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MEMBRANE TRANSPORT OF MITOXANTRONE BY L1210 LEUKEMIA CELLS*

C. PATRICK BURNS,† BRADLEY N. HAUGSTAD and JAMES A. NORTH
Department of Medicine, University of Iowa College of Medicine, Iowa City, IA 52242, U.S.A.

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Abstract—Transport of radiolabeled mitoxantrone, a new antineoplastic agent, was studied using cultured mouse L1210 leukemia cells. The initial velocity of influx remained linear for about 90 sec and was 110 pmoles/10^6 cells measured at 60 sec. The steady-state accumulation of about 480 pmoles/10^6 cells was not reached until 30 min. The unidirectional drug influx was linear from 0 to 1000 μM extracellular drug concentration. The initial uptake was relatively temperature independent between 37° and 27°, but accumulation at steady state was 17% lower at 27°. None of six metabolic inhibitors had an appreciable effect on initial uptake. Efflux was initially exponential with a half-life of 2.8 min; this efflux and the residual drug concentration plateau were not affected by KCN or verapamil. Under steady-state conditions, about 86% of the cell-associated label was contained in parent drug and the remainder in an unidentified metabolite. These studies indicate that the mechanism of mitoxantrone uptake is passive diffusion. The efflux is not energy requiring, but there is considerable tight binding of the drug to cellular structures.

Mitoxantrone (1,4-dihydroxy-5,8-bis[(2-((2-hydroxyethyl)amino)ethyl)amino] 9,10-anthracenedione; dihydroxyanthracenedione) is a synthetic [1, 2] anticancer drug which binds to DNA and RNA [3] and interferes with nucleic acid synthesis [2]. DNA topoisomerase II has been implicated in the mechanism of action [4]. Mitoxantrone was of particular early interest because it showed differences in activity in a broad spectrum of animal tumor models as compared to doxorubicin [5]. It is currently undergoing clinical trials especially in advanced breast cancer, malignant lymphoma and acute leukemia.

There is limited information available regarding its cellular pharmacology. It is taken up by human colon carcinoma cells *in vitro*, and uptake is not affected by dinitrophenol or by the calcium channel blocker verapamil [6]. A resistant subline has less drug retention as compared to a sensitive line, and this is not due to differences in efflux [6]. Another study reported the uptake of mitoxantrone by mouse L-cells using spectrophotometry [7]. Uptake was a function of time (earliest time point of 5 min) up to about 20 min where it plateaued, and it was a function of concentration between 5 and 20 μM. When the cells were permeabilized with 0.1% Triton X-100, the uptake increased by about 60%. They concluded that cellular uptake is a major factor which determines biological activity of this drug.

No detailed investigation of the mechanism of cellular drug uptake has been reported. Because of the potential clinical importance of the drug, we undertook a study to determine the basic mechanism of drug transport.

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† To whom correspondence should be addressed.

MATERIALS AND METHODS

L1210 murine leukemia cells were grown and maintained in suspension culture at 37° in medium consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), 5% fetal bovine serum (Hazleton Research Products, Denver, PA) and gentamicin sulfate (40 μg/ml) in a humid atmosphere of 5% CO_2:95% air. These cells were originally obtained from Dr. David Vistica of the National Institutes of Health. Cells were harvested during exponential growth phase and were resuspended in balanced salt solution (BSS; 132 mM NaCl, 16 mM Na_2PO_4, 5 mM KCl, 1 mM $MgSO_4$, 5.6 mM glucose, pH 7.4) for transport studies.

[^{14}C]Mitoxantrone was supplied by Dr. B. M. Silber, American Cyanamid Co., Pearl River, NY. It was received as a powder (40 Ci/mole) and was constituted with distilled water and frozen at −70° in aliquots until use. Purity after thawing and just prior to use was 99% as determined in our laboratory using high performance liquid chromatography (HPLC) [8].

Transport studies were performed by modification of techniques previously described from our laboratory [9, 10]. Studies were carried out in small vessels containing 1×10^6 cells in 0.5 ml BSS. After temperature equilibration, [^{14}C]mitoxantrone was added. In some experiments unlabeled drug was added to adjust the concentration. At the termination of incubation, the cell suspension was transferred to small tapered centrifuge tubes at 0° containing 0.25 ml *n*-butyl phthalate–corn oil (2.75:1, v:v) layered beneath 0.3 ml BSS. Rapid separation of the cells from incubation medium was accomplished by centrifugation immediately at 15,600 *g* for 30 sec in an Eppendorf 5412 centrifuge. The supernatant fraction was then aspirated, and the tip of the 1.5-ml tapered microfuge tube containing the cell pellet was severed and placed in a scintillation vial containing 0.5 ml Soluene-350 (Packard

Instruments Co., Downers Grove, IL). Neutralizer scintillation mixture (Research Products International, Elk Grove Village, IL) was added, and radioactivity was determined using a Beckman LS3133T scintillation spectrometer. Drug uptake at each concentration was corrected for rapid association with the plasma membrane which was estimated by measuring cell association of [^{14}C]mitoxantrone at 0° in a time interval of less than 3 sec.

