Biochemical Pharmacology, Vol. 28, pp. 2465–2470

0006-2952/79/0815-2465 $02.00/0

ON THE MODE OF CYTOTOXIC ACTION OF PHOTO-ACTIVATED PORPHYRINS

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(*Received* 28 *November* 1978; *accepted* 7 *February* 1979)

Abstract—Upon photo-activation, certain porphyrins can exert a substantial cytotoxic action on mammalian cells. A study of the interaction between a group of porphyrins and some transplantable murine ascitic tumors has suggested several major determinants of porphyrin toxicity. Optimally effective porphyrins are water soluble; however, they can be extracted from aqueous solution by octanol, but not by decane. Upon photo-activation, porphyrins accumulated by tumor cells catalyze cross-linking of membrane protein but not of glycoprotein. This cross-linking is associated with a decrease in cell-surface hydrophobicity and with other alterations in vital membrane propeprties.

A series of porphyrins has the interesting property of becoming localized in malignant tissue [1, 2] where, upon exposure to light, they exert a substantial cytotoxic action leading to tumor regression [3, 4]. The mode of action involves a photo-activated process leading to formation of singlet oxygen [5–7]. This reaction brings about oxidation of several compounds of biologic importance, including lipids [8–10], cholesterol [11, 12], and amino acids [5, 10, 13, 14]. Of the latter, histidine appears to be the most likely candidate for porphyrin-induced oxidation [15]. The consequences of these oxidative reactions are cross-linking of membrane protein [8, 13, 16], inhibition of membrane-bound enzymes [17], disruption of membranes (e.g. of erythrocytes [10, 12, 16]), interference with membrane transport [18], and cell death [3, 18].

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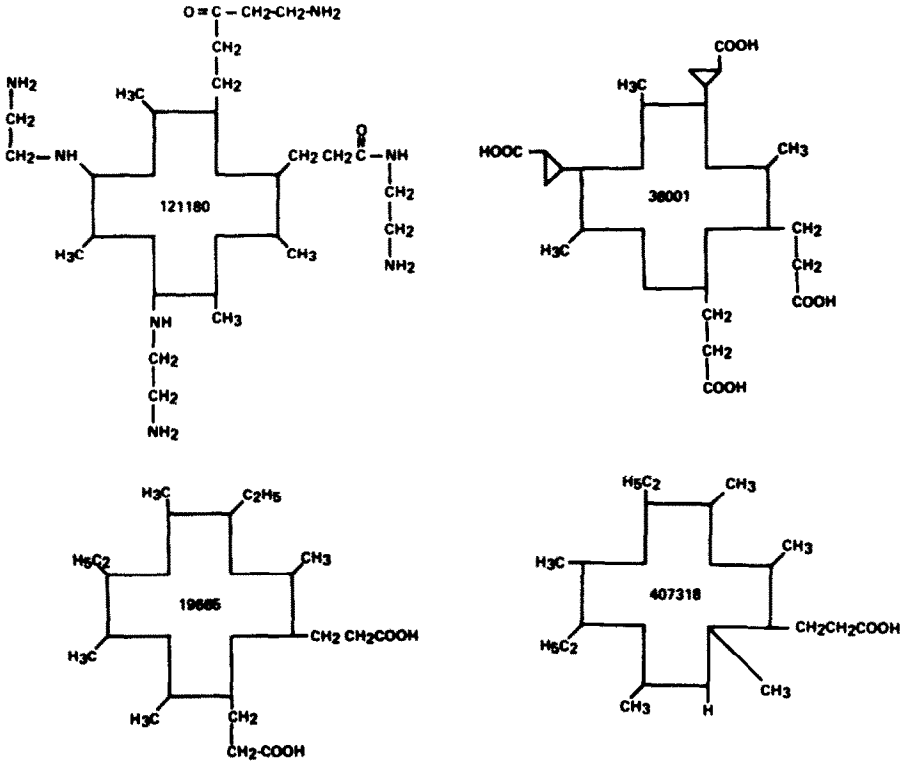

Fig. 1. Structure of porphyrins examined in this study.

