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Differential Uptake of 1-(2-Chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea and Doxorubicin by Lewis Lung Carcinoma and Ridgway Osteogenic Sarcoma[1]

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ABSTRACT

1-(2-Chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea (MeCCNU), a lipophilic substance, is therapeutically effective against many murine tumors, especially Lewis lung carcinoma (LL), but is surprisingly ineffective against Ridgway osteogenic sarcoma (ROS). Most alkylating agents and doxorubicin (DX) are active against ROS but have relatively little therapeutic activity against LL; most are water soluble. We have analyzed these differences further by measuring blood and tumor concentrations of MeCCNU and DX in LL- and ROS-bearing mice (C57BL/6J × DBA/2 F_1 and AKR/J × DBA/2J F_1, respectively) after i.p. injection of the maximum tolerated doses (MeCCNU, 40, and DX, 15 mg/kg). MeCCNU blood levels were similar in the two tumor-bearing strains, falling rapidly from 12- to 1.8-μg equivalents/ml between 1 and 6 hr and to zero by 24 hr. DX plasma levels were also similar in the two mouse strains. Presumably, differences in drug concentrations and half-life in the circulation are not the cause of the differential sensitivity. Tumor levels were more illuminating. MeCCNU concentrations were 3-fold higher in LL than in ROS. At 1, 3, 6, and 24 hr, the levels in μg equivalents/g were, respectively, 20, 13, 5, and 1.5 in LL and 5, 3, 2, and 1.7 in ROS. Conversely, ROS had approximately 50% higher concentrations of DX than did LL at each time interval. It was noted in MeCCNU assays that LL contained significantly more ether-extractable lipids than did ROS (3.1 ± 0.4 *versus* 0.67 ± 0.2 mg/g). The above results suggest that LL and ROS differ in sensitivity to the two drugs because of differences in uptake that may be related to differences in lipophilicity of the drugs and lipid content of the tumors.

INTRODUCTION

The nitrosoureas BCNU,[3] CCNU, and MeCCNU have chemotherapeutic activity against our rodent leukemias and solid tumors, especially against LL, but are strikingly ineffective against ROS (15). Especially outstanding is the effect of MeCCNU against LL. Similar results have been reported in the literature (7, 10, 11, 13). In contrast to the lipophilic nitrosoureas, most alkylating agents and DNA intercalators, *e.g.*, DX, are very active against ROS but have relatively little activity against LL (7, 13, 17, 20); they are water soluble. Thus, Goldin and Johnson (3) report that both i.p. or i.v. treatment with DX of s.c.-growing LL, early or advanced, was ineffective. In contrast, Schabel (13) and Laster (7) found that a single treatment with DX (14 mg/kg i.p.) of 12-day-old ROS tumors resulted in 7 of 8 complete regressions and a marked increase in the life span of the animals.

In this study, we attempt to correlate the differential chemotherapeutic sensitivities of LL and ROS to MeCCNU and DX with the corresponding drug concentrations in the tumor tissues and provide a possible explanation for these differences.

MATERIALS AND METHODS

Animals, Drugs, and Tumors. ROS was grown in female AKR/J × DBA/2J F_1 (hereafter called AKD2F_1) mice obtained from The Jackson Laboratory, Bar Harbor, Maine; and LL was grown in female C57BL/6J × DBA/2 F_1 (hereafter called B6D2F_1) mice from Harlan-Sprague-Dawley, Madison, Wis. In general, groups of 4 to 20 mice weighing 19 to 25 g were used.

MeCCNU was suspended in 0.5% carboxymethylcellulose in 0.9% NaCl solution. DX was dissolved in 0.9% NaCl solution. Compounds were prepared immediately prior to injection or were stored frozen in daily samples at about −20°. Both compounds were supplied by the Drug Development Branch, Drug Research and Development, Chemotherapy, National Cancer Institute, Bethesda, Md.

The MTDs of each drug were determined in tumor-free mice by plotting on probability paper the dose mortality data which had been obtained for several dose levels with 5 to 20 mice per level.

