# Autophagy and cell death signaling following dietary sulforaphane act independently of each other and require oxidative stress in pancreatic cancer

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**Abstract.** The broccoli isothiocyanate, sulforaphane (SFN), was recently identified as being capable of eliminating highly therapy-resistant pancreatic carcinoma (PC) cells without inducing toxic side effects. While SFN has been shown to stimulate autophagy or 'self-eating', it is unclear whether this catabolic process is a pro- or anti-tumorigenic response. To investigate the role of autophagy in SFN-induced cell death, established PC cell lines were treated with SFN, and the induction of autophagy was evaluated by detecting the abundance of autophagic vesicles by electron microscopy, the increase in converted LC3-II by Western blot analysis and the autophagosome puncta of GFP-LC3 by immunofluorescence. SFN-induced autophagy was suppressed by the autophagy inhibitor chloroquine, while the autophagy inducer rapamycin did not further enhance autophagy in PC cells. Importantly, neither modulator altered SFN cytotoxicity, suggesting that SFN-induced autophagy and cell death act independently of each other. In contrast, the antioxidant N-acetyl-cysteine sustained cell viability and prevented autophagy induction after SFN exposure, indicating that both signaling pathways depend on reactive oxygen species (ROS). Our studies provide a valuable new mechanistic insight into the SFN-induced elimination of PC cells and suggest that an SFN-enriched diet potentially enhances ROS-releasing chemotherapeutic agents.

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*Abbreviations:* FCS, fetal calf serum; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; SFN, sulforaphane; LC3, microtubule-associated light chain 3; AKT, AKT1-3 genes coding protein kinase α, β and γ; PI3K: phosphatidylinositol-3-kinase; CQ, chloroquine; Rapa, rapamycin; NAC, N-acetyl cysteine; ROS, reactive oxygen species; AVs, autophagic vesicles; GFP, green fluorescent protein

*Key words:* sulforaphane, autophagy, reactive oxygen species, N-acetyl cysteine, microtubule-associated light chain 3, Beclin-1, pancreatic cancer

## Introduction

Sulforaphane (SFN) belongs to the isothiocyanates (mustard oils), a group of phytochemicals providing plants with a defense mechanism against herbivores. The large botanical family of crucifers (broccoli, cauliflower, Brussels sprouts, cabbage, mustard, rapeseed, radish, horseradish, cress and wasabi) is particularly rich in glucosinolates, the stored form of isothiocyanates. Glucosinolates are hydrolyzed to isothiocyanates by plant myrosinase upon cell damage (e.g. by chewing) or alternatively (e.g. when enzymes are denatured by cooking procedures) by bacterial myrosinase of the intestinal flora. High levels of glucoraphanin, the glucosinolate precursor of SFN, are present in broccoli and cauliflower (1). SFN was first described as a potential cancer protective agent since it reduces the generation of carcinogenic metabolites from consumed xenobiotics. In line with this, several epidemiological studies have associated the consumption of cruciferous vegetables with promising potential for cancer prevention (2).

Pancreatic carcinoma (PC) has one of the worst prognoses among all human malignancies and remains the fourth leading cause of cancer-related death. Despite progress in managing other cancers, the prognosis for PC remains devastating with a mean 5-year survival rate of <1% (3). Epidemiological studies involving cruciferous vegetables and PC are less clear, and genetic differences in metabolism appear to influence the bioavailability of isothiocyanates and thus their possible cancer-protective activity (4).

SFN has attracted much attention since the discovery of its anti-tumor effects in PC and several other tumor cell lines (5-7). Cytotoxicity has been shown to rely on the modulation of different cell cycle regulators causing a $G_2M$ arrest and on the induction of intrinsic and extrinsic apoptotic cell death (8). Additionally, the generation of reactive oxygen species

(ROS) by SFN has been identified as playing a crucial role in triggering apoptosis (9). Furthermore, enhanced autophagy has been reported in prostate cancer cells in response to cellular stress of SFN-induced ROS generation, apoptosis induction and damaged mitochondria and endoplasmatic reticulum (10,11).

Macroautophagy (referred to as autophagy hereafter) provides substrates and energy to maintain cellular metabolic homeostasis by recycling proteins or impaired organelles under environmental stress conditions. This dynamic and highly conserved process is characterized by the formation of double membrane autophagosomal vesicles containing cytosolic components and organelles that deliver their engulfed cargo for lysosomal degradation by fusing with lysosomes, thus constituting so-called autolysosomes. A widely used marker for investigating autophagic activity is immunoblotting of LC3. During autophagy, in multi-step processes similar to the activation of ubiquitin, cytosolic LC3-I is converted to a phosphatidylethanolamine (PE)-anchored form called LC3-II, found in autophagosomal membranes. Within a second ubiquitin-like conjugation step required for autophagosome formation, Atg5 is linked to Atg12. In contrast to LC3-II, Atg5-Atg12 conjugates dissociate from the mature autophagosomal membrane (12). Increased LC3-II expression has been associated with the formation of autophagosomes, and thus autophagy initiation (13,14). Another known autophagy marker is Beclin-1, which together with several other proteins, forms a complex that participates in the formation of autophagosomes. Beclin-1 has been described as a Bcl-2-interacting protein and a haplo-insufficient tumor-suppressor protein required for the induction of autophagy, which has contributed to the rapidly growing interest in autophagy in cancer research (15).