Table 1. Properties of porphyrins

NSC No.	Functional groups*	Partition coefficient† Octanol:NaCl/P_i	Decane:NaCl/P_i
121180	4 amino	0.060	< 0.01
36001	4 carboxyl	0.067	< 0.01
19665	2 carboxyl	10.2	0.054
407318	1 carboxyl	33.6	0.19
26030	2 ester	144	9.5

* Exact structures are shown in Fig. 1.
† Values represent averages of five determinations and have an error of ±5 per cent.

In a previous study involving a murine leukemia cell line [18], we found that a consequence of photo-activation of certain porphyrins bound to murine leukemia L1210 cells was a marked alteration in several membrane properties, resulting in impairment of permeability barriers, inhibition of amino acid and nucleoside transport, inhibition of membrane-bound enzymes, and loss of viability. In this report, we describe further studies characterizing cell–porphyrin interactions, and develop a hypothesis concerning the nature and sequence of porphyrin-induced membrane alterations.

MATERIALS AND METHODS

Chemicals. Porphyrins were supplied by the Division of Cancer Treatment, National Cancer Institute; the structures are shown in Fig. 1 and the major properties are summarized in Table 1. All the compounds were prepared as 10 mg/ml solutions: NSC 121180 in water, others in DMF.* PEG (mol. wt 6000) was purchased from the Pierce Chemical Co., Rockville, IL, Dextran T-500 (lot 7863) from Pharmacia, Piscataway, NJ, minimum essential-Eagle's medium (spinner modification) and fetal calf serum from the Grand Island Biological Co., Grand Island NY, radioactive compounds from the New England Nuclear Corp., Boston, MA, fetuin and BSA from the Sigma Chemical Co., St. Louis, MO, lactoperoxidase and α_1-anti-trypsin from the CalBiochem Corp., Los Angeles, CA, and reagents for polyacrylamide gel electrophoresis from Eastman Organic Chemicals, Rochester, NY, and from Ortec, Inc., Oak Ridge, TN.

Cell lines. L1210 cells were grown in MEM supplemented with 10% fetal calf serum, as described before [18]. The SS-1 ascitic tumor was provided by Dr. Phillip Frost, Department of Immunology and Microbiology, Wayne State University; the line was maintained by serial transplants in Balb/C mice. The L1210 cell line is widely used in studies of modes of drug action, and was employed in our initial work [18]. The SS-1 is a transplantable non-metastasizing adenocanthoma which can also be maintained as a solid tumor for later *in vivo* experiments involving "photo-therapy."

Solubility measurements. Solubilities of porphyrins in incubation media were determined spectrophotometrically at 550 nm [8]. Decane–water and octanol–water partition of the porphyrins was measured using a 1-ml mixture containing equal volumes of solvent and NaCl/P_i, to which 5 μg of the appropriate porphyrin was added. After thorough mixing, the phases were separated by centrifugation, 300-μl portions of each phase were removed, and were mixed with 2 ml of 50% acetone containing 100 mM HCl. The optical densities of these solutions were measured at 550 nm, and the partition ratios calculated.

Incubation methodology. Cells were suspended [18] in HEPES-buffered minimal Eagle's medium (buffered with HEPES to pH 7.2) at 37° (2×10^6 cells/ml). Solutions of porphyrin in DMF (NSC 19665 or 407318) or in water (NSC 121180 and 36001) were added and incubations were continued for 10 min at 37°. The amount of DMF added did not exceed 2 μl/ml of suspension, a level which did not alter any result measured here. The cells were then exposed to light, as described previously [18], for 10 min at 22°. Control suspensions were kept in the dark during this interval. Other controls were exposed to light but contained no porphyrin. The cells were then collected by centrifugation and used for further studies.

Transport measurements. Cells treated previously with 0–20 μg/ml of porphyrin, in the light or the dark, as described above, were suspended in medium† at 37°. Radioactive cycloleucine was then added (final level 1 mM, 50,000 cpm/ml) and the incubation was continued for 5 min at 37°. The cells were then collected by centrifugation and washed once with 0.9% NaCl; then the level of intracellular radioactivity was measured by liquid scintillation counting. This procedure reduced the level of intercellular radioactivity to less than 30 cpm/cell pellet. Control incubations in the absence of porphyrin and with porphyrin in the dark were run for each cell line.

