2 by resistant cells.

INTRODUCTION

Mechanisms of resistance to the alkylating agent HN2\(^4\) include the following possibilities: increased cellular concentration of protective agents (e.g., thiol groups) that spare critical target sites from lethal injury by alkylation (2, 9, 15); the presence of enzyme(s) either to circumvent a specific metabolic block or to enhance capacity for repair [evidence of repair of alkylated DNA has been reported in mammalian cells (6, 20)]; and alteration of membrane permeability to the drug (3, 11, 12, 23, 26).

With respect to changes in membrane permeability, several conflicting reports have appeared. Wheeler and Alexander (25) found no difference in binding of \( ^{14} \)C-labeled HN2 to sensitive and resistant plasmacytomas \( {in vivo} \), and Crathorn and Roberts (5) reported no difference in binding of mustard gas-\( ^{35} \)S to DNA, RNA and protein of sensitive and resistant lymphoma cells \( {in vitro} \). However, Reid and Walker (19) showed reduced binding of sulfur mustard to resistant strain L cells, and others have demonstrated decreased uptake of HN2-\( ^{14} \)C by mammalian cells resistant to HN2 (3, 11, 12, 23, 26).

In this study, a kinetic analysis of HN2 uptake by sensitive and resistant L5178Y lymphoblasts provides evidence for a transport carrier of HN2 and suggests that resistance is due in part to alteration of that carrier. Evidence is also presented to indicate that resistance consists of more than 1 factor, since differences in binding of HN2 to DNA, RNA, and protein of sensitive and resistant cells were greater than could be accounted for by the observed changes in drug transport.

MATERIALS AND METHODS

Sensitive (L5178Y) and resistant (L5178Y/HN2-2) lymphoblasts were cultured \( {in vitro} \) as previously described (8–10). HN2 (2-chloroethyl-1,2-\( ^{14} \)C), specific activity, 1.93 or 3.1 mCi/mmol, was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. Cells were treated with 1.04 \( \times \) 10\(^{-5} \) M or 2.08 \( \times \) 10\(^{-4} \) M HN2 at 37° for 60 min. This dose is 10- to 30-fold greater than the reported cytoidal dose of HN2 (8–10); however, in the latter studies, treatment time was “infinitely” long, since the drug was not removed from the culture medium.

DNA, RNA, and protein were isolated by a modification of the phenol extraction method of Colburn and Boutwell (4). DNA was coprecipitated as a viscous gel with protein from the...
phenol phase on addition of cold methanol; the DNA gel was removed, washed successively in cold 5% trichloroacetic acid, methanol, ethanol, extracted with hot 10% NaCl solution, and reprecipitated with cold ethanol. DNA and RNA were determined by the diphenylamine and orcinol reactions, as previously reported (9), and protein was measured by the method of Lowry et al. (18). Radioactivity was measured in a Nuclear-Chicago Mark 1 liquid scintillation spectrometer with 10 ml of scintillator, consisting of 8 g of PPO, 100 mg of POPOP, 1 liter of toluene, and 500 ml of Triton X-100 (octyl phenoxypolyethoxyethanol, Sigma Chemical Co., St. Louis, Mo.). The RNA fraction was free of protein and contained less than 2% DNA contamination; the protein fraction contained less than 5% nucleic acid. However, contamination of the DNA fraction ranged from 6 to 25% protein and 8 to 30% RNA, the latter being resistant to RNase digestion. Golder et al. (13) have also shown that alkylated DNA is not readily separable from protein by phenol extraction. DNA specific activity was corrected for by measuring the specific activity and amount of protein and RNA contamination in the DNA fraction.

Uptake of HN2-14C was determined after 60 min of incubation; the cells were washed 3 times in cold Earle's solution, lysed in distilled water, and separated into cell sap and membrane fractions. Whole cells and membrane fractions were solubilized in 0.5 N NaOH, and radioactivity was determined. As others have noted, the washing procedure leads to a loss of HN2-14C from the cell (26).

