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UPTAKE AND DISTRIBUTION OF 4,4′-DIACETYL-DIPHENYL-UREA-BIS-GUANYLHYDRAZONE IN SENSITIVE AND RESISTANT SARCOMA 180 CELLS *IN VITRO**

MAIRE T. HAKALA

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

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Abstract—Sarcoma 180 cells sensitive to 4,4′-diacetyl-diphenyl-urea-bis-guanylhydrazone (DDUG) were compared with a resistant subline (S-180/DDUG) with respect to cross-resistance to methylglyoxal-bis-guanylhydrazone (CH_3G), 2-chloro-4′,4″-bis(2-imidazolin-2-yl)-terephthalanilide (NSC 38280) and vincristine (VCR); they were also compared with respect to the nature and rate of cellular uptake of DDUG and its intracellular distribution and binding.

Resistance to DDUG was found to be associated with a reduced rate of uptake of DDUG while the intracellular distribution (65–70 per cent in nuclei and 30 per cent in mitochondria) and the capacity for lipid-associated drug binding was unchanged. The uptake was a rapid passive process leading to extensive intracellular accumulation; it was not affected by CH_3G or VCR, but was inhibited by NSC 38280 or Janus Green. S-180/DDUG cells were cross-resistant to NSC 38280 but sensitive to CH_3G and VCR, while S-180/VCR cells were cross-resistant to NSC 38280 but sensitive to DDUG and CH_3G. In uptake, intracellular distribution and binding DDUG resembles NSC 38280. Combination and cross-resistance studies suggest a lack of similarity between DDUG, NSC 38280, VCR and CH_3G with respect to the site of growth inhibitory action.

THE AROMATIC bis-guanylhydrazone, 4,4′-diacetyl-diphenyl-urea-bis-guanylhydrazone (DDUG)[1] (Fig. 1) inhibits ascitic and solid L1210,[2,3] several other leukemias, a lymphoma, Ehrlich ascites carcinoma and some primary mammary tumors in mice.[4] A few solid mouse tumors were unaffected by DDUG as were all of the four rat tumors which have been tested.[4] A subline of leukemia L-1210, resistant to DDUG ,was cross-resistant to 2-chloro-4′,4″-bis-(2-imidazolin-2 yl)-terephthalanilide (NSC 38280) and vice versa. Both of these resistant sublines retained the original sensitivity to the aliphatic methylglyoxal-bis-guanylhydrazone (CH_3G).[5] Moreover, combinations of CH_3G with DDUG were synergistic against the parent L-1210 line.[5] The cellular uptake of DDUG has been studied in sensitive mouse mastocytoma P-815 and in sensitive and resistant L-1210.[6,7] The drug was taken up rapidly and there was a marked accumulation of DDUG within the cells. The present study *in vitro* is concerned with the uptake of DDUG by sensitive and resistant S-180 cells, its intracellular distribution and binding, as well as cross resistance to and combinations with other agents. Part of the results obtained have been reported in preliminary notes.[5,8,9]

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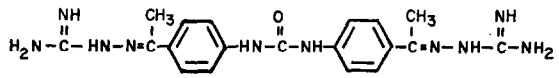
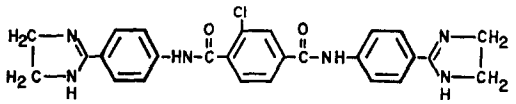


DDUG



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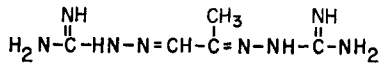

CH_3G

FIG. 1. The structural formulas of 4,4′-diacetyl-diphenyl-urea-bis-guanylhydrazone (DDUG), 2-chloro-4′,4″-bis(2-imidazolin-2 yl)-terephthalanilide (NSC 38280) and methylglyoxal-bis-guanylhydrazone (CH_3G).

MATERIALS AND METHODS

Cells. The parent S-180 cell line of Foley and Drolet[10] was maintained in this laboratory for 12 years in monolayer culture in Eagle's medium[11] supplemented with 5 per cent of horse serum. The subline resistant to DDUG, S-180/DDUG, was developed by increasing DDUG concentration gradually over a period of 2 months from 2×10^{-6} M to 10^{-5} M, which was maintained thereafter. Resistance remained unchanged after 2 months of growth in drug-free medium. The subline resistant to vincristine (S-180/VCR) was also developed gradually by increasing the concentration of vincristinesulfate over a period of 3 months from 6×10^{-9} M to 10^{-7} M, maintained thereafter. The rates of growth of the parent cells and the sublines were about the same, i.e. with a generation time of about 24 hr.

Growth inhibition. Cells, 2×10^5, in 2 ml of medium were put into each T-15 flask. The cells attached to the glass after 24 hr represent the "inoculum". At this time, the medium was replaced with a fresh medium containing the inhibitor and this was done two or three times thereafter. The experiments were terminated after 6 or 7 days and the growth was determined by a protein assay.[12] The controls grew 10 to 15-fold in 6 days and 15 to 20-fold in 7 days. Each concentration of the compound was tested in 3 vials, controls and inocula in 5.