The tumors were transplanted s.c. by means of small fragments into the right axillary region. Details of injection schedules are listed in the appropriate tables. The effects of treatment against the tumors were determined on the basis of the ratio of the average tumor diameters of treated (*T*) to control (*C*) groups (*T*/*C*). The MTD response for each drug against the 2 tumors was obtained, and for further comparison of the drug effects, the therapeutic index

$$TI = \frac{MTD}{MED}$$

was calculated. The MED is the dose of a drug which just produces a statistically significant *T*/*C* value (14). Thus, the TI is the ratio of MTD to MED, and a higher index indicates greater antitumor effect.

Measurement of Tissue MeCCNU Levels and Lipid Content. Mice bearing 14- to 17-day-old ROS and 7- to 10-day-old LL tumors measuring approximately 1 cm in diameter were used. Tumors with obvious necrosis were discarded. MeCCNU, 40 mg/kg, was given i.p. and, after various intervals up to 24 hr, groups of 4 mice were bled via the eye plexus and sacrificed, and tumor samples were taken. At each time point, MeCCNU levels in blood and tumor tissue were determined using

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[3] The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea; LL, Lewis lung carcinoma; ROS, Ridgway osteogenic sarcoma; DX, doxorubicin hydrochloride (Adriamycin); MTD, maximum tolerated dose (10% lethal dose within 4 weeks of administration); TI, therapeutic index; MED, minimum effective dose; LD_{50}, 50% lethal dose.

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the colorimetric method of Bratton Marshall as described in Ref. 9. Details of the tissue preparation, extraction, and colorimetric assay using a Beckman Model 24 spectrophotometer (absorbance measured at 540 nm) have been reported elsewhere (6, 9). Although most samples were clear when we measured their absorbance, the LL tumor samples consistently produced a cloudy emulsion (assumed to be ether-extracted lipid), and the degree of cloudiness varied. Filtering through an F-sintered glass funnel by suction did not remove the cloudiness, and therefore, we took 2 absorbance readings, one before the addition of the Bratton Marshall color reagent and the second after 10 min when the color was fully developed. The difference between them proved to be a reproducible method of measuring absorbance due to the color reaction. The weights of ether-extractable material (lipid) from the 2 tumors were found by extracting the tumors in the above manner, but with 5 times greater sample size. After evaporation of the ether, the samples were dried overnight in a vacuum dessicator, and the weight of lipid present was expressed as mg/g of tumor tissue.

Measurement of DX Levels. DX, 15 mg/kg, was given i.p., and blood and tumor samples were taken as described above. Liver samples (with gall bladder removed) were also taken. At each time point, the DX levels in plasma, tumor, and liver were determined using the method of Schwartz (18). Details of the tissue preparation, extraction, and fluorescence assay using a Perkin-Elmer MPF 44A spectrofluorometer (optimal excitation wavelength, 467 nm, and maximum emission wavelength, 592 nm) are as reported by Formelli *et al.* (2).

Preparation of Standards. Scalar amounts of MeCCNU or DX were added to blood (or plasma), liver, and tumor homogenates from control mice of both tumor-bearing strains, and the standards were extracted as described above. The calibration curves obtained were linear and almost identical for a given tissue from the 2 strains of mice. Extractability of drugs was found to be drug and tissue dependent but always greater than 90%. Drug concentrations of the experimental samples were determined by comparison with the appropriate standard curve, thus correcting for extractability of the drugs and tissue background absorbance.

Neither assay allowed for the separation of the parent drug from its metabolites, and therefore, the results are expressed as μg of drug equivalents per g of tissue or per ml of blood (plasma for DX).

Cytocidal Test. In the *in vivo* cytocidal method (1), the drugs were injected at the acute MTD and increased by 2-fold increments. The donor mice were sacrificed at 3 hr after injection, and the tumor was transplanted into 5 host mice for bioassay. The cytocidal LD_{50} dose is the dose at which 50% of the host mice show tumor take after 6 weeks of observation. Tissue assays of the drugs at or near these cytocidal LD_{50} levels were carried out 3 hr after i.p. injection as described previously.

