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Dequalinium, a topical antimicrobial agent, displays anticarcinoma activity based on selective mitochondrial accumulation

(rhodamine 123/cyanine/chemotherapy/lipophilic/cationic)

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ABSTRACT **Positively charged lipophilic compounds, such as rhodamine 123, localize in mitochondria and are selectively accumulated and retained by carcinoma cells. It has been suggested that this phenotype may be exploited for selective killing of carcinoma cells by lipophilic cations. Here we report that doubly positively charged dequalinium, which has been used for 30 years as an antimicrobial agent in over-the-counter mouthwashes, lozenges, ointments, and paints, exhibits significant anticarcinoma activity. Dequalinium is more effective than seven of eight established anticancer drugs in prolonging the survival of mice with intraperitoneally implanted mouse bladder carcinoma MB49. Dequalinium also inhibits the growth of subcutaneously implanted human colon carcinoma CX-1 in nude mice and recurrent rat colon carcinoma W163 in rats. Lipophilic cationic compounds, such as dequalinium, could comprise a unique class of anticarcinoma agents.**

Human carcinomas, particularly those of the lung, breast, pancreas, colon, and bladder, remain the major causes of death due to cancer. Defining a characteristic unique for carcinoma cells that could be exploited for the selective killing of tumors would constitute a major advance in cancer therapy. Intriguingly, a wide variety of carcinoma cells exhibit increased accumulation and retention of lipophilic cationic compounds, such as rhodamine 123, relative to untransformed epithelial cells (1–4). These compounds localize in mitochondria of living cells as a result of the electric potential across the mitochondrial membrane (2). Although rhodamine 123 has been used extensively as a relatively nontoxic stain for mitochondria in living cells (5), at higher doses it is selectively toxic toward carcinomas *in vitro* (6, 7) and *in vivo* (8).

We now report that dequalinium, a lipophilic compound with two positive charges (9), exhibits a high degree of *in vitro* and *in vivo* anticarcinoma activity. Fluorescent microscopy reveals that dequalinium localizes in the mitochondria and, at toxic doses, changes their morphology. Dequalinium is more effective than 5-fluorouracil, Cisplatin, vinblastine, bleomycin, arabinosylcytosine, methotrexate, and cylcophosphamide, but not doxorubicin (Adriamycin), in prolonging the survival of mice with i.p. implanted mouse bladder carcinoma MB49. Dequalinium also inhibits the growth of s.c. implanted tumors in rats and nude mice. We propose that lipophilic cationic compounds could comprise a unique class of agents directed against carcinomas.

MATERIALS AND METHODS

Cell Lines and Reagents. All cell lines were grown in Dulbecco's modified Eagles's medium (GIBCO) supplemented with 10% calf serum (M.A. Bioproducts, Walkersville, MD) at 37°C in 5% CO_2/95% air and 100% humidity. CV-1, an African green monkey kidney epithelial cell line, was obtained from the American Type Culture Collection (Rockville, MD). MCF-7, a human breast carcinoma cell line, was obtained from M. Rich (Michigan Cancer Foundation, Detroit). MB49, a 7,12-dimethylbenz[*a*]anthracene-transformed mouse bladder epithelial cell line, was obtained from I. C. Summerhayes (Harvard Medical School, Cambridge, MA). CX-1, a human colon carcinoma cell line, was obtained from S. D. Bernal (Harvard Medical School). W163 was explanted from a 1,2-dimethylhydrazine-induced rat colon adenocarcinoma as described (10). Dequalinium chloride was from Aldrich and was dissolved in water using a bath sonicator. Rhodamine 123, 3,3′-dihexyloxacarbocyanine iodide, 3,3′-diethyloxadicarbocyanine iodide, and methylene blue were from Eastman Kodak. 5-Fluorouracil was from Sigma. Doxorubicin (Adriamycin) was from Adria Laboratories (Columbus, OH). Vinblastine was from Eli Lilly. Cisplatin, bleomycin, methotrexate, and cyclophosphamide were from Bristol-Myers (Syracuse, NY).

