# Benzethonium Chloride: A Novel Anticancer Agent Identified by Using a Cell-Based Small-Molecule Screen

Kenneth W. Yip,[1,4] Xinliang Mao,[5] P.Y. Billie Au,[1,6] David W. Hedley,[1,4,7] Sue Chow,[1,4] Shadi Dalili,[2,5] Joseph D. Mocanu,[1,4] Carlo Bastianutto,[1,4] Aaron Schimmer,[1,5,7] and Fei-Fei Liu[1,3,4,8]

**Abstract**

**Purpose:** This study aims to identify a novel therapeutic agent for head and neck cancer and to evaluate its antitumor efficacy.

**Experimental Design:** A cell-based and phenotype-driven high-throughput screening of ~2,400 biologically active or clinically used compounds was done using a tetrazolium-based assay on FaDu (hypopharyngeal squamous cancer) and NIH 3T3 (untransformed mouse embryonic fibroblast) cells, with secondary screening done on C666-1 (nasopharyngeal cancer) and GM05757 (primary normal human fibroblast) lines. The "hit" compound was assayed for efficacy in combination with standard therapeutics on a panel of human cancer cell lines. Furthermore, its mode of action (using transmission electron microscopy and flow cytometry) and its *in vivo* efficacy (using xenograft models) were evaluated.

**Results:** Benzethonium chloride was identified as a novel cancer-specific compound. For benzethonium (48-hour incubation), the dose required to reduce cell viability by 50% was 3.8 μmol/L in FaDu, 42.2 μmol/L in NIH 3T3, 5.3 μmol/L in C666-1, and 17.0 μmol/L in GM05757. *In vitro*, this compound did not interfere with the effects of cisplatin, 5-fluorouracil, or γ-irradiation. Benzethonium chloride induced apoptosis and activated caspases after 12 hours. Loss of mitochondrial membrane potential ($\Delta\Psi_M$) preceded cytosolic $Ca^{2+}$ increase and cell death. *In vivo*, benzethonium chloride ablated the tumor-forming ability of FaDu cells, delayed the growth of xenograft tumors, and combined additively with local tumor radiation therapy. Evaluation of benzethonium chloride on the National Cancer Institute/NIH Developmental Therapeutics Program 60 human cancer cell lines revealed broad-range antitumor activity.

**Conclusions:** This high-throughput screening identified a novel antimicrobial compound with significant broad-spectrum anticancer activity.

There are generally two different approaches used for antitumor compound lead identification. The reverse chemical biology approach, or target-based approach, involves first identifying a compound that activates or inhibits a specific target (e.g., a specific protein) and then identifying the phenotype induced by the compound *in vivo* (e.g., in cells or animals; ref. 1). The forward chemical biology approach, or phenotype-based approach, starts with an outward physical characteristic or phenotype and ends with the identification of the responsible target (1). Molecules are screened for "hits" that induce a particular phenotype before the cellular target is identified. Both reverse and forward approaches have yielded clinically useful agents for cancer management.

Recently, reverse chemical biology approaches have gained significant momentum; for example, screening for compounds that perturbed Bcr-Abl identified 2-phenylaminopyrimidine (2). Imatinib mesilate, a 2-phenylaminopyrimidine derivative, is now Food and Drug Administration approved as a first-line treatment for chronic myelogenous leukemia, and also indicated for c-Kit (CD117)–positive metastatic and/or unresectable malignant gastrointestinal stromal tumors (3, 4). Molecules that inhibit Src/Abl (dasatinib, Bristol-Myers Squibb, New York, NY; for imatinib-resistant chronic myelogenous leukemia), HER1/epidermal growth factor receptor (erlotinib hydrochloride, Genentech/OSI, S. San Francisco, CA; for non–small-cell lung carcinoma), and the proteasome (bortezomib, Millenium Pharmaceuticals, Cambridge, MA; for multiple myeloma) are also, among many others, currently in clinical use (5–8). Preclinically, reverse chemical biology approaches have yielded novel inhibitors of such proteins as Bcl-2 (9) and X-linked inhibitor of apoptosis (10).

**Authors' Affiliations:** [1]Departments of Medical Biophysics, [2]Chemistry, and [3]Radiation Oncology, University of Toronto; Divisions of [4]Applied Molecular Oncology and [5]Cancer Genomics and Proteomics, Ontario Cancer Institute, [6]The Advanced Medical Discovery Institute, and Departments of [7]Medical Oncology and Hematology and [8]Radiation Oncology, Princess Margaret Hospital, University Health Network, Toronto, Ontario, Canada

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**Requests for reprints:** Fei-Fei Liu, Department of Radiation Oncology, Princess Margaret Hospital/Ontario Cancer Institute, Room 10-203, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9. Phone: 416-946-2123; Fax: 416-946-4586; E-mail: Fei-Fei.Liu@rmp.uhn.on.ca.



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Forward chemical biology approaches have also produced many common cancer therapeutics. Paclitaxel was discovered to have antitumor activity in a broad range of rodent tumors years before it was known to target microtubules (11, 12). As in the case of paclitaxel, forward chemical biology approaches often yield novel targets and pathways on identification of the active targets. Other examples include FK506 and rapamycin, which led to the discovery of the calcium-calcineurin-NFAT signaling pathway and nutrient-response signaling network involving target of rapamycin proteins, respectively (13, 14). Large-scale phenotype-based assays are currently being done by the National Cancer Institute/NIH Developmental Therapeutics Program (NCI/NIH-DTP) and the In Vitro Cell Line Screening Project (15).

Our group has extensive experience in identifying novel molecular therapies that improve outcome for head and neck cancers, ranging from adenoviral (16–20) to antisense oligonucleotide (21) approaches. In this current work, we elected to evaluate the potential usefulness of a high-throughput screening approach to determine whether novel anticancer compounds could be identified with activities against head and neck cancer.

## Materials and Methods

### Cell lines

FaDu and NIH 3T3 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured according to specifications. C666-1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Wisent, Inc., Quebec, Canada) and antibiotics (100 mg/L penicillin, 100 mg/L streptomycin) as previously described (16, 21). GM05757 and HNEpC cells were obtained from Coriell Institute for Medical Research (Camden, NJ) and PromoCell (Heidelberg, Baden-Württemberg, Germany), respectively, and cultured according to specifications.

### Small molecules

***Small-molecule screen.*** The Prestwick Chemical Library (1,120 compounds; Prestwick Chemical, Inc., Washington, DC) and the LOPAC1280 (1,280 compounds; Sigma-Aldrich Corp., St. Louis, MO) compounds were provided by the Samuel Lunenfeld Research Institute High-Throughput Screening Robotics Facility (Toronto, Ontario, Canada). The compounds were initially dissolved using the BioMek FX (Beckman Coulter, Inc., Fullerton, CA) in DMSO at a concentration of 10 mmol/L. The drugs were then diluted in sterile $H_2O$ to 0.1 mmol/L.

***All other* in vitro *assays*.** Cisplatin, 5-fluorouracil, and paclitaxel were obtained from the Princess Margaret Hospital Pharmacy Department (Toronto, Ontario, Canada) and diluted in PBS. Benzethonium chloride (Sigma-Aldrich), analogue 4 {ethanaminium, 2-[2-[[[(4-chlorophenyl)amino]carbonyl]oxy]ethoxy]-*N*,*N*,*N*-trimethyl-, chloride; Ryan Scientific, Inc., Isle of Palms, SC}, analogue 5 [(2-(2-benzoyloxy-ethoxy)-ethyl)-dimethyl-octyl-ammonium, bromide; Sigma-Aldrich], and all other small molecules were dissolved in DMSO at a concentration of 10 mmol/L, with subsequent dilutions done in $H_2O$. Analogue 2 [1-hydroxy-5′-nitro-2′-(2-(4-*tert*-octylphenoxy)-ethoxy)-2-naphthanilide; Sigma-Aldrich] was dissolved in DMSO at a concentration of 10 mmol/L, with all subsequent dilutions done in regular growth medium. Analogue 3 {benzenemethanaminium, *N*,*N*′-[1,2-ethanediylbis(oxy-2,1-ethanediyl)]bis[*N*,*N*-dimethyl-, dichloride; Ryan Scientific} was dissolved in PBS, with subsequent dilutions done in $H_2O$. The control vehicle was DMSO diluted in $H_2O$ to a concentration corresponding to the DMSO in the respective drug-treated group.