Here, we investigated autophagy in SFN-treated PC cells, assessed its importance for cell survival and describes its dependence on the generation of ROS.

## Materials and methods

*Cell culture*. The human PC cell lines, AsPc-1, Capan-1, MIA PaCa-2 and Panc-1, were purchased from ATCC and grown under standard conditions in cell culture flasks containing medium supplemented with 1% (v/v) glutamine-streptomycin solution (CC Pro, Germany), 0.2% (v/v) plasmocin (Amaxa, Biozym, Germany) and 10 or 20% (v/v) fetal calf serum (FCS) (PAA, Austria), and cultured according to ATCC instructions. All media were renewed every 2 to 3 days, and cells were passaged at 70-80% in confluent layers using accutase (PAA) and appropriate diluted and plated in fresh medium and new tissue culture flasks.

*Cell count*. Cells were detached, resuspended in three different solutions, and stained with Trypan blue (Sigma-Aldrich, Germany). Cell numbers were estimated by using a Neubauer chamber under a light microscope according to the manufacturer's instructions (Leica, Germany).

*Cytotoxicity assay*. Equal amounts of cells were plated in 96-well plates and incubated for 24 h. After the incubation time, the medium was removed and replaced in quadruplicates with fresh medium containing the drugs, SFN, chloroquine (CQ), and N-acetyl cysteine (NAC) (each from Sigma, Germany) and rapamycin (Rapa) (Biaffin, Germany) at concentrations as indicated. MTT assay was performed according to the manufacturer's instructions (Sigma, Germany). Cell viability was normalized to the untreated negative control and expressed as the mean of three independent experiments ± SEM using computer software (GraphPad Prism 5.0, USA).

*GFP-LC3*. Cells were counted, and equal numbers were dispensed into 30-mm dishes with five collagen laminated coverslips on the bottom. Transfection of GFP-LC3 (kindly provided by Dr Yamamura) was performed according to the manufacturer's instructions (PolyFect, Qiagen, Germany) and treated as indicated for 24 h. Coverslips were removed, washed three times with PBS, and cells were fixed with 2% paraformaldehyde for 15 min at room temperature. Coverslips were again washed three times, permeabilized with 0.3% Triton-X for 5 min, and washed and stained with DAPI for 3 min. Finally, the coverslips were washed three times with PBS, once with $ddH_2O$, and mounted on tissue slides with mounting medium (Fisher Scientific). Fluorescence images were captured and processed as recently described (14).

*Western blotting*. PC cell lines were seeded equally in Petri dishes, incubated for 24 h and treated as indicated for a further 24 h. The cells were harvested with a rubber policemen and transferred to 15-ml tubes. Cells were briefly centrifuged, the cell pellets were washed with PBS and again centrifuged. The cell pellets were washed by adding 1 ml cold PBS solution before being resuspended and transferred to a 1.5-ml tube. After centrifugation, the cells were incubated on ice for 20 min in lysis buffer containing 50 mM HEPES, 5 mM CHAPS, 5 mM DTT, 0.5 mM PMSF, leupeptin, pepstatin and L-leucinethiol, each at 1 mg/ml. The protein concentration was determined using the BCA method (Pierce, Rockford, IL), and 30 $\mu$g of protein homogenates was routinely loaded to a 15% SDS-PAGE gel and processed as recently described (14). The membranes were incubated with the primary anti-LC3 (MBL, Japan), anti-Beclin-1 (Santa Cruz, USA) or anti-Atg5 (Abgent, USA) antibody, followed by incubation with HRP-conjugated secondary anti-rabbit or anti-mouse antibodies (Santa Cruz) and processed as described (14). Each membrane was stripped with Western blot stripping buffer (Pierce) and re-probed with GAPDH (Cell Signaling) in order to confirm equal protein loading.

*Electron microscopy*. For the detection of autophagosome formation by electron microscopy, MIA PaCa-2 cells were fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate (pH 7.2) for 15 min at 4˚C. After scraping, cells were centrifuged at 200 x g for 10 min at 4˚C, and washed 5 times with 50 mM sodium cacodylate, followed by staining with 2% osmium tetroxide for 1-2 h at 4˚C. After washing with $dH_2O$, the samples were incubated with 0.5% uranyl acetate in $dH_2O$ overnight. After washing with $dH_2O$, samples were dehydrated using a graded series of ethanol and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate and viewed using a Zeiss EM10A electron microscope at 80 kV. The magnification indicator was routinely controlled by the use of a grating replica.

