Biochemical Pharmacology, Vol. 21, pp. 2681–2695. Pergamon Press, 1972. Printed in Great Britain

UPTAKE AND INTRACELLULAR DISTRIBUTION OF 4,4′-DIACETYLDIPHENYLUREA-BIS-(GUANYLHYDRAZONE) IN SENSITIVE AND RESISTANT LEUKEMIA L1210 CELLS*

C. DAVE† and E. MIHICH

Department of Experimental Therapeutics, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, N.Y. 14203, U.S.A.

(*Received* 16 *March* 1972; *accepted* 21 *April* 1972)

Abstract—The uptake and intracellular distribution of 4,4′-diacetyldiphenylurea-bis(guanylhydrazone) has been studied *in vitro* and *in vivo* using cells from leukemia L1210 and a subline resistant to the drug. The uptake process involved a very rapid adsorption, presumably to the cell exterior, followed by actual cellular uptake and firm binding of the drug to the cell. Both adsorption and binding occurred concurrently, although within the first few minutes adsorption predominated. The uptake exhibited first-order kinetics over a 10,000-fold range of external drug concentration with a k_{37} of about 0·5 min^{-1} and a Q_{10} of about 2·0. The intracellular-free drug concentration was found to be nearly equal to the extracellular drug concentration. Uptake was not decreased in the presence of sodium *para*-chloromercuribenzoate, 2,4-dinitrophenol, of 4,4′-diacetyldiphenylurea-mono(guanylhydrazone), or when cells were incubated in Ringer solution without calcium and glucose or killed by X-radiation. These findings support the conclusion that 4,4′-diacetyldiphenylurea-bis(guanylhydrazone) is taken up by passive diffusion.

In resistant L1210 cells, the different parameters of uptake studied were uniformly 30–50 per cent lower than those found in sensitive L1210 cells; also in this case the intracellular-free drug concentration was nearly the same as the extracellular concentration.

A large proportion of the drug taken up was bound to mitochondrial and nuclear fractions. The cellular uptake appeared to be rate-limited by binding or uptake within cellular compartments. The reduced rate of uptake in resistant L1210 was consistent with resistance and appeared to be related to alterations in the binding capacity of cellular organelles.

AMONG antileukemic bisguanylhydrazones, 4,4′-diacetyldiphenylurea-bis(guanylhydrazone) (DDUG) has the greatest activity against several mouse leukemias and tumors.[1,2] The clinical potentiality of this group of compounds is indicated by the antileukemic activity of methylglyoxal-bis(guanylhydrazone).[3,4] The chemotherapeutic effects of DDUG have not yet been thoroughly assessed in man. The mode of action of bisguanylhydrazones, in general, and of DDUG in particular has not yet been elucidated. Although drug kinetics have been studied with S-180 cells in culture,[5,6] the uptake and intracellular distribution of DDUG in target leukemic cells were unknown. This study was undertaken as a step toward the understanding of the mechanism of chemotherapeutic action of this drug. Mouse leukemia L1210 was chosen as the cell

* Supported in part by Research Grant CA-10805 from the National Cancer Institute, United States Public Health Service.

† The work reported herein was submitted in partial fulfillment of the requirements for the Ph.D. degree, State University of New York at Buffalo, U.S.A.

model in view of the known sensitivity of this leukemia to DDUG.[7,8] The results in sensitive cells were compared with those in cells from a resistant subline (L1210/DDUG).[9] Part of the data has been reported in preliminary communications.[9–11]

MATERIALS AND METHODS

Chemicals. The bismethanesulfonate salts of unlabeled DDUG and of DDUG uniformly labeled in its phenyl rings with ^{14}C (sp. act. 6·0 mc/m-mole) were obtained from Drs. A. Marxer and K. Schmid, CIBA Research Laboratories, Basel, Switzerland. The purity of DDUG and ^{14}C-DDUG was checked periodically by thin-layer chromatography on Silica gel coated "chromatogram" sheets (Eastman Kodak, Rochester, N.Y.), using three different solvent systems, namely, (A) dioxane–methanol–ammonia, s.g. 0·88 (10:20:1); (B) methyl cellosolve–ethyl acetate–acetone–triethylamine–water (3:3:2:1:1); and (C) methyl cellosolve–propionic acid–water (70:15:15). The R_f values of DDUG in solvents A, B and C are 0·22, 0·26 and 0·78 respectively. In each system the compound was found to be authentic and more than 99 per cent pure. At room temperature, DDUG in solution was stable for at least 3 weeks. A 10^{-3} M solution of DDUG could be stored in deionized water at −20° for several months without change. 4,4′-Diacetyldiphenylurea-mono(guanylhydrazone) methanesulfonate (DDUM) was obtained from CIBA Research Laboratories, Basel, Switzerland. Sodium *para*-chloromercuribenzoate (PCMB) and 2,4-dinitrophenol (DNP) were purchased from Nutritional Biochemicals, Cleveland, Ohio.

Glassware. DDUG was significantly adsorbed to a variety of plastic and glass containers.[12] Siliconization of glassware reduced such adsorption to a level compatible with 95 per cent recovery of drug in solution, and was therefore carried out throughout this study.

Leukemia cells. Leukemia L1210 was originally obtained from Dr. A. Goldin, National Cancer Institute.[2] The resistant subline L1210/DDUG was developed in this laboratory.[8] Both tumors were maintained by weekly, i.p., transplantation in female DBA/2J mice. For both *in vivo* and *in vitro* tests, 2×10^6 leukemic cells were inoculated, i.p., 3 days prior to use. Under these conditions, the leukemic cell population was least contaminated with erythrocytes.[12]

For the experiments *in vitro*, leukemic cells were separated from erythrocytes by differential centrifugation at 110 *g* for 5 min in a PR-2 model International refrigerated centrifuge. The cells were resuspended as required. For the tests *in vivo*, DDUG was injected, i.p., into leukemia-bearing mice.

