The proton pump inhibitor pantoprazole disrupts protein degradation systems and sensitizes cancer cells to death under various stresses

Yu Cao1,2, Min Chen1,2, Dehua Tang1,2, Hongli Yan3, Xiwei Ding1,2, Fan Zhou1,2, Mingming Zhang1,2, Guifang Xu1,2, Weijie Zhang4,5, Shu Zhang1,2, Yuzheng Zhuge1,2, Lei Wang1,2 and Xiaoping Zou1,2

Abstract
Proton pump inhibitors (PPIs) play a role in antitumor activity, with studies showing specialized impacts of PPIs on cancer cell apoptosis, metastasis, and autophagy. In this study, we demonstrated that pantoprazole (PPI) increased autophagosomes formation and affected autophagic flux depending on the pH conditions. PPI specifically elevated SQSTM1 protein levels by increasing SQSTM1 transcription via NFE2L2 activation independent of the specific effect of PPI on autophagic flux. Via decreasing proteasome subunits expression, PPI significantly impaired the function of the proteasome, accompanied by the accumulation of undegraded poly-ubiquitinated proteins. Notably, PPI-induced autophagy functioned as a downstream response of proteasome inhibition by PPI, while suppressing protein synthesis abrogated autophagy. Blocking autophagic flux in neutral pH condition or further impairing proteasome function with proteasome inhibitors, significantly aggravated PPI cytotoxicity by worsening protein degradation ability. Interestingly, under conditions of mitochondrial stress, PPI showed significant synergism when combined with Bcl-2 inhibitors. Taken together, these findings provide a new understanding of the impact of PPIs on cancer cells’ biological processes and highlight the potential to develop more efficient and effective combination therapies.

Introduction
Proteostasis is a necessity for cell survival when facing stress1. Two major protein degradation systems have developed to handle these tasks, the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway (ALP)2. Proteasome inhibition caused poly-ubiquitinated proteins accumulation, and then activated autophagy to eliminate protein aggregates1–6. UPS and ALP share common signaling receptors and substrates such as SQSTM17. Therefore, in the context of proteasome inhibition, the complexity of using SQSTM1 as an autophagy marker should be underscored8,9. Besides autophagy, accumulation of unfolded proteins in the endoplasmic reticulum (ER) upon proteasome inhibition, initiates a specialized response known as the unfolded protein response (UPR)10. The intensity of UPR reflects the protein overload stress. Once beyond the scope of tolerance, a terminal UPR was provoked and the irreversible damage would be brought to cancer cells under integrated stress11. Mitochondrial permeabilization is controlled by the balance of antiapoptotic and proapoptotic Bcl-2 family proteins, which set the apoptotic threshold12. In the case of proteasome inhibition, there would be a complex crosstalk between mitochondria and other organelles, and various regulations of Bcl-2 family proteins13,14. Silencing the prosurvival
pathways by Bcl-2 inhibitors would make cancer cells under integrated stress more sensitive to death. Proteasome inhibitors have been confirmed exerting a synergistic cytotoxicity when combined with Bcl-2 inhibitors.

Previous works have reported the inhibitory effects of proton pump inhibitors (PPIs) on autophagy in low pH condition, which makes PPI transformed into the active molecule to inhibit the vacuolar-type H+-translocating ATPase (V-ATPase). Moreover, Marino et al. reported that in addition to blocking the autophagic flux in low pH condition, ESOM also induced the early accumulation of autophagosomes. Thus we are wondering whether PPI has similar impacts on autophagy in neutral pH condition. Besides autophagy, the impact of PPI on another protein degradation system remains to be investigated because there was crosstalk between the ubiquitin-proteasome and autophagy-lysosome systems. A dose-dependent and time-dependent apoptotic-like cytotoxicity by PPI has been confirmed in B-cell lymphoma, melanoma, and multiple myeloma. The effect of PPI was associated with alkalization of lysosomal pH and lysosomal membrane permeabilization. Whether PPI-induced cell death was caspase dependent or not depended on tumor histology, suggesting that the specificity of the death pathway depended on the original cell type. Moreover, the impacts of PPI on Bcl-2 family members have not been investigated, and whether they were involved in PPI-induced apoptosis remains to be seen. We focused on gastric cancer cell lines for the study because our previous works about pantoprazole were about gastric cancer. In this study, at least five unexplored mechanisms have been discovered and studied. First, PPI consistently promoted autophagosome formation in both low pH and neutral pH conditions, with TM9SF4-mTOR pathway playing an important role. Second, PPI-induced autophagy with increased SQSTM1 transcription, which was mediated by oxidative stress induced-Nrf2 in both low pH and neutral pH conditions. Third, pantoprazole inhibits proteasome function via transcriptionally reducing proteasome subunits partially via inhibiting STAT3 independent of pH conditions, which contributes to the activation of UPR and ER stress. Fourth, proteasome inhibition or ER stress was responsible for the activation of PPI-induced autophagy. Last but not least, Bcl-2/Bcl-xl inhibitors such as ABT-263 and ABT-737 have synergistic interaction with PPI in gastric cancer cells in both pH 7.4 and pH 6.5 conditions, which has a broad prospect in the field of cancer treatment.

