Arch. Biochem. Biophys. **118**, 178 (1967); J. Hartmann, W. Reineke, H.-J. Knackmuss, *Appl. Environ. Microbiol.* **37**, 421 (1979); R. S. Horvath and M. Alexander, *Appl. Microbiol.* **20**, 254 (1970); W. Reineke and H.-J. Knackmuss, *J. Bacteriol.* **142**, 467 (1980); J. R. Spokes and N. Walker, *Arch. Microbiol.* **96**, 125 (1974).
11. M. J. DiGeronimo, M. Nikaido, M. Alexander, *Appl. Environ. Microbiol.* **37**, 619 (1979); R. W. Okey and R. H. Borgan, *J. Water Poll. Cont. Fed.* **37**, 692 (1965).
12. J.-M. Bollag, *Adv. Appl. Microbiol.* **18**, 75 (1974).
13. M. Alexander and B. K. Lustigman, *J. Agr. Food Chem.* **14**, 410 (1966); N. A. Shamat and W. J. Maier, *J. Water Poll. Cont. Fed.* **52**, 2158 (1980); A. Ichihara, K. Aadachi, K. Hosokawa, Y. Tkeda, *J. Biol. Chem.* **237**, 2296 (1962); N. Walker and D. Harpis, *Soil Biol. Biochem.* **2**, 27 (1970).
14. Supported in part by EPA grants R806569 and EPA-OTS subcontract T-6419 (7197)-033. We thank Peter Cornell, Mary Lou Krumme, and David Reynolds for technical assistance. Published as article No. 10158 of the Michigan Agricultural Experiment Station.

17 May 1982; revised 16 July 1982

Rhodamine-123 Selectively Reduces Clonal Growth of Carcinoma Cells in vitro

Abstract. *Rhodamine-123, a cationic laser dye, markedly reduced the clonal growth of carcinoma cells but had little effect on nontumorigenic epithelial cells in vitro. This selective inhibitory effect of Rhodamine-123 on some carcinomas is unusual since known anticancer drugs, such as arabinosyl cytosine and methotrexate, have not been shown to exhibit such selectivity in vitro.*

The fluorescent dye Rhodamine-123 (Rh-123) selectively accumulates in the mitochondria of living cells (*1*). The specific accumulation of this dye appears to depend on its cationic and permeant properties and on the high electric potential (inside negative) across the mitochondrial membrane (*2, 3*). Although all living cells we have examined thus far accumulate Rh-123 in their mitochondria, cell types differ in their ability to retain Rh-123 in dye-free medium (*3, 4*). We found that most carcinoma cells retain Rh-123 for 2 to 5 days when they are incubated in dye-free medium, whereas nontumorigenic epithelial cells and tumorigenic or nontumorigenic cells of fibroblastic, neural, or hematopoietic origin release the dye within 1 to 16 hours (*4*). It may be possible to exploit the difference in Rh-123 retention between carcinoma cells and other cell types for cancer chemotherapy (*5*). In the study described herein, we compared the effects of Rh-123 treatment on the clonal growth of carcinoma cells and nontumorigenic epithelial cells in vitro.

For these experiments we used MB 49 cells, a mouse bladder epithelial line transformed by 7,12-dimethylbenz[*a*]anthracene (DMBA) (*6*). These cells are highly tumorigenic and retain a significant amount of Rh-123 in their mitochondria for 4 days when they are incubated in dye-free medium. In contrast, primary cultures of normal bladder epithelial cells lose Rh-123 fluorescence within 2 hours (*4*). To determine whether this difference in retention results in greater inhibition of clonal growth of MB 49 cells than of normal mouse bladder epithelial cells, we treated these cells grown in vitro with Rh-123 and assayed their colony-forming ability. Exposure to Rh-123 (10 μg/ml) for 24 hours had a minimal effect on the colony-forming units (CFU) of normal mouse bladder epithelial cells (92 percent of control) (Fig. 1A), but markedly reduced the CFU of MB 49 cells (4 percent of control). The effect of Rh-123 on MB 49 cells depended on the concentration and duration of exposure. Even 6 hours of exposure to 10 μg of Rh-123 per milliliter reduced the CFU to 45 percent of control, whereas such treatment had no significant effect on the CFU of normal bladder epithelial cells.

