Targeting Ion Channel Dysregulation in Colorectal Cancer: Identification of Clofilium as a Potent Inhibitor of Tumor Growth and Metastatic Potential

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Abstract

Colorectal cancer (CRC) accounts for the second-highest number of cancer-related deaths in the United States. Targeting colon cancer channelopathies that drive proliferation and metastasis has recently emerged as a new strategy towards developing ionoceutical therapeutics. We used existing RNA seq data to compare non-cancerous colon tissue to both primary CRC samples and the colon cancer cell line COLO 205 and found many shared upregulated ion channel genes. We produced several stable cell lines with fluorescent reporters to enable our screen of 19 compounds that target the products of those genes. Nine compounds were found to reduce COLO 205 proliferation, most showing cytostatic effects. FUCCI cell cycle analysis revealed that the cytostatic concentrations increased the proportions of cells in the G0/G1 phase. COLO 205 spheroids exhibited differential responses to the compounds as compared to 2D but all compounds significantly reduced spheroid migration, invasion or both. Analysis of cytotoxic effects in three non-cancerous cell types identified clofilium (IC50 3.314 μM) as the leading candidate. Western blot analysis showed that clofilium decreased key cancer growth pathways and induced a strong upregulation of NDRG1 activity. A xenograft COLO 205 mouse model showed that clofilium treatment significantly reduced tumor volume without signs of overt toxicity.

1. Introduction

Since the early 1990s, there has been an alarming increase in colorectal cancer (CRC) rates in adults ages 20-49 in the United States [1,2]. CRC is the third most common cancer diagnosed globally and the second most deadly [3]. The majority of deaths attributed to CRC are when it metastasizes, with the 5 year survival rate being less than 20% [4]. Treatment for metastatic unresectable CRC is chemotherapy, immunotherapy, or both [4]. Due to the poor prognosis in long-term survival for metastatic CRC, more effective systemic therapies are needed.

A crucial aspect of cancer is that it is a disorder of the developmental signaling that enables cells to cooperate toward large-scale morphogenesis and maintenance of tissue and organ structures [5-8]. Coordination of growth and form in vivo is regulated in part by bioelectrical signaling [9-11], with all cells (not only neurons) regulating their bioelectric state via ion channels and pumps, and propagating those states across the tissue network via electrical synapses known as gap junctions [12,13]. Thus, bioelectrical changes due to ion channel and gap junction dysregulation have been receiving increased attention in cancer research [8,14-23]. Prior work in non-mammalian in vivo models showed that dysregulation of ionic signaling can induce a metastatic melanoma-like phenotype [24,25]; moreover, human oncogenes induce depolarization as an early component of tumorigenesis, which can be suppressed by maintaining a correct bioelectrical state [26-29].

Disruption of endogenous bioelectrical cues intersects with other drivers of cancer. For example, it has been proposed that cancer can arise from cells that are subjected to increased stress, either from inflammation or toxic insult [30]. This stress can then cause the cells in the affected area to lose gap junctional intercellular communication (GJIC) to the unaffected cells nearby, thus electrically isolating themselves and thereby depolarizing their resting membrane potential (RMP) [31,32]. The depolarized RMP drives hyperproliferation of the stressed cells and this coupled with the damaging effects of oxidative stress on their DNA can drive mutations and the development of cancer [32-34].

Strategies employing bioelectric modulation to control cancer growth focus on re-establishing GJIC [35,36], hyperpolarizing the membrane potential to levels that are no longer conducive to unchecked proliferation [27,37], or depolarizing the membrane potential even further resulting in preferential apoptosis of cancer cells [38]. To facilitate this exploration of bioelectrical controls on cancer, genetically encoded voltage indicators GEVIs [39-41], genetically encoded calcium indicators GECIs [42,43], dyes [44-46], GJIC monitoring devices [47], MEAs [48], dielectrophoresis [49], and automated patch-clamp [37,50] have been employed in the screening of ion or GJIC modulating compounds.

In this study we chose the COLO 205 colon cancer cell line since it was isolated from the ascites of a patient with metastatic adenocarcinoma [51] and also has a long history of

publication [52] and successful xenografts [53,54]. To determine the bioelectric differences between colon cancer cells and normal cells, we did voltage dye and Port-a-Patch semi-automated patch clamp system measurements on COLO 205 cells and normal epithelial colon cells under different concentrations of ions to see which ion had the most influence on each of their RMPs. We then performed a transcriptomic analysis on existing datasets to identify the ion channel-encoding genes that were most highly upregulated when compared to normal colon tissue and that were also shared between COLO 205 cells and primary CRC tumors. Using this list, we found ion modulating drugs that would either block or open those ion channels.

We chose to perform our initial screen of these ion modulating compounds using fluorescent reporters and biosensors since traditional screening assays like the MTT colorimetric assay can miss potentially therapeutic compounds due to either interference with the reduction of MTT to formazan directly or indirectly [55] or false negatives due to compounds that cause increases in cell volume, mitochondrial proliferation and activity, or result in senescent cells [55,56]. In addition, we sought to augment our studies in 2D cell culture with a characterization of the ionoceuticals' effects on 3D cancer spheroids since some important aspects of bioelectric regulation are dependent on cell-cell communication [57] and typical 2D cell culture lacks all the connections, extracellular matrix, and hypoxic gradient found in 3D models [58]. We also developed custom CellProfiler [59] pipelines and modified existing ConfluentFUCCI [60] software to analyze the results of treatment in 2D and in 3D.

We identified several FDA-approved and unapproved compounds that significantly decreased COLO 205 proliferation in 2D and migration and invasion in 3D. Our top compounds were also tested for cytotoxicity in COLO 205 cells and three other non-cancerous cell types representative of the colon epithelium, endothelium, and fibroblasts. The best compound, clofilium, from this final screen was then tested in a COLO 205 xenograft mouse model to determine efficacy in vivo. Western blot analysis of treated vs. untreated COLO 205 cells revealed how clofilium affected key cancer growth pathways, cell cycle regulators, and epithelial to mesenchymal transition (EMT).

2. Material and Methods

2.1 Reagents

Stocks of 4-Aminopyridine (4-AP) (275875, Sigma-Aldrich), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS)(4523, Tocris), amiloride hydrochloride (0890, Tocris), carvedilol (2685, Tocris), clofilium (C2365, Sigma- Aldrich), KB-R7943 (mesylate) (16352, Cayman Chemical Company), L-703606 oxalate (L119, Sigma-Aldrich), mibefradil dihydrochloride (HY-15553A, MedChem Express), ML-277 (15193, Cayman Chemical Company), NS8593 (hydrochloride) (HY-110105, MedChem Express), oxaliplatin

(2623, Tocris), pinacidil (hydrate) (15416, Cayman Chemical Company), Pyr3 (HY-108465, MedChem Express), senicapoc (29679, Cayman Chemical Company), tetraethylammonium chloride (TEA) (T2265, Sigma-Aldrich), tetrodotoxin citrate (1069, Tocris), TRAM-34 (23385, Cayman Chemical Company), VBIT-12 (31445 Cayman Chemical Company), verapamil (14288 Cayman Chemical Company), XE 991 dihydrochloride (14582, Cayman Chemica Company) were made at concentrations of 1000X in either DMSO, water, or in the case of monensin, 100% ethanol. If a 1000X stock could not be made due to maximum concentration, then no more than a total of 0.4% DMSO or 1.4% water was ever added to cell culture media.

2.2 Cell culture

COLO 205 cells (CCL-222, ATCC) were grown in RPMI 1640 media no phenol red (11835030, ThermoFisher) supplemented with 10% fetal bovine serum (FBS)(35-010-CV, Corning), 2 mM GlutaMAX (3505061, ThermoFisher) and 100 U penicillin/100 µg/mL streptomycin (15140122, ThermoFisher). Human Umbilical Vein Endothelial Cells (HUVEC) (CRL-1730, ATCC) were grown in Endothelial Growth Media-2 (CC-3162, Lonza) on 0.1% gelatin (07903, StemCell Technologies) coated plates. Human Primary Colonic Epithelial Cells (HCoEpC) (H-6047, Cell Biologics) were grown in Complete Epithelial Cell Medium with supplement kit (CECM)(H6621, Cell Biologics). Normal Colon Fibroblasts (CCD-18Co) (CRL-1459, ATCC) were grown in Eagle's Minimum Essential Medium (EMEM)(30-2003, ATCC) supplemented with 10% FBS and 100 U penicillin/100 µg/mL streptomycin. All cells were maintained at 37 °C with 5% CO_2 and passaged at 70% confluence by washing once with Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium (14190250, Thermo-Fisher) and dissociated with Accutase (AT104-500, Innovative Cell Technologies) for 15 minutes at 37 °C with 5% CO_2. Cells were then spun down at 200g for 3 minutes prior to aspiration of the dissociation reagent and resuspended in their respective medium before re-plating. Each line was initially expanded after purchase and froze down after 2 passages. COLO 205 cells were frozen in RPMI 1640 complete media with 5% Dimethyl sulfoxide (DMSO)(D2438, Sigma), HUVECs and CCD-18Co were frozen in 95% FBS with 5% DMSO, and HCoEpCs were frozen in 40% CECM with 50% FBS and 10% DMSO. After thawing, cells were used for experiments following at least one more passage but not more than 3.