Results

PPI-induced autophagosome formation via TM9SF4-mTOR pathway

The increased cytoplasmic vacuolation after PPI treatment was confirmed by transmission electron microscopy and GFP-LC3B puncta assay (Fig. 1a–c and Supplementary Figure S3a–c) in both low and neutral pH conditions. The transition from LC3B-I to LC3B-II was also enhanced in a dose-dependent manner and time-dependent manner (Fig. 1d–e and Supplementary Figure S3d), and real for different cancer cell lines (Supplementary Figure S1e). The key negative regulator of autophagy, mTOR signaling pathway and a number of autophagy regulators were significantly upregulated after PPI treatment (Fig. 1f, Supplementary Figure S2a and S2b, Supplementary Figure S3e and S3f). To determine whether they were responsible for PPI-induced autophagy, we adopted genetic and pharmacological inhibition approaches. After Atg5 and Atg7 were knocked down by siRNA, the PPI-induced LC3B-II accumulation was abolished (Supplementary Figure S2c-f and Supplementary Figure S3g). On the contrary, disrupting class III PI3K complex, neither by knocking down Beclin 1 via siRNA or inhibiting PI3K using wortmannin (WM), there was no difference (Supplementary Figure S2c and S2g, Supplementary Figure S5g). Autophagy was an important adaptation mechanism for cancer cells when exposed to an acidic environment. Cancer cells under microenvironmental stress (including low pH) use to cannibalize other cells. TM9SF4 has been recently reported as an important regulator of autophagy and cannibalism. Suppression of TM9SF4 by siRNAs attenuated the PPI-stimulated dephosphorylation of mTOR, RPS6, and 4E-BP1 (Fig. 1g, h). More important, knocking down TM9SF4 reduced LC3B accumulation after PPI treatment via reversed mTORC1 activity. In TCGA cohort, the mRNA expression level of TM9SF4 was also significantly correlated with important autophagy genes (Fig. 1i). Taken together, these data showed that PPI induced autophagosome formation via TM9SF4-mTOR pathway, consistent with recent studies.

PPI didn’t always block the autophagic flux in neutral pH

Previous works have reported PPI as a V-ATPase inhibitor in low pH condition, with no apparent increase in LC3B and SQSTM1 accumulation after Baf A1 added (Supplementary Figure S4a), increased GFP+RFP+ puncta (Supplementary Figure S4b) and reduced LysoTracker Red staining (LTR) (Supplementary Figure S4c). This could naturally explain for the accumulation of SQSTM1 after PPI treatment in low pH condition. However, in neutral pH condition, baf A1 dramatically caused further accumulation of LC3B-II by PPI, and PPI still increased the amount of LC3B-II in the presence of baf A1 (Fig. 2a). In line with the change of LC3B-II, dramatic increase in SQSTM1 was also observed (Fig. 2a). In PPI-treated cells, major parts of the LC3B-positive puncta were red, which showed the similar pattern as positive control torin 1 did (Fig. 2b). As shown in Fig. 2c, PPI resulted in a significant
Fig. 1 (See legend on next page.)
increase in acidic vesicles (LTR). In addition, the relatively predominant V-ATPase subunits exhibited an increase after PPI treatment (Fig. 2d and Supplementary Figure S4d-e), albeit to a different degree. Notably, the important regulators of lysosomal biogenesis such as TFE3 and TFEB, accompanied with key lysosomal enzymes, also showed an increased expression (Fig. 2e, Supplementary Figure S5d and S5e). These data indicated that PPI not only promoted autophagosome formation, but also induced lysosomal biogenesis. In order to examine whether the impact of PPI on V-ATPase was merely dependent on the pH condition, more PPI-treated cancer cell lines had been screened based on LTR staining in neutral pH33. Most of cell lines tested showed an increased LTR staining, with A549 exhibiting the most increment (Fig. 2f and Supplementary Figure S5f), accompanied by increased V-ATPase subunits (Supplementary Figure S5g). However, MKN45 and U2OS showed reduced LTR staining. In MKN45 cells, the accumulation of SQSTM1 was not further increased after baf A1 added (Supplementary Figure S5h), supporting autophagic flux blockage. Unexpectedly, PPI-treated MKN45 cells still showed an increase in V-ATPase subunits (Supplementary Figure S5i), indicating that PPI decreased LTR staining not via reducing V-ATPase subunits, consistent with our findings about PPI in low pH condition. Most important, the PPI-induced SQSTM1 accumulation in neutral pH was sensitive to rapamycin-induced autophagy in AGS cells, while resistant in MKN45 cells (Fig. 2g). Collectively, the impact of PPI on autophagic flux was mainly pH dependent, while regardless of the impact of PPI on LTR staining, PPI-induced lysosome biogenesis in parallel with autophagosome formation.