Uptake determinations. Two types of determinations were carried out: (A) measurement of the total uptake indicated by the drug found in the cell pellet less the amount of drug present in the extracellular space; (B) measurement of the portion of DDUG that is firmly bound to cells carried out as above, but only after the cells were washed once with drug-free medium. In both cases the wet weight of the leukemic cell pellet was corrected for extracellular fluid.

Determination in vitro *of total uptake.* Prewarmed leukemia cell suspensions were mixed with prewarmed ^{14}C-DDUG solutions, both prepared in the same medium such that when mixed together the desired final concentrations of cells ($0·5 \times 10^7$ cells/ml) and drug would be attained. The media used were either Ringer solution without Ca^{2+} and glucose,* or RPMI 1640 with 10% calf serum (RPMI 1640 CS).[13]

* NaCl (0·9%), KCl (0·024%), $NaHCO_3$ (0·02%), pH 7·4.

The medium to cell volume ratio was kept at 250 to maintain the initial drug concentration in the medium unchanged for a workable period of time. During incubation, the suspension was gently mixed in a Dubnoff metabolic shaker. Eight-ml aliquots of cell suspension were pipetted in tared, prewarmed, 15-ml centrifuge tubes about 1 min before the termination of the incubation period. At the end of the incubation, the tubes were immediately placed in a refrigerated centrifuge at 0–4° and spun for 5 min at 900 *g*. All subsequent operations were carried out at 0–4° unless otherwise specified. The supernatant containing ^{14}C-DDUG was always assayed for drug recovery. The top of the pellet and walls of the tube were rinsed once with 5–7 ml of cold drug-free medium, to be discarded, and were then dried using cotton swabs and pieces of filter paper. In a control experiment not involving any cells all the radioactivity was removed by such procedure. After equilibration to room temperature, the pellet was weighed using a semi-micro Sartorius balance. It was resuspended in 1 ml of water and quantitatively transferred to a counting vial containing 10 ml of dioxane scintillation fluid,[14] to which 3% Cab-O-Sil[15] had been added. Radioactivity was determined in a Packard liquid scintillation β-spectrometer and counts were corrected for quenching by the channel ratio method.[16]

Determination in vitro *of drug bound to cells.* For this determination the same procedure was followed as described for total uptake, except that at the end of the incubation, cell aliquots were spun for 5 min at 225 *g*. The 225 *g* pellet was resuspended in 5 ml drug-free medium and immediately recentrifuged at 900 *g* for 5 min. The supernatant was discarded and the pellet was then processed as described above for the total uptake determination.

Correction for extracellular fluid. Using ^{14}C-inulin, it was found in 17 determinations that, after centrifugation at 900 *g*, the cell pellet contained 22·7 $\pm$ 0·6 (S. E.) per cent extracellular fluid regardless of the cell line used, the time of centrifugation between 30 sec and 10 min, the weight of the pellet between 15 and 200 mg, with or without prior washing and centrifugation at 225 *g* and with or without prior treatment with 10^{-5} M DDUG for 60 min. Based on these data, pellet weights were reduced by 23 per cent. Radioactivity in the extracellular fluid was considered equivalent to that present in the supernatant after centrifugation at 900 *g*. The specific activities of DDUG in the pellet were also corrected for the presence of radioactivity in the 23 per cent extracellular fluid.

Effect of DDUG treatment and of centrifugation on L1210 cells. The L1210 cells weighed 0·86 mg $\pm$ 0·02 (S. E., 7 determinations)/10^6 cells, whereas the L1210/DDUG cells weighed 0·79 mg $\pm$ 0·04 (S. E., 5 determinations)/10^6 cells. These weights, DNA content (see below) and cell diameter remained unchanged during the uptake determinations. Three repeated centrifugations at 900 *g* and washings did not alter cell transplantability. In RPMI 1640 CS medium, the cells excluded trypan blue during the period of DDUG uptake. In Ringer solution without Ca^{2+} and glucose, cells formed minute clumping at around 60 min of incubation at 37°.

Preparation of dead cells. "Dead" cells were obtained by exposure of live cells to 5000 roentgen. Cell suspensions in Ringer at 0–4°, contained in plastic petri dishes, were exposed to X-irradiation from a G.E. Maxitron X-ray machine, model 250. The machine was set at 100 kV and 30 mA with 0·5 mm aluminum filter. At the target distance of 15 cm, the rate of irradiation in air was 2350 roentgen/min. When incubated with trypan blue in RPMI 1640 CS at 37° for 10–15 min, no unstained cell could be

detected. When transplanted, i.p., in DBA/2 mice (10^6 cells/mouse), they failed to cause death within 60 days.

Measurement of drug efflux and exchange. Cells, preincubated with ^{14}C-DDUG under specified conditions, were then centrifuged for 5 min at 225 *g* and incubated with fresh medium with or without unlabeled DDUG. The amount of ^{14}C-DDUG remaining in the cells at different times were determined as described above for total uptake.

Uptake studies in mice. Fifteen and 30 mg/kg of DDUG, containing varying quantities of ^{14}C-DDUG, were injected, i.p., into leukemia-bearing mice. Mice were sacrificed at predetermined times, 3 ml of cold RPMI 1640 was injected i.p., and cells were quickly withdrawn from the intraperitoneal cavity and collected in tared 15-ml centrifuge tubes kept at 0–4°. The DDUG bound to these cells was determined as described above.

Intracellular distribution of DDUG. Leukemic cells (5×10^6/ml) were incubated with 10^{-6} M ^{14}C-DDUG in RPMI 1640 CS for a period of 5 or 30 min at 37°, as described for total uptake. At the end of incubation, the cells were centrifuged at 225 *g* for 5 min. Osmotic shock was then applied by resuspending the cells in 0·05 M hypotonic Tris–chloride buffer at 0–4° (pH 7·6 at 0°). This buffer contained 0·005 M magnesium chloride. The cells were centrifuged again at 225 *g* for 10 min, resuspended in Tris buffer to a concentration of $2{\cdot}5 \times 10^7$ cells/ml at room temperature (22–24°), allowed to stand for 10 min and centrifuged at 500 *g* for 5 min. These centrifugations and the other steps outlined below were performed at 0–4°. The supernatant was collected and analyzed separately. The pellet was quickly resuspended in the original volume of buffer and homogenized (40 strokes) using a Dounce homogenizer with a tight-fitting pestle. Immediately thereafter 80% (w/v) sucrose solution was added to make 10% (w/v) or about 0·35 M sucrose solution. The homogenate was examined directly under phase-contrast microscope, and also under fluorescent microscope after staining with acridine orange. By the procedure outlined large membrane fragments and intact nucleii were obtained with least cytoplasmic material and minimum debris. About 60–70 per cent of intact nucleii could be counted. The remaining cells were either intact or incompletely homogenized.