2.3 Voltage Dye Staining

For attached staining, COLO 205 cells were plated at $1X10^5$ cells/mL in RPMI 1640 complete media on 0.1% gelatin coated black-walled 96 well plates for a total of $1X10^4$ cells per well and then incubated 24 h at 37 °C with 5% CO_2. HCoEpCs were plated at $2X10^5$ cells/mL in 0.1% gelatin coated black-walled 96 well plates, for a total of $2X10^4$ cells per well

and allowed to attach for 4 hours at 37 °C with 5% CO_2. All outer wells on the 96 well plates were filled with 200 µL water to act as an evaporation buffer. Attached cells were then washed 2X with Hanks' Balanced Salt Solution (HBSS) (14025092, Thermo-Fisher) before adding 2 µM Bis-(1,3-Dibutylbarbituric Acid) Trimethine Oxonol (DiBAC4(3)) (B438, ThermoFisher) dissolved in HBSS. Stock solution of DiBAC4(3) was made at 10 mM in DMSO. Cells were allowed to incubate in the dye for 45 minutes at 37 °C with 5% CO_2 before removing the dye from the entire plate and adding solution 1 of the respective ion series (Supplemental Figure 1) with 2 µM DiBAC4(3) and pre-warmed to 37 °C. This was then removed from only the last 8 columns of the plate and then a solution 2 of the respective ion series with 2 µM DiBAC4(3) was added. This was repeated for every solution of the ion series with 2 µM DiBAC4(3) each until every two columns on the plate was in a different solution of the ion series with every cell being exposed to the previous number of the series sequentially, ensuring that the cells were not exposed to extremes in ion concentrations too quickly.

For detached staining, COLO 205 cells and HCoEpCs were detached from plates and resuspended at a concentration of $1X10^5$ cells/mL in HBSS. Cells were aliquoted into five 5 mL tubes. Cells were then spun down and resuspended in HBSS with 2 µM DiBAC4(3) and incubated in a 37 °C bead bath covered to protect from light for 45 minutes. After the initial incubation, all 5 of the tubes were spun down and resuspended in solution 1 of the respective ion series with 2 µM DiBAC4(3) and pre-warmed to 37 °C. Next, 4 of the tubes were spun down and resuspended in solution 2 of the respective ion series with 2 µM DiBAC4(3) added. This was repeated for the other solutions until there were cell suspensions in each one of the solutions. Each cell suspension in its corresponding ion solution was then plated on an un-coated 96 well black-walled plate for a total of $1X10^4$ cells per well and then immediately imaged.

2.4 Electrophysiology

The Port-a-Patch (Nanion Technologies®, Munich, Germany) was used to perform whole-cell patch-clamp recordings. The experiments were performed within two hours after cells were collected. The assays were carried out using medium resistance single-hole chips (3–5 MΩ)(06 1103, Nanion Technologies).

Cells in 10 cm plates were collected at 70% confluence by rinsing with DPBS no calcium and magnesium and disassociated with Accutase for 15 min at 37 °C. RPMI 1640 media with no added FBS was added to the plate, cells were counted and then pelleted at 200g for 3 min at RT. The supernatant was discarded, and cells were re-suspended in the external standard solution (08 3001, Nanion Technologies) at $1X10^6$ cells/mL. The cells were kept until the moment of the experiment at room temperature.

The chip was primed with the following solutions (in mM), external standard solution consisting of 10 HEPES, 140 NaCl, 5 D-Glucose monohydrate, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 298

mOsmol, and pH 7.4 (NaOH). Internal potassium solution (in mM) 10 EGTA, 10 KCl, 10 NaCl, 10 K-Fluoride, 10 HEPES at pH 7.2 (KOH), and 285 mOsmol. The chip's resistance was checked to be within the 3-5 MΩ allowed range and then 10 µL of the cell suspension (in extracellular standard) was added to the well. Cell capture was promoted by holding negative pressure of −100 mbar for 20 s. After the capture, successive hyperpolarization steps from −30 mV to −100 mV were applied to foster the electrical seal formation. The seal was enhanced by transient addition of a high Ca^{2+} external standard solution (08 3012, Nanion Technologies) containing (in mM) 10 HEPES, 130 NaCl, 5 D-Glucose monohydrate, 4 KCl, 10 $CaCl_2$, 1 $MgCl_2$, 302 mOsmol, and pH 7.4 (NaOH). High Ca^{2+} solution was added for 10 seconds using a perfusion setup on the Port-a-Patch and removed by a 10 second external standard solution exchange which completely removed all the previous solution.

After the formation of the Giga-seal, a −250 mbar pressure was applied to break the membrane patch. Once in whole-cell configuration, the RMP was monitored in current-clamp configuration "I = 0", the values shown correspond to the observed mV 10 seconds after complete exchange. Any cells showing an R-series above 22 were rejected. Cells had to maintain a seal of at least 500 MΩ during the whole experiment and were also recorded in voltage-clamp mode starting at -80mV then running the KvIV Cslow protocol to characterize the different voltage-dependent conductance present in the cells and to make sure that an intact seal (R-series change < 2) was still present after all the ion solutions or drug solutions were added.

Ionic solutions (supplementary Figure 1) were added by perfusion sequentially from series number 1 to 5 for each patched cell. The solution was added by a 10 second wash out of the previous solution and the mV reading taken at the end of a 10 second wait period once the wash out was complete. At the end of the series, the external standard solution was added again, the mV recorded, and a voltage-clamp was done to test the R-series values to make sure that the seal had remained throughout all the perfusion steps. Drugs dissolved in DMSO, water, or ethanol were added to the external standard solution in different concentrations. Cells were exposed first to a carrier only control followed by increasing drug concentrations. RMP changes were recorded as described for ionic solutions.

2.5 Molecular Biology

The HypG3 Hygro ES-FUCCI plasmid was constructed as previously described. The CAG EBFP2-Nuc construct with a 3X SV40 C-terminal Nuclear Localization Signal (NLS) was subcloned from the plasmid pEBFP2-Nuc using AgeI and HpaI and was a gift from Robert Campbell (Addgene plasmid # 14893 ; http://n2t.net/addgene:14893 ; RRID:Addgene_14893)[61]. The CAG ASAP3-R3 construct was subcloned from pc3.1-CAGGS-ASAP3-mCyRFP3 using NheI and HindIII and was a gift from Michael Lin (Addgene plasmid # 193323 ; http://n2t.net/addgene:193323 ; RRID:Addgene_193323)[39]. The CAG GCaMP6f

construct was subcloned from the plasmid pGP-CMV-GCaMP6f with BglII and NotI and was a gift from Douglas Kim & GENIE Project (Addgene plasmid # 40755 ; http://n2t.net/addgene:40755 ; RRID:Addgene_40755)[62]. The CAG H2B miRFP670 construct with an H2B nuclear localization signal was subcloned from two plasmids, first PPRE-H2B-eGFP a gift from Severine Degrelle & Thierry Fournier (Addgene plasmid # 84393 ; http://n2t.net/addgene:84393 ; RRID:Addgene_84393)[63] had its eGFP removed using BamHI (blunted) and NotI and the miRFP670 from pmiRFP670-N1, a gift from Vladislav Verkhusha (Addgene plasmid # 79987 ; http://n2t.net/addgene:79987 ; RRID:Addgene_79987)[64] was removed using EcoRI (blunted) and NotI and ligated to the PPRE H2B fragment. The resulting plasmid PPRE-H2B-miRFP670 was then cut with AseI and NotI to obtain the H2B-miRFP670 fragment. All subcloned fragments were cloned into a pENTR1A plasmid with a CAG promoter and multiple cloning site (MCS) followed by an SV40 poly(A) using the same sites as were used in excising the fragment from the parent plasmid or those with compatible ends. The resulting pENTR1A CAG EBFP2-Nuc, pENTR1A CAG ASAP3-R3, pENTR1A CAG GCaMP6f, pENTR1A CAG H2B miRFP670 were then Gateway LR clonased (11791020, ThermoFisher) into the hyperactive piggyBac transposase-based, helper-independent, and self-inactivating delivery system, pmhyGENIE-3, a gift from Stefan Moisyadi[65,66]. The pmhyGENIE-3 had either a neomycin resistance gene in the backbone or a hygromycin resistance gene. The resulting plasmids HypG3 NeoBB EBFP2-Nuc, HypG3 HygroBB ASAP3-R3, HypG3 HygroBB GCaMP6f, HypG3 HygroBB H2B miRFP670 were purified using a ZymoPURE Plasmid Miniprep Kit (D4210, Zymo Research) and used for subsequent transfections.