The effects of six metabolic inhibitors on drug uptake were studied by incubating the cells with the inhibitor for 10 min at 37° prior to the addition of [^{14}C]mitoxantrone. These uptake studies with inhibitors were performed in phosphate-buffered saline without glucose.

Efflux studies were performed using L1210 cells preloaded with [^{14}C]mitoxantrone (4 μM, 30 min, 37°), which were then rapidly chilled and washed at 0°. They were resuspended in BSS at 37° to initiate efflux. Aliquots removed prior to efflux were used to estimate initial intracellular drug concentration.

The determination of intracellular metabolites was done by incubating cells with [^{14}C]mitoxantrone for 30 min at a 4 μM concentration of drug. The cells were then rapidly chilled at 0°, washed with ice-cold BSS, and disrupted in one of three ways: (a) osmotic lysis by adding water at 0° for 20 min; (b) adding ice-cold *n*-butanol to extract mitoxantrone bound to DNA [11]; (c) adding ice-cold 10% trichloroacetic acid. We then analyzed the supernatant fractions by HPLC to determine the percentage of mitoxantrone and metabolites. HPLC analysis was performed using a modification of reported methods [8]. The apparatus consisted of a Beckman model 110B delivery module, a 420 controller and a 210A sample injection valve. A Vydac TP 10 micron reverse phase column (particle size 40 μm; 25 cm × 4.6 mm i.d.) preceded by an Uptight (Upchurch Scientific Co., Oak Harbor, WA) guard column packed with pellicular C_{18} was used for analysis. Samples containing [^{14}C]mitoxantrone were spiked with cold drug and were then eluted isocratically with acetonitrile–0.2 M ammonium acetate (25:75), pH 4.0, at a flow rate of 1 ml/min. Drug elution was detected on a Beckman 163 Variable Wavelength Detector at 658 nm and recorded on a 3390A Hewlett-Packard integrator. Fractions were collected, and radioactivity was determined. In all studies, results were evaluated using a two-tailed *t*-test and were considered significant at $P < 0.05$.

RESULTS

Time course of mitoxantrone uptake. A time course of uptake of 4 μM [^{14}C]mitoxantrone by L1210 cells *in vitro* is shown in Fig. 1. After initial rapid binding, the early uptake was approximately linear for 90 sec (inset). Accordingly, later kinetic experiments were conducted using time intervals of 60 sec so that only unidirectional drug influx was measured. The initial velocity of influx measured at 60 sec was 110 pmoles/10^6 cells. The slope of the linear regression analysis was 56.35 pmoles/10^6 cells per min. Uptake entered a steady-state phase of intracellular accumulation of about 480 pmoles/10^6 cells which began at about 30 min and persisted to at least 120 min.

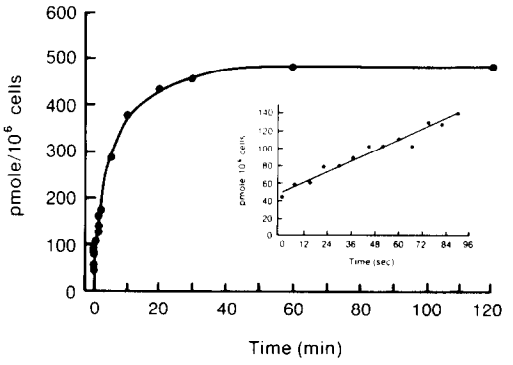

Fig. 1. Time course of mitoxantrone uptake by L1210 cells at 37°. Cells were incubated with 4 μM [^{14}C]mitoxantrone in balanced salt solution. At the termination of incubation, the cells were rapidly separated by centrifuging through an oil layer, and radioactivity of the cell pellet was determined. Each point represents the mean of six experiments. The inset shows uptake at multiple time points prior to 90 sec.