The increased SQSTM1 by PPI was transcriptionally mediated by NFE2L2

Since the accumulation of SQSTM1 by PPI was not always a result of autophagic flux blockage such as in neutral pH condition, then we measured the mRNA level of SQSTM18. PPI-induced SQSTM1 mRNA increase occurred both in low and neutral pH conditions, which indicated that this phenomenon was independent of acidic environment (Fig. 3a). Published data showed that SQSTM1 mRNA might be induced by ROS34. Cancer cells generated ROS after PPI treatment for 48 h (Fig. 3b and Supplementary Figure S6a) in both conditions as previously reported18,19. To clarify the role of ROS in SQSTM1 mRNA increase, we first checked that pre-incubation with the ROS scavenger GSH (4 mM) or NAC (5 mM) diminished the ROS (data not shown), accompanied with a reversal of cell viability (CCK8), apoptotic phenotype (cleaved PARP) and integrated stress (p-p38) (Supplementary Figure S6b and S6c). PPI-induced SQSTM1 accumulation was significantly abrogated in the presence of NAC or GSH (Fig. 3c, d). Nrf2 is a transcription factor that could be activated in response to ROS. Remarkably, PPI induced Nrf2 expression (Fig. 3e and Supplementary Figure S6d) in both pH conditions, with its increased targets (Fig. 3e, f, Supplementary Figure S6d and S6e)35. As there being antioxidant phenotype elements (AREs) in the promoter regions of SQSTM136, Nrf2 could be responsible for increased SQSTM1 mRNA. In Nrf2 deficient cancer cells, the increased SQSTM1 protein and mRNA was reversed (Fig. 3g-i). However, there was no putative AREs in the promoter regions of LC3B36. Thus, we failed to detect any significant difference of LC3B-II by PPI between normal and Nrf2-deficient cancer cells (Fig. 3g). The tight relationship between Nrf2 and SQSTM1 was not only tested in vitro, but also confirmed in gastric patients from online databases (TCGA, GSE63089, and GSE27342) (Supplementary Figure S6f). To summarize, these data suggested that Nrf2 activation increased SQSTM1 mRNA in both pH conditions, and contributed to the increased SQSTM1 protein level no matter whether PPI blocked the autophagic flux (pH 6.5) or not (pH 7.4), but was not associated with PPI-induced autophagy.
Fig. 2 (See legend on next page.)
Impaired proteasomal degradation also contributed to SQSTM1 accumulation

It appeared that Nrf2-mediated transcriptional increase could fully explain SQSTM1 accumulation in neutral pH condition without impaired autophagic degradation11. However, to our surprise, in cancer cells overexpressing HA-tagged SQSTM1 plasmid without mRNA interference, PPI increased rather than decreased the levels of HA in neutral pH condition, while it was natural in low pH condition because PPI blocked autophagic flux (Fig. 4a and Supplementary Figure S7). Although SQSTM1 was a common substrate for autophagy, it was also degraded by proteasome and accumulated after proteasome inhibition11. The SQSTM1 accumulation patterns due to autophagic flux blockage were confirmed (Fig. 4b, c), accompanied with degraded LC3B-II. Corresponding to autophagy, when UPS inhibited, SQSTM1 also accumulated (Fig. 4d), with a further accumulation upon simultaneous inhibition of autophagy (Fig. 4e). Indeed, PPI treatment prolonged the half-life of exogenous SQSTM1 protein (HA) (Fig. 4f) in neutral pH condition, which indicated that PPI impaired the proteasomal degradation of SQSTM1. It is important to notice that, both in low and neutral pH conditions (Supplementary Figure S8), PPI treatment caused the colocalization of SQSTM1 with Ubiquitin, indicating that SQSTM1 was ubiquitylated, which was the characteristic of proteasome inhibition11. More importantly, the accumulated SQSTM1 due to impaired UPS could still be cleared by autophagy (Fig. 4g), indicating no obstructed autophagic flux under proteasome inhibition. Similarly, accumulated SQSTM1 after PPI treatment could also be degraded via autophagy, while in low pH conditions the accumulated SQSTM1 couldn’t be degraded via autophagy because of autophagic flux blockage (Fig. 4h). In order to rule out the interference of mRNA changes, we measured the SQSTM1 mRNA after PPI treatment with or without autophagy inducers. There was no elevated SQSTM1 mRNA reversing back after autophagy activation (Fig. 4i), showing that the decreased SQSTM1 protein level after autophagy activation was not due to reduced transcription. To summarize, the accumulated SQSTM1 was derived from not only increased transcription via Nrf2 but also reduced proteasomal degradation in both pH conditions.

