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A superoxide-mediated MKP-1 degradation and JNK activation pathway for luteolin-induced lung cancer cytotoxicity

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Running title: MKP-1 in luteolin's anticancer activity

Abbreviations: JNK, c-Jun N-terminal kinase; MKP-1, MAP kinase phosphatase HBEC, human bronchial epithelial cells; ROS, reactive oxygen species; SOD, superoxide dismutase; BHA, Butylated hydroxyanisol; NAC, N-acetylcysteine

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Abstract

Although luteolin is implicated as a potential cancer therapeutic and preventive agent due to its potent cancer cell killing activity, the molecular mechanisms by which luteolin's cancer cell cytotoxicity is achieved have not been well elucidated. In this report, the luteolin-induced cellular signaling was systematically investigated and a novel pathway for luteolin's lung cancer killing was identified. The results show that induction of superoxide is an early and crucial step for luteolin-induced apoptotic and non-apoptotic death in lung cancer cells. The c-Jun N-terminal kinase (JNK) was potently activated following superoxide accumulation. Suppression of superoxide completely blocked luteolin-induced JNK activation, which was well correlated to alleviation of luteolin's cytotoxicity. Although luteolin slightly stimulated the JNK activating kinase MKK7, the latter was not dependent on superoxide. We further found that luteolin triggers a superoxide-dependent rapid degradation of the JNK inactivating phosphatase MKP-1. Introduction of a degradation-resistant MKP-1 mutant effectively attenuated luteolin-induced JNK activation and cytotoxicity, suggesting that inhibiting the JNK suppressor MKP-1 plays a major role in luteolin-induced lung cancer cell death. Collectively, our results unveil a novel pathway consisting of superoxide, MKP1 and JNK for luteolin's cytotoxicity in lung cancer cells, and manipulation of this pathway could be a useful approach for applying luteolin for lung cancer prevention and therapy.

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Introduction

Lung cancer is a major health concern that afflicts approximately 160,000 people each year in the United States (Jemal et al., 2010). Because most lung cancer patients are diagnosed at late disease stages when surgery is not a viable option and chemotherapy and radiation therapy are usually ineffective for lung cancer, the prognosis is very poor for most lung cancer patients (Onn et al., 2004). Therefore, development of effective prevention and therapeutic agents against lung cancer is critical for reducing morbidity and mortality.

The flavonoid luteolin (3', 4', 5, 7-tetrahydroxyflavone) has been suggested as a potential lung cancer chemoprevention and chemotherapy agent (Lin et al., 2008). Luteolin belongs to the flavone class flavonoids, a large class of polyphenols ubiquitously found in vegetables, fruits, and medicinal plants. In the past two decades, flavonoids have been shown to have anti-oxidative, anti-viral, anti-tumor and anti-inflammatory activities (Birt et al., 2001; Lin et al., 2008; Matsuo et al., 2005; Ueda et al., 2003). Epidemiological studies suggest that dietary flavonoids intake is inversely associated with risk of lung, prostate, stomach and breast cancer (Hirvonen et al., 2001; Le Marchand et al., 2000; Wright et al., 2004). Because of many confounding factors, the preventive potential of luteolin for lung cancer is not clear (Garcia-Closas et al., 1998; Hirvonen et al., 2001). However, the chemopreventive potential of luteolin has been seen in a 20-methycholanyrene-induced fibrosarcoma mouse model and the anti-cancer activity of luteolin has been well documented (Ko et al., 2002; Lee et al., 2002; Osakabe et al., 2004; Ueda et al., 2003). The anti-tumor activity of luteolin was

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attributed to its ability to induce DNA damage, cell cycle arrest, apoptosis, to suppress angiogenesis and cell survival capacity (Bai et al., 2009a; Ju et al., 2007; Lin et al., 2008; Ueda et al., 2003). As other flavonoids, luteolin is able to modulate the redox status of the cells. Depending on the cell contexts, luteolin functions as either an antioxidant or a pro-oxidant (Ju et al., 2007; Matsuo et al., 2005; Michels et al., 2005). Reactive oxygen species (ROS) are a diverse group of reactive, short-lived and oxygen-containing species, such as superoxide and H_2O_2. Besides damaging the cellular components by oxidizing DNA, protein and lipids, ROS also serve as a mediator for cell signaling (Lin et al., 2004; Starkov, 2008; Trachootham et al., 2009). We found that luteolin-induced ROS, specifically superoxide, suppresses TNF-induced NF-κB while potentiates JNK activation, which promotes TNF-induced apoptosis in lung cancer cells (Ju et al., 2007).

