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EFFECTS OF *S*-(TRITYL)-L-CYSTEINE AND ITS ANALOGS ON CELL SURFACE PROPERTIES OF LEUKEMIA L1210 CELLS

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Abstract—*S*-trityl-L-cysteine (NSC 83265) and selected analogs were examined by conventional and new techniques to identify drug effects on intracellular macromolecule biosynthesis and on cell surface properties. These compounds inhibit growth of murine leukemia cells *in vivo*, and interfere with incorporation of precursors into nucleic acid and protein in culture. The drugs also cause disorganization of cell membranes, affecting barriers to actinomycin D uptake and producing alterations in membrane charge distribution measured by a two-phase aqueous polymer system.

Several methods have previously been used by us to characterize interactions between anti-tumor agents and murine leukemia cells in culture which result in inhibition of biosynthesis of DNA [1–3], RNA [3, 4], protein [4, 5] and glycoprotein [5]. This work was based on methods cited in bibliographies of Refs. 1–5, used to characterize modes of action of anti-neoplastic agents. Recently, we have studied drug effects on cell surface properties by measuring drug-induced changes in permeability barriers, and in cell behavior in a two-phase aqueous partitioning system [6]. Among the agents producing such effects is *S*-trityl-L-cysteine, NSC 83265 (Fig. 1). This compound and several of its analogs [7] have potent anti-tumor activity agsinst the L1210 murine leukemia *in vivo*. We have compared effects of NSC 83265 and its congeners with those of other agents with known modes of action, to determine interrelationships between cell surface effects and inhibition of incorporation of precursors into nucleic acid and protein. The procedures employed here may be useful in the study of cytotoxic drug effects involving phenomena occurring at the cell surface.

MATERIALS AND METHODS

Cell cultures. L1210 murine leukemia cells were maintained in MEM-Eagle's medium, spinner modification (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum. The cells were grown in two-thirds filled sealed flasks in air. Experiments were carried out using cells suspended in HEPES-buffered medium [4] to minimize pH alterations during short-term incubations of 10^6–10^7 cells/ml.

Experimental agents. *S*-trityl-L-cysteine, NSC 83265, and its analogs were supplied by the Drug Evaluation Branch, Drug Research and Development, Division of Cancer Treatment, NCI. NSC 83265, labeled in the trityl carbon (5.3 mCi/m-mole), was supplied by the Stanford Research Institute, Stanford, Calif. These agents were dissolved in *N*,*N*′-dimethylformamide (DMF) and stored at −20°. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.), arabinosylcytosine (CalBiochem Corp., Los Angeles, Calif.), and actinomycin D (Merck, Sharpe & Dohme, West Point, Pa.) were dissolved in water and used as controls in these studies.

Radioactive substrates. Stock solutions of [2-^{14}C]thymidine, 100 μM (40 μCi/ml); [2-^{14}C]uridine, 100 μM (40 μCi/ml); and [U-^{14}C]L-leucine, 1 mM (50 μCi/ml), were prepared from compounds purchased from New England Nuclear Corp., Boston, Mass. [U-^{3}H]actinomycin D, 10 μg/ml (100 μCi/ml), was provided by the Monsanto Chemical Co., St. Louis, Mo.

Drug effects on protein and nucleic acid synthesis. Portions (1 ml) of cell suspensions containing 5 to 7×10^6 cells were warmed to 37° for 20 min; then compounds dissolved in 10 μl dimethylformamide or H_2O, or solvent alone, were added. After a specified interval, usually 10 min, stock solutions of radioactive precursors were added to obtain a final level of 1 μM uridine, 1 μM thymidine or 10 μM leucine. After an additional period of incubation, the cells were collected by centrifugation, and incorporation of label into material insoluble in 0.2 M $HClO_4$ was measured

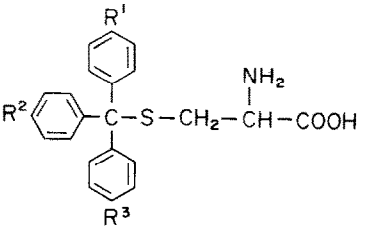

Fig. 1. Structure of *S*-(tritylthio)-L-alanine, NSC 83265, and its analogs. For the parent compound, R^1, R^2, and R^3 = H. Other derivatives are described in Table 1.

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by liquid scintillation counting. To measure intracellular levels of radioactive nucleotides, incubations were carried out at 22°, a condition which reduces DNA and RNA synthesis by 90–95 per cent without altering uptake or phosphorylation of nucleosides. Brief washing of cells with 0.9% NaCl removed extracellular radioactivity from cell pellets.

