Cancer Chemother Pharmacol (1989) 23: 151–155

Cancer Chemotherapy and Pharmacology

In vitro effect of liposome-incorporated valinomycin on growth and macromolecular synthesis of normal and *ras*-transformed 3T3 cells*

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Summary. Valinomycin is a depsipeptide antibiotic that selectively translocates potassium ion across biologic membranes. This drug has been reported to display antitumor effects, but its use has been limited by its extreme toxicity. However, its incorporation into lipid vesicles (liposomes) has resulted in a reduction in toxicity and in the enhancement of the drug's therapeutic index [4]. As a preliminary investigation of the mechanistic basis for this enhancement, the in vitro response of normal 3T3 and *ras*-transformed cells to free (VM) and liposomal valinomycin (VM-MLV) was examined. The incorporation of [^{3}H]-leucine and [methyl-^{3}H]-thymidine was used to assess macromolecular synthesis, and the MTT vital dye assay was used to measure cell survival and growth. Pretreatment of exponentially growing NIH/3T3 cells with 20 n*M* VM for 1 h decreased [^{3}H]-leucine and [methyl-^{3}H]-thymidine incorporation by 90% and 80%, respectively. However, Ha-*ras* 3T3 cells showed resistance to VM treatment with inhibitory doses in the range of 200 n*M*. At equimolar VM concentrations, VM-MLV was found to be less inhibitory than VM for protein and DNA synthesis. Specifically, marked protective activity was apparent with normal 3T3 cells. In this report we also demonstrate that VM selectively killed normal cells compared with *ras*-transformed cells grown in vitro. However, VM-MLV displayed a modest cytotoxic selectivity (3- to 4-fold) to *ras*-transformed cells. Our data suggests that first, there is good correlation between growth inhibition and inhibition of DNA and protein synthesis by VM, and second, VM-MLV exhibits a modest, selective toxicity to the *ras*-transformed 3T3 cell line as compared with nontransformed 3T3 cells, whereas free VM has the opposite selectivity.

Introduction

Valinomycin (NSC-122023) is a cyclic depsipeptide membrane-active antibiotic with selective ionophoric activity for potassium [2, 16]. It has been widely used as a mobile ionophore in studies of cell membrane function. Valinomycin has also been shown to display considerable antitumor activity in murine tumor models; however, it has not been extensively developed as an antitumor drug because of its profound host toxicity [10, 17].

We have recently shown that the incorporation of valinomycin in an appropriate liposomal carrier system can markedly reduce host toxicity, with maintenance and/or enhancement of antitumor activity [3, 4]. In this regard, the enhancement of the drug's therapeutic index might be due to alterations in the drug's tissue distribution after its incorporation into liposomes. [7, 14]. Alternatively, it is possible that valinomycin is selectively toxic to transformed cells, as has been suggested by Kleuser et al. [9], and that the incorporation of valinomycin in liposomes enhances this selectivity.

The present study was undertaken to gain a better understanding of the basis of the cellular toxicity of valinomycin. The cytotoxicity of free valinomycin (VM) in vitro is compared with that of liposomal valinomycin (VM-MLV) to normal NIH/3T3 cells as well as a transformed derivative of this line, namely, c-Ha-*ras*/3T3 cells (Ha-*ras*/3T3). We correlate the biochemical aspects characterizing this toxicity, such as the inhibition of protein or DNA synthesis, with cell growth inhibition. The results show that VM is more toxic to normal cells, whereas VM-MLV is selectively cytotoxic to 3T3 cells transformed by the *ras* oncogene. Mechanisms that could explain these phenomena are briefly discussed.