Clonogenic Assays. Clonogenic assays were performed as described (6, 7). Briefly, about 300 cells were seeded in 60-mm dishes and incubated overnight. The next day, dequalinium was added to the growth medium at various concentrations and was subsequently removed by washing the cells and adding drug-free medium after 3 hr or 1 wk. Colony-forming units were determined ≈2 wk later by staining with methylene blue (0.2%).

i.p. Tumors. Experiments were performed as described by Bernal *et al.* (8). MB49 cells (2×10^6 per mouse) were injected i.p. into male BDF_1 mice (The Jackson Laboratory), using seven mice (occasionally six or eight) per group. Drug treatments were started the next day, administered i.p. or s.c.

s.c. Tumors. MB49 cells (2×10^6 per mouse) were injected s.c. into the right flank of male BDF_1 mice on day 0. Dequalinium was administered i.p. starting on day 1 or day 8. Tumor size was measured by calipers. For recurrent rat colon carcinoma W163, 3-mm^3 pieces of tumor were implanted s.c. in the right thigh of Wistar/Furth rats (Harlan Sprague Dawley, Indianapolis, IN). The tumors were allowed to grow for 7 days and then all gross tumors were resected. The following day treatment with dequalinium was started by administering it s.c. into the left thigh. Eleven days after tumor resection the rats were sacrificed, and the recurrent tumors were excised and weighed. For human colon carcinoma CX-1, 5×10^6 tumor cells were injected s.c. into the right flank of female Swiss athymic *nu/nu* mice (Taconic Farms, Germantown, NY) on day 0. Starting on day 1, dequalinium was administered s.c. on the left side. The mice were sacrificed on day 31 and the tumors were excised and weighed.

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Abbreviations: T/C, treated/control ratio; E.I., exposure index.

Mitochondrial Localization of Dequalinium by Fluorescence Microscopy. Living CV-1 cells grown on 12-mm glass coverslips were exposed to 19 μM dequalinium in culture medium for 2.5 hr at 37°C; this was followed by exposure to 1 μM 3,3'-diethyloxadicarbocyanine iodide in culture medium for 1 min at room temperature. The cells were then washed three times with culture medium and mounted in culture medium using a silicone rubber chamber as described (1). The cells were observed and photographed using a Zeiss Photomicroscope III, Zeiss Neofluar objective lens (100×), and Kodak Ektachrome P800/1600 film with push 2 developing. Cyanine fluorescence was photographed using a Zeiss no. 14 filter set (green 510- to 560-nm excitation, 580-nm dichroic mirror, red 590-nm long-pass emission) at exposure index (E.I.) 6300. Blue excitation produced green fluorescence at a lower level. After 1 min of constant illumination the cyanine fluorescence faded so that very little red fluorescence and no green fluorescence could be observed. After the cyanine fluorescence faded, the dequalinium fluorescence was observed by illuminating the cells using a Zeiss no. 2 filter set (near-UV 365-nm excitation, 395-nm dichroic mirror, blue 420-nm long-pass emission) for 15 sec, during which time the fluorescence rapidly became brighter. Dequalinium produced the same pattern of fluorescence in the absence of cyanine. The reason for this brightening remains unclear but may be due to formation of a metastable excited state that fluoresces at longer wavelengths. The dequalinium fluorescence was photographed using a Zeiss no. 10 filter set (blue 450- to 490-nm excitation, 510-nm dichroic mirror, green 520- to 560-nm emission) at E.I. 1600. CV-1, rather than a carcinoma cell line, was used to demonstrate the mitochondrial localization of dequalinium because the mitochondria of MCF-7 cells were rapidly converted to the damaged, globular pattern under similar conditions. For experiments examining the effect on mitochondrial morphology of toxic doses of dequalinium, CV-1 cells were exposed to dequalinium (19 μM) for 24 hr; this was followed by exposure to rhodamine 123 (10 μg/ml) for 10 min at 37°C. The cells were washed and mounted in the absence of dye and photographed using the Zeiss no. 10 filter set, at E.I. 1600, with Kodak Tri-X film, developed in Kodak HC-110, dilution B, for 12 min at 25°C. Rhodamine 123 is not toxic at this dose and was used to facilitate visualization of the mitochondria for photography.

