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Supplemental Information

A High-Throughput Small Molecule Screen Identifies Ouabain as Synergistic with miR-34a in Killing Lung Cancer Cells

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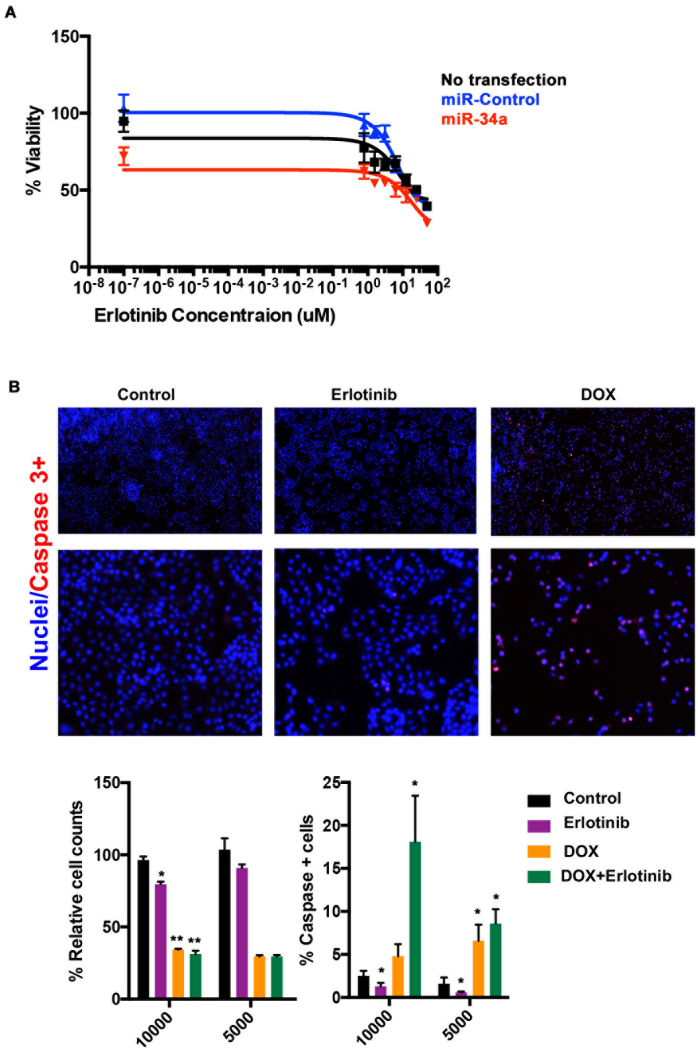
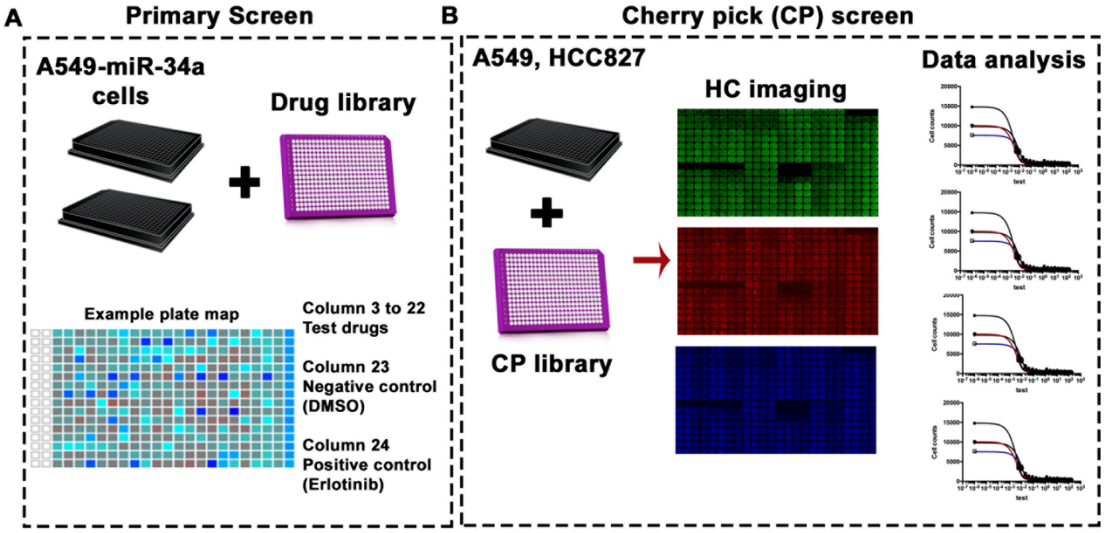
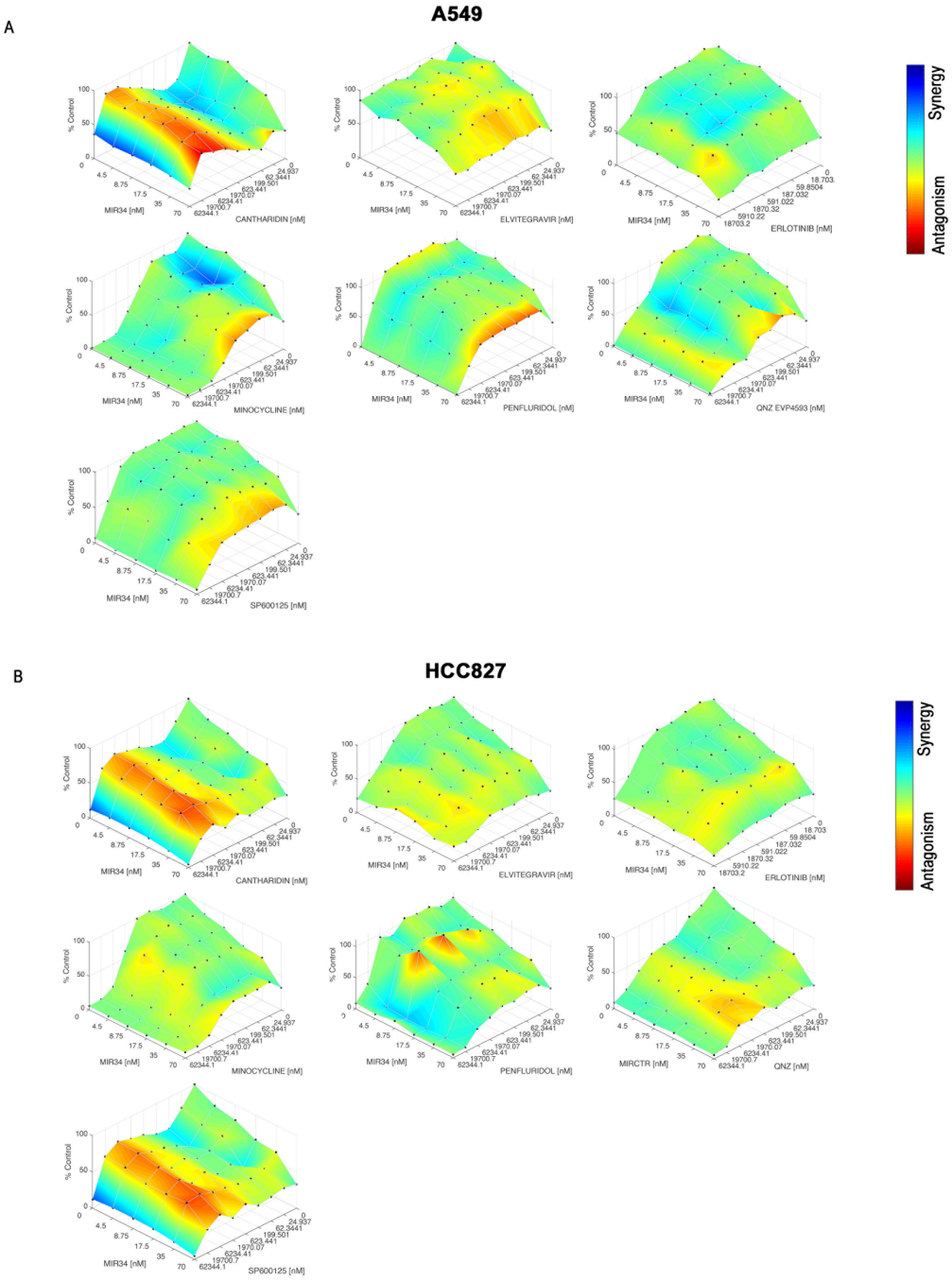


