Modulation of the Cellular Toxicity of Nitrogen Mustard in Murine Cells

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ABSTRACT

A linear increase in cell uptake of nitrogen mustard, methyl-bis(2-chloroethyl)amine (HN2), between 1 and 5 min, was observed after in vitro incubation of Ehrlich ascites tumor cells at 37°C in phosphate-buffered saline containing HN2 followed by washing in 0°C phosphate-buffered saline. After a second incubation in 37°C phosphate-buffered saline without HN2, the cells lost about one-half of the drug which had been taken up, which had not been covalently bound to macromolecules.

The basal cytotoxic effect of HN2 on the cells was determined using a standard in vitro test for cell viability. Host survival was measured after 106 HN2-treated cells were injected i.p. into recipient mice, compared with injection of 106 untreated cells into paired control mice. Five min incubation of cells in vitro with multilamellar liposome vesicles (MLV) composed of L(α)dipalmitoyl-phosphatidyl choline in the presence of HN2, significantly increased tumor cell kill and mouse survival over HN2 alone. In contrast, added Ca2+ plus HN2 decreased cytotoxicity and survival. Significant increases in host survival following MLV treatment occurred without significant increase in total HN2 uptake and could be highly correlated with increased amounts of HN2 bound to DNA.

Addition of vincristine (an inhibitor of microtubule polymerization) in the presence of HN2 also decreased the cytotoxic effect of HN2. The vincristine inhibition occurred, without altering total cell HN2 uptake, whether L(α)dipalmitoyl-phosphatidyl choline MLV were present or not. It is proposed that both Ca2+ and MLV act at membrane sites so as to alter the subcellular distribution and localization of HN2 and its accessibility to critical targets. This has been confirmed for MLV by demonstrating increased alkylation of DNA.

INTRODUCTION

Since their first application in experimental therapy, phospholipid vesicles (liposomes) have generally been regarded as inert vehicles which might favorably influence the systemic distribution and uptake of encapsulated drugs (1, 2). Manipulation of liposome composition was seen as a means of altering the systemic distribution of the drug and increasing or decreasing its biological lifetime while the placement of specific signals (antibody) on the vesicular surfaces was seen as a way of increasing the cellular selectivity of the drug. To a considerable extent, these potentiating features of liposomes have been verified, particularly in tissue culture systems (3, 4). However, in the recent past, an additional body of data has begun to emerge which suggests that liposomes, irrespective of their carrier properties, can independently modify cell permeability and drug transport. Thus, liposomes added directly to a cell culture in advance of doxorubicin increased drug uptake 3-fold (5), and empty liposomes also increased the uptake of the extracellular marker inulin (6). Mixtures of amphotericin B and empty liposomes were more cytotoxic to Candida than was the liposome-encapsulated drug (7). We have shown that liposomes promoted the uptake and effectiveness of HN22 even though no extra HN2 was either encapsulated by, dissolved in, or attached to the vesicles (8, 9). We have also demonstrated that liposomes administered in vivo as much as 3 h in advance of 1,3-bis(2-chloroethyl)-1-nitrosourea significantly potentiated its antileukemic activity whereas similar enhancement of HN2 action required simultaneous presence of drug and modulator (10, 11).

The studies reported herein extend these observations by confirming the HN2 effects (10) and relating these to intracellular distribution, while also showing the complex antagonistic action of external Ca2+ in suppressing cytotoxicity despite an enhanced drug uptake. The modulation of HN2 effectiveness by liposomes, by external Ca2+ as well as by vincristine, suggest, but do not prove, the involvement of membrane and microtubule-related pathways in intracellular drug distribution.