PPI inhibited proteasome function via reducing proteasome subunits expression

According to the results above, we considered the UPS as a new potential target of PPI. First of all, polyubiquitinated proteins increased (Fig. 5a and Supplementary Figure S9a), suggesting that PPI inhibited proteasome function. TP53, the short-lived protein degraded by proteasome2,5, accumulated after PPI treatment (Supplementary Figure S9b). Secondly, due to lacking the aromatic ketone structures, PPI might inhibit the proteasome function on the transcriptional level. We noticed that PPI affected the mRNA of several proteasome and immunoproteasome subunits with a downward trend (Fig. 5b and Supplementary Figure S9c), which contributed to proteasome activity39,40. Prior work revealed a potential link between STAT3 and its transcriptional control of immunoproteasome subunits11. Along with the inhibited transcriptional activity of STAT3 (Supplementary Figure S10a)12, PPI reduced mRNA expression of PSMB8 and PSMB9 (Supplementary Figure S10b) in both pH conditions. We also confirmed the mRNA correlation between STAT3, PSMB8 and PSMB9 in online databases (Supplementary Figure S10c). However, the definite STAT3 binding sites were only confirmed in the promoter regions of PSMB8 and PSMB941, while PPI could make other proteasome subunits downregulated. As a result, PPI maybe reduce proteasome subunits expression via far more than just inhibiting STAT3.
Fig. 3 (See legend on next page.)
ER stress/UPR was initiated upon proteasome inhibition by PPI

An inactive proteasome made unfolded proteins accumulated in the ER, and then caused ER stress/UPR. We screened for ER stress related markers, with CHOP and p- eIF2a (Ser 52) showing a dose-dependent increase (Fig. 5c, d, Supplementary Figure S11a and S11b). ER Tracker staining and quantification by flow cytometry indicated ER expansion in PPI-treated cells (Fig. 5e, Supplementary Figure S11c) 43. In addition, perturbations in Ca2+ homeostasis upon ER stress were observed as early as for 24 h (Fig. 5f and Supplementary Figure S11d). The mRNA expression of SERCA (Sarco/ER Ca2+-ATPase for influx) and IP3R (inositol trisphosphate (IP3) receptor for efflux) was also examined, revealing a decrease in SERCA (the target of thapsigargin (TG)) and an increase in the most prominent isoform, ITPR3 (Supplementary Figure S11e and S11f). Protein overload in the ER induces autophagy 44,45. Both CHOP siRNA and the IP3Rs inhibitor 2-ABP, could partially reverse the induction of LC3B-II by PPI (Fig. 5g, h) in both pH conditions, suggesting that ER stress after proteasome inhibition was located upstream of PPI-induced autophagy independent of pH conditions.

PPI sensitized cancer cells to protein overload stress

Sustained proteasome inhibition could lead to uncontrolled UPR and subsequent cell death. After pretreated with PPI for 24 h, more AGS cells underwent apoptosis (Fig. 6a) when proteasome inhibition was aggravated by bortezomib or MG132, along with more polyubiquitinated proteins accumulated (Fig. 6b). Cell viability assay showed that, in the presence of bortezomib, PPI displayed a more profound cytotoxic effect, confirmed by Bliss index (1.53 ± 0.148) (pH 7.4) (Fig. 6f) and Bliss index (1.34 ± 0.115) (pH 6.5) (Supplementary Figure S12a). As a marker for protein overload stress 43, the UPR genes also showed a further induction in combined approach. On the other side, preventing protein synthesis by cycloheximide (CHX) could lessen PPI-induced UPR and apoptosis (Fig. 6c) and poly-ubiquitinated proteins accumulation (Fig. 6d). Reducing protein synthesis by mTOR inhibitor rapamycin or torin 1 also achieved similar results (Fig. 6e). In cells with low levels of protein synthesis, the cytotoxic effect of PPI decreased, with both Bliss indexs (pH 7.4: CHX: 0.30 ± 0.09; Torin 1: 0.81 ± 0.03; pH 6.5: CHX: 0.75 ± 0.15; Torin 1: 0.77 ± 0.08) lower than 1, and the increased UPR genes reverting to normal levels (Fig. 6e). In cells with low levels of protein synthesis, the cytotoxic effect of PPI decreased, with both Bliss indexs (pH 7.4: CHX: 0.30 ± 0.09; Torin 1: 0.81 ± 0.03; pH 6.5: CHX: 0.75 ± 0.15; Torin 1: 0.77 ± 0.08) lower than 1, and the increased UPR genes reverting to normal levels (Fig. 6e). In cells with low levels of protein synthesis, the cytotoxic effect of PPI decreased, with both Bliss indexs (pH 7.4: CHX: 0.30 ± 0.09; Torin 1: 0.81 ± 0.03; pH 6.5: CHX: 0.75 ± 0.15; Torin 1: 0.77 ± 0.08) lower than 1, and the increased UPR genes reverting to normal levels (Fig. 6e). In cells with low levels of protein synthesis, the cytotoxic effect of PPI decreased, with both Bliss indexs (pH 7.4: CHX: 0.30 ± 0.09; Torin 1: 0.81 ± 0.03; pH 6.5: CHX: 0.75 ± 0.15; Torin 1: 0.77 ± 0.08) lower than 1, and the increased UPR genes reverting to normal levels (Fig. 6e). In cells with low levels of protein synthesis, the cytotoxic effect of PPI decreased, with both Bliss indexs (pH 7.4: CHX: 0.30 ± 0.09; Torin 1: 0.81 ± 0.03; pH 6.5: CHX: 0.75 ± 0.15; Torin 1: 0.77 ± 0.08) lower than 1, and the increased UPR genes reverting to normal levels (Fig. 6e). In cells with low levels of protein synthesis, the cytotoxic effect of PPI decreased, with both Bliss indexs (pH 7.4: CHX: 0.30 ± 0.09; Torin 1: 0.81 ± 0.03; pH 6.5: CHX: 0.75 ± 0.15; Torin 1: 0.77 ± 0.08) lower than 1, and the increased UPR genes reverting to normal levels (Fig. 6e). In cells with low levels of protein synthesis, the cytotoxic effect of PPI decreased, with both Bliss indexs (pH 7.4: CHX: 0.30 ± 0.09; Torin 1: 0.81 ± 0.03; pH 6.5: CHX: 0.75 ± 0.15; Torin 1: 0.77 ± 0.08) lower than 1, and the increased UPR genes reverting to normal levels (Fig. 6e). In cells with low levels of protein synthesis, the cytotoxic effect of PPI decreased, with both Bliss indexs (pH 7.4: CHX: 0.30 ± 0.09; Torin 1: 0.81 ± 0.03; pH 6.5: CHX: 0.75 ± 0.15; Torin 1: 0.77 ± 0.08) lower than 1, and the increased UPR genes reverting to normal levels (Fig. 6e).