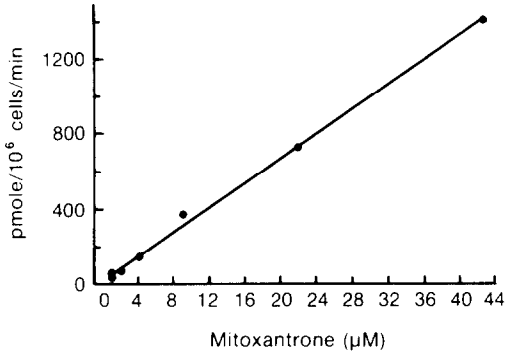

Fig. 2. Relationship of influx and extracellular concentration of mitoxantrone. Cells were incubated at various concentrations using a 60-sec initial uptake time. Each point is the mean of five experiments and is corrected for rapid binding. The equation for the linear regression analysis is $Y = 32.2X + 22.2$ ($r = 0.999$).

Rapid association of drug to cell membrane occurred and was evident by extrapolation of the time–course curve to the y-axis (Fig. 1, inset). This calculation yielded a value of 50 pmoles/10^6 cells. The temperature-independent rapid binding can also be estimated by determining uptake at 0° at as short a time point as technically feasible. Such a determination yielded a value of 44 pmoles/10)^6 cells which is similar to the calculated value. In all kinetic experiments, a 0° rapid value was determined experimentally at each concentration and subtracted from the total uptake in order to estimate intracellular uptake.

Kinetic analysis of mitoxantrone influx. The 60-sec unidirectional influx of mitoxantrone was measured over a concentration range of 1–44 μM (Fig.2). There is approximate linearity ($r = 0.996$) in this range, which encompasses the pharmacologic clinical plasma concentrations [12], estimated tissue concentrations [13], and concentration in effusions after intraperitoneal injection [12]. The line passes

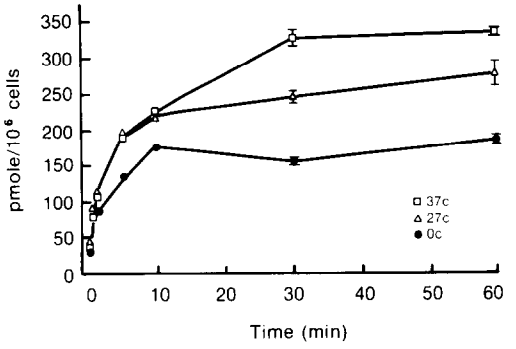

Fig. 3. Effect of temperature on mitoxantrone uptake. L1210 cells were incubated with 4 μM mitoxantrone at various temperatures. Points are the mean of triplicate determinations in a representative experiment.

through the origin, suggesting that there is no undetected high-affinity system that is saturated at the lowest concentrations used.

Uptake was determined over a wider concentration range (1, 10, 100, 250, 500, 800, 1000 μM) in selected studies. For these observations a time of 20 sec was selected in order to measure unidirectional drug influx at these higher concentrations. Studies were performed at both 37° and 0°, the latter to slow uptake and maximize any chance of observing saturation kinetics. At both temperatures, mitoxantrone uptake was a linear function of concentration up to the highest concentration studied (1 mM).

Temperature sensitivity of mitoxantrone influx. Rate of unidirectional influx at 60 sec was not temperature sensitive between 37° and 27°. In contrast, when drug accumulation at steady state was examined, there was appreciably less intracellular mitoxantrone at lower temperatures (Fig. 3). This could be due to less binding to intracellular structures at 27° and 0° as compared to 37°.

Effects of metabolic inhibitors on mitoxantrone uptake. The effects of metabolic inhibitors on mitoxantrone influx by L1210 cells are shown in Table 1. Cells were preincubated with the inhibitor for 10 min prior to the addition of labeled drug. No inhibitor had an appreciable effect on drug uptake.

Efflux of exchangeable mitoxantrone. We next examined the rate of efflux of mitoxantrone from L1210 cells that were preloaded with drug. Initial efflux was rapid at 37° and was approximately exponential for the first 60 sec (apparent first-order rate constant = $4.1 \times 10^{-3} \cdot \text{sec}^{-1}$) with a half-life of 2.82 min (Fig. 4). It then decreased rapidly, and only 30% had effluxed during the first 5 min; there was a subsequent plateau at about 50% retention. After 30 min the efflux becomes negligible, indicating that a major component of intracellular drug was tightly bound. This irreversible binding roughly correlated with retention of 43% radioactive label in the precipitate after 10% trichloroacetic acid treatment. The rate of efflux and plateau were not affected by 16 μM verapamil (Fig. 4) or 0.1 mM KCN (48% retained at 60 min). At 0° there was slow efflux; only about 17% of the drug had effluxed at 60 min (Fig. 4).

