Potential use of alexidine dihydrochloride as an apoptosis-promoting anticancer agent

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Abstract

Despite advances in surgery, radiation, and chemotherapy, novel therapeutics are needed for head and neck cancer treatment. The objective of this current study was to evaluate alexidine dihydrochloride as a novel compound lead for head and neck cancers. Using a tetrazolium-based assay, the dose required to reduce cell viability by 50% (ED_{50}) was found to be ~1.8 μmol/L in FaDu (human hypopharyngeal squamous cancer) and ~2.6 μmol/L in C666-1 (human undifferentiated nasopharyngeal cancer) cells. In contrast, the ED_{50} values were much higher in untransformed cells, specifically at ~8.8 μmol/L in GM05757 (primary normal human fibroblast), ~8.9 μmol/L in HNEpC (primary normal human nasal epithelial), and ~19.6 μmol/L in NIH/3T3 (mouse embryonic fibroblast) cells. Alexidine dihydrochloride did not interfere with the activities of cisplatin, 5-fluorouracil, or radiation, and interacted in a less-than-additive manner. DNA content analyses and Hoechst 33342 staining revealed that this compound induced apoptosis. Alexidine dihydrochloride–induced mitochondrial damage was visualized using transmission electron microscopy. Mitochondrial membrane potential ($\Delta\Psi_M$) depolarization was detectable after only 3 hours of treatment, and was followed by cytosolic Ca^{2+} increase along with loss of membrane integrity/cell death. Caspase-2 and caspase-9 activities were detectable at 12 hours, caspase-8 at 24 hours, and caspase-3 at 48 hours. FaDu cell clonogenic survival was reduced to <5% with 1 μmol/L alexidine dihydrochloride, and, correspondingly, this compound decreased the *in vivo* tumor-forming potential of FaDu cells. Thus, we have identified alexidine dihydrochloride as the first bisbiguanide compound with anticancer specificity. [Mol Cancer Ther 2006;5(9):2234–40]

Received 3/13/06; revised 6/18/06; accepted 6/29/06.

Grant support: Canadian Institutes of Health Research, Elia Chair in Head and Neck Cancer Research, and Natural Sciences and Engineering Research Council of Canada scholarship (K.W. Yip).

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doi:10.1158/1535-7163.MCT-06-0134

Introduction

Most head and neck cancers begin in the epithelia that line the mucosal surfaces of the head and neck. In the United States alone, it is estimated that >29,370 new cases and 7,320 deaths are attributed to oral cavity and pharyngeal cancers in 2005 (1). At the time of diagnosis, 10% of patients harbor distant metastases, 50% have regional disease, and only 34% have localized tumors (1). Although early detection and diagnosis, along with advances in surgery, chemotherapy, and radiation therapy, have increased survival modestly, the 5-year overall survival rate remains at ~59% (1), which underscores the need to develop novel therapeutics.

An approach commonly used for antitumor compound lead identification is to conduct a forward chemical biology screen (2). Chemicals are screened for ''hits'' that induce a particular phenotype before the cellular or protein target is identified. Such approaches often reveal novel targets and pathways. Paclitaxel, for example, was discovered to have antitumor activity in a broad range of rodent tumors several years before it was identified to target microtubules (3, 4). Studies involving rapamycin led to the discovery of the Tor protein nutrient response signaling network (5, 6). Large-scale, phenotype-based assays are currently being done by the National Cancer Institute/National Institutes of Health Developmental Therapeutics Program and the *In vitro* Cell Line Screening Project for the purpose of discovering novel therapeutic molecules (7).

Our group has extensive experience in identifying new molecular therapies that improve outcome for head and neck cancers, ranging from adenoviral (8–14) to antisense oligonucleotide (15) approaches. To this end, we have recently done a cell-based, phenotype-driven, cell viability–based high-throughput screen for novel head and neck cancer cytotoxics (16). This screen identified several known anticancer compounds from the LOPAC1280 (Sigma-Aldrich Corporation, St. Louis, MO) and Prestwick Chemical Libraries (Prestwick Chemical, Inc., Washington, DC). Significantly, several novel potential anticancer compound leads were also identified.

Alexidine dihydrochloride is perhaps the most interesting of the identified molecules. This chemical was among

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the most potent cytotoxics screened. In addition, alexidine dihydrochloride is a bisbiguanide compound (17), a class of chemicals never previously evaluated for anticancer properties. The objective of this current study is to evaluate the cancer-specific properties of alexidine dihydrochloride and to assess its mode of action in head and neck cancers.

Materials and Methods

Cell Lines

FaDu (human hypopharyngeal squamous cancer) and NIH/3T3 (untransformed mouse embryonic fibroblast) cells were obtained from American Type Culture Collection (Manassas, VA), whereas GM05757 (primary normal human fibroblast) and HNEpC (primary normal human nasal epithelial line) cells were obtained from the Coriell Institute for Medical Research (Camden, NJ) and PromoCell (Heidelberg, Germany), respectively. All cell lines were cultured according to specifications. C666-1 (human undifferentiated nasopharyngeal cancer) cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Wisent, Inc., St. Bruno, Quebec, Canada) and antibiotics (100 mg/L penicillin and 100 mg/L streptomycin) as previously described (8, 15). All experiments were conducted when cells were in an exponential growth phase.