We then compared the reductions in CFU of EJ cells (a human bladder carcinoma line), MB 49 cells, and normal mouse bladder epithelial cells that had been exposed for 24 hours or continuously to different concentrations of Rh-123 during the 2-week period of clonal cell growth (Fig. 1, B and C). Continuous exposure of normal mouse bladder epithelial cells to Rh-123 (10 μg/ml) had only a small effect on CFU. However, both EJ and MB 49 cells were susceptible to the inhibitory effects of Rh-123. Colony formation in these cells was reduced to 50 percent of control after 24 hours of exposure to 2 to 5 μg of Rh-123

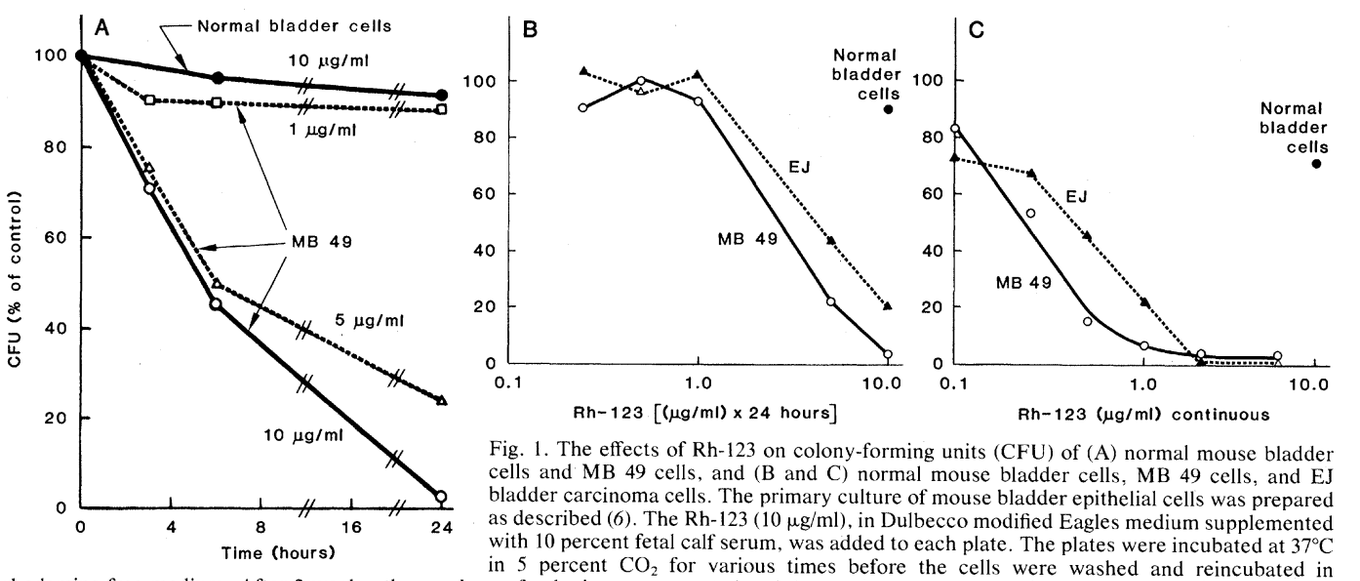

Fig. 1. The effects of Rh-123 on colony-forming units (CFU) of (A) normal mouse bladder cells and MB 49 cells, and (B and C) normal mouse bladder cells, MB 49 cells, and EJ bladder carcinoma cells. The primary culture of mouse bladder epithelial cells was prepared as described (*6*). The Rh-123 (10 μg/ml), in Dulbecco modified Eagles medium supplemented with 10 percent fetal calf serum, was added to each plate. The plates were incubated at 37°C in 5 percent CO_2 for various times before the cells were washed and reincubated in rhodamine-free medium. After 2 weeks, the numbers of colonies were counted and the results were expressed as percentages of control, with control plates normalized to 100 percent CFU. The standard error for triplicate samples was 3 to 5 percent. The MB 49 cells (a mouse bladder epithelial cell line transformed with DMBA) and EJ cells (human bladder carcinoma cell line) were plated and treated with different concentrations of Rh-123 for various times before they were washed and reincubated in fresh medium. The CFU (percentage of control) was determined as described above. The standard error for duplicate samples was 5 percent.