To measure reversibility of drug effects, cells were first incubated in medium containing drugs for 10 min, then collected by centrifugation and suspended in fresh medium at 37° for 3–30 min before addition of protein or nucleic acid precursors, or labeled actinomycin D, or before measuring the partition ratio.

In other control experiments, we measured effects of cycloheximide (a specific inhibitor of protein synthesis), cytosine arabinoside (an inhibitor of DNA synthesis), and actinomycin D (an inhibitor of RNA synthesis).

Drug effects on uptake of actinomycin D. Experiments were carried out as outlined above, except that the radioactive agent employed was actinomycin D (final level = 0.1 μg/ml, 0.05 μCi/ml). After incubations, the cells were collected by centrifugation, washed with 0.9% NaCl, and the intracellular level of radioactive actinomycin D was measured by liquid scintillation counting.

Uptake of radioactive NSC 83265. Stock solutions of labeled drug in DMF were added to cell suspensions to yield a level of 0.3 mM/ml (0.02 μCi/ml). Incubations were carried out at 0° or at 37° for 1–30 min. In some experiments, cells were incubated with labeled drug for 3 min, then suspended in fresh medium for 30 min, and exodus of the drug was measured. After incubation, cells were collected by centrifugation and washed with 0.9% NaCl to remove extracellular radioactivity. In other experiments, effects of washing with 0.2 M $HClO_4$ and formamide were assessed. Determinations of radioactivity were made by liquid scintillation counting.

Partition studies. Cell suspensions were treated with drugs for 3–10 min as outlined above. In some cases, the cells were then suspended in fresh medium for 3–30 min at 37° to assess reversibility of drug effects. Finally, the cells were suspended in 150 mM NaCl, and a 0.5-ml portion, containing 10^6 cells, was added to a partition mixture. The latter contained 5% (w/v) Dextran T-500 (Pharmacia, Piscataway, N.J.), 4% polyethylene glycol (mol. wt 6000, Pierce Chemical Co., Rockford, Ill.), 50 mM NaCl and 100 mM potassium phosphate buffer, pH 7.0 [6]. The total volume was 10 ml. This mixture was contained in small screw-cap test tubes which were inverted several times, and a sample of 0.5 ml was taken for determination of the total cell number, using a Z_F Coulter counter. The polymer phases were allowed to separate for 60 min at 4°, and the top phase was collected. A 0.5-ml sample of this phase was then removed, and the cell number determined. The number of cells in the top phase as per cent of total cell number was calculated. These experiments were performed in triplicate for each point.

Octanol–water partition studies. The partition of radioactive trityl-cysteine between octanol and water was measured as described before [8], using equal amounts of these two solvents, each of which was saturated with the other. The ratio of the drug level in octanol to the drug level in water was then measured. The octanol phase was then re-extracted with octanol-saturated water and another such determination was made. Minor water-soluble impurities were thereby removed. Subsequent re-extractions did not alter the results.

Screening data. Effects of NSC 83265 and its analogs on survival of mice bearing the L1210 leukemia were provided by the Drug Development Branch, Division of Cancer Treatment, NCI. This information, in part, has been published [7].

RESULTS

The structure of NSC 83265 (R^1, R^2, R^3 = H) is shown in Fig. 1. Two drugs were employed in which one of the phenyl substituents shown was replaced by α-naphthyl (NSC 123492) or β-naphthyl (NSC 123529) groups. The D-optical isomer, *S*-trityl-D-cys-

Table 1. Properties of *S*-trityl-L-cysteine and its analogs*

NSC No.	R^1	R^2	R^3	T/C	Drug effect on actinomycin D uptake	Partition coefficient (%)
83265	H	H	H	180†	40 ± 3‡	21.0 ± 1.0
124676	D-Isomer of NSC 83265			156	45 ± 2	20.4 ± 1.0
123493	F	H	H	193	60 ± 4	18.6 ± 1.0
126217	Br	H	H	214	70 ± 5	19.2 ± 1.0
123139	CH_3	H	H	204	45 ± 2	21.0 ± 1.5
123491	CH_3	CH_3	CH_3	210	70 ± 4	18.6 ± 1.0
124675	OCH_3	OCH_3	H	140	20 ± 2	22.8 ± 1.5
123492	α-Naphthyl‖			100	50 ± 4	22.8 ± 1.5
123529	β-Naphthyl‖			215	115 ± 8	16.8 ± 1.0

* Structural formulae are shown in Fig. 1. Data shown represent average value of four determinations; the range is shown.

† Activity *in vivo* against L1210 murine leukemia at optimal dose schedule for 9 successive days. T/C = ratio × 100 of survival of treated/control animals (days).

‡ Per cent increase in uptake of actinomycin D by cells treated with 0.3 mM drug as described in the text.