Compound Dilutions

Alexidine dihydrochloride was obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada), and dissolved in DMSO at a concentration of 10 mmol/L, with subsequent dilutions done in H_2O. Cisplatin, 5-fluorouracil, and paclitaxel were obtained from the Princess Margaret Hospital Pharmacy Department (Toronto, Ontario, Canada) and diluted in PBS. For a negative control (the vehicle alone), DMSO was diluted in H_2O to a concentration corresponding to the DMSO in the respective drug-treated group.

Cell Viability Dose-Response Curves

Cells were seeded in 96-well plates (Corning Life Sciences, Acton, MA) at 5,000 per well in 100 μL of growth medium and allowed to incubate for 24 hours. The chemicals were then added as indicated, in a total volume of 5 μL/chemical. After 48 hours, the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS; Promega Corporation, Madison, WI) was used to detect cell viability according to the specifications of the manufacturer. A 1-hour MTS incubation time was used and 490 nm absorbance was measured using a SpectraMax Plus^{384} microplate reader (Molecular Devices Corporation, Sunnyvale, CA). DMSO (0.1%)–treated cells for each respective cell line were used as a negative control, and cisplatin (166.6 μmol/L)–treated cells for each respective cell line were used as a positive control.

Where indicated, cells were irradiated immediately before compound treatment. Irradiation was done at room temperature using a ^{137}Cs unit (Gammcell 40 Extractor; MDS Nordion, Ottawa, Ontario, Canada) at a dose rate of 1.1 Gy/min.

Sub-G_1 Flow Cytometric Analysis

FaDu cells were seeded (0.3×10^6/T-25), incubated for 1 day, and treated with either alexidine dihydrochloride (4 μmol/L) or vehicle alone for 48 or 72 hours. A 72-hour time point was selected for this assay because DNA degradation occurs late in FaDu cell apoptosis. After treatment, detached and adherent cells were collected (detached cells were decanted, whereas adherent cells were trypsinized and mixed with the respective detached cells), pelleted at $200 \times g$, resuspended in a 1.5 mL hypotonic fluorochrome solution (50 μg/mL propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100; Sigma-Aldrich), and left in the dark at 4°C overnight. Flow cytometric analysis was then done (FACSCalibur, Becton Dickinson, San Jose, CA) and analyzed using FlowJo (Tree Star, Inc., San Carlos, CA). Sub-G_1 peaks were identified by ModFit LT (Verity Software House, Inc., Topsham, ME).

Morphologic Assessment of Apoptosis

Cells were seeded (0.3×10^6/T-25), allowed to incubate for 1 day, and treated as described. After 48 hours, detached and adherent cells were collected together, stained with a 10 μmol/L Hoechst 33342 (Invitrogen Corporation, Carlsbad, CA)-4% formalin-PBS solution, and visualized under UV light using a Zeiss Axioskop HBO 40 microscope (Zeiss, Thornwood, NY).

Transmission Electron Microscopy

Cells were treated with either alexidine dihydrochloride (4 μmol/L) or vehicle alone as indicated, and then processed by the University of Toronto Faculty of Medicine Microscopy Imaging Laboratory (Toronto, Ontario, Canada). Briefly, harvested cells were fixed with a Karnosky-style fixative [4% paraformaldehyde and 2.5% glutaraldehyde in a 0.1 mol/L Sorensen's phosphate buffer (pH 7.2)]. Cells were postfixed with 1% osmium tetroxide, dehydrated with ethanol, washed with propylene oxide, treated with epoxy resin polymerized at 60°C for 48 hours, sectioned on a Reichert Ultracut E microtome to 80 nm thickness, collected on 300 mesh copper grids, and counterstained with uranyl acetate and lead citrate. Analysis was done with a Hitachi H7000 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV.

Mitochondrial Depolarization, Calcium Content, and Propidium Iodide Uptake

As previously described, 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine [$DiIC_1(5)$; Invitrogen] was used to determine the mitochondrial membrane potential ($\Delta\Psi_M$), cell permeant indo-1 AM (Invitrogen) was used to determine changes in cytosolic calcium, and propidium iodide (Invitrogen) uptake was used to determine cell death (18). Briefly, FaDu cells were seeded (0.3×10^6/T-25), incubated for 1 day, and then treated with alexidine dihydrochloride (4 μmol/L) or vehicle alone. At the indicated time points, all floating and adherent cells (using trypsin) were collected and resuspended in growth medium at a concentration of 10^6/mL. $DiIC_1(5)$ (40 nmol/L final concentration) and indo-1 AM (2 μmol/L final concentration) were added to the cell

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suspensions. The cells were incubated at 37°C for 25 minutes, after which propidium iodide (1 μg/mL) was added. The cells were analyzed by flow cytometry using a Coulter Epics Elite [Beckman Coulter; $DiIC_1(5)$ excitation 633 nm, 675 ± 20 nm bandpass; indo-1 AM excitation 360 nm, emission ratio 405 nm/525 nm].

