Kinase inhibitor-induced cell-type specific vacuole formation in the absence of canonical ATG5-dependent autophagy

Susan Jose[1,#], Himanshi Sharma[1,#], Janki Insan[1,2], Varun Arora[1], Sonam Dhamija[2,3], Nabil Eid[4] and Manoj B. Menon[1,*]

[1]Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, New Delhi, 110016, India.

[2]CSIR-Institute of Genomics and Integrative Biology, Mathura Road, 110025 New Delhi, India.

[3]Academy of Scientific and Innovative Research (AcSIR), 201002 Ghaziabad, Uttar Pradesh, India.

[4]Department of Anatomy, Division of Human Biology, School of Medicine, International Medical University, 57000 Kuala Lumpur, Malaysia.

[#]These authors equally contributed to this work

*Correspondence to menon@bioschool.iitd.ac.in

Running title: SB202190 induced vacuoles and autophagy.

Abstract

Pyridinyl imidazole class p38 MAPKα/β (MAPK14/MAPK11) inhibitors including SB202190 have been shown to induce a cell-type specific defective autophagy response resulting in micron-scale vacuole formation and autophagy-dependent death. We had earlier shown that this is an off-target effect of SB202190. Here we provide evidence that the cell-type specific vacuole formation is independent of canonical autophagy pathway. While SB202190 seems to interfere with autophagic flux in many cell lines parallel to vacuolation, autophagy-deficient DU-145 cells and CRISPR/Cas9 gene-edited *ATG5* knockout A549 cells also undergo vacuolation upon SB202190 treatment. Further analyses identified the late-endosomal GTPase RAB7 as a marker for the SB202190-induced micron-scale vacuoles. A screen for modulators of SB202190-induced vacuolation revealed molecules including multi-kinase inhibitor Sorafenib as inhibitor of vacuolation. Moreover VE-821, an ATR kinase inhibitor was found to phenocopy the cell-type specific vacuolation response of SB202190. To identify the factors determining the cell-type specificity of the vacuolation response induced by SB-compounds and VE-821, we compared the transcriptomics data from vacuole forming and non-vacuole forming cancer cell lines and identified a gene expression signature which may define sensitivity of cancer cells to these small-molecule kinase inhibitors. Further analyses using the small molecule tools and the gene signature discovered here, could reveal novel mechanisms regulating this interesting phenotype relevant to anti-cancer therapy.

Key words: autophagy; SB202190; kinase inhibitor; VE-821; endosome; RAB7

Introduction

Small molecule kinase inhibitors are sought after tools for therapeutic intervention against diverse pathologies including cancer, inflammation and metabolic disorders (reviewed in (Attwood et al., 2021)) and have been developed and tested against cancer and inflammation (Bhullar et al., 2018;Zarrin et al., 2021). The discovery of p38 mitogen-activated protein kinases (MAPKs) have been closely linked with the development of kinase inhibitors to target chronic inflammatory disorders. Pyridinyl imidazole class p38 MAPKα/β (MAPK14/MAPK11) inhibitors including SB202190 and SB203580 have facilitated extensive research on the physiological roles of p38 signaling. Despite widespread use of SB202190/SB203580 as specific MAPK14/MAPK11 inhibitors, there are several additional kinase targets known for these molecules including BRAF, CK1, GAK, GSK3β, LCK, PDK1, RAF1, RIPK2 and TGFBR2 (Eyers et al., 1998;Lali et al., 2000;Bain et al., 2007). In addition, these SB compounds have also been shown to target non-kinase proteins including mediators of inflammation. We had earlier shown that SB202190 could effectively and specifically target BRAF-V600E mutant oncogenic kinase to suppress ERK1/2 activity in colorectal cancer and melanoma cells (Menon et al., 2011). A recent study has verified the use of these pyridinyl imidazole class inhibitors for dual targeting of BRAF and mTORC1 pathways against melanoma (Palusova et al., 2020). Moreover, sensitivity to SB202190 treatment was shown to be effective in predicting BRAF-activating mutations in patient-derived colorectal cancer organoids (Costa et al., 2023).

Autophagy is a catabolic process wherein cytoplasmic contents including organelles are sequestrated into autophagosomes, followed by subsequent fusion with lysosomes and degradation of contents in the autolysosomal compartments (Galluzzi et al., 2017;Klionsky et al., 2021). High rates of basal autophagy are prevalent in several cancers, which provide them resistance to metabolic stress, making autophagy a target for anti-cancer therapy (Bhat et al., 2018). Nutrient deprivation is the strongest activator of autophagy and different nutrient sensing pathways tightly regulate autophagy and cell death under nutrient deprived conditions. While mTOR signaling is the primary regulator of autophagy initiation in mammalian cells, various stages of autophagy are regulated by p38, ERK1/2, AMPK and the PI3K-III (phosphatidylinositol 3-kinase, class III) complex (Wang et al., 2009;Kim et al., 2011;Keil et al., 2013). The PI3K-III complex consisting of BECN1 protein regulates both initiation and maturation of autophagosomes, integrates signals from multiple signaling pathways and also regulates the balance between cell death and survival (Menon and Dhamija, 2018). Initiation of autophagy in response to nutrient or energy deprivation requires assembly and activation of the ULK1 (unc-51-like kinase 1) complex, which leads to the formation of a phagophore, facilitated by the ATG5-ATG12-ATG16 complex mediated lipidation of LC3/ATG8 protein. The phagophore expand and engulf autophagy cargo and form a double membrane autophagosomes which finally undergo maturation and fusion with lysosomes to form autolysosomes, degrading the contents and releasing vital metabolites (Li et al., 2020). Canonical autophagy is abrogated in ATG5- and ATG7-deficient cells, however recent evidence suggests the presence of a non-canonical pathway where RAB9 GTPase mediates the elongation of the initial autophagosomal membrane independent of ATG5-mediated LC3-lipid conjugation (Nishida et al., 2009).

Studies using SB202190 had previously established a pro-tumorigenic role for p38α/MAPK14 in colon cancer and SB202190 treatment was shown to induce cell-type specific autophagy-dependent cell death (ACD)(Comes et al., 2007;Chiacchiera et al., 2009). This initiation of ACD was attributed to a transcriptional shift from hypoxia-dependent to Foxo-3A-dependent pro-autophagic gene expression

(Costa et al., 2023). In our previous work we could show that the cell-type specific effects of SB202190 are independent of p38 inhibition and transcriptional reprogramming (Menon et al., 2011). The SB202190-induced vacuoles are unusually large to be autophagosomes and are reminiscent of a defect in autophagy clearance, resulting in enlarged vacuoles. We further investigated this cell-type specific phenotype and present evidence here that the vacuolation induced by SB compounds do not require canonical autophagy. Moreover, we identified small molecule modulators of SB202190-induced vacuolation. While multi-kinase inhibitor Sorafenib was among the molecules which inhibited SB202190-induced vacuolation, VE-821, an ATR inhibitor was found to phenocopy the cell-type specific vacuole forming effects of SB202190. We also discovered a gene expression signature which defines susceptibility of cancer cell lines to SB202190-induced autophagy-independent vacuolation.

Materials and Methods

Reagents

SB202190 (Cayman,#1000399), SB220025 (Sigma, S9070), Bafilomycin A1 (Cayman, #11038), BIRB-796 (Cayman, #10460), Leupeptin (Cayman, #14026), Chloroquine (Sigma, C6628), Apilimod (MedChemExpress, HY-14644), Vacuolin-1 (MedChemExpress, HY-118630) and all other inhibitor stocks were prepared in DMSO. Antibodies for LC3A/B (#12741), ATG5 (#12994), ATG12 (#4180) were from Cell Signalling Technology, MA, USA. Rab7 (#A12308) and Rab11a (#A3251) antibodies were from Abclonal, MA, USA and p62/SQSTM1 (#BB-AB0130) was from Bio-Bharati Life Science, West Bengal, India and GAPDH (#GTX100118) was from GeneTex, CA, USA. Goat Anti-Rabbit IgG(H+L) (HRP conjugated, #111-035-045) was from Jackson ImmunoResearch Laboratories, PA, USA and Goat Anti-Rabbit IgG (H+L)(AF488 conjugated, E-AB-1055) was from Elabscience, Wuhan, China. Phalloidin iFluor™ 647 Conjugate (#20555) was from Cayman chemicals. DMEM (#11965092), FBS (#10270106), Trypsin-EDTA (#15400054), Antibiotic-antimycotic solution (#15240062) were purchased from Thermo Fisher Scientific, MA, USA. DMEM/F12 (#AL215A), EBSS (#TL1110), Sodium pyruvate solution (#TCL015), Bovine Serum Albumin (BSA) (#MB083) and Dimethyl Sulfoxide (DMSO) (#TC185) were obtained from HiMedia Laboratories, Mumbai, India. Details of the inhibitors used in the screen is provided in Supplementary table S1. Other biochemicals were obtained from Sisco Research Laboratories (SRL), Mumbai, India.