2.6 Generation of Stable Lines

Transgenic COLO 205 cell lines were made by transfecting the cells at 70% confluence with 500 ng of the transfection grade plasmid, 1.75 µL of lipofectamine 3000, and 1 µL of P3000 reagent (L3000008, Thermo-Fisher) in 50 µL of Opti-MEM I Reduced Serum Medium (31985062, Thermo-Fisher) and then adding the entire complex to one well of a 24-well plate containing complete RPMI 1640 media. The reagent was removed after 48 h and replaced with fresh culture media. Cells were allowed to recover for 24 h prior to selecting with either 1 mg/mL G418 (10131035, Thermo-Fisher) or 150 µg/mL hygromycin B (10687010, Thermo-Fisher). Cells were selected for 2 weeks before being serially diluted into 96 well plates for clonal isolation and expansion. Only clones showing robust growth and strong expression compared to controls were chosen for additional experiments.

2.7 Proliferation and Recovery Assays

A COLO 205 stable clone sequentially transfected with HypG3 NeoBB CAG EBFP2-Nuc and HypG3 HygroBB CAG ASAP3-R3 was seeded at $2X10^4$ cells/mL in complete RPMI

1640 media for a total of 2000 cells per well of a 96 well black-walled plate. Compounds were added to complete RPMI 1640 in 4 serially diluted concentrations plus the carrier control and then added to the cells 24 h after seeding. The entire wells of each plate were scanned using a Celigo Imaging Cytometer (Revvity, Inc. Massachusetts, United States) on the initial day of compound addition and for 3 days afterwards for the proliferation assays. For the recovery assays, the compounds were removed on day 3 and replaced with complete RPMI 1640 media without carrier. Images were taken of the plates before and after the exchange of media on day 3 to account for the number of cells washed away by the exchange and the plates were imaged every day for another 3 days. Analysis of cell number and ASAP3 intensity was done by using a custom CellProfiler pipeline (COLO_205_Proliferation_Recovery_JM.cppipe). ASAP3 intensity values subtracted any bright aggregates in the cell and considered the background fluorescence values around each cell (COLO_205_ASAP_intensity_Celligo_JM.cppipe).

2.8 FUCCI assays

A COLO 205 stable clone sequentially transfected with HypG3 NeoBB EBFP2-Nuc and HypG3 Hygro ES-FUCCI was seeded on 0.1% gelatin coated 96 well black walled plate in the same way as described for the proliferation and recovery assays. Compound addition was also the same, except that the plate was then inserted into an ImageXpress Confocal HT.ai (Molecular Devices California, United States) with temperature and CO_2 control set to 37 °C and 5% respectively. Widefield images were taken overnight every 10 minutes for 16 h using a 10X Plan Apo 0.45 NA. One site in each well of a 96 well dish was imaged using four channels, including brightfield, YFP (laser excitation 520/10 nm and emission filter 562/40 nm), Texas Red (laser excitation 555 and emission filter 598/25 nm), and DAPI (laser excitation 405/20 and emission 452/45 nm). The YFP channel captured cells in the S/G2/M phases, the Texas Red channel captured cells in the G1 phase, and the DAPI channel captured the EBFP2-Nuc of all live cells with dead cells only showing a very faint signal. At the end of the 16 h, the cells were removed from the imaging system and incubated in the cell culture incubator for 8 h at 37 °C with 5% CO_2 before being put back into the imaging system for another overnight observation. This was done for three days including day 0.

2.9 FUCCI assay analysis

An automated analysis pipeline was used for processing the FUCCI timelapse images. This pipeline consisted of pre-processing the images, FUCCI analysis and cell tracking, and data plotting. For pre-processing, three channels were used. YFP, Texas Red, and DAPI were put into a CellProfiler pipeline (COLO_205_FUCCI_preprocess_JM.cppipe) to remove dead cells by creating masks from the DAPI channel that were thresholded for cells that were expressing EBFP2-Nuc levels that were consistent with live cells and applying

them to the YFP and Texas Red channel images. Brightfield images were consulted to make sure that all masks corresponded to actual cells. Then a separate Python script was used to arrange the pre-processed images into tiff stacks of the YFP or Texas Red channel for each well. The specific script can be modified depending on the naming conventions of the user's specific microscopy system. For FUCCI analysis and cell tracking, existing ConfluentFUCCI [60] open-source software was modified for our particular cell type and computational limitations. Instead of using Cellpose, we used a simple binary masking method which was possible due to the preprocessing done in CellProfiler. These masks were then fed into TrackMate, the native ConfluentFUCCI stack, and our expanded utility functions to account for unmerged cell tracks which maintained a single color for the duration of the experiment. After we generated .csv files for the tracking data, a sperate Python script was used to generate the FUCCI cell cycle proportion pie charts for 64 h and rose plots https://github.com/jordan-strasser/FUCCI.

2.10 Rose plot cell motility analysis

The .csv files containing the tracking data during the FUCCI analysis was also used to determine each cell's motility. To exclude cells that had detached from the plate, cells that moved faster than a cutoff speed were dropped from the analysis. Rose plots that tracked individual cells movement in the X and Y direction that were present during the entire first 16 h imaging session were generated and graphed using a custom python code https://github.com/jordan-strasser/FUCCI.

2.11 Live/Dead assays

COLO 205 and HCoEpCs were seeded at $2X10^4$ cells/ mL for a total of 2000 cells per well on 0.1% gelatin coated 96 well black-walled plates. CCD-18Co and HUVEC cells were seeded at $1X10^4$ cells/mL for a total of 1000 cells per well on 0.1% gelatin coated 96 well black walled plates. Compounds were added as described in the proliferation and recovery assays. Cells were incubated for 3 days at 37 °C with 5% CO_2. The LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (L3224, ThermoFisher) was added to HBSS at a 1:1000 concentration for both calcein-AM and ethidium homodimer-1 for a 4 µM and 2 µM 2X staining solution respectively. Hoechst 33342 (H3570, ThermoFisher) was also added at a concentration of 16.2 µM to the 2X staining solution. Once the 2X staining solution was made, it was added in equal volume to the cell culture media plus drug already in each well. This allowed for no loss of dead cells due to media removal or washes. The plate was then allowed to incubate at 37 °C and 5% CO_2 for 30 minutes before each entire well was scanned using a Celigo Imaging Cytometer. A custom CellProfiler pipeline was made to analyze the percentage of dead and live cells for each cell line (LIVEDEAD_COLO_205_Backgroundhigh_JM.cppipe)

(LIVEDEAD_HCoEpC_dapiforlive_modBackground_JM.cppipe)
(LIVEDEAD_ccd18co_dapiforlive_modBackground_JM.cppipe)
(LIVEDEAD_HUVEC_dapiforlive_modBackground_JM.cppipe).

2.12 Spheroid morphology and Live/Dead assays

COLO 205 cells stably transfected with HypG3 HygroBB H2B miRFP670 were used to seed 96 well Corning Elplasia plates (4442, Corning) containing 79 ultralow attachment microwells per larger well. A volume of 50 µL of complete RPMI 1640 media was added to the inside 60 wells of the plate and spun down at 500 g for 1 minute to release any pockets of air trapped in the microwells. Water was added to the outside wells to act as an evaporative barrier. Afterwards, a cell suspension volume of 100 µL was added to each well at a concentration of 4.9 $X10^4$ cells/mL for a total of about 62 cells per microwell in each larger well. This low seeding density allowed for better formation of intact spheroids over time. Plates were then incubated at 37 °C and 5% CO_2 for 7 days with 50 µL of additional media very slowly added to each well on day 3 and 100 µL of media carefully removed from the wells on day 5 and 100 µL of fresh media added. On day 7 after the initial seeding, the entire wells of each plate were imaged in the Cy5 channel and brightfield channels using a Celigo Imaging Cytometer. Following this initial imaging, all media was removed from the wells being careful not to disturb the spheroids in the microwells. Complete RPMI 1640 with different concentrations of drug and carrier were added at 150 µL per well in replicates of 4 each. After all the drugs were added, the plates were allowed to incubate for 10 minutes and then scanned with the Celigo Imaging Cytometer using the same settings as the initial scan. Plates were then incubated for 3 additional days with scans occurring every day. On the third day after the Celigo scan, the spheroids were carefully washed twice with DPBS with Ca and Mg (14040182, Thermo-Fisher) being careful not to disturb the spheroids in the bottom of each well. Then all liquid was carefully removed from each well and 100 µL of 2X LIVE/DEAD staining solution as prepared above was added to each well. A 24 well glass bottomed black-walled plate (P241.5HN, CellVis) was filled with 200 µL of 2X LIVE/DEAD staining solution per well and a large orifice pipette tip was used to gently transfer the spheroids from 4 of the 96 wells into one of the wells of the 24 plate. Once this was done, the 24 well plate was covered and placed at room temperature on an orbital rocker for 30 minutes. The entire well of each well in the 24 well plate with stained spheroids was scanned with the Celigo Imaging Cytometer using the FITC, TRITC, DAPI, Cy5, and brightfield channels. Live/dead of the spheroids were analyzed using custom cell profiler pipelines for either intact spheroids or dissociated ones (LIVEDEAD_COLO_205_spheroids_JM.cppipe) (LIVEDEAD_COLO_205_spheroids_SingleCell_JM.cppipe).