Autophagy is often activated as an important compensatory response for proteasome inhibitors 4-6,46. Further inhibiting proteasome by bortezomib promoted PPI-induced autophagy (Supplementary Figure S13a and S13b). On the contrary, when protein synthesis was abrogated or suppressed, the induced autophagy by PPI was reverted (Supplementary Figure S13c-f). Simultaneous inhibition of autophagy upon bortezomib treatment caused further accumulation of ubiquitinated proteins, and made cells more prone to apoptosis (Supplementary Figure S14a and S14b). Similarly, when the early stage of autophagy was genetically inhibited, PPI also made more cancer cells undergo apoptosis (Supplementary Figure S14c), even though the degree of increase varied in cell lines. When autophagic flux was inhibited, consistent results were achieved (Supplementary Figure S14d and S14e). Given the fact that PPI blocked autophagic flux in low pH condition, there was an interesting comparison showing that, the same amount of PPI caused more protein overload stress and UPR markers in pH 6.5 than pH 7.4, which was quantified by ER Tracker staining (Supplementary Figure S14f) and Q-PCR (Supplementary Figure S14g). Similar results were also achieved by adding HCQ into the PPI-treated cancer cells in pH 7.4, which indicating that the increased protein overload stress in pH 6.5 was due to autophagy inhibition.

These observations suggested that, based on the proteasome inhibitory effect of PPI, both proteasome and autophagy inhibitors would have a synergistic interaction with PPI, and PPI sensitized cancer cells to death under protein overload stress.
Fig. 4 (See legend on next page.)
The Bcl-2 inhibitors synergized the cytotoxicity of PPI

Similar to previous results\(^\text{18,23,24}\), in gastric cancer cells, caspase activation is involved in PPI-induced cell death, but caspase inhibition could only partially reduce PPI-induced cell death in both conditions (data not shown). Taking into account the increased mROS (Supplementary Figure S15a) and influx of calcium into mitochondria (Supplementary Figure S15b) after PPI treatment, cotargeting mitochondria together with PPI may represent a new platform to sensitize cancer cells to death. Apoptosis was guarded by Bcl-2 family proteins\(^\text{12}\). We found the interesting trend of antiapoptotic proteins increasing and proapoptic proteins decreasing (Fig. 7a, b), which may limit the pro-cell death effect of PPI. We pretreated PPI for 24 h and then added ABT-263/737 for another 24 h. This combination therapy produced a significantly advantage over each drug alone (Fig. 7c, Supplementary Figure S15c, Supplementary Figure S16a and S16c). Formal synergy analyses were performed, and the synergistic cell viability inhibitory effect was observed (Fig. 7c, d, Supplementary Figure S15c and S15d, Supplementary Figure S16c and S16d). The increased cell death was also confirmed (Supplementary Figure S15e and S15f), and the combination strategy almost totally induced mitochondrial membrane depolarization (Fig. 7e and Supplementary Figure S15f). Correspondly, more damage was brought to mitochondria (Fig. 7f)\(^\text{47}\). The synergistic cytotoxicity of combination therapy was also indicated by increased apoptosis with elevated cleaved PARP and cleaved caspase 3, accompanied with more cytochrome c releasing into cytoplasm (Fig. 7g). These data provided support that, cotargeting induced antiapoptotic Bcl-2 proteins by PPI, was an important complementary strategy for strengthening the cytotoxicity of PPI, both in low pH and neutral pH conditions.