Cell culture and Treatments

HCT116, HT29, 293T, L929, HeLa, DU145, U-87MG, MDA-MB237 and MCF7 cells were maintained in DMEM with 10% FCS. HCT116, HeLa, DU145 and MCF7 were additionally supplemented with 1mM Sodium pyruvate solution. MCF10A was maintained in DMEM/F12 + 5% Horse serum + 20 ng/ml EGF + 0.5mg/ml hydrocortisone + 100 ng/ml cholera toxin + 10 µg/ml insulin and A549, PC-3 and HaCaT cells were maintained in DMEM/F12 with 10% FCS. All cell lines other than MCF10A were maintained in the presence of 1x antibiotic-antimycotic solution (#15240062, Thermo). All Cell lines were maintained at 37 °C with 5% CO2. Cells were treated with inhibitors at indicated concentrations in 12 well plates. DMSO was used as solvent control. All treatments were performed in normal growth medium for the respective cell lines. SB202190 was used at 10 µM and BafA1 at 100 nM concentration, unless stated otherwise in the figure legends.

To delete *ATG5* gene from A549 cells, all in one CRISPR/Cas9 vector was used as described previously (Heckl et al., 2014). Previously described sgRNA sequences for *ATG5* deletion (Imam et al., 2016;Domagala et al., 2018) were synthesised as DNA oligos and cloned in pLKO5.U6-chiRNA.EF1a.hSpCas9-P2A-PAC.pre vector and lentiviral vectors were packaged using HEK293T cells,

as described previously (Menon et al., 2017). After transduction, cells were selected with puromycin (2ug/ml) and single cell clones were screened for ATG5 expression by Western blotting. Control and KO single-cell clones were isolated and further characterized.

Immunostaining and Microscopy

For experiments, cells were seeded in 12 well plates and treated with small molecule inhibitors. Live phase-contrast images were captured using camera attached to DIM150 microscope (Debro or Quasmo). Inhibitor treatments were for 24 h if not mentioned otherwise. Percentage vacuolation was calculated manually (Figure 3D) or by Image-J based image analyses as described below. For Immuno-fluorescence staining, cells were seeded on poly-L-lysine precoated glass cover slips. After treatment, cells were washed with 1X-PBS and fixed with 4% paraformaldehyde (PFA) for 2 mins at room-temperature (RT), followed by 20mins on ice. Cells were permeabilized with 0.25% Triton X-100 for 20 min at RT and blocked using 3% BSA-PBS for 1h at 4°C. Primary antibodies were used at a 1:100 to 1:200 dilution in 1% BSA–PBS for 2 h at RT. Alexa-488 labelled secondary antibodies were used at 1:250 dilution in 1% BSA-PBS at RT for 45 min, followed by DAPI (500ng/ml in PBS) staining for 10 min at RT. Imaging was performed using a Leica TCS SP8 confocal microscope with standard settings.

Image analyses for quantification of vacuolation

To quantify vacuolation in A549 cells in response to SB202190 and other inhibitors, ImageJ v1.54d (https://imagej.nih.gov/ij/) was used. For each image, the contrast was enhanced (saturated pixels=0.35%) and the green channel 8-bit image was extracted using the RGB-split tool. The threshold for each image was set manually (between 195-255 raw intensity values) to select vacuoles and the number of vacuoles was counted using the *Analyze Particle* feature (size=100-250, circularity=0.80-1.00). All these steps were recorded as a macro and run to analyse multiple images from each inhibitor treatment (4 images per treatment). To normalize the findings across experiments, the data was presented as percentage vacuolation, with the number of vacuoles in the SB202190-treated wells considered as 100%.

Western Immunoblotting

Cells were lysed directly in SDS gel loading dye after treatments and lysates separated on 12-14% SDS-PAGE gels, transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk for 1h and probed with primary antibodies for 16h and secondary antibodies for 1h. Blots were developed using ECL reagents (Westarnova 2.0, Cyanagen) and bands were visualized using ImageQuant LAS 500 (Cytiva) or Fusion Solo 6S chemiluminescence imager (Vilber).

Differential expression analyses and visualization

RNAseq read counts for cell lines in CCLE (Cancer Cell Line Encyclopedia) available on the Cancer Dependency Portal (DepMap, Release 23Q2) were downloaded on 24.07.2023 (Ghandi et al., 2019;DepMap, 2023). From the dataset, 15 cell lines with experimentally verified data for vacuolation phenotype in response to SB202190 were selected for further analyses. The RSEM values were adjusted to the nearest integer and only the genes with non-zero expression counts in ≥5 out of the 15 cell lines were used for differential gene expression analysis using DESeq2 (v1.38.3) in R (v4.2.3) (Love et al., 2014;R Core Team, 2023). DESeq results were sorted in the order of adjusted p-values and VST-normalized values for the top fifty genes were used to plot a heatmap in R. Volcano plot depicting the differentially expressed genes (|log2FC|>2 and *p*-adj <0.01) and the boxplots of selected genes were plotted using ggplot2 (v3.4.3)(Wickham, 2016).

Cell viability assays

Cell viability was measured in triplicates in 96-well plates using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. Cells were seeded at 4000 cells/well density in 96 well plates and were treated for 72hrs with indicated inhibitors. After the treatments, cells were incubated with 10ul MTT solution (5mg/ml in 1x Phosphate-buffered saline, #58945, SRL) for additional 2-3 h at 37°C. Formazan crystals were dissolved in 100ul DMSO and absorbance taken at 570nm, with 690nm as reference using a plate reader (Cytation 5, BioTek).

Gene expression analyses by real-time qPCR

RNA was isolated using the NucleoSpin RNA extraction kit (Macherey & Nagel) and reverse transcribed (Bio Bharati, BB-E0043) using random hexamer primers according to the manufacturer's instructions. qRT-PCRs were run on a CFX96 (Bio-Rad) device using TB Green Premix Ex Taq II (Tli RNase H Plus, Takara) and the gene expression was normalized to Cyclophilin and presented. Sequences of the primers used are listed in Supplementary Table S2.

Statistical Analyses

All immunoblot and microscopy results are representative of three independent results. Quantitative assays are performed in triplicates. Students 2-tailed t-tests were used to check the statistical significance of differences between sample groups in the quantitative assays. Additional details are mentioned in figure legends.

Results

SB202190-induces cell-type specific vacuoles which accompanies altered autophagy flux and is inhibited by bafilomycin A1.

We have previously shown that SB202190 and related pyridinyl imidazole family kinase inhibitors of p38α (MAPK14)/p38β (MAPK11) induce cell-type specific vacuolation attributed to defective autophagosomes (Menon et al., 2011;Menon et al., 2015). To understand the mechanisms leading to this strong phenotype, we expanded our studies to include more cell lines, in addition to those reported earlier (Table 1). While 22 out of 33 cell lines tested till date showed vacuolation upon treatment with SB202190 (10 µM for 24 h), different variants of HeLa cells gave different results. Previously, we have shown that the vacuole formation is due to off-target effects of the pyridinyl imidazole family of p38 MAPK inhibitors including SB202190 and SB203580 and is not phenocopied by more specific p38 inhibitors like BIRB796 or VX-745 (Menon et al., 2011). Interestingly, SB220025, a closely related pyridinyl imidazole-based p38 inhibitor of the same family also did not induce vacuoles (Figure 1A & 1B). This may hint towards structural differences in the pharmacophores determining the binding of the SB-family compounds to the autophagy/vacuole formation-relevant targets (Figure 1B).