§ Number of cells in top phase as per cent of total cell number. For these experiments, a control value of 24% was established.

‖ In these drugs, one benzene ring of the parent compound was replaced by an α- or β-naphthyl group.

drugs examined on survival of tumor-bearing mice teine (NSC 124676), was also tested. Effects of all are shown in Table 1. The D-isomer was only slightly less effective than NSC 83265. Halogen or methyl substituents had only minor effects on drug effectiveness; OCH_3 substituents slightly decreased activity. The β-naphthyl analog was somewhat more active then the parent compound; the α-naphthyl substitution abolished activity.

In controls, the intracellular–extracelluar distribution ratio of actinomycin D, reached after 5 min of incubation at 37°, was 12 ± 0.5. Uptake of actinomycin D was strongly promoted by many compounds tested here (Table 1); this effect was most striking in the case of NSC 123529 (the β-naphthyl derivative), and least for NSC 124675, the OCH_3-derivative. In related studies, we examined effects of cycloheximide (0.05 mM) and cytosine arabinoside (0.1 mM) on actinomycin D uptake and found no alteration thereof.

The partition of L1210 cells between phases rich in Dextran and in polyethylene glycol was also measured. The number of cells in the upper phase as per cent of total cell number was 24 ± 1% in control tubes. The partition ratio was decreased by most drugs examined, most substantially by NSC 123529. In control studies, we found no effect on the partition ratio by 0.05 mM cycloheximide, 0.1 mM cytosine arabinoside or 0.01 mM actinomycin D. These drug levels substantially inhibit protein or nucleic acid biosynthesis as described below.

The drugs tested here all inhibited incorporation of precursors into nucleic acid and protein. Values did not differ substantially within 10 min; 0.3 mM drug levels inhibited incorporation of thymidine into DNA by 65–80 per cent, of uridine into RNA by 60–80 per cent, of leucine into protein by 75–85 per cent. At the 0.05 mM level, cycloheximide inhibited protein synthesis by 90 per cent, DNA synthesis by 70 per cent, but RNA synthesis by only 10 per cent. Cytosine arabinoside (0.1 mM), within 10 min, inhibited DNA synthesis by 90 per cent with no effect on biosynthesis of DNA or protein.

Effects of NSC 83265 and its analogs were partly reversed when the drug was removed from culture media. Representative examples are shown in Table 2. Effects of NSC 83265 on protein and nucleic acid and protein biosynthesis were partly reversed, drug effects on actinomycin D uptake were wholly reversed, but the partition ratio increased to values greater than control. These effects were more striking for the β-naphthyl derivative, NSC 123529. Thirty min after removal of the drug, substantially more cells were found in the upper phase than in control studies.

It is noteworthy that NSC 83265 and NSC 123529 inhibited neither uptake nor phosphorylation of thymidine by L1210 cells (Table 3). The disorganization of the cell surface detected by other procedures did not alter nucleoside uptake, nor enzymes involved in nucleoside conversion to nucleotides.

Uptake of labeled NSC 83265 proceeded rapidly, with accumulation essentially complete within 1 min (Fig. 2). Uptake was greater at 37° than at 0°. Accumulated drug was partly lost upon suspension of preloaded cells in fresh medium at 37° (Fig. 2); however, a residue of drug could not be removed, even after 2 hr (data not shown). In other studies, we found that a brief wash of preloaded cells with formamide removed 80 per cent of the drug, but could not remove residual drug remaining after 30 min washing

Table 2. Reversibility of drug effects*

NSC No.	Drug effect	10-min Incubation, no wash	10-min Incubation, 10-min wash	10-min Incubation, 30-min wash
83265	Inhibition of protein synthesis (% of control)	23	35	55
	Inhibition of RNA synthesis (% of control)	33	53	63
	Inhibition of DNA synthesis (% of control)	34	50	75
	Actinomycin D uptake (% of control)	140	105	100
	Partition coefficient	21%	23.4%	27%
123529 and 123492	Inhibition of protein synthesis (% of control)	18 (23)	23 (26)	30 (35)
	Inhibition of RNA synthesis (% of control)	25 (28)	28 (31)	40 (47)
	Inhibition of DNA synthesis (% of control)	21 (22)	27 (29)	40 (44)
	Actinomycin D uptake (% of control)	215 (150)	140 (105)	100 (100)
	Partition coefficient	16.8 (23)%	24 (23)%	42 (24)%

* Cells were incubated for 10 min in medium containing 0.3 mM of drug, then washed in fresh medium for 10 or 30 min. Samples were taken at indicated points for measurement of incorporation of precursors into nucleic acid and protein as specified, for measurement of drug-induced promotion of actinomycin D uptake and for measurement of partition coefficient (control = 24%). Values obtained with NSC 123492 are shown in parentheses.

