J Pharmacol Sci **118**, 423 – 432 (2012)

Journal of Pharmacological Sciences

Full Paper

Activation of ERK–p53 and ERK-Mediated Phosphorylation of Bcl-2 Are Involved in Autophagic Cell Death Induced by the c-Met Inhibitor SU11274 in Human Lung Cancer A549 Cells

Ying Liu[1], Ying Yang[1], Yuan-Chao Ye[1], Qi-Feng Shi[1], Kuan Chai[1], Shin-ichi Tashiro[2], Satoshi Onodera[2], and Takashi Ikejima[1,*]

[1]*China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, 110016, PR China*

[2]*Department of Clinical and Biomedical Science, Showa Pharmaceutical University, Tokyo 194-8543, Japan*

Received October 3, 2011; Accepted January 5, 2012

Abstract. SU11274, a small molecule inhibitor of c-Met, was reported to induce apoptosis in human non-small-cell lung cancer (NSCLC) cells. However, SU11274-mediated autophagy in NSCLC cells has rarely been reported. The aim of this study was to elucidate the molecular mechanisms mediating SU11274-induced autophagy in NSCLC A549 cells. Here we reported that SU11274-induced autophagy was accompanied with an increase in the conversion of LC3-I to LC3-II and up-regulation of Beclin-1 expression. Subsequently, we also found that small interfering RNA against c-Met induced A549 cell autophagy while promotion of c-Met by hepatocyte growth factor (HGF) suppressed A549 cell autophagy. Inhibition of autophagy by 3-methyladenine (3-MA) suppressed SU11274-induced cell death, suggesting that SU11274-induced autophagy caused cell death. Further study showed that ERK and p53 were activated after SU11274 treatment. Interruption of ERK and p53 activities decreased SU11274-induced autophagy, and blocking of ERK by the specific inhibitor PD98059 suppressed SU11274-induced p53 activation. Moreover, ERK activation upregulated Beclin-1 expression through induction of Bcl-2 phosphorylation, but p53 did not induce Bcl-2 phosphorylation. In conclusion, inhibition of c-Met induced autophagic cell death, which was associated with ERK–p53 activation and ERK-mediated Bcl-2 phosphorylation in A549 cells.

Keywords: c-Met, SU11274, autophagy, Bcl-2, ERK

Introduction

NSCLC is one of the leading causes of death all over the world (1). Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a pleiotropic growth factor (2). Higher level of HGF is associated with more aggressive manifestation and a worse prognosis in NSCLC (3) and small cell lung cancer (4). HGF receptor c-Met is normally expressed by epithelial cells, and it has been found to be overexpressed and amplified in a variety of human tumor tissues (5), especially in NSCLC (6). It has been established that abnormal HGF/c-Met signaling plays a critical role in the development and progression of primary tumors and secondary metastases (7). Aberrant function of HGF receptor tyrosine kinase (RTK) c-Met is abundant in numerous human cancers (8), which makes this RTK system a highly attractive target in cancer therapy. SU11274 is a prototypic anti-Met small molecule, and it is an effective inhibitor of HGF-dependent signaling. SU11274 abrogated HGF-induced phosphorylation of c-Met and it is downstream signaling, and it is also a potential inhibitor of aberrant c-Met expression (9). Inhibition of the c-Met kinase activity by SU11274 led to a time- and dose-dependent cell growth retardation, G1 cell cycle arrest, and apoptosis (10).

Autophagy has multiple physiological functions in multicellular organisms, including lysosome-dependent protein degradation and organelle turnover (11). Other

*Corresponding author. ikejimat@vip.sina.com
Published online in J-STAGE on March 29, 2012 (in advance)
doi: 10.1254/jphs.11181FP

studies have pointed out that autophagy is also induced in the processes of many anti-cancer therapies and is considered to be a major tumor cell intrinsic resistance mechanism. Thus, autophagy is essential in modulating cell homeostasis, death, and survival. Beclin-1, a mammalian homologue of Atg 6, was known as a biological marker of autophagy.

c-Met induces the activation of numerous transduction cascades, especially PI3K/Akt and MAPKs signaling pathways (12). The ERK MAPK pathway is a typical downstream signaling factor of c-Met. For many researchers, another intriguing aspect of ERK is its potential roles in autophagic pathways (13). ERK has been shown to induce autophagy in response to a number of anti-tumor agents, such as soyasaponins in colon cancer cells (14). Inhibition of ERK was associated with decrease in autophagy and increase in cellular sensitivity to tumor necrosis factor-α (TNFα) in human breast cancer MCF-7 cells (15). ERK was shown to promote TNFα-induced autophagy in murine fibroblast L929 cells by activating p53 (16). To evaluate the impact of c-Met inhibition by SU11274 and c-Met siRNA on downstream pathways, the ERK–MAPK pathway was mainly examined in the present study.

Bcl-2, amongst other anti-apoptotic Bcl-2 family members, forms a complex with Beclin-1 and thereby abrogates autophagic signaling (17, 18). Upon starvation or drug stimuli, Bcl-2 can be phosphorylated by some upstream factors, for example, ERK, leading to release of Beclin-1 and induction of autophagy (19, 20).

It is known that inhibition of c-Met by SU11274 induces apoptosis in NSCLC cells, but whether SU11274 can induce autophagy in NSCLC cells is still unclear. Thus, the mechanism of SU11274-induced autophagy in A549 cells and the effect of c-Met inhibition by SU11274 and c-Met siRNA were investigated in the present study.

Materials and Methods

Reagents

SU11274 [(3*Z*)-*N*-(3-chlorophenyl)-3-({3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1*H*-pyrrol-2-yl} methylene)-*N*-methyl-2-oxoindoline-5-sulfonamide] was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from TBD Biotechnology Development (Tianjin, China). Monodansylcadaverine (MDC) and 3-methyladenine (3-MA) were purchased from Sigma. HGF, the ERK inhibitor PD98059, and the p53 inhibitor pifithrin-α (PFT-α) were obtained from Calbiochem (La Jolla, CA, USA). Polyclonal antibodies against c-Met, Beclin-1, LC3, ERK, p-ERK, p53, p-p53, Bcl-2, p-Bcl-2, β-actin, and horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrochemiluminescence (ECL) was from Thermo Fisher Scientific (Rockford, IL, USA).