RESULTS

The MTDs for the single and 6 daily injection schedules and the respective MTD responses and TIs are listed in Table 1. LL was much more sensitive to MeCCNU than was ROS, both by the MTD response and the TI, the TIs of LL being almost 3 times greater than those of ROS. The order of effectiveness was different for DX. It was ineffective in LL but had a TI of about 2 in ROS.

The blood concentrations of MeCCNU (and its ether-extractable nitroso-containing metabolites) were the same in LL- and ROS-bearing mice after i.p. injection of the MTD of 40 mg/kg (Chart 1). The blood levels decreased relatively slowly, and 3

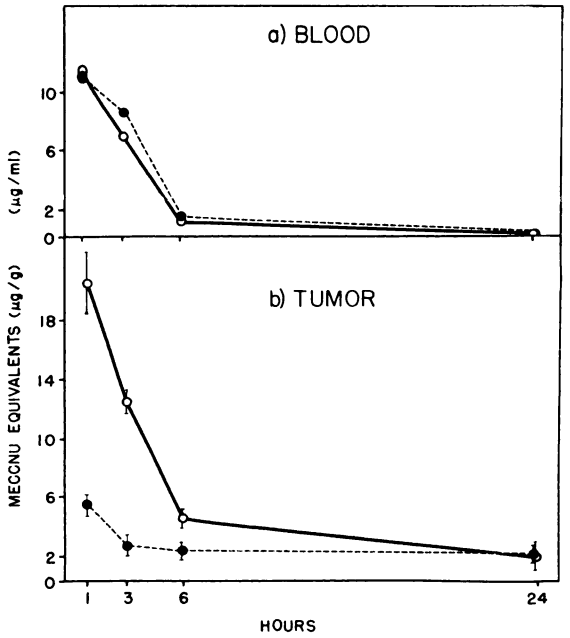

Chart 1. MeCCNU concentrations of blood and tumor from $B6D2F_1$ mice bearing LL (○) and $AKD2F_1$ mice bearing ROS (●). Mice were sacrificed, and MeCCNU levels were assayed (see "Materials and Methods") at 1, 3, 6, and 24 hr after i.p. injection of 40 mg of the drug per kg. The concentrations plotted at each time interval are expressed as MeCCNU equivalents (μg/ml or μg/g). *Points,* mean of 4 to 10 values; each value is 4 pooled mice for blood and individual mice for tumor levels. *Bars,* S.E. LL and ROS tumor levels of MeCCNU were significantly different at 1-, 3-, and 6-hr intervals ($p < 0.01$).

Table 1

Sensitivity of LL and ROS to MeCCNU and DX

The MTDs of the drugs in tumor-free mice are listed. Tumor transplantation was on Day 0. Treatment with the MTD was started on Day 1 for LL and on Day 5 for ROS. The MTD responses were determined on the basis of the treated/control (*T/C*) ratio of the tumor diameters. LL was evaluated by average tumor diameters on Day 14 and ROS, on Day 19. For these 2 tumors, the MED is the dose of the drug which produces a *T/C* of 0.7, as determined previously (14). The TI = MTD/MED. Groups of 5 to 20 mice weighing 19 to 24 g were used.

	MTD (mg/kg) for tumor-free mice		LL in $B6D2F_1$ mice			ROS in $AKD2F_1$ mice		
	F_1 B6D2F	F_1 AKD2F	MTD response (*T/C*)	MED (mg/kg)	TI	MTD response (*T/C*)	MED (mg/kg)	TI
Single i.p. injection								
MeCCNU	40	38	0	10	4	0.54	26	1.4
DX	15	14	0.87	>MTD	<1	0.15	5	2.8
Six daily i.p. injections								
MeCCNU	12.5	11.6	0.11	4.2	3	0.65	9.64	1.2
DX	2.6	2.4	0.92	>MTD	<1	0.22	1.2	2.2