We then investigated the effect of the lysosomal acidification inhibitor Bafilomycin-A1 (BafA1). SB202190-induced vacuole formation was inhibited by BafA1 treatment (Figure 1C). In addition, we monitored the levels of the autophagy substrate p62/SQSTM1 and the autophagosome marker LC3-II (lipid conjugated LC3) by immunoblotting. SB202190 treatment led to significant accumulation of the autophagy markers in A549, L929 and HEK293T cells, which was further enhanced by BafA1 treatment (Figure 1D-F). Consistent with the previous results, the pan-p38 inhibitor BIRB-796 did not alter the autophagy flux (Figure 1E & 1F). Autophagy flux analyses using the protease inhibitor leupeptin also led to an increase in LC3-II and p62 levels similar to BafA1 treatment (Figure 1F). Interestingly, the effect of SB202190 on the autophagy flux was not connected to vacuole formation, since it was also

observed in non-vacuole forming HEK293T cells (Figure 1F). This indicates the possibility that vacuole formation could be independent of alterations in the autophagy flux.

SB202190 induces vacuoles in inherently autophagy-deficient DU-145 prostate cancer cells.
In our effort to expand the cell type specific aspect of the vacuolation phenotype, we also tested the effect of SB202190 on the prostate cancer cell lines DU-145 and PC3. While PC3 cells did not display significant vacuole formation upon SB202190-treatment (Table 1), DU-145 cells formed vacuoles which were inhibited upon BafA1 co-treatment (Figure 2A). Consistent with the other cell lines, SB220025 did not induce vacuoles in this cell line. Interestingly, due to the presence of a mutation, which leads to the expression of a non-functional splice variant of ATG5 gene, DU-145 cells have previously been characterised as an autophagy-deficient cell line (Ouyang et al., 2013). We verified this by immunoblotting and there were no ATG5-ATG12 and LC3-II conjugates detectable in this cell line indicating the absence of active autophagosome biogenesis (Figure 2B). This unexpected discovery argues against the previous conclusions that the SB202190-induced micron-scale vacuoles are of autophagosome origin. To further understand the identity of these SB202190-induced acidic vacuoles, we performed immunofluorescence analyses of RAB7 and RAB11 in SB202190-treated cells. RAB7 is a small GTPase associated with late endosomes, involved in autophagosome and late-endosome fusion with lysosomes (Gutierrez et al., 2004;Langemeyer et al., 2018). The large vacuoles induced by SB202190 were clearly labelled by RAB7 in both autophagy-proficient HT29 cells and autophagy-deficient DU-145 cells (Figure 2C). Similar immunofluorescence analyses for RAB11, a marker for recycling endosomes (Lock and Stow, 2005) did not reveal any redistribution upon SB202190-treatment (Supplementary Figure S1). Thus, SB202190 induced vacuoles are swollen RAB7 positive vesicular compartments, which are sensitive to BafA1, but not obligately dependent of autophagy.

Gene-edited A549 cells confirm autophagy-independent formation of SB202190-induced vacuoles.
To conclusively prove the lack of involvement of canonical autophagy pathways in the generation of SB202190-induced micron-scale vacuoles, we performed CRISPR/Cas9-mediated gene editing in vacuole forming A549 lung adenocarcinoma cells. CRISPR/Cas9 all-in-one vector expressing Cas9 and sgRNA against *ATG5* gene was used to generate canonical autophagy-deficient cells (Supplementary Figure S2). After gene deletion, two independent clonal cell lines of each genotype (wild type – WT, ATG5-knockout – KO) were compared. The *ATG5* KO cells displayed higher basal levels of LC3-I and p62/SQSTM1 (Figure 3C). Moreover, upon induction of autophagy by amino-acid starvation, there was no detectable change in autophagic flux in the *ATG5*-KO clones compared to the control cell lines (Figure 3A). When SB202190 was used to alter the autophagy pathway, we observed similar results, with the *ATG5*-KO cell lines neither displaying p62/SQSTM1 degradation, nor inducing LC3-II conjugation (Figure 3B). We then monitored SB202190-induced vacuole formation in these cell lines. While there were clonal differences in the extent of vacuole formation, both WT and *ATG5*-KO A549 cell lines displayed significant vacuolation in response to 24 h of SB202190 treatment (Figure 3C & 3D). Immunofluorescence analyses again revealed RAB7 labelled compartments in A549 cells upon SB202190-treatment independent of the presence of ATG5. These data conclusively prove that even in an autophagy proficient cell line like A549, SB202190-induced vacuole formation is independent of the canonical autophagy pathway. Unlike colon cancer cells (Chiacchiera et al., 2009;Menon et al., 2011), we did not observe autophagy-related gene expression changes in A549 cells (Supplementary Figure S3), and, therefore the role of the *ATG5*-deletion on gene expression could not be analysed further.

SB202190-induced vacuole formation is modulated by multiple small molecule inhibitors including the multi-kinase inhibitor Sorafenib.

Since the vacuoles are not of autophagy origin, we investigated other possible pathways involved in the biogenesis of SB202190-induced vacuoles. For this, we co-treated A549 cells with a panel of diverse small molecule inhibitors including modulators of cytoskeleton/vesicular trafficking and kinase inhibitors (Supplementary Table S1). As expected, lysosomotropic agents like BafA1 and chloroquine inhibited the vacuole formation induced by SB202190 (Figure 4A). Interestingly, among the 54 small molecules tested, we also obtained significant suppression of vacuole formation by several additional compounds including kinase inhibitors (Figure 4A & Supplementary Figure 4). While suppression of vacuolation was associated with cell death and major morphological changes in case of some molecules (indicated by asterisks in Figure 4A), the compounds MS-049 (PRMT4/6 inhibitor), Gefitinib (EGFR tyrosine kinase inhibitor) and Sorafenib (multiple tyrosine kinase inhibitor) inhibited vacuole formation in A549 cells without apparent toxicity upon co-treatment with SB202190 for 24 h (Figure 4B). Interestingly, four compounds including (i) pan-p38 inhibitor BIRB-796 (BIRB796), (ii) the calcineurin phosphatase inhibitor Cyclosporin-A (CsA), (iii) ferroptosis inducer Erastin and (iv) ATR kinase inhibitor VE-821 significantly enhanced SB202190-induced vacuole formation (Figure 4B). We also performed similar experiments in HT29 cells, where the vacuole formation was more pronounced at 24 h (Figure 4C). While Sorafenib inhibited the extent of SB202190-induced vacuolation in HT29, the effects were less pronounced. The inhibitory effect of MS-049 and gefitinib was restricted to a reduction in the large-sized vacuoles (Figure 4C). This confirms the idea that there are cell-type specific differences in the pathways contributing to the vacuolation phenotype. Previous studies have shown that SB202190 induced significant viability loss in vacuole forming cell lines and there was significant apoptotic death upon suppression of vacuolation/autophagy. Interestingly, treating cells with a combination of sorafenib and SB202190 led to a small but significant increase in cell death compared to sorafenib alone (Supplementary Figure S5).

VE-821 phenocopy the cell-type specific effects of SB202190 and induce RAB7-positive vacuoles.

We then focused on the small molecules which had positively modulated SB202190-induced vacuolation in the screen. When A549 cells were treated with Cyclosporin-A, Erastin or BIRB-796, no micron-scale vacuoles were detected (data not shown). Interestingly, treatment of A549 cells with the ATR inhibitor VE-821 alone was sufficient to induce vacuoles similar to SB202190 (Figure 4D). More importantly, the modulators of SB202190-induced vacuolation identified above had similar effects on VE-821-induced vacuolation, suggesting that these vacuoles are of similar origin. To further support this notion, we monitored RAB7 localisation in VE-821 treated A549 cells. Immunofluorescence staining and confocal microscopy revealed that the VE-821 treated vacuoles were decorated by RAB7 (Figure 4E), similar to the the SB202190-induced vacuoles (Figure 2C & 2D). We then investigated whether V-821-induced vacuolation displayed a similar cell-type specificity as SB202190. The cell-type specificity profile of VE-821 perfectly matched with that of SB202190 in all nine cell lines tested (Table 2 and Supplementary Figure S6). These data clearly support the hypothesis that these two inhibitors interfere with the same or similar pathways to induce a cell-type specific enlargement of a RAB7-positive vesicular compartment, independent of canonical ATG5-dependent autophagy. In addition, we observed enhanced SB202190- and VE-821-induced vacuolation when co-treated with the pan p38 inhibitor BIRB796 indicating a role of p38 inhibition in facilitating vacuolation (Figure 4C & 4D).