Materials and methods

Materials. Valinomycin (NSC-122023) was obtained from Natural Products Branch, National Cancer Institute (Bethesda, Md); CH (cholesterol), PS (phosphatidyl serine) and MTT (thiazolyl blue) were purchased from Sigma Chemical Co. (St. Louis, Mo); DMPC (dimryistoylphosphatidyl choline) was purchased from Aventi Polar Lipids, Inc. (Birmingham, Ala). Radiochemicals used in the incorporation inhibition studies, including [methyl-^{3}H]-thymidine (sp. act. 42 Ci/mmol) and [^{3}H]-leucine (sp. act. 110 Ci/mmol) were obtained from ICN Pharmaceuticals, Inc. (Irvine, Calif) and Amersham International Ltd. (Amersham, England), respectively. Lipid purity was confirmed by thin-layer chromatography on silica-coated plates. All other chemicals were reagent-grade.

* This work is supported by NCI grant CA 47044 (R.J.)

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Abbreviations: VM, valinomycin; ara-C, 1-β-D-arabinofuranosyl cytosine; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (thiazolyl blue); CH, cholesterol; PS, phosphatidyl serine; DMPC, dimyristoylphosphatidyl choline; MLV, multilamellar liposomes; NIH/3T3, nontransformed mouse fibroblasts; Ha-*ras*/3T3, *ras* oncogene-transformed mouse fibroblasts; α-MEM, alpha minimal essential medium; FCS, fetal calf serum; IC_{50}, 50% inhibition of cell growth

Preparation of liposomes. Sterile multilamellar lipid vesicles (MLVs, liposomes) were prepared by mixing 10 parts DMPC, CH, and PS (molar ratio 10:4:1) with 1 part VM in chloroform and drying the sample under vacuum using a Buchi rotary evaporator. Liposomes were formed by the addition of 10 ml hot (50° C) HEPES buffer (20 m*M*, pH 7.4) followed by mechanical shaking in a water bath for 1 h. The nonincorporated VM was removed by sedimenting the vesicles at 11,000 rpm in a Beckman JA-21 centrifuge and resuspending the pellet in 10 ml HEPES buffer. This washing was repeated three times. The resulting pellet was then diluted with HEPES buffer to the required volume. Our previous experience with such methods suggests that this procedure gives rise to multilamellar vesicles in the 1-to 2- μm size range [4]. The amount of VM incorporated in MLVs was determined by colorimeteric assay as previously described [4].

Cells and culture conditions. NIH/3T3 and Ha-*ras*/3T3 cells were routinely maintained in monolayer cultures in alpha-minimal essential medium (α-MEM) (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS) (Hazelton) and 1% (v/v) antibiotic (Gibco) at 37° C in humidified air containing 5% CO_2. Under these conditions, the cell density was maintained at $1 \times 10^4 - 5 \times 10^5$ cells/cm^2.

Drugs. Valinomycin stock solutions were prepared at $10^{-3} - 10^{-4}$ *M* in ethanol, and 20-μl aliquots from the appropriate dilutions were added to the tissue-culture plates. The final concentration of ethanol in culture was always $< 0.1\%$, which is essentially nontoxic to cells.

Cytotoxicity and growth assay. Cells from exponentially growing cultures were plated in 24-well plates (Corning) in α-MEM + 10% FCS at about 8×10^3 cells/well, and cell counts were carried out using an Electrozone/Celloscope particle counter (Particle Data, Inc., Elmhurst, Ill). Following overnight incubation, valinomycin (free or liposomal) was added to quadruplicate wells and allowed to incubate at 37° C for 1, 6, or 24 h. After drug exposure, the cells were washed twice with prewarmed PBS and 2 ml drug-free medium was added. The plates were then incubated for 5 days and the surviving cell fraction was determined by the MTT vital dye assay; absorbance was measured with an Automated Microplate Reader (Biotek Instruments, Inc., Winooski, Vt) as previously described by Carmichael et al. [1]. The precision of this method using quadruplicate determinations is ± 10% (SD). The concentration causing 50% growth inhibition (IC_{50}) was calculated by plotting the surviving cell fraction vs drug concentration and extrapolating the concentrations of valinomycin producing 50% inhibition of growth compared with the control cultures.