In kinetic studies of drug uptake, cells were incubated at 37° with HN2-14C at doses varying from 1.04 X 10^-4 M to 2.08 X 10^-4 M. At the end of incubation, the cells were chilled to 4° and centrifuged in Hopkin's vaccine tubes through a layer of 0.25 M sucrose to remove extracellular radioactivity and simultaneously avoid the leaching effect of conventional washing (7). The "washed" cells were solubilized in 0.5 N NaOH, and radioactivity was determined. The time course of HN2 uptake by sensitive and resistant cells treated with 1.04 X 10^-4 M HN2-14C was linear for at least 60 min and thereafter reached a plateau (Chart 1). All subsequent kinetic studies were terminated at 60 min to ensure that initial uptake velocity was being examined.

RESULTS

Binding of HN2-14C to DNA, RNA, and protein of L5178Y lymphoblasts was significantly lower (p < 0.001) for all 3 fractions isolated from resistant cells relative to that observed in sensitive cells (Chart 2). The mean ratio of specific activity (in sensitive/resistant cells ±S.E.) was greatest for DNA (8.20 ± 1.34), lowest for protein (2.80 ± 0.21), and intermediate for RNA (4.21 ± 0.27); the 3 ratios differed significantly from each other (p < 0.02), suggesting a difference in the distribution pattern between the 2 cell lines. Correction of the DNA fraction for RNA and protein contamination did not alter the relative difference in binding between DNA of sensitive and resistant cells. Similar results were observed for RNA and protein when the dose was reduced 20-fold to 1.04 X 10^-5 M, but at this dose the level of radioactivity in the DNA fraction of resistant cells was too low to yield reproducible results.

The data on binding of HN2 to DNA must be interpreted with great care because of 2 limitations: (a) there was heavy contamination of the DNA fraction with RNA and protein, and (b) extraction of DNA with hot 10% NaCl may lead to loss of alkylated purines (17). If the same degree of loss occurred from DNA of sensitive and resistant cells, the relative ratio of specific activities would remain unchanged; however, a different rate of labilization of bound HN2 would alter this ratio. Assuming a molecular weight for DNA of 5 X 10^6 and

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**Chart 1.** Time course of the uptake of HN2-14C by sensitive (L5178Y) cells (○) and resistant (L5178Y/HN2-2) cells (●), treated with 1.04 X 10^-4 M HN2-14C.

**Chart 2.** The specific activity (μmoles HN2-14C/μg) of DNA, RNA, and protein in sensitive (L5178Y) and resistant (L5178Y/HN2-2) lymphoblasts treated with 2.08 X 10^-4 M HN2-14C for 60 min at 37°. (See text for details of extraction procedure and correction of DNA specific activity for contamination with RNA and protein.)
Transport Carrier for Nitrogen Mustard

Chart 3. Density-gradient centrifugation profile of RNA extracted from sensitive (LS178Y) and resistant (LS178Y/HN2-2) lymphoblasts treated with 2.08 × 10⁻⁴ M HN2-¹⁴C for 60 min at 37°. RNA extracted by phenol (4) was dissolved in 0.1 M NaCl solution, 0.01 M Tris buffer, 0.001 M EDTA, and 0.2% sodium dodecyl sulfate at pH 7.4, layered on a 15 to 30% sucrose gradient and centrifuged at 16,000 rpm for 16 hr at 28° in a Beckman L-2 ultracentrifuge with the SW 25.2 rotor. —, A₂₆₀; —, radioactivity (cpm).

Using the data in Chart 2, with the above limitations in mind, the number of moles of HN2 per DNA molecule was estimated at 7.5 for sensitive cells and 0.9 for resistant cells.

RNA isolated from sensitive and resistant cells was fractionated by sucrose density gradient centrifugation. In all RNA fractions the specific activity of sensitive cells was greater than that of resistant cells (Chart 3). The specific activity was fairly uniform over the 28 S, 18 S, and 4 S RNA components; a possible exception was the higher activity observed in the 4 S to 18 S interval in the RNA profile of sensitive cells that might represent preferential alkylation of messenger RNA.

The uptake of nitrogen mustard by whole cells and the distribution of drug between cell sap and membrane fractions is shown in Table 1. The uptake of HN2 was 3-fold greater in sensitive than in resistant cells exposed to 1.04 × 10⁻⁵ M HN2-¹⁴C, but it was only 1.68 times greater when the dose of drug was increased to 2.08 × 10⁻⁴ M. The radioactivity of cell sap was approximately 7- to 10-fold greater than that of membranes in both sensitive and resistant cells.

