Cell-Based Screening of Focused Bioactive Compound Libraries: Assessing Small Molecule Modulators of the Canonical Wnt Signaling and Autophagy-Lysosome Pathways

Leading Light™ Wnt Reporter Assay (ENZ-61001)
Screen-Well™ Autophagy Library (Cat No. BML-2837)
ProteoStat® Aggresome Detection Kit (Cat No. ENZ-51035)
Cyto-ID® Autophagy Detection Kit (Catalog No. ENZ-51031-K200)

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ABSTRACT

Wnt signaling has been extensively investigated as an effector of diverse physiological processes including neuronal development and plasticity, bone development and formation, as well as pathological diseases, such as cancer and neurodegeneration. Recently, autophagy has been shown to down-regulate the Wnt signal transduction pathway via targeted degradation of a key signaling protein, Dishevelled [Nature Cell Biology 12,781–790(2010)]. Given the known relationship of both the Wnt pathway and autophagy to oncology and neurodegenerative disease, understanding the interplay between the two pathways is of significant biomedical importance. Consequently, an autophagy pathway library, consisting of 96 compounds with defined autophagy-inducing or -inhibitory activity, was evaluated using a battery of cell-based assays relevant to the two pathways. A high-throughput mammalian cell-based assay utilizing a transcription-based luciferase reporter of Wnt/β-catenin signaling was employed as well as fluorescence-based assays for detection of autophagy and intracellular protein aggregate accumulation. The highlighted battery of assays should provide insight into regulatory cross-talk between the canonical Wnt and autophagy-lysosome pathways.

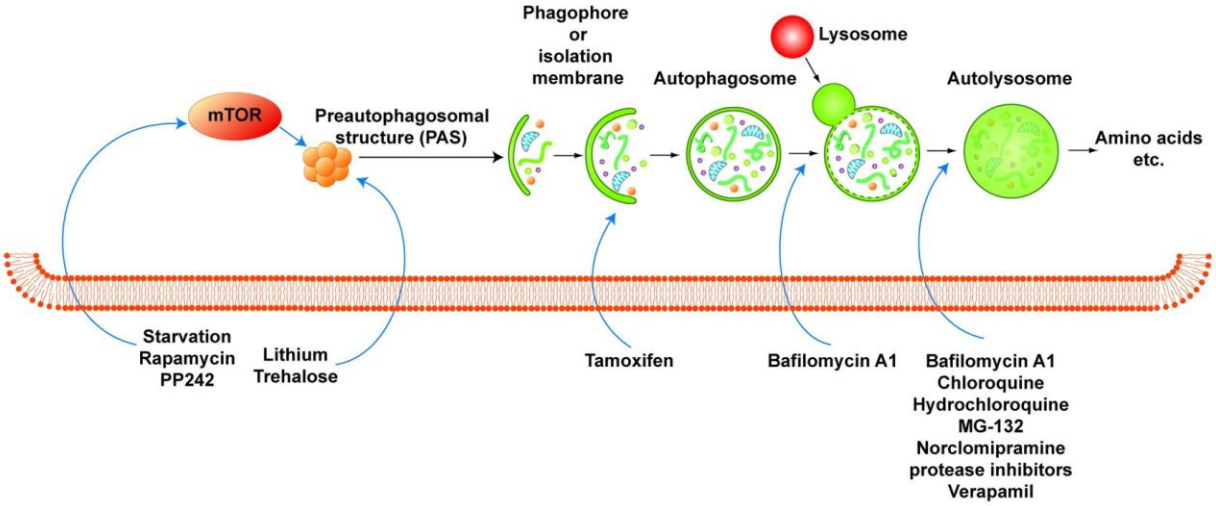

Figure 1: Schematic depiction of autophagy. Cytosolic material is sequestered by an expanding membrane sac, the phagophore, resulting in the formation of a double-membrane vesicle, an autophagosome. The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded in the autolysosome. Various regulators of autophagy are also depicted.

BACKGROUND

In eukaryotic cells, autophagy is a highly conserved self-digestion process to promote cell survival in response to nutrient starvation and other metabolic stresses. Deregulation of autophagy has been associated with a variety of human diseases, including cancer. During autophagy, double-membrane vesicles, called autophagosomes, are formed to deliver cytoplasmic materials to lysosomes, where the encompassed cargos are ultimately degraded (Figure 1). Autophagy is regulated by cell signaling such as the mammalian target of rapamycin (mTOR) pathway. However, the significance of autophagy in modulation of signal transduction is unclear.