Incorporation studies. For the determination of DNA and protein synthesis, cells of exponentially growing cultures at 3×10^5 cells/well were preincubated at 37° C for 1 h with various concentrations of valinomycin (free or liposomal), followed by a 30-min pulse with [methyl-^{3}H]-thymidine (25 μCi/ml) for DNA synthesis or [^{3}H]-leucine (10 μCi/ml) for protein synthesis. Cells were then washed twice with ice-cold PBS and incubated for 1 h at 0° C with ice-cold 1 *N* $HClO_4$. The acid-insoluble fraction retained on each well was washed twice with ice-cold H_2O and brought into solution with 0.5 ml 1 *N* NaOH and 1% SDS, then counted in a BetaTrac 6895 liquid scintillation counter (Tracor Analytic, Inc., Elk Grove Village, Ill). The studies were carried out on quadruplicate samples and the results were expressed as the percentage of initial values relative to the control counts. Cyclohexamide (3×10^{-7} *M*) and Ara-C (6×10^{-5} *M*) were used as standards for protein and DNA synthesis inhibition.

Results

The cytotoxic effect of 1-, 6-, or 24-h exposure of NIH/3T3 and Ha-*ras*/3T3 cells to different concentrations of VM is shown in Fig. 1. With 1-h exposure, a concentration of 12 n*M* VM produced 50% growth inhibition in normal 3T3 cells, whereas 6–7 times that dose was needed for transformed cells. The 1-h growth-inhibition curve for NIH/3T3 cells shows a shoulder at VM concentrations between 1 and 10 n*M*, with about 30%–40% inhibition compared with only 15% inhibition at these doses for trans-

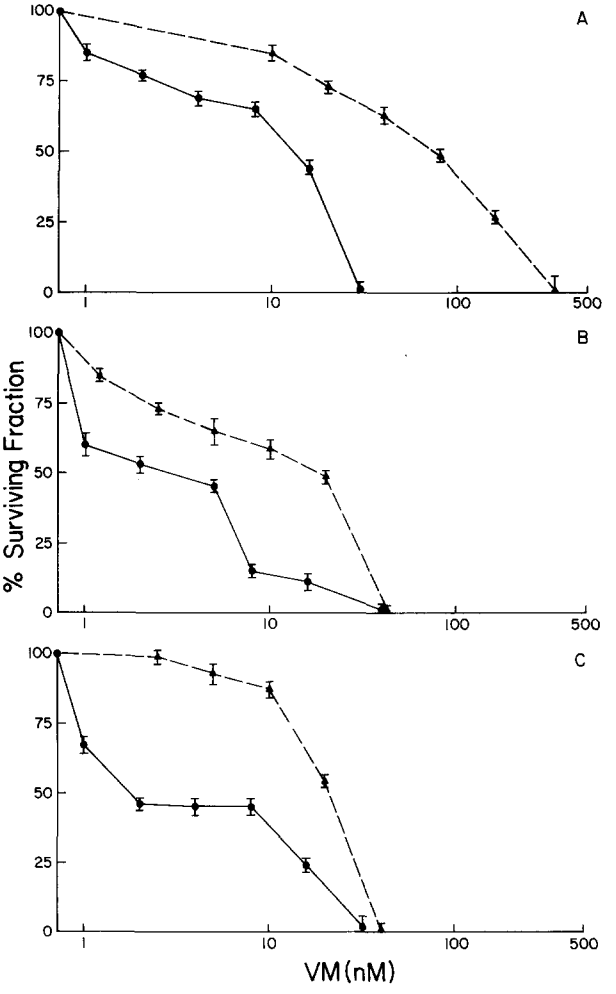

Fig. 1. Effects of valinomycin on cell survival. Surviving fraction of NIH/3T3 (●) and Ha-*ras*/3T3 (▲) cells as a function of VM concentration. Exponentially growing cells (8×10^3/well) were exposed to VM for 1 h (*A*), 6 h (*B*), or continuously for 24 h (*C*), followed by growth for 5 days in drug-free medium. The assay conditions are described in *Materials and methods. Points,* mean of quadruplicate determinations from three to four experiments; SD < 10%