^{14}C-DDUG uptake. Each Roux flask was inoculated with 40 million cells (5×10^5/ml). After 24 hr the medium was poured off and replaced with 80 ml of similar growth medium warmed up to 36° but containing ^{14}C-DDUG uniformly labeled in the phenyl rings (3000 cpm/ml) and the unlabeled carrier. The Roux flasks were then incubated at 36° for periods ranging from 15 min to 48 hr. At the end of the incubation the medium was poured off and the cell layer was rinsed three times rapidly with 30 ml of cold, drug-free medium. This washing was found to have no effect on the cellular DDUG content. The cells were then scraped off with a rubber policeman and centrifuged directly in preweighed counting vials for 10 min at 850 *g*. The walls of the vials were wiped dry, the cell pellet was weighed, 10 ml of scintillation solution[13] added and the samples were counted in a Packard Scintillation counter. Quenching was estimated by inspection of channel ratios and was corrected for. An annoying characteristic

of DDUG is the adherence of it to many kinds of surface structures such as glass, teflon and cellophane. To overcome this complication, the samples of the medium were taken directly from the Roux flask at the start of the incubation and provided the values for the zero time DDUG concentration. Only by using this procedure was it possible to recover all of the radioactivity in the incubation system. In studies concerning the effect of temperature on the rate of uptake, the medium and the Roux flasks containing the cells were pre-adjusted to the desired temperatures by placing them in water of that temperature. To free the resistant cells of the bound DDUG, they were grown in drug-free medium for 7–13 days prior to their use in uptake studies. Assuming one cell division per day, one would expect less than 1 per cent of the bound drug to remain after 7 days.

Calculation of results. The cell pellet described above has been shown in previous studies[14] to consist of 33 per cent of extracellular medium, 18 per cent of dry weight and the remainder (49 per cent) of intracellular water. To calculate the DDUG content of the cells, the weight of the extracellular medium (33 per cent) was subtracted from the weight of the pellet. Similarly, the calculation of the drug concentration in the intracellular water was based on the 49 per cent figure mentioned above. The counting efficiency in these studies varied from 50 to 100 per cent depending on the sample.

Subcellular fractionation. Freshly harvested S-180 cells, even though relatively large in size (19μ in diameter), were very hard to break without destroying the nuclei. A satisfactory method was a modification of that by Bach and Johnson.[15] A 0·1 to 0·3 g pellet of cells was allowed to swell for 30 min at 0° in 5 ml of distilled H_2O and was homogenized in Potter–Elvehjem tube with 8 passes of a teflon pestle with a clearance of 0·004″–0·006″. To avoid lysis, the mixture was immediately adjusted to 0·25 M sucrose, 0·005 M Mg acetate, 0·1 M KCl and 0·005 M Tris-HCl, pH 7·8, by adding 5 ml of a doubly concentrated solution. The nuclei (plus any unbroken cells) were centrifuged at 600 *g* for 15 min. The sediment was washed twice with 5 ml of the single strength solution described above and centrifuged for 10 min at 850 *g*. The resulting nuclear pellet contained clean, unbroken nuclei with less than 1 per cent unbroken cells, as determined by microscopic examination. The supernatant and washings were combined and centrifuged at 12,000 *g* for 15 min in a Spinco model L-65B ultracentrifuge to sediment the mitochondria, and for a further 90 min at 150,000 *g* to separate the microsomes. The final supernatant was considered to represent the diluted cell sap.

Chemicals. 4,4′-Diacetyl-diphenyl-urea-bis-guanylhydrazone dimethane sulfonate (DDUG) was obtained from CIBA Co., Basel, Switzerland; the 2-chloro-4′,4″-bis-(2-imidazolin-2-yl) terephthalanilide hydrochloride (NSC compound No. 38280) from the CCNSC. The phosphatides, phosphatidyl-L-serine, phosphatidyl-inositide, L-α-cephalin (synthetic), L-α-lecithin (egg, chromatographically pure), cerebroside (beef brain) and sphingomyelin were products of General Biochemicals, Chagrin Falls, Ohio. Triton X-100 (alkyl phenoxy-polyethoxy ethanol) was the product of Rohm & Haas Co., Philadelphia, Pa. Vincristine sulfate (Oncovin) was the commercial product of Eli Lilly & Co.; 4-acetamido, 4′-isothiocyanostilbene-2,2′-disulphonic acid (SITS) a product of Nutritional Biochemicals Co.; and Janus Green B, 3-(diethylamino)-7[*p*-(dimethylamino)-phenyl]azo-5-phenylphenazinium chloride, a product of Allied Chemical Corp.