### Small-molecule high-throughput screening

The BioMek FX and the Samuel Lunenfeld Research Institute High-Throughput Screening Robotics platform were used for cell seeding, treatment, and viability assessment. FaDu or NIH 3T3 cells were cultured to 85% confluency, trypsinized, and resuspended in growth medium at 25,000/mL. Cells were seeded onto 96-well plates (Corning Life Sciences, Acton, MA) at 5,000 per well (200 μL) and allowed to incubate for 24 hours at 37°C, 5% $CO_2$, 95% humidity. Small molecules were then added to a final concentration of ~5 μmol/L. Cells treated with 0.1% DMSO were used as a negative control and cells treated with 166.6 μmol/L cisplatin were used as a positive control. Forty-eight hours later, 100 μL of growth medium were removed from each well. 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 Corp., 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 Corp., Sunnyvale, CA). This screen was done twice independently, with readings that differed by >10% excluded. Mean results were visualized using Cluster and TreeView (Michael Eisen, Lawrence Berkley National Laboratory, Berkley, CA).

### Cell viability dose-response curves

Cells were seeded in 96-well plates 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. After 48 hours, MTS assay was done according to the specifications of the manufacturer, with DMSO (0.1%)–treated cells as negative control and cisplatin (166.6 μmol/L)–treated cells as positive control.

Where indicated, cells were irradiated immediately before small-molecule 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.

### Morphologic assessment of apoptosis

Cells were seeded ($0.3 \times 10^6$ per T-25 flask), allowed to incubate for 1 day, and treated as indicated. After 48 hours, detached and adherent cells were collected, stained with a 10 μmol/L Hoechst 33342 (Invitrogen Corp., Carlsbad, CA)-4% formalin-PBS solution as previously described (16, 21), and visualized under UV light using a Zeiss Axioskop HBO 40 microscope (Zeiss, Thornwood, NY).

### Caspase activity

Cells were seeded ($0.4 \times 10^6$ per well in six-well plates), allowed to incubate for 1 day, and treated as indicated. Afterwards, 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, San Jose, CA).

### Mitochondrial depolarization, calcium content, and propidium iodide uptake

$DiIC_1(5)$ (1,1′,3,3,3′,3′-hexamethylindodicarbocyanine; Invitrogen) was used to determine 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 as previously described (22). Briefly, FaDu cells were seeded ($0.3 \times 10^6$ per T-25 flask), allowed to incubate for 1 day, and then treated with benzethonium chloride or vehicle alone (0.1% DMSO) as indicated. After 24 hours, 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 suspensions. The cells were incubated at 37°C for 25 minutes and propidium iodide (1 μg/mL) was then 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 405/525 nm].

### Transmission electron microscopy

Cells were treated as outlined above and transmission electron microscopy was done 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.

### *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

Cells were seeded ($2 \times 10^6$ per T-75 flask), incubated for 1 day, and treated as indicated. After 48 hours, the cells were harvested and implanted into the left gastrocnemius muscle of SCID mice ($2.5 \times 10^5$ 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.

### Treatment of established FaDu xenograft tumors

FaDu cells were injected into the left gastrocnemius muscle of SCID mice ($2.5 \times 10^5$ in 100-μL growth medium per mouse). The leg diameter of a normal mouse was <7 mm. When the tumor-plus-leg diameter reached 7.25 mm, the mice were randomized into one of the following groups: (*a*) PBS, (*b*) benzethonium chloride, (*c*) radiation-plus-PBS, and (*d*) radiation-plus-benzethonium chloride. On days 1 to 5, the mice were given one i.p. injection (100 μL bolus) daily of either PBS or benzethonium chloride (5 mg/kg in PBS).

Where indicated, 4-Gy local tumor radiation therapy was delivered on days 2 and 5 before the i.p. injection. Briefly, the mice were immobilized in a Lucite box and the tumor-bearing mouse leg was exposed to 100 kV (10 mA) at a dose rate of 10 Gy/min.

Tumors were measured at least thrice per week. The animals were sacrificed when the tumor-plus-leg diameter reached either 14 mm (for nonirradiated tumors) or 13 mm (for irradiated tumors) as per Animal Care Committee guidelines. Irradiated tumors were sacrificed earlier due to radiation-induced moist desquamation and erythema. Three independent experiments were done, with three mice per group for each experiment.

### *In vivo* safety assays

Xenograft tumors were established in SCID mice as outlined above. When the tumor-plus-leg-diameter reached 7.25 mm, the mice were randomized into two groups (six mice per group). Mice were treated with one i.p. injection (100 μL) daily for 5 days with either PBS or benzethonium chloride. Twenty-four hours after the last injection, mouse blood was collected for serum blood biochemistry analyses (done by Vitatech, Mississauga, Ontario, Canada) and all major organs were harvested for H&E staining (done by Pathology Research Program Services, Toronto General Hospital, University Health Network, Toronto, Ontario, Canada).

### NCI/NIH DTP *in vitro* cell line screening

The efficacy of benzethonium chloride on a panel of 60 human cancer cell lines was evaluated by the NCI/NIH DTP (Bethesda, MD) according to their standard operating procedures.[9] Briefly, cells were seeded onto 96-well plates (day 1), allowed to incubate for 24 hours, and then treated with benzethonium chloride (day 2). After 48 hours, relative protein amounts (using sulforhodamine B) were measured (day 4). $GI_{50}$ (the drug concentration resulting, on day 4, in a 50% reduction in the net protein increase from day 2 to day 4), TGI (the drug concentration resulting in total growth inhibition on day 4 relative to day 2), and $LC_{50}$ (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment on day 4 compared with the beginning on day 2) were reported.

### Statistical analyses

Unless otherwise stated, all experiments were done thrice independently and data are reported as mean ± SE. The *Z* factor was used to evaluate the high-throughput screening dynamic range as previously reported (23). Mean and range (minimum and maximum) values are reported for the serum blood biochemistry analyses. The Mann-Whitney *U* test was used for comparing xenograft treatment groups.

## Results

***Initial high-throughput screening.*** To identify novel potential therapeutic agents for head and neck cancer, FaDu cells (human hypopharyngeal squamous cancer; ~21 hours doubling time) were treated with compounds from the Prestwick Chemical Library and LOPAC1280 for 48 hours (*Z* factor = 0.70). FaDu cell viability was detected using the MTS assay (Fig. 1). A counter-screen was done using untransformed NIH 3T3 mouse embryonic fibroblasts. NIH 3T3 cells were used as a preliminary "normal" cell line because they are adherent, easily manipulated, well characterized, and grow at approximately the same rate as FaDu cells (~20 hours doubling time). Compounds that decreased FaDu cell viability by ≥50%, but decreased NIH 3T3 cell viability by ≤5%, were selected as potential hits (Table 1).

The potential hit compounds belonged to the following broad classes of agents: antimicrobial, cytoskeleton/extracellular matrix, ion pump, neurotransmission, phosphorylation, or others (including apoptosis, DNA metabolism, multidrug resistance, and photosensitizer). The cytoskeleton/extracellular matrix and phosphorylation classes contained known anticancer agents such as paclitaxel. The ion pump class (mainly cardiac glycosides) and neurotransmission class might induce cardiac and neural toxicities, respectively. Thus, we decided to focus on the antimicrobial class for potential anticancer agents.

***Final screen for anticancer efficacy.*** We did a dose-response evaluation of the potential hit antimicrobial compounds to confirm the initial high-throughput screening results. In both FaDu and NIH 3T3 cells, the effective dose required to decrease cell viability by 50% after 48 hours ($ED_{50}$) was determined

[9] http://dtp.nci.nih.gov/index.html.

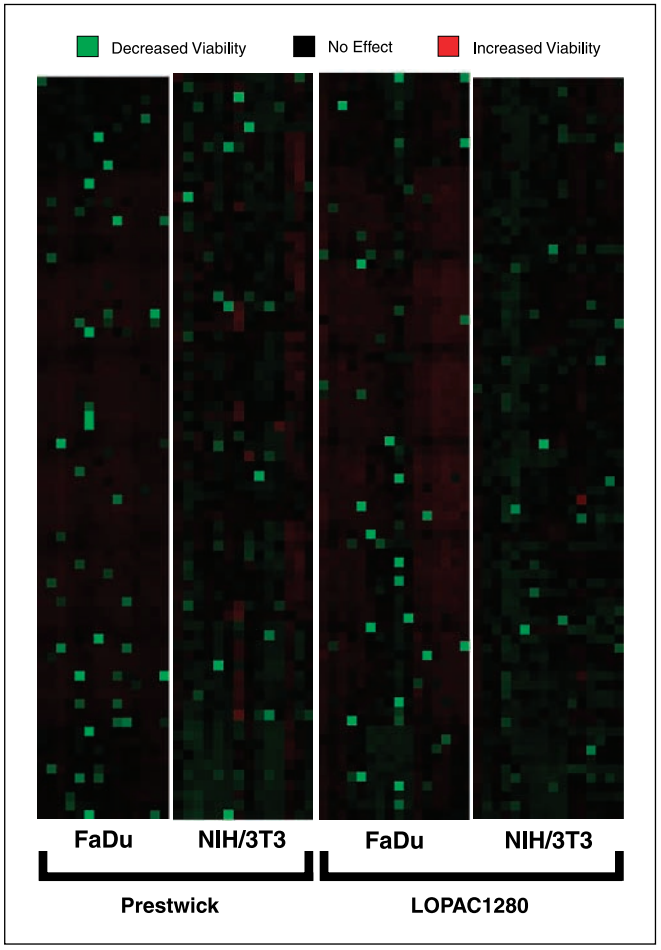


**Fig. 1.** Initial high-throughput screening for novel potential anticancer compounds. FaDu and NIH 3T3 cells were treated with compounds from the Prestwick Chemical Library (1,120 compounds) and LOPAC1280 (1,280 compounds) for 48 hours; after which, cell viability was assayed (MTS). The columns on the heat map represent successive plates of the respective chemical library. The rows represent successive wells. The screen was done twice independently. The potential hit compounds are identified in Table 1.