A distinct gene-expression signature defining sensitivity to SB202190-induced vacuole formation.
The autophagy interfering activity of VE-821 has been reported previously and is also attributed to off-target effects rather than a consequence of inhibiting its primary target ATR (Bowler et al., 2020). However, it was interesting that SB202190 and VE-821 displayed similar cell type specificity in our studies. SB202190-induced autophagy and colon-cancer specific autophagy-dependent death was originally proposed to be dependent on a gene expression switch between hypoxia-dependent and pro-autophagic Foxo3a-dependent gene expression (Chiacchiera et al., 2009). To understand the gene expression signature which determines the sensitivity of cell lines to formation of these RAB7+ micron-scale vacuoles, we utilized the transcriptomics datasets available from the Cancer Cell Line Encyclopaedia (CCLE) (Ghandi et al., 2019). Out of the 23 human cell lines tested for the SB202190-induced vacuolation response, gene expression data was available for 16 cell lines. We excluded HeLa cells from the analyses due to the variations in the vacuolation phenotype associated with different strains of this cell line and the remaining 15 lines included 10 vacuole-forming and 5 non-vacuolating cell lines. We then identified differentially expressed genes between these two groups of cell lines. The analyses revealed an interesting array of genes differentially expressed between the groups of cell lines defined by their susceptibility to SB202190-induced vacuole formation (Figure 5A and 5B). A total of 128 genes (*p value* ≤ 0.01) were differentially expressed between the two groups (Supplementary Table S3), of which 35 were upregulated and 93 downregulated in vacuole-forming cell lines in comparison to the cell lines not susceptible to SB202190-induced vacuolation. Interestingly, the top candidates upregulated in vacuole-negative cell lines included several proteins with vesicular and membrane localisation like LAMP5, AEBP1, RELN etc (Figure 5C). These genes potentially block vacuole formation and define a signature of cancer cell lines based on the basal endo-lysosomal transport and autophagy flux in these cancer cells.

Discussion

SB202190 was originally reported to induce a colon-cancer specific autophagy-dependent cell death, and this was demonstrated to be an effective anti-cancer approach using colon tumor models in mice (Comes et al., 2007;Chiacchiera et al., 2009). Reprogramming from a hypoxia driven transcriptional program to Foxo3-dependent gene expression was proposed as the underlying mechanism (Chiacchiera et al., 2009). Our previous work had revealed that SB202190 influences autophagy flux cell-type specifically independent of gene expression changes and inhibition of p38 MAPK activity (Menon et al., 2011). Here, we have further expanded our analyses and present evidence that the cell type-specific vacuolation response induced by SB202190 proceeds even in the absence of the canonical autophagy mediator ATG5. In the search to identify the signaling pathways facilitating the development of these micron-scale RAB7-positive compartments, we identified small molecules including the multi-kinase inhibitors Sorafenib and Gefitinib and the PRMT4/6 inhibitor MS-049 as inhibitors of SB202190-induced vacuole formation. SB202190 and Sorafenib seems to have an additive effect on inducing cell death in A549 and HT29 cells (Supplementary Figure S5). Our screen also discovered the ATR kinase inhibitor VE-821 as a small molecule which phenocopies the SB202190-induced cell-type specific vacuolation response. By comparing the transcriptome datasets of vacuole forming and non-vacuole forming cells, we have identified a gene expression signature which may define the sensitivity of human cancer cell lines to SB202190/VE-821 induced vacuolation. These findings take us closer to the understanding of this strong cell type-specific response affecting vesicular transport and sorting.

The pyridinyl imidazole class of MAPK14/p38α-MAPK11/p38β-inhibitors led to the discovery of these kinases as master regulators of inflammation (Lee et al., 1994). Over the past three decades, the use of SB compounds in cell culture and animal models was a key method in the characterisation of this pathway. Despite studies revealing off-targets (Bain et al., 2007;Karaman et al., 2008), these compounds have been essential tools in p38 MAPK research. Considering the autophagy interfering activity of these compounds, we had recommended to avoid the use of these class of p38 inhibitors in characterization of the role of p38 in autophagy research (Menon et al., 2015;Klionsky et al., 2021). Even though genetic studies have revealed a role for p38 MAPKs and the downstream kinase MK2 in regulation of autophagy (Wei et al., 2015), several of the phenotypes on autophagy and lysosomal biogenesis reported using SB-compounds can be attributed to these off-target effects (Schwartz et al., 2018;Sase et al., 2023). Interestingly, SB202190 was shown to induce TFE3/TFEB activation and regulate autophagy and lysosome biogenesis independent of p38 inhibition (Yang et al., 2020). Recent studies indicate that the effect of SB202190 on TFEB nuclear localisation is mediated by p38-independent EIF2AK3/PERK activation (Do et al., 2022). Unlike the micron-scale vacuole formation, these effects on TFEB and PERK activation were not reported to be cell-type specific. We have identified SB220025, an SB-compound closely related to SB202190 as a potential pyridinyl imidazole compound and p38 inhibitor with no effect on the vacuolation response. Future comparative studies using SB220025 and SB202190 will be useful in identifying the molecular target(s) of SB202190 relevant to cell-type specific vacuole formation.

Expanding the cell-type specificity profile of SB202190 to over 30 cell lines have given us important insights into potential mechanisms of this unique phenotype. Moreover, the differential gene expression analyses have revealed interesting candidates relevant to lysosomal, endosomal and autophagy pathways, which may be used to understand the susceptibility of cancer cell lines to compounds interfering with endosomal/autophagosome maturation. Based on data from The Human Protein Atlas (https://www.proteinatlas.org)(Thul et al., 2017), the top differentially expressed genes show subcellular localisation corresponding to vesicular compartments (AEBP1, RELN, LAMP5, DLK1, NLRP2, MYOM3) or the cytoplasmic membrane (ANPEP, IGFL2) The knockdown of NLRP2, a gene preferentially expressed in vacuole-forming cells was shown to induce vacuole formation and cell death in HUVECs (Human Umbilical Vein Endothelial Cells) (Zhang et al., 2019). Interestingly, UBE2QL1, a ubiquitin conjugating enzyme less expressed in SB202190-sensitive cell lines was shown to be important to maintain lysosomal integrity and facilitate the autophagic clearance of damaged lysosomes termed lysophagy - (Koerver et al., 2019). Monitoring the sensitivity of various cancer cell lines to lysophagy inducers and correlating the responses to the cell-type specificity of vacuolation will be an interesting avenue for future research. Another target, relevant to endosomal transport and downregulated in vacuole forming cells is the lysosome-associated membrane protein-5 gene (LAMP5)(Figure 5C). Originally discovered as brain-associated LAMP-like molecule (BAD-LAMP), LAMP5 has now been shown to play role in haematological and epithelial malignancies (Wang et al., 2019;Umeda et al., 2022). Interestingly, LAMP5 was shown to suppress lysosomal degradation of oncogenic fusion proteins promoting mixed-lineage leukemia (Wang et al., 2019). This autophagy/lysosomal activity suppression by endosomal LAMP5 may be functionally relevant in preventing accumulation of micron-scale vacuoles in non-vacuole forming cells.