EXPERIMENTAL

Growth inhibition of various sublines of S-180 cells. The DDUG resistant S-180 cells were cross-resistant to NSC 38280 but retained their sensitivity to CH_3-G and Vincristine (VCR) (Table 1). On the other hand, the VCR-resistant subline was cross-resistant to NSC 38280 but retained the sensitivity to DDUG and CH_3-G. The synergism of CH_3G and DDUG in inhibiting the growth of the parent S-180 cells is shown in Fig. 2. Thus, $\frac{1}{6}$ of the ID_{50} dose of CH_3G with $\frac{1}{3}$ that of DDUG were equivalent to individual doses alone. No synergism was observed with combinations of DDUG and CH_3G against S-180/DDUG cells. The additive effects of combinations of NSC 28380 with CH_3G or DDUG become apparent from Fig. 3.

TABLE 1. GROWTH INHIBITION OF VARIOUS SUBLINES OF S-180 CELLS

Compound	μM Required for 50 per cent inhibition*		
	Parent S-180	S-180/DDUG	S-180/VCR
DDUG	0·8	9·0	0·8
CH_3G	6·0	8·0	6·5
NSC 38280	1·0	5·0	12·0
Vincristine SO_4	0·005	0·005	0·1

* S-180/DDUG had been maintained in a medium containing 10^{-5} M DDUG, S-180/VCR in a medium containing 10^{-7} M Vincristine sulfate. All of the cell lines were inoculated in a drug-free medium 24 hr prior to the exposure to the compounds for 6 or 7 days.

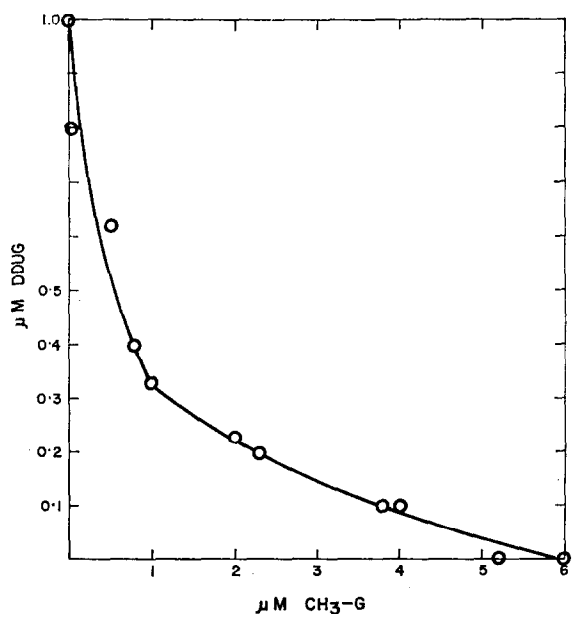

FIG. 2. Synergistic inhibition of the growth of S-180 cells *in vitro* by combinations of DDUG and CH_3G. The points indicate concentrations required to inhibit the growth by 50 per cent.

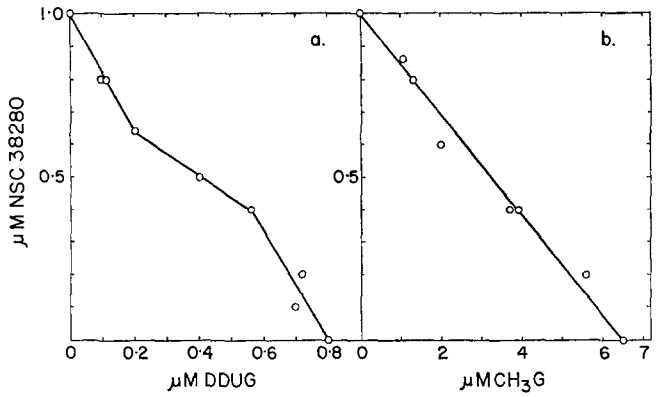

FIG. 3. The additive effects of combinations of NSC 38280 with DDUG (a) or CH_3G (b) in inhibiting the growth of S-180 cells. Each point signifies the 50 per cent growth inhibitory combination.

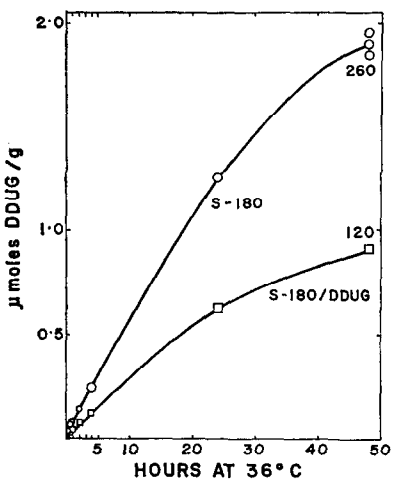

FIG. 4. Time dependence of cellular uptake of DDUG by sensitive and resistant S-180 cells at 10 μM concentration of DDUG. The numbers next to the curves at 48 hr indicate the ratio of cellular and extracellular concentrations.

Time dependence of DDUG uptake by sensitive and resistant cells. Uptake at 10μM DDUG is compared in sensitive and resistant S-180 cells in Fig. 4, which shows that the rate of DDUG uptake was much smaller in the resistant than in the sensitive cells. Saturation was not reached even after 48 hr when drug was concentrated 120- and 260-fold in the resistant and sensitive cells respectively. When the medium was changed at 48 hr, the uptake still continued for another 48 hr without signs of saturation.