2.13 Spheroid morphology analysis

Each stitched image from the Celigo was put through a custom CellProfiler pipeline (COLO_205_Spheroid_analysis_IntenShapeFilter_H2BmiRFP670_JM.cppipe) to identify the individual spheroids in the well. The initial image of the spheroids prior to adding the drug was used to identify any spheroids that had been disturbed or lost during the complete media exchange and to remove any doublets from the analysis. Locations for each spheroid were used to track each individual spheroid's change in shape and intensity over the 3 days of treatment. CellProfiler outputs were parsed with a python script to make plots of all intensity measurements divided by all shape measurements. These were then compared to find a plot that captured the differences in the concentrations of the drugs https://github.com/Fw-Franz/Spheroid-Analysis.

2.14 Spheroid migration assays and analysis

Spheroids were made using COLO 205 cells stably transfected with HypG3 HygroBB H2B miRFP670 in 24 well Corning Elplasia plates (4441, Corning). Each well of the Elplasia plate was filled with 500 µL of complete RPMI 1640 media and spun down at 500 g for 1 minute to remove bubbles. The cells were seeded at 34,392 cells/mL with 1mL of cell suspension being added to each well. This gave roughly 62 cells per each of the 554 ultralow attachment microwells in the larger well. The plate was then incubated at 37 °C and 5% CO_2 for 10 days with 500 µL of additional media very slowly added to each well on day 3 and 1 mL of media carefully removed and replaced with fresh media in each well on day 5, day 7, and day 9. On day 10 the spheroids were removed from the wells using a wide orifice pipette tip and filtered through a 40 µm cell strainer (TC70-SWM-40 Stellar Scientific) pre-coated with FBS and rinsed 3X with DPBS with Ca and Mg to remove any lose single cells. Spheroids were then transferred into a 60 cm dish precoated with FBS and rinsed with DPBS to avoid sticking and filled with complete RPMI 1640. Each spheroid was individually picked using an EVOS FL microscope in the tissue culture hood and placed in the center of each of the inner 60 wells in a 96-well black-walled plate pre-coated with 0.1% gelatin solution in water and filled with 150 µL of complete RPMI 1640 with drug concentrations and carrier controls already added. Each plate had water in the outer wells to serve as an evaporative barrier. The plates were allowed to equilibrate in the incubator for 10 minutes prior to being imaged with an EVOS M7000 using the 4X objective and Cy5 filter. The plates were placed back in the incubator at 37 °C and 5% CO_2 and allowed to sit for 4 days without being disturbed. On the fourth day the plates were imaged using the same settings as before. Images from day 4 were analyzed using a custom CellProfiler pipeline that identified all cells that had migrated from the spheroid combined them into a single object converted to an image and s convex hull was created to approximate the entire area filled with migrated cells (COLO_205_Spheroid_migration_JM.cppipe). The area of the spheroid on day 0 was subtracted from the entire area on day 4.

2.15 Spheroid invasion assays and analysis

Spheroids were made and maintained the same as for the migration assays with the only difference that they were harvested on day 9 after seeding. Harvesting was also the same as the migration assays. Collagen gels were made using collagen Type I solution (C3867-1VL, Sigma-Aldrich), 10X RPMI 1640 made from powder (R8755-10X1L, Sigma-Aldrich), 1N NaOH, and complete RPMI 1640 for a gel at a final concentration of 3 mg/mL and allowed to sit at 4 °C for one hour to crosslink. Then after the hour, the gel was diluted to 1 mg/mL with complete RPMI 1640 and 50 µL was added to each of the inner 60 wells of a 96 well black-walled plate to create a bed to place the spheroids. The plate was then incubated at 37 °C and 5% CO_2 for 1 hour to solidify the gel. The rest of the gel was kept on ice for that hour. Spheroids were then added individually to the center of each well on top of the gel bed and another 50 µL of collagen was added on top. The plate was then incubated for another hour to solidify the top layer and then 100 µL of 2X concentration of each drug and carrier in complete RPMI media was added on top. The plate was then imaged using an EVOS M7000 with the 10X objective and Cy5 and brightfield filters. The plate was allowed to incubate at 37 °C and 5% CO_2 and allowed to sit for 2 days after which it was imaged again using the same settings as before. Images from day 2 were analyzed using a custom CellProfiler pipeline with the option for some manual adjustment of what was an object (COLO_205_Spheroid_invasion_analysis_EditOption_Draw.cppipe). The entire area composed of the spheroid and invading cells was subtracted from the area of the spheroid on day 0. The invading areas were all normalized to control.

2.16 Western Blots

COLO 205 cells were seeded at $1X10^5$ cells/mL in complete RPMI 1640 in 10 cm tissue culture dishes for a total of 1 million cells per dish. Cells were incubated at 37 °C and 5% CO_2 for 24 hours and then media was removed and replaced with 10 mL of complete RPMI 1640 containing drug or carrier control. Cells were incubated and treated for 72 hours, after which the cells (both adherent and suspended) were harvested, washed with DPBS, pelleted and resuspended in 2.5X volume of CellLytic M (C2978, Sigma-Aldrich) supplemented with 100 mM sodium fluoride,1.5 mM sodium orthovanadate, and 1% protease inhibitor (P8340, Sigma-Aldrich). Lysis was done according to the manufacturer's protocol. Protein quantification was done using the Qubit broad range protein assay (A50668, Thermo-Fisher) and measured using the Qubit 4 Fluorometer (Q33238, Thermo-Fisher). Protein was loaded at 15 µg per well using Mini-PROTEAN TGX Stain-Free Precast Gels (4568026, 4568041, Bio-Rad). The protein ladder used was MagicMark XP (LC5602, Thermo-Fisher). Proteins in gel were transferred to low fluorescence PVDF membranes (1620260, Bio-Rad) using Trans-Blot Turbo system (1704150EDU, Bio-Rad). Total protein was recorded for each blot using stain-

free technology and Bio-Rad ChemiDoc Touch Imaging System (12003153). Blots were blocked in tris buffered saline (TBS) (12498, Cell Signaling Technology (CST)) with 0.1% Tween-20, 5% dried milk, and 5% donor goat serum overnight at 4 °C on a rocking platform. Primary antibodies were the following: Akt (pan)(C67E7) rabbit mAb (4691, CST), cleaved caspase-3 (Asp175) rabbit pAb (9661, CST), E-Cadherin (24E10) rabbit mAb (3195, CST), GSK-3β (D5C5Z) rabbit mAb (12456, CST), LC3B rabbit pAb (2775, CST), Ndrg1 (D8G9) rabbit mAb (9485, CST), NF-κβ p65 (D14E12) rabbit mAb (8242, CST), p21 Waf1/Cip1 (12D1) rabbit mAb, p27 Kip1 (SX53G8.5) mouse mAb (3698, CST), p38 MAPK (D13E1) rabbit mAb (8690, CST), p44/42 MAPK (Erk1/2) (137F5) rabbit mAb (4695, CST), phospho-Akt (Ser473)(D9E) rabbit mAb (4060, CST), phospho-p44/42 MAPK(Erk1/2) (Thr202/Tyr204) rabbit pAb (9101, CST), phospho-GSK-3β (Ser9) (D85E12) rabbit mAb (5558, CST), phospho-p38 MAPK (Thr180/Tyr182) (D3F9) rabbit mAb (4511, CST), phospho-NDRG1 (Thr346) (D98G11) rabbit mAb (5482, CST), phospho- NF-κβ p65 (Ser536) (93H1) rabbit mAb (3033, CST), phospho-SAPK/JNK (Thr183/Tyr185) (81E11) rabbit mAb (4668, CST), SAPK/JNK rabbit pAb (9252, CST), and p53 (7F5) rabbit mAb (2527, CST). Each was added at 1:1000, except phospho-Akt which was added at 1:2000, in either CST TBST with 5% bovine serum albumin (BSA)(A9647, Sigma-Aldrich) or 5% dry milk depending on the antibody (see CST protocol for each). Blots were incubated overnight at 4 °C while rocking. Blots were washed on the following day with high salt TBS (1706435, Bio-Rad) with 0.1% Tween-20 for 10min, 3X. Blots were then incubated for one hour at room temp with secondary of goat anti-rabbit IgG HRP conjugate (7074, CST) or horse anti-mouse IgG HRP conjugate (7076, CST) added to blocking buffer at 1:2000. Blots were washed again with high salt TBST 10min, 3X. Chemiluminescent detection was done with Bio-Rad Clarity ECL substrate (1705061, Bio-Rad). Blots were analyzed using Image Lab software and normalized to total protein from stain-free blot image.