Cell culture

The A549 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 0.03% L-glutamine (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin and the cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Cell growth inhibition assay

The inhibition of cell growth was measured by MTT assay as described previously (21). Briefly, the cells were dispensed in 96-well flat-bottom microtiter plates (NUNC, Roskilde, Denmark) at a density of 1×10^4 cells per well. After 24-h incubation, they were treated with various concentrations of SU11274, and cultured for 24 h. Then, MTT (5 mg/ml) was added to each well for 3 h, and the resulting crystals were dissolved in DMSO. Optical density was measured by MTT assay using a plate microreader (Tecan Spectra, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows:

Cell inhibitory ratio (%) = (A492 control − A492 sample) / (A492 control − A492 blank) × 100

Flowcytometric analysis of autophagy

A549 cells were dispensed in a 25-ml culture flask at a density of 3×10^5 per flask. After 24 h incubation, they were pre-treated with 3-MA, PD98059, or PFT-α for 1 h and then co-incubated with 100 nM SU11274 for 24 h. The cells were harvested by trypsin and rinsed with PBS. Collected cells were suspended with 0.05 mM MDC at 37°C for 40 min. Then the samples were analyzed by FACScan flowcytometer (Becton Dickinson, Franklin Lakes, NJ, USA) (22).

The analysis of MDC staining

A549 cells were seeded in 24-well culture plates at a density of 2.5×10^4 per well and cultured for 24 h. The cells were treated with 100 nM SU11274 for 24 h and then incubated with 0.05 mM MDC at 37°C for 20 min; after staining, the fluorescent changes were observed by fluorescence microscopy (Olympus, Tokyo) (23).

Autophagy assay

A549 cells (2.5×10^4/well) were inoculated in 24-well

culture plates overnight and then were transfected with 2 μg of GFP-LC3 expression plasmid (Sigma) using lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h, the cells were treated with 100 nM SU11274 at 37°C for 24 h, and then GFP-LC3 fluorescence was observed by fluorescence microscopy. Characteristic punctate GFP-LC3 signaling was considered to indicate that cells were undergoing autophagy (24).

Western blot analysis

A549 cells (1×10^6) were pretreated with various concentrations of SU11274 or 3-MA, PD98059, or PFT-α 1 h before 100 nM SU11274 treatment and then cultured for 24 h. Both adherent and floating cells were collected and lysed. Briefly, cell pellets were resuspended with lysis buffer consisting of 50 mM Hepes (PH 7.4), 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM edetic acid, 1 mM PMSF, 10 mg/L aprotinin (Sigma), and 10 mg/L leupeptin (Sigma) and lysed at 4°C for 1 h. After centrifugation at 12,000 × *g* for 15 min, the protein content of the supernatant was determined by the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equivalent amounts of total proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analyses were performed as described before (25).

siRNA transfection

siRNAs against human c-Met and control siRNA were purchased from Invitrogen. According to the manufacturer's instructions, cells were transfected with siRNAs at a final concentration of 30 nM using Lipofectamine 2000 (Invitrogen). The transfected cells were used for subsequent experiments 24 h later (26).

Statistical analysis

All results and data were confirmed in at least three separate experiments. Data are expressed as means ± S.D. The data were analyzed by ANOVA using Statistics Package for Social Science (SPSS) software (version 13.0; SPSS, Chicago, IL, USA), and the post-hoc test was employed to assess the statistical significance of the difference between the control and treated groups. *P*-values of < 0.05 were considered statistically significant.

Results

SU11274 time- and dose-dependently induced autophagy in A549 cells

As shown in Fig. 1A, SU11274 inhibited A549 cell growth in a dose-dependent manner. To confirm whether SU11274-treatment could induce autophagy, the cells were treated with SU11274 ranging from 100 to 800 nM for 24 h, and the ratio of MDC-positive cells was determined by flowcytometry. SU11274 exerted an autophagy-inducing effect on A549 cells from 100 to 800 nM (Fig. 1B, n = 3). SU11274 at 100 nM showed a marked up-regulation of autophagyic ratio, so 100 nM was chosen as the tested concentration in this study. SU11274 treatment increased the autophagic ratio in a time-dependent manner (Fig. 1C, n = 3). We then examined the expression of autophagic markers, Beclin-1 and MAP-LC3. SU11274-treatment enhanced the level of Beclin-1 and the conversion of LC3-I to LC3-II in a time- and dose-dependent manner (Fig. 1D, E, n = 3). We further aimed to determine whether c-Met was involved in SU11274-induced autophagy. Accordingly, we treated A549 cells with c-Met small interfering RNA (siRNA), and the autophagic ratio was measured by flowcytometry. As shown in Fig. 1F, the percentage of MDC-positive cells was increased after transfection with c-Met siRNA. Meanwhile, HGF-treatment markedly decreased SU11274-induced autophagy (Fig. 1G). These results demonstrate that inhibition of c-Met by both the chemical inhibitor SU11274 and c-Met siRNA could induce autophagy in NSCLC A549 cells.

Inhibition of autophagy decreased SU11274-induced cell death in A549 cells

To further detect SU11274-induced A549 cell autophagy, the morphologic changes were examined by MDC staining. As shown in Fig. 2Aa, intense punctate MDC fluorescence was observed in SU11274-treated cells, and 3-MA reduced the number of MDC-labeled fluorescent particles. In addition, SU11274-induced autophagy was also examined by transient transfection of cells with an expression plasmid containing GFP-LC3. Recruitment of LC3-II to the autophagosomes is characterized by the punctate pattern of its localization. The vehicle-treated control cells exhibited diffused and weak LC3-associated green fluorescence. However, the cells treated with SU11274 for 24 h showed a characteristic punctate pattern of LC3, while this process was reversed by 3-MA (Fig. 2Ab). The result of flowcytometric analysis also confirmed that the autophagic ratio was decreased by 3-MA (Fig. 2B, n = 3). Then the expression of Beclin-1 and the conversion of LC3-I to LC3-II were suppressed by 3-MA (Fig. 2C, n = 3). Further, compared with the SU11274 alone–treated group, 3-MA-pretreatment decreased SU11274-induced cytotoxicity (Fig. 2D, n = 3). These results further confirmed that SU11274 induced autophagy in A549 cells, and the inhibition of autophagy contributed to down-regulation of cell death.