Discussion

Previous works have shown that the accumulation of LC3B and SQSTM1 by PPI in low pH condition, was associated with the inhibitory effect of PPI on V-ATPase. However, according to our work, in neutral pH condition where PPI was unable to inhibit V-ATPase, PPI could still cause LC3B and SQSTM1 accumulation. These results showed that the impacts of PPI on autophagy were not merely the autophagic flux blockage in low pH condition\(^\text{49}\). Recent studies also found that TM9SF4 could regulate autophagy initiation in response to nutrient starvation by inhibiting the nutrient-sensing kinase complex mTORC1\(^\text{30}\). Indeed, we found that PPI markedly reduced the mTOR activity consistent with previous reports\(^\text{25}\), as indicated by dephosphorylation of mTOR, 4E-BP1 and RPS6. Intriguingly, TM9SF4-siRNAs substantially attenuated the PPI-induced mTOR inactivation, and more important LC3B accumulation. These data provide strong evidence that TM9SF4 acts through mTOR to facilitate autophagosomes formation induced by PPI in different pH conditions.

SQSTM1 accumulation in neutral pH condition after PPI treatment without V-ATPase inhibition also made the actual sources of increased SQSTM1 confusing\(^\text{9}\). Consistent with the increased SQSTM1 mRNA in esomiprazole-treated EAC cells\(^\text{22}\), PPI elevated SQSTM1 mRNA via Nrf2 in both pH conditions dependent on ROS generation\(^\text{35,36}\). Our inertial thinking usually assumed SQSTM1 accumulation to be totally mediated by Nrf2 especially in neutral pH condition, but the accumulation of exogenously expressed SQSTM1 made us aware of the potential decreased protein degradation by other system, such as UPS. In our hands, PPI inhibited proteasome function via reducing various proteasome subunits expression, even though the responsible transcription factors besides STAT3 must be elucidated in future. The colocalization of SQSTM1 with Ub indicated that, proteasome function was inhibited after PPI treatment, and then SQSTM1 was ubiquitylated and mediated the transient aggregation of ubiquitinated proteins, such as proteasome, aggresome, and ALIS formation to regulate the proteostasis\(^\text{7}\). The specific progress mediated and
Fig. 5 (See legend on next page.)
structure formed by SQSTM1 after PPI treatment needs further investigations.

Proteostasis is a key requirement for cancer cell viability and stress adaptation, with protein degradation systems maintaining the balance. Our results indicated that heightened protein synthesis was a prominent source of PPI-induced autophagy. To summarize, PPI treatment sensitized cancer cells to PPI-induced cell death. Future studies that characterize the specific pathways mediating synthetic lethality following PPI and Bcl-2 inhibitors may provide additional strategies to enhance cancer cell death.

Materials and methods

Cell culture

The human cancer cell lines including AGS, HGC27, HeLa, Panc-1, HCT116, A549, and U2OS were purchased from the Cell Resource Center, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). MKN45 was purchased from the Cell Resource Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). HuccI was obtained from the Japanese Collection of Research Bioreresources (JCRB) (Tokyo, Japan). The cell lines were originally authenticated in China Center for Type Culture Collection (CCTCC) by Short Tandem Repeat (STR) profiling and passed less than 6 months in the lab. These cells are routinely maintained in the laboratory of our group.

The acid cell culture medium (pH 6.5) was obtained by the addition of 1 M HCl solution as previously reported. The pH was estimated by the use of a FE20 FiveEasy Plus pH Meter (METTLER TOLEDO Instruments (China), Shanghai, China).

Cell viability assay and quantitative methods for drug interaction analysis

Different cancer cells (5000 cells per well) were seeded into 96-well plates and then allowed for adherence and culture overnight at 37 °C. PPI was dissolved in complete medium (CM), and other different reagents were dissolved in optimal solvent such as DMSO, whose final

(see figure on previous page)
Fig. 6 (See legend on next page.)
concentration was guaranteed to be less than 1%. The order in which the drugs were added, and the time of sustained exposure, were described in detail in the section of Results and Figure legends. Hundred microliter CCK-8 working solutions were added into each well of 96-well plates after treatment, and plates were incubated at 37 °C for another 1–1.5 h. The absorbance at 450 nm was measured, and the percentage of cell viability or cell viability inhibition was calculated by using the absorbance values of untreated control cells (C), and drug-treated cells (T) as follows: T/C × 100 for cell viability, where untreated cells set as 100%, and (C−T)/C × 100 for cell viability inhibition, where untreated cells set as 0%. All experiments were carried out in quintuplicate for technical repetition, and performed in triplicate for biologic repetition.

To assess the synthetical effect of pantoprazole with other drugs, we adopted two methods for determining the drug interaction under different circumstances. One is to calculate the ratio of observed [Eobs(x,y)] and expected [Eexp(x,y)] responses according to the Bliss method\(^5\). The formula for predicting expected effect is as follows: Eexp(x,y) = Ex + Ey − (Ex*Ey), where Ex and Ey are each separate drug effect. It should be noted that the Bliss index of 1, <1 or >1 indicates additive, antagonistic or synergistic effects, respectively. CI is an another quantitative measure for determining the degree of drug interaction with synergism (CI<1), additive effect (CI = 1), or antagonism (CI > 1) for a given final measurement, which is different from the Bliss index\(^6\). CIs were calculated by using CalcuSyn software program (BIOSOFT, Cambridge, UK).