Although luteolin has been shown to induce and potentiate apoptosis in cancer cells, the precise mechanisms by which luteolin kills lung cancer cells is not well elucidated. Understanding the cell signaling mechanisms of luteolin will undoubtedly facilitate the application of this flavonoid for lung cancer chemoprevention and chemotherapy. In this report, we identify a novel pathway that involves superoxide production, MKP1 degradation and JNK activation as the main mechanism for luteolin's cytotoxicity in lung cancer cells. Modulating this pathway could be a useful approach for applying this agent for lung cancer prevention and therapy.

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Materials and Methods

Reagents and antibodies

Luteolin was from Cayman Chemical (Ann Arbor, MI). Butylated hydroxyanisol (BHA), N-acetylcysteine (NAC), Necrostatin-1, z-VAD and cycloheximide were purchased from sigma (St. Louis, MO). The JNK inhibitor SP600125, p38 inhibitor SB203580, ERK inhibitor U0126, IKK inhibitor II, PKC inhibitor Ro31, Gö6796 and proteasome inhibitor MG132 were from Calbiochem (La Jolla, CA). The following antibodies were used for Western blot: anti-phospho-JNK (Invitrogen, Camarillo, CA), anti-MKP-1, -ubiquitin, - HA (Omni-probe) and anti-SKP-2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-MKK7 (Abcam, Cambridge MA), anti-JNK1 (BD Biosciences, San Diego, CA), anti-β-tubulin (Sigma, St. Louis, MO). Dihydroethidium(DHE) was purchased from Invitrogen (Carlsbad, CA). The pRSV-LacZ plasmid was described previously (Lin et al., 1999). The MKP-1EE plasmid was constructed by PCR-directed mutagenesis using the pLenti6/V5-MKP-1 plasmid as a template (a gift from Dr. C. Chen from Albany Medical College) to generate a MKP-1 mutant, in which the two serine residues at positions 359 and 364 were replace by glutamic acid residues (Liu et al., 2009). The PCR primers are 5'-ttggatcccATGGTCATGGAAGTGGGCAC-3' and 5'-ttctcgagTCAGCAGCTGGGTTCGGTCGTAATGGGTTCCTGAAGGTAGCTCG CGCAC-3'. The PCR products was digested by BamHI/XhoI and cloned into the pcDNA 3.1/HisB vector (Invitrogen), resulting in pcDNA-MKP-1EE. The construct was verified by DNA sequencing.

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Cell culture, Transfection and X-gal staining

The human lung cancer cell line H460 and A549 were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 with 10% fetal bovine serum, 1 mmol/L glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells grown in 12-well plates were transfected with pcDNA -MKP-1EE with FuGENE HD according to manufacturer's instruction (Roche, Indianapolis, IN). Twenty-four hours after transfection, cells were treated as indicated in each figure legend. For X-gal staining, Cells were co-transfected with pRSV-LacZ and pcDNA-MKP-1EE or empty vector with FuGENE HD. Thwenty four hour after transfection, the cells were treated as indicated in figure legend. Cells were washed with PBS once and fixed in 1% paraformaldehyde and stained as described(Lin et al., 1999). Cells were visualized and photographed under a microscope.

Cytotoxicity assay

Cytotoxicity was determined using a lactate dehydrogenase (LDH) release-base cytotoxicity detection kit (Promega, Madison, WI). Cells were seeded in 48-well plates at 70–80% confluence. After culture overnight, cells were treated as indicated in each figure legend. LDH release was determined and cell death was calculated as described previously (Wang et al., 2006). In order to morphologically study cell death, H460 cells were cultured on cover slides and pretreated with luteolin (40 μM) for 36 hours or remained untreated. Cells were stained with 50 μg/ml of acridine orange and 50 μg/ml ethidium bromide (EB), and immediately visualized and photographed under a fluorescent microscope (Chen et al., 2007).

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Western blot

Cells were harvested and lysed in M2 buffer (20 mM Tris-HCl, pH7.6; 0.5% NP-40; 250 mM NaCl; 3 mM EGTA; 3 mM EDTA; 2 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride; 20 mM β-glycerophosphate; 1 mM sodium vanadate; and 1 μg/ml leupeptin). Equal amounts of protein extracts were resolved in 12% SDS-PAGE and the proteins of interest were probed by Western blot and visualized by enhanced chemiluminescence according manufacturer's instructions (Amersham, Piscataway, NJ) (Bai et al., 2009b).

