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NOTE

Metacycloprodigiosin induced cell death selectively in β-catenin-mutated tumor cells

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Wnt signaling pathway is known as a proliferation signaling, which has crucial roles in the regulation of diverse processes, including embryonic development.[1] This proliferation signaling is regulated by β-catenin destruction complex, consists of β-catenin itself, glycogen synthase kinase 3 (GSK-3) and casein kinase 1 (CK1), the scaffolding protein Axin, the adenomatous polyposis coli and the E3-ubiquitin ligase β-TrCP.[2] In the absence of Wnt ligands, β-catenin is degraded by destruction complex phosphorylated by CK1 and GSK-3.[3] On the other hand, in the presence of Wnt ligands, the complex formation is inhibited, and then, β-catenin is stabilized and enters the nucleus, leading to the binding to T-cell factor to activate the transcription of Wnt target genes, such as *c-myc* and *cyclin D1*.[4,5] Mutation of the β-catenin gene, *CTNNB1*, would cause stabilization and nuclear accumulation,[6] resulting in disruption of a large number of cellular functions that may be important in tumor development. β-Catenin is mutated in a wide variety of tumors,[7] and is detected up to 10% of all sporadic colon carcinomas and 20% of hepatocellular carcinomas.[8] Several small molecules targeting Wnt signaling pathway have been developed for the patients with mutant β-catenin. However, to date there are still no effective therapeutic compounds. Previously, we have reported that MEK inhibitor, SMK-17, induced G1 cell cycle arrest in BRAF mutant tumor cells, but it induced apoptosis in tumor cell lines harboring β-catenin mutations.[9] Moreover, tumor regression in response to multiple daily oral administration of SMK-17 was observed only in active β-catenin mutant xenograft models, without significant body weight loss.[9] These results demonstrated that SMK-17 exhibited synthetic lethality with β-catenin mutation. Unfortunately, when we found this effect of SMK-17, development of SMK-17 in pharmaceutical company has already dropped out. Therefore, we started screening for other compounds than MEK inhibitor. We first screened more than 2000 microbial extracts for the compounds that induced apoptosis in β-catenin-mutated HCT116 cells, but not in A375 cells harboring wild-type β-catenin. As a result, we found one *streptomyces* strain produced such active compound, which was isolated from the extracts of *streptomyces* cultures following bioassay-guided fractionation; both silica gel column chromatography and reverse-phase HPLC were utilized. Spectroscopic analysis by NMR and MS revealed this active compound to be metacycloprodigiosin (mcPG) (Figure 1a).[10] Flow cytometric analysis of propidium iodide staining revealed that the subG1 population in β-catenin-mutated HCT116 cells was significantly increased upon treatment with 0.1 μM mcPG for 48 h, whereas the subG1 population in A375 cells expressing wild-type β-catenin was only slightly increased, indicating that mcPG induced apoptosis in HCT116 cells, but not in A375 cells (Figure 1a). We further examined whether mcPG induced apoptosis selectively in β-catenin mutant tumor cells by using several types of human tumor cells in detail. For this, we selected 12 tumor cells including 5 β-catenin mutant tumor cells (HCT116 cells: S45 deletion, LS-174T cells: S45F, SW48 cells: S33Y, HepG2 cells: W25-I140 deletion and Colo-205 cells: N287S), and these tumor cells were treated with 0.1, 0.3, 1.0, 3.0 and 10 μM mcPG for 48 h and the subG1 populations were measured by flow cytometer (Supplementary Table S1). The extent of apoptosis induction was calculated using the area under the curve, which represents the area under the line in plots of concentrations of each chemical against subG1 population, and *Z*-score. As shown in Figure 1b, mcPG induced apoptosis not only in HCT116 cells but also in LS-174T, SW48 and HepG2 cells, which are all β-catenin mutant tumor cells. Individual value plots of mcPG confirmed that β-catenin-mutated cell lines were significantly sensitive to mcPG, compared with cell lines harboring wild-type β-catenin (Figure 1c). On the other hand, significant differences were not observed in cell lines harboring KRAS and p53 mutations, and BRAF mutation seemed to be a negative factor of mcPG-induced apoptosis. Because prodigiosin analogs are reported to inhibit vacuolar-type H^+-ATPase (V-ATPase),[11,12] we next examined whether other V-ATPase inhibitors, bafilomycin A1 (BMA)[13] and concanamycin A (CMA),[14] also could induce apoptosis selectively in β-catenin mutant tumor cells. As shown in Figure 1d, BMA or CMA showed a similar cell type-dependent apoptosis-inducing profile to mcPG, suggesting that V-ATPase inhibitors induced apoptosis selectively in β-catenin-mutated tumor cell lines.