Caspase Activity

Cells were seeded (0.4×10^6 per well in six-well plates), incubated for 1 day, and treated as indicated. Afterward, cells were stained using the CaspGLOW *In situ* Caspase Staining kits (BioVision, Mountain View, CA) for caspase-9, caspase-8, caspase-3, and caspase-2 activity according to the specifications of the manufacturer. Analysis was done using flow cytometry (FACSCalibur, CellQuest software, Becton Dickinson).

Clonogenic Cell Survival Assays

FaDu cells were seeded in six-well plates (Nalge Nunc International, Rochester, NY) at densities of 10^2 to 10^4 per well in 3 mL growth medium and incubated overnight at 37°C to allow for cell attachment. Alexidine dihydrochloride or the vehicle control was added at the specified concentrations in a total volume of 50 μL. After 48 hours, the drug-containing medium was replaced with fresh growth medium and the plates were incubated at 37°C. Eleven days after seeding, colonies were fixed in 70% ethanol, stained with 10% methylene blue, and colonies of ≥50 cells were counted. The surviving fraction was determined as the ratio of the number of colonies in the treated sample to that of the nontreated control sample. Triplicate wells were set up for each condition and the experiment was done twice.

In vivo Experiments

All animal experiments were conducted in accordance with the guidelines of the Animal Care Committee, Ontario Cancer Institute, University Health Network (Toronto, Ontario, Canada). In all cases, 6- to 8-week-old severe combined immunodeficient (SCID) BALB/c female mice were obtained from the Animal Research Colony, Ontario Cancer Institute.

Tumor Formation Experiments

FaDu cells were seeded (2×10^6/T-75 flask), incubated for 1 day, and treated with either alexidine dihydrochloride (4 μmol/L) or vehicle alone. After 48 hours, the cells were harvested and implanted into the left gastrocnemius muscle of SCID mice (2.5×10^5 cells in 100 μL growth medium per mouse). The mice were monitored for tumor formation at least thrice per week for 100 days. Three independent experiments were done, with three mice per group for each experiment.

Statistical Analyses

All experiments were done thrice independently and all data are reported as mean ± SE unless otherwise stated.

Results

Anticancer Specificity of Alexidine Dihydrochloride

We have previously used a cell-based, phenotype-driven, MTS-based, high-throughput screen of the LOPAC1280 (Sigma-Aldrich) and Prestwick Chemical Library to identify novel potential therapeutic agents for head and neck cancer (16). This preliminary screen identified alexidine dihydrochloride as a possible head and neck cancer–specific therapeutic (Fig. 1A). Thus, we did alexidine dihydrochloride dose-response evaluations on two cancer and three untransformed cell lines to confirm the preferential toxicity of the compound in head and neck cancer cells. The dose required to reduce cell viability by 50% (ED_{50}) was ~1.8 μmol/L in FaDu (~21 hour doubling time) and ~2.6 μmol/L in C666-1 (~48 hours doubling time) cells (Fig. 1B). In contrast, the ED_{50} values were much higher in untransformed cell lines, specifically at ~8.8 μmol/L in GM05757 (~20-hour doubling time), ~8.9 μmol/L in HNEpC (~67-hour doubling time), and ~19.6 μmol/L in NIH/3T3 cells (~20-hour doubling time). A therapeutic window between malignant cells and nonmalignant cells thus exists with this compound.

Combination Therapy

To evaluate the effect of combining traditional head and neck cancer therapeutic agents with this novel anticancer compound, FaDu cells were simultaneously treated with a low dose of either cisplatin (1.9 μmol/L), 5-fluorouracil (5 μmol/L), or γ-radiation (10 Gy), along with increasing doses of alexidine dihydrochloride (Fig. 2A). In turn, FaDu cells were simultaneously incubated with a low dose of alexidine dihydrochloride (1.2 μmol/L) and with increasing doses of cisplatin or 5-fluorouracil (Fig. 2B and C). It is apparent from these dose-response curves that alexidine

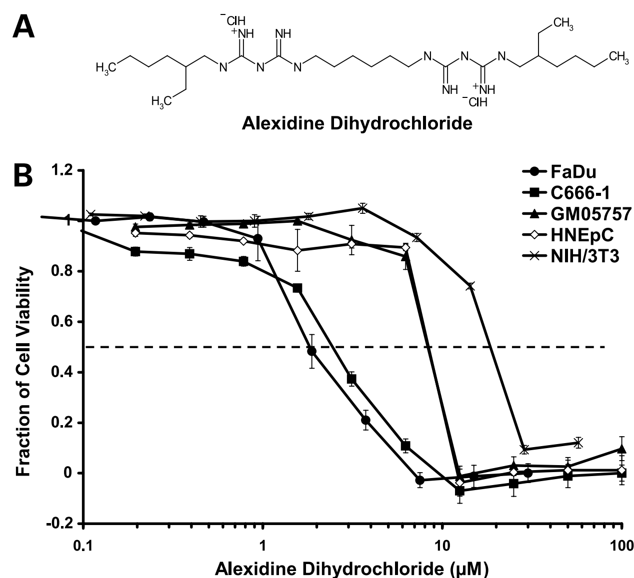

Figure 1. Alexidine dihydrochloride is preferentially toxic to head and neck cancer cells. **A,** chemical structure of alexidine dihydrochloride. **B,** dose-response (cell viability) curves were determined for alexidine dihydrochloride in two cancer (FaDu, C666-1) and three normal (GM05757, HNEpC, and NIH/3T3) cell lines. The cell viability assays were done 48 h after drug treatment. Each experiment was done thrice independently. *Points,* mean; *bars,* SE. *Dotted line,* 50% viability.