Table 1. Effects of metabolic inhibitors on [^{14}C]mitoxantrone influx by L1210 cells *in vitro*

Inhibitor	Concentration of inhibitor (mM)	Percentage of control (%)
Tunicamycin	0.012	132 ± 9
Antimycin A	0.01	101 ± 9
Verapamil	0.016	96 ± 6
Iodoacetic acid	1.00	95 ± 7
KCN	0.10	93 ± 4
NaF	10.00	87 ± 6

Cells were preincubated with inhibitor in glucose-free medium for 10 min at 37°, and then 5 μM [^{14}C]mitoxantrone was added and the 60-sec uptake was determined. Values are expressed as a percentage of influx in the absence of inhibitor and are the mean of three to six determinations. All are corrected for rapid binding. The absolute values for uptake in the controls ranged from 120 to 200 pmoles/10^6 cells/min in this series of inhibitor experiments.

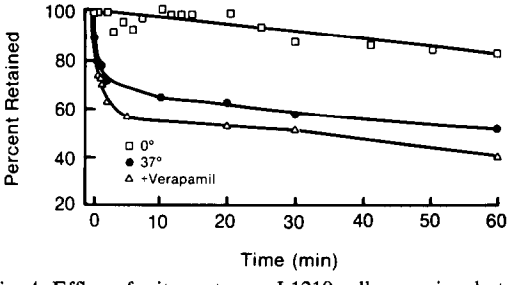

Fig. 4. Efflux of mitoxantrone. L1210 cells were incubated with 4 μM [^{14}C]mitoxantrone for 30 min at 37°. Cells were then chilled to 0° and washed. Efflux was measured by determining residual radioactivity after various times at 37° or 0°. For some studies, efflux was measured at 37° in the presence of 16 μM verapamil.

Intracellular metabolism of mitoxantrone. L1210 cells were preloaded with [^{14}C]mitoxantrone and washed at 0°. The cells were treated with one of the following to release drug: cold distilled water, 10% trichloroacetic acid or *n*-butanol for 20 min. The soluble fractions containing released drug were obtained by centrifugation (1000 *g* for 15 min) and contained 37% (water), 57% (trichloroacetic acid) or 71% (*n*-butanol) of the recovered radioactivity respectively. Each supernatant fraction was spiked with cold mitoxantrone and analyzed by HPLC. Regardless of the treatment used to release drug from the cells, the majority of the radioactivity coeluted with mitoxantrone at 3.8 min (*n*-butanol 84%, trichloroacetic acid, 87%, water 85%). In each there was a second labeled compound detected at about 10 min. It contained 13–16% of the radioactivity and likely represented a mitoxantrone metabolite.

DISCUSSION

Unidirectional influx of mitoxantrone was relatively slow, reaching steady-state conditions after about 30 min. The process by which the drug entered the L1210 cells was nonsaturable when analyzed over

the pharmacologic range of drug concentration (1–1000 μM) used in this study. This suggests that the mechanism of uptake was passive diffusion; the findings that transport was not impeded by a wide choice of metabolic inhibitors and was only partially temperature dependent are consistent with diffusion. Other anticancer drugs such as nitrosoureas [14], chlorambucil [15], busulfan [16], procarbazine [17], hexamethylmelamine [18] and hydroxyurea [19] enter by such a mechanism.

An alternative argument could be made that the uptake of the mitoxantrone was by facilitated diffusion. The drug binds to cellular components within the cell, and this may facilitate diffusion across the membrane. In fact, concentration studies below the K_m of such a process would give results suggestive of passive diffusion. We believe that the data best fit passive diffusion for the following reasons: (1) The uptake of drug as a function of concentration remained linear over a broad concentration range up to 1 mM. This concentration was 500-fold greater than that achieved in the plasma of humans after intravenous injection and 10- to 20-fold greater than that in peritoneal fluid after intracavitary injection of mitoxantrone [12]. (2) The temperature dependence of the uptake of mitoxantrone was limited as compared to other drugs which are taken up by facilitated diffusion such as cytosine arabinoside and 5-fluorouracil [20, 21] and more like that of drugs transported by passive diffusion such as procarbazine, busulfan, chlorambucil or hydroxyurea [15–17, 19].

Although we did not determine intracellular drug concentration directly, we can estimate the amount of drug available to cytosol. With an intracellular water of 0.247 μl/10^6 L1210 cells [22], it can be calculated from our results that intracellular mitoxantrone concentration is 3.8 μM at steady state in medium containing 4 μM drug. This cell to medium drug concentration ratio of near unity is compatible with influx by passive diffusion. If this estimation is correct, it also suggests that there is no appreciable electrochemical gradient for exchangeable drug across the cell membrane. However, it should be noted that the calculation does not take into account the fact that a portion of the mitoxantrone was not in true aqueous solution.