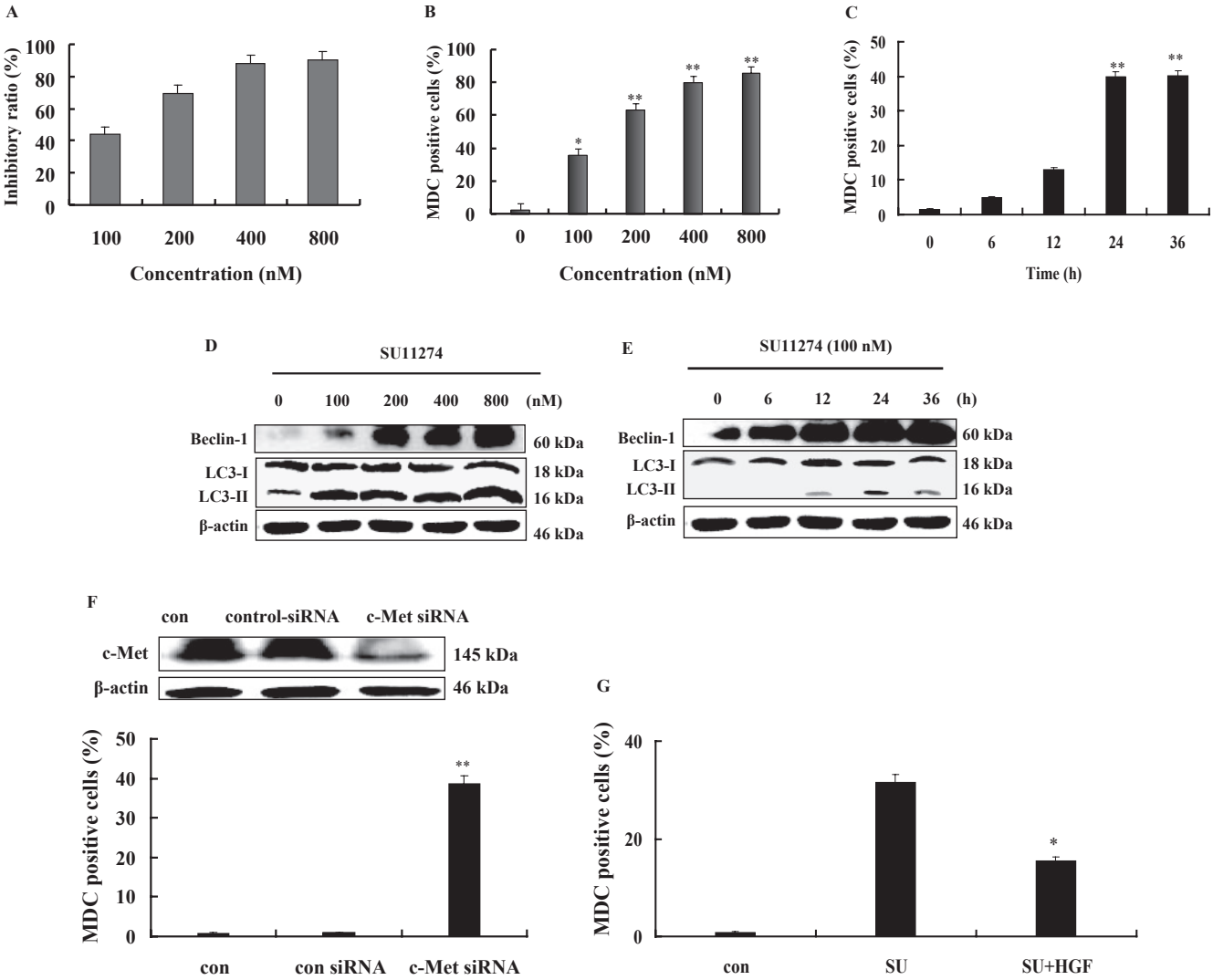

Fig. 1. SU11274 induced autophagy in A549 cells. A: The cells were treated with SU11274 at various doses for 24 h and the cell growth inhibition was measured by MTT assay. B: The autophagic ratio was examined by flowcytometric analysis. C: The cells were treated with 100 nM SU11274 for the indicated time periods. The autophagic ratio was measured by flowcytometric analysis. The data are presented as the mean ± S.E.M. of the results for three independent experiments. *$P < 0.05$, **$P < 0.01$ vs. control group. D and E: The cells were treated with 0, 100, 200, 400, or 800 nM SU11274 for 24 h (D) or incubated with 100 nM SU11274 for 0, 6, 12, 24, or 36 h (E); and the levels of Beclin-1 and MAP-LC3 were detected by western blot analysis. β-Actin was used as an equal loading control. F: The cells were transfected with c-Met or control siRNA for 24 h, and the c-Met level was examined by western blot analysis (upper panel). The cells were transfected with c-Met or control siRNA for 24 h, and the autophagic ratio was examined by flowcytometric analysis (n = 3) (lower panel). Values are expressed as the mean ± S.D. **$P < 0.01$ vs. control group. G: The cells were treated with 100 nM SU11274 in the presence or absence of 0.5 ng/ml HGF for 24 h, and the autophagic ratio was measured by flowcytometric analysis. Values are expressed as the mean ± S.D. *$P < 0.05$ vs. SU11274 group.

