DNA-Damaging Imidazoacridinone C-1311 Induces Autophagy followed by Irreversible Growth Arrest and Senescence in Human Lung Cancer Cells

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ABSTRACT

Imidazoacridinone 5-diethylaminoethylamino-8-hydroxyimidazoacridinone (C-1311) is an antitumor inhibitor of topoisomerase II and FMS-like tyrosine kinase 3 receptor. In this study, we describe the unique sequence of cellular responses to C-1311 in human non-small cell lung cancer (NSCLC) cell lines, A549 and H460. In A549 cells, C-1311 (IC₅₀ = 0.08 μM) induced G1 and G2/M arrests, whereas H460 cells (IC₅₀ = 0.051 μM) accumulated predominantly in the G1 phase. In both cell lines, cell cycle arrest was initiated by overexpression of p53 but was sustained for an extended time by elevated levels of p21. Despite prolonged drug exposure (up to 192 hours), no apoptotic response was detected in either cell line. Instead, cells developed a senescent phenotype and did not resume proliferation even after 2 weeks of post-treatment, indicating that C-1311–triggered senescence was permanent. When cell cycle arrest was evident but there were no signs of senescence, C-1311 significantly induced autophagic cells. Pharmacological inhibition of autophagy by 3-methyladenine profoundly reduced the senescent phenotype and slightly sensitized cancer cells to C-1311 by increasing cell death, suggesting a link between both autophagy and senescence. However, a small interfering RNA–mediated knockdown of the autophagy-associated Beclin 1 and ATG5 genes attenuated but failed to block development of senescence. Taken together, our studies suggest that in NSCLC, a C-1311–induced senescence program is preceded and corroborated but not exclusively determined by the induction of autophagy.

INTRODUCTION

Antitumor 5-diethylaminoethylamino-8-hydroxyimidazoacridinone (C-1311) (Fig. 1A) belongs to a group of imidazoacridinone derivatives sharing structural similarities with anthracyclines like mitoxantrone or anthracyclines like doxorubicin (Cholody et al., 1992). In phase II clinical trials, C-1311 displayed significant activity toward advanced breast cancers refractory to taxane treatment (Capizzi et al., 2006). Initially, the drug was characterized as a topoisomerase II inhibitor/ poison (Składanowski et al., 1996) and DNA intercalator, which covalently crosslinks DNA after metabolic activation (Burger et al., 1999; Dziegielewski and Konopa, 1999). Recent studies indicate that C-1311 also exerts its cytotoxicity through selective inhibition of the FMS-like tyrosine kinase 3 receptor (Chau et al., 2006) and NAD(P)H quinone oxidoreductase 2 (Nolan et al., 2010), and decreases tumor angiogenesis by downregulating the hypoxia-inducible factor-1a/vascular endothelial growth factor pathway (Paradziej-Lukowicz et al., 2011). In addition to its antineoplastic properties, C-1311 has shown preliminary activity against rheumatoid arthritis and multiple sclerosis (Karlik and Ajami, 2006). Consistent with the multitargeted activity of C-1311, the response of cancer cells to C-1311 varied among cells examined. In lymphoblastic leukemia cells, C-1311 induced a unique (for DNA-damaging drugs) sequence of events involving G2/M arrest followed by mitotic catastrophe, which in turn precipitated apoptosis (Skwarska et al., 2007). The classic apoptotic pathway was triggered in ovarian cancer and osteogenic sarcoma cells (Zaffaroni et al., 2001). Conversely, in human colon, melanoma, and bladder cancer cell lines, the C-1311 cell-killing mechanism was not restricted to apoptosis alone (De Marco et al., 2007; Smith et al., 2010). It is currently apparent that tumor cells respond to therapy through multiple pathways, with the main ones being apoptosis, necrosis, senescence, and autophagy (Portugal et al., 2006).
The importance of autophagy in regulating cancer development and cancer therapy outcome is now better appreciated (Di et al., 2009; Hoare et al., 2011). Autophagy contributes to the cytotoxic effects of ionizing radiation (IR), since it mediates subsequent cell death via apoptosis or necrosis (Gewirtz, 2009). Induction of autophagy has been reported in response to anticancer agents, such as adriamycin (Di et al., 2009), dexamethasone (Laane et al., 2009), or elisidepsin (Ling et al., 2011). However, there is extensive evidence for autophagy serving an opposite, cytoprotective function in cancer treatment (Gewirtz, 2009). Autophagy delayed apoptosis in solid tumors after DNA damage induced by camptothecin (Abedin et al., 2007), antimitotic MG-2477 (3-cyclopropylmethyl-7-phenyl-3H-pyrrolo[3,2-f]quinolin-9(6H)-one) (Viola et al., 2012), or IR (Apel et al., 2008).