The homogenate was centrifuged at 900 *g* for 10 min. From the supernatant, mitochondria were separated as the 10,000 *g* pellet and microsomes as the 100,000 *g* pellet.[12] In each case the centrifugation was carried out for 1 hr. Each of the pellets was resuspended in Tris buffer containing 1·13 M KCl, and recentrifuged. The first 100,000 *g* supernatant fluid (S_{100}) was also retained for analysis.

A procedure based upon isopycnic centrifugation[17] was developed[12] to separate various cellular fractions present in the 900 *g* pellet. The pellet was resuspended in Tris buffer containing 10% sucrose and layered on top of a density gradient containing one layer each of equal volumes of 35, 45 and 55% (w/v) sucrose solution in Tris buffer. This was centrifuged at 900 *g* for 15 min; the bands of cellular material sedimenting at the junctions of 10–35 (35 S), 35–45 (35R) and 45–55 (45R)% sucrose layers were aspirated. The pellet (55R) formed at the bottom of the tube itself was also separated. Each fraction was diluted to about 10% sucrose concentration and separately relayered on the same density gradient, recollected from the appropriate density region and pooled with comparable fractions. The fractions were once again diluted to about 10% sucrose concentration, centrifuged at 900 *g* in tared tubes and weighed

as described for the uptake procedures. The 35 S fraction consisted mainly of broken membrane fragments, the 35 R of whole or nearly intact membrane ghosts, the 45 R of incompletely broken or intact cells and considered as "broken" cells and the 55 R of intact nucleii. The identification was carried out by phase-contrast and fluorescent microscopy.

Measurements of total DNA,[18] RNA,[18] protein[19] and lipid[20,21] contents were made on whole cells and on the various cellular fractions obtained. The L1210 cells contained 9·4 ± 1·1 μg DNA, 15·2 ± 1·5 μg RNA, 119·9 ± 15·7 μg protein and 27·5 ± 0·9 μg lipid/10^6 cells (S. E., 5 determinations). Corresponding values were similar for L1210/DDUG cells, except that the protein content was about half of that found in L1210 cells. No gross changes occurred in these values as a result of osmotic shock. Only the nuclear fraction (55 R) contained detectable DNA. Considerably higher concentrations of RNA were found in the microsomal fraction and the S_{100} supernatant than in the other fractions. The lipid content in the S_{100} supernatant was two orders of magnitude greater than that in nucleii.

Identity of "cell-bound" radioactivity. ^{14}C-DDUG (6 mg/kg) was injected, i.p., into mice bearing L1210 or L1210/DDUG 1 hr prior to sacrifice. Leukemia cells were collected as described above for the studies *in vivo*, and the pellet was resuspended in 1 ml of water. Nearly 90 per cent of the cell-associated radioactivity was recovered in three consecutive extractions with cellosolve at room temperature. The pooled extracts were chromatographed as described above and gave only one radioactive spot which co-chromatographed with authentic samples of DDUG.

RESULTS

Experiments in vitro. The results indicate that the total uptake of DDUG by L1210 or L1210/DDUG cells occurs according to the following sequence of events. Initially the drug is rapidly adsorbed on the cells. Drug entry into cells follows at a relatively slower rate and takes place by passive diffusion. This diffusion seems to be rate-limited by the subsequent firm binding of DDUG to cell organelles. Consistently, the uptake of the drug was less in the resistant than in the sensitive cells.

The uptake of DDUG by L1210 and L1210/DDUG cells is shown in Fig. 1. In this and three other experiments, the same batches of cells were used for the measurement of total uptake and bound drug. The total uptake of DDUG was characterized, in each case, by a very rapid phase. Subsequent to this phase, the uptake was linear as a function of time for at least 60 min in L1210 cells. The total uptake in L1210/DDUG varied during the first hour of incubation. This was seen in three out of five experiments and may represent experimental variations. The proportion of drug that could be washed (see below) relative to the total uptake was considerably higher at the earlier times of incubation.

Evidence for adsorption. At the concentration of 1 $\mu\mu$mole of drug/μl of medium, the total uptake was 18 $\mu\mu$moles of drug/mg of L1210 cells at 4 min and about 80 $\mu\mu$moles/mg at 120 min (Fig. 1). Bound drug values were 6 $\mu\mu$moles/mg at 4 min and 65 at 120 min. Thus, the difference between total and bound drug was 12–15 $\mu\mu$moles/mg both at 4 and 120 min. A constant of similar magnitude was also obtained at 0° (data not shown). At the same drug concentration in the medium, the total uptake in L1210/DDUG cells was about 10 $\mu\mu$moles/mg at 4 min and 42 at 120 min; bound