Detection of superoxide

Cells were seeded in 12-well plates, cultured overnight, and then treated as indicated in each figure legend. Thirty minutes before collecting cells, DHE (5 μM) was added. Superoxide was detected by flow cytometry with FACSCalibur and analyzed with the CellQuest program (both from BD Biosciences) as reported previously (Ju et al., 2007).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted with the RNAeasy kit (Qiagen, Valencia, CA). One microgram of RNA from each sample was used as a template for cDNA synthesis with a reverse transcription kit (Promega). An equal volume of cDNA product was used in the PCR. The primers used were: MKP-1 5'-CCCCGGATCCAATGGTCATGGAAGTGGGCACC-3' and 5'-CCCCGAATTCGGAGCTGGGTTCGGTCGTAATGGGTTCCTGAAGGTAGCT-3'; β-actin, 5'-CCAGCCTTCCTTCCTGGGCAT-3' and

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5'-AGGAGCAATGATCTTGATCTTCATT-3'. The reaction condition was 94°C, 45 s; 55°C, 40 s; and 72°C, 45 s. For MKP-1 and β-actin, the cycles for PCR were 27 and 21, respectively. PCR products were run on 2% agarose gel with 0.5 μg/ml ethidium bromide, visualized, and photographed (Bai et al., 2009a).

Statistical analysis

Data are expressed as mean ± SD. Statistical significance was examined by one-way analysis of variance pairwise comparison. $P<0.05$ was considered statistically significant.

Results

Luteolin-induced cytotoxicity is dependent on superoxide

Luteolin-induced cytotoxicity in a dose-dependent manner was detected in H460 and A549 cells, confirming its anticancer activity in lung cancer cells (Fig. 1A). The dying and dead cells showed both apoptotic and necrotic morphologies, suggesting that luteolin-induced cancer cell death was associated with both apoptosis and necrosis (Suplemental Figure 1). Pretreatment of the cells with either the pan-caspase inhibitor z-VAD that suppresses apoptosis or the necrosis inhibitor necrostatin-1 effectively suppressed luteolin-induced cytotoxicity, and combination of these two inhibitors caused a potentiated inhibition of cell death (Fig. 1B). These results confirmed that luteolin is capable in killing lung cancer cells and determined that induction of both apoptosis and necrosis contributes to luteolin's anticancer property.

Our previous results showed that luteolin induces superoxide, which is pivotal for blocking TNF-induced NF-κB and promoting TNF-induced apoptosis in lung cancer

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cells (Ju et al., 2007). To investigate if induction of superoxide contributes to luteolin-induced cancer cell death, ROS scavengers BHA and NAC were used to block luteolin-induced superoxide accumulation. Both reagents potently suppressed luteolin-induced cytotoxicity, which was associated with effectively scavenge of luteolin-induced superoxide (Fig. 1C, 1D). These results establish that luteolin induces lung cancer cell death through induction of cellular superoxide accumulation.

Superoxide-dependent JNK activation is required for Luteolin-induced cytotoxicity

Because ROS are potent JNK activators and our previous studies found that luteolin potentiates TNF-induced lung cancer cell death involving JNK (Ju et al., 2007), we investigated whether luteolin by itself kills lung cancer cells through JNK activation. Indeed, luteolin potently induced JNK activation in both A549 and H460 cells, although in a relatively slow kinetic; starting at 2 hr post luteolin exposure (Fig. 2A). The pivotal role of JNK in luteolin-induced cytotoxicity was demonstrated by JNK inhibition with the selective JNK inhibitor SP600125, which almost completely blocked luteolin-triggered cell death (Fig. 2B). As controls, the inhibitors for p38 (SB 203580), ERK (U0126) and NF-κB (IKK inhibitor II) had no detectable effect on luteolin-induced cytotoxicity (Fig. 2B). Scavenging superoxide with either BHA or NAC completely suppressed luteolin-induced JNK activation, suggesting that luteolin stimulates JNK through superoxide generation (Fig. 2C). These results unveil a pathway involving superoxide and JNK by which luteolin kills lung cancer cells.

Luteolin activates JNK through suppressing MKP-1 expression

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To investigate the mechanism by which luteolin activates JNK in lung cancer cells, we first investigated if luteolin activates the MAPKKKs and MAPKKs upstream of JNK. Under the conditions that luteolin strongly induced JNK, there was no detectable activation of MAKKKs including MEKK1, ASK1 and TLK. Similarly, MKK4, the MAPKK that is able to mediate JNK activation, was not stimulated by luteolin either (data not shown). Another MAPKK, MKK7, was moderately activated by luteolin in both A549 and H460 cells (Fig. 3A). However, the ROS scavengers BHA and NAC, which completely blocked luteolin-induced JNK activation (Fig. 2C), had no effect on luteolin-stimulated MKK7 activation (Fig. 3B). These results suggest MKK7 has subtle contribution to luteolin-induced JNK activation in lung cancer cells, and luteolin stimulates JNK is unlikely through direct stimulating the MAKKK/MAPKK/JNK cascade.