Given that autophagy may enhance tumor cell viability (Young et al., 2009), as well as tumor dormancy and disease recurrence (Gewirtz, 2009), it has become clear that the complex pathways linking autophagy and apoptosis also encompass cellular senescence (Hoare et al., 2011). Under physiologic conditions, senescence and autophagy have been postulated to be a part of the same metabolic program, known as the 'senescence-autophagy axis.'

![Chemical structure of imidazoacridinone C-1311](left panel) and concentration-dependent growth inhibition in A549 and H460 cells after 72 h C-1311 treatment (right panel; raw data not shown). Data represents the IC_{50} and IC_{80} mean values from three independent experiments (± S.D.). (B and C) Cell cycle profiles of A549 and H460 cells exposed to C-1311 at IC_{80} doses for the indicated times. Representative flow cytometry analyses (B) and quantification of cells in cell cycle phases (C). Data represent mean values from three experiments (± S.D.).
as the autophagy-senescent transition, in which autophagy mediates the onset of stress-induced senescence (Capparelli et al., 2012). Autophagy that occurs during senescence may shape senescence-mediated cytokine production, which in turn may facilitate the clearance of senescent cells by the immune system (Rosenfeldt and Ryan, 2011). An opposite concept, the senescence-autophagy transition, implicates aging and senescence as triggers for autophagy, which could provide recycled nutrients that "fuel" tumor growth (Capparelli et al., 2012). From a therapeutic perspective, it is not clear whether senescence is irreversible, transient but succeeded by cell death, or transient but with potential for proliferative recovery (Gewirtz, 2009). There is only limited scientific literature addressing the role of autophagy in tumor senescence after drug or IR treatment. Recent studies have shown that chronic apoptotic stress and DNA damage triggered by IR initiate cytoprotective autophagy and regulate cell survival through senescence in human lung carcinoma (Singh et al., 2012). Similarly, the inhibition of Bcr-Abl tyrosine kinase by imatinib blocked the BCR-ABL/AKT/mammalian target of rapamycin pathway and consequently induced autophagy and senescence in chronic myeloid leukemia (Drullion et al., 2012). In contrast, although the inhibition of the mammalian target of rapamycin pathway by ruxolitinib or rapamycin increased autophagy, it led to suppression of cellular senescence (Demidenko et al., 2009).

Given that autophagy has conflicting roles in the regulation of cell survival and cell death outcomes, it is thus important to define its positive and negative effects in response to autotumor therapies involving new agents. In this context, we studied the action of imidazoacridinone C-1311 in two human non-small cell lung cancer (NSCLC) cells, namely A549 and H460. We selected the human lung cancer models since they were particularly sensitive to C-1311 in the National Cancer Institute in vitro screening system. This work describes a new and complex mechanism of cell growth inhibition after C-1311 treatment that involves cross-talk between autophagy and senescence.

Materials and Methods

Chemicals and Reagents. Imidazoacridinone C-1311 was synthesized at the Gdański University of Technology (Gdańsk, Poland) (Chodły et al., 1992). C-1311 was prepared as a 50 mM stock in 50% (v/v) ethanol, stored at −20°C, and freshly dissolved in water before use. Other chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Cell Lines and Culture Conditions. Human NSCLC cell lines, A549 and H460, were obtained from American Type Culture Collection (Manassas, VA). Mycoplasma-free cells were maintained in F12K (A549 cells) and RPMI 1640 medium (H460 cells) supplemented with 10% fetal bovine serum (Cytogen, Florham Park, NJ), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich), at 37°C in a humidified incubator. For all assays, cells were grown to 60–70% confluence in 100-mm Petri dishes unless stated otherwise; the medium was then changed to medium containing C-1311 and cells were allowed to grow for the indicated time.

Growth Inhibition Assay. Cell growth inhibition studies were performed using a Coulter Counter (model ZBI; Beckman Coulter, Fullerton, CA). Cells were seeded in 24-well plates (2 × 10^5/well) and incubated with various concentrations of C-1311 for 72 hours. At the end of drug treatment, adherent cells were trypsinized, growth medium was added, and single cell suspensions were carefully pipetted. Cells were then counted in a Coulter Counter and sigmoidal dose-response curves were plotted to determine IC50 and IC80 values for the drug.

Cell Cycle Analysis. After C-1311 treatment, cells were trypsinized and pooled with floating cells, washed twice with ice-cold phosphate-buffered saline (PBS), and fixed in 70% (v/v) ethanol overnight at −20°C. DNA content was assessed by staining cells with propidium iodide (PI) in the presence of RNase A and monitored by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) as described previously (Skwarska et al., 2007). Cell distribution was determined with WinMDI software (Scripps Research Institute, San Diego, CA).