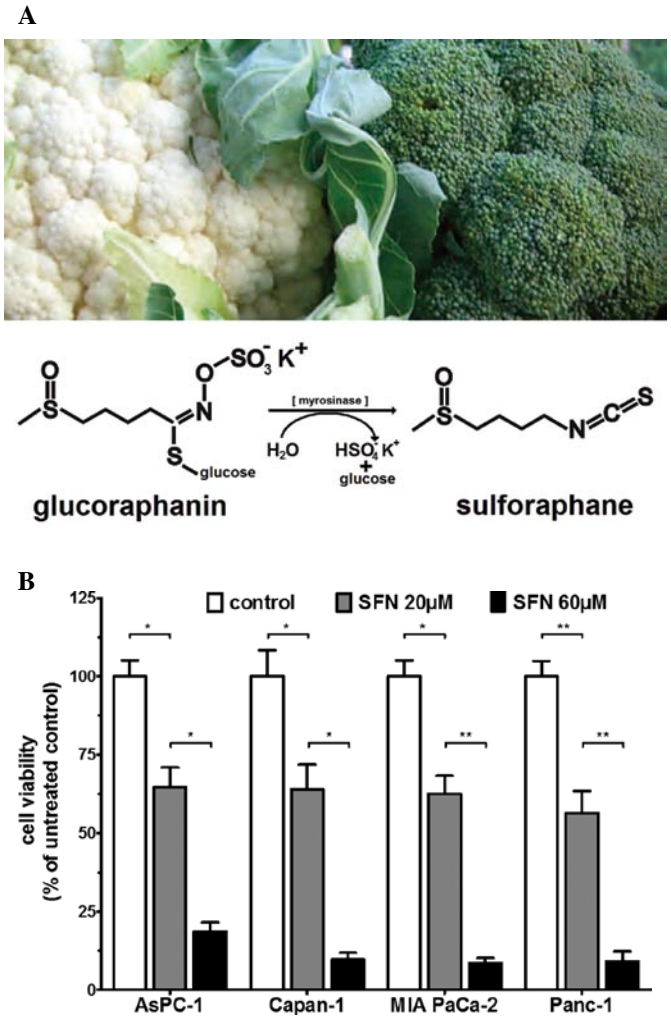


Figure 1. Dietary isothiocyanate SFN has potent cytotoxic effects on PC cells. (A) An image of cruciferous vegetables cauliflower and broccoli together with the chemical equation of the hydrolization of their main glucosinolate, glucoraphanin, to SFN. (B) Relative cell viability was plotted as the mean ± SEM, determined by multiple independent MTT assays (n=3) of four PC cell lines (AsPC-1, Capan-1, MIA PaCa-2, Panc-1) untreated or treated for 24 h with SFN (20 or 60 $\mu$M) ($^{*}0.01 \leq p < 0.05$; $^{**}p < 0.01$).

*Statistical analysis*. Statistical analysis was performed using the Student's t-test for each experimental group. Results were considered significantly different when the obtained p-value was <0.05. Statistical calculations were performed using Prism software from GraphPad.

## Results

*SFN induces cell death in human PC cell lines*. SFN, an isothiocyanate enriched in broccoli and other vegetables of the cruciferous family (Fig. 1A), has been shown to suppress growth of human PC cell lines, MIA PaCa-2 and Panc-1, by arresting cells in the $G_2$-M phase and by inducing apoptosis (6,7). We confirmed and extended these results using metastatic AsPC-1 (established from human ascite fluid) and Capan-1 (originating from a liver metastasis) established PC cells (Fig. 1B). All four investigated human PC cell lines were sensitive to SFN cytotoxicity.

*SFN initiates autophagy in human PC cell lines*. To examine whether autophagy is involved in the elimination of PC cells by SFN, we performed electron microscopy (TM) analysis using MIA PaCa-2 cells. Untreated MIA PaCa-2 cells mainly showed endosomes and lysosomes (Fig. 2A), without any appearance of autophagic vesicles (AVs). In contrast, SFN treatment resulted in the formation of typical AVs (Fig. 2B), while Rapa treatment resulted in reduced formation of AVs (16). We extended our EM observation by determining the conversion of LC3-I to LC3-II – the classical approach for autophagy induction – and the expression of Beclin-1 by immunoblotting. Both are known to be essential for the initiation and expansion of autophagic membranes (15). We found that PC cell lines, MIA PaCa-2 and Panc-1, showed a higher level of conjugated LC3-II compared to DMSO negative control cells (Fig. 2D). The detection of LC3-I was less efficient due to a higher sensitivity of the primary anti-LC3 antibody to its LC3-II isoform, as previously suggested (13). Our results indicate that SFN cytotoxicity increased autophagosome formation. Interestingly, we found that Beclin-1 expression was consistently attenuated after SFN treatment, indicating a possibly different modulation of Beclin-1 expression during the 24 h exposure time of SFN.

We confirmed our observation by applying the GFP-LC3 plasmid in order to visualize LC3-II-containing autophagosomal structures, as described previously (17). Panc-1 cells were transiently transfected with GFP-LC3 for 24 h and subsequently exposed to SFN cytotoxicity. Immunofluorescence images showed strong GFP-LC3 puncta, and resemble the LC3-II-containing autophagosomes after SFN exposure (Fig. 2E). Our data provide evidence that SFN treatment promotes the initiation of autophagy in PC cells.