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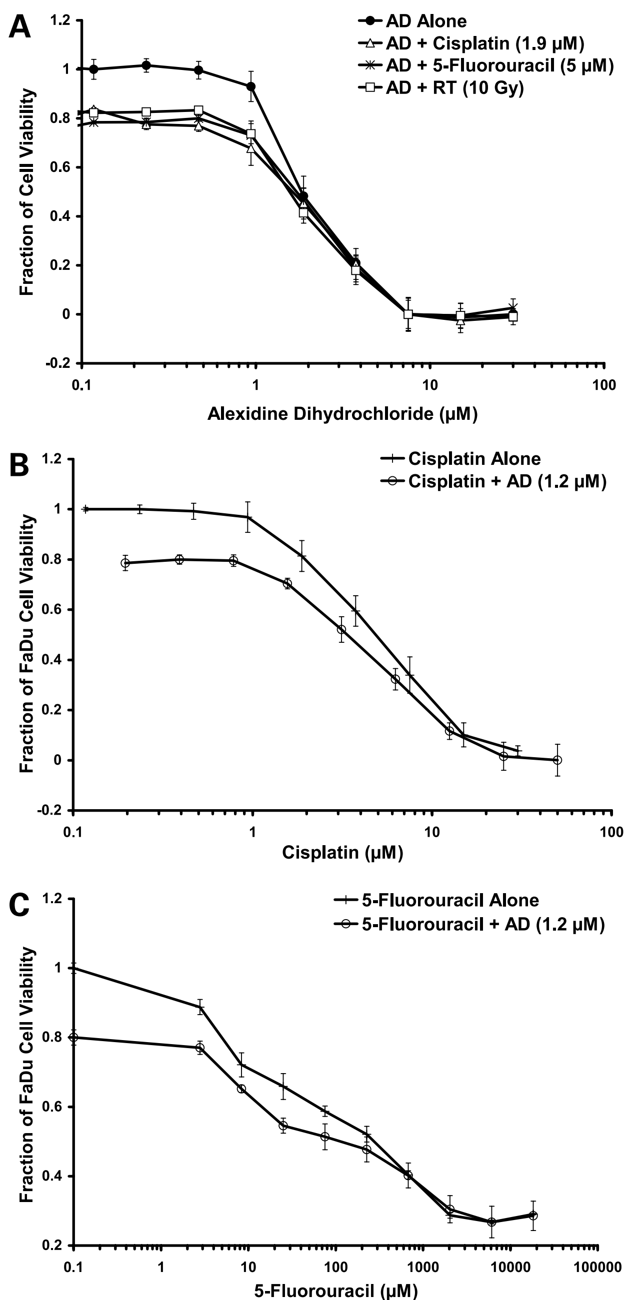

Figure 2. The effect of combining alexidine dihydrochloride (*AD*) with either cisplatin, 5-fluorouracil, or radiation. **A,** FaDu cells were treated with a sensitizing dose of either cisplatin, 5-fluorouracil, or radiation along with increasing doses of AD. **B,** FaDu cells were treated with a sensitizing dose of AD and increasing doses of cisplatin. **C,** FaDu cells were treated with a sensitizing dose of AD and increasing doses of 5-fluorouracil. In **A**, **B**, or **C**, AD does not interfere with the actions of standard anticancer agents. The cell viability assays were done 48 h after treatment. Each experiment was done thrice independently. *Points,* mean; *bars,* SE.

dihydrochloride does not interfere with the actions of cisplatin, 5-fluorouracil, or γ-radiation. The interactions are possibly additive at lower doses of the respective compounds, but seem to be less than additive at most doses.

Alexidine Dihydrochloride Induces Apoptosis

In an effort to determine the mode of cell death induced by alexidine dihydrochloride, flow cytometric DNA content analyses was done on FaDu cells treated with 4 µmol/L alexidine dihydrochloride (the dose required to reduce cell viability by 75% after 48 hours, or ED_{75}). There was minimal change in G_1-G_0, S, or G_2-M phase (or aneuploid) populations at 12, 24, 48, or 72 hours (Fig. 3A, data not shown). However, in alexidine dihydrochloride–treated cells, the sub-G_1 population increased, indicative of apoptosis (Fig. 3A). Hoechst 33342 staining of alexidine dihydrochloride–treated (for 48 hours) cells revealed nuclear condensation and blebbing, consistent with apoptotic nuclear morphology (Fig. 3B). Necrosis was not observed. Transmission electron microscopy was used to further visualize the subcellular morphologic characteristics of apoptosis. Chromatin condensation and morphologic abnormalities in the mitochondria were observed (Fig. 3C). The rough endoplasmic reticulum, which can also induce apoptosis (19), did not seem to be affected.