Cell partitioning studies. After exposure to 0–20 μl of porphyrin in the light or the dark, as described above, cells were suspended in 0.9% NaCl and 0.1-ml portions, containing 5×10^4 cells, were mixed with 9.9 ml of partitioning mixture [19]. An initial cell count was taken by dilution of a 1-ml portion of the mixture with 9 ml of diluent. Cell number was determined with a Coulter electronic particle counter. The phases were then allowed to separate at room temperature, and 1 ml

* Abbreviations: DMF, *N,N'*-dimethylformamide; PEG, polyethylene glycol; BSA, bovine serum albumin; NaCl/P_i, phosphate-buffered saline (20 mM sodium phosphate, pH 7.0, 140 mM NaCl); MEM, minimal essential Eagle's medium (spinner modification); and SDS, sodium dodecyl sulfate.
† HEPES-buffered MEM was used throughout.

was removed from the upper phase. The cell number was determined as before, and the number of cells in the upper phase was calculated as per cent of total cell number.

The partitioning system used in these measurements was composed of 5% (w/v) Dextran T-500, 4% PEG, 0.001% PEG–palmitate [20], 10 mM sodium phosphate buffer (pH 7.0) and 140 mM NaCl.

Cross-linking measurements. Cross-linking of membrane proteins was detected after exposure of cells to porphyrins, as described above. L1210 cells were disrupted, nuclei were discarded [21] and the cell membranes were isolated [22]. SS-1 cell membranes were isolated as described by Wright *et al.* [23]. Membranes were then dissolved in 25% sucrose containing 2.5% SDS, 25 mM Tris buffer at pH 8, 2.5 mM EDTA, 10 mM mercaptoethanol and 0.0005% bromphenol blue. The SDS-protein ratio was 4:1. Electrophoresis was carried out in 0.5 × 75 mm cylindrical gels containing 5% acrylamide and 0.1% bis-acrylamide, in a buffer of 50 mM Tris (pH 8), 0.1% SDS and 2.5 mM EDTA [24]. A current of 5 mA/gel was uded for the 2-hr electrophoresis runs. The gels were stained with Brilliant Coomassie Blue.

Cross-linking of membrane glycoproteins was measured using cells labeled with ^{125}I by the lactoperoxidase method [25]. The post-nuclear fraction was extracted with lithium diiodosalicylate, dialyzed, and analyzed on polyacrylamide gels as described above.* The gels were fractionated into 1-mm slices and radioactivity was measured after solubilization, using liquid scintillation counting.

Cross-linking in a "cell-free" system was measured by mixing 0.5-mg portions of BSA or α_1-antitrypsin, 100 μg of porphyrin and 100 μl of water. After exposure to light for 10 min, as described previously [18], the mixture was dialyzed against 50 mM Tris–Acetate buffer at pH 7.5 to remove DMF and excess porphyrin, and then analyzed on 5% polyacrylamide gels. Control mixtures were treated as described above, but kept in the dark. After staining with Coomassie Blue, gels were examined for the appearance of bands of high molecular material, an indication of cross-linking.

RESULTS

Properties of the porphyrins. The structural features of the five porphyrins studied here are shown in Table 1, with exact structures shown in Fig. 1. NSC 26030 is the dimethyl ester of NSC 19665. An increase in hydrophobicity was found (Table 1) as the number of ionizable functional groups decreased. NSC 121180 and 36001 are highly water soluble; NSC 19665 and 407318 are less soluble in water at 37° (20 μg/ml and 5 μg/ml, respectively), but far more soluble in octanol than are NSC 121180 and NSC 36001. Only the ester NSC 26030 is substantially soluble in decane, and this compound is not detectably soluble in water.

Table 2. Effects of different porphyrins on cycloleucine transport by ascitic tumor cells

Porphyrin*	L1210	SS-1
None (– DMF)	102†	100
None (+ DMF)	100	100
None (+ DMF, dark)	101	99
26030	100	100
121180	99	100
36001	100	98
19665	10	28
407318	20	31
19665 (dark)	98	99

* Structures shown in Fig. 1. Porphyrin levels were 10 μg/ml.

† Transport of cycloleucine is shown as per cent of control cells (0.2 per cent DMF present) after treatment with porphyrin in the presence of light. Numbers represent averages of five determinations and are subject to an error of ± 10 per cent of values shown. Cells were irradiated as described in the text. The notation (dark) indicates non-irradiated controls.