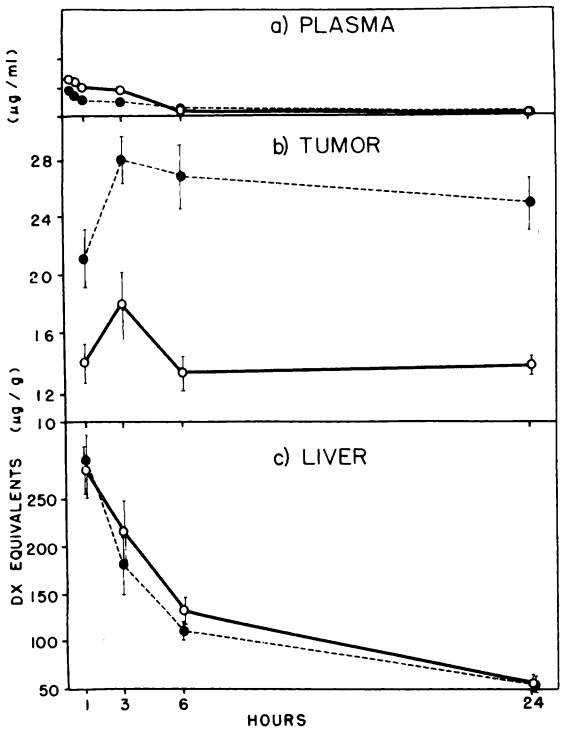

Chart 2. DX concentrations of plasma, tumor, and liver from B6D2F$_1$ mice bearing LL (○) and AKD2F$_1$ mice bearing ROS (●). Mice were sacrificed, and DX levels were assayed (see "Materials and Methods") at 1, 3, 6, and 24 hr after i.p. injection of the drug. Plasma levels were also assayed at 15 and 30 min. The concentrations plotted at each time interval are expressed as DX equivalents (μg/ml or μg/g). For tumor and liver assays, each *point* represents the mean of 3 to 5 individual mice; *bars*, S.E. For plasma assays, each *point* represents the value of 4 pooled mice. LL and ROS tumor levels of DX were significantly different at 1-, 3-, 6-, and 24-hr intervals ($p < 0.05$).

hr after drug injection, they were still approximately 8-μg equivalents/ml. In contrast to the blood, the levels of MeCCNU equivalents in LL and ROS tumor tissues differed greatly. One hr after injection, they were 5 μg/g in ROS but 4 times greater in LL. At 3 hr, the concentrations in ROS were 3 and in LL 13 μg/g, and 24 hr after injection, they were close to zero.

The DX-equivalent concentrations in plasma, tumor, and liver at different times after i.p. injection of 15 mg/kg are shown in Chart 2. The drug levels in plasma of both mouse strains were similar. After 15 min, the plasma of ROS-bearing mice was 1.7-μg equivalents/ml, and of LL mice, it was 2.7 μg/ml; 6 hr after injection, the DX levels were no longer measurable. In the tumor tissue, the drug concentrations increased until 3 hr after injection when they reached the highest value in both tumors, 18-μg equivalents/g for LL and 28 μg/g for ROS. The tissue levels showed a gradual drop from 3 to 6 hr after administration and then remained relatively constant beyond 24 hr after injection, when the LL values were 14-μg equivalents/g, and the ROS values were 27 μg/g.

The DX levels in liver were similar in the LL- and ROS-bearing mice. One hr after drug administration, the concentration was about 280 μg/g but then sharply decreased, reaching 54 μg/g by 24 hr.

In the *in vivo* sterilization experiments (Table 2), the single i.p. doses of drug required to sterilize 50% of the tumors (cytocidal dose, LD_{50}) were in agreement with the differential antitumor effects shown in Table 1. MeCCNU is more effective

Table 2

In vivo cytocidal effects and tumor concentrations in LL and ROS

Three hr after i.p. injection of the drug, tumor pieces were transplanted s.c. into 5 hosts in 2 to 3 separate tests. Viability was evaluated by percentage of tumor take. The cytocidal LD_{50} dose is the dose at which 50% of the host mice show tumor take after 6 weeks of observation. The tumor tissue concentrations were determined 3 hr after i.p. injection of drug.