SU11274 induced ERK-p53 activation in the progression of autophagy

To investigate the effects of SU11274 on p53 accumulation and ERK activation, the levels of p53 and ERK expression were examined by western blot analysis. The expression of ERK and p53 significantly decreased after SU11274-treatment, but the levels of phosphorylation of ERK and p53 were markedly elevated in a time-dependent manner (Fig. 3A, n = 3). Transfected A549 cells with c-Met siRNA promoted phosphorylation of ERK and p53, and inhibited the expression of ERK and p53 (Fig. 3B). The above findings showed that ERK and p53 were activated during SU11274-induced A549 cell autophagy. To investigate the role of ERK and p53 in SU11274-induced autophagic cell death, the cells were pre-treated with the ERK inhibitor PD98059 or p53 inhibitor PFT-α for 1 h,

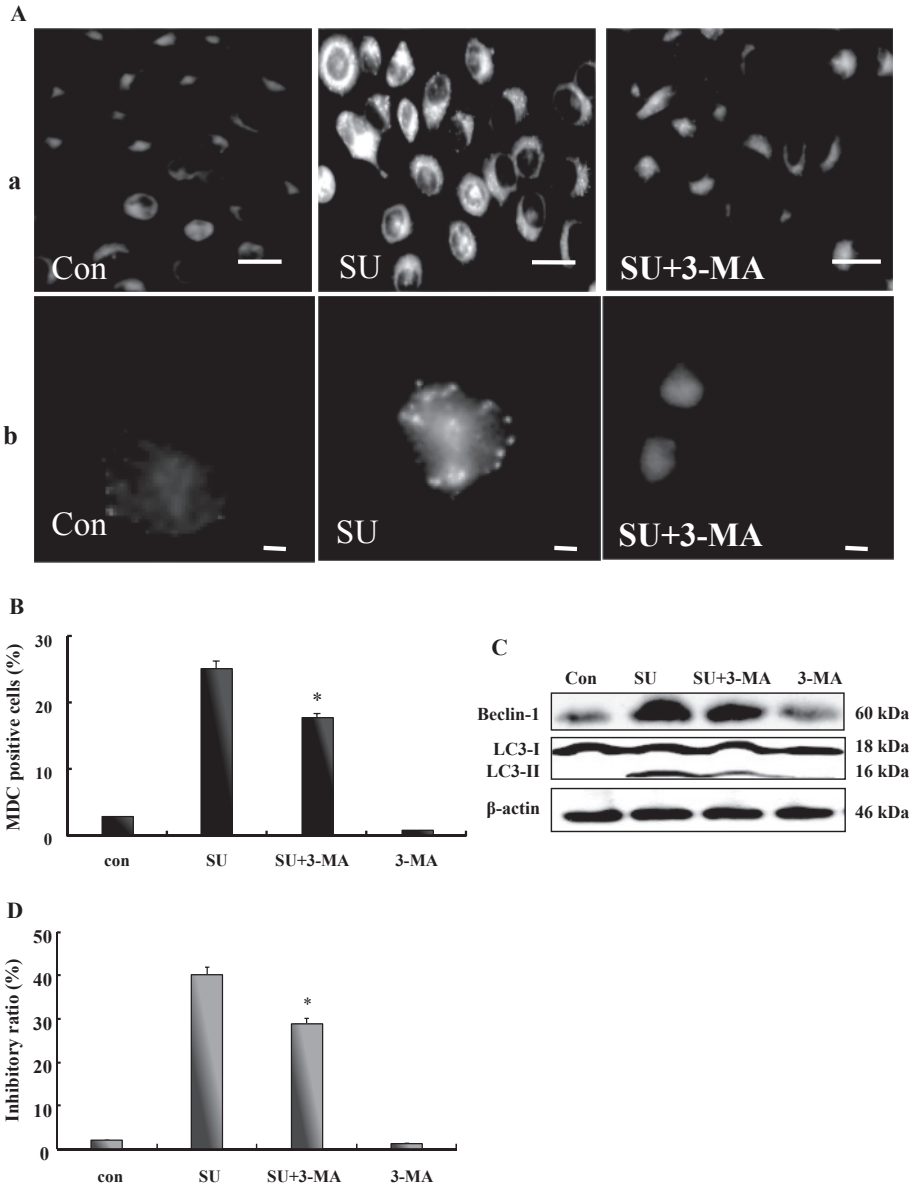

Fig. 2. Inhibitory effect of 3-MA on SU11274-induced autophagy. A: The cells were incubated with 100 nM SU11274 for 24 h, in the presence or absence of 0.4 mM 3-MA. a: The fluorescent images of A549 cells that were labeled with MDC staining, scale bar = 20 μm. b: The cells were transfected with GFP-LC3 for 24 h and then observed by fluorescent microscopy, scale bar = 30 μm. B: Autophagic ratio was measured by flowcytometric analysis. C: Levels of Beclin-1 and MAP-LC3 were detected by western blot analysis and β-actin was used as an equal loading control. D: Inhibitory ratio was measured by MTT assay. The data are presented as the mean ± S.E.M. of the results for three independent experiments. $^*P < 0.05$ vs. SU11274 group.

and SU11274-induced cell cytotoxicity was measured. As shown in Fig. 3C, these inhibitors decreased SU11274-induced cytotoxicity; meanwhile, the autophagic ratio was reduced by PD98059 or PFT-α (Fig. 3D). Next, the level of autophagy-associated Beclin-1 and LC3 were also assessed by western blot analysis. As shown in Fig. 3E, the level of Beclin-1 and conversion of LC3-I to LC3-II both decreased after PD98059 or PFT-α administration. Inhibition of ERK by using PD98059 could decrease p53 levels, but suppression of p53 by using PFT-α failed to alter ERK levels. These results indicated that ERK existed in the upstream of p53 (Fig. 3F, n = 3). P53 was a positive regulator of autophagy, and SU11274-induced ERK activation might contribute to p53's function.

ERK but not p53 activation upregulated Beclin-1 expression and induced Bcl-2 phosphorylation

The ERK signaling pathway was reported to regulate Bcl-2 expression or its phosphorylation to control the cell fate (27). A current model suggests that Bcl-2 constitutively interacts with Beclin-1 and prevents Beclin-1 from initiating autophagy (28). To explore the involvement of ERK activation in the modulation of Bcl-2, ERK inhibitor was used to co-treat the cells with SU11274 for 24 h, and the levels of Bcl-2 and p-Bcl-2 were examined by western blot analysis. As shown in Fig. 4A, phosphorylation of Bcl-2 was markedly increased, while the expression of Bcl-2 was suppressed after SU11274 administration. However, treatment with the ERK inhibitor PD98059 obviously reversed the above-mentioned phe-