0036-8075/82/1210-1117$01.00/0

per milliliter (Fig. 1B) and by continuous exposure to 0.2 to 0.5 μg of Rh-123 per milliliter (Fig. 1C).

We also compared the effects of Rh-123 on the CFU of other carcinoma and nontumorigenic cell lines, all of which have been confirmed to be of epithelial origin by studies of immunofluorescence with keratin antibody. The carcinoma cells retained Rh-123 longer than nontumorigenic epithelial cells (*4*). BSC 1, a nontumorigenic line of monkey kidney epithelial cells (Fig. 2A) and CCL 34, a nontumorigenic dog kidney epithelial line (Fig. 2B) were relatively insensitive to Rh-123. In contrast, CCL 51, a mouse breast carcinoma line (Fig. 2A) and HUT 23, a human lung adenocarcinoma line (Fig. 2B), were very sensitive to the inhibitory effects of Rh-123, with colony formation being reduced to 50 percent of control by continuous exposure to 0.2 to 0.5 μg of Rh-123 per milliliter.

The clonal growth of MCF-7 cells (human breast carcinoma line) and Ehrlich ascites cells (mouse carcinoma line) was also reduced to 50 percent of control after continuous exposure to Rh-123 (0.5 μg/ml), whereas the clonal growth of Pt K1 (a nontumorigenic marsupial kidney line) and CRL 1521 (a normal human skin fibroblast line) was greater than 80 percent under similar conditions. However, the clonal growth of all the cell lines we tested was not reduced by 10 minutes of exposure to 10 μg of Rh-123 per milliliter. Thus, the conditions required for specific staining of mitochondria (*1, 2*), for measurements of Rh-123 retention (*3, 4*), and for cell viability assays (*3, 7*) were not inhibitory to the cells.

Unlike Rh-123, arabinosyl cytosine (Ara-C) and methotrexate (cell cycle–specific anticancer drugs) were not selectively inhibitory for carcinoma cells in vitro (Fig. 2, C and D). Since BSC 1, CCL 34, CCL 51, and HUT 23 had similar doubling times, it appeared that Ara-C and methotrexate inhibited the clonal growth of cycling tumorigenic and nontumorigenic epithelial cells. These results also suggest that the selective inhibition of CFU by Rh-123 was not due to differences in cell-cycle kinetics between carcinoma and nontumorigenic epithelial cells.

The ability of carcinoma lines to retain Rh-123 may have been important in their sensitivity to Rh-123. However, this prolonged dye retention was unlikely to have been the sole mechanism for the selective inhibitory effects since continuous treatment with Rh-123 also resulted in a much greater reduction of CFU in carcinoma cells than in nontumorigenic epithelial cells. Some possible explanations for these observations are (i) that certain cellular components or organelles (possibly mitochondria) of carcinomas are very sensitive to the inhibitory effects of Rh-123; (ii) that nontumorigenic cells have an active mechanism for excluding Rh-123 from the vulnerable cellular compartment in the face of continued presence of Rh-123 in the culture medium; or (iii) that nontumorigenic cells are capable of inactivating Rh-123. Nonetheless, mitochondria are the likely targets for the inhibitory action of Rh-123 since they selectively accumulate the dye (*1–3*). Rhodamine-123 may disrupt some mitochondrial functions, such as the translocation of adenosine diphosphate (*8*), proton ejection (*9*), or electron transport (*10*). In previous studies the cytostatic effects of Rh-123 on the L 1210 leukemia line (*11*) and of rhodamine 6G on the mink fibroblast line CCL 64 (*12*) were described.

