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The problem of pyridinyl imidazole class inhibitors of MAPK14/p38α and MAPK11/p38β in autophagy research

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In addition to its established role in inflammation, the stress-activated p38 MAP kinase pathway plays major roles in the regulation of cell cycle, senescence, and autophagy. Robust studies could establish mechanistic links between MAPK11-MAPK14/p38 signaling and macroautophagy converging at ATG9-trafficking and BECN1 phosphorylation. However, several reports seem to monitor MAPK11-MAPK14/p38-dependence of autophagy exclusively by the use of the SB203580/SB202190 class of MAPK14/MAPK11/p38α/β inhibitors. In this "Letter to the editor" we present data to support our claim that these inhibitors interfere with autophagic flux in a MAPK11-MAPK14/p38-independent manner and hence should no longer be used as pharmacological tools in the analysis of MAPK11-MAPK14/p38-dependence of autophagy. We propose a general guideline from *Autophagy* with regard to this issue to avoid such misinterpretations in the future.

Dear Editor,

An article by Zhong et al.[1] analyzed the role played by stress-induced MAPK11-MAPK14/p38 signaling in the expression of autophagy-related (*ATG*) genes and concludes that MAPK11-MAPK14/p38 isoforms α, β, γ, and δ (MAPK14/11/12/13) are involved in the transcription of *ATG* genes in response to a novel anticancer copper complex. We have serious concerns regarding the title and conclusions of this publication, which should be discussed to preserve the high standards of *Autophagy*.

Our major point concerns the analysis of the role of the MAPK11-MAPK14/p38 pathway in the regulation of autophagy by the pyridinyl imidazole class MAPK14/p38α-MAPK11/p38β-inhibitor SB203580. Several technically robust publications in the past decade have conclusively established a context-dependent role for the stress-activated MAPK11-MAPK14/p38 pathway in the regulation of MTOR signaling and autophagy.[2-4] Furthermore, a connection between the MAPK14/p38α-MAPK11/p38β-activated protein kinase MAPKAPK2/MK2 and autophagy was established recently via demonstrating phosphorylation of BECN1/Beclin-1 at serine 90, using a dominant-negative mutant of MAPK14/p38α instead of MAPK11-MAPK14/p38 inhibitors.[5] However, we are deeply concerned about the use of a class of pyridinyl imidazole inhibitors, such as SB203580 and SB202190, in monitoring the role of MAPK14/p38α-MAPK11/p38β signaling in autophagy, because we had previously reported that these compounds alter autophagic flux and pro-autophagic gene expression in a cell type-specific, MAPK14/p38α-MAPK11/p38β-independent manner.[6] In the figure panels (**Fig. 1A-H**), we provide additional data to support our claims that:

1. SB202190 and SB203580, but not the structurally nonrelated and more potent MAPK11-MAPK14/p38 inhibitor BIRB-796,[7] induce vacuoles (**Fig. 1A**) characterized as acidic compartments (**Fig. 1B**) in HT29 cells in a 3-methyladenine (3MA)-sensitive manner (**Fig. 1C**) indicating a compound-specific, MAPK11-MAPK14/p38-independent autophagic response.[6,8,9]

2. SB202190 does induce vacuole formation in about 70% of the cell lines analyzed when used at very low concentrations (**Table 1**), but induces accumulation of the autophagy substrate SQSTM1/p62 and lipid-conjugated MAP1LC3B (LC3-II) also in cells, which display no vacuole formation, in a compound-specific, MAPK11-MAPK14/p38-independent manner (**Fig. 1E and F**). As expected from the structural similarity, SB203580 gave results very similar to SB202190 albeit with less potency (**Fig. 1G and H**). In contrast, BIRB-796 did not affect the levels of autophagy substrates (**Fig. 1E–H**), although it effectively blocked MAPK14/p38α-MAPK11/p38β signaling as monitored by stress-induced downstream phosphorylation events (**Fig. 1D**) already at low concentrations.

Because of the MAPK11-MAPK14/p38-independent interference with autophagy, the SB-compounds should no longer be used as pharmacological tools in the analysis of MAPK11-MAPK14/p38-dependence of autophagy.