Reagents and drugs
Pantoprazole sodium in powder (PANTOLOC) was purchased from Takeda GmbH (Germany). Bafilomycin A1 (Baf-A1, S1413), Hydroxychloroquine Sulfate (HCQ, S4430), Rapamycin (Rapa, S1039), Wortmannin (WM, S2758), and Nutlin-3a (S8059), were purchased from Selleck Chemicals (Houston, TX, USA). N-acetyl-L-cysteine (NAC, A7250), 2',7'-Dichlorofluorescin diacetate (DCFHDA, D6883), L-Glutathione reduced (GSH, G4251), Thapsigargin (TG, T9033), and 2-Aminoethyl diphenylborinate (2-APB, D9754) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Torin 1 (HY-13003), Tunicamycin (Tu, HY-A0098), Cycloheximide (CHX, HY-12320), Bortezomib (borte, HY-10227), and MG-132 (HY-13259) were purchased from MedChem Express (Shanghai, China). LysoTracker Red DND-99 (L7528), and Fluo-4-AM (F23917), Rhod-2-AM (R1245MP), MitoSOX® Red (M36008), MitoTracker® Green (M7514), MitoTracker® Red CMXRsos (M7512), and JC-1 Mitochondrial Membrane Potential Dye (T3168) were purchased from Molecular Probes (USA). ABT-263 (Navitoclax) (A3007) and ABT-737 (A8193) were purchased from Apex Bio (Houston, TX, USA). Lipofectamine 3000 (L3000008) and Lipofectamine RNAiMAX (13778150) were purchased from Invitrogen.

Plasmids, siRNAs and transfections
The pBABE-puro-EGFP-LC3 was purchased from Addgene (22405, deposited by Jayanta Debnath (University of California, San Francisco, CA, USA)). Full-length HA-tagged SQSTM1 and HA-tagged Ubiquitin plasmids were kind gifts from Dr. Mingming Zhang (Nanjing, China). For plasmids transfection, AGS and HeLa cells were seeded into 6-well plates at a density of 20–25 × 10^4 cells/well for overnight and transiently transfected using Lipofectamine 3000 according to the manufacturer’s instructions. The siRNA oligonucleotides targeting Atg5, Atg7, Beclin 1, Nrf2, CHOP, and negative control siRNA were all chemically synthesized by RiboBio (Guangzhou, China) and resuspended in RNase-free water to a stock concentration of 20 μM. The siRNA sequences were detailed in Supplementary Table 1. AGS, HGC27, and HeLa cells were reversely transfected as follows: 5 μl of 20 μM siRNA and 5 μl Lipofectamine RNAiMAX were mixed with 400 μl of Opti-MEM (Gibco) in sterilized...
Fig. 7 (See legend on next page.)
1.5 ml EP tubes. 1.6 ml of cell suspension including 30 × 10⁴ cells in complete growth medium without antibiotics was first added into each well of six-well plate. After incubation for 20 min at room temperature, the siRNA-lipid complex was then added gently into cells in a spiral pattern. This gave a final siRNA working concentration of 50 nM.

**Western blot analysis and antibodies**

Cells were lysed in RIPA buffer (Beyotime Biotechnology, P0013B) containing EDTA-free protease inhibitor cocktail (Roche, 04693159001) on ice for 30 min, and then centrifuged for 15 min (13,000 × g, 4 °C). The supernatant was collected, and protein concentrations were determined by BCA Protein Assay Kit (KeyGEN BioTECH, KG0903). Equal amounts of protein (at least 30 μg) were separated on 8–12% SDS-PAGE and then electrophoretically transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 4 h, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:3000 dilutions) for 2 h. Signals generated by enhanced chemiluminescence (Millipore) were recorded on an X-ray film. The pBABE-puro-EGFP-LC3 plasmid was transiently transfected in AGS and HeLa cells. After 48 h of transfection, the cells underwent designated treatments for further experiments. The GFP-LC3B dots were observed and captured under cellSens fluorescence microscope (Olympus, Tokyo, Japan). The average number of GFP-LC3B dots per cell was determined from three independent experiments. AGS cells were grown on collagen-precoated glass coverslips directly in 24-well plates. After appropriate treatments, cells were fixed with 30%–100%, and gradually infiltrated with epoxy resin (GENMED, GMS11012). Ultra-thin sections were obtained and stained with 4% uranyl acetate and lead citrate, and examined in a transmission electron microscope (JEM-200CX, JEOL, Japan).

**Immunofluorescence**

The pBABE-puro-EGFP-LC3 plasmid was transiently transfected in AGS and HeLa cells. After 48 h of transfection, the cells underwent designated treatments for further experiments. The GFP-LC3B dots were observed and captured under cellSens fluorescence microscope (Olympus, Tokyo, Japan). The average number of GFP-LC3B dots per cell was determined from three independent experiments. AGS cells were grown on collagen-precoated glass coverslips directly in 24-well plates. After appropriate treatments, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% normal goat serum for 1 h, incubated with primary antibodies and subsequently corresponding Alexa Fluor-labeled secondary antibodies, mounted using the Prolong Gold Antifade reagent with DAPI (Sigma), and viewed using a cellSens fluorescence microscope.