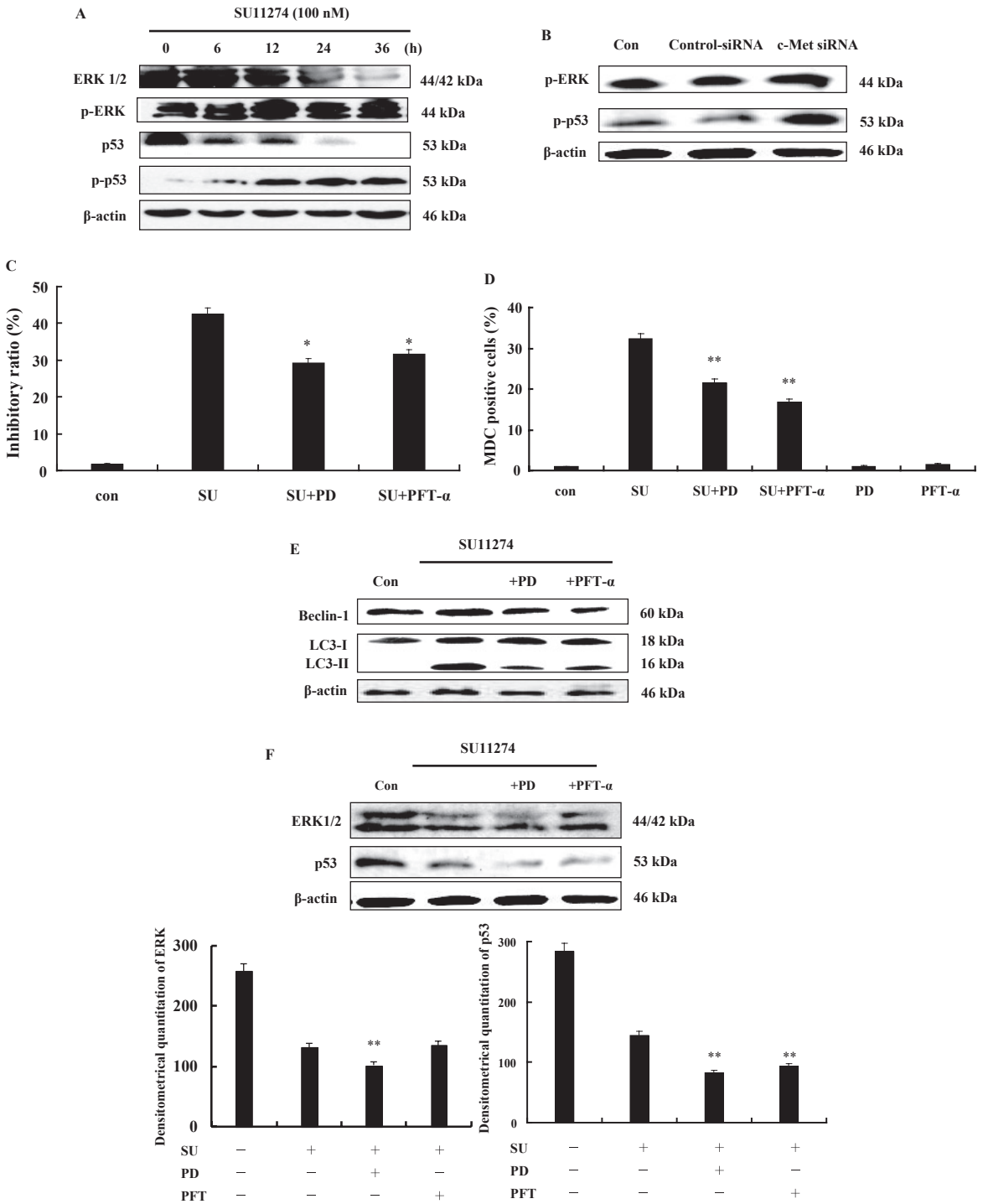

Fig. 3. ERK–p53 signal mediated SU11274-induced autophagy in A549 cells. A: The cells were treated with 100 nM SU11274 for the indicated time periods; and the levels of ERK, p-ERK, p53, and p-p53 were detected by western blot analysis. B: The cells were transfected with c-Met or control siRNA for 24 h and the levels of p-ERK and p-p53 were examined by western blot analysis. C: The cells were incubated with or without 5 μM ERK inhibitor (PD98059) or 20 μM p53 inhibitor (PFT-α) for 1 h before 100 nM SU11274 administration. After 24 h, the inhibitory ratio was measured by MTT assay. The data are presented as the mean ± S.E.M. of the results for three independent experiments. *$P < 0.05$ vs. SU11274 group. D: Autophagic ratio was measured by flowcytometric analysis. The data are presented as the mean ± S.E.M. of the results for three independent experiments. **$P < 0.01$ vs. SU11274 group. E: Protein levels of Beclin-1 and MAP-LC3 were detected by western blot analysis. F: The cells were pretreated with or without 5 μM PD98059 or 20 μM PFT-α for 1 h before 100 nM SU11274 administration; then the protein levels of p53 and ERK were detected by western blot analysis (upper panel), and the density of ERK or p53 in each group was detected through Bandscan 5.0 software (Glyko, Novato, CA, USA) (lower panel). **$P < 0.01$ vs. SU11274 group.