Transport studies. In control tubes, we observed an uptake of 1750 ± 150 cpm of cycloleucine during a 5-min incubation at 37° (a distribution ratio of approx. 3, calculated as described in ref. 26). The rate of uptake was linear for at least 5 min.

The effects of 10 μg/ml levels of different porphyrins on two murine ascitic tumor cells are shown in Table 2. The highly water-soluble porphyrins, NSC 121180 and NSC 36001, had no effect at this concentration. NSC 26030 was insoluble in water and was also ineffective. NSC 19665 and 407318 inhibited subsequent cellular capacity for cycloleucine transport when porphyrin-treated cells were exposed to light. The extent of this effect varied in the two cell lines; the L1210 cell was inherently more porphyrin-sensitive.

Partitioning studies. When an untreated population of L1210 cells was partitioned between the phases of the partitioning system described above, 25.1 per cent of the cells appeared in the upper phase. If the PEG–palmitate ligand was omitted from the system, less than 2 per cent of the cells partitioned into the upper phase. The SS-1 cells behaved similarly; 18.3 per cent of the cells population was found in the upper phase of the complete system.

Upon exposure to 10 μg/ml of porphyrin NSC 121180 in the dark or the light, the number of cells partitioning into the upper phase was increased (Table 3). This has been taken as a measure of hydrophobic interactions between the cell surface and the PEG–palmitate ligand [19, 20], and indicates that a porphyrin–cell interaction has served to mask certain hydrophilic cell-surface sites. Upon photo-activation, NSC 121180 was inactive, altering neither cycloleucine transport nor gel patterns of membrane proteins (see below).

Upon exposure to light, the number of cells treated with NSC 19665 or 407318 which partitioned into the upper phase was decreased (Table 3), indicating a marked decrease in cell-surface hydrophobicity.

Porphyrin-induced membrane protein cross-linking. Treatment of SS-1 or L1210 cells with 10 μg/ml levels of NSC 19665 or NSC 407318 followed by exposure to light led to substantial cross-linking of membrane protein, detected by analytical gel electrophoresis. Re-

* We have independently assessed the effectiveness of this technique, using a cell-membrane preparation labeled with [^{3}H]fucose (*Molec. Pharmac.*, in press). At least 75 per cent of high molecular weight label appeared in the dialyzed salicylate extract of the post-nuclear fraction.

Table 3. Effects of porphyrins on partitioning behavior of ascitic tumor cells

Porphyrin NSC No.	Conditions*	Partition coefficient+ L1210	SS-1
Control	Dark	25.1	18.0
121180	Light	27.0	20.1
	Dark	28.3	19.9
36001	Light	23.3	18.4
	Dark	24.5	18.2
19665	Light	17.4	13.5
	Dark	25.0	18.0
407318	Light	19.4	14.3
	Dark	24.4	17.9

* Cells were treated with 10 μg/ml of porphyrin in the light or the dark for 10 min at 22°, and then the partition coefficient was measured.

+ Shown as per cent of total cells which partition into the upper phase. All numbers shown were subject to error of 10 per cent of values shown; data represent an average of four determinations for each value.

sults obtained with control SS-1 cells and NSC 19665- and 121180-treated cells are shown in Fig. 2. No cross-linking was found when 10 μg/ml levels of NSC 121180, 36001 or 26030 were employed. A similar result was obtained when membrane proteins of SS-1 cells were labeled via the lactoperoxidase radioiodination procedure, the cells then treated with photo-activated porphyrins (NSC 19665 or 407318), and the membrane proteins isolated and analyzed by gel electrophoresis (Fig. 3). In contrast, we found no cross-linking of ^{125}I-labeled glycoprotein from cell membranes which had been isolated by the lithium diiodosalicylate procedure (Fig. 3).

Cross-linking in vitro. When purified preparations of BSA or α_1-antitrypsin were exposed to 1 mg/ml concentrations of NSC 19665, 121180 or 36001, in the presence of light, a marked amount of cross-linking was detected by subsequent gel electrophoresis (Fig. 4). A 1 mg/ml drug level is found in L1210 cells following exposure to 10 μg/ml of porphyrin for 10 min at 37° [18].