We next investigated if the JNK inactivating mechanism is interfered by luteolin. Because MKP-1 is the main phosphatase that inactivates JNK and is implicated in lung cancer's resistance to therapy (Wu, 2007), we examined the effect of luteolin on MKP-1 protein expression in lung cancer cells. The MKP-1 protein levels rapidly decreased in luteolin-exposed A549 and H460 cells (Fig. 4A). It is remarkable that MKP-1 decrease preceded JNK activation in both A549 and H460 cells. MKP-1 protein declined at 30 min and diminished at 1 hr (Fig.4A) while JNK was activated at 2 h (Fig. 2A) in H460 cells. The similar trend was also clearly seen in A549 cells, where MKP-1 reduced at 1 hr and diminished at 2 hr while JNK activation began at 2 hr (Fig. 2A, 4A). These results strongly suggest that suppression of MKP-1

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contributes to luteolin-induced JNK activation.

Luteolin suppresses MKP-1 expression through destabilizing the MKP-1 protein

To address the mechanism by which luteolin induces MKP-1 down-regulation, we first checked the mRNA expression levels in luteolin-treated cells. As shown in Fig. 4B, there was no detectable effect of luteolin on MKP-1 mRNA levels, suggesting that it is unlikely that luteolin regulates MKP-1 expression via affecting transcription or RNA stability. Then we examined protein stability by shutting off protein synthesis with cycloheximide (CHX) and examining the MKP-1 levels at different time points. In A549 cells, after CHX treatment, MKP-1 level declined and showed a half-life of 25 min. However, when the cells were treated with CHX combined with luteolin, the MKP-1 levels decreased much faster, showing a half-life of 7.8 min, suggesting that luteolin suppresses MKP-1 expression through destabilizing the MKP-1 protein (Fig. 4C, 4D).

Luteolin triggers superoxide-mediated ubiquitination and proteasomal degradation of MKP-1 protein

The above results suggest that MKP-1 protein degradation accounts for the major mechanism underlying luteolin-induced MKP-1 down-regulation. Then we investigated if MKP-1 down-regulation is mediated by superoxide. Indeed, the ROS scavengers BHA and NAC completely blocked luteolin's effect on MKP-1 expression, strongly suggesting that ROS is the main mediator for luteolin-induced MKP-1 degradation (Fig. 4D). In contrast, the inhibitors for ERK (U0126), p38 (SB203580), JNK (SP600125), PKC (Ro31 and Go6796) and NF-κB (IKK inhibitor II) had

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marginal effect on luteolin-induced MKP-1 decrease, suggesting the corresponding signaling pathways play a minor role, if there is any, in this effect of luteolin (Fig. 4D). All the inhibitors are effective in blocking their respective pathways (Li et al., 2011)(data not shown). Together with the results that luteolin induced superoxide accumulation, and BHA and NAC completely blocked luteolin-induced JNK activation (Fig. 1D, 2C), these findings strongly suggest that luteolin stimulates JNK activation predominantly through superoxide-mediated degradation of MKP-1. The preteatment with the preatesome inhibitor MG132 fully restored MKP-1 expression in the luteolin treated cells (Fig. 4D), suggesting the MKP-1 protein is degraded in the proteasome. Collectively, these results suggest that luteolin triggers superoxide-mediated proteasomal degradation of MKP-1 protein.

Overexpression of a degradation-resistant MKP-1 mutant suppresses luteolin-induced cytotoxicity in lung cancer cells

To further validate the role of MKP-1 in luteolin-induced cytotoxicity in lung cancer cells, a MKP-1 mutant that is resistant to proteasomal degradation was constructed and used for rescuing luteolin-induced cell death in A549 cells. To this end, we substituted the serine residues at positions 359 and 364, which are critical for ERK-mediated stabilization (Brondello et al., 1999; Liu et al., 2009), with two glutamic acidic residues to create MKP-1EE to mimic the phophorylated conformation. The replacement of serine with glutamic acidic residues is a well-accepted approach to mimic a phophorylated conformation of a protein(Puri et al., 2000). The MKP-1EE protein was confirmed to be much more stable than the

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endogenous wild type MKP-1 in luteolin-treated cells (Fig. 5A). Consistently, overexpression of MKP-1EE significantly suppressed luteolin-induced JNK activation (Fig. 5B), which was well correlated to effective attenuation of luteolin-induced cell death (Fig. 5C). These results strongly substantiate the conclusion that MKP-1 degradation is the main mechanism by which luteolin activates JNK and kills lung cancer cells.

