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# BIOCHEMICAL EFFECTS OF THE VINCA ALKALOIDS—IV

## STUDIES WITH VINLEUROSINE*

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**Abstract**—Exposure of sarcoma 180 cells to vinleurosine *in vitro* inhibited the uptake of uridine-$^3$H by the cells, as well as incorporation of this nucleoside into RNA, of 2′-deoxycytidine-$^3$H into DNA, of glutamic acid-$^{14}$C into proteins and of acetate-$^{14}$C into lipids. Incorporation of acetate into phospholipids was the parameter most sensitive to the inhibitory effect of the alkaloid. Similar effects were obtained when mice bearing the ascitic tumor were treated with vinleurosine and the incorporation of precursors was studied *in vivo*.

VINLEUROSINE is one of four indole alkaloids with antineoplastic activity that have been isolated from the periwinkle plant (*Vinca rosea* Linn.).[1] Although it is an effective inhibitor of the growth of murine tumors,[2] the compound has been disappointing in clinical trials,[3,4] possibly because of binding by plasma proteins or some other form of inactivation.

Vinleurosine has been reported to inhibit the incorporation of formate-$^{14}$C into nucleic acids in suspensions of rat thymus cells,[5] but no other more detailed biochemical studies have been undertaken.

The present paper describes the results of experiments carried out with sarcoma 180 cells to explore the metabolic effects of vinleurosine in more detail. It represents part of a continuing program aimed at evaluating the biochemical parameters involved in the action of compounds that arrest cell division in metaphase.

## MATERIALS AND METHODS

*Chemicals.* Vinleurosine sulfate was provided through the generosity of Doctors J. G. Armstrong and J. M. McGuire of Eli Lilly & Company, Indianapolis, Ind. Acetate-2-$^{14}$C, uridine-5,6-$^3$H and glutamic acid-3,4-$^{14}$C were purchased from the New England Nuclear Corp., 2′-deoxycytidine-$^3$H from Schwartz Bio-Research Inc. and uridine-5-$^3$H from Tracer-lab Inc.

*Tissues.* Sarcoma 180 (S180) cells in the ascites form were maintained in Swiss white mice (CD-1; Charles River Breeding Laboratories, No. Wilmington, Mass.) and harvested 4–6 days after intraperitoneal inoculation ($4 \times 10^6$ cells). Ascitic fluid was separated by centrifugation and erythrocytes were lysed by suspending the cell pellet in 4 volumes of distilled water. Isotonicity was restored with 4·5% saline, and, after further washing with isotonic saline, the cell pellet was suspended

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in Eagle's basal medium supplemented with 10% of dialyzed fetal calf serum (Microbiological Associates, Bethesda, Md.).

*Uptake of labeled precursors* in vitro. Tumor cells (0·05 ml) were incubated in supplemented medium for 1 hr, with or without vinleurosine. At the end of this preincubation, precursors were added per 2 ml of vol. as follows: 2′-deoxycytidine-$^{3}$H and uridine-$^{3}$H, 0·2 μc, 0·01 μmole; acetate-$^{14}$C and glutamic acid-$^{14}$C, 1 μc, 0·3 μmole.

Runs were terminated by cooling to 0° and centrifuging the cells through 0·25 M sucrose solution in Shevky-Stafford and McNaught centrifuge tubes to remove them from the incubation medium as described previously.[6] The procedures employed to separate alkaline hydrolysates of RNA, hot-acid extracts of DNA and residual protein were identical to those described in earlier studies.[6,7] In experiments involving uptake of acetate-2-$^{14}$C, the pellet obtained after centrifuging the cells through isotonic sucrose solution was extracted with ethanol–ether (3:1, v/v). The extract was evaporated to dryness and lipids were extracted from the residue with petroleum ether. These extracts were dried over anhydrous sodium sulfate and were either evaporated in the vials used for counting radioactivity or processed further for separation of neutral lipids and phospholipids. Radioactivity was measured with a Packard Tri-Carb scintillation counter.