*Neither inhibition nor induction of autophagy influences SFN-induced cytotoxicity*. In order to further evaluate the role of SFN-induced autophagy signaling we investigated whether CQ and Rapa modulate the induction of autophagy and cytotoxicity in response to SFN. The anti-malarial drug CQ has been shown to inhibit autophagy at a late stage, causing an accumulation of autophagosomes (18). First, we determined the expression of LC3 and Atg5, another known essential autophagic protein involved in autophagosome formation (Fig. 3A) (12). SFN increased both LC3 and Atg5 levels in comparison to the DMSO-treated controls, indicating an induction of autophagy. In contrast, single treatment with CQ showed no Atg5 signal but rather an increased level of LC3-II, indicating an accumulation of LC3-II-positive autophagosomes rather than an induction of autophagy. Rapa exposure alone resulted in a smaller amount of LC3-II and Atg5, supporting our previous EM evaluation.

We next co-treated MIA PaCa-2 and Panc-1 cells with SFN with or without CQ or Rapa (Fig. 3B). Single CQ treatment increased the LC3-II level, which was less pronounced after single Rapa exposure. In contrast, co-treatment of SFN with CQ or Rapa potentiated LC3-II expression. Cells treated with SFN and CQ caused robust LC3-II signals, most probably based on synergistic effects of autophagy induction by SFN and autophagosome accumulation by CQ. Co-treatment with SFN and Rapa showed marginally higher LC3-II than SFN alone, implicating that Rapa only slightly increases