	Cytocidal LD_{50} (mg/kg)	Dose (mg/kg)	Tumor concentration (μg/g)
MeCCNU			
LL	66	40	12.4 ± 0.9
ROS	158	160	12.2 ± 0.7
DX			
LL	>150	150	89 ± 11
ROS	135	150	238 ± 66

against LL than ROS; therefore, as expected, the cytocidal LD_{50} dose of MeCCNU was also much smaller for LL than for ROS. Also, the dose of 40 mg/kg for LL caused the same tissue concentration of MeCCNU equivalents as the 4-fold-higher LD_{50} cytocidal dose for ROS. In contrast, for DX, the cytocidal LD_{50} doses were greater for LL than for ROS. The dose of 150 mg/kg caused more than 2-fold-higher tumor concentration in ROS than in LL.

For the quantitative determination of the ether-extractable lipids in LL and ROS, 3 to 5 separate experiments were averaged. LL contained 3.1 ± 0.4 mg lipids per g, and ROS contained 0.67 ± 0.2 mg per g ($p < 0.01$).

DISCUSSION

The chemotherapeutic results in Table 1 confirm our previous findings (Footnote 4; Ref. 15) and those of other workers (3, 7, 13) on the differential sensitivities of LL and ROS to MeCCNU and DX.

The clearance of each drug from the blood circulation was the same in both mouse strains used, suggesting that neither plasma half-life nor concentration is the cause of the differential chemotherapeutic sensitivities.

Sensitivity of a tumor to a drug obviously involves both tumor and drug characteristics. A major difference between MeCCNU and DX is their lipophilicity, the octanol-water partition coefficient for MeCCNU being 3.3 (4) and DX base, only 0.52.[5]

At the MTD, MeCCNU levels in LL reached 4 times those of ROS, indicating that drug uptake is more efficient in LL. The relative antitumor effects are in agreement with the well-established observation that cytotoxicity is related to the concentration of the antitumor agent and duration of exposure. It is interesting that, although there is a 4-fold difference between the MeCCNU cytocidal LD_{50} doses of LL and ROS, the concentrations 3 hr after injection of these doses are almost the same in the 2 tumors (Table 2). This indicates that ROS is basically as sensitive as LL to MeCCNU, but the differential sensitivity observed at the MTD is due to poor uptake of MeCCNU by ROS. A reason for this became evident during the Bratton Marshall assay, where the LL tissue contained approximately 4 times more ether-extractable lipids than did the ROS samples. This finding might help explain the great sensitivity of LL

[4] F. A. Schmid, unpublished data.

[5] F. Arcamone, personal communication.

to the clinically used nitrosoureas BCNU, CCNU, and especially MeCCNU.

The better antitumor effect of DX against ROS can similarly be related to the higher DX concentration in ROS as compared with LL; in agreement is the finding that the high dose of 150 mg/kg (Table 2) also caused higher tumor concentration in ROS than in LL. The fact that DX rapidly disappears from the plasma (22) suggests that any factor affecting its rate of uptake, such as high lipid content of LL or fragile membrane of ROS (16), may be a cause of the differential concentrations.

ROS is considered to be a good predictive tumor system for the clinical use of diverse agents (13). It is relatively sensitive to most clinically used chemotherapeutic agents. LL, in contrast, is refractory to the well-known chemotherapeutic agents with a few exceptions, especially the nitrosoureas (5).

The degrees of lipid solubility and their antitumor activity against LL both follow the same order: MeCCNU > CCNU > BCNU (4, 10–12).[4] Several investigators showed a correlation between the lipophilic character of the nitrosoureas and their antitumor activity, especially against brain tumors (4, 8, 12, 21). First, the specific activity of the nitrosoureas on the brain tumors was wholly attributed to the ability of the lipophilic drugs to cross the blood-brain barrier, but more recent investigations (4, 8, 19) stress the fact that brain is a lipophilic tissue and thus is probably more easily permeable to lipid-soluble drugs. Lipophilic drugs probably enter both the tumor and the surrounding normal brain, preventing the formation of a concentration gradient and permitting time for the agent to take effect (19).

We have shown that LL and ROS differ in sensitivity to MeCCNU and DX because of differences in uptake and suggest that the differential uptake is related to the lipophilicity of the drugs and the lipid content of the tumors. However, this does not discount the possibility that other factors such as repair are equally if not more important.

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