(Table 2). To further evaluate the effect of the potential anticancer compounds on normal cells, the $ED_{50}$ was determined in GM05757 cells (primary normal human fibroblast; ~20 hours doubling time). Finally, the $ED_{50}$ was determined for C666-1 cells, an undifferentiated nasopharyngeal cancer cell line (~48 hours doubling time). Alexidine dihydrochloride, benzethonium chloride, berberine chloride, dequalinium chloride, and methyl benzethonium chloride were thus identified to be selectively toxic to human pharyngeal cancer cells but not to normal human or mouse cells. Benzethonium chloride, along with its analogue, methyl benzethonium chloride, had a favorable therapeutic index. Thus, we decided to further evaluate the anticancer potential of benzethonium chloride.

As a final determination of benzethonium chloride normal cell toxicity, we evaluated this compound on HNEpC cells, a primary normal human nasal epithelial line (~67 hours doubling time; Fig. 2A). For benzethonium chloride (48-hour incubation), the $ED_{50}$ values (Fig. 2A, *dotted line*) for FaDu and C666-1 (both human cancer) cell lines were ~3.8 and ~5.3 μmol/L, respectively. In contrast, for the "normal" cells represented by HNEpC, GM05757, and NIH 3T3, the $ED_{50}$ values were ~8.7, ~17.0, and ~42.2 μmol/L, respectively, showing a clear therapeutic index.

***Combination therapy.*** To evaluate the effect of combining benzethonium chloride with traditional head and neck cancer therapeutic agents, FaDu cells were simultaneously incubated with a low dose of either cisplatin (1.9 μmol/L), 5-fluorouracil (5 μmol/L), or γ-radiation (10 Gy) and with increasing doses of benzethonium chloride (Fig. 2B). FaDu cells were also simultaneously incubated with a low dose of benzethonium chloride (2 μmol/L) and with increasing doses of either cisplatin or 5-fluorouracil (Fig. 2C and D). It is apparent from these dose-response curves that benzethonium chloride does not interfere with cisplatin, 5-fluorouracil, or γ-radiation. The interaction is possibly additive, particularly at lower doses of cisplatin or 5-fluorouracil.

***Benzethonium chloride induces apoptosis and caspase activation.*** In an effort to determine the mode of cell death induced by benzethonium chloride, cell cycle and apoptosis analyses were done. For FaDu cells treated with 9 μmol/L benzethonium chloride (the dose required to reduce cell viability by 75% after 48 hours, or $ED_{75}$), flow cytometric DNA content analyses showed no change in $G_1$-$G_0$, S, or $G_2$-M phase (or aneuploid) populations at 12, 24, or 48 hours (data not shown). However, Hoechst 33342 staining revealed nuclear condensation and blebbing, indicative of apoptosis, after 48 hours of treatment (Fig. 3A). Necrosis was not observed. Transmission electron microscopy was used to visualize the subcellular morphologic characteristics of apoptosis, such as nuclear condensation and membrane blebbing (Fig. 3B). Interestingly, autophagy of the mitochondria and rough endoplasmic reticulum swelling were also observed after 24 or 48 hours of benzethonium chloride treatment.

Benzethonium chloride–induced caspase activation was evaluated in FaDu cells treated for 12, 24, or 48 hours (Fig. 3C). Caspase-2, caspase-8, and caspase-9 activations were observed as early as 12 hours whereas caspase-3 activation was not detected until 48 hours.

***$\Delta\Psi_M$ depolarization and cytosolic $Ca^{2+}$ increase.*** To further investigate the role of the mitochondria and rough endoplasmic reticulum in benzethonium chloride–mediated cell death, $\Delta\Psi_M$ depolarization and cytosolic $Ca^{2+}$ increase (which may be due the endoplasmic reticulum or $Ca^{2+}$ membrane channels) were evaluated. FaDu cells treated (9 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). As treatment time increased, the proportion of cells with depolarized mitochondria increased (6.2% at 3 hours, 8.4% at 12 hours, and 19.9% at 24 hours, versus 2.6% at 24 hours with vehicle alone; Fig. 3D, *box A*). Moreover, increased cytosolic $Ca^{2+}$, indicated by the indo-1 AM 405/525 nm ratio, could be observed in cells with depolarized mitochondria. Loss of membrane integrity and cell death, indicated by propidium iodide uptake, also increased with incubation time (4.1% at 3 hours, 6.2% at 12 hours, and 11.4% at 24 hours, versus 1.7% at 24 hours with vehicle alone; Fig. 3D, *box C*). Thus, loss of $\Delta\Psi_M$ occurs before both cytosolic $Ca^{2+}$ increase and loss of membrane integrity/cell death in benzethonium chloride–treated cells. Notably, in benzethonium chloride–treated FaDu cells, $\Delta\Psi_M$ hyperpolarization could also be observed (a phenomenon

commonly observed during apoptosis; refs. 24, 25). The relative mean fluorescence readings in polarized cells after 3, 12, and 24 hours of treatment were 52.8, 64.6, and 99.2, respectively (37.8 at 24 hours with vehicle alone; Fig. 3D, *boxes B* and *D*).

***Elimination of tumor formation.*** To determine whether benzethonium chloride had any ability to reduce tumorigenicity, FaDu cells were treated with 9 μmol/L benzethonium chloride for 48 hours ($ED_{75}$) and then injected into the left gastrocnemius muscle of SCID mice ($2.5 \times 10^5$ per mouse; Fig. 4A). Such treated mice did not develop tumors even after 100 days, whereas mice injected with 0.1% DMSO-treated FaDu cells developed tumors after only 10 days. Thus, benzethonium chloride effectively eliminates the tumor-forming potential of FaDu cells.

***Growth delay in established xenograft tumors.*** Therapeutic experiments were conducted to determine whether benzethonium chloride could treat established FaDu tumors in SCID mice. Once the tumor-plus-leg diameter reached 7.25 mm, the mice were treated with benzethonium chloride (dissolved in PBS). The dosing regimen (5 mg/kg i.p. bolus × 5 days) has not been optimized for absorption, distribution, metabolism, or excretion, but a delay in tumor growth was nevertheless observed. One day after the five treatment doses, the tumor-plus-leg diameter between the PBS and benzethonium chloride–treated groups differed by >1 mm ($P < 0.05$; Fig. 4B). Benzethonium chloride–treated mice survived for 3.5 days more than PBS-treated mice, which was a statistically significant delay ($P < 0.05$; Fig. 4C). In comparison, a single i.p. dose of paclitaxel (30 mg/kg) increases the survival of FaDu tumor–bearing SCID mice (over controls) by 3.9 days (26). The comparable benefit to paclitaxel and the ability to completely eliminate tumor formation suggest that benzethonium chloride is a highly effective anticancer agent *in vivo*.

When combined with local tumor radiation therapy, benzethonium chloride seemed to have a modest additive effect. The tumor-plus-leg diameter between the PBS-plus-local radiation therapy and the benzethonium chloride-plus-local radiation therapy groups differed by ~1 mm ($P < 0.05$; Fig. 4B). The benzethonium chloride-plus-local radiation therapy group survived for an additional 5 days when compared with the PBS-plus-local radiation therapy group ($P < 0.05$; Fig. 4C).