RESULTS

Dequalinium in Clonogenic Assays. We compared the effect of dequalinium (chemical structure, Fig. 1) using a clonogenic assay on MCF-7, a human breast carcinoma cell line, and CV-1, an untransformed African green monkey kidney epithelial cell line. CV-1 was used since no "normal" human epithelial cell line is currently available. These two cell lines have been used previously as examples of carcinoma and normal epithelial cell lines with phenotypes of long and short retention of lipophilic cationic compounds, respectively (5). These cell lines contain keratin, typical of epithelial cells, and have comparable growth rates. The IC_{50} obtained by clonogenic assay of a given lipophilic cationic compound for MCF-7 is in most cases very similar to those obtained for various other human carcinoma cell lines, including human colon carcinoma CX-1, human bladder carcinoma EJ, human pancreatic carcinoma PaCa-2, human cervical carcinomas HeLa and CaSki, human lung carcinoma A549, human adrenal cortex carcinoma SW-13, and human prostate carcinoma PC-3. Likewise, the IC_{50} values obtained using CV-1 are similar to those obtained using PtK-2, BSC-1, and MDCK, the untransformed epithelial cell lines.

The IC_{50} values of dequalinium by clonogenic assay are 2 μM for MCF-7 and 250 μM for CV-1 when the cells were exposed to dequalinium for 3 hr. Thus, dequalinium is

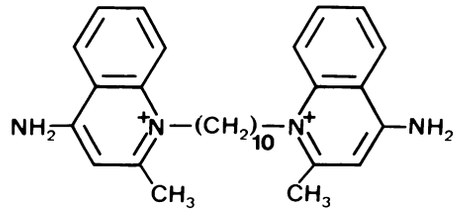

FIG. 1. Structure of dequalinium.

125-fold more toxic to MCF-7 than to CV-1 under these conditions. However, when the cells were exposed to dequalinium for 1 wk continuously, the selectivity was almost completely abolished, with IC_{50} values of 0.3 μM for MCF-7 and 0.8 μM for CV-1. Based on these results, *in vivo* experiments were performed.

Prolongation of Survival with i.p. Tumors. Dequalinium was tested in mice against i.p. implanted mouse bladder carcinoma MB49, a 7,12-dimethylbenz[*a*]anthracene-transformed mouse bladder epithelial cell line. MB49 cells exhibit the phenotype of increased uptake and prolonged retention of lipophilic cationic compounds such as rhodamine 123. In one experiment, dequalinium was effective in prolonging survival with the treated/control ratio (T/C, traditionally expressed as %) = 252% when 4 mg/kg was injected i.p. every other day from day 1 to day 25 (tumor implanted on day 0) and T/C = 210% when 2 mg/kg was injected daily from day 1 to day 25 (Fig. 2). Similar experiments with varying doses (at least 2 mg/kg) and schedules (at least until day 21) were performed independently 25 times over 3 yr. Twelve of these experiments produced a T/C > 200%. No toxic death occurred in these experiments. The average of all 25 experiments was T/C = 190%, which was more effective than seven of eight antineoplastic agents tested (Table 1). All of these agents were administered i.p.

Dequalinium also prolonged survival when administered s.c., although to a lesser degree (T/C = 150%; 10 mg/kg on days 1–3, 5, 7, 9, and 11). Administration s.c. of dequalinium induced local irritation at the site of the injections, which limited the number of injections that could be administered.

Inhibition of Growth of s.c. Tumors. Dequalinium was also effective against tumors implanted s.c. In three model systems, mouse bladder carcinoma MB49 in BDF_1 mice (Fig. 3), rat colon carcinoma W163 in Wistar/Furth rats, and human colon carcinoma CX-1 in nude mice (Fig. 4), dequalinium significantly inhibited the growth of subcutaneous tumors. The fact that dequalinium was effective on recurrent W163 tumors, and on MB49 when treatment was delayed for 8 days, indicates that the drug is not acting by inhibiting tumor implantation but is actually

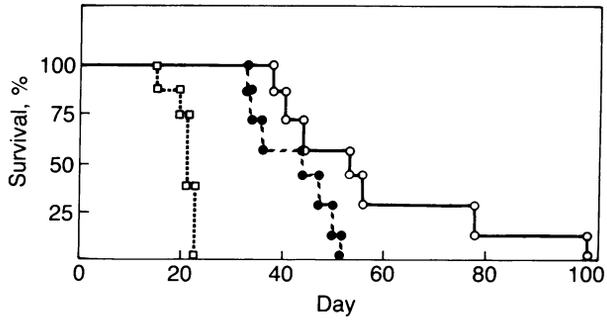

FIG. 2. Prolongation of survival of mice implanted i.p. with mouse bladder carcinoma MB49 by dequalinium. □, Untreated control; ●, dequalinium at 2 mg/kg, daily, days 1–25, T/C = 210%; ○, dequalinium at 4 mg/kg, every other day, days 1–25, T/C = 252%. The control group for this experiment contained eight mice and each group that received dequalinium had seven mice.