**Live-cell imaging for autophagic flux**

The mRFP-GFP-LC3 adenoviral particles were purchased from HanBio (Shanghai, China). Cells (10 × 10⁴ cells per well) were seeded into 6-well plates and then allowed for adherence overnight at 37 °C. Then cells were infected with adenoviral particles at a MOI of 100 in complete medium without antibiotics; after infection for 24 h, the medium was replaced with fresh medium with antibiotics, and the cells were cultured for another 24 h before various treatments. Imaging was performed on a
Leica SP8 Confocal System (Carl Zeiss, Germany). All image acquisition settings were kept same.

**Quantitative real-time PCR with reverse transcription (qRT–PCR)**

After designated treatments, total RNA was isolated from cells using RNAiso Plus Reagent (Takara, 9109). Approximately 1000 ng RNA was reversely transcribed into cDNAs using 5 × PrimeScript RT Master Mix (Takara, RR036A) in a 10 μl reaction mixture. The reaction conditions were: 37 °C for 15 min, 85 °C for 5 s. Quantification of gene expression was performed using the SYBR Premix Ex TaqTM II kit (TaKaRa, Kyoto, Japan) on the LightCycler 96 system (Roche, Mannheim, Germany). The reaction conditions were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All reactions were performed in triplicate. Primers used in real-time PCR experiments were shown in Supplementary Table 3.

**Detection of cell death**

Cells were seeded in a six-well plate at a density of 30 × 10⁴ cells per well, and then exposed to appropriate treatments for indicated times. The cell death was quantitatively and qualitatively analyzed by the following approaches: (1) PI staining (BD Biosciences, 556463) assay by FACS according to the manufacturer’s protocol; (2) the level of PARP and caspase-3 cleavages were detected by

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Fig. 8 Schematic illustration of proposed mechanisms. PPI inhibited proteasome function, and induced SQSTM1 elevated autophagy as a compensatory response for impaired proteasomal degradation. PPI promotes autophagic flux in neutral pH condition while blocks autophagic flux in low pH condition. When cancer cells were under proteins overload stress caused by proteasome inhibitors and autophagic flux blockers, or mitochondrial stress caused by Bcl-2 inhibitors, the cytotoxicity of PPI would be significantly increased.
western blotting. Both the floating and adherent cells were collected, and then washed twice with phosphate buffered saline (PBS). After harvested, cells were immediately resuspended in 300 μL PBS buffer followed by incubation with propidium iodide (PI; final concentration of 5 μg/ml) for 10 min. The percentage of PI-positive cells, in the name of dead cells, was quantified by BD Accuri™ C6 (BD, Becton, Dickinson, USA).

**JC-1 assay**

AGS and HGC27 cells were pretreated with PPI for 24 h, and then incubated with or without Bcl-2 inhibitors (ABT-263 or ABT-737) for another 24 h. Adherent cells were digested by trypsin and collected with floating cells. Harvested cells was washed twice with PBS, and then incubated with JC-1 dye for 25 min at 37°C. The cells with loss of mitochondrial membrane potential (ΔΨm), which showed decreased red fluorescent J-aggregates, were detected by BD FACSAria II (BD, Becton, Dickinson, USA). Data were analyzed by FlowJo software (Version 7.6.5, Tree Star Inc., Ashland, Oregon).

**LysoTracker Red staining**

After the planned treatments, cells were harvested and incubated with 50 nM LysoTracker Red DND-99 (LTR, L7528) in PBS for 30 min to label acidic organelles in live cells. After twice washing, cells were suspended in PBS and transferred into 1.5 ml EP tubes. The fluorescence intensities of at least 10,000 cells/sample were measured by BD FACSAria II (BD, Becton, Dickinson, USA).

**Dysfunctional mitochondria labeling**

For labeling mitochondria, the cells were incubated with 100 nM MitoTracker Green FM (total mitochondria) and 400 nM MitoTracker Red CMXRos (functional mitochondria) for 25 min at 37°C. The labelled cells were then washed twice with PBS followed by resuspended in PBS for flow cytometry. The subpopulation was distinguished by BD Accuri™ C6 (BD, Becton, Dickinson, USA). Dysfunctional mitochondria was reflected by decreased red fluorescence intensity under the same green fluorescence intensity.

**Statistical analysis**

All data from western blot and flow cytometry analysis were representative of at least three independent experiments. Data were analysed with GraphPad Prism 6 Software to generate curves and bar charts. Statistical analyses were performed using Student t test (two tailed) or ANOVA (analysis of variance). The appropriate tests for multiple comparisons in ANOVA were specifically described in the corresponding Figure legends. *p < 0.05, **p < 0.01, and ***p < 0.001 were all considered statistically significant, while p > 0.05 was considered not significant (n.s).
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