**In vivo *safety.*** As stated earlier, the absorption, distribution, metabolism, or excretion properties of this compound have not been optimized, and yet a biological effect (i.e. therapeutic benefit) was still observed. The body weights of the treated mice were therefore measured to provide an overall indicator of benzethonium chloride safety and toxicity (Fig. 4D). The benzethonium chloride–treated mice experienced a transient decrease (~1 g) in weight compared with control mice,

**Table 1.** Potential anticancer compounds from an MTS screen using Prestwick Chemical Library and LOPAC1280 compounds

| Class | Drug | FaDu (%) | NIH 3T3 (%) |
|---|---|---|---|
| Antimicrobial | Alexidine dihydrochloride | 25.7 | 97.4 |
| | Benzethonium chloride | 30.1 | 99.8 |
| | Berberine chloride | 29.9 | 101.1 |
| | Dequalinium dichloride | 32.7 | 97.4 |
| | Methyl benzethonium chloride | 38.5 | 98.8 |
| Cytoskeleton and ECM | Fenbendazole | 44.5 | 101.6 |
| | Paclitaxel | 26.1 | 95.0 |
| Ion pump | Clofilium tosylate | 39.9 | 96.6 |
| | Digitoxigenin | 29.2 | 100.6 |
| | Digoxin | 7.9 | 103.0 |
| | Lanatoside C | 9.5 | 100.9 |
| | Ouabain | 14.4 | 104.0 |
| | Proscillaridin A | 10.8 | 116.2 |
| | Strophantine octahydrate | 8.8 | 97.8 |
| Neurotransmission | 3-(1*H*-imidazol-4-yl)propyl... | 21.5 | 100.3 |
| | Indatraline hydrochloride | −2.9 | 100.6 |
| | Ivermectin | −4.1 | 102.1 |
| | Loperamide hydrochloride | 11.1 | 97.8 |
| | LY-367265 | 41.2 | 100.0 |
| | Zaprinast | 43.5 | 97.3 |
| Phosphorylation | 7-Cyclopentyl... | 15.9 | 97.8 |
| | Cantharidin | 22.7 | 108.4 |
| | LY-294002 hydrochloride | 43.6 | 97.7 |
| | ML-7 | 20.4 | 95.9 |
| Other | | | |
| Apoptosis | Sanguinarine | 43.0 | 107.6 |
| DNA metabolism | Azathioprine | 49.2 | 100.6 |
| Multidrug resistance | Ketoconazole | 48.9 | 100.5 |
| Photosensitizer | Verteporfin | −0.2 | 101.8 |

NOTE: These are compounds that decreased FaDu cell viability by ≥50% and NIH 3T3 cell viability by ≤5% after 48 hours.
Abbreviations: ECM, extracellular matrix; 3-(1*H*-imidazol-4-yl)propyl, 3-(1*H*-imidazol-4-yl)propyl di(pfluorophenyl) methyl ether hydrochloride; 7-cyclopentyl, 7-cyclopentyl-5-(4-phenoxy)phenyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-ylamine.

**Table 2.** Potential anticancer compounds from an MTS screen using Prestwick Chemical Library and LOPAC1280 compounds

| Drug | FaDu (μmol/L) | C666-1 (μmol/L) | NIH 3T3 (μmol/L) | GM05757 (μmol/L) |
|---|---|---|---|---|
| Alexidine dihydrochloride | 1.8 | 2.6 | 19.6 | 8.8 |
| Benzethonium chloride | 3.8 | 5.3 | 42.2 | 17.0 |
| Berberine chloride | 6.6 | 33.2 | >100 | 69.3 |
| Dequalinium dichloride | 4.0 | 2.8 | >100 | 10.2 |
| Methyl benzethonium chloride | 3.6 | 4.9 | 41.5 | 16.0 |

NOTE: The $ED_{50}$ for the antimicrobial compound subset in two cancer (FaDu and C666-1) and two normal (NIH 3T3 and GM05757) cell lines.

although weight in this latter group of untreated mice continued to decline, likely due to progressive tumor growth.

Histologic and biochemical analyses were conducted on benzethonium chloride–treated mice for a more detailed evaluation of the safety and toxicity profile. No toxicity could be observed via histologic evaluation of the major organs, including brain, heart, intestine, kidney, liver, lung, pancreas, spleen, and thymus (data not shown). Biochemical analyses revealed slight perturbations in albumin, globulin, glucose, and urea levels, but no other changes were observed (Table 3). A therapeutic index thus seems to exist for benzethonium chloride in cancer treatment.

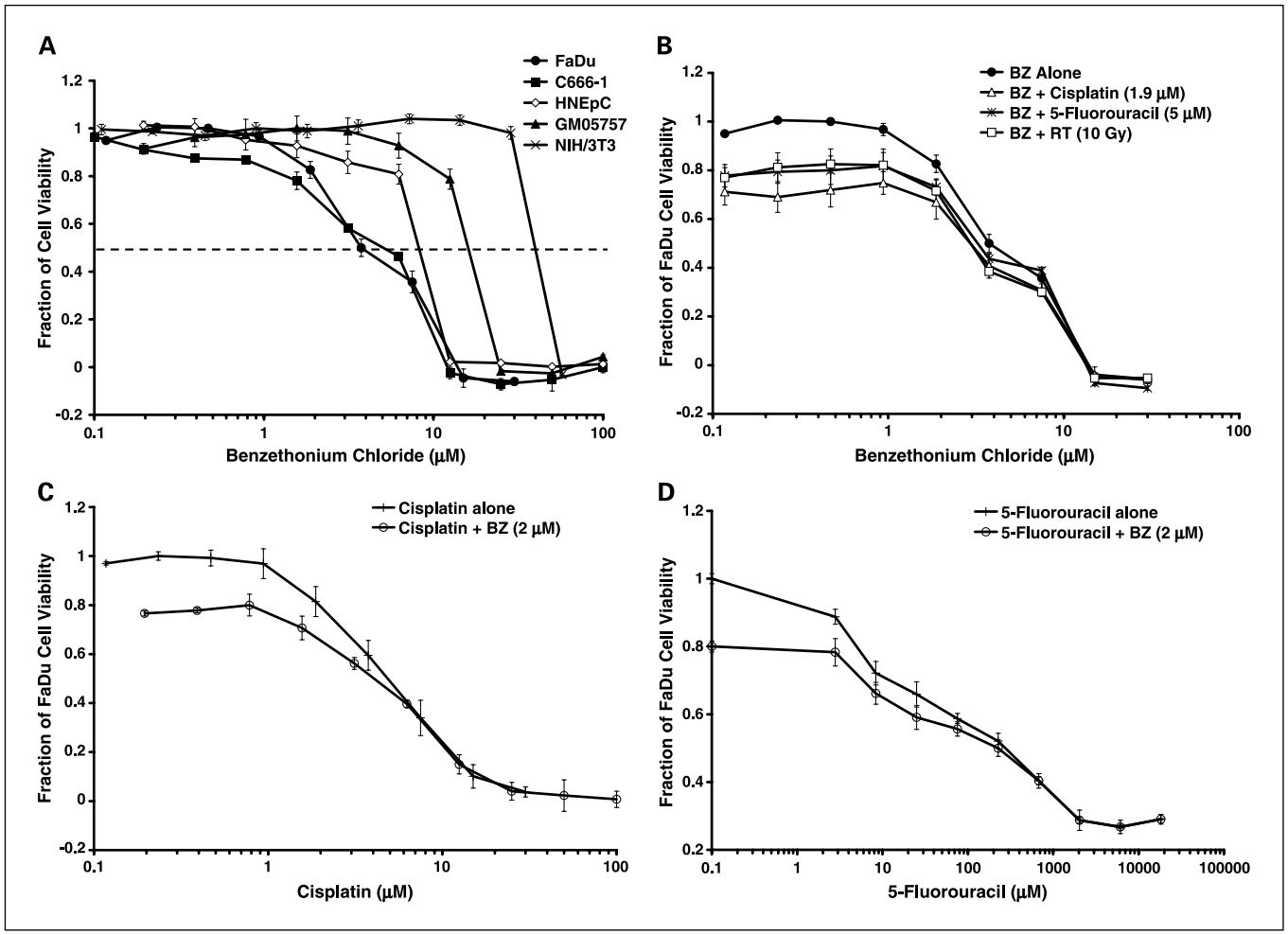


**Fig. 2.** Characterization of benzethonium chloride as a novel anticancer agent. *A,* dose-response (cell viability) curves were determined for benzethonium chloride in two cancer (FaDu and C666-1) and three normal (NIH 3T3, GM05757, and HNEpC) cell lines. Dotted line, 50% viability. *B,* the effect of combining benzethonium chloride (*BZ*) with a sensitizing dose of cisplatin, 5-fluorouracil, or radiation (*RT*) on the viability of FaDu cells. *C,* the effect of combining cisplatin with a sensitizing dose of benzethonium chloride in FaDu cells. *D,* the effect of combining 5-fluorouracil with a sensitizing dose of benzethonium chloride in FaDu cells. In (*B*), (*C*), or (*D*), benzethonium chloride did not interfere with commonly used anticancer agents. The MTS assays were done 48 hours after drug treatment. Each experiment was done thrice independently. Points, mean; bars, SE.