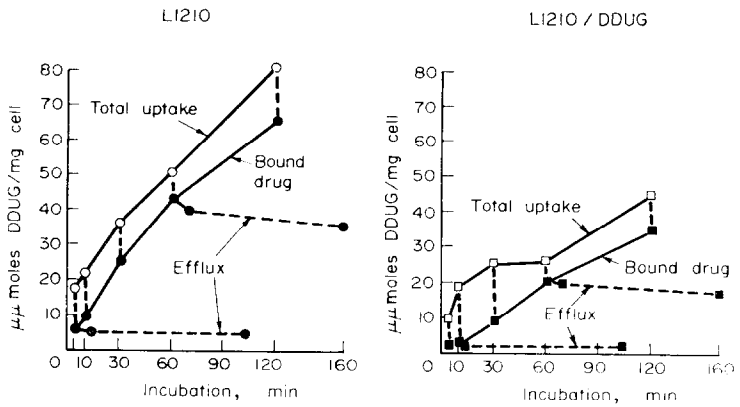

FIG. 1. Uptake and efflux of DDUG. Leukemic cells (5×10^6/ml) were incubated with ^{14}C-DDUG at 10^{-6} M concentration in RPMI 1640 CS medium at 37°. At the indicated time points, aliquots of cell suspensions were withdrawn to measure total uptake (○——○ and □——□). Other aliquots taken from the same flask at each of these time points were centrifuged, resuspended in DDUG-free medium, and incubated at 37°. The drug remaining in the cells was determined at various times after this reincubation. Bound drug is shown by ●——● and ■——■. Efflux is shown by ●- - -● and ■- - -■.

drug values were 2 μμmoles/mg at 4 min and 35 at 120 min. Thus, in this case, the difference was a constant of 7–8 μμmoles of drug/mg of cells.

As shown in Fig. 1, the amount of drug lost from cells on a single transfer into drug-free medium was significant in comparison to the concentration in extracellular fluid. On further incubation in drug-free medium at 37°, however, only a small proportion of the remaining drug, if any, was released from cells, as shown by the "efflux" lines. The remaining drug was associated with the cells even after three successive washings in drug-free medium, regardless of the time and temperature of previous incubation with DDUG. The cell-bound ^{14}C-DDUG could not be exchanged with unlabeled drug

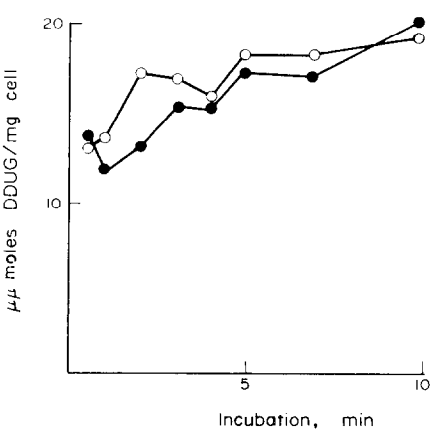

FIG. 2. Uptake of DDUG within 10 min. Total uptake of ^{14}C-DDUG at 10^{-6} M concentration by L1210 cells (5×10^6/ml) incubated in RPMI 1640 CS at 37° (● and ○ correspond to two different experiments).

when the cells were transferred to a medium containing a 100 times higher concentration of unlabeled DDUG (data not shown).

To study the rapid phase of uptake, the total uptake of DDUG at 10^{-6} M by L1210 cells was measured at very short intervals (Fig. 2). At 4 min, the rapid rate of uptake clearly decreased. Measurements of total DDUG uptake by L1210 and L1210/DDUG at 4 min are shown in Table 1. Considerable differences were observed between sensitive and resistant cells. At 37° in RPMI 1640 CS medium, the difference was statistically significant at the 5 per cent probability level by the Student *t*-test.[22]

TABLE 1. RAPID PHASE OF UPTAKE OF DDUG BY L1210 CELLS *in vitro* UNDER DIFFERENT CONDITIONS OF INCUBATION*

Cells	Incubation medium	Total uptake of DDUG at 4 min ($\mu\mu$mole/mg cell ±S. E.) 0°	37°
L1210	RPMI 1640 CS	11·4 ± 2·1 (6)	15·0 ± 2·4 (16)
	Ringer†	16·1 (1)	25·0 ± 4·1 (4)
L1210/DDUG	RPMI 1640 CS	5·2 (2)	8·9 ± 0·5 (3)

* The total uptake of ^{14}C-DDUG (at 10^{-6} M) by leukemia cells ($0{\cdot}5 \times 10^7$ ml) was determined as described Materials and Methods. Numbers in parentheses indicate the number of determinations.

† Without Ca^{2+} and glucose.

Since the fraction of drug represented by the difference between total uptake and bound drug was more or less constant throughout the whole incubation period, was relatively large at 4 min, was removable by a single "cell-wash", was temperature independent, and could not be ascribed to drug efflux, it seems to represent primarily the amount of DDUG adsorbed to the cell. Moreover, in agreement with the Freundlich equation of adsorption at heterogeneous surfaces,[23] the uptake at 4 min showed a direct proportionality to the external DDUG concentration (Fig. 3). No indication of saturation occurred up to 10^{-4} M concentration of DDUG. Higher concentrations could not be used because of the limited solubility of the drug.

Mechanism of entry. For the measurement of the uptake rates, only the linear segment of the uptake curve between 10 and 30 min of incubation was considered, which excluded the rapid phase. At different DDUG concentrations, at 37° higher rates of total uptake were observed in Ringer without any Ca^{2+} and glucose than in RPMI 1640 CS. In either case, a linear dependence of uptake rate was observed over a 10,000-fold range of drug concentrations (Fig. 4). In most of the experiments not involving various drug concentrations, the 10^{-6} M concentration of DDUG was used. From the data in Fig. 4, the apparent first-order rate constant of uptake at 37° (k_{37}) was calculated. From a series of such experiments at three other temperatures, the respective k_T values* were determined and the activation energy (E_A) of the uptake was obtained using the Arrhenius equation.[23] Similarly, Q_{10}† was also calculated as the increase in the k_T for increase in temperature from 27° to 37°. The values of k_{37}, Q_{10}

* Apparent first-order rate constant of uptake at T° or designated temperature.

† k_{37}/k_{27} ratio.