Table 3. Drug effects on nucleoside uptake and phosphorylation*

Drug	Level (mM)	Thymidine (cpm)	Nucleotides (cpm)
Controls		300	1200
NSC 83265	0.3	280	1180
NSC 123529	0.3	285	1130

* Cells were incubated with specified drugs for 10 min at 22°, then 1 mM (final level) of radioactive thymidine was added for an additional 5 min at the same temperature. Incorporation of label into acid-soluble and acid-insoluble pools was measured as outlined in Ref. 9.

in the culture medium. These data were not altered when the drug labeled with ^{14}C in the amino acid moiety was employed.

The octanol–water distribution of NSC 83254 was 45:1, indicating the compound to be highly lipid soluble. The drug is essentially insoluble in water, but dissolves readily in warm dimethylformamide.

DISCUSSION

The procedures described here, involving measurement of drug effects on actinomycin D uptake and on partition of cells between aqueous polymer phases, are intended to identify drug–cell interactions which directly affect the cell surface, as contrasted with interactions primarily leading to inhibition of nucleic acid or protein biosynthesis. In the present study, cytosine arabinoside and cycloheximide, agents which rapidly lead to inhibition of DNA and protein synthesis, respectively, have little effect on cell surface properties, as measured here, within 10 min. In contrast, trityl-L-cysteine and its analogs both affect macromolecule biosynthesis and cell surface phenomena.

Disruption of barriers to actinomycin D uptake [10] by detergents was previously shown [6] to lead to enhanced uptake of the drug. The procedure involved is simple, and can readily be used to screen large numbers of agents for action at the cell surface. Another procedure involves the measurement of the partition of cells between two aqueous polymer phases. This procedure has been described before [11–13] and apparently detects alterations in surface and subsurface charge patterns. We have found [6] that non-ionic detergents, like agents studied here, cause a substantial movement of cells from the top layer to the interface.

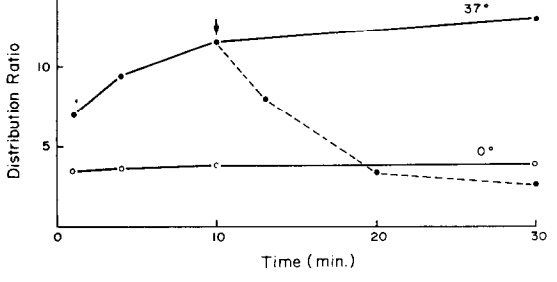

Fig. 2. Uptake of NSC 83265, labeled in the trityl carbon by L1210 cells. Incubations were carried out at 0° (-○-) or at 37° (-●-). Exodus of drug from preloaded cells during subsequent washing at 37° (--●--) is also shown. For exodus experiments, cells were suspended in fresh medium at the point indicated by the arrow. The distribution ratio is of drug concentration in intracellular to that in extracellular water. Data obtained with drug labeled in the amino acid moiety did not significantly differ from results shown here.

The drugs tested here did not affect thymidine uptake nor its conversion to a nucleotide pool which is retained by cells after washing with 0.9% NaCl [14]. Considerable disorganization of the cell membrane can apparently occur without affecting nucleoside transport.

The role of drug–cell surface interactions in cytotoxicity is, at present, unknown. Pharmacologic properties of NSC 83265, which might be compared with phenomena observed here, have not been fully explored. Experiments to determine whether certain types of drug toxicity are correlated with drug-induced disorganization of cell surfaces are now underway. The present data do show that such disorganization may have delayed as well as immediate cytotoxicity. Effects of NSC 83265, and an analog, on actinomycin D uptake appear to be readily reversible; drug effects on macromolecule biosynthesis may be slowly reversible, but drug-induced alteration in partition of cells between polymer phases was found to "overshoot" control values (Table 2) after removal of the drug from the nucleus. This may be related to the apparent tight binding of the drug to cell components (Fig. 2). "Alkylation" of macromolecules by the trityl component of NSC 83265 has been suggested [7], but the observed binding of the drug labeled in either the amino acid or the trityl moiety does not support this suggestion.

The octanol–water distribution of *S*-trityl-L-cysteine (40:1) shows the compound to be readily lipid soluble. Association of the drug with lipid components of the cell could also play a role in the observed tight binding of the drug to L1210 cells.

The relation between drug effects on macromolecule synthesis and effects on the cell surface appears to be remote. Drug effects on the cell surface appear to be a new and hitherto unexplored phenomenon. The relevance of such measurements with respect to anti-tumor action of specific agents remains to be established.

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