Efflux of exchangeable drug was rapid for about 60 sec, but then the rate quickly decreased and about half of the intracellular drug remained within the cell even after 60 min. The lack of effect of KCN and verapamil suggests that the mechanism of efflux is not energy requiring. An appreciable proportion of cellular [^{14}C]mitoxantrone was trichloroacetic acid, water or *n*-butanol insoluble so it may be bound to cellular structures. The exact nature of the binding of mitoxantrone is not known. It is fairly tight as suggested by failure to solubilize all of the radioactivity after precipitation of nucleic acids and proteins by trichloroacetic acid. The non-exchangeable drug is likely non-covalently bound to DNA or other macromolecules. In this regard, Taylor *et al.* [23] have demonstrated that aminoanthraquinones bind to two distinct low molecular weight macromolecular lipids of L1210 cells. The cell-associated drug may be binding to one or both such macromolecules. Two binding sites with different affinities may relate to the partial retention demonstrated in efflux studies or the temperature-dependent uptake.

REFERENCES

1. K. C. Murdock, R. G. Child, P. F. Fabio, R. B. Angier, R. E. Wallace, F. E. Durr and R. V. Citarella, *J. med. Chem.* **22**, 1024 (1979).
2. R. K. Johnson, R. K. Zee-Cheng, W. W. Lee, E. M. Acton, D. W. Henry and C. C. Cheng, *Cancer Treat. Rep.* **63**, 425 (1979).
3. J. Kapuscinski, Z. Darzynkiewicz, F. Traganos and M. R. Melamed, *Biochem. Pharmac.* **30**, 231 (1981).
4. K. M. Tewey, T. C. Rowe, L. Yang, B. D. Halligan and L. F. Liu, *Science* **226**, 466 (1984).
5. R. K. Johnson, M. G. Broome, W. S. Howard, S. F. Evans and D. F. Pritchard, in *New Anticancer Drugs: Mitoxantrone and Bisantrene* (Eds. M. Rozencweig, D. D. Von Hoff and M. J. Staquet), p. 1. Raven Press, New York (1983).
6. K. C. Murdock, R. E. Wallace, R. J. White and F. E. Durr, in *The Current Status of Novantrone* (Ed. C. A. Coltman), p. 3. Park Row Publishers, John Wiley & Sons Medical Group Co., New York (1985).
7. A. Nishio and E. M. Uyeki, *Cancer Res.* **43**, 1951 (1983).
8. Y. M. Peng, D. Ormberg, D. S. Alberts and T. P. Davis, *J. Chromat.* **233**, 235 (1982).
9. C. P. Burns, D. G. Luttenegger, D. T. Dudley, G. R. Buettner and A. A. Spector, *Cancer Res.* **39**, 1726 (1979).
10. C. P. Burns and D. T. Dudley, *Biochem. Pharmac.* **31**, 2116 (1982).
11. G. T. Bowden, R. Roberts, D. S. Alberts, Y. Peng and D. Garcia, *Cancer Res.* **45**, 4915 (1985).
12. D. S. Alberts, Y. M. Peng, G. T. Bowden, W. S. Dalton and C. Mackel, *Investl New Drugs* **3**, 101 (1985).
13. G. Ehninger, B. Proksch, G. Heinzel, E. Schiller, K. Weible and D. L. Woodward, *Investl New Drugs* **3**, 109 (1985).
14. A. Begleiter, H. P. Lam and G. J. Goldenberg, *Cancer Res.* **37**, 1022 (1977).
15. B. T. Hill, *Biochem. Pharmac.* **21**, 495 (1972).
16. D. Kessel, M. Myers and I. Wodinsky, *Biochem. Pharmac.* **18**, 1229 (1969).
17. H. P. Lam, A. Begleiter, W. Stein and G. J. Goldenberg, *Biochem. Pharmac.* **27**, 1883 (1978).
18. A. Begleiter, J. Grover and G. J. Goldenberg, *Cancer Res.* **40**, 4489 (1980).
19. J. S. Morgan, D. C. Creasey and J. A. Wright, *Biochem. biophys. Res. Commun.* **134**, 1254 (1986).
20. D. Kessel and S. B. Shurin, *Biochim. biophys. Acta* **163**, 179 (1968).
21. G. J. Goldenberg and A. Begleiter, in *Handbook of Experimental Pharmacology* (Eds. B. W. Fox and M. Fox), Vol. 72, p. 241. Springer, Berlin (1984).
22. F. M. Sirotnak, S. Kurita and D. J. Hutchison, *Cancer Res.* **28**, 75 (1968).
23. R. F. Taylor, L. A. Teague and D. W. Yesair, *Cancer Res.* **41**, 4316 (1981).