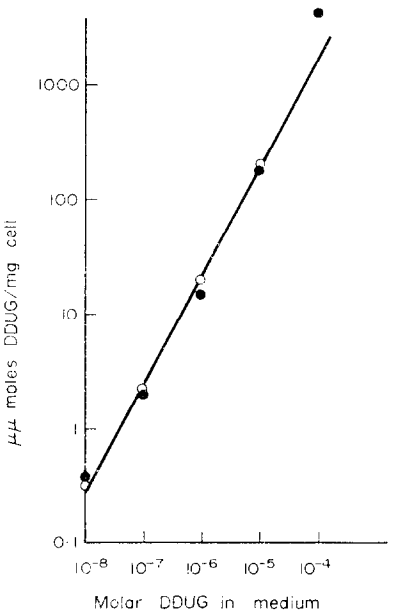

FIG. 3. Total uptake of DDUG by L1210 cells at 4 min: dependence on drug concentration. L1210 cells (5×10^6/ml) were incubated in RPMI 1640 CS (●——●), and in Ringer without Ca^{2+} and glucose (○——○) at 37° together with ^{14}C-DDUG at different concentrations.

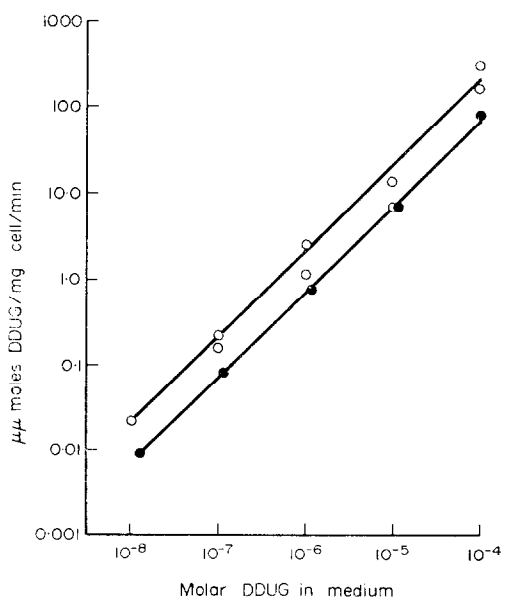

FIG. 4. Rate of uptake of DDUG by L1210 cells. L1210 cells (5×10^6/ml) were incubated in RPMI 1640 CS (●——●) and in Ringer solution without Ca^{2+} and glucose (○——○) at 37° together with ^{14}C-DDUG at different concentrations. The rate of uptake was determined from the total uptake between 10 and 30 min of incubation (linear phase of uptake).

and E_A for total uptake and bound drug obtained for L1210 and L1210/DDUG are shown in Tables 2 and 3. The k_{37} values of total uptake by L1210 cells increased to 2·44 min^{-1} in Ringer medium, this being possibly related to the nutritional inadequacy of this medium. The k_{37} values of total uptake and bound drug as determined in RPMI 1640 CS did not appear to be different from each other. Although the Q_{10} and E_A values for total DDUG uptake by L1210 and L1210/DDUG cells were not significantly different, the k_{37} values for total uptake and bound drug were lower for L1210/DDUG than for L1210 cells, the difference being significant at 1 and 0·2 per cent levels of probability[22] respectively (Tables 2 and 3).

TABLE 2. EFFECT OF INCUBATION TEMPERATURE ON THE TOTAL UPTAKE OF DDUG IN LEUKEMIA CELLS*

Cells	Medium	k_{37}‡ (min^{-1})	Q_{10}	E_A (Kcal)
L1210	RPMI 1640 CS	0·52 ± 0·03 (12)	2·12 (2)	13·7 (2)
	Ringer†	2·44 (2)	2·37 (1)	15·7 (1)
L1210/DDUG	RPMI 1640 CS	0·37 ± 0·03 (4)	1·79 (1)	10·6 (1)

* L1210 cells (0·5 to 1·0 × 10^7/ml) were incubated with ^{14}C-DDUG (10^{-6} M) at four different temperatures ranging between 0 and 37°. The k_T values were calculated from linear portion of the total uptake curve at each temperature. Q_{10} and energy activation energy (E_A) are expressed as average ±S. E. (No. of determinations).

† Q_{10} and E_A values are based upon k_0 and k_{37} only.

‡ The values used for the calculations were obtained from 10- and 30-min data points.

As shown in Fig. 5, the total uptake of DDUG by X-irradiated cells at 0° or 37° in RPMI 1640 CS was not different from that by non-irradiated cells. Pretreatment of L1210 cells with 10^{-4} M PCMB, or 10^{-4} M DNP did not result in a significant change in total drug uptake. Simultaneous incubation of L1210 cells with 1·2 × 10^{-6} M DDUM had no effect (Table 4).

Evidence for binding. The firm binding of DDUG to cells was indicated by the efflux experiments described above (see Fig. 1). The rate-limiting process related to binding

TABLE 3. EFFECT OF INCUBATION TEMPERATURE ON THE AMOUNT OF DDUG BOUND IN LEUKEMIA L1210 CELLS*

Cells	k_{37}, bound ±S. E. (min^{-1})	Q_{10}	E_A (Kcal)
L1210	0·48 ± 0·028 (4)	2·42 (2)	16·1 (2)
L1210/DDUG	0·29 ± 0·017 (4)	1·90 (2)	11·7 (2)

* L1210 cells (0·5 × 10^7/ml) were incubated with ^{14}C-DDUG (10^{-6} M) in RPMI 1640 CS at four different temperatures ranging between 0 and 37°. The k_T values were calculated from the linear portion of the curve for bound drug at each temperature. Q_{10} and activation energy (E_A) were calculated from different k_T values as described in the test. The results are expressed as average value (No. of determinations).

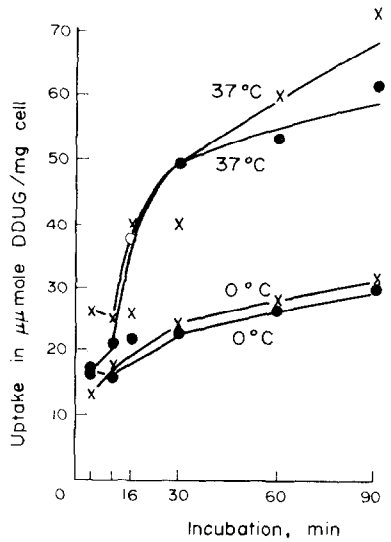

FIG. 5. Uptake of DDUG by non-irradiated (●——●) and X-ray killed (×——×) L1210 cells. Non-irradiated and 5000 roentgen X-irradiated L1210 cells (5×10^6/ml) were incubated with ^{14}C-DDUG at 10^{-6} M concentration in RPMI 1640 CS medium at 0° and at 37°. The total uptake was determined at the time points indicated.

of DDUG to cellular organelles was demonstrated in studies of the intracellular distribution of the drug.