Western Blotting. Cells were detached by trypsinization, pooled with floating cells and washed twice with ice-cold PBS. Cells were suspended in lysis buffer [50 mM Tris-HCl (pH 7.4), 5 mM EDTA 1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), 0.5% sodium deoxycholate, 50 mM NaF, 50 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate] and kept for 20 minutes on ice with brief vortexing every 5 minutes. Lysates were then centrifuged at 14,000g for 15 minutes at 4°C. Denatured protein (approximately 30 μg) was subjected to SDS-PAGE, transferred to nitrocellulose membranes using a semidy blotting apparatus, and probed with primary antibodies. Mouse anti-p53, anti-microtubule-associated protein 1A/1B-light chain 3 (LC3) I/Ii, and anti-β-actin antibodies were purchased from Sigma-Aldrich. Mouse anti-Beclin, rabbit anti-p21, and rabbit anti-p62 antibody were from Cell Signaling Technology (Beverly, MA). Secondary anti-mouse horseradish peroxidase–linked antibody was purchased from Sigma-Aldrich and Cell Signaling Technology. For each Western blot, chemiluminescence detection was performed using enhanced chemiluminescence system reagents (Thermo Fisher Scientific, Waltham, MA). Densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD).

Detection of Apoptosis. Induction of apoptosis was determined qualitatively and quantitatively. Briefly, after drug treatment, both trypsinized and floating cells were pooled, washed twice with ice-cold PBS, spun onto microscopic slides, fixed in methanol/glacial acetic acid (3:1) for 15 minutes and stained with 1 μg/ml 4′,6-diamidino-2-phenylindole for 5 minutes. Nuclear morphologic changes were detected under a Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) with a 40× objective and photographed using an AxioCam digital camera (Zeiss, Jena, Germany). For apoptosis quantification, cells (5 × 10^5) were stained with Annexin V–fluorescein isothiocyanate in combination with PI using the Annexin-V–FITC Staining Kit (Roche Diagnostics). After flow cytometry analysis, Annexin-V–fluorescein isothiocyanate versus PI staining was plotted.

Senescence-Associated β-Galactosidase Staining for Senescence. Senescence-associated β-galactosidase (SA-β-gal) expression was examined as a marker for senescence (Dimri et al., 1995). Briefly, after drug treatment, cells were washed with PBS, fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 5 minutes, and incubated overnight at 37°C in a fresh X-gal staining solution containing 1 mg/ml 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 40 mM citrate-phosphate buffer (pH 6.0), 150 mM NaCl, and 2 mM MgCl2. The next day, cells were rinsed with PBS and examined under a bright-field microscope. SA-β-gal–positive (blue-stained) flattened cells with increased granularity were regarded as senescent. The percentage of senescent cells was determined after counting cells from 10 randomly selected fields (approximately 500 cells). Representative fields were photographed with a 20× objective.

Acridine Orange Staining of Acidic Vesicular Organelles. As a marker of autophagy, the presence of acidic vesicular organelles (AVOs) was examined by acridine orange staining. Within acidic vesicles, acridine orange forms aggregates that emit bright red fluorescence, whereas cytoplasm remains bright green (Traganos and Darzynkiewicz, 1994). Briefly, after the indicated time points after C-1311 treatment, live cells growing on cover slides were incubated for 15 minutes with medium containing 0.5 μg/ml acridine orange. Next,
cells were rinsed with PBS and immediately analyzed using a fluorescence microscope (Olympus BX60) with a 40× objective and were photographed using an AxioCam digital camera (Zeiss). The levels of AVOs were assessed by counting cells that displayed considerable bright red fluorescence from 10 randomly selected fields.

**Autophagy Inhibition by RNA Silencing.** Transfection of cells with Beclin 1 small interfering RNA (siRNA), ATG5 siRNA, or scrambled RNAi oligonucleotide (Ambion Life Technologies, Grand Island, NY) was performed with siRNAiMAX Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Briefly, 1 day before transfection, cells were seeded in 60-mm Petri dishes at the density to give approximately 30% confluence at the time of transfection. Cells were then treated with a mixture of siRNAiMAX Lipofectamine and siRNA for 24 hours, subsequently supplemented with a complete growth medium, and exposed to C-1311 for different periods of time. The efficiency of siRNA-mediated knockdown of Beclin 1 and ATG5 was determined by Western blotting at various times and the inhibition of autophagy was confirmed by acridine orange staining.