MATERIALS AND METHODS

Liposome Preparation. Liposomes are MLV which were made by evaporating CHCl3 from a solution of L(α)dipalmitoyl-phosphatidyl choline, leaving a thin DPC film using a stream of dry N2. The N2 flow was then continued for an additional 2 h to remove residual traces of solvent. PBS (154 mM NaCl-6.2 mM KCl-1.5 mM NaH2PO4-9.4 mM Na2HPO4, pH 7.4) was then added to the flask. MLV were allowed to form spontaneously with shaking; then the suspension was homogenized by hand using a tight-fitting Teflon pestle in a glass homogenizer tube. The concentration of phospholipid in this suspension was analyzed using a technique for lipid phosphorus (13). The liposomes were analyzed for purity by 2-dimensional thin-layer chromatography by the method of Rouser and Fleischer (14) in which a 4- or 10-Å aliquot was spotted onto an anhydrolysed 20×20-cm silica gel plate (Redicat; Supelco, Bellefonte, PA) developed in one direction with chloroform:methanol:20% aqueous ammonia (65:35:5), removed, dried for 10 min, then developed perpendicularly to the first direction with chloroform:acetone:methanol:acetic acid:water (4:2:1:1:0.5). The plate was then blotted and developed with either chromic acid spray followed by heating at 180°C or with Dragendorff reagent to detect choline (15). Duplicates done in this manner yielded single spots having a characteristic Rf and identical final position for DPC.

Association of Liposomes and HN2. The degree of association of MLV, made of L(α)DPC, and HN2 was measured using a procedure in which the HN2 was exposed to an increasing MLV concentration. MLV, 3.4 µmol, were suspended in 3 ml of a solution containing 1.1 M sucrose, 1.0 µmol, of HN2, and 7.5 nmol of [3H]HN2 (30 µCi/µmol), dispersed either in PBS or 10 mM Tris-buffered saline, pH 7.4, and added to a 5.8-ml nitrocellulose centrifuge tube. Atop the solution was layered an identical 0.8 M sucrose solution with no MLV. Samples of these solutions were taken for lipid phosphate analysis (when Tris buffer was used) and for scintillation counting. Tubes were then centrifuged at 105,000 × g for 90 min in the SW 65 rotor of a Beckman L-2 65-B ultracentrifuge at 5°C, resulting in upward migration of the MLV from the denser sucrose solution through the lighter one to form a cap atop the lighter solution. The bottom of the tube was punctured and 10-drop fractions were collected. MLV in the last 0.2 ml did not flow spontaneously from the tube but were collected by pipet from the top. A 0.1-ml aliquot of each fraction was analyzed for either lipid phosphate or for [3H]HN2.

Several variations of the composition were tested to examine the effects of Ca2+ and of different molecular species of HN2 on this partition. Solutions containing 250 µM Ca2+ were compared to Ca2+-free controls. All solutions were kept cold (0-5°C); analysis of alkylating activity by the γ-4-nitrobenzyl pyridine method (16) showed that less than 15% of the alkylating activity was lost. Similarly, we tested the...
MODULATION OF HN2 EFFECTIVENESS

effects of 37°C, 2-h incubation on alkylating activity and liposomal association with or without Ca²⁺.

Cell Preparation. A 0.1-ml undiluted 6-day ascites cell inoculum was harvested after 4 days from the peritoneal cavities of ICR Swiss mice. Following cervical and sacral spinal dislocations, the cells were flushed into a Petri dish via a ventral laparotomy with PBS, then were washed at least 4 times at 5°C to remove contaminating RBC as well as loosely bound Ca²⁺. A cell count was done using a hemocytometer, diluting cells as required. One-ml samples were taken in duplicate and used to determine dry weight (17, 18) and protein concentration (19).