Tumors of the lung, breast, and colon are still the major causes of deaths due to cancer in the United States (*13*), and new and more selective drugs are needed to combat these tumors. The system we have described in this report would be useful for screening the anticarcinoma activity of additional rhodamine analogs before they are tested in animals. It would be of interest to determine whether some carcinomas, particularly those that do not retain Rh-123 (*4*), are resistant to the inhibitory effects of Rh-123. Whether Rh-123 can prolong the survival of mice implanted with carcinomas also remains to be investigated.

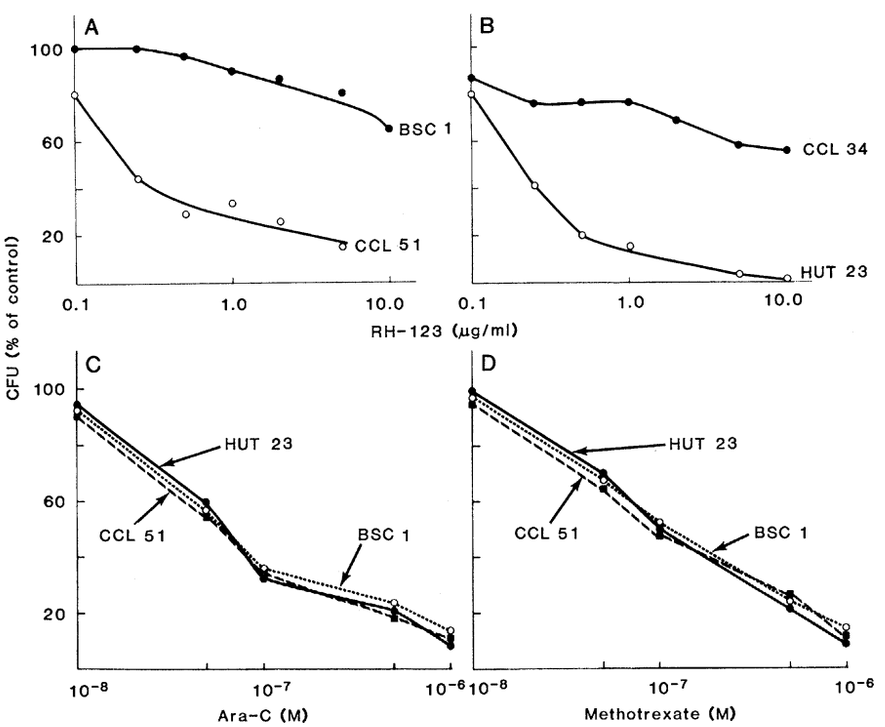

Fig. 2. The effect of Rh-123 on CFU of (A) BSC 1 cells, a nontumorigenic monkey kidney epithelial line, and CCL 51 cells, a mouse breast carcinoma line, and (B) CCL 34 cells, a nontumorigenic dog kidney epithelial line, and HUT 23 cells, a human lung adenocarcinoma line. The effect of (C) Ara-C and (D) methotrexate on CFU of BSC 1, CCL 51, and HUT 23 cell lines.

SAMUEL D. BERNAL
THEODORE J. LAMPIDIS
IAN C. SUMMERHAYES
LAN BO CHEN
Sidney Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115

References and Notes

1. L. V. Johnson, M. L. Walsh, L. B. Chen, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 990 (1980).
2. L. V. Johnson, M. L. Walsh, B. J. Bockus, L. B. Chen, *J. Cell Biol.* **88**, 526 (1981).
3. L. B. Chen, I. C. Summerhayes, L. V. Johnson, M. L. Walsh, S. D. Bernal, T. J. Lampidis, *Cold Spring Harbor Symp. Quant. Biol.* **46**, 141 (1981).
4. I. C. Summerhayes, T. J. Lampidis, S. D. Bernal, J. J. Nadakavukaren, K. K. Nadakavukaren, E. L. Shepherd, L. B. Chen, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5292 (1982).
5. T. J. Lampidis, S. D. Bernal, I. C. Summerhayes, L. B. Chen, *Ann. N.Y. Acad. Sci.*, in press.
6. I. C. Summerhayes and L. M. Franks, *J. Natl. Cancer Inst.* **62**, 1017 (1979); I. C. Summerhayes and L. B. Chen *Nature (London)* **298**, 462 (1980); I. C. Summerhayes, Y. S. E. Cheng, T. T. Sun, L. B. Chen, *J. Cell Biol.* **90**, 63 (1981); I. C. Summerhayes and L. B. Chen, *Cancer Res.* **42**, 4098 (1982).
7. S. D. Bernal, H. M. Shapiro, L. B. Chen, *Int. J. Cancer* **30**, 219 (1982).
8. A. R. L. Gear, *J. Biol. Chem.* **249**, 3628 (1974).
9. T. Higuti, S. Niimi, R. Saito, S. Nakasima, T. Ohe, I. Tani, T. Yoshimura, *Biochim. Biophys. Acta* **593**, 463 (1980).
10. S. Zigman and P. Gilman, Jr., *Science* **208**, 188 (1980).