formed 3T3 cells. Only at very high concentrations (> 0.5 μ*M*) did VM produce an essentially 100% inhibition for Ha-*ras*/3T3 cells during 1-h exposure, whereas 60 n*M* VM produced 100% inhibition for NIH/3T3 cells. With 6-h exposure (Fig. 1 B), the 50% inhibitory concentrations for VM were 2.5 and 20 n*M* in 3T3 and *ras*-transformed cells, respectively. VM almost completely arrested the growth of both cells at 60 n*M*. With 24-h exposure (Fig. 1 C), the 50% growth inhibition for NIH/3T3 and Ha-*ras*/3T3 cells were 1.8 and 20 n*M*, respectively. The 3T3 cells displayed a pronounced shoulder at doses below 10 n*M*.

Growth inhibition by VM-MLV was also dose- and time-dependent (Fig. 2). Biphasic dose-response curves occurred with the liposome-incorporated drug, and a small population (10%–20%) of resistant cells was noted. In contrast to the situation with VM, Ha-*ras*/3T3 cells were not markedly more resistant than 3T3 cells to VM-MLV; indeed, liposomal VM was slightly more toxic to Ha-*ras*/3T3 than to 3T3 cells. With 1-h exposure (Fig. 2 A), the normal 3T3 cells were unaffected by VM-MLV concentrations up to 50 n*M* and continued to grow normally. However, treatment with 120 n*M* VM-MLV, at which concentration 50% of the 3T3 cells were arrested, also arrested the growth of the *ras*-transformed cells by about 90%. Thus, at the shorter exposure time (1 h), the selective cytotoxicity of VM to the transformed cells was enhanced by its incorporation in liposomes. Likewise, the 50% growth-inhibitory doses for transformed and normal 3T3 cells treated with VM-MLV for 6 h (Fig. 2 B) were 15 and 22 n*M*, respectively. For longer exposure time (24 h, Fig. 2 C), the IC_{50} of VM-MLV was about 2 n*M* for Ha-*ras*/3T3 cells and 5 n*M* for 3T3 cells.

In conjunction with the growth-inhibition study, the effect of valinomycin (free and liposomal) on DNA and protein synthesis in normal and transformed cells was measured using the incorporation of radioactive thymidine or leucine in acid-insoluble material (Figs. 3, 4). Normal cell protein and DNA synthesis were dramatically inhibited following 1-h treatment with VM and 30-min pulse labeling with the radioactive precursors. For VM, the inhibition of DNA and protein synthesis in normal 3T3 cells occurred at concentrations at which *ras*-transformed cells were relatively unaffected. For example, 90% inhibition of [^{3}H]-leucine incorporation in normal 3T3 cells was observed when 20 n*M* VM was used (Fig. 3 A), a dose that has no effect on *ras*-transformed cells (Fig. 4 A). The same dose inhibited 80% of [^{3}H-methyl]-thymidine incorporation in the DNA of normal 3T3 cells but had no noticeable effect on *ras*-transformed cells (Table 3), as indicated in Fig. 3 B and 4 B, respectively. Higher doses of VM failed to inhibit macromolecular synthesis completely, and a plateau was reached in each case.

The use of liposomal valinomycin caused a dramatic rightward shift in the macromolecular synthesis dose-response curves for 3T3 cells but less of a shift for Ha-*ras*/