Modification of HN2 Uptake into Cells. Uptake of HN2 was measured in 15-ml Corex centrifuge tubes in which 5 ml PBS containing 2 mM glucose was preincubated with washed cells, 0.6–1.0 mg dry weight at 37°C for 5 min. The reaction was started by adding 24 μM HN2 and 1 nM [³H]HN2 (1.6 × 10⁶ cpm) to cell suspensions, then terminated at intervals from 10 s to 5 min, by adding 6 ml ice-cold PBS and placing the tube in an ice bath. The cells were washed (0–5°C) to remove extracellular HN2. Such cells then contained bound plus free intracellular HN2. Separation of bound from free HN2 was achieved by reincubating one of a pair of tubes containing cold-washed cells at 37°C for 4 min in drug-free PBS allowing “free” HN2 labeled components to efflux. Measurements at each time interval yielded, total, free, and bound HN2. The resulting data are presented on a plot showing bound plus free and bound HN2 as a function of incubation time. The statistical significance of uptake relative to that at 10 s was determined using Student’s t test. This procedure was applied to tubes which contained glucose and HN2, and a glucose solution was added to glucose-free tubes for comparison. Uptake of HN2 into cells preincubated with a glucose solution was not different from uptake into glucose-free cells.

Modification of HN2 Effectiveness on Cell Viability. After in vitro treatment, 10⁵ ascites cells were injected into 20-g female HA/ICR mice, and mouse survival was used to indicate tumor cell viability. This method of tumor cell viability analysis is highly sensitive; injection of one viable tumor cell has been shown to be lethal (20) and with a cell doubling time of 18 h (21) would lead to a lethal cell number, 2 x 10⁶, mouse within 30 days. Injection of 10⁵ untreated tumor cells has been shown to kill all recipient mice within 40 days (22). We used such an assay in the experiments reported here. Following the injection of cells, surviving mice were counted each day for 60 days. 1.5x as long as needed based on the tumor growth rate (20, 21). Analysis of the cell uptake of HN2 and its effectiveness were done together. Measurements of uptake required the use of a radiotracer. That tracer was omitted from the incubation medium bathing cells to be reincubated for viability analysis. In all other respects, the treatments were the same. Eight mice were injected for each treatment and between 3 and 5 separate experiments were done. Statistical analysis of survival curves of drug treated cells, legitimate only when injection of untreated cells indicated that cell viability was not impaired by the washing and incubation procedures, were done using the χ²-square test.

HN2 Distribution. Cells were processed and then incubated (37°C) at a density of 1 × 10⁶ cells/ml in PBS supplemented with HN2, HN2 plus Ca²⁺ or HN2 plus t(a)DPC MLV. The cells were then cold washed, incubated a second time in PBS (37°C), cold washed again, and analyzed. These analyses indicated bound HN2 (as cpm [³H]HN2) in each fraction. Approximately 10⁶ cells containing 1–1.5 mg DNA were used to isolate DNA by the procedure of Marmur (23). Isolation was conducted in 15 ml conical glass centrifuge tubes to permit concentration of the formed product in a small volume (1–2 ml) from which it was possible to spool out 0.5–1.0 mg DNA fibers. Only one treatment with RNase and pronase was used. All DNA samples showed 260/280 absorbance ratios >1.80. DNA (0.1–0.2 mg) was assayed for bound [³H]HN2 by scintillation counting.

Estimates of binding to protein and RNA were made by a combination of Schneider (24) and Ogur-Rosen (25) procedures. A 0.1-aliquot of the homogenate to be used for DNA isolation was precipitated in the cold with 0.2 value of 25% TCA, centrifuged, and washed free of excess TCA with 1% TCA followed by 95% ethanol. The nucleoprotein pellet was dissolved in 0.2–0.5 ml cold NaOH, allowed to stand in the cold for 10 min, then precipitated with 1 N HClO₄. This precipitate was extracted for 30 min at 100°C with 1 N NaOH. The supernatant was recovered, perchlorate removed as KClO₄, and the deoxyribonucleotide-containing fraction was used to obtain a crude confirmatory estimate of the DNA radioactivity. The protein fraction was washed thoroughly with pH 9.0 carbonate buffer and then dissolved in 1.0 ml 1.0 N NaOH with heat and assayed for protein by the Biuret procedure (19) as well as for radioactivity.