Mitochondrial Membrane Potential Depolarization

To further investigate the mechanism of apoptosis in alexidine dihydrochloride-mediated cell death, $\Delta\Psi_M$ depolarization and cytosolic Ca^{2+} increase (which may be due to damage to the endoplasmic reticulum or Ca^{2+} plasma membrane channels) were evaluated. FaDu cells treated (4 µmol/L) for 3, 12, or 24 hours were simultaneously stained with $DiIc_1(5)$ ($\Delta\Psi_M$), indo-1 AM (Ca^{2+}), and propidium iodide (membrane integrity/cell death; Fig. 3D). The proportion of cells with depolarized mitochondria increased as treatment time increased (Fig. 3D, *column A*). Increased cytosolic Ca^{2+}, indicated by the indo-1 AM 405 nm/525 nm ratio, could be observed in cells with depolarized mitochondria. Propidium iodide uptake, indicating loss of membrane integrity and cell death, also increased with incubation time (Fig. 3D, *column C*). Notably, in alexidine dihydrochloride-treated FaDu cells, $\Delta\Psi_M$ hyperpolarization could be observed, a phenomenon commonly observed during apoptosis (20, 21). The relative mean fluorescence readings in polarized cells after 3, 12, and 24 hours of treatment were 44.0, 83.5, and 130.1, respectively (37.8 at 24 hours with vehicle alone; Fig. 3D, *columns B* and *D*).

Alexidine Dihydrochloride Induced Caspase Activation

To evaluate the biochemical characteristics of alexidine dihydrochloride–induced apoptosis, caspase activation was evaluated in FaDu cells treated with 4 µmol/L of compound for 12, 24, or 48 hours (Fig. 3E). At 12 hours, caspase-2 activation was observed, along with slight activation of caspase-9. More extensive caspase-9 activation, combined with caspase-8 activation, were observed at 24 hours, with caspase-3 activation following at 48 hours.

Elimination of Tumor Formation

To assay the effect of alexidine dihydrochloride on clonogenic survival, FaDu cells (10^2–10^4 per well) were seeded and then incubated with this compound for 48 hours. Nine days later, colonies were counted (Fig. 4A).

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Colony formation was reduced to <5% with 1 μmol/L alexidine dihydrochloride.

Next, FaDu cells were treated with 4 μmol/L alexidine dihydrochloride for 48 hours (ED_{75}) and then injected into the left gastrocnemius muscle of SCID mice (2.5×10^5 per mouse) to determine whether this compound had the ability to prevent *in vivo* tumor formation. These mice did not develop tumors even after 100 days, whereas mice injected with 0.1% DMSO–treated FaDu cells started developing tumors after only 10 days (Fig. 4B). Thus, alexidine dihydrochloride effectively eliminates the tumor-forming potential of 2.5×10^5 cells FaDu cells in SCID mice.

Discussion

This current study, to our knowledge, is the first to evaluate the tumor-specific properties of alexidine dihydrochloride. There has been no known previous report of the anticancer efficacy of such bisbiguanide compounds. Alexidine dihydrochloride possesses anticancer efficacy in human hypopharyngeal squamous (FaDu; head and neck squamous cell carcinoma) and in human nasopharyngeal (C666-1; undifferentiated epithelial) cancers, with minimal toxicity to GM05757, HNEpC, or untransformed mouse embryonic fibroblasts. *In vitro*, this compound does not interfere with chemotherapy or radiation, and induces

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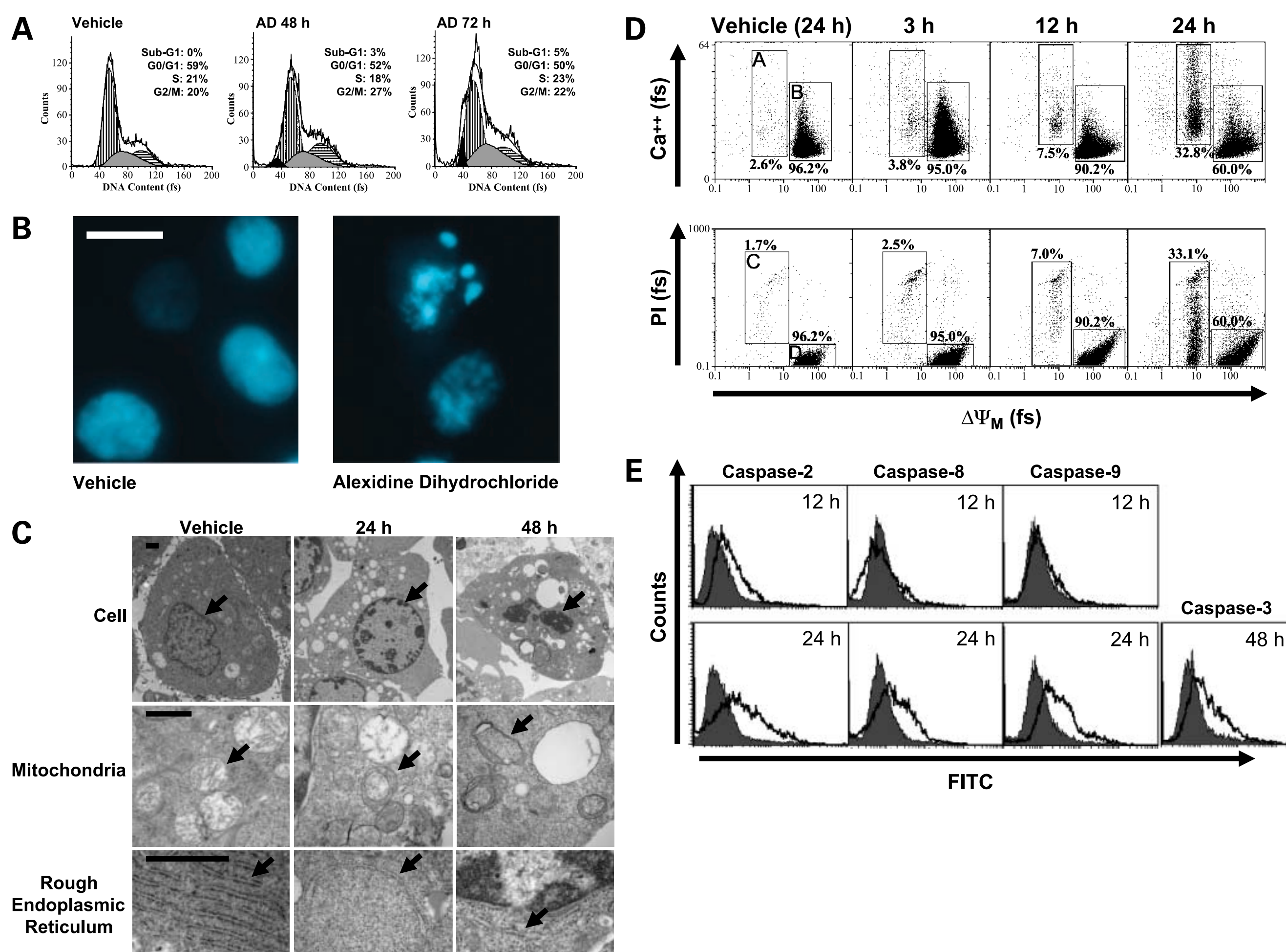