Table 1. Effect of dequalinium and antineoplastic drugs on survival of mice implanted i.p. with mouse bladder carcinoma MB49

Drug	Median survival (T/C, %)
Dequalinium	190
Doxorubicin	4 of 7 survivors
5-Fluorouracil	163
Cisplatin	162
Vinblastine	156
Bleomycin	125
Arabinosylcytosine	125
Methotrexate	110
Cyclophosphamide	100

Dosage and regimen for other anticancer agents were as follows: doxorubicin, 2.5 mg/kg, daily, days 1–11; 5-fluorouracil, 20 mg/kg, daily, days 1–9; Cisplatin, 2 mg/kg, daily, days 1–9; vinblastine, 2 mg/kg, days 1, 2, 14, and 27; bleomycin, 6 mg/kg, daily, days 1–9; arabinosylcytosine, 200 mg/kg, daily, days 1–5; methotrexate, 2 mg/kg, daily, days 1–9; cyclophosphamide, 90 mg/kg, day 1. The doses for Cisplatin, 5-fluorouracil, cyclophosphamide, and bleomycin used here were similar to the recommended doses by the National Cancer Institute for other carcinoma models in mice (11). The doses for the other drugs used were comparable to those reported in the literature.

affecting tumor growth. In all cases the site of drug administration was not the site of tumor implantation.

Mitochondrial Localization and Morphological Changes. As revealed by epifluorescence microscopy, dequalinium fluorescence occurs predominantly in the mitochondria of epithelial cells. The staining pattern is identical to that produced by a cationic cyanine dye previously shown (2) to localize in mitochondria (Fig. 5). Prolonged exposure to dequalinium drastically alters the morphology of the mitochondria. The mitochondria, which were originally filamentous (Fig. 5), become globular and appear primarily around the nucleus, as visualized by staining with rhodamine 123 (Fig. 6). Rhodamine 123 does not alter the morphology of mitochondria under these conditions (1, 2). To be described elsewhere, these morphologically altered mitochondria exhibit impaired respiration and ATP production. The membranous endoplasmic reticulum, as visualized by staining with 3,3′-dihexyloxacarbocyanine iodide (12), appears unchanged (data not shown).

DISCUSSION

Dequalinium has been available over-the-counter for about 30 yr as an antimicrobial agent in topical ointments, oral and vaginal paints, sore-throat lozenges, and mouthwash. In addition to its antimicrobial toxicity, dequalinium's two delocalized positive charges and its C-10 aliphatic chain made it an attractive candidate for testing the selective killing of carcinoma cells. It was postulated that because dequalinium had two positive charges compared to the single charge on rhodamine 123, the selective accumulation, retention, and selective toxicity would be increased. The Nernst equation predicts that the equilibrium concentration of a doubly positive cation in response to a negative membrane potential is the *square* of that of a monovalent cation. Although equilibrium would not be achieved *in vivo*, this illustrates the powerful effect that doubling the charge could have on the relative rate and extent of accumulation and retention of lipophilic cations.