Wnt signaling has key functions in development, tissue self-renewal and tumorigenesis. Binding of Wnt ligands to their cell-surface receptors activates several signaling pathways. In the canonical pathway, Wnt-initiated binding of Dishevelled (Dsh) to Frizzled and of axin to LRP5/6 results in the disassembly of the β-catenin destruction complex and consequently leads to the accumulation of β-catenin in the nucleus. Together with the transcription factors of the lymphoid enhancer-binding factor/T-cell factor family, β-catenin regulates the transcription of Wnt target genes.

Dsh also has key functions in the non-canonical Rho/c-Jun N-terminal kinase (JNK) planar cell polarity and Ca2+-dependent Wnt pathways. However, the mechanism governing the activity and stability of Dsh is not fully understood. It has been suggested that phosphorylation by casein kinases can potentiate Dsh activity and that the stability of Dsh proteins is influenced by ubiquitination-dependent proteasomal degradation. Recent studies suggest that autophagy negatively regulates Wnt signaling by promoting Dsh degradation. Von Hippel–Lindau protein-mediated ubiquitination is critical for the binding of Dsh2 to p62. This binding step facilitates the LC3-mediated autophagosome recruitment of Dsh2 under starvation. Ubiquitinated Dsh2 aggregates are ultimately degraded through the autophagy–lysosome pathway. A reverse correlation between Dsh expression and autophagy is observed in late stages of colon cancer development, indicating that autophagy may contribute to the aberrant activation of Wnt signaling in tumor formation.

MATERIALS AND METHODS

A Luminescent Cell-Based Microplate Assay for HTS of Compounds Modulating Autophagy

Leading Light™ Wnt Reporter Assay cells were seeded in white-wall 96-well plates and treated overnight with compounds at a concentration of 1μM from the Screen-Well® Autophagy library. To determine the effect of the Wnt antagonists, the same cells were simultaneously treated with 100 ng/mL of recombinant Wnt3a protein. The next day, the activity of compounds was detected using luciferase reagents (supplied in the kit). To account for the toxicity of screened compounds, standard MTT test was performed in parallel on the cell samples treated in the same manner.

A Fluorescent Cell-Based Microplate Assay for HTS of Compounds Modulating Autophagy

HeLa cells were seeded in black-wall 96-well plates and treated with 1 μM compounds from the Screen-Well® Autophagy library overnight. Each compound was screened in triplicate. The following day, cells were stained with Cyto-ID® Autophagy Green Detection Reagent and Hoechst 33342 nuclear stain (to account for the toxicity of screened compounds and to normalize the green signal). The accumulation of autophagic vesicles was detected using a BioTek Synergy™ Mx multiplate fluorescence reader. Cyto-ID® Autophagy Green fluorescence was measured using ex/em 480/530 settings and Hoechst 33342 fluorescence was measured with ex/em 340/480 settings. To quantitate autophagic vesicles formation, the ratio of normalized green signal of treated cells vs mock-treated (DMSO) was calculated for each compound.

A Flow Cytometry Assay for Screening of Compounds Modulating Autophagy

Cell Lines used: HeLa, Jurkat, Leading Light™ Wnt reporter cell line. Cells were treated overnight with select compounds (a total of 32) from the Screen-Well® Autophagy library at a concentration of 1μM. The following day, cells were washed and stained with Cyto-ID® Autophagy Green Detection Reagent. Accumulation of autophagic vesicles was analyzed by flow cytometry using blue (488nm) laser and FITC or PE channel for the signal detection. Results were quantified using Kolmogorov-Smirnov statistical assay for the fluorescence signal of treated vs. mock-treated cells and presented as D-values. D>0.2 was accepted as a positive autophagic value.