Figure 3. Alexidine dihydrochloride induces cancer cell apoptosis. **A,** an increase in the sub-G_1 population (*black*) of cells was visible after 48 h or 72 h of alexidine dihydrochloride treatment. The percentage of sub-G_1 cells is shown. *Horizontal stripes,* G_0-G_1 fraction; *gray,* S fraction; *vertical stripes,* G_2-M fraction. *fs,* fluorescent signal. **B,** Hoechst 33342 was used to visualize condensed areas of chromatin and nuclear blebbing, which are indicative of apoptosis, in alexidine dihydrochloride–treated FaDu cells after 48 h. *White bar,* 10 μm. **C,** transmission electron microscopy was used to further visualize the mode of alexidine dihydrochloride–induced cell death. Nuclear condensation (*top row, arrow*) and mitochondrial abnormalities (*middle row, arrow*) were observed with treatment. The rough endoplasmic reticulum (*bottom row, arrow*) seemed to be unaffected. *Black bar,* 1 μm. **D,** treated cells were stained with $DilC_1(5)$ ($\Delta\Psi_M$), indo-1 AM (cytosolic Ca^{2+}), and propidium iodide (cell viability). Example gatings for quantification are shown. *Column A,* gated on mitochondrial membrane potential ($\Delta\Psi_M$) depolarized cells; *column B,* gated on $\Delta\Psi_M$ polarized cells; *column C,* gated on cells that were $\Delta\Psi_M$ depolarized and dead; *column D,* gated on cells that were $\Delta\Psi_M$ polarized and viable. Loss of $\Delta\Psi_M$ occurs before cytosolic Ca^{2+} increase and cell death in alexidine dihydrochloride–treated cells. **E,** using a fluorescent caspase inhibitor peptide-based assay (CaspGLOW), alexidine dihydrochloride–induced caspase activation was observed. *Gray,* vehicle-treated cells; *white,* alexidine dihydrochloride–treated cells. In all cases, 4 μmol/L alexidine dihydrochloride (ED_{75}) was used; vehicle represents 0.1% DMSO. Each experiment was done thrice independently.