Concentration dependence of DDUG-uptake. After 48-hr incubation of parent S-180 cells at 1·7 μM DDUG when the viability of the cells (lack of staining with trypan blue) was 96 per cent, the drug concentration in the medium was reduced by 43 per cent. Because of this extensive exhaustion of the extracellular DDUG, the rate of uptake was determined after 15 min of incubation. The initial velocities of uptake (v)

were proportional to the extracellular concentration between 0·5 and 11 μM (Table 2). At 100 μM DDUG there was a break in the linearity with an abrupt increase in the velocity of uptake. Since 100 μM is about one hundred times the 50 per cent growth inhibitory concentration, it is proposed that this apparent rapid "uptake" reflected nonspecific membrane damage. Indeed, further on it will be demonstrated that subcellular particles, when not within the living cells, bind DDUG instantly. The average velocity constant (K) for the sensitive S-180 as well as for S-180/VCR cells was 0·3 min^{-1}. The concentration dependence of the rate of uptake for S-180/DDUG cells was also linear, but with a slope half of that for the sensitive cells ($K = 0{\cdot}15\ min^{-1}$).

TABLE 2. RATE OF UPTAKE OF DDUG BY S-180 CELLS

μM DDUG in medium	Rate of uptake (v)* (m-moles/g/min)	K†
0·46	$0{\cdot}108 \times 10^{-6}$	0·24
0·81	$0{\cdot}314 \times 10^{-6}$	0·39
0·90	$0{\cdot}335 \times 10^{-6}$	0·37
1·7	$0{\cdot}506 \times 10^{-6}$	0·30
1·7	$0{\cdot}635 \times 10^{-6}$	0·37
10·5	$2{\cdot}34 \times 10^{-6}$	0·22
10·8	$2{\cdot}98 \times 10^{-6}$	0·28
100·0	$52{\cdot}0 \times 10^{-6}$	0·52
100·0	$60{\cdot}0 \times 10^{-6}$	0·60

* Determined after 15 min of incubation and calculated per gram of cells (free of extracellular medium).
† K represents the constant for $v = K \times C$, when C is the molar concentration of drug in the medium.

Intracellular accumulation of DDUG. Not only was the rate of cellular uptake of DDUG rapid but the intracellular accumulation of the drug was also quite marked. Already after 15 min of incubation at 0·5–10 μM DDUG, the drug content of the cells was four to six times higher than that of the medium. The results obtained after 48 hr of incubation (Table 3) show that the DDUG content of the cells could be

TABLE 3. INTRACELLULAR ACCUMULATION OF ^{14}C-DDUG*

S-180 subline	Initial medium† μM DDUG	Cell pellet (mg)	Ratio of DDUG concentration (cells/medium)	Per cent of total DDUG in cells
Parent				
	0·46 (2)	120·5	822	45·3
	1·7 (1)	164	550	43·0
	10·5 (5)	156	248	24·5
Resistant				
	10·5 (5)	164	113	13·5

* Determined after 48 hr of incubation in 80 ml of medium.
† The numbers in parentheses indicate the number of separate experiments, the results being averages of these.

several hundred times that of the medium and that 150 mg of S-180 cells contained 25–45 per cent of the total drug present in the 80-ml experimental system.

Temperature vs. DDUG uptake. The rate of ^{14}C-DDUG uptake was studied at 5, 15, 25, 31 and 40° using 1 μM concentration of DDUG and two time intervals at each temperature. Shorter incubations (15 and 30 min) were utilized at higher temperatures: longer ones, 30 and 60 min, at lower temperatures. The results for S-180 and S-180/DDUG cells are shown in Fig. 5. The rate of DDUG-uptake in both cells was only slightly dependent on temperature increasing by 70 per cent with every 10° rise in temperature. These results are summarized in Table 4.

Effect of various compounds on the uptake of DDUG. The effect of 10 different compounds on uptake of DDUG by S-180 cells was examined. As is seen in Table 5, only NSC 38280 and Janus Green were able to slow down the uptake. The concentration dependence of these inhibitions is illustrated in Figs. 6 and 7. It is significant that the aliphatic bis-guanylhydrazone, CH_3G, had no effect on DDUG uptake; neither did the compound, SITS, which has been used for specific labeling of outer plasma membranes.[16] On the other hand, Janus Green, which is a selective stain for

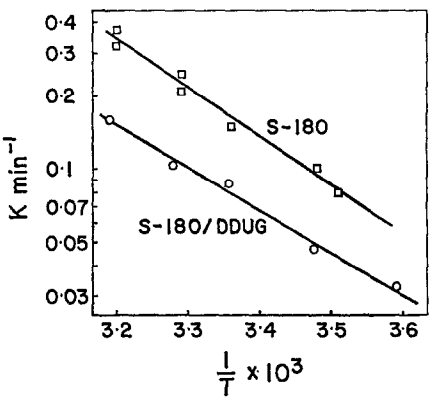

FIG. 5. Effect of temperature on the rate of DDUG uptake by the sensitive and resistant S-180 cells. The rate constant is K min^{-1} when the rate is expressed in m-moles per g cells per min and the extracellular concentration in moles per liter.