RESULTS

The uptake and binding of [³H]HN2 by Ehrlich cells during in vitro incubation is presented in Fig. 1. Uptake increases linearly with time from 0–5 min. Binding, measured as [³H]HN2 retained after 0°C washing and 37°C reincubation, increased steadily from 40 s–5 min although the early measurements were variable. There is a constant partition of [³H]HN2 from the free compartment to the bound compartment, at a ratio of 2:1. Throughout this period, free drug can be recovered upon reincubating the cells at 37°C, in keeping with expectations as to transport by the choline carrier (26) and not consistent with instantaneous covalent binding of the HN2. In fact, the uptake data suggest a two-stage process whereby HN2, carrier bound, is maintained in such a fashion that two-thirds of the unbound drug is transferred to acceptor, nondiffusible molecules or nonexchanging compartments, while up to one-third continues to be available to the carrier for return to the external medium.

Before proceeding to further study the effects of liposomes on uptake of [³H]HN2 by the Ehrlich cells in vitro, we wished to establish the extent, if any, to which the drug was taken up by liposomes, either by solution in the lipid phase or by entrapment in the vesicular aqueous phase. The liposomes were subjected to flotation through a sucrose layer. From Table 1, it can be seen that although >99% of the phospholipid floated to the top of the sucrose, the concentration of HN2 was uniform throughout and was uninfluenced by the presence of Ca²⁺.

![Fig. 1. Uptake of HN2. Cells were preincubated in PBS with glucose (2 mM) at 37°C for 10 min. The reaction was started by adding 2.4 × 10⁻⁴ M HN2 and a tracer amount of [³H]HN2 (1.6 × 10⁶ cpm; 1.6×10⁻⁷ mol), stopped by cold dilution, and the cells were washed free of extracellular HN2. The cells in one tube of each pair were added to 10 ml scintillation fluid, counted, and the results were presented as bound plus free HN2, cpm/mg dry weight (○). Cells in the second tube were reincubated at 37°C for 4 min in PBS to allow efflux of unbound cellular HN2 and the reaction was stopped as before. The cells were washed once with cold PBS, then counted, and the results are presented as bound HN2 (●). Liner joining points were calculated by least squares analysis, having r > 0.98 and P < 1 × 10⁻⁴. Horizontal lines above and below symbols, SE.

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Concentrating liposomes through HN2-containing sucrose solutions does not alter the amount of liposome-associated HN2. HN2 results are given as the percentage of the HN2 concentration of each component solution before the gradient was assembled. Their HN2 concentrations were the same. No HN2 hydrolysis represents experiments done in the cold in which 86 ± 2.0% (N = 6) of the alkylating activity was retained. For HN2 hydrolysis, the buffered sucrose solutions containing HN2 were incubated at 37°C for 120 min, then cooled, and the gradients were made. Experiments in which phosphate was measured were performed using a 10 mM Tris buffer rather than phosphate. In those experiments, the phosphate concentration of the sucrose solutions below the lipid cap were not significantly different from that of buffered sucrose.

Table 1 Nonassociation of nitrogen mustard and liposomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cellular HN2 content (cpm/mg protein)</th>
<th>% of basal HN2 uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN2</td>
<td>1567 ± 112</td>
<td>100</td>
</tr>
<tr>
<td>HN2 + Ca²⁺</td>
<td>2051 ± 84</td>
<td>131</td>
</tr>
<tr>
<td>HN2 + LoDPC MLV</td>
<td>1847 ± 152</td>
<td>118</td>
</tr>
<tr>
<td>HN2 + LoDPC MLV + Ca²⁺</td>
<td>1853 ± 94</td>
<td>118</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Table 2 Effect of liposomes and Ca²⁺ on HN2 uptake