2.17 qRT-PCR

COLO 205 cells treated with drug and carrier were prepared the same as for westerns except that washed cell pellet was then flash frozen in liquid nitrogen prior to RNA extraction using RNeasy Mini Kit (74106, Qiagen) with on column DNase treatment (79254, Qiagen). RNA was then cleaned using the Monarch RNA Cleanup Kit (T2040L, New England Biolabs) quantified and 1 µg was converted to cDNA using Iscript gDNA Clear cDNA Synthesis Kit (1725035, Bio-Rad). Final concentration of cDNA was 50 ng/µL, which was used for subsequent qPCR using SYBR green master mix (A25742, Applied Biosystems). QuantStudio 3 real-time PCR system (A40393, Thermo-Fisher) was used to run and analyze samples. Multiple housekeeping genes were initially screened for each treatment to determine the most stable and at least two housekeeping genes were used per treatment for analysis.

2.18 COLO 205 xenograft model

All animal work was approved by the Institutional Animal Care and Use Committee and was performed in collaboration with Takeda biosciences. The COLO 205 cells were implanted by injecting a single cell suspension ($1X10^6$ cells in 100 µL PBS) subcutaneously in the right flank over the rib cage of male athymic nude mice (weighing 20-30 g). Tumors were measured using calipers two times a week. Treatment began two weeks later when the tumors reached around 150 mm^3 . Animals were randomly divided into 4 groups with 6 animals per group. Clofilium was administered by *i.v.* injection in a saline solution using three dosing regimens: 4 mg/kg daily, 2 mg/kg daily, and 4mg/kg every 3 days. The control group was given saline solution every day. The weights of the mice were taken every 2 days. Nutragel was given from day 10 to 12 across dosing groups due to some weight loss in the highest dosing group. All mice were taken down after 18 days on the study due to ulceration in the vehicle control group. Tumors samples were taken from each animal and placed in an optimal cutting temperature (OCT) compound and frozen on dry ice and stored at -80 °C.

3. Results

3.1 HCoEpC and COLO 205 resting membrane potentials are most affected by potassium and have different responses to ion changes when attached vs. unattached.

We sought to determine which ions in the extracellular media would have the greatest effect on the RMP of colon cancer cells. The RMP of normal colon epithelial cells (HCoEpCs) and COLO 205 cells under different extracellular ion concentrations in a series of defined and osmotically controlled and pH balanced salt solutions were made. Surprisingly, these recordings showed no statistical difference between the changes in RMP observed in normal colon epithelial cells and the COLO 205 cells (Supplementary Figure S1). However, they did show that potassium was the ion that had the largest effect on RMP in both cell types, with all other ions tested also showing significant effects on RMP. Since the resting membrane recordings were done with a Port-a-patch semi-automated patch clamp system, which reads the RMP of cells in suspension, a state that has been shown to depolarize breast and kidney epithelial cells [67,68], DiBAC4(3) staining of both cell types in suspension and attached were done as outlined in [45] and results compared to each other (Supplementary Figure S2-S6). Attached COLO 205 cells stained with DiBAC4(3) were significantly more responsive to changes in calibration solutions, showing a depolarization with increasing potassium and concomitant decreasing sodium. This appeared to be dependent on the changing potassium levels, as they were also significantly more responsive in their depolarization to potassium increases than HCoEpCs but not significantly different in their depolarization than HCoEpCs to sodium decreases. COLO 205 cells also did not significantly respond to increases in chloride, showing a slight hyperpolarization but they did significantly differ in their response

to chloride when compared to HCoEpCs which had more of a depolarizing response, but not a significant one. The HCoEpCs and COLO 205 suspended cells that were analyzed for membrane potential changes in DiBAC4(3) showed significant differences in their responses when compared to one another which contradicted the findings from the Port-a-Patch. The differences between the two suspended cell lines were not as great as they were when the cells were attached for the calibration solutions but were about the same for the potassium and chloride solutions. Interestingly, the HCoEpCs and the COLO 205 cells had the opposite trends in membrane potential changes with decreasing chloride when suspended than they did while attached. Attached DiBAC4(3) stained COLO 205 cells exposed to decreasing chloride ions showed a slight hyperpolarization as opposed to the large hyperpolarization when suspended cells were analyzed with the port-a-patch. To eliminate the potential confounding effects of the DiBAC4(3) dye and the time cells were kept in the different ion solutions, we also tested the changes in RMP on suspended cells dyed with DiBAC4(3). We found that both HCoEpCs and COLO 205 cells responded differently to the calibration solutions (potassium increasing with decreasing sodium) when attached vs. suspended and that these changes were also evident when only the potassium ion was increased, but not the sodium ion, although less so in HCoEpCs. We also found that COLO 205 cells respond significantly differently to decreasing chloride when attached vs. suspended while HCoEpCs do not. Neither COLO 205 or HCoEpCs responded differently to sodium ions when attached vs. suspended. Thus, we conclude that potassium ions are the main driver of the RMP in both cancerous and non-cancerous colon epithelial cells and that cells that are attached have significant differences in how they respond to ions than cells that are suspended.

3.2 GEO database searches revealed that many potassium conducting ion channel associated genes are upregulated in colon cancer.

To identify electrogenic targets that explain and allow modification of the cancer phenotype, we sought to determine the transcriptional electrome of colon cancer. Two studies in the gene expression omnibus (GEO) database, which is a public repository of gene expression analysis data made available by the National Institutes of Health (NIH) were analyzed using GEO2R for ion channel genes that were significantly upregulated in colon cancer (Figure 1). The first study from GSE195985 [69] was used to compare COLO 205 cells or primary CRC to those of patient derived normal colon tissue (set A). The second study from GSE59760 [70] was used to compare primary CRC (set B) to patient derived normal colon tissue (set B). The COLO 205 comparison, primary CRC set A comparison, and primary CRC set B comparison all showed most of the upregulated ion channel associated genes coded for proteins belonging to ion channels that had a capacity to conduct potassium. When the upregulated channels for all three of these comparisons were searched for overlap, the

greatest overlap was between the genes upregulated in primary CRC set A and set B. The COLO 205 cell line had the most upregulated ion channels that were not shared by the primary CRC sets and a majority of those were potassium conducting. Thirteen ion channels genes were found to be shared between all three comparisons, and of these thirteen genes, seven encoded sodium conducting ion channels, seven that conduct potassium, six calcium and only two that conduct chloride. six of the thirteen genes, encoded for ion channels capable of conducting more than one type of ion. Of the shared genes, the highest expression in all three groups was PACC1, which encodes the proton activated chloride channel. The second highest expressed gene was GRIN2D, which encodes for a subunit of the N-methyl-D-aspartate (NMDA) receptor, a ligand gated cation channel (Supplemental Tables 1-3). Overall, more genes encoding potassium conducting ion channels were found upregulated in colon cancer when compared to normal colon tissue.

3.3 Initial screen of ion modulating drugs show multiple compounds inhibit proliferation of COLO 205 cells.

Once we had identified the upregulated ion channel genes shared by both the COLO 205 cell line and primary CRC samples, we chose 19 drugs to target those associated ion channels and conducted proliferation assays with treated COLO 205 cells containing the ASAP3-R3 genetically encoded voltage reporter and the EBFP2 nuclear fluorescent reporter. Cells were treated for three days with images taken every day to track cell number and changes in RMP. The results from these preliminary screens are shown in Supplemental Table 4. Of the compounds tested, nine showed high efficacy against COLO 205 proliferation (Figure 2A and Supplementary Figure S7). Table 1 lists the most effective compounds, their known targets, and FDA approval status. These initial screens gave a list of drugs that blocked potassium, sodium, and calcium conducting channels in near equal ratios.

Table 1. Compounds with Highest Efficacy on Proliferation of COLO 205 cells.

Compound	**Targets**	**Status and Ref.**
L-703606	NALCN [71]	Not FDA approved, animal studies done. [72]
Pyr3	TRPC3 [73], Orai [74]	Not FDA approved, animal studies done. [73]
Mibefradil	Cav3.1-3.3 [75], L-Type calcium channels [76],Cav2.2 [77], IP3R [78], Orai [79], Nav1.2 [80], Nav1.4-1.5 [80], Nav1.7 [80], ClC [81], Kir6.1-6.2(KATP)	FDA approved for hypertension and angina, then withdrawn. [86]