**Statistical Analysis.** Statistical differences were determined using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Unless indicated, the results are presented as the mean ± S.D. of three independent experiments. To compare the differences between groups, data were analyzed using one-way analysis of variance followed by a Bonferroni/Dunn test. \( P < 0.05 \) and \( P < 0.01 \) were considered significant.

### Results

**C-1311 Induces G1 or G2-M Cell Cycle Arrest in NSCLC Cells.** Imidazoacridinone C-1311 induced a concentration-dependent inhibition of cell growth in both A549 and H460 cells after 72-hour exposure; however, no significant difference in the sensitivity to the drug was found between the two tested cell lines. IC_{50} values for A549 and H460 cells varied between 0.014 ± 0.002 and 0.016 ± 0.004 \( \mu \)M, respectively, whereas IC_{50} values producing significant antiproliferative effects ranged between 0.080 ± 0.02 and 0.051 ± 0.004 \( \mu \)M (Fig. 1A). To further address the series of cellular responses, all subsequent experiments were performed using C-1311 at the IC_{50} concentration with incubation times up to 192 hours.

Flow cytometry analysis of cell cycle progression revealed that the response of A549 cells to C-1311 was predominantly characterized by G2-M arrest (Fig. 1B). The accumulation of cells in the G2-M transition was already visible after 24 hours of treatment, with a concomitant reduction of cells in the G1 phase. For the next 168 hours, approximately 30% and approximately 50% of the total cell population remained in the G1 and G2-M phases, respectively, whereas the S phase was mostly depopulated. Interestingly, only few cells (approximately 10%) underwent apoptosis based on a sub-G1 population (Fig. 1C). In contrast, C-1311 exposure led to the substantial accumulation in the G1 phase in H460 cells, although G2-M arrest was also evident (Fig. 1, B and C). At 48 hours, the G1 fraction reached approximately 60%, whereas only approximately 15% of the total cell population remained at the G2-M transition. This cell cycle distribution was sustained for prolonged drug exposure (up to 192 hours). Similarly to the A549 cell line, the apoptotic sub-G1 population remained small and did not increase throughout the 192 hours of treatment.

**Effect of C-1311 on p53 and p21 Proteins in NSCLC Cells.** Consistent with the DNA-damaging properties of C-1311, Western blot analysis indicated that treatment of NSCLC cells resulted in overexpression of p53 protein and transcriptional activation of p53 target, p21 protein. In A549 cells, the p53 level increased within 24 hours and remained high throughout the next 3 days, after which it progressively decreased (Fig. 2A). A similar pattern was observed in H460 cells, although the accumulation of p53 that occurred in response to C-1311 was slightly delayed and noted from 48 hours (Fig. 2B). Treatment of A549 cells with C-1311 caused a gradual and substantial increase in p21 (Fig. 2A). In contrast, the expression of p21 in H460 cells significantly increased within 24–48 hours up to a maximum at 72 hours, after which it decreased and subsequently remained constant (Fig. 2B).

**C-1311-Induced Cell Cycle Arrest Is Not Followed by Activation of the Apoptotic Pathway in NSCLC Cells.** According to the cell cycle profile, a sub-G1 fraction, did not significantly increase in both tested NSCLC cell lines despite the pronounced drug treatment. A further assessment of nuclear morphology supported a conclusion of minimal apoptosis induced by C-1311 in A549 and H460 cells. Fluorescence microscopy of 4’,6-diamidino-2-phenylindole–stained cells indicated abnormally enlarged nuclei in A549 cells upon prolonged C-1311 treatment, whereas nuclear morphology of H460 cells was similar to that observed in control conditions (Fig. 3A). Importantly, only occasional nuclear condensation and chromatin fragmentation were detected in the two studied lines. Consistent with these results, neither significant change in mitochondrial membrane potential nor cellular blebbing was observed (data not shown).

![Flow Cytometry Analysis](image)
To further confirm that apoptosis was not triggered by C-1311, NSCLC cells were analyzed for Annexin V/PI staining by flow cytometry. Annexin V⁺/PI⁻ cells, indicative of apoptosis, were not significantly increased in either cell line despite the prolonged time of C-1311 exposure (Fig. 3B). Although the majority of cells remained alive upon continuous drug treatment, H460 cells exhibited a slight decrease in viability starting from 96 hours of treatment, represented by a single-positive PI staining indicative of necrosis (Fig. 3B, right panel).