The vacuoles induced by SB-compounds were originally considered to be autolysosomes (Comes et al., 2007). While autophagy flux analyses in HT29 cells revealed a defective autophagic response associated with LC3-II/p62 accumulation, YFP-LC3 puncta were seen proximal to the micron-scale vacuoles, but did not completely colocalize with them (Menon et al., 2011). We had previously shown that SB202190 may alter autophagy flux also in non-vacuole forming cells (Menon et al., 2015). Here we show that while autophagy proficient cells like HT29, HCT116 and A549 show significant alterations in autophagy flux, the vacuole formation is independent of ATG5 and LC3 lipid conjugation as observed in DU-145 and ATG5-deficient A549 cells. This argues against an obligate role for canonical autophagy in SB-induced vacuolation, while autophagy pathway may contribute to the enlarged vesicles in autophagy proficient cells. RAB9 GTPase has been associated with a non-canonical autophagy pathway in the absence of ATG5, but confocal analyses did not reveal consistent colocalization of RAB9 with SB202190-induced vacuoles (data not shown). In contrast, these compartments are positive for RAB7 GTPase, a late endosomal marker. Confirming potential endosomal origin of these compartments, a recent study had observed RAB7-positive vacuoles in SB203580/SB202190-treated melanoma cells (Palusova et al., 2020). They also indicated similarities between SB202190-induced vacuoles and the vacuolar compartments induced upon the inhibition of the lipid kinase PIKfyve. More recently, another study has suggested PIKfyve as a potential target of SB202190 relevant for vacuolation, based on the similarities in the phenotypes induced by inhibitors of PIKfyve and SB202190 (Wible et al., 2023). Interestingly, the small molecule inhibitors identified in our screen failed to abrogate vacuolation induced by PIKfyve inhibitors (Supplementary Figure S7). This is supportive of the hypothesis that PIKfyve inhibitors and SB202190 inhibit similar vesicular transport processes, rather than the same target kinase. Consistent with this, a previous study has shown synergistic effect of p38 and PIKfyve inhibitors against cancer cell proliferation *in vitro* and *in vivo* (O'Connell and Vassilev, 2021). In our hands, the ATR inhibitor VE-821 seems to accurately phenocopy the vacuolation response and cell-type specificity shown by SB202190. Unfortunately, the autophagy-interfering activity of VE-821 and related inhibitors were also shown to be independent of ATR (Bowler et al., 2020). Further studies using VE-821, PIKfyve inhibitors, SB202190 and SB220025 could reveal novel mechanisms and targets responsible for this interesting phenotype relevant to anti-cancer therapy.

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Author contributions

Conceived and designed the experiments: MBM, SJ and HS. Performed the experiments: SJ, HS, VA and SD. Provided reagents and protocols: VA and NE. Analyzed the data: SJ, HS, JI, SD & MBM. Writing of the manuscript: MBM, SD, HS, JI and NE. All read and approved the submitted version of the manuscript.

Conflict of interests

The authors declare no conflict of interests.

Table 1. Cell-type specificity of SB202190-induced vacuole formation

No	Cell line	Species	Cell type	Vacuoles
1	**A549**	Human	lung carcinoma	+
2	AGS	Human	gastric adenocarcinoma	+
3	BHK21	Hamster	adult kidney fibroblast	+
4	**C2C12**	Mouse	myoblast	-
5	Caco-2 Bbe	Human	colorectal adenocarcinoma	+
6	**DU-145**	Human	prostate carcinoma	+
7	**HaCaT**	Human	keratinocyte	-
8	**HCT 116**	Human	colorectal adenocarcinoma	+
9	**HEK293T**	Human	embryonic kidney	-
10	**HeLa**	Human	cervical adenocarcinoma	-/+
11	hMSC	Human	primary mesenchymal stem cells	+
12	**HT29**	Human	colorectal adenocarcinoma	+
13	**Huh7**	Human	hepatocellular carcinoma	-
14	HUVEC	Human	primary endothelial cells	+
15	IEC6	Rat	small intestinal epithelium	+
16	L929	Mouse	fibrosarcoma	+
17	**LN229**	Human	glioblastoma	+
18	**MCF-10A**	Human	Mammary epithelial	+
19	**MCF7**	Human	breast adenocarcinoma	-
20	**MDA-MB231**	Human	breast carcinoma	+
21	MEF-T	Mouse	embryonic fibroblast	+
22	**NCI-H460**	Human	lung carcinoma	+
23	**NIH 3T3**	Mouse	embryonic fibroblast	-
24	NMuMG	Mouse	mammary epithelial	+
25	**PC-3**	Human	prostate adenocarcinoma	-
26	RAW 264.7	Mouse	monocytic	+
27	RGM1	Rat	gastric epithelium	+
28	Sh-SY5Y	Human	neuroblastoma	-
29	SW480	Human	colorectal adenocarcinoma	+
30	**U87MG**	Human	glioblastoma	+
31	WM1617	Human	melanoma	-
32	WM793	Human	melanoma	-
33	**4T1**	Mouse	breast carcinoma	+

Notes: The cell lines tested in the present study are shown in bold. Others have been reported in previous studies (Menon et al., 2011;Menon et al., 2015). -/+ indicate presence of difference results from different sublines of the same cell line.

Table 2. Comparison of cell-type specificity between SB202190 and VE-821-induced vacuole formation

No	Cell line	SB202190	VE-821
1	A549	+	+
2	HEK293T	-	-
3	HT29	+	+
4	DU-145	+	+
5	PC-3	-	-
6	HaCaT	-	-
7	U87MG	+	+
8	MDA-MB231	+	+
9	HCT116	+	+

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Figure Legends

Figure 1. SB202190-induced cell type specific vacuolation accompanies suppression of autophagy flux and is inhibited by Bafilomycin A1. A. Indicated cell lines were treated with SB202190 (10 µM), SB220025 (10 µM) or solvent control (DMSO) for 24 h and images were acquired using a 20x phase-contrast objective. **B.** Structures of pyridinyl imidazole class inhibitors SB202190 (upper panel) and SB220025 (lower panel). **C.** Effect of Bafilomycin A1 (BafA1) treatment on SB202190-induced vacuolation in HT29, HCT116 and A549 cells. **D.** HCT116 cells were treated with BafA1 and/or SB202190 for indicated time-points and lysates were probed with p62 and LC3 antibodies to monitor autophagy flux. GAPDH is shown as control. **E.** A549, HeLa and L929 cells were treated with indicated small molecules for 24 h and lysates were probed for LC3, p62 and GAPDH as in panel D. **F.** HEK293T cells were treated as indicated for 24 h and lysates were analysed by immunoblotting.

Figure 2. Autophagy-deficient DU-145 cells induce RAB7-positive vacuoles in response to SB202190. A. DU-145 cells were treated as indicated for 24 h and imaged using a 20x phase contrast objective to observe vacuoles. **B.** Lysates from DU-145 cells were probed with p62, LC3 and ATG12 antibodies to monitor autophagy. GAPDH is shown as control. **C & D.** Confocal microscopy analyses of RAB7 localisation in DU-145 (C), HT29 and A549 (D) cells treated with SB202190 or solvent control (DMSO) for 24 h.

Figure 3. Canonical autophagy and LC3-conjugation is dispensable for SB202190-induced vacuolation. A. EBSS (Earle's Balanced Salt Solution) treatment-induced autophagy was monitored in *ATG5 deficient*-A549 cells by probing the degradation of autophagy adapter and substrate p62 and the autophagy marker – lipid conjugated lower band of LC3 (LC3-II). Bafilomycin A1 is used as a control to determine autophagic flux and DMSO as solvent control. EBSS was for 3 hrs, while control cells were in complete DMEM/F12 medium. **B.** Western Blot analysis shows LC3-II accumulation in WT cells co-treated with SB202190 (20 µM) and BafA1. **C & D.** Clone-specific differences are seen in the number of vacuoles but vacuoles were observed in both WT and ATG5 KO cells(C). **E.** SB202190-induced vacuoles are GTPase Rab7 positive and are independent of the presence or absence of ATG5 in the cells.

Figure 4. Screen for modulator of SB202190-induced vacuoles identify Sorafenib and VE-821
A. To screen for small molecule modulators of SB202190-induced vacuolation, A549 cells were treated with a panel of inhibitors in combination with SB202190 (10 µM) for 24 h. Vacuole formation was quantified and represented taking SB202190 alone treated as 100%. The molecules showing significantly lower or higher vacuolation are indicated with red and green bars respectively. * indicates loss of viability. **B & C.** A549(B) and HT29(C) cells were treated with SB202190 (10 µM) in combination with indicated inhibitors and phase contrast images were taken after 24 h. **D.** A549 cells were treated with VE-821 (10 µM) alone or in combination with indicated inhibitors for 24 h. **E.** A549 cells treated for 24 h with DMSO control or VE-821 (10 µM) were subjected to confocal immunofluorescence analyses to detect RAB7 positive vesicles. DNA (DAPI staining) and F-actin (phalloidin staining) are shown as control.