Another concern regards the findings and title of the paper, the latter of which explicitly states that MAPK11/12/13/14 are involved in the transcriptional

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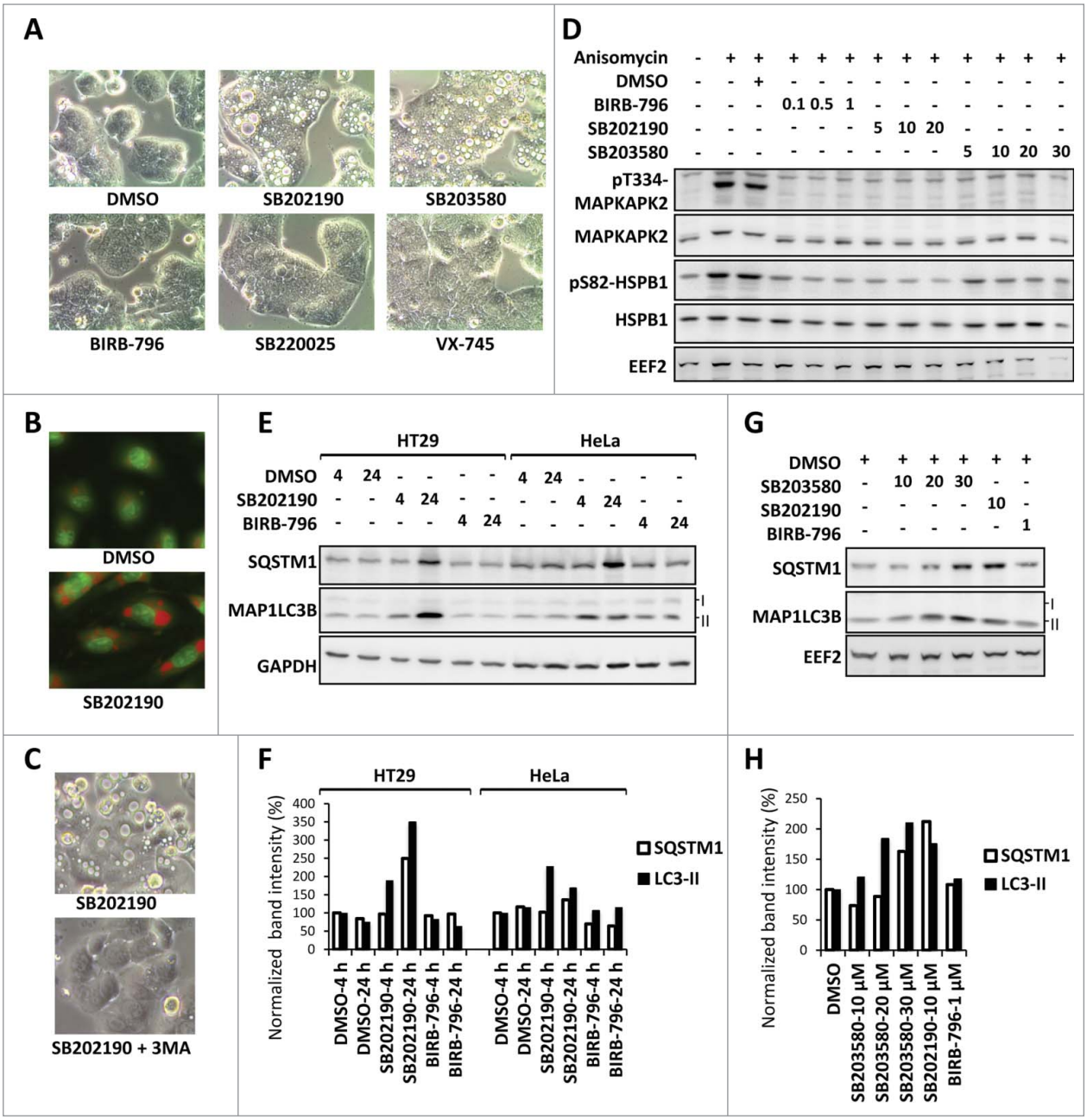