TABLE 4. COMPARISON OF DDUG UPTAKE IN SENSITIVE AND RESISTANT S-180 CELLS

Cell line	Cellular uptake of DDUG			
	Rate constant* K at 36°	Energetics† Q_{10}	E K cal	Cells/medium (48 hr, 10 μM)
S-180	0·3	1·73	10·5	260
S-180/DDUG	0·15	1·67	8·5	120

* Rate of uptake (v) is directly proportional to concentration (C) of DDUG; v (m-moles/g/min) = $K \times C$ (M).

† Measured at temperatures between 5° and 40°; see Fig. 5.

TABLE 5. EFFECT OF VARIOUS COMPOUNDS ON CELLULAR UPTAKE OF DDUG*

Compound	S-180 subline		
	S-180	S-180/DDUG	S-180/VCR
Iodoacetamide	−		
2,4-Dinitrophenol	−		
Choline chloride	−		
Spermine	−		
Spermidine	−		
CH_3G	−		
NSC 38280	+	+	±†
SITS	−	−	
Janus Green	+		

* Cells were incubated for 2–4 hr in a medium containing 0·5–1·0 μM DDUG, including ^{14}C-DDUG, and 0·1–100 μM of the compounds, except VCR, which was used at 0·1–10 μM. Minus sign indicates lack of effect, plus sign inhibition of DDUG uptake.

† One hundred μM NSC 38280 caused only 30 per cent reduction in DDUG uptake (1 μM) by S-180/VCR cells in 2 hr.

mitochondria, 90 per cent being bound by a specific lipoprotein fraction,[17] was about as effective as NSC 38280 in slowing down DDUG uptake. NSC 38280 inhibited the uptake of DDUG by S-180/DDUG to the same degree as in S-180 cells, while in S-180/VCR the uptake was only slightly affected. No interference by VCR could be observed with respect to DDUG uptake by S-180/DDUG cells.

Intracellular distribution of ^{14}C-DDUG. The intracellular distribution of DDUG was determined in sensitive and resistant cells (Table 6). Even though the total amount of DDUG in the resistant cells was only half of that in sensitive cells, in both sublines the nuclear pellet contained 65–70 per cent of the total drug, mitochondria 30 per cent and microsomes and cellular sap only little. The intracellular distribution of

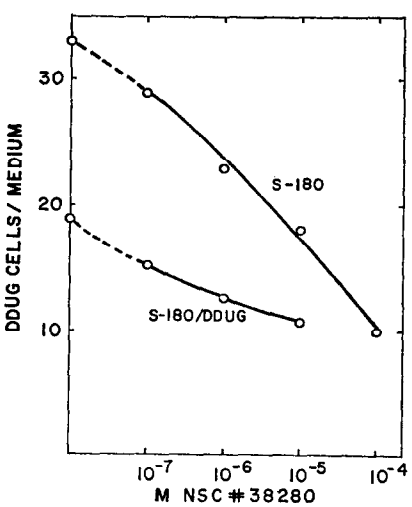

FIG. 6. Inhibition of DDUG (1 μM) uptake in S-180 and S-180/DDUG cells by varied concentration of NSC 38280; incubation for 4 hr at 36°.

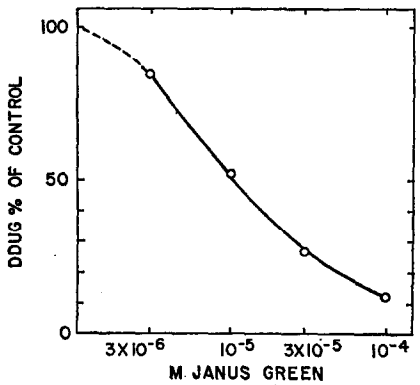

FIG. 7. Inhibition of DDUG (1 μM) uptake in S-180 cells by varied concentrations of Janus Green; incubation for 2 hr at 36°. In this case the use of internal standards was necessary to correct for the interference in ^{14}C-counting caused by the color of Janus Green.

TABLE 6. INTRACELLULAR DISTRIBUTION OF ^{14}C-DDUG IN SENSITIVE (S) AND RESISTANT (R) S-180 CELLS*

Cell fraction	mg		Relative DDUG concentration†		dis./min per cent of total		Centrifugation
	S	R	S	R	S	R	
Cell pellet	262	283	100	100	100	100	10 min
	263	287	100	100	100	100	850 *g*
Nuclear pellet	177	130	104	142	70	65	10 min
	172	157	102	119	67	65	850 *g*
Mitochondria	56	88	127	96	27	30	15 min
	71	97	114	86	31	29	12,000 *g*
Microsomes	56	66	6·3	5·4	1·3	1·3	90 min
	64	59	2·8	10·8	0·7	2·2	150,000 *g*
Cellular sap	128	139	3·4	7·3	1·6	3·6	
	129	141	3·8	7·7	1·9	3·7	

* Monolayer cultures were incubated for 48 hr in 80-ml stock medium supplemented with 10^{-5} M DDUG containing ^{14}C-DDUG to give an initial dis./min of 4000–7000 per ml of medium.