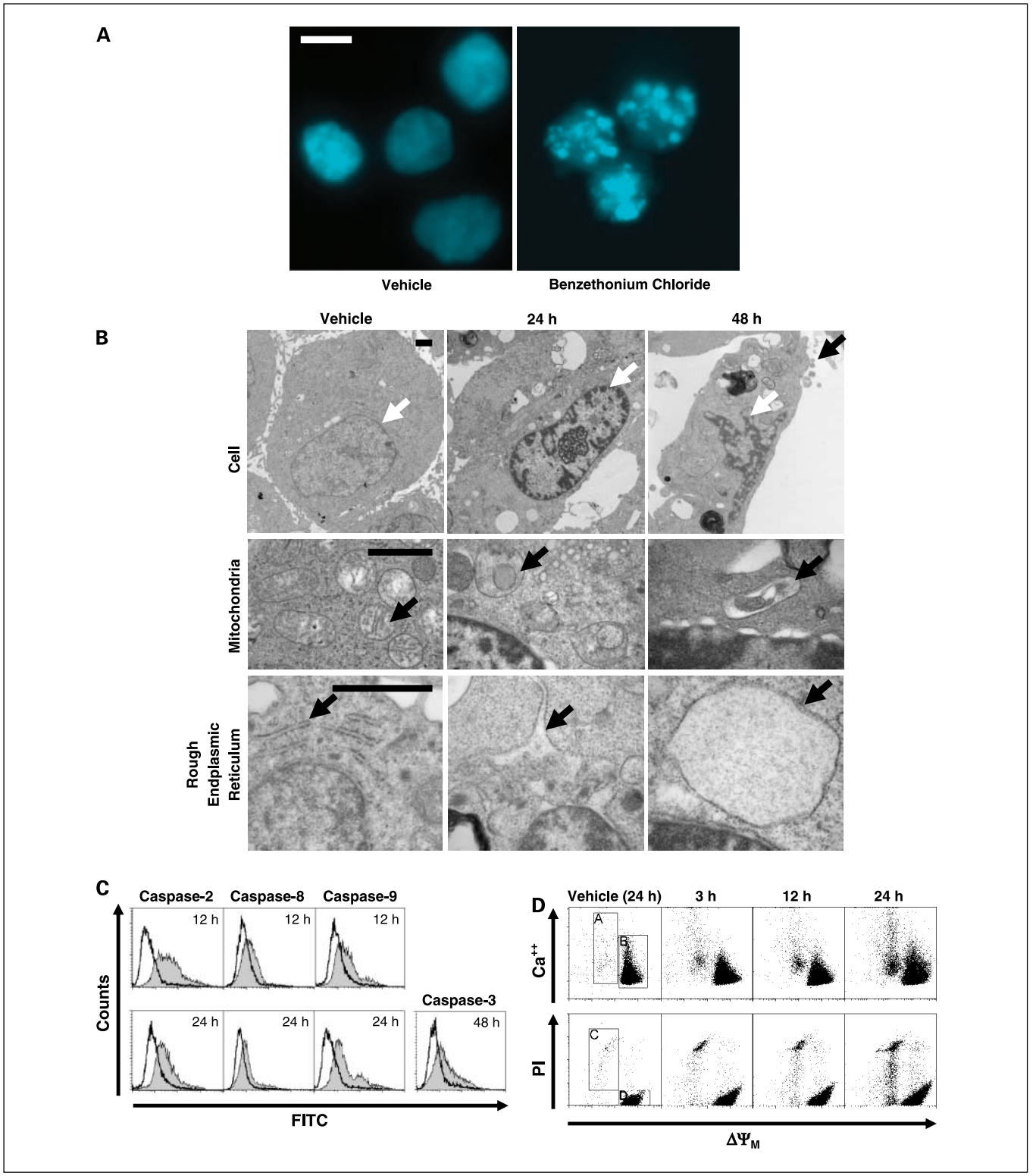


**Fig. 3.** Benzethonium chloride induces cancer cell apoptosis. *A,* condensed areas of chromatin and nuclear blebbing were observed in benzethonium chloride – treated (9 μmol/L) FaDu cells after 48 hours. Hoechst 33342 was used for nuclear staining. Bar, 10 μm. *B,* transmission electron microscopy was used to visualize the mode of cell death induced by benzethonium chloride. Nuclear condensation (*top*; *white arrow*) and membrane blebbing (*top*; *black arrow*), indicative of apoptosis, were visualized. Autophagy of the mitochondria (*middle*; *arrow*) and progressive rough endoplasmic reticulum swelling (*bottom*; *arrow*) were also visible. Bar, 1 μm. *C,* using a fluorescent caspase inhibitor peptide-based assay (CaspGLOW), benzethonium chloride – induced caspase-2, caspase-8, and caspase-9 activation could be observed after 12 hours. Caspase-3 activation was detected at 48 hours. White, vehicle-treated cells; gray, benzethonium chloride – treated cells. *D,* treated cells were stained with $DiIC_1(5)$ ($\Delta\Psi_M$), indo-1 AM (cytosolic $Ca^{2+}$), and propidium iodide (*PI*; cell viability). Example gatings for quantification are shown. Box A is gated on $\Delta\Psi_M$ depolarized cells; Box B is gated on $\Delta\Psi_M$ polarized cells; Box C is gated on cells that were $\Delta\Psi_M$ depolarized and dead; Box D is 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 benzethonium chloride – treated cells. In all cases, 9 μmol/L benzethonium chloride ($ED_{75}$) was used, and each experiment was done thrice independently. Vehicle represents 0.1% DMSO.