The uptake of HN2 by both sensitive and resistant cells obeyed simple Michaelis-Menten kinetics. When applied to transport phenomena, Vₘₐₓ, the maximal rate of drug uptake depends on the number and mobility of transport sites; Kₘ, the Michaelis constant, represents substrate concentration at one-half maximal velocity and is related to the affinity of the transport site for drug (14). The data summarized in Table 2 show that in resistant cells the Kₘ was higher and the Vₘₐₓ was lower than in sensitive cells, indicating that transport was less efficient in resistant cells. Considerable variation in Kₘ and Vₘₐₓ was noted; therefore, in subsequent studies simultaneous controls were routinely used.

Evidence suggesting that uptake of HN2 was an active process was the finding that transport of both intact and hydrolyzed HN2 proceeded "uphill" against a concentration gradient as high as 35-fold. The intracellular-extracellular distribution of drug in sensitive cells is shown in Chart 4. At very low drug concentrations, the gradient reached a limiting value; as the drug concentration increased, the ratio dropped, and at high concentrations the ratio approached another plateau. The Z-shaped curve is characteristic of a carrier-mediated transport system, as discussed in detail by Bihler (1).

Further evidence for an active process was that uptake of HN2 was almost completely inhibited at 4° (Chart 5) and partially inhibited by DNP and ouabain (Chart 6). The inhibitory effects of both DNP and ouabain were slight but highly significant (t test comparing the difference of slopes, p < 0.001).

Alterations in the transport of HN2 due to intracellular binding of drug would not be expected with the hydrolyzed derivative. HN2 was hydrolyzed in 0.1 N NaOH at 60° for 2 hr, the pH was then adjusted to 7.5, and the solution was filtered through a Millipore membrane. HN2-OH had no

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>HN2-¹⁴C (1.04 × 10⁻⁵ M)</th>
<th>LS178Y/HN2-2 (mean ± S.E.)</th>
<th>HN2-¹⁴C (2.08 × 10⁻⁴ M)</th>
<th>LS178Y/HN2-2 (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell sap</td>
<td>100.1 ± 8.2</td>
<td>35.2 ± 2.0</td>
<td>552.4 ± 56.3</td>
<td>337.0 ± 29.3</td>
</tr>
<tr>
<td>LS178Y/HN2-2</td>
<td>11.1 ± 0.3</td>
<td>3.7 ± 0.5</td>
<td>81.7 ± 5.1</td>
<td>39.5 ± 3.5</td>
</tr>
<tr>
<td>Whole cell</td>
<td>114.7 ± 6.8</td>
<td>38.2 ± 1.8</td>
<td>634.0 ± 60.0</td>
<td>376.4 ± 32.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Kₘ (X 10⁻⁴ M)</th>
<th>Vₘₐₓ (moles/min/cell X 10⁻¹⁷)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS178Y</td>
<td>1.44 ± 0.27</td>
<td>4.78 ± 0.90</td>
</tr>
<tr>
<td>LS178Y/HN2-2</td>
<td>1.87 ± 0.34</td>
<td>3.76 ± 0.80</td>
</tr>
</tbody>
</table>

a The values shown are the mean ± 1 S.D. for 30 determinations on the sensitive (LS178Y) line and 16 determinations on the resistant (LS178Y/HN2-2) line.

b Significance of the difference of means, determined by the t test.
cytocidal activity when tested against sensitive and resistant lymphoblasts in vitro, confirming earlier reports that the hydrolyzed compound is inactive as an alkylating agent (10, 21). In sensitive cells with 14C-labeled HN2-OH serving as sub-

strate, uptake followed Michaelis-Menten kinetics and was competitively inhibited by intact HN2. Conversely, HN2-OH acted as a competitive inhibitor to the uptake of HN2-14C. Similar results were obtained with resistant cells. For both the active and hydrolyzed drug, the Km for the compound serving as substrate was similar to the K_i for that same drug acting as inhibitor (Table 3). This finding confirms the competitive nature of the reciprocal inhibition.