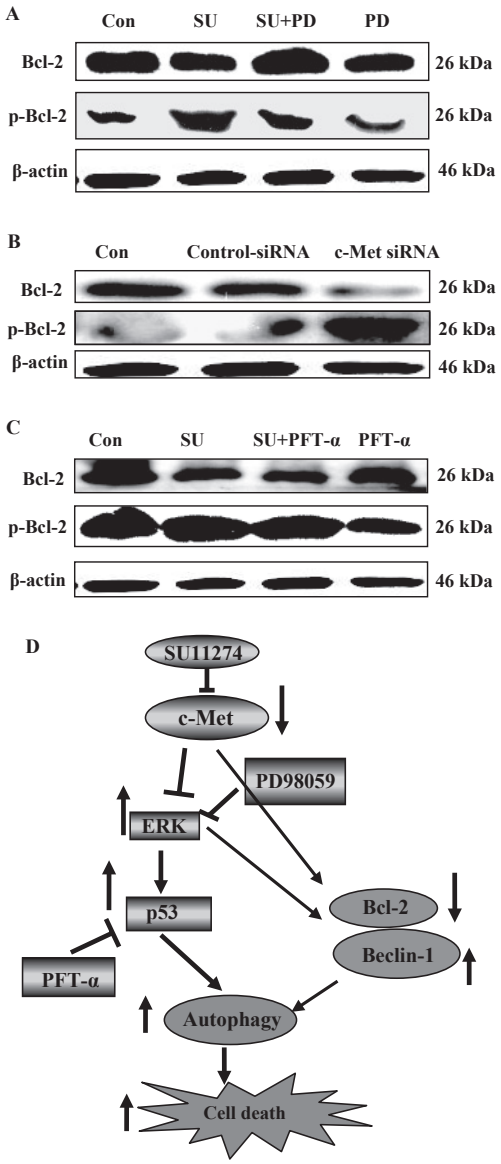

Fig. 4. Effects of ERK or p53 inhibitor on the expressions of Bcl-2 and p-Bcl-2 in A549 cells. A: The cells were incubated with or without 5 μM PD98059 for 1 h followed by 100 nM SU11274 administration, and the expression of Bcl-2 and p-Bcl-2 were detected by western blot analysis, with β-actin used as an equal loading control. B: The cells were transfected with c-Met or control siRNA for 24 h, and then the levels of Bcl-2 and p-Bcl-2 were examined by western blot analysis. C: The cells were treated with or without 20 μM PFT-α for 1 h followed by 100 nM SU11274 administration, and the expression of Bcl-2 and p-Bcl-2 were detected by western blot analysis. β-Actin was used as an equal loading control. D: Suggested molecular mechanisms by which SU11274 induces A549 cell autophagy. Up-arrow represents the increased expression and down-arrow represents the decreased expression.

nomena, suggesting that ERK activation could repress the binding of Beclin-1 to Bcl-2. Furthermore, we knocked down the expression of c-Met by specific siRNA. As shown in Fig. 4B, this treatment up-regulated phosphorylation of Bcl-2 and down-regulated the expression of Bcl-2. To elucidate whether p53 played a role in regulating Bcl-2 phosphorylation, PFT-α was used to suppress p53 activity. As shown in Fig. 4C, the expression of Bcl-2 and p-Bcl-2 were not conspicuously changed by PFT-α administration, indicating that p53 did not mediate the regulation of Bcl-2. Therefore, we confirmed that activated ERK augmented Beclin-1 release through Bcl-2 phosphorylation. However, activation of p53 failed to alter Bcl-2 activity.

To summarize all the aforementioned results: suppression of c-Met by SU11274 treatment triggered autophagy in A549 cells, which was mediated by activation of p53 under regulation of ERK. Moreover, the activated ERK also down-regulated the binding of Beclin-1 and Bcl-2 by promoting Bcl-2 phosphorylation and then evoked autophagy. This promoted autophagy augmented SU11274-induced cell death (Fig. 4D).

Discussion

In small cell lung cancer (SCLC) and NSCLC cells, c-Met was found to be overexpressed (29). Ichimura et al. reported that c-Met was overexpressed in all 11 NSCLC cell lines, 34 of 47 adenocarcinomas and 20 of 52 squamous cell carcinomas in their study (30). Siegfried et al. showed that the expression of c-Met was two- to ten-fold higher than in adjacent normal lung tissue in 25% of NSCLC tumors (31). In lung carcinoma, c-Met was expressed in 47% of tumor tissues and significantly correlated with survival in a univariate analysis (32). This notion suggests a key role for potential c-Met inhibitors in cancer therapy. Indeed, various anti-c-Met approaches, including several small molecules, are currently in clinical trials (33). PF-2341066 was identified as a potent and ATP-competitive inhibitor of the catalytic activity of c-Met kinase. It inhibited human GTL-16 gastric carcinoma cell growth and induced apoptosis. It also inhibited human NCI-H441 NSCLC cell proliferation and migration (34). PHA665752, a selective small molecule c-Met inhibitor and an effective inhibitor of phosphorylation on c-Met, specifically inhibited cell growth, induced apoptosis, and caused cell cycle arrest in H441 NSCLC cells and BxPC-3 pancreatic carcinoma cells (35). Recent studies demonstrate that SU11274 is also an effective inhibitor of both HGF-dependent and independent signaling (36). Inhibition of c-Met kinase activity reduced cell growth, induced G1 cell cycle arrest or apoptosis and inhibited c-Met-dependent signaling. The identification of SU11274 as an effective inhibitor of c-Met tyrosine kinase activity illustrates the potential therapeutic use of targeting c-Met in cancers associated

with activated forms of this kinase.

The transduction of signaling and subsequent biological effects of c-Met has been shown to be important in the epithelial–mesenchymal transition and regulation of cell migration, invasion, cell proliferation, and survival (37). Binding of HGF to the c-Met extracellular ligand-binding domain results in receptor multimerization and phosphorylation of multiple tyrosine residues at the intracellular region. Tyrosine phosphorylation at the c-Met juxtamembrane, catalytic, and cytoplasmic tail domains regulates the internalization, catalytic activity, and docking of regulatory substrates, respectively (38). Activation of c-Met results in the binding and phosphorylation of adaptor proteins such as Gab-1, Grb2, and Shc and subsequent activation of signal transducers such as PI3K, PLC-γ, STATs, ERK 1/2, and FAK (39).

MAPKs are a family of serine/threonine protein kinases that are activated by mitogens or stress conditions, and MAPKs play an essential role in a diverse array of cellular functions, including cell growth, differentiation, apoptosis, and autophagy (40). One common effect observed is the inhibition of constitutive or HGF-stimulated ERK phosphorylation in each cell line. The activation of ERK through the Grb2–SOS–Ras cascade has been linked with uncontrolled cell proliferation (41). Recent studies suggest that ERK regulates the maturation of autophagic vacuoles. ERK is associated with the formation of large cytoplasmic vacuoles (42). ERK is reported here to be activated in response to SU11274 for the induction of autophagy. So contradicting results have been reported in these findings, since the activation of the ERK cascade is originally associated with proliferation, differentiation, and survival of the cells. The ERK pathway is predominantly activated through a Ras-dependent mechanism and required for cell proliferation and survival. In this study, inhibition of ERK with PD98058 inhibited SU11274-induced autophagy, indicating that ERK mediated this process. All the results indicated that ERK was activated by a signal different from the Ras/Raf pathway, and the activated ERK could mediate SU11274-induced autophagy. Further, we demonstrated that SU11274-induced ERK activation contributed to p53 activation, which subsequently induced autophagy in A549 cells.