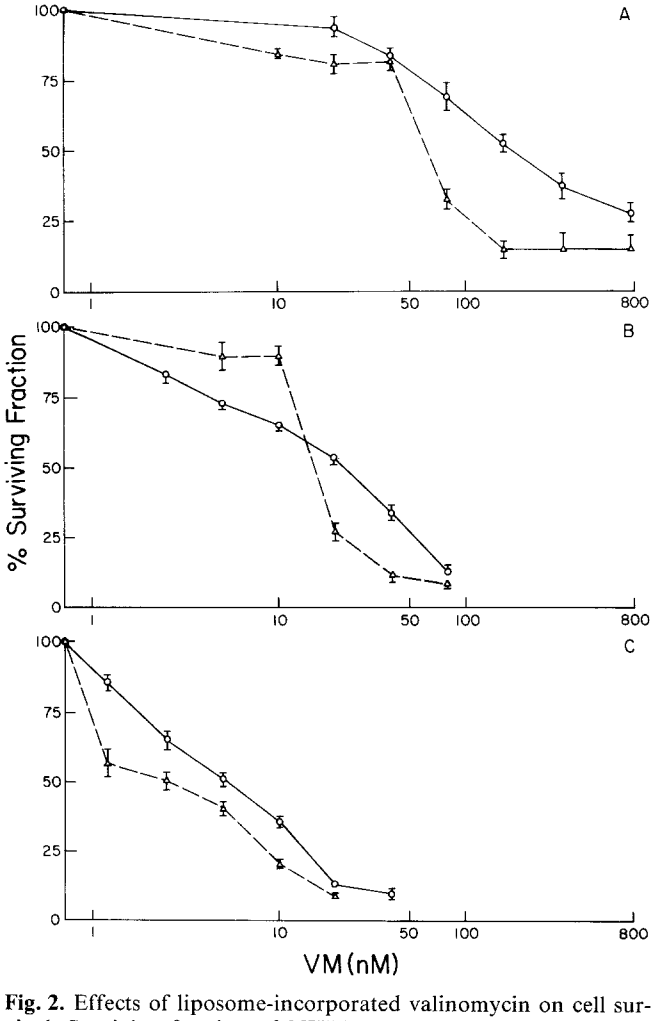

Fig. 2. Effects of liposome-incorporated valinomycin on cell survival. Surviving fraction of NIH/3T3 (○) and Ha-*ras*/3T3 (△) cells as a function of VM-MLV concentration. Conditions were as described in Fig. 1, except that liposomal valinomycin was used. *Points*, mean of quadruplicate determinations from two to four experiments; SD < 10%

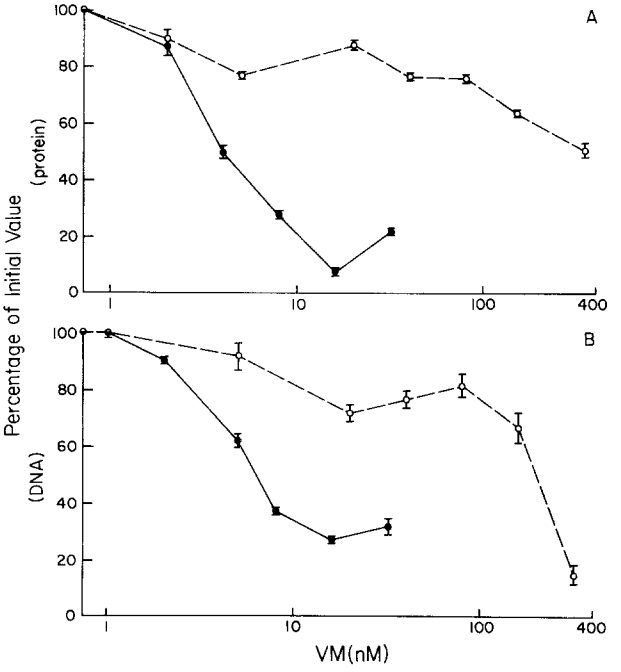

Fig. 3. Effects of various concentrations of VM (●) or VM-MLV (○) on the incorporation of [^{3}H]-leucine (*A*) or [methyl-^{3}H]-thymidine (*B*) into acid-insoluble material in NIH/3T3 cells. Each point represents the average value from at least three separate experiments; SD < 10%