*Incorporation of precursors* in vivo. Mice bearing 4- or 5-day-old growths of S180 ascites tumor received intraperitoneal injections of vinleurosine (2 mg/kg) followed 24 hr later by radioactive precursors (4 μc, 0·2 μmole); 30–60 min were allowed for metabolic utilization. The mice were killed, the ascitic fluid was removed, red cells were lysed by exposure to hypotonic saline and the tumor cells were extracted with cold 0·5 M perchloric acid and prepared for hydrolysis of DNA and RNA in the usual way.[6–8]

*Estimations.* Protein was determined by the biuret reaction[9] with bovine serum albumin fraction V as a standard. DNA was measured by the method of Burton[10] with 2′-deoxyadenosine as a standard, or by measuring ultraviolet absorbance of hydrolysates at 260 mμ as for RNA. Lipid was quantitated on a weight basis by evaporating solutions to dryness in aluminum pans.

*Fractionation of lipids.* Lipid extracts, dissolved in chloroform, were applied to 5 × 0·5 cm columns of silicic acid equilibrated with the same solvent. Neutral lipids were eluted with chloroform (30 ml) and the phospholipids with increasing concentrations of methanol in chloroform: 20%, cephalin; 38%, lecithin; 60%, sphingomyelin; and 100%, lysolecithin.[11]

## RESULTS

*Studies* in vitro. The uptake of deoxycytidine-$^{3}$H and its incorporation into DNA were followed in cell suspensions. Although uptake of the nucleoside into the acid-soluble fraction was not significantly affected by vinleurosine, its incorporation into DNA was profoundly inhibited (Fig. 1). In marked contrast, studies of the effect of vinleurosine on the disposition of uridine-$^{3}$H by S180 cells indicated that both uptake of the precursor and its incorporation into RNA were equally inhibited (Fig. 2). This result differs somewhat from those of experiments reported elsewhere,[12] in which the related alkaloid, vinblastine, markedly inhibited the incorporation of uridine into RNA by human leukosarcoma cells, but had much less effect on uptake of the nucleoside. Examination of the effect of vinleurosine on the metabolism of glutamic

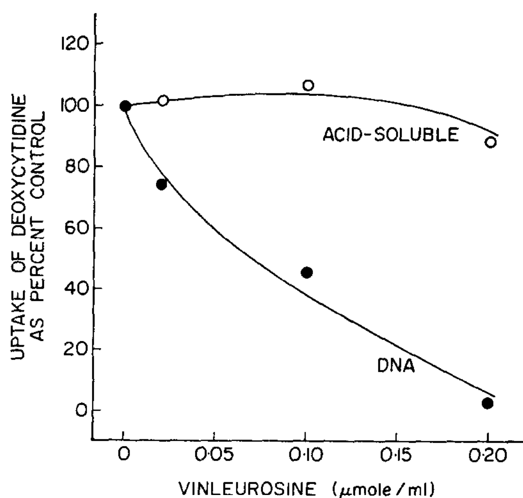


FIG. 1. The uptake of 2′-deoxycytidine-$^3$H and its incorporation into DNA by S180 cells as a function of vinleurosine concentration. Cells (0·05 ml) were incubated for 1 hr with vinleurosine and then deoxycytidine-$^3$H (0·2 μc; 0·01 μmole) was added. Data represent the amount of nucleoside taken up in 30 min, expressed as the per cent of that seen in the absence of vinleurosine. Values are derived from uptakes at 15 and 30 min (duplicate samples) and are the means of 2 experiments.

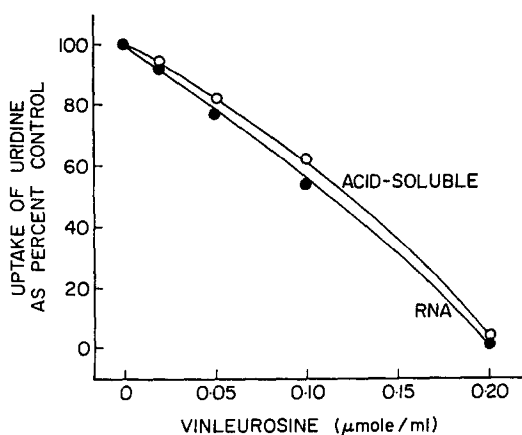


FIG. 2. The uptake of uridine-5,6-$^3$H and its incorporation into RNA by S180 cells as a function of vinleurosine concentration. Incubation conditions were as for the experiment in Fig. 1, but with uridine-$^3$H (0·2 μc; 0·01 μmole) substituted for deoxycytidine. Values, which are the means of 3 experiments, were derived from uptakes at 15 and 30 min for duplicate samples.