Most cations are unable to pass through cell membranes without specific carrier systems due to the large free energy barrier posed by the hydrophobic interior of the membrane. To be selectively accumulated and retained by the mitochondria of carcinoma cells, a cationic compound requires sufficient lipophilicity and delocalization of its positive charges to reduce the free energy change when moving from an aqueous to a hydrophobic environment. This allows the compound to cross the plasma and mitochondrial membranes

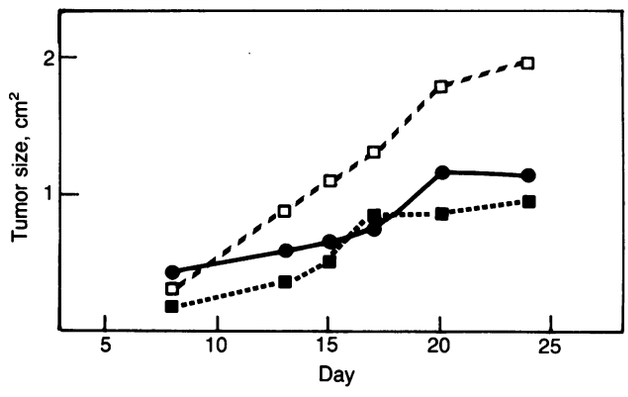

FIG. 3. Inhibition of growth of mouse bladder carcinoma MB49, implanted s.c., by dequalinium. The size of tumors is expressed as the length × width in cm². □, Untreated control. Dequalinium was administered i.p., 2 mg/kg daily, starting on day 1 for 10 days, followed by 1 mg/kg daily thereafter (■) or starting on day 8 for 9 days, followed by 1 mg/kg daily thereafter (●). Note that on day 8 all mice had measurable tumors.

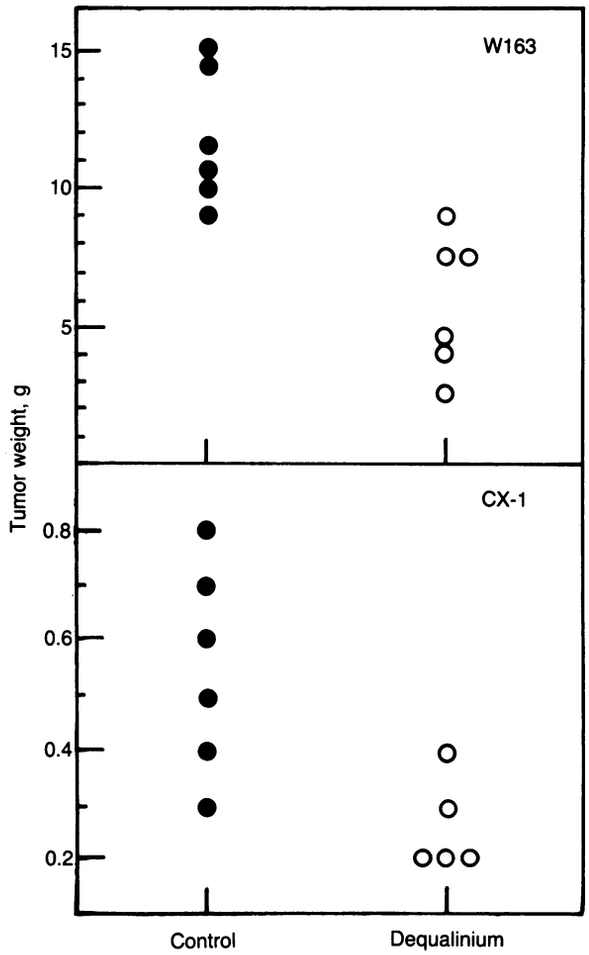

FIG. 4. Inhibition of growth of recurrent rat colon carcinoma W163, implanted s.c. in rats, and human colon carcinoma CX-1, implanted s.c. in nude mice, by dequalinium. (*Upper*) For W163, dequalinium was administered at 4 mg/kg s.c. on days 1–4 and 7–10. The recurrent tumors were excised and weighed on day 11. (*Lower*) For CX-1, dequalinium was administered at 5 mg/kg s.c. on days 1–3, 6–10, 14, 15, 17, 20–22, 24–27, and 30. Tumors were excised and weighed on day 31. All groups had six animals each. One mouse treated with dequalinium died because of toxicity.