11. Z. Darzynkiewicz, F. Traganos, L. Staino-Coico, J. Kapuscinski, M. R. Melamed, *Cancer Res.* **42**, 799 (1982).
12. L. V. Johnson, I. C. Summerhayes, L. B. Chen, *Cell* **28**, 7 (1982).
13. J. Higginson and C. Muir, in *Cancer Medicine*, J. Holland and E. Frei, III, Eds. (Lea & Febiger, Philadelphia, ed. 2, 1982), pp. 297–328.
14. Supported by grants from the American Cancer Society and National Cancer Institute to L.B.C. S.D.B. is supported by a Biomedical Research Support Grant, T.J.L. by an NCI Young Investigators Award, and L.B.C. by an American Cancer Society Faculty Research Award. A. F. Gazdar, NCI, provided HUT 23 and M. Rich, Michigan Cancer Foundation, provided MCF-7.

21 June 1982

The Sperm Attractant of the Marine Brown Alga *Ascophyllum nodosum* (Phaeophyceae)

Abstract. *Spermatozoids of the intertidal seaweed* Ascophyllum nodosum *(Fucales, Phaeophyceae) are attracted to eggs prior to fertilization. The attractant has been isolated and its structure identified as 1(3E,5Z,8Z)-undecatetraene (finavarrene). The relation of finavarrene to sex hormones in other brown algae is discussed.*

Chemotactic attraction of spermatozoids by eggs is a well-known phenomenon in algae, mosses, and ferns (*1*). Some marine algae in the order Fucales, which clearly show sperm chemotaxis, were studied as early as 1854 (*2*). However, it was not until 1972 that advances in analytical methods permitted the identification of the pheromones involved in this reaction. Sperm attractants are now known from five genera of marine brown algae: *Ectocarpus* (*3*), *Cutleria* (*4*), *Dictyota* (*5*), *Desmarestia* (*6*), and *Fucus* (*7*) (Fig. 1). In all cases, the sperm attractants are olefinic hydrocarbons, and their molecular size and structure appear to parallel phylogenetic relationships (*8*). Since the attractant in the genus *Fucus* (Fucales) is a straight-chain octatriene and those of all other brown algae studied (in other orders) are monocyclic C_{11} molecules, we decided to study another member of the order Fucales, *Ascophyllum nodosum* (L.) Le Jol., which forms large intertidal populations in many areas of the North Atlantic. It is dioecious, and sperm chemotaxis has been reported in this species (*2*).

Mature receptacles of female *Ascophyllum nodosum* plants, collected in various sheltered locations of Galway Bay, Ireland, between 10 and 30 April 1982, were processed on the German research vessel *Friedrich Heincke*. The receptacles were soaked in water for 5 minutes and dried at 20°C. Receptacles that produced oogonial masses within a few hours were submerged in seawater (10°C) in an illuminated cold room. Eggs soon were liberated and were allowed to sediment through clean seawater to remove debris. Suspensions of clean eggs were pipetted into extraction flasks filled with 2 liters of seawater decontaminated by stripping. Low molecular hydrophobic compounds were removed from suspensions of living eggs by the closed-loop stripping technique (*9*), and adsorbed on a filter bed containing 2 mg of activated carbon. After 12 hours, substances were eluted from the filter with 30 μl of dichloromethane and subjected to analytical and preparative glass-capillary gas chromatography. The extracts contained a single compound that was produced by eggs for as long as 36 hours. In 16 trials, the average yield of this compound in an hour was 4.5 ± 2.0 ng per 10^6 eggs.