**C-1311 Induces Irreversible Senescence in NSCLC Cells.** Since both A549 and H460 cells exhibited persistent cell cycle arrest, prolonged expression of p53 and p21, as well as minimal apoptosis, we next tested the possibility that induction of senescence was the major response of these NSCLC cells to C-1311. Cells were treated with C-1311 and stained for SA-β-gal, a marker of senescence. In A549 cells, increased SA-β-gal staining was visible within 48 hours of treatment. After 8 days of continuous drug exposure, more than 50% of the cells displayed SA-β-gal activity and flattened and enlarged morphology that was consistent with senescence phenotype (Figs. 1B and 4A). Although H460 cells also stained positively for SA-β-gal (Figs. 1B and 4A), the percentage of cells undergoing senescence was 2-fold lower than in A549 cells. Importantly, we did not observe proliferative recovery over 2 weeks post-treatment in either cell line (data not shown), which suggests that C-1311–induced growth arrest and senescence were irreversible processes.

**C-1311–Induced Senescence of NSCLC Cells Is Preceded by Autophagy.** Many studies recently established that irradiation- or drug-induced DNA damage, next to nutrient starvation, reactive oxygen species, or hypoxia, induces autophagy (Kroemer et al., 2010). Because these findings suggest that autophagy can enhance cellular survival during genotoxic stress, we next examined the possible involvement of this process in the response of NSCLC cells to C-1311 treatment. We first examined the effect of C-1311 on the development of AVOs, which are considered typical characteristics of autophagy (Paglin et al., 2001). Representative images of A549 and H460 cells treated with C-1311 and stained with acridine orange show an increase in cell size and the presence of bright red fluorescent puncta indicative of AVO development (Fig. 5A). Quantitative analysis showed approximately 40% of A549 cells and over 50% of H460 cells with AVOs as early as 24 hours after drug exposure (Fig. 5B). By the third day of treatment, approximately 80–90% of cells from both lines appeared to be undergoing autophagy.

In addition to AVO detection, we next determined the conversion of cytoplasmic LC3-I protein (18 kDa) to the autophagosomic membrane-bound form of LC3-II (16 kDa).
As shown in Fig. 5C, the immunoblot analysis revealed that C-1311 treatment led to the accumulation of LC3-II protein in both tested cell lines. Importantly, the appearance of LC3-II protein was already visible after 24 hours of C-1311 exposure, whereas senescence characteristics were not yet detected at this time point.

Although the total level of LC3-II is known to correlate well with the number of autophagosomes (Eskelinen et al., 2002), it was also important to verify whether C-1311–induced accumulation of LC3-II was caused by the enhanced formation of AVOs and did not result from interference of vesicle clearance. To this end, we incubated lung cancer cells with C-1311 in the presence of ammonium chloride, a nonspecific lysosomal inhibitor that alters the lysosomal pH, thus preventing maturation of autophagic vacuoles (Eskelinen et al., 2002; Mizushima et al., 2010). As shown in Fig. 5D, LC3-II levels were significantly increased in C-1311–treated cells in the presence of ammonium chloride compared with that in the absence of the inhibitor, indicating that C-1311–induced accumulation of LC3-II was a result of enhanced formation of autophagosomes and autophagy induction. To further corroborate those observations, we monitored autophagy-mediated degradation of p62 protein. p62 (SQSTM1) is a ubiquitin-binding scaffold protein that upon autophagy binds directly to LC3-II and shuttles proteins to autophagosomes (Seibenhener et al., 2004). Since p62 is itself degraded by autophagy and accumulates when autophagy is inhibited, p62 may be used as a marker to study autophagic flux (Bjerkøy et al., 2009). As shown in Fig. 5E, significant degradation of p62 protein was already visible in A549 cells within 72 hours of treatment. The autophagic flux induced by C-1311 was detected between 144 and 168 hours after drug treatment in H460 cells, although it was attenuated compared with A549 cells.