	[82], Kv1.5 [83],Kv10.1 [84] Kv channels [85]	
Clofilium	Slick [87], Slack [87], Slo3 [88], Kv1.5 [89], TASK-2 [90,91], NMDA receptors [92], hERG [93-95], hEAG [93], Kv7.1/mink [96], Na^+/K^+-ATPase [92], BTX-activated sodium channels [97], L-type calcium channels [92,98], Kir6.1-6.2(KATP) [99]	Not FDA approved, animal and human studies done. [100-103]
Carvedilol	Kir6.1-6.2(KATP) [104], Kv1.3 [105], Kv1.5 [106], Kv2.1 [107], Kir2.1-2.3 [108], Kv4.3 [109], Kir3.1/3.4(KACh) [104], Kv7.1 [110], hERG [111,112], RYR2 [113,114], Nav1.5-1.6 [115,116], IP_3R [117], TASK-1 [118], TREK-1 [119], TREK-2 [119], L-type calcium channels [120], HCN1-2 [121], HCN4 [121]	FDA approved for heart failure. [114,122]
KB-R7943	ASIC1-3 [123], nAChRs [124], NMDA receptor [125], NCX [126], TRPC3 [127], TRPC5-6 [127], MCU [128], RYR1-2 [129], mitochondrial complex 1 [125], SOCE [130], $Ca_V1.2$ [131], $Na_V1.2$ [132], $Na_V1.7$ [132], hERG [133], Kir3.1/3.4(KACh)[134], Kir2.x [135], Kir6.1-6.2(KATP) [134]BK(activates) [136]	Not FDA approved, animal studies done. [137]
NS 8593	Kca2.1-2.3 [138], TRPM7 [139], $Na_V1.5$ [140]	Not FDA approved, animal studies done. [141-143]
Verapamil	Cav1.2 [144], Cav1.3 [145], Cav3.1 [146], nAChRs [147], Kv1.1-1.4 [148-150], Kv1.5 [149], Kv4.3 [151], Kir2.1-2.3 [152], Kir6.1-6.2(KATP) [153,154], TREK [155], BK [156], hERG [149,157,158], ENaC [159]	FDA approved for angina, arrhythmias, and hypertension.
XE 991	Kv4.3 [160], Kv7.1/mink [161], Kv7.1-7.3 [160,162,163], Kv1.2/1.5 [164], Kv2.1/Kv9.3 [164], hERG [165], EAG1 [160], nAChRs [166]	Not FDA approved, animal studies done. [167,168]

3.4 Toxicity tests on COLO 205 cells show that many compounds are cytostatic.

To test if the reduced proliferation seen in the initial screens was due to a cytotoxic effect or cytostatic effect, a live/dead stain was done on treated COLO205 cells after three days of incubation in the drug (Figure 4D-4E and Supplementary Figure S8). Drugs L-703606, pyr3, mibefradil, clofilium, carvedilol, KB-R7943, and verapamil, were cytotoxic to COLO 205 cells at their highest concentrations. However, mibefradil, clofilium, carvedilol, KB-R7942, and verapamil also showed cytostatic effects at lower concentrations with the most significant of these being clofilium which showed cytostatic effects at concentrations 5-20 μM with little to no cytotoxicity on COLO205 cells at those concentrations. NS 8593 and XE 991 were both cytostatic at their highest concentrations, showing very little cell death in COLO 205 cells.

3.5 FUCCI cell cycle tracking finds most compounds work by increasing the proportion of cells in G1/G0.

To investigate the cytostatic effect of the compounds and determine what part of the cell cycle was being affected, a COLO 205 cell line with a genetically encoded ES-FUCCI [169] cell cycle reporter and EBFP-Nuc fluorescent reporter was used to track individual cells and report real time changes in cell cycle proportions over a 64 h period of time (Figure 3 and Supplemental Figures S9-S13). This data was also used to generate 2D migration rose plots for each compound and its control (Figure 3 and Supplemental Figures S14). Oxaliplatin, a known inhibitor of colon cancer cells and which is known to arrest cells in the G2/M phase [170], was used as a positive control for the analysis. A modified version of the software, ConfluentFUCCI [60] was used to analyze the time series. The analysis correctly identified the G2/M phase arrest of oxaliplatin (Supplemental Figure S13) on COLO 205 cells and found that treatment with all the most effective compounds tested from Table 1 showed an increase in the G1/G0 cell cycle phase. Most notable among these was clofilium, which almost completely arrested all the cells in the G1/G0 phase at 20 μM and over half at 10 μM. Only mibefradil showed a very active 2D migration rose plot as compared to control, with the other compounds showing little to no increased migratory activity.

3.6 Cytoplasmic calcium concentrations were not elevated for most top compounds

Since cytoplasmic calcium levels have been shown to be important to proliferation, all compounds were applied in 2D to a COLO 205 cell line with a GCaMP6f genetically encoded cytoplasmic reporter and EBFP2-Nuc fluorescent reporter. Images were taken immediately after application and for two days afterwards. No significant differences in cytoplasmic levels were seen except for in the concentrations that were known to be cytotoxic to the cells (Figure 2G and Supplementary Figure S15). This indicated that calcium levels were not a significant driver of the changes in proliferation that were seen.

3.7 Top compounds showed a significant immediate depolarizing effect on COLO 205 resting membrane potential but not on that of normal colon epithelial cells.

To investigate whether the effects these drugs had on cell proliferation was linked to their bioelectric state, the top compounds added to a balanced salt solution were applied in increasing concentration to COLO 205 cells and HCoEpCs patched using the Port-a-Patch semi-automated planar patch clamping system (Figure 2C-2D and Supplemental Figure S16). Application of L-703606, mibefradil, clofilium, carvedilol, KB-R7943 and XE 991 all showed at least one significant depolarization upon application of increasing concentrations of drug to COLO 205 cells. However, none of the drugs that were applied resulted in a significant depolarization of HCoEpCs. Although not significant, XE 991 application to HCoEpCs did show a clear trend of depolarization. This preferential depolarization of COLO 205 cells as compared to normal colon epithelial cells show that the bioelectric effects of the drugs are specific to the cancerous state.

3.8 COLO 205 cells show hyperpolarization of resting membrane potential during prolonged incubation in cytostatic concentrations of the top compounds.

To monitor the long-term changes in resting membrane potential of the COLO 205 cells while they were being treated with the top compounds in complete media, we used the GEVI ASAP3-R3. Surprisingly, most of the top compounds which elicited an immediate depolarization upon application, resulted in a gradual hyperpolarization over the three-day incubation period (Figure 2E and Supplemental Figure S17). This hyperpolarization was most apparent at the cytostatic concentrations of each compound, while the cytotoxic concentrations showed either a strong depolarization followed by death or a slight depolarization followed by hyperpolarization and then a depolarization before death. These results show that compounds that were initially depolarizing were hyperpolarizing after prolonged incubation and that cytostatic concentrations showed the strongest hyperpolarization.

3.9 Recovery tests on COLO 205 cells show that some compounds have a lasting inhibition even after exposure ceases.

To determine whether these bioelectric treatments would result in a lasting therapeutic, we investigated whether their effects on proliferation are transient or stable. Recovery assays on the treated COLO 205 cells were done by removal of the compound after three days treatment and replacement with untreated complete media. The cell proliferation was monitored for another three days to see how well the cells recovered from their cytostatic state (Figure 2B and Supplementary Figure S18). Cells pre-treated with XE 991

recovered immediately, even at the highest concentration tested. NS 8593 had a lasting effect on COLO 205 proliferation at its highest concentration showing that the cytostatic effect of this compound is present even after removal. Of the compounds showing cytotoxicity on COLO 205 cells, recovery at the concentrations that were not notably cytotoxic was fastest for cells pretreated with L-703606, followed by another quick recovery by mibefradil pretreated cells. The slowest recovery was for cells pretreated with clofilium, which showed very little recovery for concentrations 10-20 μM. This was followed by the recovery of cells that had been pretreated with KB-R7942 at 10 μM. Next pyr3 also showed low recovery at 5 μM followed by carvedilol pretreatment at 10 μM showing a slightly slower recovery than verapamil pretreatment at 50 μM. These results showed that many of our top compounds had lasting efficacy at their cytostatic doses even three days after their removal from the cells.

3.10 Differential toxicity was found in COLO 205 spheroids when compared to 2D results.

We wanted to find out if our top compounds would have the same efficacy in a model that more closely resembles colon cancer tumors *in vivo*. To this end, 3D spheroids were made from COLO 205 cells, which are not amenable to spheroid formation, by seeding at a very low density and allowing them to grow for one week before being treated with the top compounds. A COLO 205 cell line containing an H2B miRFP670 fluorescent reporter, expressing only in the nucleus, was used to track the morphological changes of the spheroids over the three-day treatment with each drug followed by a live/dead stain at the end of the treatment period (Figure 4A-4E and Supplemental Figure S19 and S8). NS 8593 and XE991, which both did not show any cytotoxicity to COLO 205 cells in 2D, also did not show very much cytotoxicity in 3D, however their morphological plots over the three-day period did show changes for the highest two and three concentrations tested, respectively. L-703606, pyr3, KB-R7942, mibefradil, verapamil, and carvedilol treated spheroids all showed less toxicity in 3D than in 2D, with the lowest concentration that was toxic in 2D not being toxic in 3D. All the morphological plots of spheroids treated with these compounds had large differences. Clofilium treated spheroids showed a very different toxicity profile in 3D when compared to 2D, with all but the lowest concentration of clofilium completely dissociating the spheroids (Figure 4C) and toxicities increasing for concentrations 10-20 μM. These findings highlight that there are significant differential effects when COLO 205 cells were treated in 3D vs. 2D.