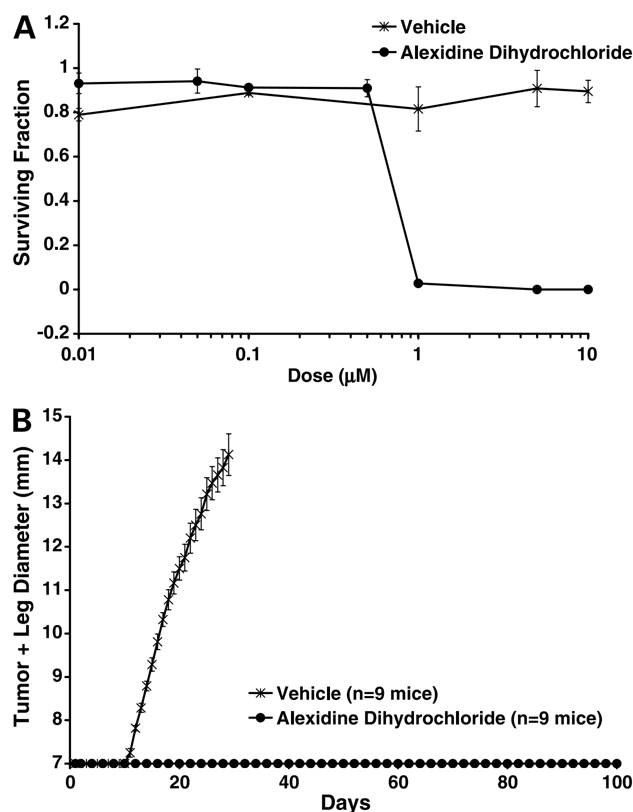

Figure 4. **A,** alexidine dihydrochloride (48-h incubation) reduces the clonogenic survival of FaDu cells. **B,** alexidine dihydrochloride eliminates the tumor-forming ability of 2.5×10^5 FaDu cells in SCID mice. FaDu cells, treated with 0.1% DMSO (vehicle) or alexidine dihydrochloride for 48 h, were injected into the left gastrocnemius muscle of SCID mice (2.5×10^5 per mouse). Alexidine dihydrochloride–treated cells could not form tumors (followed for 100 d). The experiment was done thrice, with a total of three mice in each group per experiment.

apoptosis. *In vivo*, this compound decreases the tumor-forming potential of FaDu cells.

Alexidine dihydrochloride has been previously used in mouthrinses (17). This compound is positively charged, and such cations are attracted to the negatively charged bacterial cell walls (17). In fact, gram-positive bacteria are more sensitive to cations because they are more negatively charged (17). Alexidine dihydrochloride induces lipid-phase separation and domain formation at bacterial membranes (17). Interestingly, mitochondria are thought to be evolutionarily derived from bacteria, and are one of the most negatively charged biological membranes (22, 23). Many cationic lipophilic toxins preferentially accumulate in the mitochondrial matrix of cancer cells, which generally have higher plasma and mitochondrial membrane potentials, as well as more mitochondria compared with normal epithelial cells (24, 25). With alexidine dihydrochloride treatment, we observed loss of $\Delta\Psi_M$ after only 3 hours, caspase-9 activation after 12 hours, and transmission electron microscopy–visible mitochondrial alterations at 24 hours. Thus, damage to the mitochondria occurs early in alexidine dihydrochloride–induced death. Alexidine dihydrochloride–induced lipid-phase separation and domain formation potentially represents a novel anticancer therapeutic mechanism of action.

Caspase-2 was more active after 12 hours of treatment than the mitochondria-activated caspase-9. This may reflect either a differential sensitivity in the caspase activation assay, differing amounts of active caspase types necessary for cell death, or an alternate alexidine dihydrochloride target. Caspase-2 has been controversially implicated in genotoxic stress, endoplasmic reticulum stress, death receptor ligation, and in heat shock–induced apoptosis (26). Endoplasmic reticulum damage was not visible in our transmission electron microscopy studies, although further studies will be required to identify other potential targets of alexidine dihydrochloride.

Head and neck cancers are treated with traditional therapeutic modalities, including surgery, radiation, cisplatin, and/or 5-fluorouracil (27). Alexidine dihydrochloride did not interfere with the action of cisplatin, 5-fluorouracil, or radiation, but the interaction seemed to be less than additive. Clonogenic-based assays, a more complete range of dose-response curves, and further analyses will be required to fully elucidate the efficacy of the combinations *in vitro* (28). Further studies will also be required to evaluate the efficacy of combining alexidine dihydrochloride with other therapeutics. The observation that alexidine dihydrochloride led to caspase-8 activation (after 24-hour treatment) and apoptosis in FaDu cells, a cell line known to be resistant to tumor necrosis factor–related apoptosis-inducing ligand therapy due to a homozygous deletion in the death receptor *DR4* gene (preventing caspase-8 activation; ref. 29), suggests that an alexidine dihydrochloride–tumor necrosis factor–related apoptosis-inducing ligand combination might have significant therapeutic potential.

FaDu cells represent a highly aggressive cell line *in vivo*. These cell lines form tumors after only 10 days with the injection of 2.5×10^5 cells, and increase tumor-plus-leg diameter by ~0.45 mm/d. Merely 4 μmol/L alexidine dihydrochloride was able to eliminate the tumor-forming ability of 2.5×10^5 cells in SCID mice. Using this compound as a drug lead, future studies should focus on enhancing its solubility and bioavailability for further *in vivo* treatment and safety studies.

Alexidine dihydrochloride is chemically similar to chlorhexidine, another bisbiguanide compound. Chlorhexidine possesses chlorophenol end groups, as opposed to the ethylhexyl end groups in alexidine (17). Chlorhexidine affects membrane integrity at low concentrations, and causes the cytoplasm to congeal at higher concentrations (17). Cellular leakage induced by this compound is not responsible for cellular inactivation, but is a consequence of cell death (17). The difference in end-groups between these two compounds is thought to be responsible for the more rapid bactericidal action of alexidine dihydrochloride, as well as the ability of this biguanide to produce lipid domains (30, 31). Evaluating the anticancer potential of

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chlorhexidine, other bisbiguanide compounds and alexidine analogues will provide for a more complete structure-activity relation profile.

In conclusion, our study has identified alexidine dihydrochloride as a novel anticancer drug lead. This compound, as well as other compounds from the bisbiguanide family and alexidine analogues, warrant further study.

Acknowledgments

We thank Melania Pintilie for her statistical advice.