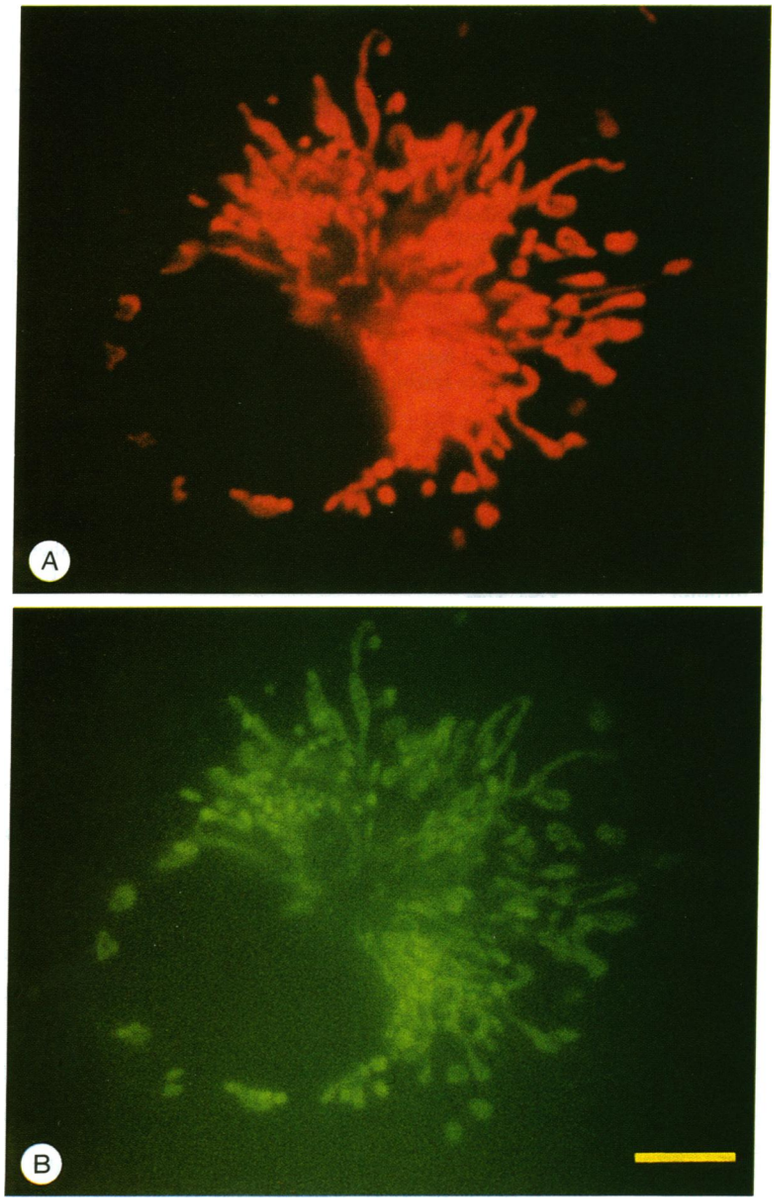

FIG. 5. Mitochondrial localization of dequalinium. (*A*) 3,3′-Diethyloxadicarbocyanine fluorescence in a living CV-1 cell. (*B*) The same cell after bleaching of the cyanine fluorescence, which was followed by near-UV excitation of dequalinium fluorescence. (Bar = 15 μm.)

passively and be accumulated in response to membrane potential. The results reported here confirm the idea that the anticarcinoma activity of rhodamine 123 is neither unique nor necessarily related to its specific chemical structure. Anticarcinoma activity can be exhibited in varying degrees by other lipophilic cationic compounds with different chemical structures. The fact that the selectivity exhibited by dequalinium was most marked when a shorter exposure time was employed is consistent with the hypothesis that selective retention by carcinomas contributes to the selective toxicity.

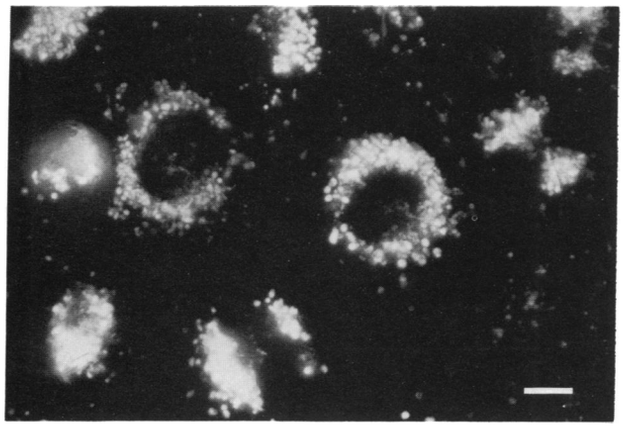

FIG. 6. Fluorescence micrograph of rhodamine 123-stained CV-1 cells that were exposed to a cytotoxic level of dequalinium (19 μM for 24 hr). (Bar = 25 μm.)