acid-$^{14}$C indicated that, as with the other vinca alkaloids and colchicine,[6] both the uptake of this amino acid and its incorporation into protein were depressed (Fig. 3). In the case of acetate-$^{14}$C, only the incorporation into lipids was examined (Table 1); vinleurosine depressed this uptake. Separation of several lipid fractions on columns of silicic acid showed that this inhibitory effect was much greater in the phospholipids than in the neutral fats; lecithin biosynthesis tended to be somewhat more sensitive (Fig. 4).

*Studies* in vivo. Since the production of biochemical changes *in vitro* may not necessarily be indicative of events that occur *in vivo* after administration of a drug, the various parameters described above were measured in mice 25 hr after treatment with vinleurosine (2 mg/kg). Labeled precursors were injected and 30–60 min were

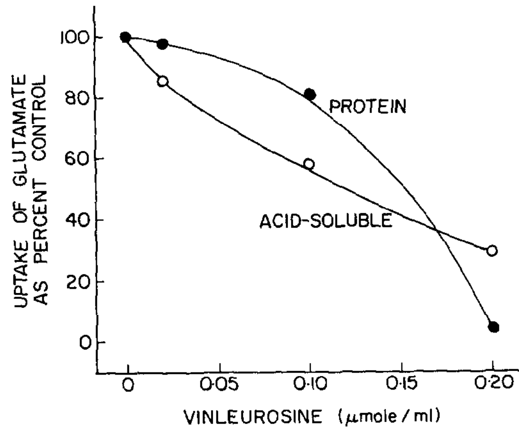


FIG. 3. The uptake of glutamic acid-3,4-$^{14}$C as a function of vinleurosine concentration. After a preliminary incubation with or without vinleurosine, glutamic acid-$^{14}$C (1 μc; 0·3 μmole) was added to the suspension of cells (0·05 ml) in a final volume of 2 ml. Values, which are the means of 2 experiments expressed as per cent of control, were derived from duplicate figures for uptake at 15 and 30 min.

TABLE 1. EFFECT OF VINLEUROSINE UPON THE UPTAKE OF ACETATE INTO LIPIDS OF S180 CELLS *IN VITRO**

| Vinleurosine (μmole/ml) | Time (min) | Incorporation (cpm/0·05 ml cells) |
|---|---|---|
| 0 | 30 | 630 |
| | 60 | 1305 |
| 0·02 | 30 | 344 |
| | 60 | 817 |
| 0·05 | 30 | 210 |
| | 60 | 467 |
| 0·1 | 30 | 70 |
| | 60 | 138 |

* Cells were preincubated with vinleurosine for 1 hr before addition of acetate-2-$^{14}$C (1 μc; 0·3 μmole) in a final volume of 2 ml. After separation of labeled medium, the cell pack was extracted with ethanol–ether (3:1), the extract was evaporated to dryness and lipids were dissolved in petroleum ether.

allowed for metabolic utilization. It can be seen (Table 2) that the incorporation of these precursors into all cellular components was depressed in mice receiving vinleurosine compared with saline-treated animals. The time course for the development of these effects was not explored.