Figure 5. A gene expression signature defining the sensitivity of cancer cell lines to SB202190-induced vacuolation. A. Heatmap showing the expression (VST normalized values) of top 50 differentially expressed genes (after sorting by adjusted *p*-values) between the vacuole-forming (vacuole positive) and non-vacuolating (vacuole negative) cell lines. The vacuole-forming cell lines are indicated in red and the non-vacuole forming cell lines are indicated in green. **B.** Volcano plot representing the differential expressed genes identified in the study. The interesting candidate genes showing differential expression are indicated. **C.** Boxplots of VST normalized values, representing expression of select differentially expressed genes across 15 cell lines (red: vacuolation positive and green: vacuolation negative).

Figure 1

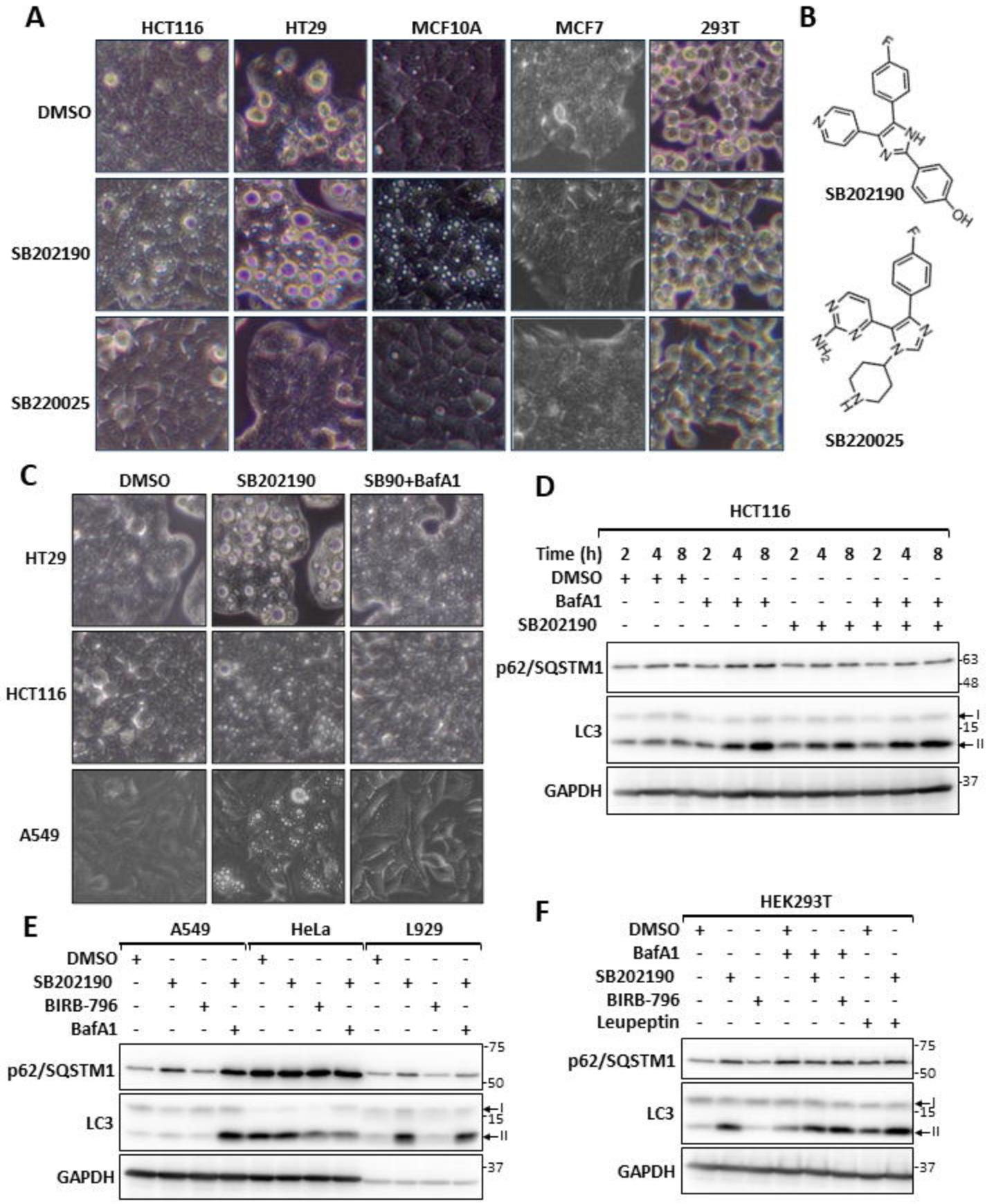

A
HCT116
HT29
MCF10A
MCF7
293T
DMSO
SB202190
SB220025
B
SB202190
SB220025
C
DMSO
SB202190
SB90+BafA1
HT29
HCT116
A549
D
HCT116
Time (h) 2 4 8 2 4 8 2 4 8 2 4 8
DMSO + + + - - - - - - - - -
BafA1 - - - + + + - - - + + +
SB202190 - - - - - - + + + + + +
p62/SQSTM1
63
48
LC3
I
15
II
GAPDH
37
E
A549
HeLa
L929
DMSO + - - - + - - - + - - -
SB202190 - + - + - + - + - + - +
BIRB-796 - - + - - - + - - - + -
BafA1 - - - + - - - + - - - +
p62/SQSTM1
75
50
LC3
I
15
II
GAPDH
37
F
HEK293T
DMSO + - - + - - + -
BafA1 - - - + + + - -
SB202190 - + - - + - - +
BIRB-796 - - + - - + - -
Leupeptin - - - - - - + +
p62/SQSTM1
75
50
LC3
I
15
II
GAPDH
37