† The relative DDUG concentration is based on dis./min/mg of the total cell pellet taken as 100. It ought to be noted that the actual DDUG content of resistant cells and their fractions is about half that of sensitive cells when grown in similar conditions.

^{14}C-DDUG was similar also in S-180/VCR cells (data not shown). The DDUG content per milligram of the nuclear and mitochondrial pellets was about equal and of the same magnitude as that of the cell pellets. The mitochondrial fraction of the resistant cells was noted to have a slight trend to a lower specific activity as compared with the nuclear pellet. This might have been caused by increased weight (swelling?) and did not affect the percentage intracellular distribution. The actual DDUG concentration of the cell sap in S-180 and S-180/DDUG was equal.

The rate of uptake of DDUG by the resistant cells was only half of that by sensitive cells either because of altered intracellular capacity for DDUG-accumulation or of some change at the cell membrane. When isolated subcellular fractions of sensitive

and resistant cells were incubated directly with the compound, they took it up far more rapidly than did the whole cells, clearly indicating a cell membrane barrier. In one case when 136 mg nuclei of S-180 cells was kept in ice for 2 hr in 5 ml of medium containing 5×10^{-7} M DDUG, 90 per cent of the total drug was found in the pellet. Moreover, the relative capacity of the isolated cell fractions to accumulate the drug resembled that found in whole cells, microsomes picking up very little of the drug as compared with nuclei and mitochondria. No difference was observed between the sensitive and resistant cells in respect to the capacity of the subcellular fractions to take up drug at 0° (Table 7). Therefore, it seems likely that the change associated with DDUG resistance which results in reduced rate of cellular uptake of the compound involves the membrane rather than the subcellular compartments.

TABLE 7. UPTAKE OF ^{14}C-DDUG BY ISOLATED SUBCELLULAR FRACTIONS*

Cell fraction	Weight (mg)	dis./min/mg
Sensitive S-180		
Nucleus	202	330
Mitochondria	131	176
Resistant S-180		
Nucleus	271	301
Mitochondria	126	143

* The pellets were suspended in 20 ml of ice-cold Eagle's medium containing 10^{-6} M DDUG and immediately separated by centrifugation in cold. Consequently, the length of exposure at 0° for nuclei was 13 min, for mitochondria 26 min. The change in DDUG concentration of the medium in these conditions was minimal.

Extractability of ^{14}C-DDUG from S-180 cells. DDUG taken up by S-180 cells was hard to remove. Extraction by neutral sucrose-buffer solutions even from broken cells was minimal as indicated by the very low drug content (2–4 per cent of total) in the high speed supernatant of the cell homogenate (Table 6). However, if the subcellular fractionation of DDUG containing cells was performed in the presence of a detergent (0·125 per cent Triton X-100), the high-speed supernatant contained 44–56 per cent of the total DDUG, mainly from nuclear fraction. Since Triton X-100 is a surface active agent, this finding strongly suggested that some lipid-containing structures, possibly nuclear and mitochondrial membranes, might be involved in the binding of DDUG. DDUG in the Triton X-100 extract of the cells was in a freely dialyzable form. It was also observed that 40 per cent of the total DDUG was held by the cellophane dialysis membrane. This provided additional evidence for the affinity of DDUG to surface structures in general.

Additional observations on the pattern of extraction of DDUG from cellular material (Tables 8 and 9) are in accord with a possible lipid association of DDUG. Thus, DDUG was extracted poorly by HCl or urea while NaOH was about as effective as the detergent Triton X-100. The best extraction of DDUG was provided by a lipid

TABLE 8. EXTRACTION OF CELLULAR ^{14}C-DDUG

DDUG-containing cells	Conditions for extraction	Per cent extracted
S-180/DDUG	0·1 M Tris, pH 8·0, 10 min at 100°	5·3
	0·1 N HCl, 60 min at 37°	14·3
	0·1 N NaOH, 60 min at 37°	64
	2nd, 60 min at 37°	16
S-180	0·1 N NaOH, 60 min at 37°	52
	0·1 N NaOH, 10 min at 100°	49
	4 M Urea in 0·1 M Tris, pH 8·0, 60 min at 37°	17·4

TABLE 9. EFFECT OF INORGANIC IONS ON THE EXTRACTABILITY OF ^{14}C-DDUG FROM CELL HOMOGENATE* BY LIPID SOLVENT

Supplement in the aqueous phase	Per cent of total DDUG extracted	Distribution ratio (Upper/lower)
None	10·8	1·9
0·1 N HCl	83·0	5·0
0·1 N NaOH	96·0	5·7
0·1 M PO_4, pH 7·0	76·4	133·0