Rhodamine 123 (13) and dequalinium (unpublished data) inhibit ATP production by isolated rat liver mitochondria *in vitro*, and dequalinium interferes with bacterial respiration (14). If the primary target for dequalinium is the mitochondrion, vital metabolic functions such as nucleic acid and protein synthesis could be inhibited secondarily due to inhibition of mitochondrial ATP production. However, the mechanism of cell killing or growth inhibition by dequalinium could involve sites in addition to mitochondria. Dequalinium binds to DNA (15), inhibits calmodulin (16), and has surface-active properties (17). The fact that dequalinium fluorescence is not observed in the nucleus (or in other organelles) does not preclude the possibility that dequalinium might be binding to the DNA and that the nucleus may also be one of the target sites. Although a much higher concentration of dequalinium

would be expected in the mitochondria due to the high mitochondrial membrane potential, a concentration might exist in the nucleus sufficient to inhibit nuclear function but not noticeably fluoresce relative to the bright mitochondria. Other effects, such as quenching by DNA and nucleoproteins, might also prevent visualization in the nucleus.

The distinction between the mechanisms of selectivity and the sites of cytotoxic action could be important for the development of clinically useful agents. The mitochondrial fluorescence of dequalinium that we observed suggests that the mechanism of selectivity is indeed mitochondrial. This selectivity is apparently a result of the increased uptake and retention of lipophilic cations by the mitochondria in carcinoma cells in response to the mitochondrial membrane potential (3, 4, 18). It is unnecessary to postulate that the target of dequalinium is more sensitive in tumor cells. This distinction allows the possibility of using several lipophilic cationic drugs that exploit the same mechanism of selectivity—i.e., increased accumulation by the mitochondria—but aim at different targets, such as the cytoskeleton, the endoplasmic reticulum, or the Golgi apparatus. Conceivably, the mitochondria could serve also as a reservoir for the slow release of a single multitargeted lipophilic cation that inhibits vital cellular functions elsewhere in the cell.

To evaluate the efficacy of lipophilic cationic compounds, it is crucial to choose an animal model system that is sensitive to this class of compounds. The majority of carcinoma (but not leukemia or sarcoma) cell lines exhibits the phenotype of long retention of rhodamine 123, as do the tumor types used in the experiments reported here (3). Likewise, most human primary explanted carcinomas from a variety of tissues retain rhodamine 123 for a prolonged period of time (unpublished data). In contrast, L1210 leukemia, B16 melanoma, and Lewis lung carcinoma, which are commonly used for screening potential cancer drugs, do not retain rhodamine 123 *in vitro* and do not respond to it *in vivo* (8). Therefore, lipophilic cationic compounds, which depend upon selective uptake and retention by carcinomas to display maximal antitumor activity, have been passed over as chemotherapeutic agents. Dequalinium was tested by the National Cancer Institute >10 yr ago against the L1210 leukemia using standard screening protocols and was deferred from development based on a lack of activity (M. Wolpert, personal communication).

In light of the chemiosmotic hypothesis of Mitchell (19), it is reasonable that the prolonged retention of lipophilic cationic compounds by carcinoma cells is largely due to a high mitochondrial membrane potential. We have recently provided evidence supporting this view (18). It is possible that an elevated membrane potential may be related to abnormal bioenergetics in cancer cells (20).

Although existing cancer drugs are directed primarily at the nucleus and DNA synthesis of the cell, other cellular organelles and processes have been largely bypassed as targets for cancer chemotherapy. Antimitochondrial activity has been proposed as a basis for chemotherapy (20), and balancing lipophilicity and hydrophilicity has been emphasized in designing potential antitumor agents (21). The antitumor activity of two agents, methylgloxalbis(guanylhydrazone) (22) and lonidamine (23), has been attributed to antimitochondrial effects. However, the selective accumulation by mitochondria of toxic cationic compounds has not yet been purposefully exploited. Dequalinium and rhodamine 123 could be the prototypes of a unique class of antineoplastic agents specifically aimed at carcinomas.

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