TABLE 2. EFFECT OF TREATMENT WITH VINLEUROSINE (VLR) UPON THE INCORPORATION OF PRECURSORS BY S180 ASCITES CELLS *IN VIVO**

| Fraction | Precursor | Treatment | Incorporation (cpm/$10^7$ cells) |
|---|---|---|---|
| DNA | Deoxycytidine | Control | 4356 |
| | | VLR | 1492 |
| | Thymidine | Control | 2654 |
| | | VLR | 1425 |
| RNA | Uridine | Control | 3115 |
| | | VLR | 1989 |
| Protein | Glutamic acid | Control | 1595 |
| | | VLR | 690 |
| Lipids | Acetate | Control | 124 |
| | | VLR | 76 |

* Tumor-bearing mice received an i.p. injection of vinleurosine (2 mg/kg) 24 hr before the radioactive precursors (4 $\mu$c; 0·2 $\mu$mole). Times allowed for metabolic utilization were 30 min for 2′-deoxycytidine-$^3$H, thymidine-methyl-$^3$H and uridine-5-$^3$H, and 60 min for acetate-2-$^{14}$C and glutamic acid-3,4-$^{14}$C.

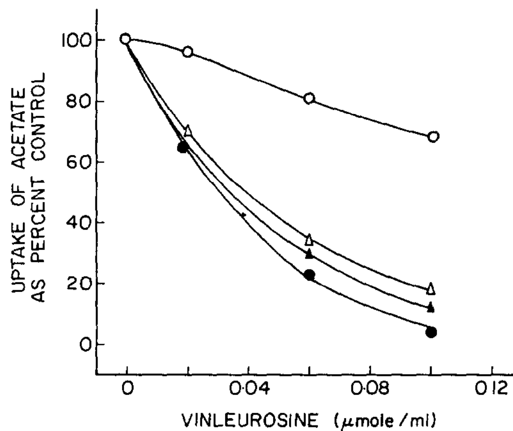


FIG. 4. The uptake of acetate-2-$^{14}$C into the lipids of S180 cells as a function of vinleurosine concentration. Cells (0·05 ml) were incubated for 1 hr with or without vinleurosine and then acetate-$^{14}$C (1 $\mu$c, 0·3 $\mu$mole) was added per 2 ml of incubation medium. After removing the medium by centrifuging the cells through isotonic sucrose solution, lipids were extracted with ethanol–ether (3:1) and processed as described in Methods. Curves represent: ○—○, neutral lipids; △—△, cephalin (phosphatidyl ethanolamine + phosphatidyl serine); ▲—▲, sphingomyelin; ●—●, lecithin (contains some inositol phosphatide). Data are the means of 2 experiments involving duplicate determinations at 30 and 60 min.

## DISCUSSION

The present data serve to expand the available information concerning the metabolic effects of agents that arrest cell division in metaphase. In its effects on the incorporation of precursors into DNA, RNA and protein, vinleurosine resembles colchicine and the other vinca alkaloids.[8,13–18] The extent to which such effects are direct (through inhibition of the respective polymerase enzymes) or indirect cannot yet be determined. In our hands, inhibition of RNA polymerase by vinca alkaloids requires relatively high levels of these agents.

On the other hand, synthesis of lipids is an area to which little attention has been given in terms of the action of antineoplastic agents, despite the importance of lipids in critical membrane phenomena. Yet, as we have seen, it is on incorporation of acetate into phospholipids that vinleurosine exerts its most pronounced inhibitory effects. There may well be important implications of this finding. Preliminary experiments indicate that it is not vinleurosine alone, but also vincristine that is able to act in this metabolic area. With this alkaloid the specificity for phospholipid appears greater in that, while inhibition occurs at as low or at lower concentrations, neutral lipid synthesis is unaffected. Several lines of evidence suggest that lipid effects may be of great importance in the action of vinca alkaloids. The existence of cross resistance between vincristine and the phthalanilides,[19] which are involved in complexes with lipids,[20] is one such line of evidence. Another is the association of demyelination with the neurotoxicity produced by vincristine,[21] since the myelin sheath is rich in phospholipid. In addition, it is also possible that the lipid effects are related to neurotoxicity rather than to cytotoxicity, which may stem from the other metabolic effects or binding to spindle precursor proteins.[22] In this case, it might be possible to dissociate the toxicity from the anti-tumor action, either by synthesis of new agents or by development of selective antagonists. The success of current modes of therapy in childhood leukemia is such that even a modest increase in the therapeutic index of a compound such as vincristine might lead to a significant incidence of long-term survivors.

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