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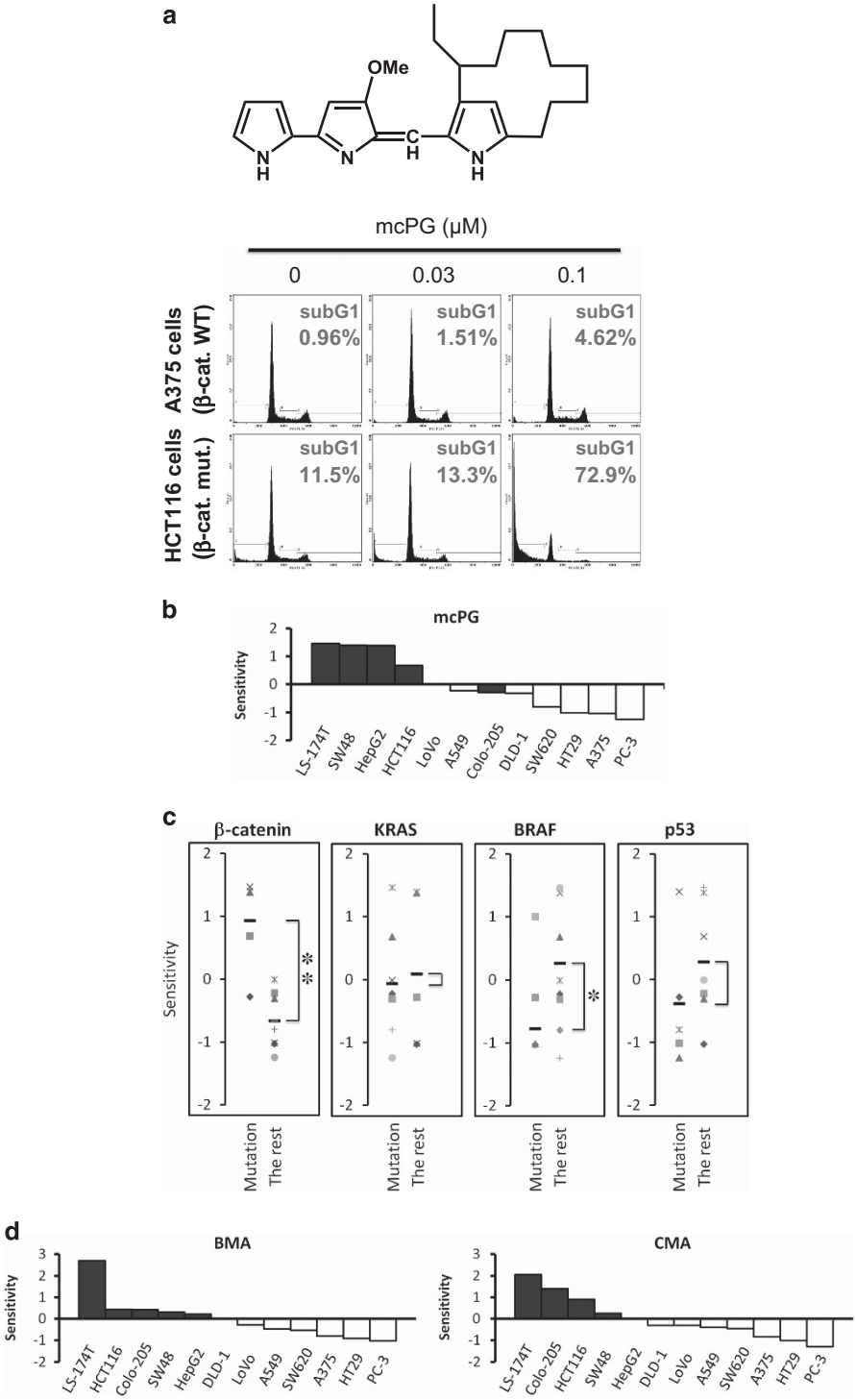