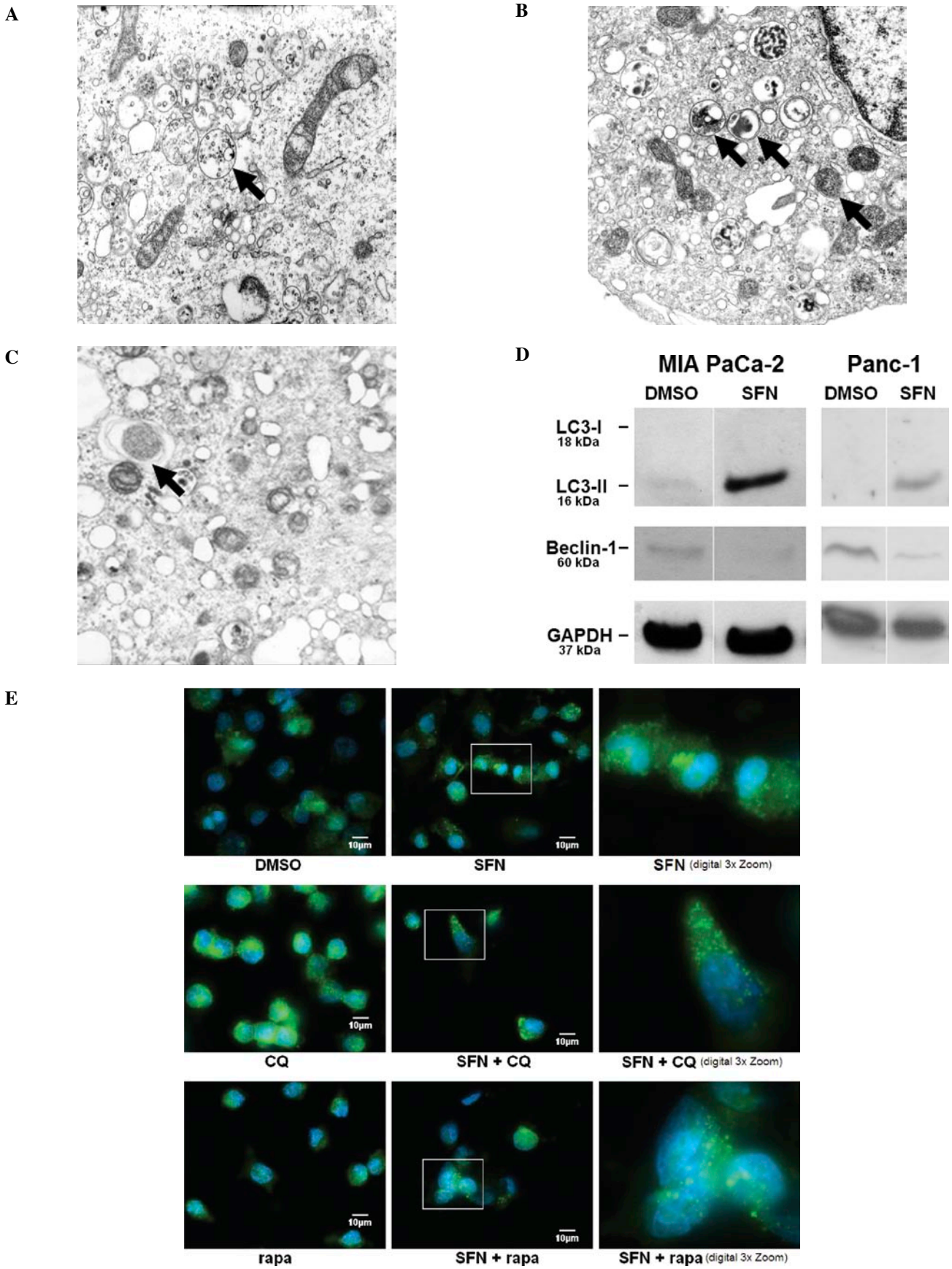


Figure 2. SFN induces the formation of autophagic vesicles in PC cells. (A-C) EM images were captured with a Zeiss EM10A electron microscope at 80 kV at a magnification of x20,000. (A) Untreated MIA PaCa-2 cells exhibited endosomes (arrow), and no signs of AVs were visible. (B) Exposure to 10 $\mu$M SFN induced multiple AVs, such as autophagosomes and autolysosomes (arrows), while (C) only a few AVs were noted (arrow) upon treatment with 20 $\mu$M Rapa. (D) PC cell lines were treated with 40 $\mu$M SFN for 24 h. Cell lysates were subjected to Western blotting for LC3. Blots were stripped and re-probed for Beclin-1 and for GAPDH as the loading control. (E) Panc-1 cells were transiently transfected with GFP-LC3 plasmids for 24 h and subsequently treated for 24 h with 40 $\mu$M SFN and co-treated with 100 $\mu$M CQ and 20 $\mu$M Rapa, respectively. Images were captured with DAPI and GFP fluorescence channels using a Zeiss Axiovert 200M microscope.

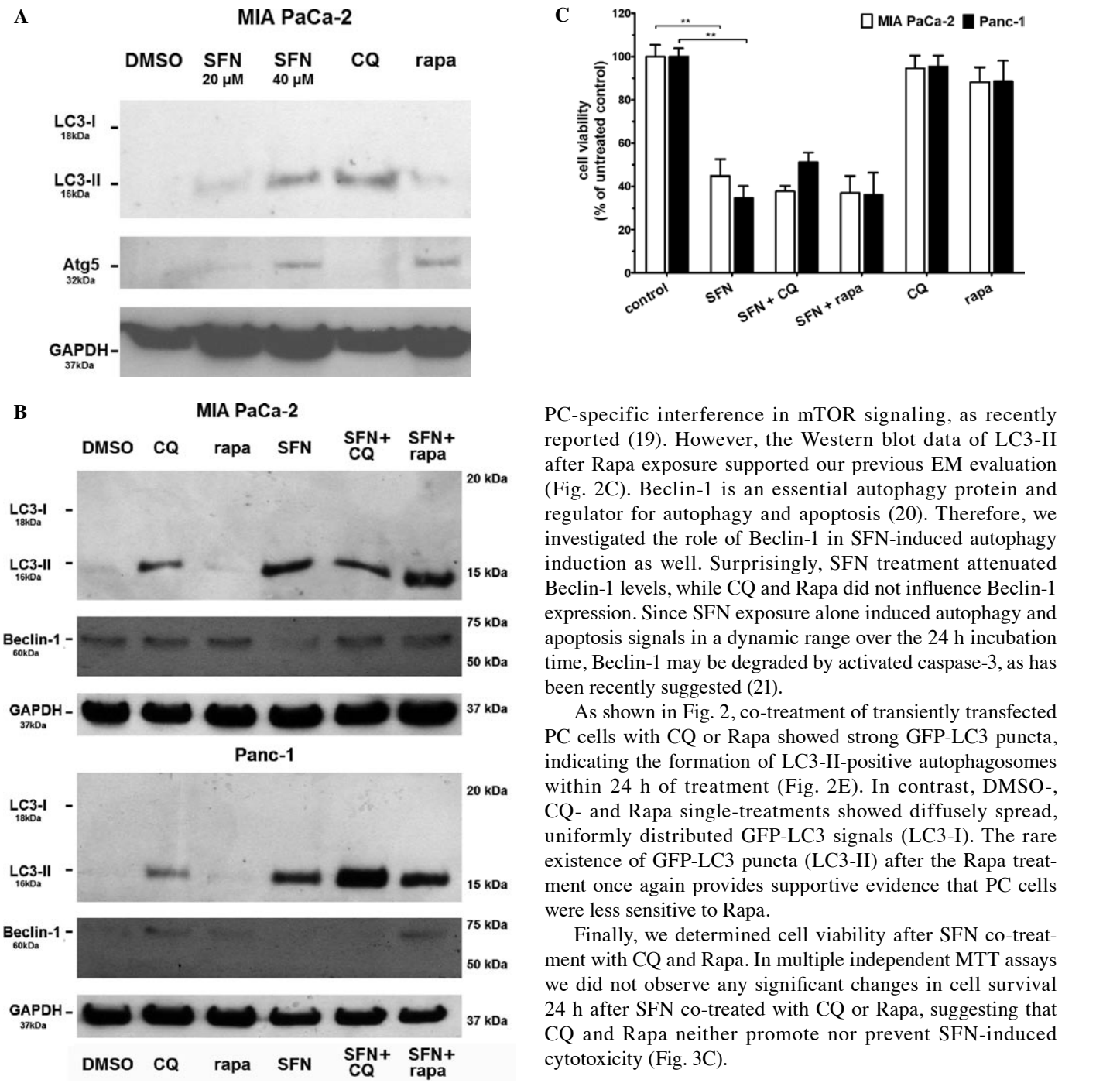


Figure 3. SFN-induced autophagy is modulated by CQ and Rapa. (A) MIA PaCa-2 cells were exposed to SFN (20 and 40 $\mu$M), CQ (100 $\mu$M) and Rapa (20 $\mu$M) for 24 h. Cell lysates were prepared for SDS-PAGE and immunoblotted for LC3 and after were re-stripped for Atg5 and GAPDH. (B) MIA PaCa-2 and Panc-1 cells were treated with 40 $\mu$M SFN with and without co-treatment with 100 $\mu$M CQ or 20 $\mu$M Rapa for 24 h. Cell lysates were then subjected to Western blot analysis to detect LC3, stripped and re-probed for Beclin-1 and finally assessed for the loading control GAPDH. (C) Relative cell viability was plotted as the mean ± SEM, determined by multiple independent MTT assays (n=3) for MIA PaCa-2 and Panc-1 cells treated for 24 h with 40 $\mu$M SFN and/or co-treated with 10 $\mu$M CQ and 20 $\mu$M Rapa ($^{**}$p<0.01 versus controls).