To study the biological effect of the compound, the fractions were collected after emergence from the gas-chromatographic column in a micro-ampule placed directly on the column exit and cooled with dry ice. The ampule contained a strip of cover-glass on which there were droplets of Vaseline. This preparation was tested with freshly released spermatozoids of *A. nodosum* following the procedure described for *Fucus serratus* (*10*). Spermatozoids were strongly attracted to the Vaseline droplets impregnated with the egg product, indicating that the isolated compound represented the native hormone.

Detailed analytical studies on this substance and comparison by gas chromatography–mass spectrometry with synthetic references (*11*) revealed that it is 1(3*E*,5*Z*,8*Z*)-undecatetraene (Table 1). This compound had been reported as a constituent in vegetative thalli of the brown alga *Dictyopteris plagiogramma* (*12*). It is also secreted by the gametes of another brown alga, *Spermatochnus paradoxus*, but no biological function was detected in this case (*13*). Now that a highly specific biological activity of this undecatetraene has been detected, we suggest "finavarrene" as a trivial name, referring to Finavarra, a locality in Galway Bay where the isolation work was carried out. The molecular size of finavarrene is identical to that of related substances in other brown algae in which the attractants are all C_{11} olefines. *Fucus*, with a C_8 molecule, appears to be an exception. *Ascophyllum* and *Fucus*, both members of the order Fucales in the subclass Cyclosporidae, share the straight-chain character of their attractants, whereas all sex hormones in algae of the subclass Phaeophycidae are monocyclic cyclopentane or cycloheptane derivatives.

Spermatochnus paradoxus, a member

Table 1. Analytical data for the *Ascophyllum* egg product (finavarrene), a synthetic sample (*11*), and retention indices for two additional undecatetraenes.

Compound	Retention index	
	OV 73 (120°C)	UCON 75H (160°C)
Gas chromatography		
Ascophyllum (finavarrene)	1182.0 ± 0.1	1389.2 ± 0.7
1(3*E*,5*Z*,8*Z*)-undecatetraene	1181.8 ± 0.2	1389.0 ± 0.2
1(3*E*,5*E*,8*Z*)-undecatetraene	1193.1 ± 0.4	1409.0 ± 0.2
1(3*Z*,5*E*,8*Z*)-undecatetraene	1190.0 ± 0.1	1400.0 ± 0.1
Mass spectrometry		
Mass/charge 148 (M^+), 119, 105, 91, 79 (100 percent)		

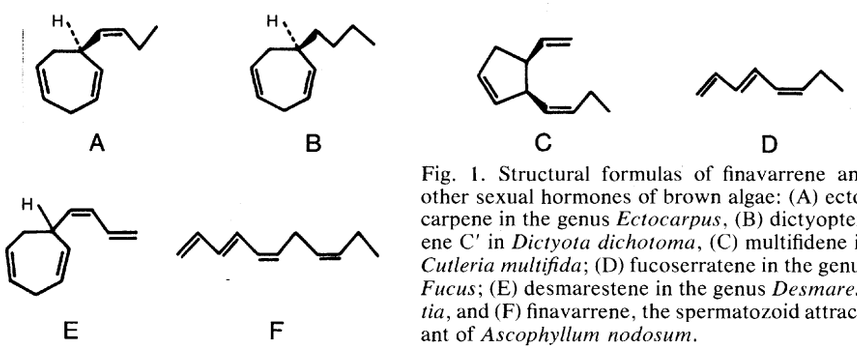

Fig. 1. Structural formulas of finavarrene and other sexual hormones of brown algae: (A) ectocarpene in the genus *Ectocarpus*, (B) dictyopterene C′ in *Dictyota dichotoma*, (C) multifidene in *Cutleria multifida*; (D) fucoserratene in the genus *Fucus*; (E) desmarestene in the genus *Desmarestia*, and (F) finavarrene, the spermatozoid attractant of *Ascophyllum nodosum*.

0036-8075/82/1210-1119$01.00/0