After 30-min of incubation, both in L1210 and L1210/DDUG, a large proportion of drug was bound to the particulate matter, relative to the 9–11 per cent present in the S_{100} fraction. Only quantitative differences occurred in the distribution of DDUG

TABLE 4. TOTAL UPTAKE OF DDUG BY L1210 CELLS IN THE PRESENCE OF PCMB, DNP OR DDUM*†

Group	k_{37} (min^{-1})	Total uptake at 60 min ($\mu\mu$mole/mg cell)
Control	0·48	65·7
PCMB (10^{-4} M)	0·45	62·7
DNP (10^{-4} M)	0·58	66·2
DDUM ($1{\cdot}2 \times 10^{-6}$ M)	0·50	66·8

* Cells ($0{\cdot}5 \times 10^7$/ml) were pre- and post-incubated with either PCMB or DNP. Pre-incubation was carried out in Ringer–Locke solution for 30 min at 37°. Post-incubation was carried out with PCMB, DNP or DDUM in RPMI 1640 CS medium containing 10^{-6} M ^{14}C-DDUG at 37°. Results of one experiment are shown. Similar results were obtained in two more experiments.

† PCMB = sodium *para*-chloromercuribenzoate, DNP = 2,4-dinitrophenol, DDUM = 4,4′-diacetyldiphenylurea-mono(guanylhydrazone) methanesulfonate.

between different organelles of the same cell line or same organelle of different cell lines. The differences in the DDUG bound to different fractions from L1210 and L1210/DDUG cells corresponded approximately to the differences observed in the uptake of the drug by intact cells. The order of concentration of DDUG in micromicromoles per milligram of wet weight of different cell fractions was mitochondria > nuclei > "intact" membranes > microsomes > "broken" membranes for L1210 cells. For L1210/DDUG cells, the order was mitochondria > nuclei > microsomes > "intact" membranes > "broken" membranes (Table 5). Since nuclei and mitochondria would make up the bulk mass of cells, binding of DDUG to these organelles would account for a large proportion of cell-bound drug. In both L1210 and L1210/DDUG the total recovery was 55–65 per cent of drug present in "shocked" cells. The losses were not unexpected, paricularly because of the multistep fractionation procedure used.

TABLE 5. INTRACELLULAR DISTRIBUTION OF DDUG IN LEUKEMIA L1210 CELLS*

	DDUG in			
	L1210 cells		L1210/DDUG cells	
Fraction	Concentration ($\mu\mu$mole/mg)	Total amount ($\mu\mu$mole/10^6 cells)†	Concentration ($\mu\mu$mole/mg)	Total amount ($\mu\mu$mole/10^6 cells)†
"Broken" membranes (35S)	6·5	0·11	7·3	0·38
"Intact" membranes (35R)	14·6	0·96	8·0	0·65
Nuclei (55R)	17·0	3·80	12·4	1·12
Mitochondria	27·8	2·16	19·6	1·87
Microsomes	12·0	0·50	8·6	0·30
S_{100}		0·78		0·55
		Total 8·31		Total 4·87
"Broken" cells (45R)	16·6	2·37	9·3	1·10
		Total 10·68		Total 5·97
"Shocked" cells	12·3	16·2	8·8	10·8
Total recovery		65·9%		55·3%

* Leukemia cells (5×10^6/ml) were incubated at 37° with 10^{-6} M ^{14}C-DDUG solution, in RPMI 1640 CS medium for 30 min. The fractions were obtained as described in Materials and Methods. The ^{14}C-radioactivity in different fractions was also determined and expressed as micromicromoles of DDUG per milligram of the fraction and micromicromole per quantity of the fraction contained in 10^6 cells. S_{100} fraction is the supernatant fluid obtained after centrifugation at 100,000 *g* for 2 hr. Each value is an average of three to five experiments.

† Values based upon 70 per cent cells homogenized (see Materials and Methods).

After 5-min incubation the pattern of drug distribution was qualitatively similar to that found at 30 min. In the two cell lines the rates at which drug accumulation occurred in the nuclear and mitochondrial fractions between 5 and 30 min (Table 6) were similar in magnitude to the rates obtained from the bound drug values in the cellular uptake experiment (see Table 3). The concentration of DDUG in the S_{100}

TABLE 6. RATE OF BINDING OF DDUG IN FRACTIONS OF LEUKEMIA CELLS*

Fraction	Rate ($\mu\mu$mole/mg/min) L1210	L1210/DDUG
"Washed" cells	0·52	0·26
"Shocked" cells	0·31	0·20
Nuclei	0·41	0·29
Mitochondria	0·61	0·20
S_{100}†	<0·01	<0·01

* Leukemia cells were incubated with 10^{-6} M DDUG for 5 and 30 min at 37°, homogenized, fractionated and analyzed for DDUG content in each fraction as described under Table 5. Each value is an average of two experiments.

† Rate expressed as micromicromoles/10^6 cells per min.

fraction, however, remained unaltered, as shown by the very small rates of uptake in this compartment (Table 6).