In a sense, the ERK signal provides a protective pathway by which some growth factors (such as HGF) prevent cell death, and this promotion of cell survival goes through several mechanisms including the regulation of anti-apoptotic Bcl-2 family members (43). Recent work has also demonstrated that ERK promotes apoptosis and cell death in response to certain chemotherapeutic agents, and this effect is partially due to the increased phosphorylation of Bcl-2 (44), Moreover, decrease in the binding of Beclin-1 to Bcl-2 promoted autophagy (45). Hence, our findings, together with these results, supported the notion that ERK is controversial in the regulation of autophagy. It has been reported that during starvation and drug stimuli, Bcl-2 becomes phosphorylated, and the expression of Bcl-2 is suppressed by phosphorylation of Bcl-2, and then causes its dissociation from Beclin-1, resulting in induction of autophagy. We have reported here that both p53 and ERK are activated by SU11274 in a time dependent manner. It might be that ERK or p53 becomes phosphorylated, and the expression of ERK or p53 is suppressed by phosphorylation of ERK or p53. SU11274 decreased the expressions of ERK, p53, and Bcl-2 in a similar mechanism, and the present study demonstrated that activation of ERK and p53 is important for SU11274-induced autophagy in A549 cells. Then, ERK increases phosphorylation of Bcl-2 but decreases the expression of Bcl-2. All those results indicate that ERK is activated by SU11274, and the activated ERK could mediate SU11274-induced autophagy.

LC3 is now widely used to monitor autophagy (46). Beclin-1, another powerful tool to detect autophagy, promotes autophagy associated with inhibition of cellular proliferation and tumorigenesis (47). In this study, SU11274-induced autophagy was manifested by increased conversion of LC3-I to LC3-II and Beclin-1 expression. Meanwhile, we found that SU11274 increased Beclin-1 activation partially attributed to promoting the phosphorylation of Bcl-2 by activated ERK and then augmented autophagy in A549 cells.

In general, SU11274's suppressive effect on c-Met expression facilitated ERK and p53 activation and subsequently induced autophagy; meanwhile, activated ERK promoted Beclin-1 release through phosphorylation of Bcl-2. These findings provide new information of a signaling pathway among HGF/c-Met, ERK–p53, and ERK–Bcl-2 in the regulation of the autophagic process, and the promotion of autophagy might shed light on how to increase tumor cell sensitivity to SU11274 in the clinical treatment of lung cancer.

Acknowledgment

This research was funded by the National Key Scientific Project for New Drug Discovery and Development (2009ZX09301-012), P.R. China.

References

1 Bonomi P. Matrix metalloproteinases and matrix metalloproteinase inhibitors in lung cancer. Semin Oncol. 2002;29:78–86.

2 Li L, He D, Yang J, Wang X. Cordycepin inhibits renal interstitial myofibroblast activation probably by inducing hepatocyte growth factor expression. J Pharmacol Sci. 2011;117:286–294.

