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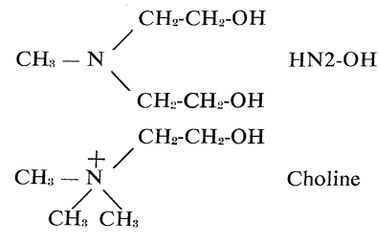
Transport of Nitrogen Mustard on the Transport-Carrier for Choline in L5178Y Lymphoblasts

Abstract. *The uptake of choline by L5178Y lymphoblasts occurs by a carrier mechanism and is an active process. Transport of nitrogen mustard and its hydrolyzed derivative is mediated by this same carrier. This finding is an example of drug transport by a carrier whose primary function is transport of a naturally occurring substrate.*

Transport of the alkylating agent nitrogen mustard (HN2) by L5178Y lymphoblasts was found to be an active and carrier-mediated process (*1*, *2*). The nature and identity of the transport carrier were investigated, since the existence of a specific carrier for a toxic and unphysiologic compound such as HN2 was considered unlikely. This report presents evidence of a transport-carrier for choline in L5178Y lymphoblasts which also serves as a carrier for HN2. This finding has interesting pharmacological implications regarding mechanisms of drug transport, in that it demonstrates drug transport by a carrier system whose primary function is transport of a naturally occurring substrate. We also suggest that in the field of cancer chemotherapy choline may have a role to play as a protective agent against HN2.

Transport studies were performed on suspension cultures of L5178Y lymphoblasts incubated in vitro at 37°C as described (*2*). Incubations were terminated by rapid chilling to 4°C and centrifugation of the cells in Hopkins' vaccine tubes through a layer of 0.25*M* sucrose to remove extracellular radioactivity. The washed cells were solubilized in 0.5*N* NaOH, and radioactivity was determined by liquid scintillation spectrometry. [1,2-^{14}C]Choline chloride, specific activity 2.0 mc/mmole, was obtained from New England Nuclear, Boston, and [1,2-^{14}C]HN2, specific activity 3.1 mc/mmole, from Mallinckrodt Chemical Works, St. Louis; the hydrolyzed derivative [^{14}C]HN2-OH was prepared by alkaline hydrolysis of the parent compound in 0.1*N* NaOH at 60°C for 2 hours. Transport of the inactive hydrolyzed derivative occurred by the same mechanism as the active drug (*2*), and was preferred to HN2 in that transport could be investigated without the complications of alkylation reactions.

In competition experiments 3-*O*-methyl-D-glucose, phlorizin, phloretin, and several amino acids representative of the various amino acid transport systems were without effect on the transport of [^{14}C]HN2-OH. There is a striking structural similarity between choline, HN2, and HN2-OH:



Therefore we studied the transport of choline and its interaction with the transport of HN2.

The time course of choline and HN2-OH uptake closely followed that reported for HN2 in that uptake was linear for at least 60 minutes and thereafter reached a plateau (*2*). Accordingly, all kinetic studies were terminated at 60 minutes to ensure that initial uptake velocity was being examined. Transport of [1,2-^{14}C]choline chloride by L5178Y lymphoblasts proceeded "uphill" against a concentration gradient of over 40-fold, obeyed simple Michaelis-Menten kinetics, and was competitively inhibited by HN2 (Fig. 1). From an analysis of ten such experiments the K_m (mean ± S.E.) for choline was $2.51 \pm 0.29 \times 10^{-5}M$ and the V_{max} $3.08 \pm 0.22 \times 10^{-17}$ mole/min per cell. When applied to transport phenomena, V_{max}, the maximal rate of uptake, depends on the number and mobility of transport sites; K_m, the Michaelis constant, represents substrate concentration at one-half maximal velocity and is related to the affinity of the transport carrier for drug (*2*, *3*). The K_m for choline was lower than that for HN2-OH ($6.86 \pm 0.67 \times 10^{-5}M$) and HN2 ($13.5 \pm 0.50 \times 10^{-5}M$), suggesting that choline is the preferred transport substrate; the V_{max} for HN2-OH was $4.90 \pm 0.43 \times$

Table 1. Transport of [^{14}C]choline chloride, [^{14}C]HN2, and [^{14}C]HN2-OH by L5178Y lymphoblasts in vitro. K_m, V_{max}, and K_i were calculated from the linear regression equations as described (*2*).

Inhibitor	V_{max} ($\times 10^{-17}$ mole/min per cell)	K_m ($\times 10^{-5}M$)	K_i ($\times 10^{-5}M$)
Choline as substrate			
Control	3.19	1.95	
HN2	3.15	4.15	8.86
Control	3.18	2.59	
HN2-OH	2.85	7.62	5.36
Control	2.36	2.20	
Ethanolamine	2.87	4.57	9.26
Control	2.23	1.74	
Hemicholinium-3	2.43	3.25	11.5
HN2 as substrate			
Control	5.59	16.12	
Choline	4.27	26.93	14.9
HN2-OH as substrate			
Control	3.62	7.76	
Choline	3.91	28.92	3.66
Control	4.27	4.75	
HN2	3.20	10.29	17.8
Control	2.63	6.38	
Ethanolamine	2.59	10.74	14.6
Control	3.81	6.84	
Hemicholinium-3	3.68	9.55	25.0