Supplemental Figure 1: Standardization of the assay for measuring cell number and caspase activity after treating cells with miR-34a and small molecules. Related to Figure 2. **A)** Viability curves from A549 cells treated with miR-34a compared to controls and increasing doses of erlotinib. B) Nuclei stained using Hoechst 33342 and activated caspase 3 positive cells stained using Nucview 549, images were taken at 200x. The top panel shows A549 cells treated with miR-control, miR-34a, erlotinib or combination of miR-34a and erlotinib. The bottom panel shows quantified data from nuclei and activated caspase 3 positive cell counts in two different cell plating conditions. *p<0.05, **p<0.01, Student's t test. Bars and errors bars represent the means and the corresponding SEMs for $n \geq 3$



Supplemental Figure 2: Outline of the high throughput screen. Related to Figure 3. A) Schematic of the primary screen with A549 cells with expression of miR-34a, under the control of the tetracycline responsive element. A plate map of screen setup is shown. B) An outline of the cherry pick screen and identification of top candidate molecules to combine synergistically with miR-34a is shown.



Supplemental Figure 3: Synergy plots of miR-34a and the top 8 molecules in treated lung cancer cells A549 (A) and HCC827 (B). Related to Figure 3. Lowe's additivity combination index was calculated and plotted as heatmap using Combenefit data analysis tool.

Supplemental Tables

Name of the Library	Description (Source: ICCB-Longwood)
NIH Clinical Collection 1 and 2	This collection was assembled by the National Institutes of Health (NIH) and is comprised of molecules that have a history of use in human clinical trials
Microsource1	These have reached clinical trial stages in the USA. Each compound has been assigned USAN or USP status and is included in the USP Dictionary
LOPAC1	Collection of 1280 pharmacalogically-active compounds from Sigma, USA
Biomol4	Known and well-characterized bioactivities and undergone safety and bioavailability testing
MSDiscovery1	This is a collection of compounds newly available at MicroSource (part of their Pharmakon 1600 library) and not previously represented in ICCB-L Known Bioactives libraries
Selleck-3.33mM	This library contains some FDA-approved compounds and most of Selleck's inhibitors, active pharmaceutical agents, and chemotherapeutic agents
eMolecules	Small molecules included in this library were identified via text mining from a variety of sources including ChEMBL, ClinicalTrials and DrugBank by chemists at eMolecules
Prestwick2 Collection	This library contains 1120 off-patent compounds that have been selected for structural diversity, collective coverage of multiple therapeutic areas, and known safety and bioavailability in humans
Cayman Biolipid	Included are prostaglandins, thromboxanes, cannabinoids, D-myo–inositol-phosphates, phosphatidylinositol-phosphates, sphingolipids, inhibitors, receptor agonists and antagonists, ceramide derivatives, and several other complex polyunsaturated fatty acids
Biomol ICCB known bioactives	The collection includes many classes of compounds including ion channel blockers, GPCR ligands, second messenger modulators, nuclear receptor ligands, actin and tubulin ligands, kinase inhibitors, protease inhibitors, gene regulation agents, lipid biosynthesis inhibitors
Tocriscreen MiniLibrary	A collection of 1120 biologically active compounds
Chembridge KINAcore	These molecules have total of 34 targets (ADK, Aurora, BRAF, CDK, CDK2, CDK5, CHK1, C-MET, CSFR1, EGFR, FGFR, GRK2, GSK, HER, IKK, IRAK, JAK, JNN, LCK, MYT-1, P38 MAP, PDGFR, PI3K, PKA, PKB, PKC, PLK1, RAF, SRC, SYK, TGFR, TIE2, TK, VEGFR) by 16 different scaffolds, with each scaffold having a minimum of 5 targets