3.11 Top compounds inhibit the 3D migration and invasion of COLO 205 spheroids.

Due to the differential efficacy of the drugs we observed, we used our 3D model to test the migration and invasion of treated COLO205 spheroids by placing them on gelatin coated plates or in a collagen gel, respectively. Spheroid migration onto the coated plate was

quantified after four days of treatment and invasion was quantified after 2 days treatment (Figure 4F-4I and Supplementary Figure S20). KB-R7942 and verapamil treated spheroids showed a significant decrease in 3D migration for all concentrations tested and a decrease in invasion for all concentrations tested but with only the highest two concentrations being significant. Clofilium treatment significantly decreased 3D migration close to zero at all concentrations tested. The 3D invasion was significantly reduced in the highest concentration, 40 μM, was not reduced for 20 μM, but was then significantly reduced for 10 μM and non-significantly reduced at 5 μM. This bimodal invasive response to clofilium was consistent across trials. Carvedilol treatment showed a significant decrease in migration and invasion for the top two concentrations tested while L-703606 treatment only showed a significant decrease in migration and invasion for the highest concentration tested. Pyr3, mibefradil, and XE 991 treatment all significantly reduced migration but not invasion, with pyr3 treated spheroids showing significantly decreased migration for all concentrations tested and mibefradil and XE 991 treatment only showing significant decreases for their highest concentrations. NS 8593 treated spheroids showed a decrease in migration at the highest concentrations, but it was not significant. However, invasion with NS 8593 treated spheroids was significantly reduced for the highest concentrations. Overall, most of our top compounds were capable of decreasing 3D spheroid migration, invasion, or both.

3.12 Top compounds were nontoxic to non-cancerous cells at concentrations that were still effective at reducing COLO 205 proliferation.

Although effective on COLO 205 cells in 2D and 3D, we wanted to make sure that our top compounds were not toxic to cells representative of normal colon epithelia, endothelia, and fibroblasts. HCoEpCs, HUVECs, and CCD-18Co cells, were chosen to represent these three cell types, respectively. Cells were tested for toxicity after three days of treatment by using a live/dead assay (Figure 5 and Supplementary Figure S21). Significantly toxic compounds were those that had a significant toxicity of more than 30%. KB-R7942 treated cells showed significant toxicity at the three highest concentrations in HUVECs and CCD-18Cos and only for two of the highest concentrations in HCoEpCs. Mibefradil treated cells showed significantly toxic responses for the highest two concentrations in all three of the cell types, as did cells treated with verapamil, carvedilol, and pyr3. Pyr3 also showed more toxicity in HCoEpC cells with a third concentration being significantly toxic as well. L-703606 treatment was significantly toxic at its highest concentration only for HUVECs and CCD-18Cos. Clofilium treatment was significantly toxic at its highest concentration for CCD-18Cos and was just over the cutoff for significant toxicity for HUVECs. XE 991 and NS 8593 were not toxic to any of the non-cancerous cell types tested. The TD50 was calculated for the HCoEpCs and used to calculate an in vitro therapeutic index (TI) (Supplementary Table 4). These results showed that some of our top compounds were not toxic to non-cancerous

cells at concentrations that were still effective at reducing COLO 205 proliferation. Clofilium was the best of these with the lowest toxicities in non-cancerous cells for most of its effective concentrations against COLO 205 cells in 2D and 3D.

3.13 COLO 205 cells treated with clofilium show decreased activity of key cancer growth pathways and increased senescence markers.

To elucidate the potential mechanism by which clofilium exerts its effects on proliferation, invasion, and migration of COLO 205 cells, we analyzed its effects via western blot on four canonical cancer pathways, PI3/AKT/mTOR, Ras/MAPK, NF-κβ, and Wnt/β-catenin and various other proteins (Figure 6). Protein lysate comparison from clofilium treated and control treated COLO 205 cells in 2D cells showed a significant decrease in total AKT protein and a significant decrease in phosphorylated AKT (pAKT), with the ratio of pAKT/AKT showing a decrease in comparison to control of almost 50% indicating an inactivation of the AKT pathway. Total SAPK/JNK protein was slightly but significantly elevated in treated cells while phosphorylated SAPK/JNK (pSAPK/JNK) was significantly decreased. The ratio of pSAPK/JNK to total SAPK/JNK decreased by 66%, indicating a decrease in SAPK/JNK activity. Total p38 did not significantly change upon treatment, but phosphorylated p38 (phos-p38) significantly decreased and the ratio of phos-p38 to total p38 decreased by 53%, indicating a decrease in p38 activity. The ERK1/2 protein was also slightly decreased in treated cells but not significantly so. Phosphorylation of ERK1/2 (pERK1/2) stayed the same, however when looking at the ratio of pERK1/2 to total ERK1/2, there was a slight increase of 34%, indicating an activation of this pathway. To probe the changes in the NF-κβ pathway, we found that total NF-κβ levels did not significantly change but phosphorylated levels did decrease significantly. The ratio of pNF-κβ/ NF-κβ decreased by 77%, showing an inactivation of this pathway. To probe into the WNT/β-catenin pathway, we tested for changes in GSK-3β and NDRG1 activity, both known to downregulate this pathway [171,172]. We found that total GSK-3β protein was significantly elevated in treated cells and phosphorylated GSK-3β (pGSK-3β) was decreased. The ratio of pGSK-3β to total GSK-3β decreased by 66%, indicating an increase in GSK-3β activity. Strikingly NDRG1 activity was found to be highly upregulated in treated cells by 768%. We also checked the phosphorylation status of NDRG1 at Thr346, known to mark cytoplasmic NDRG1 [173] and found that it was increased in treated cells, but when looking at the pNDRG1/NDRG1 ratio we found a 75% decrease in the treated cells. Since NDRG1 is also known to upregulate p21 activity in CRC [174], we checked the expression of p21 Waf1/Cip1and found it to be highly expressed in treated cells with an increase of 353%. This increase in p21 Waf1/Cip 1, a known marker for senescence led us to also look at p27 Kip1 expression, also a marker of senescence [175], and we found a significant 35% increase in treated cells. To see if the increase in NDRG1 could be caused by a DNA damage stress response, we looked at p53

expression and found a significant decrease of 82% in treated cells. We also looked at the EMT down regulator, E-cadherin, and found that levels significantly increased by 54% upon treatment. Checking for LC3B-II expression, we found that it increased by 1142%, indicating that the treated cells are upregulating their autophagy response. To see if the cells were becoming apoptotic, we also checked cleaved caspase 3 but did not find any expression in either the control or the treated cells. Overall, we found that clofilium treatment of COLO 205 cells significantly downregulated many canonical cancer pathways involved in cell growth, increased senescence markers, showed a decrease in EMT, increased autophagy, and showed no signs of apoptosis or DNA damage stress response.

3.14 Clofilium COLO 205 xenografts showed reduced tumor volume after treatment with clofilium and low toxicity.

Having a solid understanding of the effect that clofilium had in 2D and 3D, we next grew COLO 205 xenografts in athymic nude male mice to see its effect in an *in vivo* model. When tumors had reached an average of 150 mm^3, mice were treated with varying doses of *i.v*. injected clofilium either daily or every 3 days. Those mice treated with 4 mg/kg daily, the highest dose, saw a significant decrease in tumor volume as compared to the vehicle control after 6 days of treatment (Figure 7). On day 10, since some weight loss was observed in treated mice, Nutra-Gel was given to all mice until day 12. The study was terminated on day 18 due to the control mice developing ulcerations. Mean tumor volume in the group of mice treated with the highest dose of clofilium was reduced by 37% after 18 days as compared to the vehicle control with no overt signs of toxicity besides a slight decrease in weight (2%) by the end of the study (Supplementary Figure S22). Thus, daily treatment with clofilium is capable of significantly reducing *in vivo* tumor volume and is well tolerated.

4. Discussion

4.1 Method of resting membrane potential assessment.

During our investigation of RMP changes in response to different ion concentrations we saw a marked difference in our results when using the Port-a-Patch semi-automated patch clamp system, which requires cells to be in suspension, and our DiBAC4(3) staining of cells that were attached. There are three likely sources of this difference, one being that in suspension, epithelial cells are known to lose some of their potassium channels thus depolarizing them [68], two that the cells that were attached were exposed to the ion solutions for a longer period (10 minutes vs 10 seconds), and three that the DiBAC4(3) is altering native ion channel activity (activation of BK channels [176]). To account for two of these variables, we also did DiBAC4(3) measurements on suspended cells to see if there was still a difference in

RMP response between attached and suspended cells when using voltage dyes. Our results showed that there were still significant differences and that they were primarily seen in the COLO 205 cells. HCoEpCs did show some differences as well, but mainly due to changes in potassium concentrations, showing less of a response when suspended. These findings suggest that caution should be taken when comparing electrophysiological measurements of RMP to dye measurements of relative RMP, and that different kinds of electrophysiological measurements (traditional vs. Port-a-Patch) could give different results just based on the state of the cell (attached vs. suspended) [177]. Past research has shown that trypsinization of cells can affect ion channel functionality [178] and that the volume changes experienced by cells in suspension could also affect the open or closed states of their ion channels [179]. Although Accutase was used in this study, it still has proteolytic activity but has not been reported to alter ion channel function.