DISCUSSION

One of the critical determinants of cytotoxicity of the photo-activated prophyrins is their relative solubility in

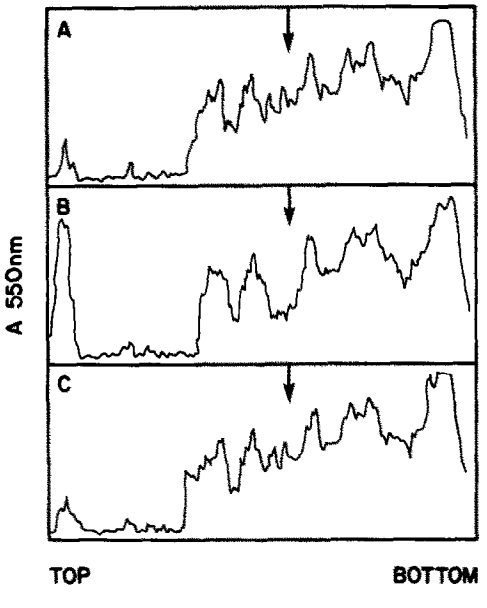

Fig. 2. Densitometer tracings of SDS gels of SS-1 membrane fractions after staining with Coomassie Blue. The gels were scanned at 550 nm. The arrow indicates the position of a bovine serum albumin standard. Each gel contained 125 μg of membrane protein. Panel A: control SS-1 cells. Panel B: cells treated with 10 μg/ml of NSC 19665 and irradiated. Panel C: cells treated with 10 μg/ml of 121180 and irradiated.

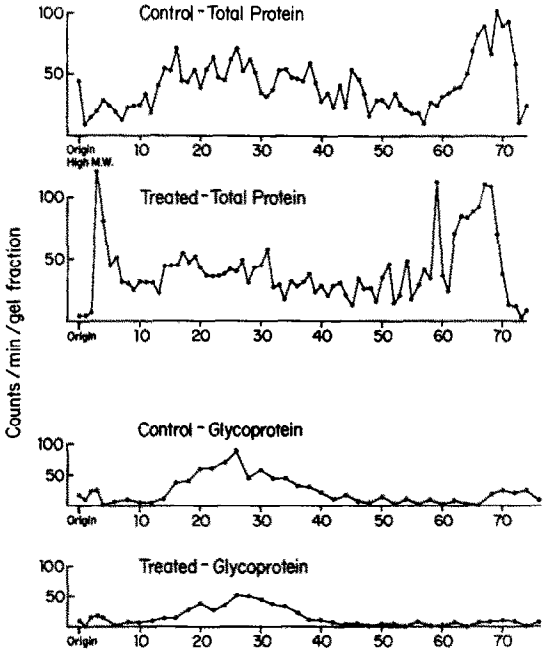

Fig. 3. Analysis of ^{125}I-labeled membrane protein and glycoprotein on SDS–acrylamide gels. High mol. wt material is to the left. From the top: membrane protein from control SS-1 cells; membrane protein from cells treated with 10 μg/ml of NSC 19665 (light); membrane glycoprotein from control cells; and membrane glycoprotein from porphyrin-treated cells.

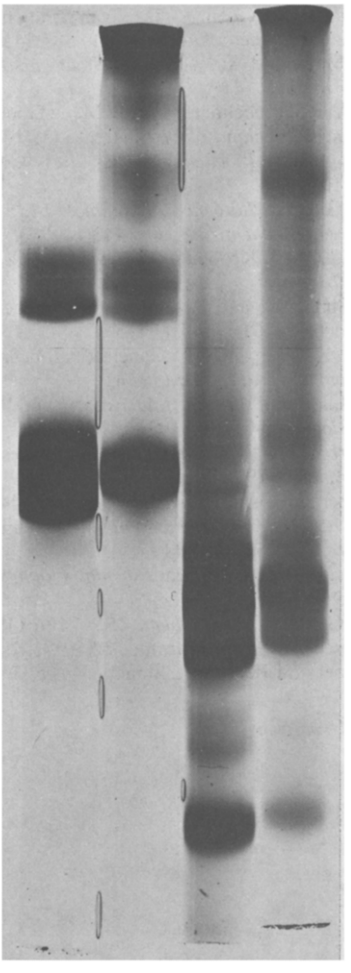

Fig. 4. Analysis of proteins on SDS–acrylamide gels. From left: BSA + porphyrin (dark), BSA + porphyrin (light), α_1-antitrypsin + porphyrin (dark), α_1-antitrypsin + porphyrin (light). Each gel contained 500 μg of protein previously treated with 1 mg/ml of porphyrin in the presence or the absense of light, as described in the text.