Influence of Ca²⁺ and LoDPC MLV on cellular HN2 content. Incubation for 5 min as per text. Only Ca²⁺-stimulated uptake was significantly greater than basal uptake (P = 0.006). The data are from 4-6 experiments, the same as were used to prepare Fig. 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of basal HN2 uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN2</td>
<td>100</td>
</tr>
<tr>
<td>HN2 + 20 µM Ca²⁺</td>
<td>96 ± 1.3</td>
</tr>
<tr>
<td>HN2 + LoDPC MLV</td>
<td>96.2 ± 1.2</td>
</tr>
<tr>
<td>HN2 + LoDPC MLV + 20 µM Ca²⁺</td>
<td>97.5 ± 4.8</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Fig. 2 shows the manipulation of cells in vitro (incubations and washing) has not decreased the viability or growth rate of the Ehrlich cells. All control mice are killed with an average median survival time of about 23 days which is no different than the median survival time due to unmanipulated cells (22). All in vitro treatments with HN2 produce increased survival due to the cytotoxicity of the drug and this in turn is modified by the treatments with liposomes described in the legend to Fig. 2. Liposomes increase the HN2 survival effect significantly (60-90%), while Ca²⁺ depresses the survival (60-45%). Combining liposomes with Ca²⁺ reverses the inhibitory effects of Ca²⁺ on HN2 almost completely and slightly decreases the MLV enhancement of cytotoxicity.

Data in Table 2 seek to correlate the changes in HN2 cytotoxicity with changes in permeability as shown by the in vitro uptake of HN2. Ca²⁺ significantly stimulates the uptake of HN2. Liposomes slightly increase HN2 uptake but the effect is not statistically valid, but liposomes do prevent a Ca²⁺-induced increase in HN2 uptake. Thus, the uptake results do not support a liposomal role in increasing HN2 transport; furthermore, calcium ions, which increase uptake, somewhat modify the coupling between increased uptake and HN2 effect. Since this might be due to effects on the binding of HN2, we compared the survival of washed cells (bound plus free HN2) with that of washed and reincubated cells (bound HN2) corresponding to the upper and lower lines, respectively, in Fig. 1. Table 3 shows the percentage of 60-day survivors for these two treatments and that bound plus free HN2 and bound HN2 were equally effective; accordingly, free, exchangeable drug had little or no effect.

The altered relations between uptake and effect observed with Ca²⁺ suggested that the internal distribution of the HN2 might be altered. We sought to test this using vincristine, an antimicrotubule drug known to inhibit secretion (27) and alter intracellular membrane relationships (28, 29). Table 4 shows that vincristine has no effect on uptake in the presence or absence of Ca²⁺ or MLV; nonetheless, vincristine decreases the effectiveness of HN2 and antagonizes the enhancement of HN2 activity produced by liposomes (Table 5). It is interesting to note that vincristine was 0.61 µM, which is the concentration of vincristine which have been shown to modify HN2 uptake and effect. None of the changes in content was significantly different from the basal level measured after incubation in HN2 alone. The concentration of vincristine was 0.61 µM. N = 3 experiments.

Table 3 Comparison of cytotoxicity of total HN2 versus bound HN2 in cells

Effects of bound and bound plus free HN2 are not significantly different under a variety of treatment conditions. Data on survival due to bound plus free drug are taken from Fig. 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of mice surviving after being inoculated with treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN2</td>
<td>75 (7/12)</td>
</tr>
<tr>
<td>HN2 + LoDPC MLV</td>
<td>71 (7/10)</td>
</tr>
<tr>
<td>HN2 + 20 µM Ca²⁺</td>
<td>38 (5/16)</td>
</tr>
<tr>
<td>HN2 + LoDPC MLV + 20 µM Ca²⁺</td>
<td>38 (5/16)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of mice surviving per number treated.