A Fluorescence Microscopy Assay of Intracellular Protein Aggregate Accumulation

HeLa cells were seeded on the microscope slides and treated with select compounds from the Screen-Well® Autophagy library at a concentration of 1μM. The following day, cells were washed, fixed with paraformaldehyde, permeabilized, and stained with ProteoStat® Aggresome Detection Reagent and Hoechst 33342 nuclear stain. Aggregated protein accumulation was detected using fluorescent microscopy (standard Texas Red filter set for the aggresome signal and DAPI filter set for the nuclear signal imaging)

RESULTS

Detecting Wnt Modulators Within the Screen-Well® Autophagy Library

Leading Light™ Wnt Reporter Assay cells were seeded in 96-well plates and treated as described in Materials and Methods. Simultaneous treatment with recombinant Wnt3a protein was performed to assay Wnt antagonists. Figure 2A shows Wnt agonists detected through screening of the Screen-Well® Autophagy library. Recombinant Wnt3A at a concentration of 100ng/mL was used as a positive control. Results are presented as an average +/- Standard Error (SE)-fold induction of luciferase signal over mock-treated (DMSO) cells. Figure 2B shows Wnt antagonists detected through screening of the Screen-Well® Autophagy library. Results are presented as an average +/- SE-decrease of luciferase signal compared to a signal from rWnt3a-treated cells. In both assays, three or more independent experiments were performed.

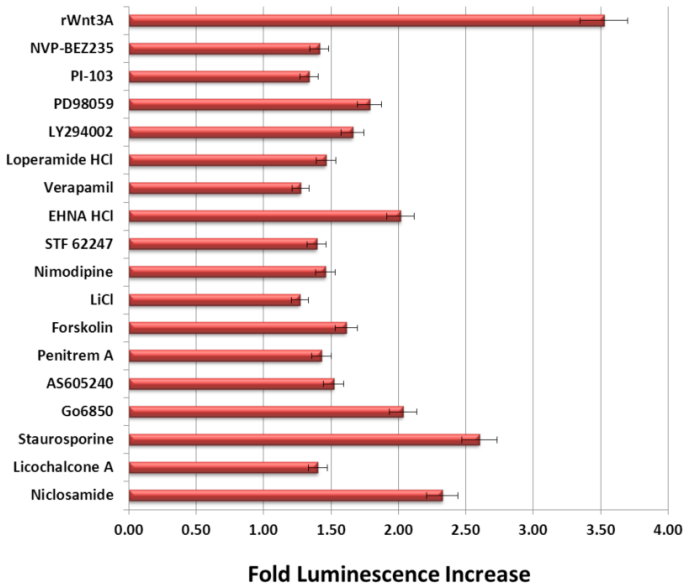

Figure 2A. Wnt agonists identified by screening Autophagy Library in the Leading Light™ Wnt Reporter Assay. Results are displayed as fold induction relative to mock (DMSO)-induced activation.

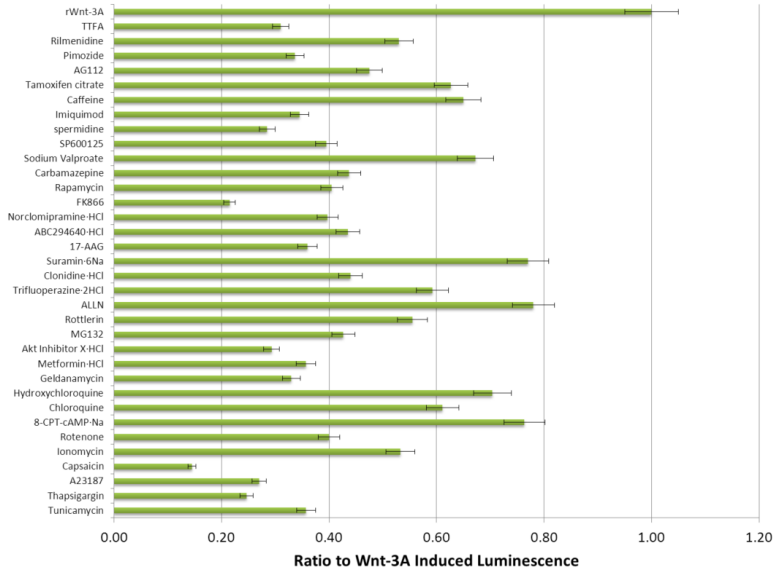

Figure 2B. Wnt antagonists identified from Screen-Well Autophagy Library, using the Leading Light™ Wnt Reporter Assay. Results are displayed as the ratio of luminescence relative to activation with rWnt3a.