LC3-II, which is in accordance with Rapa as a moderate autophagy inducer in PC cells. Rapa treatment alone induced less LC3-II, indicating that Rapa is less capable of enhancing autophagosome formation as a possible consequence of PC-specific interference in mTOR signaling, as recently reported (19). However, the Western blot data of LC3-II after Rapa exposure supported our previous EM evaluation (Fig. 2C). Beclin-1 is an essential autophagy protein and regulator for autophagy and apoptosis (20). Therefore, we investigated the role of Beclin-1 in SFN-induced autophagy induction as well. Surprisingly, SFN treatment attenuated Beclin-1 levels, while CQ and Rapa did not influence Beclin-1 expression. Since SFN exposure alone induced autophagy and apoptosis signals in a dynamic range over the 24 h incubation time, Beclin-1 may be degraded by activated caspase-3, as has been recently suggested (21).

As shown in Fig. 2, co-treatment of transiently transfected PC cells with CQ or Rapa showed strong GFP-LC3 puncta, indicating the formation of LC3-II-positive autophagosomes within 24 h of treatment (Fig. 2E). In contrast, DMSO-, CQ- and Rapa single-treatments showed diffusely spread, uniformly distributed GFP-LC3 signals (LC3-I). The rare existence of GFP-LC3 puncta (LC3-II) after the Rapa treatment once again provides supportive evidence that PC cells were less sensitive to Rapa.

Finally, we determined cell viability after SFN co-treatment with CQ and Rapa. In multiple independent MTT assays we did not observe any significant changes in cell survival 24 h after SFN co-treated with CQ or Rapa, suggesting that CQ and Rapa neither promote nor prevent SFN-induced cytotoxicity (Fig. 3C).

*SFN-induced cytotoxic autophagy depends on oxidative stress*. Since many cytotoxic drugs exert their cytotoxicity via the release of ROS and induce parallel autophagy, we investigated whether ROS might also be important for the mode of action of SFN. Examination of cell viability by MTT assay revealed that the addition of NAC to a cytotoxic SFN dose sustained cell viability in MIA PaCa-2 and Panc-1 cells (Fig. 4A). We then further determined whether NAC co-treatment modulates autophagy induction by immunoblots for LC3 and Beclin-1. Our results showed that SFN-induced LC3-II expression was completely suppressed by NAC co-treatment in MIA PaCa-2 and Panc-1 cells (Fig. 4B). Interestingly, SFN-mediated reduction of Beclin-1 still persisted after co-treatment with NAC. Our data suggest that scavenging ROS prevents SFN-induced cytotoxicity and autophagy in PC cells and that ROS appear to be a key prerequisite for