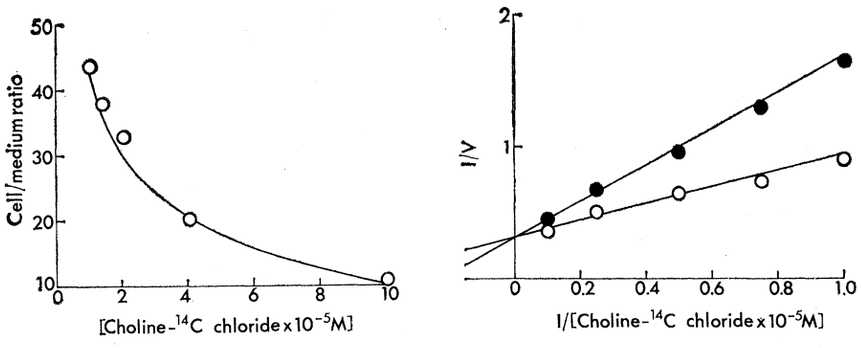


Fig. 1. Uptake of [1,2-^{14}C]choline chloride by L5178Y lymphoblasts incubated in vitro for 1 hour at 37°C. (Left) The cell/medium distribution ratio of [1,2-^{14}C]choline chloride plotted against concentration of choline in the medium. (Right). Lineweaver-Burk plots of the uptake of [1,2-^{14}C]choline chloride by L5178Y lymphoblasts: control, ○; and with $1 \times 10^{-4}M$ HN2 as inhibitor, ●. The linear regression equation of the control plot was $y = 0.6128x + 0.3135$, with a correlation coefficient of 0.9940, and that with inhibitor present was $y = 1.3155x + 0.3173$, with a correlation coefficient of 0.9993. V, velocity of drug uptake expressed as 10^{-17} mole per minute per cell.

10^{-17} mole/min per cell, and that for HN2, $4.60 \pm 0.14 \times 10^{-17}$ mole/min per cell.

The inhibition constant (K_i) for HN2 as inhibitor of choline transport was $8.86 \times 10^{-5}M$, which was lower than the K_m for HN2 as substrate (Table 1). Conversely, choline served as competitive inhibitor to [^{14}C]HN2 transport and the K_i was $14.9 \times 10^{-5}M$, which was approximately sixfold greater than the K_m for choline as substrate. These differences between K_m and K_i suggest that interaction of HN2 with the carrier involves more than simple attachment to the transport site. The HN2 may cause modification of the carrier through alkylation of adjacent sites on the cell membrane resulting in reduced affinity between carrier and substrate; this is supported by the observation that the affinity of the carrier for choline is reduced in cells first treated with $1 \times 10^{-4}M$ HN2 for 1 hour ($K_m = 8.13 \times 10^{-5}M$) compared to cells first treated with the nonalkylating HN2-OH ($K_m = 2.50 \times 10^{-5}M$).

Some reduction in transport capacity may also occur, since V_{max} was reduced in cells first treated with HN2 ($V_{max} = 1.32 \times 10^{-17}$ mole/min per cell) compared to cells first treated with HN2-OH ($V_{max} = 2.45 \times 10^{-17}$ mole/min per cell), which is consistent with direct alkylation of the active site on the carrier. To avoid these complicating effects in subsequent experiments we employed the hydrolyzed derivative HN2-OH.

Choline transport was competitively inhibited by HN2-OH, ethanolamine, and hemicholinium-3 (Table 1). The hemicholinium-3 has been described as an inhibitor of choline transport in other tissues (*4*). Choline also served as a competitive inhibitor to the transport of [^{14}C]HN2-OH with a $K_i = 3.66 \times 10^{-5}M$, which was similar to the K_m obtained with choline as substrate. Similarly, the K_m for HN2-OH serving as substrate approximated the K_i for that agent acting as inhibitor ($K_m = 7.76 \times 10^{-5}M$ and $K_i = 5.36 \times 10^{-5}M$). This similarity of K_m and K_i for a compound acting as substrate or inhibitor confirms the competitive nature of the mutual inhibition between choline and HN2-OH. Nitrogen mustard, ethanolamine, and hemicholinium-3 also acted as competitive inhibitors of HN2-OH transport.

The above findings suggest that the entry of choline into L5178Y lymphoblasts occurs by a carrier mechanism and is an active process; furthermore, transport of HN2 and its hydrolyzed derivative appears to be mediated by this same carrier.

The observation that a toxic and unphysiologic compound (HN2) enters the cell on the transport-carrier for a natural substrate (choline), may be another example of a general pattern of drug transport. Similar interactions include active transport of 5-fluoro- and 5-bromouracil on the pyrimidine carrier in rat intestine (*5*), transport of 8-mercapto- and 8-bromoadenine on the purine carrier in polymorphonuclear leukocytes (*6*), methotrexate transport on the folic acid carrier in L1210 leukemia cells (*7*), and competitive inhibition of ouabain transport by steroid in rat liver slices (*8*). In each case the drug was a close structural analog of the natural substrate.

The structural similarity between choline, acetylcholine, and HN2 has been noted previously, and the cholinergic and paralytic effects of HN2 on the central and autonomic nervous system have been described (*9*).

In the field of cancer chemotherapy choline might serve as a protective agent against HN2. An immediate practical application could be the intravenous administration of choline to prevent systemic toxicity following the local administration of HN2 by regional perfusion or intracavitary instillation. Initial experiments indicate that choline protects L5178Y lymphoblasts against the cytocidal effect of HN2 in vitro. It may be possible to use choline in proper temporal sequence with HN2 in vivo to increase its therapeutic index by preferentially rescuing normal marrow stem cells. Such an approach in which the antimetabolite methotrexate has been used with its specific antidote, folinic acid, has resulted in more effective treatment of leukemia and cancer (*10*).

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11. Unlabeled HN2-hydrochloride (Mustargen) was supplied by Dr. W. Dorian (Merck, Sharp and Dohme, Dorval, Quebec). G.J.G. is a clinical research associate of the National Cancer Institute of Canada, and I.B. is a medical research associate of the Medical Research Council of Canada. Supported by a grant from the National Cancer Institute of Canada.

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