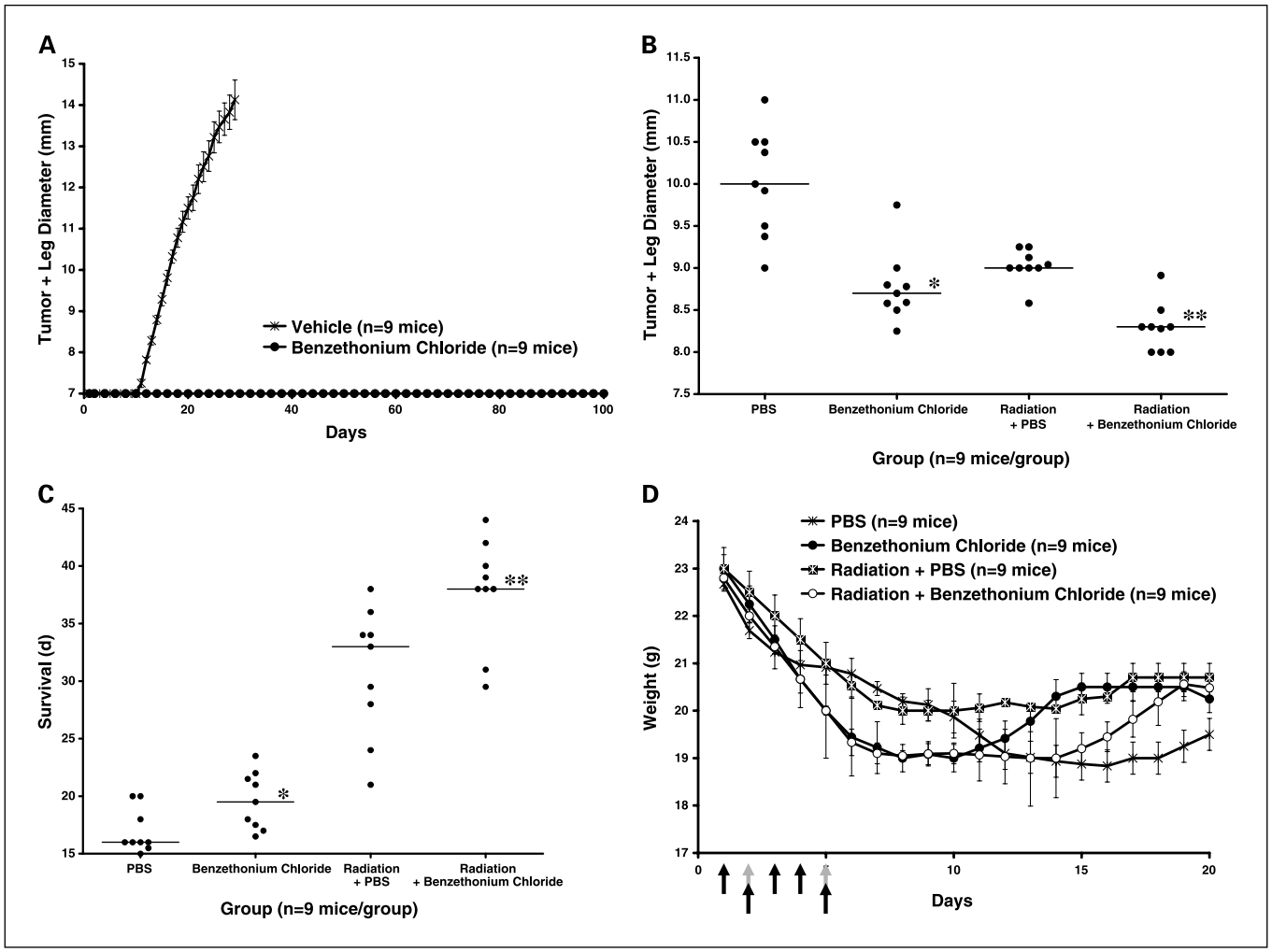


**Fig. 4.** *In vivo* efficacy of benzethonium chloride. *A,* FaDu cells, treated with 0.1% DMSO (vehicle) or benzethonium chloride for 48 hours, were injected into the left gastrocnemius muscle of SCID mice ($2.5 \times 10^5$ per mouse). After 100 days, benzethonium chloride – treated cells could not form tumors. *B,* FaDu tumors were established in SCID mice. When tumor-plus-leg diameter grew to 7.25 mm, the mice were treated with PBS or benzethonium chloride (dissolved in PBS). Injections were administered once a day for 5 days (5 mg/kg i.p. bolus, five total injections per mouse) and 4-Gy local tumor radiation therapy was delivered on days 2 and 5 before i.p. injections. Tumor-plus-leg diameter was measured 24 hours after the last injection. Benzethonium chloride delayed tumor growth. The median for each group is shown. *, **, $P < 0.05$, significant difference when compared with the PBS-treated group and radiation-plus-PBS – treated group, respectively. *C,* the mouse survival times for the mice treated in (*B*) were also measured. Benzethonium chloride increased survival times. The median for each group is shown. *, **, $P < 0.05$, significant difference when compared with the PBS-treated group and radiation-plus-PBS – treated group, respectively. *D,* FaDu tumors were established and treated as in (*B*). Benzethonium chloride induced a transient decrease in mouse body weight. Black arrow, benzethonium chloride injection (5 mg/kg i.p. bolus); gray arrow, local tumor radiation therapy (4 Gy). Each experiment was done thrice, with a total of three mice in each group per experiment.

***Anticancer efficacy in other cancer cell lines.*** The NCI DTP evaluated benzethonium chloride on the NCI DTP 60 cancer cell lines to determine the efficacy of this compound in other human malignancies (Table 4). For the majority of cancer cell lines, the $GI_{50}$ and TGI concentrations were achievable at low doses. The GM05757 $ED_{50}$ for benzethonium chloride was 17.0 μmol/L and can be used for an approximate comparison. Therefore, as indicated in Table 4, benzethonium chloride should be highly effective in almost all leukemia, several lung and colon cancer cell lines, as well as in melanoma and ovarian cancers. COMPARE (i.e., NCI computerized analyses of relative cell line sensitivity) analyses of these results revealed no highly related compounds and no related compounds with known function (data not shown).

***Chemical analogs.*** Benzethonium chloride represents a potential anticancer compound scaffold or "lead," and might therefore have more efficacious analogues or chemical derivatives. As a preliminary assay, commercially available benzethonium analogues were evaluated on FaDu cells (Fig. 5A and B). Only analogues 1 and 5 reduced FaDu cell viability (Fig. 5B). For analogue 1 (methyl benzethonium chloride), the FaDu $ED_{50}$ was 3.6 μmol/L, versus 16.0 μmol/L for GM05757 (Table 2; Fig. 5C). For analogue 5, the FaDu $ED_{50}$ was 36.0 μmol/L (Fig. 5B), versus >100.0 μmol/L for GM05757 (Fig. 5C), indicating that this analogue retained its cancer-specific properties but would need to be used at a much higher dose.

## Discussion

In this current study, we did a rapid, cell-based, and phenotype-driven small-molecule screen of the Prestwick

Chemical Library (1,120 compounds) and LOPAC1280 (1,280 compounds) to identify novel potential head and neck cancer–specific agents. Benzethonium chloride was revealed as the novel hit compound after $ED_{50}$ values were determined for two cancer (FaDu and C666-1) and two normal (NIH 3T3 and GM05757) cell lines. Further experiments revealed that benzethonium chloride induced cell death via apoptosis, activating caspase-2, caspase-8, caspase-9, and caspase-3 and inducing loss of $\Delta\Psi_M$. This compound ablated the tumor-forming ability of FaDu cells *in vivo*, delayed the growth of xenograft tumors, and displayed *in vivo* additivity with local radiation therapy. Evaluation of benzethonium chloride on a panel of 60 human cancer cell lines representing various tissue origins revealed a broad range of antitumor efficacy. This study reports a new high-throughput screening and is the first known published report of the identification and characterization of benzethonium chloride as a novel cancer-specific agent.

Our high-throughput screening technique employed the use of a tetrazolium-based (MTS) assay using FaDu and NIH 3T3 cells. The MTS dynamic range may be smaller than other assays, such as those that use sulforhodamine B, Alamar Blue, or clonogenic-based methods. However, the speed and low manipulation requirements of this assay reduce error, rendering it useful for high-throughput screening. In addition, larger dynamic ranges are not required for the identification of hits by our criteria (≥50% cell viability reduction in FaDu and ≤5% cell viability reduction in NIH 3T3). Notably, the cells were treated with compounds for 48 hours. The rapidly proliferating nature of the cells selected for screening and counter-screening (~20.5 hours doubling time), as well as the criteria set for potential hits, reduced bias from cytostatic or cell cycle–arresting agents. FaDu cells also represent a clinically relevant model line for the study of head and neck cancers (27, 28). The compound libraries were screened at ~5 μmol/L, although future screens and counter-screens might use different concentrations.

The high-throughput screening employed in this study identified several known anticancer agents in the compound libraries. Paclitaxel and ketoconazole are currently in clinical use; the latter typically administered as third-line treatment for prostate cancer (29). Verteporfin, a known photosensitizer, has also been evaluated in the clinic (30). Cantharidin is the active ingredient in *Mylabris* (blister beetle) and is used in traditional Chinese medicine to treat cancer (31). Cantharidin and sanguinarine have been recently shown to be cancer-specific cytotoxic agents (31, 32). The ion channel class of drugs identified by the high-throughput screening is composed mainly of cardiac glycosides (digitoxigenin, digoxin, lanatoside C, ouabain, proscillaridin A, and strophantine octahydrate). The cancer-specific action of these compounds has previously been reported, and chemically modified derivatives lacking cardiac activity have been identified (33, 34). Similarly, high-throughput screening hits from the neurotransmitters have been investigated as cancer cell cytotoxics (35–37). Because our high-throughput screening successfully identified known cancer-specific therapeutics in a rapid, cell-based manner, this type of forward chemical biology approach can be further used for large-scale screening of other compound libraries.

The *in vitro* efficacy and cancer selectivity of benzethonium chloride in pharyngeal cancers were comparable to those observed for paclitaxel. Although benzethonium chloride was more toxic to HNEpC cells than to GM05757 cells, HNEpC "normal" cells do not grow well *in vitro* and are

**Table 3.** Biochemical serum analyses for mice treated with either PBS or benzethonium chloride

| Biochemistry | PBS | Benzethonium chloride |
|---|---|---|
| | Average (range) | Average (range) |
| Total protein (g/L) | 53 (51-54) | 52 (48-56) |
| Albumin (g/L) | 36 (35-37) | 32 (31-33) |
| Globulin (g/L) | 16 (15-17) | 20 (16-23) |
| Albumin/globulin ratio | 2.3 (2.2-2.4) | 1.7 (1.4-2.0) |
| ALP (units/L) | 183 (171-200) | 108 (94-140) |
| ALT (units/L) | 110 (60-180) | 107 (57-170) |
| AST (units/L) | 600 (340-775) | 577 (354-1,330) |
| CK (units/L) | 10,230 (6,490-17,610) | 9,688 (4,654-26,390) |
| Amylase (units/L) | 2,573 (2,230-3,000) | 1,901 (1,677-2,140) |
| Glucose (mmol/L) | 8.9 (7.8-10.0) | 5.5 (3.7-9.8) |
| Urea (mmol/L) | 6.3 (5.6-6.6) | 4.3 (3.3-5.3) |
| Creatinine (μmol/L) | 18 (14-21) | 14 (12-20) |
| Sodium (mmol/L) | 146 (144-149) | 151 (148-152) |
| Potassium (mmol/L) | 8.8 (8.6-9.0) | 8.8 (8.5-9.0) |
| $Na^+/K^-$ ratio | 16 (16-17) | 16 (15-17) |
| Chloride (mmol/L) | 110 (107-112) | 112 (109-113) |
| Calcium (mmol/L) | 2.53 (2.46-2.64) | 2.59 (2.51-2.65) |
| Bicarbonate (mmol/L) | 12 (11-12) | 14 (12-16) |

NOTE: FaDu tumors were established in the left gastrocnemius muscle of SCID mice. When tumor-plus-leg diameter grew to 7.25 mm, the mice were treated with PBS or benzethonium chloride (5 mg/kg i.p. bolus × 5 days). One day after the last injection, serum was obtained for biochemical analyses (six mice per group). The underlined values indicate the only values that seem to be mildly abnormal.
Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase.