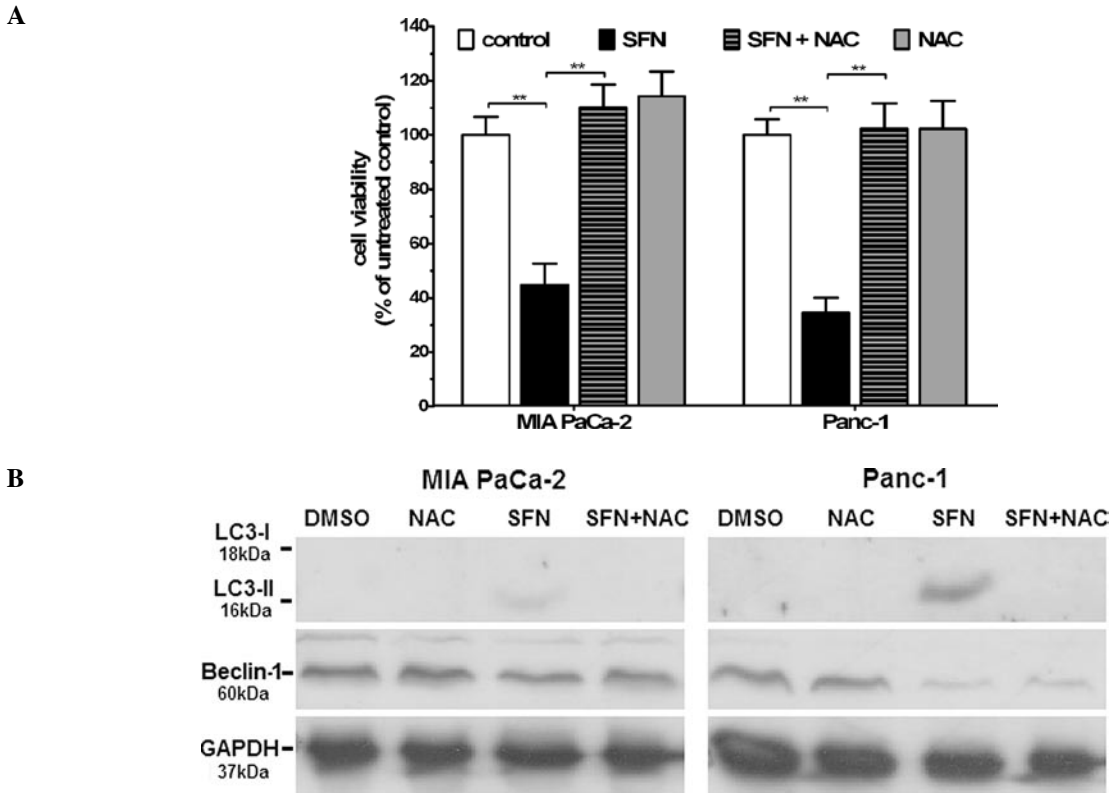


Figure 4. SFN-induced cytotoxicity depends on oxidative stress. (A) MIA PaCa-2 and Panc-1 cells were treated for 24 h with 40 $\mu$M SFN or co-treated with NAC 2 mM. Relative cell viability was plotted as the mean ± SEM, determined by multiple MTT assays (n=3) ($^{**}p<0.01$). (B) Western blot analysis of MIA PaCa-2 and Panc-1 cells treated with 40 $\mu$M SFN or co-treated with 2 mM NAC as indicated. Cell lysates were immunoblotted for LC3, stripped and re-probed for Beclin-1 and GAPDH.

SFN-induced cell death and autophagy, while the detailed mechanism of interaction remains to be determined. However, these data indicate that co-treatment of cancer cells with SFN and ROS-inducing cytotoxic agents may result in synergistic effects.

## Discussion

In the present study, we report for the first time that the naturally occurring isothiocyanate sulforaphane induces autophagy in PC cells. We showed an abundance of AVs in electron microscopy as a consequence of SFN-induced cytotoxicity, an increase in the autophagosome-associated LC3-II and Atg5 in Western blot analysis, and an aggregation of GFP-LC3 puncta, indicating autophagosome formation. Consistent with other reports, SFN is a potent cytotoxic agent and can be effectively used in the *in vitro* treatment of PC as well as for other malignancies (5-8). Concentrations of SFN used in previous reports usually vary between 10-100 $\mu$M. Plasma concentrations of SFN in humans who have consumed a portion of standard broccoli soup (100 g florets microwaved for 90 sec) reach 2-3 $\mu$M which is excreted almost entirely in the urine 24 h later (22). Therefore, we assume that higher SFN plasma levels may be reached by consuming broccoli sprouts, which contains 100 times higher levels of glucoraphanin (23), or by the intake of synthetic glucoraphanin or purified SFN. Several phase I clinical studies have already established the safety of 3-day old broccoli sprouts as a suitable oral or parenteral SFN source for SFN administration in humans (24).

*The role of autophagy in cancer progression and cancer therapy*. In prostate and colon cancer cells, autophagy is activated concomitantly with SFN-induced apoptotic cell death and acts as a cytoprotective mechanism, as inhibition of autophagy potentiates apoptotic cell death (10,25). In PC, the role of autophagy in carcinogenesis and the question of whether autophagy promotes or attenuates cancer cell death in response to anti-cancer treatment remains unknown. Originally, it was suggested that PC cells lose their autophagic capacity in the course of malignant transformation as found in an animal model with azaserin-induced pancreatic adenocarcinoma in rats. In this model, autophagy was only highly active in premalignant stages (26,27). These data are in contrast to our findings, which showed basal-autophagy activity in PC cells. Our data are supported by another more recent study, in which LC3 expression correlated well with a short disease-free period and poor patient prognosis in human PC, suggesting that autophagy was activated and promoted tumor cell survival in response to the typical hypovascular conditions in PC (28,29). On the other hand, gemcitabine, the standard chemotherapy for PC, has been shown to induce autophagy and potentiate apoptotic cell death in PC, while autophagy blockage attenuated apoptotic cell death. This suggests that induction of autophagy promotes gemcitabine-induced apoptotic cell death (30).

*SFN-induced cell death and autophagy signaling are independent of each other.* In order to evaluate the potential role of autophagy in SFN-mediated cell death, we co-treated PC cells with CQ and Rapa. The anti-malarial drug CQ is known for its lysosomotropic effects and blocks autophagosomal-lysosomal fusion at a late stage of autophagy signaling, facilitating the accumulation of autophagosomes (18). Here, we showed enhanced LC3-II levels after CQ exposure, indicating an accumulation rather than an induction of autophagy, as suggested by unaltered Atg5 expression, known to be essential for autophagosome formation. In combination with standard chemotherapy, CQ has been reported to enhance anti-cancer therapy efficiency in different cancer cell types (31,32). However, CQ treatment alone has been shown to induce apoptosis in several malignancies, indicating that CQ itself induces apoptotic cell death in cancer cells (31,33,34). In our experimental settings, CQ treatment did not influence SFN-induced cell death. Our results suggest that CQ co-treatment inhibited autophagy execution while SFN-mediated cytotoxicity was neither prevented nor augmented by CQ.