Inhibition of Autophagy with 3-Methyladenine Decreases C-1311–Induced Senescence and Sensitizes NSCLC Cells to Drug Treatment. Data obtained thus far indicated that C-1311–induced autophagy occurred before massive senescence. We used 3-methyladenine (3-MA), a widely applied pharmacological inhibitor of autophagosome formation, to further determine whether autophagy was a necessary step in the development of senescence after C-1311 treatment (Mizushima et al., 2010). A549 and H460 cells were incubated with 3-MA for 1 hour before the addition of C-1311, and the inhibitor was present during the entire C-1311 exposure. As shown in Figs. 6A and 7A (top panels), 3-MA treatment led to a significant decrease in C-1311–induced acridine orange staining in both tested cell lines, confirming inhibition of autophagy. We next tested whether cotreatment with 3-MA could affect the onset of senescence in C-1311–treated cells. We found that the presence of 3-MA markedly suppressed C-1311–induced senescence in A549 and H460 cells, as detected by SA-β-gal staining (Figs. 6A and 7A, lower panels). Importantly, such profound reduction in cellular senescence was accompanied by an increase in the dying population of C-1311–treated cells. In A549 cells, interference with autophagy resulted in a 15% increase in the extent of apoptosis after 168 hours of C-1311 exposure (Fig. 6B). In contrast to A549 cells, the addition of 3-MA to C-1311–treated H460 cells caused a 10% (statistically significant) increase in necrotic cells, as detected by the Annexin-V assay (Fig. 7B).
Fig. 5. C-1311 induces autophagy in A549 and H460 cells. (A) Detection of C-1311–induced AVOs accumulation by acridine orange staining. Representative slides under a fluorescent microscopy show AVOs displaying red bright fluorescence staining in cells after C-1311 exposure. (B) Quantification of the extent of autophagy was performed by counting cells with bright red fluorescence from 10 randomly selected fields. Data represent the mean ± S.D. of two independent experiments. (C) Accumulation of LC3-II in cells treated with C-1311 estimated by Western blot. The protein level was monitored based on densitometric analysis and expressed as a fold-change relative to protein level in untreated cells. Data are representative of two independent experiments. (D) Ammonium chloride–induced accumulation of autophagosomes manifested by the enhanced appearance of the LC3-II band, indicating that autophagy occurs in both lung cancer cell types after C-1311 exposure (n = 2). (E) Western blot analysis of p62 and β-actin.
Downregulation of Autophagy Beclin 1 and ATG5 Proteins May Attenuate the Onset of C-1311–Induced Senescence in NSCLC Cells. The studies presented thus far suggested that autophagy triggered in lung cancer cells after C-1311 treatment not only preceded the development of the senescent phenotype but also protected cells against drug-induced death. To further verify whether autophagy was a necessary step in the induction of senescence by C-1311, we complemented our observations with a genetic approach using siRNA against Beclin 1, a protein involved in autophagic vesicle formation (Liang et al., 2001). Because lung cancer cells permanently stopped their proliferation within the first 72 hours of C-1311 treatment, we were able to perform effective knockdown of Beclin 1 even during prolonged drug exposure (between 96 and 168 hours), when the induction of senescence was clearly visible. As shown in Fig. 8A, Beclin 1 siRNA transfection led to a downregulation of the Beclin-1 protein level in C-1311–treated lung cancer cells, as assessed by Western blotting. The inhibition of autophagy was further confirmed by the reduction of acridine orange staining for AVO formation; X-Gal, 5-bromo-4-chloro-3-indolyl B-D-galactosidase staining for senescent cells.

Fig. 6. Effect of blocking autophagy with 3-MA in C-1311–treated A549 cells. Cells were treated with C-1311 alone or with C-1311 in combination with 3-MA (5 mM). (A) Upper panel, the presence of AVOs as a marker of autophagy was monitored under fluorescence microscope. Cells were stained with acridine orange and counted from 10 randomly selected fields. (A) Lower panel, inhibition of autophagy with 3-MA suppress development of C-1311–induced senescence. Senescence was estimated based on expression of SA-β-gal and cell morphology. Flattened and enlarged cells with blue coloration were counted under bright-field inverted microscope from 10 randomly selected fields. Data represent mean ± S.D. of three independent experiments. **P < 0.01 compared with untreated cells. (B) Inhibition of autophagy with 3-MA enhances cytotoxic effect of C-1311 by switching cell response from senescence to apoptosis. Representative histograms of Annexin-V/PI uptake (upper panel) and flow cytometry analysis after treatment of A549 cells with C-1311 in the presence of 3-MA. Data represent mean ± S.D. of two independent experiments. **P < 0.01 compared with C-1311 or 3-MA alone. AO, acridine orange staining for AVO formation; X-Gal, 5-bromo-4-chloro-3-indolyl B-D-galactosidase staining for senescent cells.
results suggest that autophagic Beclin 1 only partially elicits its effect on senescence upon C-1311 treatment and this effect strongly depends on the type of cancer cells.