Experiments in mice. The uptake of DDUG by L1210 and L1210/DDUG cells in DBA/2 mice is shown in Table 7. Consistent with the results obtained *in vitro*, the uptake by L1210/DDUG cells was about half that by L1210 cells. The difference between cell lines was of borderline significance at 30 min ($0{\cdot}1 < P > 0{\cdot}05$) and was not significant at 6 hr ($P > 0{\cdot}1$) because of inherent variability. The total amount of DDUG was about 140 nmoles in the ascites fluid of L1210 and about 200 nmoles in the ascites fluid of L1210/DDUG-bearing mice 30 min after an i.p. dose of 500 nmoles (15 mg/kg). Assuming 100 mg cells ($\approx 1 \times 10^8$) on an average, the uptake at 30 min represents 20 per cent of total dose for L1210 cells and 12 per cent for L1210/DDUG cells. In either case, nearly 50 per cent of DDUG was not recovered from the peritoneal cavity, probably consequent to distribution in the animal.

TABLE 7. UPTAKE OF DDUG IN LEUKEMIA L1210 CELLS *in vivo* AFTER I.P. INJECTION OF DDUG*

Cell line	Dose (mg/kg)	No. of mice	Time of sacrifice (hr)	DDUG bound to cells ($\mu\mu$mole/mg cell ±S. E.)
L1210	15	9	0·5	944 ± 135
	15	4	6·0	1324 ± 619
	30	6	0·5	2003 ± 534
	30	4	4·0	2313 ± 867
L1210/DDUG	15	9	0·5	590 ± 107
	15	4	6·0	621 ± 50

* Female DBA/2J mice weighing 18–20 g were inoculated with 2×10^6 leukemia cells, i.p., 72 hr prior to ^{14}C-DDUG injection. The drug uptake was determined as described in Materials and Methods.

DISCUSSION

As shown in this report, the uptake of DDUG involves a rapid phase of uptake, followed by a relatively slower, temperature dependent, first-order transport process ($k_{37} = 0{\cdot}5\ min^{-1}$). The rapid phase of uptake probably represents, to a large extent, adsorption phenomena, as evidenced by the similarities of k_T, Q_{10} and E_A values for total uptake and bound drug. The occurrence of drug adsorption as a possible primary event has also been observed for methotrexate.[24] Once DDUG is transported into cells, very little can be washed out or exchanged with unlabeled drug, this indicating firm binding. Indeed only with lipid solvents could the drug be extracted, even from broken cells. Although the precise nature and the site of cellular binding are unknown, it seems fairly certain from results of TLC analysis that the binding does not involve any covalent linkage or any prior modification of the DDUG molecule in either L1210 or L1210/DDUG cells.

The linear increase of the rate of uptake over a wide range of external DDUG concentrations could represent uptake due either to passive diffusion or to a carrier-mediated transport with K_m greater that 10^{-3} M. The latter possibility could not be tested directly since the solubility of DDUG reached saturation at 10^{-4} M both in RPMI 1640 CS medium and in saline. Although, the low Q_{10} cannot distinguish unequivocally between these two alternatives, it seems to exclude that an energy-dependent transport is involved. The lack of inhibition of uptake by DNP and PCMB suggests that transport is not coupled with oxidative phosphorylation, and that if there is a carrier for DDUG, the transport does not depend on an active SH group. The lack of competition with DDUM could mean that the postulated carrier has a much higher binding affinity for the DDUG molecule as compared to that for the DDUM molecule. Lack of saturation of the rate of uptake up to 10^{-4} M DDUG concentration rules out the possibility that the value for apparent K_m for DDUG is less than 10^{-4} M. If K_m for DDUM was less than that for DDUG, it would have resulted in a decreased rate and amount of uptake of DDUG when equimolar concentrations of DDUG and DDUM were used. A greater transport occurred in a nutrient-free medium, and this increase could not be due to changes in active efflux since no evidence for the existence of this mechanism was found. Furthermore, the uptake of DDUG was similar in X-irradiated and normal L1210 cells.

The over-all results discussed above suggest that transport of DDUG occurs by passive diffusion. Passive diffusion of DDUG was also reported to occur in S-180 cells *in vitro*[5,6] with a k_{37} 40 per cent smaller than that reported here for L1210.

Mice weighing 20 g received 15 or 30 mg/kg of DDUG, namely 0·5 or 1·0 μmole of drug each. Assuming a final fluid volume of 0·5 to 2·0 ml in the i.p. cavity 72 hr after the i.p. inoculation of 2×10^6 L1210 cells, the final concentration of DDUG would be between $2{\cdot}5 \times 10^{-4}$ to 1×10^{-3} M under ideal conditions. Such a concentration, under the *in vitro* conditions of incubations in RPMI 1640 CS medium for 30 min at 37°, would result in about 375–1500 $\mu\mu$moles DDUG bound/mg cell pellet. The results actually obtained in mice agree with the expectations based upon the *in vitro* data. This correlation strongly suggests that the uptake of DDUG by leukemic cells in mice also occurs by passive diffusion. Since 50 per cent of the injected DDUG was found in the peritoneal cavity of both L1210 and L1210/DDUG-bearing mice, the differences in uptake between these cell lines represent differences in rates of uptake. It should be noted that the doses of DDUG used considerably prolonged the survival of mice

bearing parent L1210 cells but had no effect on the survival of mice bearing the resistant cells.[2]

The intracellular "free" drug concentrations could be determined from the amount of DDUG present in the S_{100} fraction, based on the assumption that this amount indeed represents the "free" drug in the intracellular fluid of intact cells.[6] The total water content in L1210 and L1210/DDUG cells was found to be 83·1 per cent by volume of wet cell weight. This proportion of water was assumed to be the maximum intracellular water content. For L1210 cells, if 0·78 $\mu\mu$mole DDUG/10^6 cells in the S_{100} fraction were to be present in 83·1 % of 0·86 mg (10^6 cell weight), the intracellular concentration of "free" DDUG 30 min after incubation with 10^{-6} M DDUG would amount to $1{\cdot}09 \times 10^{-6}$ M. Similarly the intracellular "free" DDUG concentration in L1210/DDUG would amount to $0{\cdot}84 \times 10^{-6}$ M. These values are nearly equal to the extracellular concentration experimentally used. The equilibration of "free" intracellular and extracellular drug concentrations occurs in less than 5 min after incubation with DDUG, as shown by the very small rates of uptake in the S_{100} fraction. This *per se* indicates that the rate-limiting step in the accumulation of the drug is intracellular uptake and/or binding to organelles.