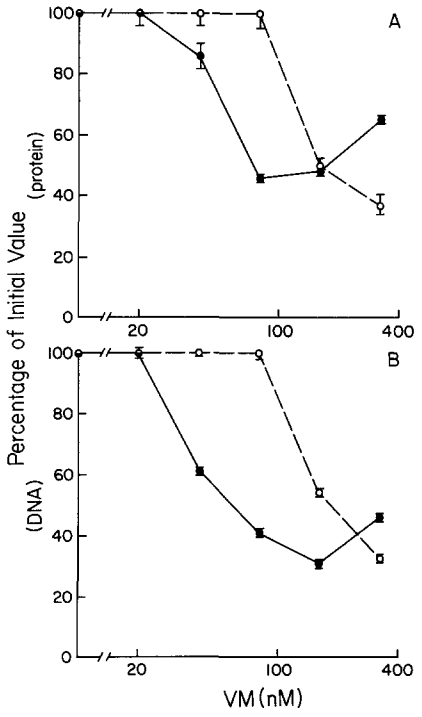

Fig. 4. Conditions were as described in Fig. 3, except that Ha-*ras*/3T3 cells were used. Each point represents the mean value from four separate experiments; SD < 10%

3T3 cells (Figs. 3, 4). The incorporation of radioactive precursors in the acid-insoluble fraction of the transformed cells was reduced by approximately 50% with 170 n*M* VM-MLV, a dose that produced only a 20% reduction in the incorporation of [^{3}H]-leucine or [^{3}H-methyl]-thymidine in the protein or DNA of normal 3T3 cells.

Discussion

Mobile ionophores (e.g., valinomycin) are a subclass of a heterogeneous group of antibiotics that bind metal ions or organic cations to form lipid-soluble complexes, which can then shuttle back and forth across the cell membrane, increasing transmembrane cation movements and disrupting a multitude of cellular functions. Therefore, these compounds may have potential use as cytotoxic agents in cancer therapy; however, very few ionophores have been therapeutically developed. The likely reason for this is that ionophores can be extremely toxic to organs and tissues that rely on ion gradients for their functions, such as the heart, kidney, and CNS. We have recently shown that the toxicity of valinomycin can be reduced, with maintenance and/or enhancement of its antitumor activity, by its incorporation in liposomes [4]. In the present study we found that VM is more toxic to normal cells than to their *ras*-transformed counterparts, but this toxicity is reduced by the use of liposomes. Indeed, the *ras*-transformed cells are slightly more sensitive than normal 3T3 cells to growth inhibition by liposomal valinomycin.

In terms of growth inhibition (Table 1), VM shows profound toxicity to normal 3T3 cells compared with transformed cells. In all cases, this selective toxicity is time- and dose-dependent. Kleuser et al. [9] have also recently demonstrated that daily exposure of 3T3 cells to 2 n*M* VM resulted in substantial growth inhibition. In contrast to our findings, however, this group reported that VM displayed selective growth inhibition for transformed cells. The incorporation of VM in liposomes (VM-MLV) results in a marked (> 10-fold) reduction in the toxicity of the drug to normal 3T3 cells, especially during short exposures. However, the reduction in toxicity to Ha-*ras*/3T3 cells measured by the inhibition of macromolecular synthesis was only marginal (3-fold), although growth-inhibition studies indicated that VM-MLV was actually more toxic than VM. Thus, the incorporation of VM in liposomes results in a modest, selective toxicity of the drug to the transformed cell line (Tables 2, 3).

Table 1. IC_{50} values for growth inhibition by 1-, 6-, or 24-h exposure to either VM or VM-MLV: exponentially growing cells were plated in tissue-culture 24-well plates, allowed to grow overnight, then treated with either VM or VM-MLV for various times and allowed to grow for 5 days in drug-free medium. Cell survival was determined by the MTT vital dye assay. In this case the IC_{50} value represents the concentration of either VM or VM-MLV that inhibited growth by 50% over 5 days

Period of exposure (h)	Cell line	IC_{50} (nM) for VM		IC_{50} (nM) for VM-MLV		Dose ratio[b] VM	Dose ratio[b] VM-MLV
1	NIH/3T3	12	(8)[a]	120	(8)	7.0	0.5
	Ha-*ras*/3T3	90	(16)	60	(16)		
6	NIH/3T3	2.5	(8)	22	(8)	8.0	0.75
	Ha-*ras*/3T3	20	(16)	15	(16)		
24	NIH/3T3	1.8	(8)	5	(8)	10.0	0.5
	Ha-*ras*/3T3	20	(16)	2.5	(16)		