References

1. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. CA Cancer J Clin 2005;55:10 – 30.

2. Stockwell BR. Chemical genetics: ligand-based discovery of gene function. Nat Rev Genet 2000;1:116 – 25.

3. Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J Am Chem Soc 1971;93:2325 – 7.

4. Schiff PB, Fant J, Horwitz SB. Promotion of microtubule assembly *in vitro* by Taxol. Nature 1979;277:665 – 7.

5. Brown EJ, Albers MW, Shin TB, et al. A mammalian protein targeted by G_1-arresting rapamycin-receptor complex. Nature 1994;369:756 – 8.

6. Shamji AF, Nghiem P, Schreiber SL. Integration of growth factor and nutrient signaling: implications for cancer biology. Mol Cell 2003;12: 271 – 80.

7. Holbeck SL. Update on NCI *in vitro* drug screen utilities. Eur J Cancer 2004;40:785 – 93.

8. Yip KW, Li A, Li JH, et al. Potential utility of BimS as a novel apoptotic therapeutic molecule. Mol Ther 2004;10:533 – 44.

9. Li AA, Ng E, Shi W, et al. Potential efficacy of p16 gene therapy for EBV-positive nasopharyngeal carcinoma. Int J Cancer 2004;110:452 – 8.

10. Chia MC, Shi W, Li JH, et al. A conditionally replicating adenovirus for nasopharyngeal carcinoma gene therapy. Mol Ther 2004;9:804 – 17.

11. Li JH, Shi W, Chia M, et al. Efficacy of targeted FasL in nasopharyngeal carcinoma. Mol Ther 2003;8:964 – 73.

12. Lee AW, Li JH, Shi W, et al. p16 gene therapy: a potentially efficacious modality for nasopharyngeal carcinoma. Mol Cancer Ther 2003;2:961 – 9.

13. Liu FF. Novel gene therapy approach for nasopharyngeal carcinoma. Semin Cancer Biol 2002;12:505 – 15.

14. Li JH, Chia M, Shi W, et al. Tumor-targeted gene therapy for nasopharyngeal carcinoma. Cancer Res 2002;62:171 – 8.

15. Yip KW, Mocanu JD, Au PY, et al. Combination bcl-2 antisense and radiation therapy for nasopharyngeal cancer. Clin Cancer Res 2005;11: 8131 – 44.

16. Yip KW, Mao X, Au PY, et al. Benzethonium chloride: a novel anticancer agent identified utilizing a cell-based small molecule screen. In press 2006.

17. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 1999;12:147 – 79.

18. Schimmer AD, Hedley DW, Chow S, et al. The BH3 domain of BAD fused to the Antennapedia peptide induces apoptosis via its α helical structure and independent of Bcl-2. Cell Death Differ 2001;8: 725 – 33.

19. Xu C, Bailly-Maitre B, Reed JC. Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest 2005;115:2656 – 64.

20. Vander Heiden MG, Chandel NS, Schumacker PT, Thompson CB. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Mol Cell 1999;3:159 – 67.

21. Pham NA, Hedley DW. Respiratory chain-generated oxidative stress following treatment of leukemic blasts with DNA-damaging agents. Exp Cell Res 2001;264:345 – 52.

22. Dyall SD, Brown MT, Johnson PJ. Ancient invasions: from endosymbionts to organelles. Science 2004;304:253 – 7.

23. Burlacu A. Regulation of apoptosis by Bcl-2 family proteins. J Cell Mol Med 2003;7:249 – 57.

24. Davis S, Weiss MJ, Wong JR, Lampidis TJ, Chen LB. Mitochondrial and plasma membrane potentials cause unusual accumulation and retention of rhodamine 123 by human breast adenocarcinoma-derived MCF-7 cells. J Biol Chem 1985;260:13844 – 50.

25. Leprat P, Ratinaud MH, Julien R. A new method for testing cell ageing using two mitochondria specific fluorescent probes. Mech Ageing Dev 1990;52:149 – 67.

26. Tu S, McStay GP, Boucher LM, Mak T, Beere HM, Green DR. *In situ* trapping of activated initiator caspases reveals a role for caspase-2 in heat shock-induced apoptosis. Nat Cell Biol 2006;8:72 – 7.

27. DeVita VT, Hellman S, Rosenberg SA, editors. CANCER Principles & Practice of Oncology, 7th ed. New York: Lippincott Williams & Wilkins; 2005.

28. Greco WR, Bravo G, Parsons JC. The search for synergy: a critical review from a response surface perspective. Pharmacol Rev 1995;47: 331 – 85.

29. Ozoren N, Fisher MJ, Kim K, et al. Homozygous deletion of the death receptor DR4 gene in a nasopharyngeal cancer cell line is associated with TRAIL resistance. Int J Oncol 2000;16:917 – 25.

30. Chawner JA, Gilbert P. Interaction of the bisbiguanides chlorhexidine and alexidine with phospholipid vesicles: evidence for separate modes of action. J Appl Bacteriol 1989;66:253 – 8.

31. Chawner JA, Gilbert P. A comparative study of the bactericidal and growth inhibitory activities of the bisbiguanides alexidine and chlorhexidine. J Appl Bacteriol 1989;66:243 – 52.

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