Figure 1. MAPK11-MAPK14/p38-independent effects of SB202190/SB203580 in autophagy. (**A**) SB202190 and SB203580 (10 μM each) but not the other more specific MAPK11-MAPK14/p38 inhibitors (SB220025, 10 μM; BIRB-796, 1 μM; or VX-745, 10 μM) induce large vacuoles in HT29 cells (24 h treated). (**B**) The SB202190-induced vacuoles are acidic compartments as shown by strong acridine orange staining in primary HUVECs. (**C**) Autophagy inhibitor 3-MA suppresses SB-induced vacuolation in HT29 cells. (**D**) The efficacy of BIRB-796, SB202190, and SB203580 to inhibit MAPK14/p38α-MAPK11/p38β signaling in HeLa cells was compared by monitoring their effect on stress-induced phosphorylation of the direct MAPK14/p38α-MAPK11/p38β substrate MAPKAPK2 at Thr334 (T334) and of the downstream target HSPB1/HSP27 at Ser82 (S82). The membrane was reprobed with MAPKAPK2, HSPB1 and EEF2 (eukaryotic translation elongation factor 2) antibodies as loading controls. Cells were treated with the indicated concentrations of inhibitors (μM) prior to 30 min anisomycin (10 μg/ml) stimulation. (**E and F**) The off-target effect of SB202190 in autophagy is independent of cell-type specific vacuolation. In both, vacuole-positive HT29 and vacuole-negative HeLa cells (see **Table 1**), long-term SB202190 treatment (10 μM for 4 or 24 h) leads to the accumulation of autophagy substrates SQSTM1 and lipid conjugated MAP1LC3B (LC3-II) (**E**). Quantified band intensities for LC3B-II and SQSTM1 normalized to that of the loading control (GAPDH) are shown (**F**). (**G and H**) Dose-dependent (10-30 μM) effect of SB203580 on autophagy in HeLa cells demonstrated by monitoring the levels of SQSTM1 and MAP1LC3B (LC3-II) at 24 h treatment (**G**). Quantified band intensities for lipid conjugated MAP1LC3B (LC3-II) and SQSTM1 normalized to the loading control (EEF2) are shown (**H**).

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

No	Cell line	Species	Cell type	Vacuoles
1	AGS	Human	gastric adenocarcinoma	+
2	A549	Human	lung carcinoma	+
3	BHK21	Hamster	adult kidney fibroblast	+
4	C2C12	Mouse	myoblast	–
5	Caco-2 Bbe	Human	colorectal adenocarcinoma	+
6	HCT 116	Human	colorectal adenocarcinoma	+
7	HEK293T	Human	embryonic kidney	–
8	HeLa	Human	cervical adenocarcinoma	–
9	hMSC	Human	primary mesenchymal stem cells	+
10	HT29	Human	colorectal adenocarcinoma	+
11	HUVEC	Human	primary endothelial cells	+
12	IEC6	Rat	small intestinal epithelium	+
13	L929	Mouse	fibrosarcoma	+
14	MCF-10A	Human	mammary epithelial	+
15	MEF-T	Mouse	embryonic fibroblast	+
16	NIH 3T3	Mouse	embryonic fibroblast	–
17	NMuMG	Mouse	mammary epithelial	+
18	RAW 264.7	Mouse	monocytic	+
19	RGM1	Rat	gastric epithelium	+
20	Sh-SY5Y	Human	neuroblastoma	–
21	SW480	Human	colorectal adenocarcinoma	+
22	WM1617	Human	melanoma	–
23	WM793	Human	melanoma	–

The table depicts the cell-type specificity of SB202190-induced autophagy-dependent vacuole formation. Cells were treated with 5 μM SB202190 for 12 h. Vacuoles were clearly visible in most of the cell lines after approximately 2 h of SB202190 treatment.

response induced by the copper complex. These conclusions are exclusively based on the use of the inhibitor SB203580, which targets only MAPK14/p38α and MAPK11/p38β.[10] Hence, the title statement about MAPK13/p38δ and MAPK12/p38γ is not justified by the data presented and should be corrected.

Materials and methods

SB203580 (Calbiochem, 559389), SB220025 (Sigma, S9070), BIRB-796 (Axon Medchem, 1358), VX-745 (Philip Cohen, University of Dundee) and SB202190 (Axon Medchem, 1364) stocks were prepared in DMSO (Carl Roth, 4720.4). Primary antibodies used were: MAP1LC3B (Cell Signaling Technology, 3868), pT334-MAPKAPK2 (Cell Signaling Technology, 3007), MAPKAPK2 (Cell Signaling Technology, 3042), pS82-HSPB1 (Cell Signaling Technology, 2401), SQSTM1 (BD Biosciences, 610833), HSPB1 (Santa Cruz Biotechnology, sc-1048) and GAPDH (Chemicon international, MAB374). Secondary antibodies used were goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2005), donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, sc-2033) and goat anti-rabbit IgG (Dianova, 111-035-003). Acridine orange (Sigma, 158550), anisomycin (Sigma, A9789) and 3-methyladenine (Calbiochem, 189490) were purchased as indicated. Cell culture, microscopy and immunoblotting procedures were followed as described previously.[6]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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