[a] Numbers in parentheses represent the number of determinations used to calculate the IC_{50} values

[b] 50% inhibitory concentration of Ha-*ras*/3T3 compared with that of NIH/3T3 cells

In the intact cell, it seems most probable that valinomycin exerts its effects in at least two distinct ways: by its action on the K^+ permeability and transmembrane voltage of the cell membrane [11, 19], and by uncoupling oxidative phosphorylation in the mitochondria [6, 15], the latter effect perhaps being more important in terms of cytotoxicity [5]. At this point we do not have a clear idea as to why VM should be more toxic to 3T3 cells than to their *ras*-transformed counterparts. Differences in mitochondrial sensitivity or in plasma membrane properties leading to increased cell penetration by VM might be possible explanations. It is interesting that Sklar [18] has reported resistance to cisplatin in *ras*-tranformed 3T3 cells, although there is no indication that similar mechanisms are involved. The reduction in VM toxicity to 3T3 cells via liposomal incorporation might be explained by the extremely lipophilic nature of VM; its inclusion in liposomes provides an alternate binding site, thus reducing the availability of the drug to the cells.

In Ha-*ras*/3T3 cells, the incorporation of VM in liposomes fails to reduce toxicity and even enhances it to some degree, thus reversing the pattern observed in 3T3 cells. It is possible that the *ras*-transformed cells have a higher endocytotic capability than 3T3 cells and thus tend to accumulate liposomal VM [12, 13]. Alternatively, there may be differences in the plasma membrane lipid characteristics of 3T3 and Ha-*ras*/3T3 cells that tend to favor the parti-

Table 2. IC_{50} values for the inhibition of [^{3}H]-leucine incorporation into protein in NIH/3T3 or Ha-*ras*/3T3 cells: 5×10^5 cells in logarithmic growth were plated in tissue-culture 24-well plates and treated with various concentrations of either VM or VM-MLV for 1 h, followed by a 30-min pulse with [^{3}H]-leucine (10 μCi/ml). The amount of radioactivity incorporation in acid-insoluble material was then measured as described in *Materials and methods*. The IC_{50} value represents the concentration of drug that inhibited the incorporation of radioactive precursors into acid-insoluble material of NIH/3T3 or *ras*-transformed cells by 50%

Cell line	IC_{50} (n*M*) for		Dose ratio[b]	
	VM	VM-MLV	VM	VM-MLV
NIH/3T3	4 (8)[a]	350 (8)	13	0.5
Ha-*ras*/3T3	52 (8)	160 (8)		

[a] Numbers in parentheses represent the number of determinations used to determine the IC_{50} values
[b] 50% inhibitory concentration of Ha-*ras*/3T3 compared with that of NIH/3T3 cells

Table 3. IC_{50} values for the inhibition of [methyl-^{3}H]-thymidine incorporation into DNA in NIH/3T3 or Ha-*ras*/3T3 cells. Conditions were as described in Table 2, except that [^{3}H-methyl]-thymidine (25 μCi/ml) was used as radioactive precursor

Cell line	IC_{50} (n*M*) for		Dose ratio	
	VM	VM-MLV	VM	VM-MLV
NIH/3T3	6 (8)[a]	200 (8)	9.7	0.9
Ha-*ras*/3T3	58 (8)	180 (8)		

[a] Numbers in parentheses represent the number of determinations used to determine the IC_{50} values
[b] 50% inhibitory concentration of Ha-*ras*/3T3 compared with that of NIH/3T3 cells

tioning of liposomal VM into the membranes of the transformed cells; precedents exist for the selective partitioning of lipophilic drugs into membranes of different cell types [8]. Another significant question is whether these observations represent a difference common to all pairs of normal and transformed fibroblasts, a difference between *ras*-transformed cells and their normal counterparts, or just a clonal variation in the 3T3 population. We are expanding this preliminary study to explore these important questions.

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Received May 31, 1988/Accepted August 1, 1988