Since Beclin 1–independent autophagy has been reported in the literature (Scarlatti et al., 2008), we next silenced the critical autophagic protein ATG5. As shown in Fig. 9A, ATG5 siRNA transfection led to a significant downregulation of the ATG5 protein level in C-1311–treated lung cancer cells, as assessed by Western blotting. This was accompanied by the reduction of AVO formation in ATG5 knockdown cells exposed to C-1311 (Fig. 9B). However, as with studies using Beclin 1–silenced cells, blocking ATG5 function only attenuated C-1311–induced senescence (Fig. 9, C and D). After 144 hours of C-1311 exposure, downregulation of ATG5 in A549 cells resulted in an approximately 25% inhibition of senescence compared with ATG5-positive cells (Fig. 9C, upper and lower panels). Similar to A549 siATG5 cells, C-1311–induced senescence was also reduced in H460 siATG5 cells; however, those cells responded by a significant higher decrease in SA-β-gal staining. That is, after 144 hours of C-1311 treatment, the level of senescence in ATG5-silenced H460 cells was reduced by approximately 40% relative to cells expressing ATG5. However, for both A549 and H460 cell lines, the suppression of senescence by the knockdown of ATG5 could not be sustained after prolonged drug treatment (Fig. 9, C and D). After 168 hours of C-1311 exposure, the extent of senescence in ATG5 knockdown cells was similar to that in cells with functional ATG5 protein.

Discussion

Over the last few years, it has become clear that autophagy, a major intracellular degradation system, and senescence, a cellular program of terminal growth arrest, are closely
linked and are induced by numerous forms of stress, including anticancer drugs and radiation (Di et al., 2009). However, there are still relatively few studies evaluating the senescence response to DNA damage in the context of autophagy (Singh et al., 2012). Here we demonstrated for the first time that a promising topoisomerase II and FMS-like tyrosine kinase 3 kinase inhibitor, imidazoacridinone C-1311, induced autophagy in two NSCLC cell lines, namely A549 and H460, and that this process is linked with the acquisition of senescent phenotype. Previous studies showed that C-1311 treatment in leukemic and solid tumor cells led to cell death via apoptosis or mitotic catastrophe followed by secondary apoptosis (De Marco et al., 2007; Skwarska et al., 2007). The identification of autophagy precipitating delayed yet massive senescence as an alternative element in the action of C-1311 provides a novel insight into the complex mechanism of C-1311–induced cellular response that may be substantial for its anticancer activity.
In our studies, the early response of A549 and H460 lung cancer cells to C-1311 exposure involved prolonged cell cycle arrest, indicating a cytostatic effect of the drug. Although both analyzed cancer cell lines exhibited similar IC₅₀ and IC₈₀ values, we could identify a differential form of cell cycle arrest between A549 and H460 cells followed by C-1311 treatment. The response of A549 cells to C-1311 was characterized by G1 and significant G2-M arrests, whereas H460 cells accumulated predominantly in the G1 phase of the cell cycle. Importantly, in both cell lines, a described pattern of cell cycle distribution was sustained for extended drug exposure (up to 192 hours) and was accompanied by an early increase in p53 level and p21 induction. The fact that p53 levels in A549 and H460 cells initially increased during drug exposure and decreased after prolonged time of treatment suggests that p53 is required to induce both G1 and G2-M blocks, but not to maintain them. Consequently, even when p53 protein was significantly reduced, p21 levels remained elevated long enough to provide cell cycle arrest for the extended time of C-1311 treatment.

In A549 and H460 cells exposed to C-1311, cell cycle arrest appears to be potentiated by G1 and G2/M checkpoint mediators, such as p53 and p21, which also are crucial in promoting senescence (Gewirtz et al., 2008). At the cellular level, cell morphology became flattened and enlarged after prolonged drug treatment, suggesting that A549 and H460 cells underwent senescence in a time-dependent manner in response to C-1311–induced DNA damage. The senescent phenotype was further confirmed by the robust presence of cells stained positive for β-galactosidase activity. Furthermore, cell cycle analysis (lack of significant sub-G1 phase increase), corroborated by the fluorescent microscopy (lack of apoptotic bodies).
and flow cytometry analysis of apoptotic markers (lack of Annexin-V–positive cells), indicated essentially no apoptotic response in both A549 and H460 cells after C-1311 treatment. Importantly, although the development of senescence was much more intense in A549 cells compared with H460 cells, both cell lines did not resume proliferation even after 2 weeks post-treatment in a drug-free medium. Together, these results show that this compound is capable of inducing permanent and irreversible senescence in lung cancer cell lines that do not activate the apoptotic pathway after C-1311 treatment.