Figure 1 Increasing subG1 population selectively in β-catenin-mutated HCT116 cells is caused by vacuolar-type H^+-ATPase (V-ATPase) inhibition. (**a**) A375 and HCT116 cells were treated with the indicated concentrations of metacycloprodigiosin (mcPG) for 48 h. SubG1 population was measured by flow cytometer. (**b**) Twelve cell lines were treated with mcPG (0.1, 0.3, 1.0, 3.0 and 10 μM) for 48 h, and subG1 population was measured by flow cytometer. Sensitivity represents *Z*-score of mcPG against 12 cell lines. *Z*-score was calculated based on the values from area under the curve (AUC). Black Bar; β-catenin-mutated tumor cell line. (**c**) Individual value plots show the *Z*-score of mutated type and the rest cell lines in tumor-related proteins including β-catenin, KRAS, BRAF and p53. *P*-values were obtained by performing a Student's *t*-test for group comparisons. $*P<0.05$ and $**P<0.01$. (**d**) Twelve cell lines were treated with the concentrations of bafilomycin A1 (BMA) (1.0, 3.0, 10, 30 and 100 nM) and concanamycin A (CMA) (0.1, 0.3, 1.0, 3.0 and 10 nM). SubG1 population was measured by flow cytometer. Sensitivity represents *Z*-score of BMA and CMA against 12 cell lines. *Z*-score was calculated based on the values from AUC. Black Bar; β-catenin-mutated tumor cell line. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Next we asked the possibility that this selective apoptosis in β-catenin mutant tumor cells caused by V-ATPase inhibitors is related to the difference of the sensitivity to V-ATPase inhibitors between β-catenin mutant tumor cells and β-catenin wild-type tumor cells. The low pH of intracellular acidic organelles including lysosomes is maintained by V-ATPases, and this acidification is detected as an orange fluorescence when the cells are stained with acridine orange, a weak base fluorescent reagent.[15] When HCT116 and A375 cells were incubated with acridine orange, intracellular organelles of these cells were stained with orange fluorescence, whereas the orange fluorescence in these organelles almost completely disappeared upon treatment with mcPG (0.1 μM) for 4 h, indicating that this concentration of mcPG inhibited V-ATPase not only in HCT116 cells but also in A375 cells (Supplementary Figure S1A). Moreover, this concentration of mcPG is comparable to that of the induction of apoptosis in HCT116 cells, but they failed to induce apoptosis in A375 cells. Similar results were obtained when BMA (3.0 nM) or CMA (0.1 nM) were used instead of mcPG (Supplementary Figure S1B). Thus, although inhibition of V-ATPase activity is not sufficient for the induction of apoptosis, apoptosis induction by V-ATPase inhibitors in β-catenin-mutated tumor cells was closely correlated to its V-ATPase inhibitory activities.

V-ATPase inhibitors induced apoptosis selectively in β-catenin mutant tumor cells, therefore, we next examined whether expression of the active form of β-catenin in tumor cells harboring wild-type β-catenin could induce apoptosis following V-ATPase inhibitor treatment. Because most of the human cancer cell lines contained mutations in β-catenin at codons 33, 37, 41 and 45 that altered potential CK1 or GSK-3β phosphorylation site,[3] the β-catenin mutants (S37A and S45A)[9,16] were transfected into HEK-293T cells harboring wild-type β-catenin, and tested for apoptosis-inducing activity of V-ATPase inhibitors. As shown in Figure 2, mcPG did not induce apoptosis in HEK-293T cells but it induced apoptosis in mutant β-catenin-expressing HEK-293T cells, as judged from Poly (ADP-ribose) polymerase (PARP) cleavage. These results suggested that actively mutated β-catenin is exactly the key factor of V-ATPase inhibitor-induced apoptosis. V-ATPase is a complex multisubunit protein devoted to the transport of protons from the cytoplasm toward intracellular compartments and from inside to outside of the cell through the cytoplasmic membrane.[17]

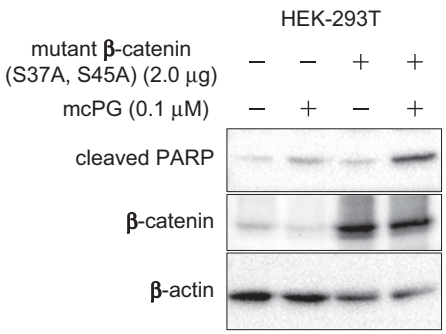

Figure 2 Mutated β-catenin-overexpressed cells become more sensitive to vacuolar-type H^+-ATPase (V-ATPase) inhibitors. HEK-293T cells were transiently transfected with control vectors or mutant β-catenin (S37A and S45A) plasmids. Then, cells were treated with 0.1 μM of metacycloprodigiosin (mcPG) for 24 h, and cell lysates were prepared for western blot analysis to detect the expression of β-catenin and cleaved PARP.

V-ATPase contributes to lower extracellular pH thus activating extracellular metalloproteinases that promote tumor cell survival, motility and invasion, resulting in enhanced malignancy ability. In addition, upregulation or overexpression of V-ATPase is frequently observed in several types of solid tumors and the causative acidic microenvironment offers an advantage in tumor progression, chemoresistance and metastatic behavior.[18] Therefore, the diverse functions of the V-ATPase in tumor survival and metastasis make it an attractive potential target in the development of anticancer drugs. Indeed, several papers have reported that V-ATPase inhibitors showed antitumor effects *in vitro* and *in vivo*. We also previously reported that V-ATPase inhibitors potentiated the cytotoxicity of anticancer drugs in Bcl-2/Bcl-xL-overexpressing tumor cells,[19] and V-ATPase inhibitors induced apoptosis in epidermal growth factor (EGF) receptor-overexpressing tumor cells only when the cells were stimulated with EGF.[20] However, so far, selective apoptosis-inducing ability to mutant β-catenin in tumor cells by V-ATPase inhibitors have not been reported; therefore, this is the first report to find that V-ATPase inhibitors exhibited synthetic lethality with active β-catenin mutant in tumor cells. The mechanism by which V-ATPase inhibitor selectively induced apoptosis in cell lines harboring active mutated β-catenin is currently under investigation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on *The Journal of Antibiotics* website (http://www.nature.com/ja)