Figure 2

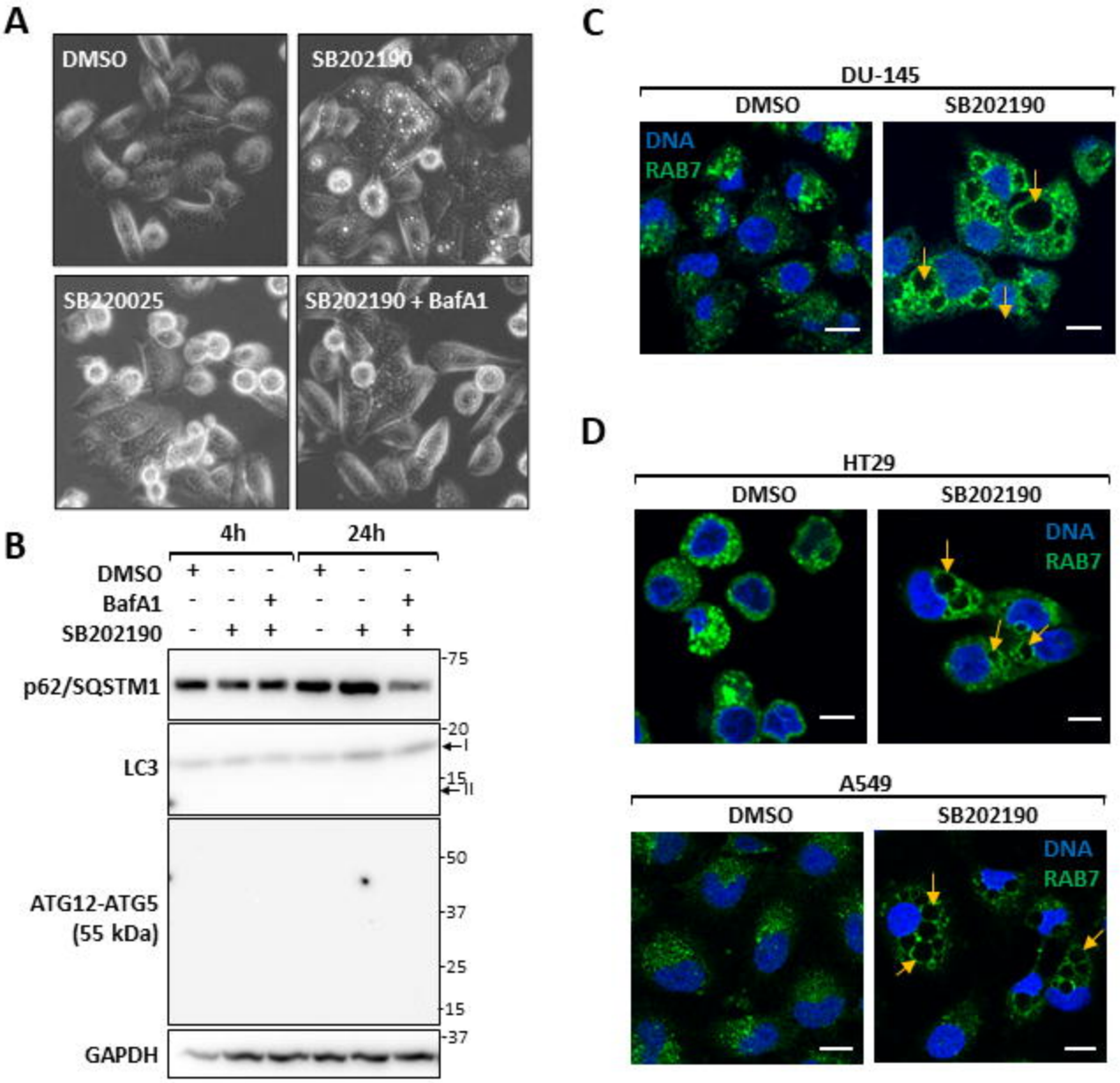

A
DMSO
SB202190
SB220025
SB202190 + BafA1
B
4h
24h
DMSO + - - + - -
BafA1 - - + - - +
SB202190 - + + - + +
p62/SQSTM1
LC3
ATG12-ATG5
(55 kDa)
GAPDH
75
20
I
15
II
50
37
25
15
37
C
DU-145
DMSO
SB202190
DNA
RAB7
D
HT29
DMSO
SB202190
DNA
RAB7
A549
DMSO
SB202190
DNA
RAB7

Figure 3

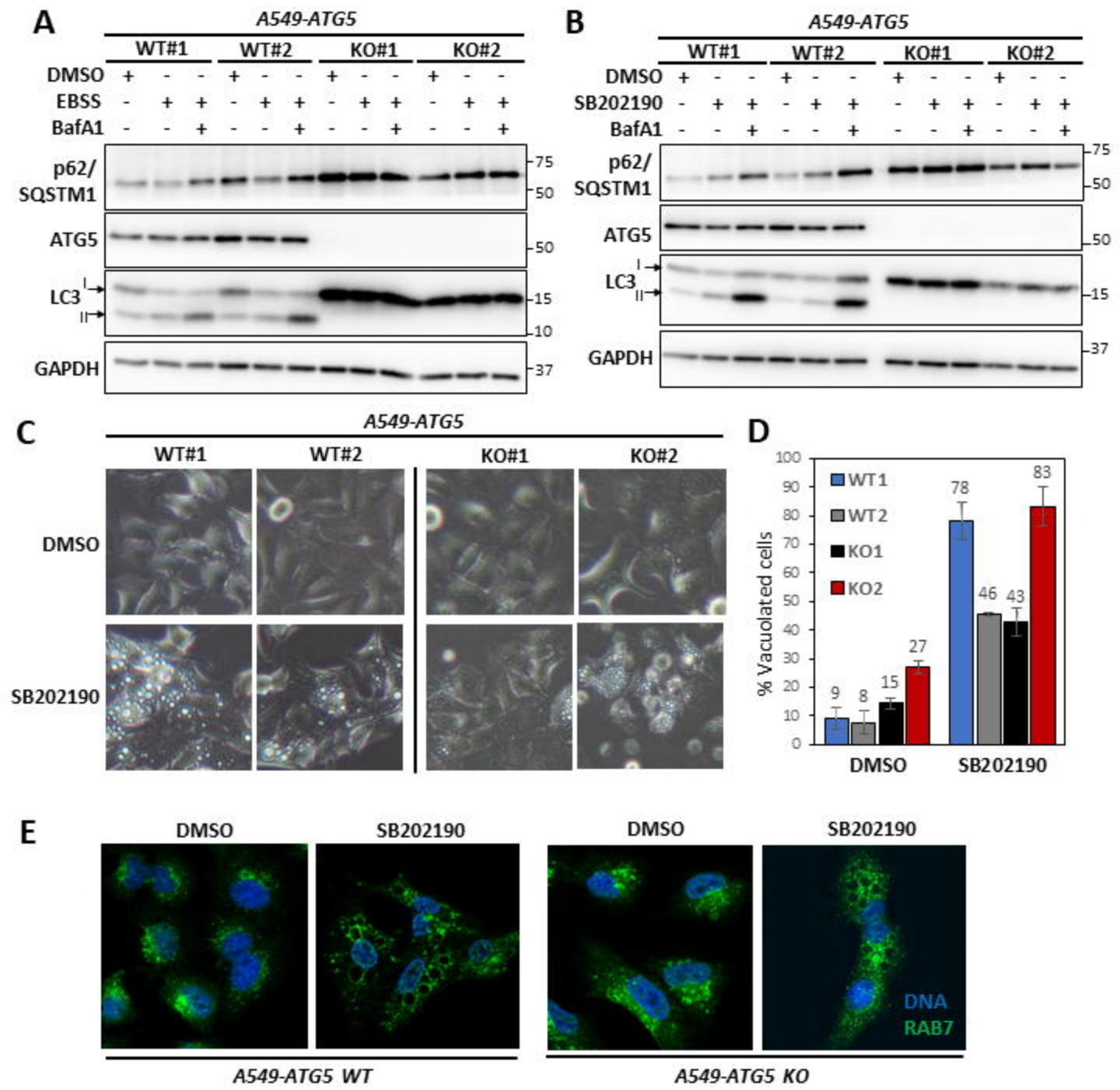

A
A549-ATG5
WT#1
WT#2
KO#1
KO#2
DMSO
EBSS
BafA1
p62/ SQSTM1
ATG5
LC3
I
II
GAPDH
75
50
15
10
37
B
A549-ATG5
SB202190
C
DMSO
SB202190
D
% Vacuolated cells
WT1
WT2
KO1
KO2
9
8
15
27
78
46
43
83
E
DMSO
SB202190
DNA
RAB7
A549-ATG5 WT
A549-ATG5 KO