**Table 4.** NCI/NIH DTP human tumor cell line screen

| Cell panel | Cell line | $GI_{50}$ (μmol/L) | TGI (μmol/L) | $LC_{50}$ (μmol/L) |
|---|---|---|---|---|
| Leukemia | CCRF-CEM | 2.0 | 6.3 | 25.1 |
| | HL-60(TB) | 0.8 | 6.3 | 100.0 |
| | K-562 | 2.0 | 15.8 | 100.0 |
| | MOLT-4 | 2.0 | 6.3 | 79.4 |
| | RPMI-8226 | 1.3 | 3.2 | 100.0 |
| | SR | 2.0 | 4.0 | 100.0 |
| Non–small-cell lung | A549/ATCC | 7.9 | 31.6 | 100.0 |
| | EKVX | 4.0 | 15.8 | 100.0 |
| | HOP-18 | 2.5 | 7.9 | 25.1 |
| | HOP-62 | 10.0 | 31.6 | 100.0 |
| | HOP-92 | 2.0 | 4.0 | 63.1 |
| | NCI-H226 | 5.0 | 15.8 | 100.0 |
| | NCI-H23 | 1.6 | 6.3 | 79.4 |
| | NCI-H322M | 10.0 | 25.1 | 63.1 |
| | NCI-H460 | 3.2 | 12.6 | 100.0 |
| | NCI-H522 | 1.6 | 5.0 | 100.0 |
| | LXFL 529 | 1.3 | 2.5 | N/A |
| Small-cell lung | DMS 114 | 1.3 | 3.2 | 31.6 |
| | DMS 273 | 1.6 | 2.5 | 5.0 |
| Colon | COLO 205 | 1.6 | 2.5 | 5.0 |
| | DLD-1 | 4.0 | 20.0 | 100.0 |
| | HCC-2998 | 1.6 | 4.0 | 25.1 |
| | HCT-116 | 1.6 | 4.0 | 7.9 |
| | HCT-15 | 20.0 | 50.1 | 100.0 |
| | HT29 | 1.6 | 3.2 | 6.3 |
| | KM12 | 1.6 | 3.2 | N/A |
| | KM20L2 | 4.0 | 20.0 | 79.4 |
| | SW-620 | 2.5 | 10.0 | 100.0 |
| Central nervous system | SF-268 | 2.0 | 5.0 | 100.0 |
| | SF-295 | 10.0 | 31.6 | 79.4 |
| | SF-539 | 2.5 | 6.3 | 100.0 |

(*Continued on the following page*)

only viable for ~5.4 doubling times. Benzethonium chloride did reduce FaDu and C666-1 cancer cell viability at even lower concentrations and was safe when used *in vivo*. Thus, a therapeutic window and cancer specificity exist with this compound.

FaDu cells represent a highly aggressive cell line *in vivo*, forming tumors after only 10 days with the injection of $2.5 \times 10^5$ cells and increasing tumor-plus-leg diameter by ~0.45 mm/d. *In vivo*, five i.p. doses of benzethonium chloride at 5 mg/kg extended mouse survival by 3.5 days, with the largest difference in mean tumor-plus-leg diameter between treated and control mice being >1 mm. Previous studies have found that a single i.p. dose of paclitaxel (30 mg/kg) increases FaDu tumor–bearing SCID mouse survival by 3.9 days (26), which is comparable to the benefit achieved with benzethonium chloride. Paclitaxel is currently used in head and neck cancer clinics (38, 39). Benzethonium chloride could also completely ablate the tumor-forming ability of FaDu cells, indicating that improving drug distribution would render this compound highly effective. Further studies on the absorption, distribution, metabolism, or excretion of benzethonium chloride will enable the optimization of the dosing regimen and route of administration.

Head and neck cancers are treated with traditional therapeutic modalities, including surgery, radiation, cisplatin, and/or 5-fluorouracil (40). Benzethonium chloride combined additively with radiation therapy *in vivo*, and would likely interact similarly with cisplatin or 5-fluorouracil. Although the benzethonium chloride-radiation therapy combination was evaluated *in vivo*, clonogenic-based assays will be required to fully elucidate the efficacy of this combination *in vitro*. Further elucidation of the mechanism of action of benzethonium chloride would also be required to rationally select therapeutics for combination treatment. For example, benzethonium chloride activated caspase-8 and induced apoptosis in FaDu cells. This cell line possesses a homozygous deletion in the death receptor *DR4* gene, but not in *KILLER/DR5*, rendering it resistant to TNF-related apoptosis-inducing ligand therapy (41). Thus, a benzethonium chloride/TNF-related apoptosis-inducing ligand combination might provide significant therapeutic opportunities.

Benzethonium chloride effectively inhibited the growth of a large number of cancer cell lines from the NCI/NIH DTP panel at concentrations lower than the $ED_{50}$ for GM05757 (~17.0 μmol/L). Comparisons between the two assays, however, need to be interpreted with caution. The MTS $ED_{50}$ values were calculated relative to both cells treated with 0.1% DMSO (negative control) and cells treated with 166.6 μmol/L cisplatin (positive control) for 48 hours, representing the dynamic range of the assay. The NCI/NIH DTP used a sulforhodamine B assay and calculated $GI_{50}$, TGI, and $LC_{50}$ values relative to untreated cells before the 48-hour treatment incubation.

**Table 4.** NCI/NIH DTP human tumor cell line screen (Cont'd)

| Cell panel | Cell line | $GI_{50}$ (μmol/L) | TGI (μmol/L) | $LC_{50}$ (μmol/L) |
|---|---|---|---|---|
| | SNB-19 | 3.2 | 12.6 | 100.0 |
| | SNB-75 | 2.5 | 5.0 | 31.6 |
| | SNB-78 | 1.6 | 4.0 | 100.0 |
| | U251 | 1.6 | 3.2 | 100.0 |
| | XF498 | 0.4 | 1.6 | N/A |
| Melanoma | LOX IMVI | 1.6 | 2.5 | N/A |
| | MALME-3M | 1.6 | 4.0 | 7.9 |
| | M14 | 2.0 | 5.0 | 100.0 |
| | M19-MEL | 1.6 | 3.2 | N/A |
| | SK-MEL-2 | 2.0 | 5.0 | 100.0 |
| | SK-MEL-28 | 2.0 | 4.0 | 7.9 |
| | SK-MEL-5 | 1.3 | 2.5 | N/A |
| | UACC-257 | 1.6 | 3.2 | 6.3 |
| | UACC-62 | 1.3 | 2.5 | 5.0 |
| Ovarian | IGROV1 | 1.6 | 3.2 | N/A |
| | OVCAR-3 | 1.6 | 6.3 | 100.0 |
| | OVCAR-4 | 2.0 | 10.0 | 100.0 |
| | OVCAR-5 | 6.3 | 20.0 | 50.1 |
| | OVCAR-8 | 3.2 | 12.6 | 100.0 |
| | SK-OV-3 | 7.9 | 25.1 | 100.0 |
| Renal | 786-0 | 5.0 | 25.1 | 100.0 |
| | A498 | 15.8 | 50.1 | 100.0 |
| | ACHN | 12.6 | 25.1 | 63.1 |
| | CAKI-1 | 10.0 | 31.6 | 100.0 |
| | RXF 393 | 3.2 | 10.0 | 100.0 |
| | RXF-631 | 12.6 | 31.6 | 100.0 |
| | SN12C | 3.2 | 12.6 | 100.0 |
| | TK-10 | 4.0 | 15.8 | 79.4 |
| | UO-31 | 20.0 | 39.8 | 79.4 |

NOTE: Cells were seeded onto 96-well plates (day 1), incubated for 24 hours, and then treated with benzethonium chloride (day 2). After 48 hours, relative protein amounts were measured (day 4). For comparison, the GM05757 $ED_{50}$ for benzethonium chloride was 17.0 μmol/L. The experiment was done by the NCI/NIH DTP, Division of Cancer Treatment and Diagnosis.

COMPARE analysis of the NCI/NIH DTP benzethonium chloride $GI_{50}$, TGI, and $LC_{50}$ values did not identify any highly related compounds, or any related compounds with known function, confirming that this hit seems to be a novel anticancer therapeutic.