Table 4 Influence of vincristine on cell HN2 uptake in presence of substances which have been shown to modify HN2 uptake and effect

No change in content was significantly different from the basal level measured after incubation in HN2 alone. The concentration of vincristine was 0.61 µM. N = 3 experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HN2 content (cpm/mg protein)</th>
<th>% of basal HN2 uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN2</td>
<td>1637 ± 160&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td>HN2 + vincristine</td>
<td>1538 ± 215</td>
<td>91</td>
</tr>
<tr>
<td>HN2 + vincristine + Ca²⁺</td>
<td>1828 ± 117</td>
<td>112</td>
</tr>
<tr>
<td>HN2 + LoDPC MLV + vincristine</td>
<td>1632 ± 79</td>
<td>100</td>
</tr>
<tr>
<td>HN2 + LoDPC MLV + Ca²⁺</td>
<td>1750 ± 164</td>
<td>107</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SE.
Table 5 Vincristine-Ca\(^{2+}\) and liposome interaction

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% survivors</th>
<th>% change from HN2 control</th>
<th>Relative uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>HN2</td>
<td>64</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>HN2 + Ca(^{2+})</td>
<td>47</td>
<td>-20</td>
<td>1.3*</td>
</tr>
<tr>
<td>HN2 + vincristine</td>
<td>37</td>
<td>-40</td>
<td>1.05</td>
</tr>
<tr>
<td>HN2 + vincristine + Ca(^{2+})</td>
<td>62</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>HN2 + L(a)DPC MLV</td>
<td>93</td>
<td>+53</td>
<td>1.1</td>
</tr>
<tr>
<td>HN2 + vincristine + L(a)DPC MLV</td>
<td>37</td>
<td>-40</td>
<td></td>
</tr>
<tr>
<td>HN2 + vincristine + Ca(^{2+}) + L(a)DPC MLV</td>
<td>88</td>
<td>+48</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* Significantly increased uptake, at P = 0.006.

Table 6 Distribution of bound \[^3H\]HN2 in Ehrlich cell subcellular fractions following in vitro incubation

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Total uptake (pmol [^3H]HN2/10^10 cells)</th>
<th>Protein</th>
<th>Soluble fraction</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C, 5 min</td>
<td>1775 ± 275 (100)</td>
<td>870 ± 760 (55)</td>
<td>(100) (53) (45) (100) (100) (33.3)</td>
<td>(136) (55) (126) (44) (55) (14)</td>
</tr>
<tr>
<td>+ [[^3H]HN2 + Ca(^{2+})]</td>
<td>2220 ± 130 (125)</td>
<td>1180 ± 955 (30)</td>
<td>(100) (53) (45) (100) (100) (33.3)</td>
<td>(136) (55) (126) (44) (55) (14)</td>
</tr>
<tr>
<td>37°C, 5 min</td>
<td>1770 ± 50 (95)</td>
<td>835 ± 670 (72)</td>
<td>(95) (53) (88) (43) (131) (4.6)</td>
<td>(136) (55) (126) (44) (55) (14)</td>
</tr>
<tr>
<td>+ [[^3H]HN2 + L(a)DPC MLV]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

...note that Ca\(^{2+}\), despite its own inhibitory effect on cytotoxicity, exercises a liposome-sparing effect, neutralizing the vincristine inhibition and restoring the liposome enhancement of HN2 cytotoxicity.

We next investigated the actual distribution of the bound HN2 within the Ehrlich cells. These data are presented in Table 6 and shows both Ca\(^{2+}\) and liposomes have significant effects on the localization and molecular targeting of the HN2. Ca\(^{2+}\) increases the total uptake and retention, causing 36% more drug to interact with protein and a similar increase in the acid soluble products (26%) while reducing by almost one-half (45%) the quantity of drug which binds to the DNA. These absolute changes in binding induced by Ca\(^{2+}\) reflect little relative change in distribution, except a small increase in protein binding, which dominates the macromolecular binding (>95%). In the case of liposomes, there is a decrease in the amount of drug retained in the soluble fractions accompanied by both a relative and absolute increase in transfer to the DNA. The percentage of binding to DNA, as shown in Table 6, Column 5, is highly correlated with HN2 effectiveness as shown by mouse survival in Table 3, Column 2 (r = 0.97).

We have measured only total binding of HN2 to DNA at a time interval too short for appreciable repair (30); however, previous work (22) clearly demonstrated statistically significant correlations between dosage, alkylation, cross-linking and cytotoxicity. The limited data on correlation between binding and cytotoxicity in the paper suggest that the current data also conform to those regularities.