Figure 4

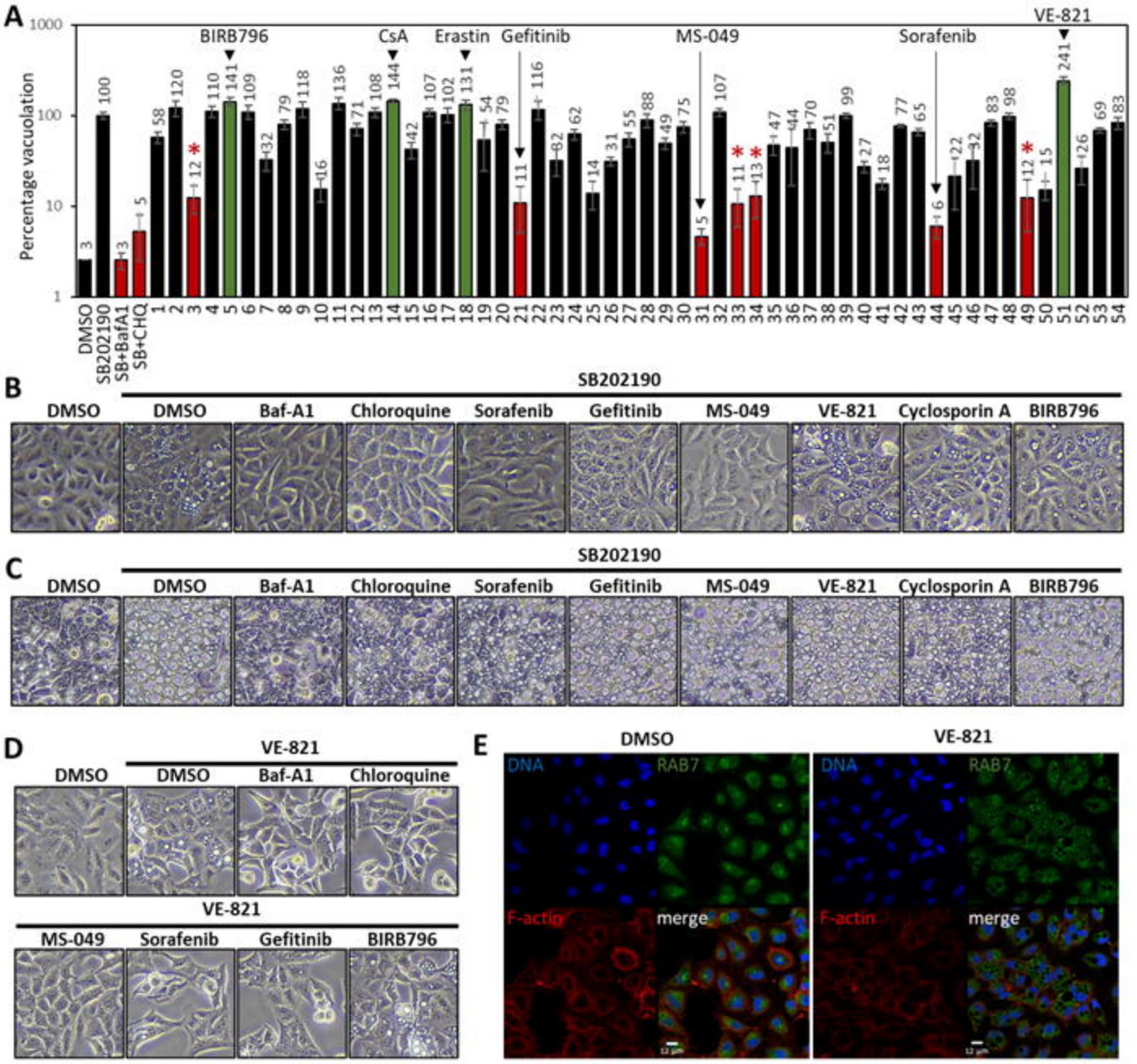
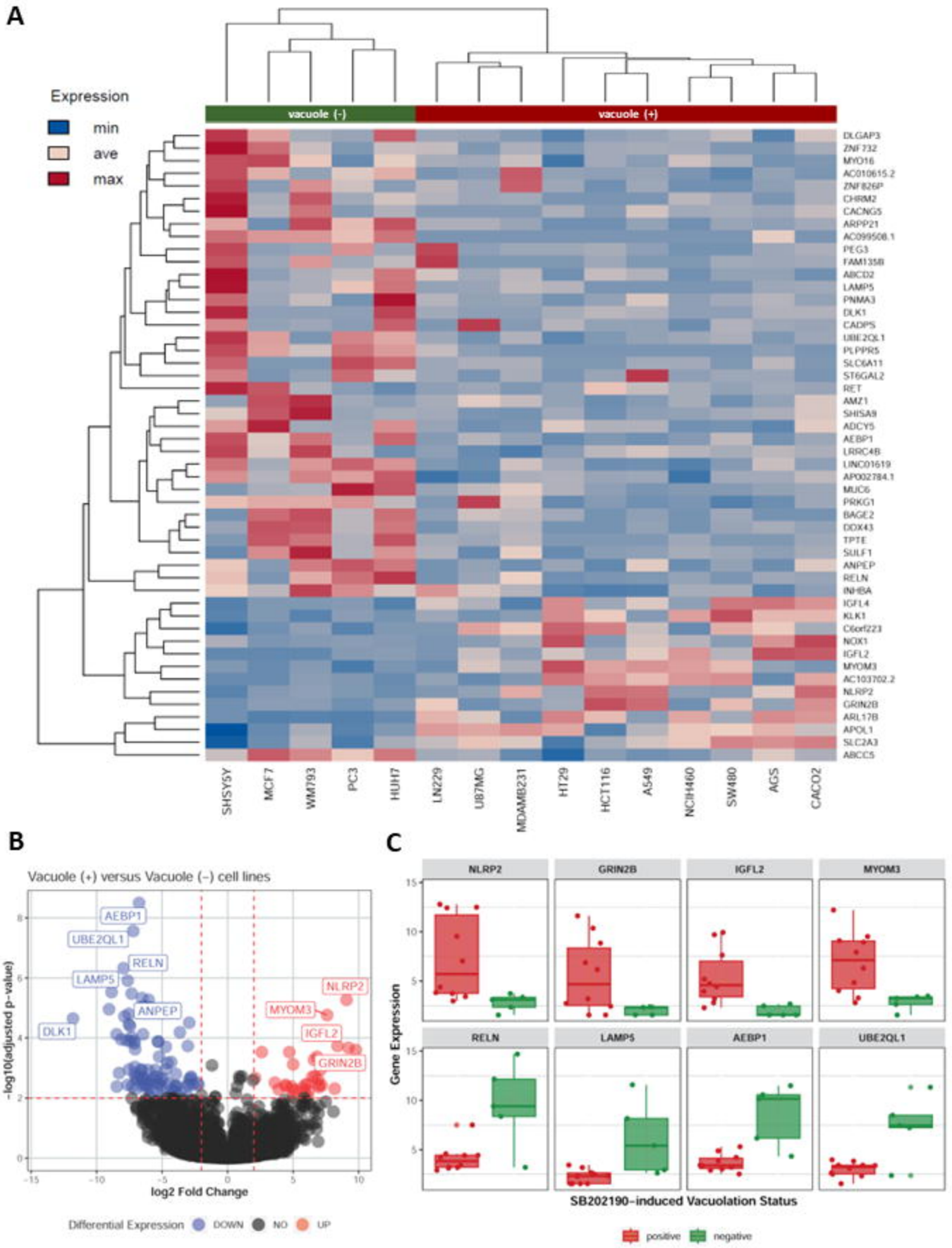

A
Percentage vacuolation
1000
100
10
1
BIRB796
CsA
Erastin
Gefitinib
MS-049
Sorafenib
VE-821
DMSO
SB202190
SB+BafA1
SB+CHQ
B
SB202190
DMSO
DMSO
Baf-A1
Chloroquine
Sorafenib
Gefitinib
MS-049
VE-821
Cyclosporin A
BIRB796
C
SB202190
DMSO
DMSO
Baf-A1
Chloroquine
Sorafenib
Gefitinib
MS-049
VE-821
Cyclosporin A
BIRB796
D
VE-821
DMSO
DMSO
Baf-A1
Chloroquine
VE-821
MS-049
Sorafenib
Gefitinib
BIRB796
E
DMSO
VE-821
DNA
RAB7
F-actin
merge
DNA
RAB7
F-actin
merge

Figure 5


A
Expression
min
ave
max
vacuole (-)
vacuole (+)
DLGAP3
ZNF732
MYO16
AC010615.2
ZNF826P
CHRM2
CACNG5
ARPP21
AC099508.1
PEG3
FAM135B
ABCD2
LAMP5
PNMA3
DLK1
CADPS
UBE2QL1
PLPPR5
SLC6A11
ST6GAL2
RET
AMZ1
SHISA9
ADCY5
AEBP1
LRRC4B
LINC01619
AP002784.1
MUC6
PRKG1
BAGE2
DDX43
TPTE
SULF1
ANPEP
RELN
INHBA
IGFL4
KLK1
C6orf223
NOX1
IGFL2
MYOM3
AC103702.2
NLRP2
GRIN2B
ARL17B
APOL1
SLC2A3
ABCC5
SHSY5Y
MCF7
WM793
PC3
HUH7
LN229
U87MG
MDAMB231
HT29
HCT116
A549
NCIH460
SW480
AGS
CACO2
B
Vacuole (+) versus Vacuole (–) cell lines
AEBP1
UBE2QL1
RELN
LAMP5
ANPEP
DLK1
NLRP2
MYOM3
IGFL2
GRIN2B
-log10(adjusted p-value)
log2 Fold Change
Differential Expression
DOWN
NO
UP
C
NLRP2
GRIN2B
IGFL2
MYOM3
RELN
LAMP5
AEBP1
UBE2QL1
Gene Expression
SB202190-induced Vacuolation Status
positive
negative