The mitochondrial and the nuclear fractions of both L1210 and L1210/DDUG cells account for nearly 70–80 per cent of the cellular drug uptake, and the rates at which the drug is bound to these fractions are of the same order as the rates obtained from the bound drug values after uptake by intact cells. This also suggests that the intracellular concentration of "free" drug is nearly equal to the extracellular drug concentration, and thus supports the conclusion that intracellular uptake and/or binding to cell organelles is the rate-limiting step in drug accumulation.

In view of the above discussion, it appears that the reduced rate of entry of DDUG in L1210/DDUG may be related to alterations in the physico–chemical characteristics of intracellular sites such that less drug is bound. It is likely that one or more of these binding sites is a site of action of the drug. In this context, it should be mentioned that both *in vitro* and in mice, the incorporation of thymidine into DNA, a principal biochemical target of DDUG, was 30–50 per cent less inhibited in L1210/DDUG than in L1210 cells under conditions similar to those employed in this study.[25] If the nucleus were the gross site of action of DDUG, the reduced nuclear drug binding in L1210/DDUG could satisfactorily explain the basis for resistance. The gross changes in the total protein content of L1210/DDUG cells seem consistent with possible changes in the physico-chemical characteristics of these cells with respect to parent L1210 cells.

The data presented in this report indicate that a cationic compound like DDUG can be transported into cells by a process of passive diffusion. This phenomenon is possibly related to the fact that the drug binds to lipid moieties[6] as also confirmed by its extractability with organic solvents.[12] The cellular drug distribution does not parallel the total lipid content of cell fraction, however. For example, in the S_{100} fraction, the amount of DDUG per microgram of lipid is far smaller than in the nuclear fraction, possibly because of the different nature of the lipids. DDUG diffusion into cells may be favored by the initial adsorption of the drug to the exterior to the cell membrane, adsorption which in turn may be facilitated by possible ionic interactions with the negatively charged cell surface.[26]

Although a reduction in carrier-mediated transport has been proposed as a mechanism of resistance in some cases,[27] a reduction of passive diffusion cannot be readily

suggested as a specific cause for resistance. In the case of terephthalanilide NSC 60339, another cationic drug, it was shown that sensitivity of leukemia P-388 correlates with extent of lipophilic binding of the agent in the target cells; in this case resistance was associated with an increased efflux of drug.[28,29] It was also postulated[6] that the reduction of DDUG uptake in resistant S-180 culture cells is a consequence of altered membrane lipid constitution. The results of the present study are consistent with the view that resistance of L1210 cells is related to a reduction in the rate and extent of uptake and/or binding of the drug to intracellular structures. The example provided herein may also be relevant in the case of other cationic agents, such as the terephthalanilides, the stilbamidines, or ethidium bromide.

REFERENCES

1. E. Mihich and J. Gelzer, *Cancer Res.* **28,** 553 (1968).
2. E. Mihich and A. I. Mulhern, *Cancer Res.* **28,** 354 (1968).
3. W. Regelson and J. F. Holland, *Cancer Chemother. Rep.* **11,** 81 (1961).
4. W. Regelson and J. F. Holland, *Cancer Chemother. Rep.* **27,** 15 (1963).
5. J. R. Gillette and M. T. Hakala, *Proc. Am. Ass. Cancer Res.* **9,** 24 (1968).
6. M. T. Hakala, *Biochem. Pharmac.* **20,** 81 (1971).
7. E. Mihich, *Proc. ninth Int. Cancer Congress*, Tokyo, p. 317 (1967).
8. E. Mihich, *Cancer, N.Y.* **20,** 880 (1967).
9. C. Dave and E. Mihich, *Fedn Proc.* **28,** 389 (1969).
10. E. Mihich, C. Dave, J. Soucek, A. I. Mulhern and M. J. Burgett, *Proc. sixth Int. Congress Chemother.*, Tokyo, p. 336 (1969).
11. C. Dave, J. Ehrke and E. Mihich, *Pharmacologist* **12,** 302 (1970).
12. C. Dave, Ph.D. Thesis, State Univ. of N.Y. at Buffalo, June (1970).
13. G. E. Moore, *J. Am. med. Ass.* **199,** 519 (1967).
14. G. A. Bruno and J. E. Christian, *Analyt. Chem.* **33,** 1216 (1961).
15. D. G. Ott, *Nucleonics* **17** (9), 106 (1959).
16. R. J. Herberg, *Packard Tech. Bull.* **15,** Packard Technical Instruments Co., Downer's Grove, Ill. (1961).
17. L. Warren, M. C. Glick and M. K. Nass, *J. Cell Biol.* **68,** 269 (1966).
18. W. C. Schneider, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 3, p. 680. Academic Press, New York (1957).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
20. J. M. L. Folch and G. H. S. Stanley, *J. biol. Chem.* **226,** 497 (1957).
21. J. B. Marsh and D. B. Weinstein, *J. Lipid Res.* **7,** 574 (1966).
22. G. M. Clarke, *Statistics and Experimental Design*, p. 52. American Elsevier, New York (1969).
23. F. Daniels and R. A. Alberty, *Physical Chemistry*, pp. 289 and 342. John Wiley, New York (1966).
24. I. D. Goldman, N. S. Lichtenstein and V. T. Oliverio, *J. biol. Chem.* **243,** 5007 (1968).
25. J. Soucek, M. J. Ehrke and E. Mihich, *Cancer Res.* **30,** 2187 (1970).
26. L. Weiss and M. T. Hakala, *Cancer Res.* **31,** 1369 (1971).
27. A. Y. Divekar and M. Hakala, *Molec. Pharmac.*, **7,** 663 (1971).
28. D. W. Yesair, W. I. Rogers, P. E. Baronowsky, I. Wodinsky, P. S. Thayer and C. J. Kensler, *Cancer Res.* **27,** 314 (1967).
29. D. W. Yesair and C. HoFook, *Cancer Res.* **28,** 314 (1968).