Development of senescence with stable cell cycle arrest and active cell metabolism, in contrast to apoptosis, is typically a delayed response involving multiple effector mechanisms (Young et al., 2009). With regard to senescence, the role of autophagy as an early adaptive pathway after genotoxic stress and enhancing cancer cell survival is still controversial and poorly understood. Our results suggest that a C-1311–induced senescence program may be, in part, mediated by an autophagic mechanism. In both A549 and H460 cell lines, typical hallmarks of autophagy, including AVOs staining and the conversion of LC3-I to LC3-II form, were observed within first 48 hours of drug exposure (e.g., when cell cycle arrest was evident but when there were no signs of senescence). Moreover, the progressive reduction of the p62 protein level, that is normally degraded during autophagy and is consistent with autophagy flux, indicated that in our cellular system autophagy was not only initiated but also underwent completion. Although it was not directly tested, it is feasible that autophagy may cooperate with G1 and G2/M checkpoints at the early time point of C-1311 exposure to allow the cells time to repair DNA damage. Strong support of this hypothesis is derived from studies of Katayama et al. (2007), in which single and double-strand DNA breaks triggered by temozolomide (DNA alkylator) or etoposide (topoisomerase II inhibitor), respectively, activated signaling pathways that control the G2 checkpoint as well as the autophagic process, suggesting the connection between DNA damage, cell cycle arrest, and autophagy. Furthermore, Gewirtz (2009) postulated that if, autophagy is sufficient to maintain cells in a prolonged growth arrest and protected state in response to chemotherapy, this process may be succeeded by senescence in which cells remain metabolically active but unable to return to the cell cycle. With regard to this model, our studies not only indicate that C-1311–induced autophagy lies upstream of senescence but also suggest that autophagy may facilitate the latter to occur. This conclusion is supported by findings in which pharmacological inhibition of autophagy using 3-MA caused a profound reduction of senescence in C-1311–treated lung cancer cells. Importantly, the suppression of the senescent phenotype was accompanied by a small, but statistically significant, enhancement of cell death. This in turn suggests that in our model, autophagy not only may contribute to the establishment of senescence but to some extent can also protect cells against drug-induced death. Interestingly, as revealed by Annexin-V/PI staining, A549 cells cotreated with C-1311 and 3-MA were more competent to die through apoptosis, whereas H460 cells underwent necrosis at the same conditions. We have not yet established an explanation of this discrepancy except that compared with the A549 cell line, H460 cells were initially more susceptible to trigger necrosis and less prone to undergo senescence after only C-1311 treatment.

Pharmacological inhibition of autophagy indicated a close association between autophagy and senescence after C-1311 exposure. However, studies involving siRNA knockdown of autophagy-related Beclin 1 and ATG5 do not fully corroborate these observations. In A549 cells, downregulation of Beclin 1 and ATG5 decreased C-1311–induced senescence, whereas in H460 cells, only targeting ATG5 reduced development of the senescent phenotype. Importantly, silencing of Beclin 1 and ATG5 attenuated but did not entirely block the ability of cells to undergo senescence after C-1311 treatment. Moreover, after prolonged drug exposure (168 hours), the extent of senescence returned to the level similar to that in cells expressing functional Beclin 1 and ATG5 proteins despite suppressed autophagy. Although the outcome of autophagy inhibition upon C-1311–induced senescence may vary by the type and genetic background of cancer cells, it seems that autophagy is not the sole requirement for senescence to occur in our models. These data are in agreement with a recent report from Goche et al. (2012), in which blocking autophagy by pharmacological and genetic approaches could not abrogate senescence in response to doxorubicin (topoisomerase II inhibitor) or camptothecin (topoisomerase I inhibitor) treatment in breast and colon carcinoma cells. Despite collateral regulation of autophagy and senescence via common signaling pathways involving induction of p53 or p21, these responses were not interdependent because senescence occurred when autophagy was suppressed (Goche et al., 2012). Taking the above studies together with our own, it is becoming apparent that when senescence and autophagy are triggered after genotoxic therapy, the latter may only facilitate development of senescence.

In conclusion, our study reports for the first time that DNA-damaging anticancer imidazoacridinone C-1311 is able to trigger both autophagy and senescence in A549 and H460 lung cancer cells. However, although autophagy and senescence may cross-talk to determinate the cell fate after C-1311 exposure and autophagy may contribute to the establishment of senescence, it is evident that cells can also undergo senescence even when autophagy is inhibited. The ongoing debate in the literature regarding the clinical importance of drug-induced senescence points to both positive and negative aspects of that process. On one hand, the possibility to induce senescence in cancer cells is very exciting because this is the first barrier against tumorigenesis (Drullion et al., 2012). On the other hand, there is a high risk that a subpopulation of senescent cells, however small, could ultimately recover proliferative function, leading to disease recurrence (Gewirtz, 2009). The unclear relationship between senescence and autophagy further complicates this picture. Here we demonstrate that upon C-1311–induced senescence, the inhibition of autophagy by 3-MA stimulates lung cancer cell death, whereas genetic ablation of Beclin 1 or ATG5 only attenuates development of a senescence phenotype. Such diverse outcomes observed when C-1311 is combined with genetic or pharmacologic inhibitors of autophagy highlight the potential pitfalls intrinsic to the use of modulators of autophagy together with cytotoxic agents in general.

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