Forskolin	Rapamycin	Timosaponin A-III	NVP-BEZ235	PI-103	AS605240	Lithium Chloride	L-690,330	Carbama-zepine	Sodium Valproate	Verapamil·HCl	Anisomycin
(±)Bay K8644	Loperamide ·HCl	Amiodarone·HCl	Nimodipine	Nitren-dipine	Niguldipine	Penitrem A	Ionomycin	**Rotenone**	TTFA	**Nocodazole**	PD-98059
Norclomipra mine·HCl	Trifluopera-zine·2HCl	Sorafenib tosylate	Niclosamide	Ro lerin	Caffeine	Me ormin·HCl	Clonidine ·HCl	Rilme-nidine	2',5'-Dideoxyade nosine	3-MA	AICAR
Hydroxy-chloroquine	Pimozide	STF-62247	Spermidine	FK-866	Tamoxifen citrate	Minoxidil	Imiquimod	Ima nib mesylate	AG112	Rolipram	Quinine HCl·2H2O
Chloroquine	SB202190	**Brefeldin A**	**Tunicamycin**	**Thapsigargin**	A23187	Capsaicin	Dihydrocapsa icin	Glucosamine HCl	DTT	Pifithrin-μ	Tolazamide
SP600125	ABC294640 ·HCl	Lico-chalcone A	Curcumin	Plumbagin	**6-Gingerol**	Akt Inhibitor X·HCl	PMSF	MG132	ALLN	Deoxy-cholate·Na	SB-216763
Wortmannin	17-AAG	**Gelda-namycin**	C1	Z36	Rockout	Go6850	2-Deoxyglucose	Etoposide	SMER28	Trehalose	EHNA·HCl
LY294002	C2-dihydrocera mide	Temozo-lomide	Resveratrol	**Stauro-sporine**	Bafilomycin A1	Dibutyryl cAMP·Na	Cyclo-heximide	SU11652	Suramin ·6Na	DMSO	8-CPT-cAMP·Na

Figure 3. Screening of autophagy induction using Cyto-ID Autophagy Detection dye and Autophagy compound library in a microplate-based assay. Light. shaded cells correspond to 1.5-2-fold increase in signal relative to DMSO, with compounds inducing >2 fold increase in signal indicated by dark green shading.

Screen-Well® Autophagy Library compound screening using a fluorescence cell-based microplate assay

HeLa cells were seeded in 96-well plates, treated and stained. Accumulation of autophagic vesicles was detected by analyzing the fold increase of fluorescence relative to mock-treated (DMSO) cells. The plate layout (Figure 3) shows specific compounds that were screened. The filled wells correspond to a 1.5 - 2-fold increase in fluorescent signal. The names of the compounds yielding more than a 2-fold fluorescent increase are in bold. The scheme represents the results from three independent experiments.

Flow cytometry-based screening of compounds modulating autophagy and Wnt pathway in live cells

Screening of compounds was also evaluated by flow cytometry using the Cyto-ID® Autophagy Green Detection Reagent. The results of screening Wnt antagonists are presented in Table 1A, Wnt agonists in Table 1B, and select compounds that are not known to modulate Wnt pathway (inactive compounds) are presented in Table 1C, as a control. D-values were calculated using a Kolmogorov-Smirnov statistical analysis of three independent experiments (Threshold value D>0.2 is accepted for positivity, shown in red). SE values do not exceed 15%.

Table 1A Antagonists

Wnt Antagonists	Jurkat	HeLa	Leading Light™
Geldanamycin	0.51	0.35	ND
Tunicamycin	0.24	0.38	0.22
Rottlerin	0.26	0.37	0.04
Thapsigargin	0.55	0.37	ND
A23187	0.47	0.22	ND
Capsaicin	0.41	0.05	0.04
Akt Inhibitor XHCl	0.39	0.50	0.01
Ionomycin	0.47	0.21	ND
Rotenone	0.51	0.33	ND
MG132	0.42	0.47	ND
Rapamycin	0.31	0.22	0.09
Chloroquine	0.40	0.12	ND
Hydroxychloroquine	0.24	0.20	0.07
Norclomipramine HCl	0.30	0.16	ND
8-CPT-cAMP-Na	0.26	0.04	ND
Metformin HCl	0.18	0.09	0.38
SP600125	0.40	0.12	0.19
Wortmannin	0.57	0.10	0.08
Anisomycin	ND	0.66	0.43
Nocodazole	0.26	0.51	ND
Brefeldin A	ND	0.62	ND
UT Control	0.01	0.02	0.01