4.2 Potassium's role in setting the resting membrane potential changes.

RMP measurements of COLO 205 cells and HCoEpCs in response to different ion concentrations showed no differences between the cancerous and non-cancerous cells when measured using the Port-a-Patch. However, our Port-a-Patch measurements did show that potassium was the ion that had the most effect on the resting membrane potential, followed by chloride, then sodium. Our RMP measurements using DiBAC4(3) did find that there was a difference in how attached COLO 205 cells and HCoEpCs responded to potassium, although we cannot discount the possibility that the DiBAC4(3) itself was interfering with native potassium channel conduction. Regardless, DiBAC4(3) also found that COLO 205 and HCoEpCs cells depolarized more strongly in increasing concentrations of potassium ions when attached or suspended. It should be noted that DiBAC4(3) did not find any significant changes in RMP when chloride or sodium ions were changed for either cell type, which is strongly in contradiction to the large hyperpolarizing effects of decreased chloride ions and strongly depolarizing effects of increased sodium ions seen in both cell types when patched. This contradiction in the results needs to be explored more fully.

4.3 Decreased proliferation induced by application of ion channel targeting drugs

Our analysis of existing data sets in the GEO database resulted in our testing 19 drugs that were known to block those channels corresponding to the ion channel genes that were upregulated when colon cancer samples were compared to normal tissue. Chloride conducting channels that were strongly upregulated in COLO 205 cells or shared with primary CRC were targeted with the broad-spectrum chloride channel blocker DIDS (4,4-diisothiocyanatostilbene-2,2-disulfonic acid) [180]. However, DIDS had no significant effect on proliferation. This lack of effect could be due to ineffective blocking of these channels by DIDS. Even though DIDS was reported to block PAC1 [181], ClC-5 [182] and ClC-7 [183], there are

some conflicting reports with regards to ClC-5 [184]. Further studies with more specific blocking agents will be needed to rule out chloride channel importance to colon cancer proliferation.

The sodium channel blocker tetrodotoxin (TTX) also did not show a marked change in COLO 205 proliferation, which suggests that TTX sensitive NaV [185] channels are not crucial for growth. In addition, amiloride which blocks the ENaC sodium channel [186] was also not effective at reducing proliferation. Blocking the NALCN sodium leak channel with L-703606 was effective, but this effect could also be attributed to its blocking effect on the neurokinin-1 receptor (NK1R) [187] which would interfere with substance P (SP) signaling [72], which has been shown to be upregulated in CRC especially lymph node metastasis [188]. However, levels of NK1R and SP were significantly lower in COLO 205 cells and primary CRC than in normal colon tissue according to the GEO dataset GSE195985 and not significantly changed according to the GEO dataset GSE59760 (data not shown).

We also tested senicapoc and TRAM-34 which are known to inhibit KCa3.1, a potassium channel that we found was highly upregulated in primary CRC as compared normal colon tissue and that was recently found to be necessary to generate periodic calcium activity that drove MAPK and NF-κβ pathways in glioblastoma networks [42]. However, neither of these inhibitors influenced COLO 205 proliferation which has highly upregulated KCa2.1 channels instead. When these were targeted with NS8593 we did see a significant reduction in proliferation in 2D but not in 3D. Interestingly, another recent study looking at fast dynamic RMP fluctuations in breast cancer cells found that apamin, which blocks KCa2.1-2.3, was able to reduce RMP fluctuations and that the presence of those fluctuations were much more common in cancerous breast epithelial cells than their non-transformed counterparts [46]. The role of KCa2.1 in COLO 205 calcium or voltage transients has yet to be determined.

Following the lines of calcium dependent RMP changes, we also tested pyr3 a known inhibitor of TRPC3 [73] and Orai [74] channels. Treatment with this drug also inhibited proliferation in 2D but not in 3D. Instead, we found that our best treatments in 3D had multiple ion channel targets but all shared the ability to block calcium and potassium conducting channels. It seemed that this combination of activity was necessary since XE 991, which blocks Kv [160] channels and hERG [165] was effective in 2D proliferation but not on 3D growth. Verapamil, KB-R7943, carvedilol, and mibefradil all block calcium and potassium channels and all showed effectiveness on 3D growth, except the effectiveness was less in 3D than in 2D. Clofilium was interesting in that it was the only drug tested that showed more effectiveness in 3D than in 2D and it was the only one of the drugs that blocked calcium, potassium, sodium, and specifically the Na/K/Atpase[92].

4.4 Hyperpolarization's role in cytostatic response to incubation with top compounds

One of the most surprising findings of this study was that all the top compounds showed significant hyperpolarization of the resting membrane potential upon prolonged incubation in concentrations of drug that were known to by cytostatic. Whether this was due to the arrest of the cell cycle or if it was the driver of the arrest is unknown. Previous work in the MCF-7 breast cancer cell line has found that Kca3.1 is the driver of hyperpolarization during the progression from G1 into S phase [189]. None of the top effective compounds block Kca3.1, so we can speculate that the hyperpolarization that is being observed could be due to Kca3.1 activity, but the exact mechanism of how each of the top compounds arrests the cell cycle at G0/G1 still needs to be determined.

4.5 Possible mechanisms of clofilium's ability to reduce CRC growth, migration, and invasion.

Clofilium treatment of COLO 205 cells showed a drastic increase in NDRG1, a protein that has received a lot of recent attention due to its role as a metastasis suppressor [172-174,190-194]. Upregulation of NDRG1 has been shown to stabilize β-catenin at the membrane resulting in its reduced translocation to the nucleus, which in turn reduces CRC growth, invasiveness, and stemness [195]. It is also been shown to induce differentiation in CRC cell lines [194]. We showed that the high increase in NDRG1 was also accompanied by a large increase in p21 levels, which agrees with other studies finding that NDRG1 can stabilize p21 or transcriptionally activate it [174,193]. This increase in p21 expression agrees with our FUCCI analysis that clofilium strongly arrested COLO 205 cells in G1. Since NDRG1 has been shown to be upregulated by the DNA damage stress response gene p53 [196], we tested whether p53 was upregulated in treated cells but found instead that it was downregulated. Interestingly a recent study which identified clofilium combined with pazopanib as a promising treatment for malignant rhabdoid tumors, also found that clofilium on its own was an inducer of endoplasmic reticulum (ER) stress [197]. ER stress has been shown to increase the expression of NDRG1 [198] and decrease the expression of p53 [199] via GSK-3β activity, which agrees with our findings. However, a more detailed look into how clofilium induces ER stress in COLO 205 cells will need to be done. Previous studies on clofilium have also found that clofilium induced apoptosis in human promyelocytic leukemia cells [200] but we did not find evidence for this in the COLO 205 cell line at the concentration tested.

4.6 Limitations and future outlook.

Our study investigated the effect in 2D and in 3D of a variety of ion channel modulating drugs that were known to target the products of the upregulated ion channel genes in CRC. We confined our study to the COLO 205 cell line due to its well-known status as a particularly aggressive CRC cell line. We plan to examine more cancer cell lines that are representative of the different molecular subtypes to see if the top compounds we found in

this study have a broad effect. Clofilium is a promising therapeutic due to its effectiveness *in vivo* and published studies showing that it is well tolerated in humans [100]. Some concerns over hERG inhibition may be raised, but both carvedilol and verapamil are also hERG inhibitors and are currently FDA approved. Indeed, new protocols for identifying which hERG inhibitors would be potentially problematic in humans have been developed to not lose out on promising therapeutics [157]. Future studies will explore each of clofilium's ion channel targets and their contribution to the phenotypes we observed. Indeed, it may be that targeting specific combinations of ion channels is the key to unlocking the potential of ion modulating drugs in cancer treatment. Given the emerging computational tools for modeling bioelectric states [201-204], novel reporters, [205-207] animal models for tracking electrophysiological states in vivo [208,209], and improving understanding of the information flows between normal and transformed cells [42,210,211], we foresee an important and expanding role for ionoceuticals in cancer medicine [20,211-213].

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Author Contributions:

J.D.M and M.L. conceived the idea and designed the experiments. J.D.M., S.H., J.S., S.A.P., M.M., J.A.C., Z.M.W., B.G.C., A.T., J.S.I., L.S., and A.Y.L. carried out the experiments or made plasmids needed for the experiments. J.D.M., J.S., and F.K. developed the analysis pipelines and graphed the data. J.D.M., J.S., L.S., A.T., and A.Y.L. manually reviewed all image analysis outputs for errors. J.D.M. wrote the manuscript. M.L. contributed to the final version of the manuscript and supervised the project.

Data Availability Statement:

All CellProfiler pipelines used in this work are specified in the materials and methods and can be found at https://github.com/JuanitaMathews/Tufts-projects. The customized FUCCI analysis pipeline is found in the materials and methods as well as the python scripts for rose plots and plotting spheroid intensities and shapes.

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