different solvents. The ineffectiveness of NSC 26030, a dimethyl ester, is presumably related to the relative insolubility of the compound in aqueous media. The ineffectiveness of NSC 121180 and NSC 36001 was associated with a high degree of solubility in water, but a relatively poor solubility in more hydrophobic solvents. These data suggest that the optimally effective porphyrins must be concentrated in hydrophobic regions of the cell membrane in order to catalyze subsequent membrane perturbations. Since the non-carboxyl moiety of the porphyrin is decane-soluble (Table 1), concentration of carboxyl-substituted porphyrins at a hydrophobic–hydrophilic interface in the cell membrane is likely. In a related study, Suwa *et al.* [11] found that porphyrin-induced oxidation of cholesterol was found only if both porphyrin and cholesterol were incorporated into liposomes; non-liposmal porphyrin was ineffective. The lifetime of the singlet oxygen intermediate must be very short, with detectable alterations in the cell membrane produced only if the porphyrin is bound to appropriate membrane sites prior to photo-activation.

The cell lines examined here had a slightly different degree of sensitivity to the cytotoxic effects of the octanol-soluble porphyrins, NSC 19665 and 407318 (Tables 2 and 3). These data suggest that each cell membrane may have a characteristic pattern of porphyrin binding.

In this study, we found that cross-linking of membrane proteins could readily be demonstrated after treatment of cells with NSC 19665 or 407318. However, cross-linking of ^{125}I-labeled membrane glycoprotein extracted with lithium diiodosalicylate could not be shown. The latter extraction technique was employed, since Tsai *et al.* [21] had demonstrated that other procedures do not detect certain glycoprotein components of the membrane. Since proteins and glycoproteins could be cross-linked in a cell-free system, membrane glycoprotein components may be located at sites at which cytotoxic porphyrins are not bound in sufficient concentration to catalyze cross-linking, or such glycoproteins may be distant from membrane components to which they could be cross-linked.

Girotti [27] showed that singlet oxygen-catalyzed cross-linking of the erythrocyte membrane could not be dissociated by SDS or -SH reagents at 100°. Girotti [8] also found no evidence of porphyrin-induced cross-linking of membrane glycoprotein. Considering other reports, he concluded that the mobility of membrane glycoprotein might preclude *in situ* cross-linking reactions.

Dubbelman *et al.* [15] demonstrated porphyrin-induced cross-linking of proteins in cell-free system, and implicated histidine residues as the likely site of photo-oxidation. The cross-linking reaction is then believed to involve a nucleophilic addition of free amino groups to the oxidized histidine. Such a reaction could proceed in the absence of other postulated intermediates [8, 9, 11] resulting from porphyrin-induced oxidation of membrane components.

The behavior of cells in the two-phase system containing PEG-palmitate has been used previously to characterize drug-induced alterations in membrane properties [28, 29] which result in an altered cell-surface hydrophobicity. In the present study, only the hydrophilic porphyrin 121180 altered the cell-surface hydrophobicity in the dark, indicating masking of hydrophilic cell-surface sites. The more hydrophobic porphyrins, NSC 19665 and 407318, did not affect cell partitioning behavior until the cells were irradiated. The resulting decrease in partition ratio upon irradiation indicates a singlet oxygen-mediated decrease in cell-surface hydrophobicity. This effect may be related to the cross-linking phenomenon, a more rigid membrane structure impairing access of cellular hydrophobic sites to the aqueous environment.

We conclude that concentration of porphyrin at a relatively hydrophobic membrane site is required before photo-induced toxicity can occur. The consequences of photo-toxicity include a decrease in cell-surface hydrophobicity, impaired membrane transport, and cross-linking of membrane protein. The drug-induced alterations, therefore, include not only inactivation of membrane-bound enzymes [17] but also alterations in the capacity of cell surface to interact with its environment.

Acknowledgements—We thank Gwynn Smith, Joanne Blah-

nik and Cheryl Murphy for excellent technical assistance, and Gwendolyn Dozier for manuscript preparations. This work was supported by Grant CA-23378 from the National Cancer Institute, NIH, DHEW.

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