Supplemental Table 1: List of compound libraries used in the study and content description of each of the library set from ICCB-Longwood small molecule screening facility. Related to Figure 3

Number	Compound Names	Fold change		Number	Compound Names	Fold change	
		Total cell counts	Caspase 3+ counts			Total cell counts	Caspase 3+ counts
1	SDM25N	0.2446	1.9599	26	Doxycycline hyclate	0.6408	1.7633
2	Pyrithione zinc	0.4158	2.9319	27	WHI-P154	0.645	0.9309
3	Digitoxin	0.4238	1.5281	28	Pimozide	0.645	0.9095
4	Doxifluridine	0.4508	2.0884	29	Quinacrine	0.6483	1.6384
5	Ftorafur	0.4627	3.7194	30	SP-600125	0.6549	1.6197
6	L-703,606 Oxalate	0.4845	0.2814	31	Dioscin	0.658	1.3874
7	Cladribine	0.4854	5.5569	32	(R)-SLV 319	0.6628	0.4677
8	Clofazimine	0.5146	1.3694	33	5-Nonyloxytryptamine	0.6638	1.9585
9	Ouabain	0.5365	2.2016	34	T 0070907	0.6671	2.8365
10	Azacitidine	0.5556	2.9755	35	SNAP 5089	0.6678	1.8827
11	Terfenadine	0.562	4.339	36	BVT 948	0.6719	1.772
12	Bay 11-7821	0.5682	4.0154	37	Phorbol	0.6758	1.6242
13	Darapladib	0.5774	1.5815	38	Penfluridol	0.6767	1.1929
14	Mibefradil	0.5822	0.6806	39	Ionomycin	0.6794	0.9221
15	3,4-DAA	0.5849	0.6874	40	Xylazine HCl	0.6807	1.3269
16	Go6976	0.5909	1.0553	41	PFK15	0.6814	0.506
17	Cantharidin	0.5923	2.5958	42	QNZ EVP4593	0.6834	3.233
18	Lanatoside c	0.5998	2.3108	43	Meclocycline	0.6838	2.9329
19	Tannic acid	0.6021	1.4585	44	Minocycline HCl	0.6889	1.8871
20	HMB-Val-Ser-Leu-VE	0.6096	0.7812	45	ARQ 621	0.6895	0.42
21	Elvitegravir	0.6108	1.2621	46	NNC 05-2090 Hcl	0.6939	1.5537
22	Cetrimonium Bromide	0.6155	3.2117	47	Stattic	0.6976	0.9635
23	Sertraline hydrochloride	0.6205	0.9981	48	GSK J4 HCl	0.6982	1.3702
24	Nifedipine	0.6242	1.7792	49	1-Linoleoyl Glycerol	0.6983	0.4773
25	Lacidipine	0.6353	1.5741	50	PD 158780	0.6988	1.7043

Supplementary Table 2: Molecules used in cherry pick screen along with the % change in total cell counts and caspase 3+ cell counts. Comparison was done between control cells and miR-34a induced cells. Related to Figure 3.