3 Francesca DB, Michela F, Andrea R. Receptor tyrosine kinases as targets for cancer therapy. Cancer Ther. 2004;20:317–328.
4 Onitsuka T, Uramoto H, Nose N, Takenoyama M, Hanagiri T, Sugio K, et al. Acquired resistance to gefitinib: the contribution of mechanisms other than the T790M, MET, and HGF status. Lung Cancer. 2010;68:198–203.
5 Wader KF, Fagerli UM, Holt RU, Stordal B, Borset M, Sundan A, et al. Elevated serum concentrations of activated hepatocyte growth factor activator in patients with multiple myeloma. Eur J Haematol. 2008;81:380–383.
6 Moon WS, Park HS, Yu KH, Park MY, Kim KR, Jang KY, et al. Expression of betacellulin and epidermal growth factor receptor in hepatocellular carcinoma: implications for angiogenesis. Hum Pathol. 2006;3:1324–1332.
7 Tomiya T, Nishikawa T, Inoue Y, Ohtomo N, Ikeda H, Tejima K, et al. Leucine stimulates HGF production by hepatic stellate cells through mTOR pathway. Biochem Biophysl Res Commun. 2007;358:176–180.
8 Birchmeier C, Gherardi E. Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. Trends Cell Biol. 1998; 10:404–410.
9 Mahtouk K, Tjin EP, Spaargaren M, Pals ST. The HGF/MET pathway as target for the treatment of multiple myeloma and B-cell lymphomas. Biochim Biophys Acta. 2010;1806:208–219.
10 Zimmer Y, Vaseva AV, Medova M, Streit B, Blank-Liss W, Greiner RH, et al. Differential inhibition sensitivities of MET mutants to the small molecule inhibitor SU11274. Cancer Lett. 2010;289:228–236.
11 Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell. 2004;6:463–477.
12 Lee WJ, Chen WK, Wang CJ, Lin WL, Tseng TH. Apigenin inhibits HGF-promoted invasive growth and metastasis involving blocking PI3K/Akt pathway and β4 integrin function in MDA-MB-231 breast cancer cells. Toxicol Appl Pharmacol. 2008; 226:178–191.
13 Duan WJ, Jin XY, Li QS, Tashiro S, Onodera S, Ikejima T. Silibinin induced autophagic and apoptotic cell death in HT1080 cells through a reactive oxygen species pathway. J Pharmacol Sci. 2010;113:48–56.
14 Li D, Wu LJ, Tashiro S, Onodera S, Ikejima T. Oridonin-induced A431 cell apoptosis partially through blockage of the Ras/Raf/ERK signal pathway. J Pharmacol Sci. 2007;103:56–66.
15 Ye YC, Yu L, Wang HJ, Tashiro S, Onodera S, Ikejima T. TNFα-induced necroptosis and autophagy via supression of the p38-NF-κB survival pathway in L929 cells. J Pharmacol Sci. 2011; 117:160–169.
16 Xu S, Zhao Y, Yu L, Shen X, Ding F, Fu G. Rosiglitazone attenuates endothelial progenitor cell apoptosis induced by TNF-α via ERK/MAPK and NF-κB signal pathways. J Pharmacol Sci. 2011; 117:265–274.
17 Huang J, Wu L, Tashiro S, Onodera S, Ikejima T. Reactive oxygen species mediate oridonin-induced HepG2 apoptosis through p53, MAPK, and mitochondrial signaling pathways. J Pharmacol Sci. 2008;107:370–379.
18 Klionsky DJ. The molecular machinery of autophagy: unanswered questions. Cell Sci. 2005;118:7–18.
19 Kanzawa T, Kondo Y, Ito H. Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res. 2003; 63:2103–2108.
20 Kessel D, Reiners JJ Jr, Hazeldine ST, Polin L, Horwitz JP. The role of autophagy in the death of L1210 leukemia cells initiated by the new antitumor agents XK469 and SH80. Mol Cancer Ther. 2007;6:370–379.
21 Yu Y, Chiba Y, Sakai H, Misawa M. Possible involvements of nuclear factor-κB and activator protein-1 in the tumor necrosis factor-α–induced upregulation of matrix metalloproteinase-12 in human alveolar epithelial A549 cell line. J Pharmacol Sci. 2010; 112:83–88.
22 Zeng J, Xia S, Zhong W, Li J, Lin L. In vitro and in vivo effects of ketamine on generation and function of dendritic cells. J Pharmacol Sci. 2011;117:170–179.
23 Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008;132:27–42.
24 Mizushima N, Yoshimori T. How to interpret LC3 immunoblotting. Autophagy. 2007;3:542–545.
25 Wang HJ, Tashiro SI, Onodera S, Ikejima T. Inhibition of insulin-like growth factor 1 receptor signaling enhanced silibinin-induced activation of death receptor and mitochondrial. J Pharmacol Sci. 2008;3:260–269.
26 Pushparai PN, Melendez AJ. Short interfering RNA (siRNA), a novel therapeutic tool acting on angiogenesis. Clin Exp Pharmacol Physiol. 2006;33:504–510.
27 Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 2005;122:927–939.
28 Wang ZH, Xu L, Duan ZL, Zeng LQ, Yan NH, Peng ZL. Beclin 1-mediated macroautophagy involves regulation of caspase-9 expression in cervical cancer HeLa cells. Gynecol Oncol. 2007; 107:107–113.
29 Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C, et al. Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo. Nat Med. 1999;5: 662–668.
30 Ichimura E, Maeshima A, Nakajima T, Nakamura T. Expression of c-met/HGF receptor in human non-small cell lung carcinomas *in vitro* and its prognostic significance. Jpn J Cancer Res. 1996; 87:1063–1069.
31 Siegfried JM, Weissfeld LA, Luketich JD, Weyant RJ, Gubish CT, Landreneau RJ. The clinical significance of hepatocyte growth factor for non-small cell lung cancer. Ann Thorac Surg. 1998;6:1915–1918.
32 Christensen JG, Burrows J, Salgia R. c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention. Cancer Lett. 2005;225:1–26.
33 Christensen JG, Schreck R, Burrows J, Kuruganti P, Chan E, Jeffrey PL, et al. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes *in vitro* and exhibits cytoreductive antitumor activity *in vivo*. Cancer Res. 2003;11: 7345–7355.
34 Zou HY, Li Q, Lee JH, Arango ME, McDonnell SR, Yamazaki S, et al. An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms. Cancer Res. 2007; 9:4408–4417.
35 Puri N, Khramtsov A, Ahmed S, Nallasura V, Hetzel JT, Jagadeeswaran R, et al. A selective small molecule inhibitor of c-Met, PHA665752, inhibits tumorigenicity angiogenesis in mouse lung cancer xenografts. Cancer Res. 2007;8:3529–3534.

36 Underiner TL, Herbertz T, Miknyoczki SJ. Discovery and development of small molecule c-Met inhibitors: evolution and profiles of clinical candidates. Anticancer Agents Med Chem. 2010; 10:7–27.
37 Graveel C, Su Y, Koeman J, Wang LM, Tessarollo L, Fiscella M, et al. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. PNAS. 2004;101:17198–17203.
38 Moon WS, Park HS, Yu KH, Park MY, Kim KR, Jang KY, et al. Expression of betacellulin and epidermal growth factor receptor in hepatocellular carcinoma: implications for angiogenesis. Hum Pathol. 2006;3:1324–1332.
39 Gobert Gosse S, Bourqin C, Liu WQ, Garbay C, Mouchiround G. M-CSF stimulated differentiation requires persistent MEK activity and MAPK phosphorylation independent of Grb2–Sos association and phosphatidylinositol 3-kinase activity. Cell Signal. 2005;11:1352–1362.
40 Suzuki K, Hino M, Kutsuna H. Selective activation of p38 mitogen-activated protein kinase cascade in human eutrophils stimulated by IL-1β. J Immol Lunol. 2001;167:5940–5947.
41 Pierre S, Bats AS, Coumoul X. Understanding SOS (Son of Sevenless). Biochem Pharmacol. 2011;9:1049–1056.
42 Yang SJ, Chang SC, Wen HC, Chen CY, Liao JF, Chang CH. Plumbagin activates ERK1/2 and Akt via superoxide, Src and PI3-kinase in 3T3-L1 cells. Eur J Pharmacol. 2010;638:21–28.
43 Francesca DB, Michela F, Andrea R. Receptor tyrosine kinases as targets for cancer therapy. Cancer Ther. 2004;2:317–328.
44 Singh S, Upadhyay AK, Ajay AK, Bhat MK. p53 regulates ERK activation in carboplatin induced apoptosis in cervical carcimoma: a novel target of p53 in apoptosis. FEBS Lett. 2007;581: 289–295.
45 Nakata K, Suzuki Y, Inoue T, Ra C, Yakura H, Mizuno K. Deficiency of SHP1 leads to sustained and increased ERK activation in mast cell, thereby inhibiting IL-3-dependent proliferation and cell death. Mol Immunol. 2011;48:472–480.
46 Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, et al. Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci U S A. 2006;103:4952–4957.
47 Zalckvar E, Berissi H, Mizrachy L, Idelchuk Y, Koren I, Eisenstein M, et al. DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep. 2009; 10:285–292.