* S-180 cells were grown for 15 hr in a medium containing 1 μM DDUG (including ^{14}C-DDUG). The cells (120 mg each) were collected, frozen and then homogenized in 2 ml of the aqueous phase. Cell homogenate was mixed with 10 ml of chloroform–methanol (2:1) for 60 sec using Vortex mixer. The upper (CH_3OH–H_2O) and lower phases (CH_3Cl–H_2O) were separated by centrifugation for 20 min at 3000 rev./min. The residual pellet, as well as 1-ml samples of the upper and lower phases, were counted.

solvent,[18] chloroform–methanol–water (2:1:0·6), if the water phase was supplemented with inorganic ions. This solvent can be separated into a more polar upper (methanol–H_2O) and less polar lower phase (chloroform–H_2O). As is seen in Table 9, the presence of phosphate altered the distribution of DDUG markedly in favor of the upper phase, suggesting an attraction between DDUG and inorganic phosphate.

When cellular material was not involved, DDUG was distributed in the lipid solvent system almost entirely into the upper methanol–H_2O layer, and more so if the H_2O phase contained buffers (pH 7·0) such as phosphate, Tris buffer, citrate, glycylglycine or imidazole. However, the addition of certain phospholipids into the chloroform phase reversed the DDUG distribution completely in favor of the lower chloroform–H_2O layer (Table 10). Two phosphilipids, L-α-lecithin and spingomyelin, which differ from the others by containing choline in place of a more neutral side-chain, had no effect on the DDUG distribution. It thus appears that DDUG has an affinity to phospholipids other than those containing a choline moiety. It is probable that this is a charge-oriented affinity. The positively charged DDUG is apparently drawn toward the negatively charged phosphate whenever the latter is not shielded by the bulky, positively charged choline moiety.

TABLE 10. EFFECT OF VARIOUS PHOSPHOLIPIDS ON ^{14}C DDUG DISTRIBUTION IN LIPID SOLVENT SYSTEM

Phosphatide*	Side chain	cpm/ml		Concentration (upper/lower)
		Upper phase	Lower phase	
None		1720	48	36
L-α-lecithin (egg)	Choline	1820	45	40
Sphingomyelin	Choline	1610	74	22
L-α-cephalin (synthetic)	Ethanolamine	170	550	0·31
Phosphatidyl-inositide	Inositol	68	580	0·12
Phosphatidyl-L-serine	Serine	36	500	0·07
Cerebroside (beef brain)	Galactose	41	590	0·07

* Phosphatides were dissolved into chloroform (1 mg/ml) and 2 parts of this solution were combined with 1 part of methanol. This mixture (10 ml) was shaken with 2 ml of 10^{-5} M DDUG-H_2O solution (containing the label) and separated into upper (methanol–H_2O) and lower (chloroform–H_2O) phases by centrifugation for 20 min at 3000 rev./min.

DISCUSSION

Several animal tumors *in vivo* are known to be either naturally resistant to DDUG or have been deliberately selected for resistance.[4] In the present study we have compared the DDUG resistant S-180 cells, developed *in vitro*, with the sensitive parent cells. These two are similar in size and rate of growth but differ 2-fold in the rate of cellular uptake of DDUG. The rate of cellular uptake of DDUG was generally very rapid, being forty times faster in S-180 cells than the uptake of amethopterin.[14] It was also established that there was no difference between those sublines in the capacity of the isolated subcellular fractions to take up the compound. Thus, in this particular case it is suggested that some alteration in the plasma membrane resulting in slower uptake of DDUG must have occurred in the resistant cells. The cellular uptake was compared in another system involving resistance to DDUG, namely L-1210 leukemia *in vivo*.[7] The rate of uptake in that case remained unchanged irrespective of resistance to DDUG. No metabolic conversion of DDUG was observed in sensitive and resistant L-1210 cells or rat liver *in vivo*.* Although this aspect was not investigated in S-180 cells, it seems unlikely that a fundamental difference in this regard would exist.

The mode of uptake of DDUG by S-180 cells is passive diffusion as indicated by the low energy of activation (9–11 kcal) and lack of inhibitors of energy metabolism (2,4-dinitrophenol) or SH-reagents (iodoacetamide). The extensive intracellular accumulation of DDUG, far over that in the surrounding medium, fails in this case to provide evidence for an active uptake process because most of the intracellular drug is firmly bound and the free DDUG does not exceed the concentration in the medium. In a study on DDUG uptake in P-815 mouse mastocytoma cells, it was concluded that DDUG was accumulated in the cell water.[6] However, this cell water was the supernatant fluid of sonicated cells after centrifugation for 15 min at 3000 rev./min. Such low-speed supernatant probably contained not only the mitochondria but also disintegrated nuclei resulting from sonication.