The immunosuppressive Rapa inhibits mTOR, resulting in reduced cell growth and proliferation as well as induction of autophagy (35). Rapa has been shown to enhance the efficiency of commonly clinically used chemotherapeutic agents in many cancer types (36). In our study, Rapa alone or in combination with SFN resulted in a smaller increase in AVs and showed no significant difference in LC3 conversion compared to either SFN single treatment or the negative control. Moreover, co-treatment of SFN and Rapa did not influence cell viability as determined by MTT assay. Induction of autophagy by Rapa seems to be less efficient in PC cells as a possible consequence of constitutively stimulated mTOR by AKT, which is typically overexpressed in PC (37,38). Accordingly, Rapa resistance has been reported to be dependent on the expression of AKT and PTEN in different cell lines (39,40). In line with the data presented here, Rapa and its analogues showed rather disappointing effects in clinical studies, and enhancing its efficiency by additional inhibition of PI3K-AKT signaling is currently under intense investigation for PC (19,41).

We found that neither blockage of autophagy by CQ nor induction by Rapa influenced the cell viability of SFN-treated PC cells, suggesting that autophagy does not affect SFN-induced cell death in PC. Further studies are required to elucidate the potential death modulating role of autophagy in PC.

*SFN-induced autophagy and cell death depends on ROS formation in PC.* In our study, cell viability of established PC cells exposed to a cytotoxic SFN concentration was completely abolished by adding the ROS scavenger NAC. Moreover, our antioxidant co-treatment prevented the induction of autophagy in PC cells, as shown by LC3 Western blot analysis. Our findings suggest that SFN-induced cell death and autophagy induction in PC seem to be dependent on the generation of ROS.

Similar to our data, scavenging ROS by NAC was reported to suppress cytotoxic effects of SFN in different types of cancer cells (6,42,43). In prostate cancer cells, SFN-induced cytotoxicity was associated with a massive generation of mitochondria-derived ROS, which is believed to act upstream of cell cycle arrest, apoptosis and autophagy (9-11). These results are consistent with our findings for NAC-co-treated PC cells and indicate that cytotoxicity and autophagy induction by SFN are very likely the consequence of ROS generation.

On the other hand, NAC has recently been shown to decrease cellular uptake of SFN by forming NAC conjugates (44). In fact, SFN is metabolized and predominantly excreted as NAC conjugates *in vivo* (45). However, SFN-NAC conjugates as well as SFN itself have been shown to induce growth arrest and apoptosis, in particular at higher concentrations and longer exposure times, indicating that the use of NAC to prevent SFN-induced ROS generation requires further evaluation (46,47). SFN has been described as a potent inducer of detoxifying phase II enzymes, such as glutathion-S-transferase, NAD[P]H:quinone oxidoreductase and UDP-glucuronosyl-transferase, which exhibit antioxidant activity (48). We showed that NAC addition prevents SFN cytotoxicity and autophagy induction. Since SFN seems to trigger the formation of ROS as a primary mode of action, we suggest that cytotoxicity and autophagy are caused by ROS as secondary effects of SFN. Consequently, the induction of phase II enzymes could occur as part of an antioxidant cellular response. Based on these data, we propose that a combination of an SFN diet with common chemotherapeutics or radiation, both also known to induce ROS, may result in synergistic effects and improve therapeutic efficacy.

Surprisingly, the attenuation of Beclin-1 expression induced by SFN exposure was not altered by NAC co-treatment, indicating that this effect might be independent or upstream of ROS generation in response to SFN. Beclin-1 has recently been described as a substrate of activated caspase-3, and truncated Beclin-1 cannot execute its pro-autophagic activity (49). Beclin-1 can also interact within its BH3 domain with anti-apoptotic Bcl-2 or Bcl-XL, leading to a decreased availability for autophagy (49). Since SFN has been reported to downregulate Bcl-2 and apoptosis in prostate cancer, which was recently found to be ROS independent, we suggest that SFN directly or indirectly decreases Beclin-1 availability in PC, independent of the formation of ROS (50).

In conclusion, the cytotoxic effects and induction of autophagy in PC cells by SFN are dependent on oxidative stress. Inhibition of SFN-induced autophagy by CQ did not change cell survival significantly, implicating that autophagy does not influence cell survival in PC. The autophagy inducer Rapa did not alter cell survival and did not further potentiate autophagy over existing enhanced basal levels of autophagy in PC. It also did not affect cell viability. In contrast, scavenging of ROS by NAC co-treatment protects against the cytotoxic effects of SFN and prevents autophagy induction, indicating that SFN-induced autophagy is dependent on the formation of ROS. Surprisingly, SFN attenuates Beclin-1 availability upstream of ROS generation, suggesting a feasible down-regulation by SFN similar to that reported for Bcl-2. Since chemotherapeutic drugs and radiation therapy are potent inducers of ROS, our data suggest that synergistic therapeutic effects can be achieved by combining conventional therapy with SFN-enriched dietary schedules.

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