In addition to HN2-OH, several other structural analogs of HN2 were examined for inhibitory activity on HN2 transport. The monofunctional analog HN1 also acted as a competitive inhibitor of HN2-14C transport; K_m was increased, and V_max was not reduced. The K_i for sensitive cells was 2.99 × 10^{-4} M and that for resistant cells was 1.78 × 10^{-4} M (Table 4); these values were an order of magnitude greater than the K_i of

\[
K_i \text{ was determined from Lineweaver-Burk plots with the formula: apparent } K_m \text{ (from } x \text{ intercept of curve with inhibitor present)} = \frac{K_m}{1 + [I]/K_i}, \text{ where } K_m \text{ was obtained from the control slope in the absence of inhibitor and } [I] \text{ was the concentration of inhibitor, } i.e., \text{, hydrolyzed HN2 (14).}
\]
HN2-OH. Analogs of HN2 that did not inhibit drug uptake were chlorambucil (phenylbutyric acid-nitrogen mustard), melphalan (L-phenylalanine-nitrogen mustard), and cyclophosphamide; the latter was also inactive as an inhibitor when preincubated with mouse liver homogenate for 1 hr at 37°C. These findings suggest that the transport mechanism for HN2 demonstrates chemical specificity.

The data in Table 3 suggested that HN2-OH may be the preferred transport form. Therefore, a simultaneous comparison of transport of intact and hydrolyzed HN2 was undertaken (Chart 7). The \( V_{\text{max}} \) with hydrolyzed drug was 4.94 \( \times 10^{-17} \) mole/min/cell, which was similar to that observed with active drug in this and previous experiments (Table 2). However, the \( K_m \) with hydrolyzed drug was 5.19 \( \times 10^{-5} \) M, which was approximately one-third that observed with the active compound. The lower \( K_m \) may indicate a greater affinity of the carrier for hydrolyzed drug.

This poses the question of whether hydrolysis of HN2 may occur rapidly outside the cell and is a prerequisite of transport into the cell. Assuming that only the hydrolyzed form is transported, the "hypothetical dose" of hydrolyzed drug required to provide uptake equivalent to that observed with HN2-\(^{14}C\) was calculated (see Chart 7 and Table 5). It may be seen that 47.9% of the drug would have to be in the hydrolyzed form (for the entire 60-min incubation period) to account for the observed uptake. With reactivity with \( \gamma \)-(4-nitrobenzyl) pyridine as an index of alkylating activity, the half-life of HN2 in tissue culture medium at 37°C was 75 min (10). From this, the average degree of hydrolysis during a 60-min incubation period was estimated to be 24% (i.e., average dose available over 60 min = extent of hydrolysis at 30 min). Thus transport of the hydrolyzed form accounts at most for 50% of the observed uptake, and therefore transport of other forms of HN2 must also occur. Since the Epstein reagent does not distinguish between the difunctional and monofunctional compound (22), the other transport forms may include the active compound, the cyclic ethyleneimmonium ion, and the monohydroxyl-monofunctional derivative.

### DISCUSSION

In a previous report, the slow development of resistance and the requirement for repeated exposure of cells to drug suggested that resistance to HN2 was multifactorial (9). Rutman's group (3, 23) concluded that permeability changes alone were inadequate to explain the degree of resistance. The difference in uptake of sensitive and resistant cells (Table 1) is inadequate to explain the greater differences in binding to DNA, RNA, and protein (Chart 2) or the 30-fold difference in drug resistance previously described (9). Part of the discrepancy between uptake and binding to macromolecules may be due to other factors, such as elevated thiols, which have been found in resistant cells (2, 8, 9, 15). The superior repair capacity of resistant cells, which involves excision of alkylated fragments of DNA, may also account for the lower level of binding to DNA in resistant cells (6, 20, 26).

On the other hand, this study along with similar reports in the literature (3, 11, 12, 23, 26) clearly establishes that resistance to HN2 is characterized by reduced uptake of drug. Kinetic analysis provided the following evidence in support of a carrier mechanism for HN2 transport: uptake followed saturation kinetics, displayed chemical specificity, and exhibited mutual competitive inhibition between 2 transport substrates (with similar values for \( K_m \) and \( K_i \)). The Z-shaped plot of cell-medium ratio against extracellular concentration of drug was also consistent with a carrier mechanism (1).