Drug name	Growth inhibition 30 (GI30)		% Decrease in GI30	Increase in Caspase 3+ cells
	DMSO Control	DOX +		
Minocycline HCl	14.15	1.42	89.96466431	Yes
Digitoxin	0.062	0.017	72.58064516	Yes
Ouabain	0.021	0.003	85.71428571	Yes
Meclocycline	9.63	2.882	70.07268951	Yes
Cantharidin	2.479	0.734	70.39128681	Marginal
Doxycycline	13.62	4.539	66.67400881	Yes
Mibefradil	13.177	4.958	62.37383319	Marginal
Phorbol	30.858	0.009	99.97083414	Yes
Penfluridol	33.22	4.832	85.45454545	Yes
SP-600125	19.548	3.62	81.48148148	Yes
T 0070907	33.22	11.442	65.55689344	Marginal
Nifedipine	35.5	12.029	66.11549296	Yes
Lacidipine	33.22	7.254	78.16375677	Yes
QNZ - EVP4593	10.776	2.954	72.58723088	Yes
Elvitegravir	3.811	0.819	78.50957754	Yes

Supplementary Table 3: List of molecules used in combination index calculations. GI30 calculated from dose response of individual drugs selected from cherry pick screen. Related to Figure 3.

Transparent Methods

Cell line maintenance, microRNA mimic transfections, and establishing cell lines for screen

All cell lines used in the study were maintained in 5% CO2, 37^0 C standard cell culture incubators, with weekly media change and close monitoring. All the cell lines used in the study were obtained from American type cell culture collection (ATCC) and maintained in RPMI1640 medium supplemented with 15% FBS and antibiotics. All cell lines were routinely tested for mycoplasma. Lung cancer patient derived xenograft (PDX) was obtained and processed as previously discussed (Zhang et al., 2019). All research involving PDX studies were granted ethical approval by the Beth Israel Deaconess Medical Center. Prior to miRNA transfections, spheroid cultures were established from PDX primary cultures in geltrex (Gibco) as support matrix (25 µl/well) mixed with complete growth media (20 µl/well) in 96-well non-treated clear plates (Corning). The complete growth media composition is published previously (Zhang et al., 2019). For each 96 wells containing spheroids, 10^3 cells were plated in the above mixture of Matrigel and media, incubated for 7 days before transfection with miRNA control and miR-34a as per below protocol.

For miRNA transfections, RNAiMAX Lipofectamine (Life Technologies) was used as a transfection agent, and the ratio of RNAiMAX to a specific miRNA was 2:1 (nM to ul). The concentration of miRNAs used for transfection was set to 40nM. Briefly, miRNA mimics for miR-34a and control miRNA (Life Technologies) were diluted in Opti-MEM media and liposomal complex was prepared by adding RNAiMAX reagent. The mixture was incubated for 15 min before adding to the cell mixture in complete media and incubated for the specific time frame of the studies mentioned below.

For the small molecule screen, A549 and HCC827 cells were transduced with lentiviral particles containing miR-34a overexpression system under tight control of a tetracycline responsive element (TET, doxycycline inducible). Using constitutive eGFP, cells were selected in two rounds for actively transduced cells. For inducing miR-34a expression, doxycycline (Sigma Aldrich) was added at different doses in 6 well plate cultures of A549 and HCC827 cells previously transduced with lentiviral particles containing miR-34a over expression plasmid. Using qPCR, induction of miR-34a expression and downstream target CD44 (Liu et al., 2011) and SRC (Adams et al., 2016) mRNA changes were measured.

High-throughput screen (HTS) to identify miR-34a synergistic small compounds

HTS was standardized and performed against 9908 small molecule compounds which are part of biologically active small molecule collection at the ICCB Longwood screening facility (Harvard Medical School). In 384 well plates, assays to count the total number of cells and activated caspase using Nucview 549 probe (Biotium) were standardized. As a positive control, Erlotinib at IC50 dose for A549 cells was used in every screening plate (two replicates per compound). Post plating A549-miR-34a cells with 1 ug/ml of doxycycline, compounds were transferred to individual wells using a pin-transfer system, and the plates were incubated at 37°C in 5% CO_2 for 72 hr. After the incubation period, cell plates were removed, washed with PBS and incubated with Hoechst 33342 (Thermofisher) and Nucview 549 (Biotium) for 10min and cells were fixed with 4% paraformaldehyde. Signals from each well were quantified