**DISCUSSION**

Our data show that added L(a)DPC MLV, Ca\(^{2+}\), and vincristine modulate the *in vitro* cytotoxicity of Ehrlich ascites cells by HN2 as measured by i.p. growth and mouse survival. These effects include changes in HN2 uptake, intracellular distribution, and DNA alkylation. These give insight into the possible cellular mechanisms behind the altered cytotoxicity and provide a basis for additional in-depth investigation. The effects include an uncoupling between uptake and cytotoxicity with proportionate changes in HN2-DNA binding and suggest the existence of hierarchical elements in the control of this process as well.

The mechanism of HN2 action requires the existence of cryptic or sequestered intracellular forms of HN2 which participate in transport and distribution, since a significant fraction of the HN2 which is taken up from an extracellular solution travels through the cytoplasm to the nucleus and bifunctionally alkylates DNA (22). This "HN2" retains its activity despite the presence of a vast stoichiometric excess of highly reactive binding sites which could destroy bifunctional reactivity. Therefore, some of the HN2 appears to be protected during transit. Our data offer evidence that, after 10-40 s of 37°C incubation, HN2 is present in the cell in a form accessible to the extracellular solution but not yet bound to macromolecules. Such intracellular HN2 seems to exemplify a form which has been sequestered and is being temporarily withheld from reactive targets. A precedent for such a sequestered form can be found in the 0°C binding of HN2 to DNA in *in vitro* (31).

Calcium added to incubated cells increases HN2 uptake but paradoxically decreases cytotoxicity; this paradox is reconciled by the observation that decreased DNA alkylation occurs, as compared to cells incubated in HN2 without Ca\(^{2+}\). This suggests that Ca\(^{2+}\) is acting on intracellular mechanisms to direct the increased intracellular HN2 away from the nucleus. Liposomes, in contrast, do not seem to alter plasma membrane HN2 uptake but, instead, disproportionately increase the alkylation of DNA compared to controls, also suggesting an alteration of a connection between uptake and subcellular distribution. In these two cases the uptake and distributive processes are differentially affected. The data which suggest that intracellular HN2 is protected taken together with the modulatory effects of Ca\(^{2+}\) and liposomes suggest a coordinate cell membrane involvement in uptake and distribution. Furthermore, the data show that Ca\(^{2+}\) and L(a)DPC are both able to control the distribution phase and that the L(a)DPC stimulatory action overcomes the Ca\(^{2+}\) inhibition.

Vincristine and increased external Ca\(^{2+}\) reduce HN2 effectiveness but by different mechanisms, since they antagonize each other. This antagonism permits reexpression of the liposome stimulation of HN2 cytotoxicity which would otherwise be inhibited by vincristine. This also suggests that vincristine has actions which alter HN2 uptake and distribution. A specific interaction of *Vinc* alkaloids, which may be relevant to our observations, has been reported in investigations on brain microtubules where calcium-calmodulin inhibited the vinblastine binding to tubulin (32); in turn, vincristine binding to the calcium-calmodulin-microtubular complex inhibited microtubular phosphodiesterase (33). Each of those effects would serve to maintain microtubular integrity. The effects of liposomes on these *in vitro* microtubular interactions have not been examined. While action of vincristine is pleiotropic, its known effects on microtubule integrity (34), and on the organization of the Golgi apparatus (28, 29), suggest that influences on drug transport and intracellular distribution could be anticipated.
The results presented in this paper direct one’s attention to the possible participation of cell membrane systems in the distribution of HN2. These pathways can be modulated to alter the effects of HN2 and other nuclear-interactive drugs.

ACKNOWLEDGMENTS

We thank Howard Landa, Susan Glassman, John Sinnigen, Marc Blumstein, Narayan Avadhani, Ann Jeglum, and Eleanor Arrington for their help with various aspects of this work.

REFERENCES

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