Michaelis-Menten kinetics imply interaction with a limited and constant number of reactive sites and alkylation reactions are unlikely to follow this pattern. Therefore the finding of Michaelis-Menten kinetics on analysis of HN2 uptake suggests, not only existence of a carrier mechanism, but also that interaction of carrier and drug is rate-limiting for overall uptake.

Several findings indicate that transport was an active process: transport of both intact and hydrolyzed drug proceeded uphill against a concentration gradient, uptake was markedly inhibited at 4°C and was partially inhibited by...
Table 5

<table>
<thead>
<tr>
<th>Actual dose of HN2-14C</th>
<th>Hypothetical dose of HN2-OH-14C</th>
<th>% hydrolysis of HN2-14C to provide hypothetical dose of HN2-OH-14C</th>
<th>[HN2-OH]/[HN2]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.048</td>
<td>0.09</td>
<td>53.3</td>
<td>0.09</td>
</tr>
<tr>
<td>0.096</td>
<td>0.22</td>
<td>43.6</td>
<td>0.22</td>
</tr>
<tr>
<td>0.24</td>
<td>0.46</td>
<td>52.2</td>
<td>0.46</td>
</tr>
<tr>
<td>0.48</td>
<td>1.02</td>
<td>47.1</td>
<td>1.02</td>
</tr>
<tr>
<td>0.96</td>
<td>2.22</td>
<td>43.2</td>
<td>2.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>X = 47.9</strong></td>
<td></td>
</tr>
</tbody>
</table>

- Actual dose of HN2-14C used to determine drug uptake by sensitive cells (see Chart 7).
- The assumption is made that transport of HN2-14C occurs only in the hydrolyzed form. The concentration of hydrolyzed HN2 (hypothetical dose) required to provide uptake equivalent to that observed after HN2-14C was determined by extrapolation to the curve for HN2-OH-14C and then reading the concentration from the abscissa (see - - - in Chart 7).

Kessel et al. (16) attributed the concentrative accumulation of HN2 in L1210 cells to extensive binding of drug to cell components, a process that was reported to be temperature sensitive. That the pattern of saturation kinetics reported here was not merely an index of binding to cell components was clearly demonstrated by the finding that hydrolyzed HN2, which is not an alkylating agent (10, 21), was transported against a concentration gradient of up to 35-fold and acted as a competitive inhibitor of HN2 transport.

In resistant cells, the elevated $K_m$ implies a decreased affinity of the carrier for drug, and the lower $V_{max}$ may indicate a decreased number and/or mobility of transport sites. An analogous mechanism of carrier-mediated transport of amethopterin has been described in L1210 leukemia cells (14, 24) with alteration of the binding properties of the carrier in an amethopterin-resistant mutant (24).

The 3-fold lower $K_m$ of the hydrolyzed drug at first hand suggests that its affinity for the carrier may be greater than that of the active compound. However, interpretation of the data is quite complex; the higher $K_m$ observed with intact HN2 may reflect a lower effective concentration of the drug due to loss by alkylation of constituents in the medium or on the cell membrane. Another possibility is allosteric modification of the carrier due to alkylation of adjacent sites on the membrane. Finally, the lower $K_m$ with hydrolyzed drug could mean that this form alone is transported, and that hydrolysis is a prerequisite for transport of HN2. That the active form is transported into the cell prior to hydrolysis was suggested by the finding that initial uptake of HN2-14C was linear without a lag period. Secondly, a comparison of uptake kinetics and rate of hydrolysis of HN2 indicated that transport of forms other than the hydrolyzed species must occur. Thirdly, binding of drug to DNA, RNA, and protein requires transport of the active form into the cell. Finally, the correlation between transport kinetics and drug sensitivity also implies transport of the active compound.

It may be concluded that at least part of the drug is transported in the active form. The high reactivity of HN2 places unusual demands on the hypothetical carrier. While it is possible that carrier function may be modified by alkylation of neighboring membrane sites, formation of a covalent bond with the reactive site cannot be part of the transport process, since the latter depends on the formation of a reversible carrier-drug complex.

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