Our data suggest that benzethonium chloride might possibly induce the following chain of events: First, benzethonium chloride or a target of benzethonium chloride dysregulates the mitochondria or rough endoplasmic reticulum. This induces initiator caspase activation within 12 hours. $\Delta\Psi_M$ hyperpolarization is followed by depolarization. By 24 hours, loss in cell membrane integrity begins and cytosolic $Ca^{2+}$ increases. The nucleus begins to condense; rough endoplasmic reticulum swelling continues, and this structure is now visibly "ballooned." The depolarized mitochondria are no longer functional, hence autophagy commences. Finally, at 48 hours, membrane blebbing occurs. This chain of events suggests that damage to the mitochondria or rough endoplasmic reticulum might possibly mediate the benzethonium chloride mechanism for cancer specificity. Benzethonium is cationic, and such intracellular cationic molecules are reported to induce swelling of the rough endoplasmic reticulum (42, 43). Other novel anticancer agents also target this organelle, such as brefeldin A (44). Brefeldin A was present in our high-throughput screening, reducing FaDu cell viability to 0.9% and reducing NIH 3T3 cell viability to 85.0% (data not shown). This compound has been reported to induce endoplasmic reticulum swelling (44), although to a lesser extent than observed in this study with benzethonium chloride. If benzethonium chloride indeed initializes apoptosis via the rough endoplasmic reticulum, then caspase-8 might be the upstream initiator caspase, as it can be activated at this organelle (45). Furthermore, the $Ca^{2+}$/propidium iodide/$\Delta\Psi_M$ flow cytometry data (Fig. 3D) might be explained by the fact that functioning mitochondria might buffer any $Ca^{2+}$ released by early endoplasmic reticulum damage (46). By 24 hours, further $Ca^{2+}$ release or caspase activation induces $\Delta\Psi_M$ depolarization, discharging buffered $Ca^{2+}$ into the cytosol.

Future studies will attempt to elucidate the upstream mechanisms of action of benzethonium chloride. Benzethonium chloride, a quaternary ammonium compound, is a natural product that can be found in commercially available grapefruit seed extracts (47). This compound has been used as an antiseptic and as a preservative for ketamine (Ketalar), the Anthrax vaccine, thrombin, orphenadrine (Norflex), and butorphanol (Stadol). Benzethonium chloride damages phospholipid membranes and may possibly affect $\alpha_7$ and $\alpha_4\beta_2$ neuronal nicotinic acetylcholine receptors (48, 49). Alterations of choline phospholipid metabolism in tumor progression have been reported (50), thereby possibly accounting for the cancer specificity of benzethonium

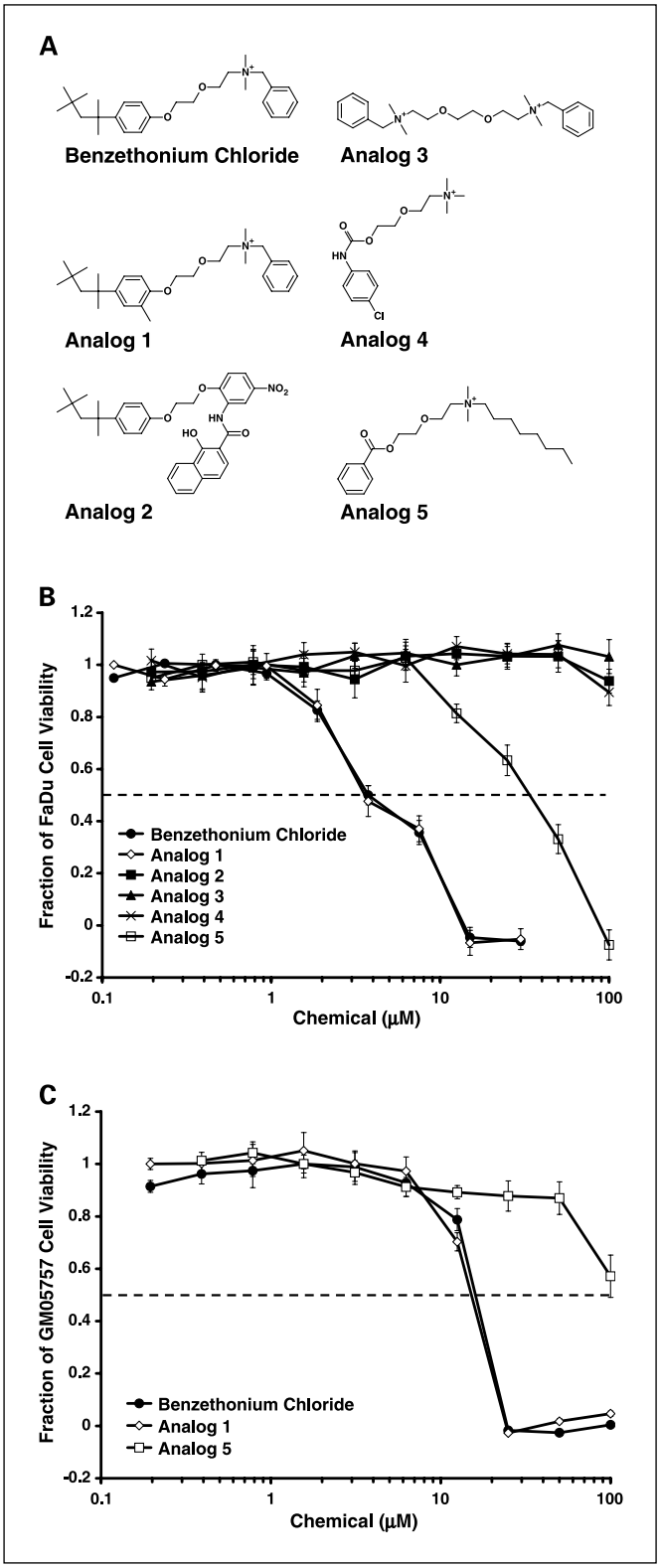


**Fig. 5.** Commercially available benzethonium chloride analogues. *A,* structures of benzethonium chloride, methyl benzethonium chloride (analogue 1), analogue 2, analogue 3, analogue 4, and analogue 5. *B,* dose-response (cell viability) curves for benzethonium chloride and analogues 1 to 5 in FaDu cells after 48 hours. *C,* dose-response (cell viability) curves for benzethonium chloride and analogues 1 to 5 in GM05757 cells after 48 hours. Analogue 1 has a similar dose-response profile to benzethonium chloride in both cell lines. Analogue 5 retains preferential cancer toxicity but needs to be used at higher concentrations. Experiments were done thrice. Points, mean; bars, SE.

chloride. Other potential benzethonium chloride targets might be identified via covalent labeling, peptide sequencing, and subsequent cloning. These techniques have been used to identify protein targets of many natural products, such as FK506, cyclosporin, and rapamycin (13, 14). Other target identification methods may use a yeast "three-hybrid" system, DNA microarrays, or proteomics (1). Pattern-matching algorithms may determine whether benzethonium chloride treatment has the same molecular signature as a particular gene deletion.

Benzethonium analogues 1 and 5 maintained cancer-specific properties. Analogue 1, methyl benzethonium chloride, had a dose-response profile similar to that of benzethonium chloride. The similar activity of analogue 1 to benzethonium chloride indicates that *ortho*-substitutions on the phenoxy aryl ring do not diminish the anticancer activity of the compound. This hypothesis would require further testing via the substitution of bulkier groups at this position (e.g., a *tert*-butyl group). In addition, the efficacy of introducing *meta*-substitutions should also be determined. The inability of analogue 2 to reduce the viability of FaDu cells is most likely due to its noncationic nature. Benzethonium chloride and analogue 1 are, in contrast, cationic. The charged species requirement for the activity of this compound is indicative of a stable ion-pair interaction between the molecule and its target. Analogue 2 also has a more sterically hindered structure, with more hydrogen-bond donors and acceptors. Analogue 3, albeit doubly cationic, is not an active analogue, likely due to the symmetrical and dimerlike nature of the molecule. Thus, $C_2$-symmetrical molecules are likely not active analogues. Analogue 4, although cationic, is lacking a benzyl side chain on the nitrogen in comparison with benzethonium chloride, which could be responsible for its loss of activity; however, the amide group may not be beneficial for activity either, as it is also present in the inactive analogue 2. Higher doses of analogue 5 were required to produce cancer cytotoxicity but the therapeutic index (relative to GM05757 cells) seemed to be similar to that of benzethonium chloride. This analogue is missing the benzyl group on the nitrogen, indicating that a longer alkyl chain can restore activity to the molecule (in place of an aryl ring). The optimal length of the alkyl chain to produce higher potency can be investigated by testing analogues with different carbon chain lengths. Further studies are needed on a wider range of analogues with different substituents at various positions to draw solid conclusions about the structure-activity relationships of these molecules.

In conclusion, we have designed and did a rapid, cell-based, and phenotype-driven small-molecule screen for anticancer agents. Benzethonium chloride was characterized as a novel anticancer compound possessing both *in vitro* and *in vivo* efficacy, and definitely warrants further evaluation.

## Acknowledgments

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