Some similarities were noted between DDUG and NSC 38280 and some differences between these compounds and CH_3G. Thus, DDUG resistant S-180 cells were cross-resistant to NSC 38280 but sensitive to CH_3G. Several mouse leukemias resistant to

* C. DAVE and E. MIHICK, personal communications.

terephthalanilides have been found to be sensitive to CH_3G.[19–22] Furthermore, combinations of DDUG and CH_3G acted in a synergistic manner in inhibiting the growth of the parent S-180 cells while the combinations of NSC 38280 and DDUG were additive. Additional evidence for some similarity between DDUG and NSC 38280 was provided by the observation that NSC 38280 effectively inhibited the uptake of DDUG while CH_3G did not. Also, the intracellular distribution of DDUG (30 per cent in mitochondrial, 70 per cent in nuclear fraction) is similar to that reported for NSC 38280.[23,24] NSC 38280 has been shown to be associated with cellular lipids.[23,25] Similar conclusions are reached for DDUG on the basis of the types of solvents required for its extraction. Also, when S-180 cells became resistant to DDUG by reducing the rate of uptake of the compound, they became resistant to NSC 38280 possibly by the same mechanism. Unlike the combinations of DDUG with CH_3G, the combinations of NSC 38280 with CH_3G did not inhibit the growth of S-180 cells in a synergistic manner. This is in contrast to findings of other investigators who have shown a synergistic antitumor activity with combinations of NSC 38280 and CH_3G.[19,26] The cross-resistance of vincristine resistant S-180 cells to NSC 38280 and their sensitivity to DDUG revealed another point of difference between the two compounds. It is suggested that DDUG and NSC 38280 are similar in respect to cellular uptake and intracellular binding, but evidence is lacking to suggest a similarity in their biochemical mode of action.

It remains to be elucidated whether the DDUG bound to the nuclear and mitochondrial fractions is responsible for its inhibitory effect on cellular multiplication. One can estimate that at an extracellular concentration of DDUG (0·46 μM) which inhibited growth of S-180 cells only slightly (20 per cent in 6 days), the cells after 2 days in such a medium contained 0·21 μmoles of DDUG per g. At a concentration (1·7 μM) which in 2 days inhibited growth by 50 per cent (60 per cent in 6 days) the cells contained 0·53 μmoles of DDUG per g. In resistant cells a much higher extracellular concentration (10·5 μM) which was necessary for equal inhibition (60 per cent in 6 days) resulted in 1·0 μmole of DDUG per g of cells in 2 days. As was mentioned in the text, the amount of DDUG in the cells continued to increase almost linearly when fresh medium was applied after 2 days. Therefore, the values as shown represent only a fraction of the actual amount of DDUG which would be found at 6 days when a fresh medium had been applied two more times. Even without taking this into consideration, it is clear that the amount of the total cellular DDUG necessary for growth inhibition is very high. It also seems that almost half of this DDUG in sensitive cells is perfectly harmless to the cells. In resistant cells the amount of DDUG was twice that in sensitive cells after 2 days incubation in equally inhibitory concentrations of DDUG. This might indicate a second change (intracellular) associated with resistance to DDUG, in addition to that which seems to involve the plasma membrane. The lack of synergism with combinations of DDUG and CH_3G in inhibiting the growth of the resistant cells (unlike the sensitive cells) might also reflect such change.

From a theoretical point of view, the observations made in the two-phase solvent system of Folch *et al.*[18] were intriguing. In the absence of phospholipids, DDUG was found mainly in the upper methanol–H_2O phase and the presence of lecithin or sphingomyelin in the chloroform phase had no effect on this distribution. However, noncholine phospholipids caused a striking shift in DDUG distribution from the

upper to the lower chloroform–H_2O phase which then resembled more the distribution observed in the extracts of cells. It seems that the positively charged DDUG is attracted by the negatively charged phosphate of phospholipids whenever it is not shielded or neutralized by the bulky, positively charged choline moiety. Inorganic phosphate alone in the methanol–H_2O phase caused a shift in distribution of DDUG in cellular extracts from the lower to the upper methanol phase, indicating affinity between inorganic phosphate and DDUG. In 1961, when Hirt and Berchtold[27] introduced phthalanilides, these were specifically intended to be phosphatide blockers. A certain selectivity in the formation of phosphatide–phthalanilide (NSC 57153) complexes was also observed by Yesair *et al.*[28] Choline containing phospholipids make up most (59 per cent) of the membrane phospholipids in the plasma membranes of mouse L-cells grown *in vitro*.[29] On the other hand, large differences were observed among different cell types in this respect. It is proposed that the noncholine phospholipids in plasma membranes attract DDUG and aid its passage into the subcellular compartments. This would be in accord with the passive uptake of DDUG and the competition observed with NSC 38280. To explain the alteration in the plasma membrane resulting in reduced rate of uptake in the resistant cells, one could suggest that in the plasma membranes of the DDUG-resistant cells the choline-containing phospholipids are more numerous or spacially so arranged that less attraction for DDUG or NSC 38280 is manifested.

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