Table 1B Agonists

Wnt Agonists (B)	Jurkat	HeLa	Leading Light™
Verapamil-HCl	0.22	0.15	0.06
Forskolin	0.25	0.12	ND
Licochalcone A	0.33	0.31	0.05
LY294002	0.35	ND	ND
Go6850	0.23	ND	0.05
Penitrem A	ND	0.17	ND
PD 98059	0.21	0.15	0.07
Niclosamide	ND	0.82	ND
UT Control	0.01	0.02	0.01

Table 1C Controls

Inactive Compounds (C)	Jurkat	HeLa	Leading Light™
3-MA	0.31	0.31	
Rolipram	0.16	0.20	0.04
(+) Bay K8644	0.24	0.11	0.13
UT Control	0.01	0.02	0.01

A fluorescence microscopy assay of intracellular protein aggregate accumulation in cells treated with autophagy-modulating compounds

HeLa cells were seeded, treated with 1 µM compounds, and stained with ProteoStat® Aggresome Detection Reagent and Hoechst 33342 nuclear stain as described in the Materials and Methods section. Aggregated protein accumulation was detected using fluorescent microscopy (Figure 4).

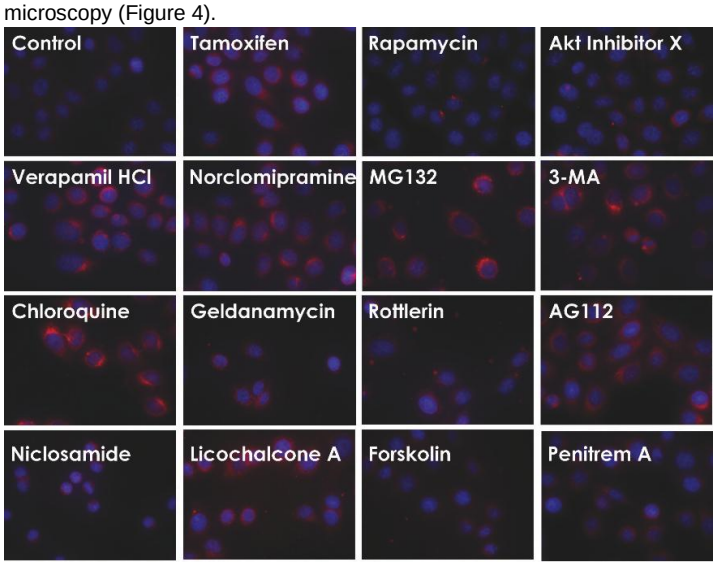

Figure 4. Aggregate accumulation (red) in HeLa cells following treatment with autophagy-modulating compounds (60X magnification).

CONCLUSIONS

The Screen-Well® Autophagy compound library was screened using the Leading Light™ Wnt Reporter Assay Kit. Sets of Wnt agonists and antagonists were identified among the compounds. Most of the Wnt antagonists identified by this screen are known to be positive modulators of autophagy pathway or mitochondrial poisons.

The Screen-Well® Autophagy Compound library was also screened in live cells using Cyto-ID® Green Detection Reagent and fluorescence microplate assay. A select group of compounds that increased accumulation of autophagic vesicles was identified through this work. The correlation between the set of Wnt antagonists and the set of positive autophagy modulators defined by the plate autophagy assay (23 out of 42 compounds) was greater than 50%. Interestingly, 6 compounds of the 42 (14%) identified by the plate autophagy assay were also found to be Wnt agonists. A discrepancy between the two sets could be attributed to cell line phenotype differences, non-optimized treatment conditions or to different threshold settings for the two assays.

Additional data corroborating the link between Wnt down-regulation and accumulation of autophagic vesicles were obtained using flow cytometry. Out of 21 screened Wnt antagonists, 15 compounds (70%) induced autophagic vesicles increase (green fluorescence) in more than two cell lines. Out of 8 screened Wnt agonists, only licochalcone A induced an increase in green fluorescence (from autophagic vesicles) in both Jurkat and HeLa cell lines. It was noted that all compounds tested induced an increase in fluorescence in the Jurkat cell line.

Non-Wnt modulators demonstrated variable effects on the autophagic vesicles increase. The set of compounds, which both down-regulated Wnt and induce accumulation of autophagic vesicles, was analyzed for its ability to increase protein accumulation and aggresome formation in the cells. The results suggest that Wnt antagonists have variable effects on the protein aggregation in the cells. These effects are dependent on the stage at which the compounds target the autophagy signaling cascade. Given the known connections of both the Wnt pathway and autophagy to cancer, understanding the interplay between the two is of clear medical importance.

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