using Acumen eX3 high content imager (TTPLabtech) and data plotted as % total cells and % caspase positive cells compared to control DMSO treatments. The Z-factor was used for quality control purposes and each plate's positive control and negative control readings were used for calculations. A Z-factor of 0.5 was used as cutoff for quality control of each plate and assay. The top 10% of compounds with the highest decrease in viability and further cutoff of an increase in caspase 3+ cells (n=58) were used for a cherry pick screen with a dose response curve. Further combination index analysis on the top selected candidates were performed with miR-34a or miR-control at increasing doses in a matrix of increasing dose of compounds. Data analysis using Combenefit (Di Veroli et al., 2016) tool with Loews additivity combination calculations were carried out. The top two compound with significant synergy points were used for further analysis. Raw data from the screen deposited at Pubchem with AID 1347080.

Immunoblotting and Quantitative real-time PCR

For Western blotting, lysates from cultured cells were prepared using modified RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton, 0.5% deoxycholate) plus Halt phosphatase and protease inhibitor cocktail (Pierce Biotechnology) at 1x concentration. The protein concentrations were determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology). Lysates were loaded and separated on SDS–PAGE. Proteins were transferred to a nitrocellulose membrane by semidry electrophoresis (Bio-Rad Laboratories) overnight, blocked with 5% BSA for 1 h and then incubated at 4 °C overnight with primary antibody against LC3A/B 1:500 (Novus Biologicals). After washing with TRIS-buffered saline with Tween 20, the membrane was incubated with horseradish peroxidase-conjugated horse anti-Mouse or Rabbit IgG (1:2,000, GE Healthcare) for 2 h. Horseradish peroxidase was visualized using an enhanced chemiluminescence detection kit (Pierce Biotechnology). To confirm equal sample loading, the blots were probed with an antibody specific for β-actin (0.1 μg ml^{-1}; Sigma).

For mRNA and miRNA quantification, total RNA was isolated using the Direct-zol™ RNA MiniPrep Plus kit following the manufacturer protocol (Zymo Research). For mRNA measurements, 1,000 ng of RNA was used to synthesize complementary DNA using a Verso cDNA kit (Thermo Scientific), as per the manufacturer's instructions. Analysis of mRNA levels was performed on a Roche 384 Real-Time PCR System (Roche) using pre-validated (Qiagen) primers specific for each of the genes. Semi-quantitative real-time PCR was done with reverse-transcribed RNA and 100 ng μl^{-1} sense and antisense primers in a total volume of 10 μl. For miRNA quantification TaqMan miRNA assays (Life Technologies) were used and reverse transcription, real-time PCR were carried out, according to the manufacturer's instructions. *RNU6B* (for mature miRNAs) or *18S* (mRNAs) were used as a housekeeping gene.

Detection of autophagy in miR-34a and ouabain treated cells

Measurement of autophagy in cells treated with control miRNA and miR-34a with or without ouabain was carried out using the Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit (Thermo Fisher Scientific) as described in the manufacturer's instructions. The RFP-GFP-LC3B sensor enables the detection of LC3B positive, neutral pH autophagosomes in green fluorescence (GFP) and LC3B positive acidic pH autophagolysosome in red fluorescence (RFP). The cells were grown on chamber slides with transfection of miR-34a or miR-control. Following transfections,

cells were treated with ouabain (30nM) or DMSO control and incubated with BacMam Reagents containing the RFP-GFP-LC3B overnight. Treatment was done for total of 24hrs and cells were washed with PBS, nuclei were stained with Hoechst 33342. Fluorescent images were taken using confocal microscopy (Carl Zeiss Meditec). LC3B positive autophagosomes (green) and LC3B positive autophagolysosome (red) were analyzed as ratio of percentage of RFP and GFP positive cells compared total cells per field (200x).

Statistics

For all the data presented, graphs were plotted using Prism (Graphpad Software). For all statistical comparisons, Student's t test or ANOVA were used with multiple comparison tests. P value for significance was set at minimum of 0.05. Data is representative of triplicate experiments, unless otherwise mentioned in figure legends.