Discussion

In an attempt to explore the mechanisms for luteolin's cancer prevention and therapy potential, we have systematically examined the main pathways that are involved in luteolin-induced cancer cell cytotoxicity. We found that luteolin-induced cytotoxcity (apoptosis and necrosis) in lung cancer cells is associated with induction of superoxide and the subsequent activation of JNK and the rapid MKP-1 degradation triggered by superoxide underlies the main mechanism of JNK activation. Thus, our results identify a novel luteolin-activated pathway consisting of superoxide, MKP-1 and JNK for lung cancer cell death (Fig. 6) and manipulation of this pathway could be a useful approach for applying luteolin for lung cancer prevention and therapy.

The luteolin-induced cytotoxicity in lung cancer cells is associated with both apoptosis and necrosis, which was demonstrated by morphological (AO/EB staining, Suplemental Figure 1), pharmacological (chemical inhibitors, Fig. 1B) and biochemical (caspase activation, data not shown) experiments. Removal superoxide by ROS scavengers BHA or NAC dramatically protected cells against

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luteolin-induced cell death, clearly showing that the pro-oxidative but not the anti-oxidatvie property of luteolin is involved in lung cancer cell death. Indeed, luteolin's anti-oxidative activity is mainly underlying the mechanisms of its cytoprotective effect in normal tissues (Lin et al., 2008; Lopez-Lazaro, 2009). This is not surprising, because under certain conditions many polyphenols act as as either pro-oxidants or anti-oxidants. For example, quercetin is an antioxidant for brain cell survival whereas is a pro-oxidant for cancer cell death (De Marchi et al., 2009; Thangasamy et al., 2007). Recent reports provided clear evidence for luteolin's pro-oxidant property (Ju et al., 2007; Matsuo et al., 2005). Particularly, we found luteolin induces superoxide accumulation to block TNF-induced NF-κB activation and potentiate apoptosis (Ju et al., 2007).

Our results further reveal that JNK plays a key role downstream of superoxide in mediating luteolin's cytotoxicity. As a main MAP kinase in response to a variety of extracellular stimulations and intracellular stresses, JNK is usually activated through the activation of a MAPKKK-MAPKK-JNK kinase cascade (Lin and Dibling, 2002). In this study, we did not detect any effect of luteolin on ASK1, MEKK1 and MLK, the MAPKKKs that are involved in ROS-mediated JNK activation (data not shown) . Although we detected a moderate activation of MKK7, the JNK activating MAPKK, it appears that the JNK activation cascade contributes subtly to luteolin-induced JNK activation. This view is supported by that scavenging superoxide completely blocked luteolin-induced JNK activation while had undetectable effect on MKK7 activation in lung cancer cell lines. Instead, we determined that luteolin causes an early and

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dramatic reduction of the main JNK inactivating phosphatase MKP-1 preceding JNK activation. The reduction of MKP-1 is mediated by superoxide, because removal of superoxide with BHA and NAC completed blocked luteolin-induced MKP-1 reduction. Luteolin did not affect MKP-1 mRNA expression while the proteasome inhibiter MG132 fully restored MKP-1 protein expression in luteolin treated cells, suggesting that transcription of MKP-1 gene is unlikely involved in luteolin's inhibiting effect on MKP-1 whereas the enhancement of proteasomal degradation underlies the main mechanism of this function of luteolin. Importantly, the introduction of a degradation-refractory MKP-1 mutant effectively suppressed luteolin-induced cell death. Although the defined mechanism by which luteolin induces MKP-1 degradation requires further investigation, the results from this study establish a novel pathway that involves superoxide, MKP-1 and JNK in luteolin-induced cytotoxicity in lung cancer cells. It is worthy noting that MKP-1 has been suggested to play an important role in cancer cell survival and inhibition of which is implicated in cancer prevention and chemosensitization (Chen et al., 2005; Liao et al., 2003; Wang et al., 2007; Wu, 2007).

In summary, our results unveil a novel pathway consisting of superoxide, MKP-1 and JNK for luteolin's cytotoxicity in lung cancer cells. This property of luteolin renders it not only a candidate for lung cancer therapy, but also for lung cancer chemoprevention, which warrants further studies in animal models.

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Author Contribution

Participated in research design: Lin and Tang

Performed data analysis: Bai and Lin.

Wrote the manuscript: Bai and Lin.

Conducted experiments: Bai, X. Xu, Q. Wang, S.Xu, Ju, Chen. X. Wang and He.

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Footnotes:

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Figure legends

Figure 1. Superoxide is required for luteolin-induced apoptosis and necrosis in lung cancer cells. A, H460 and A549 cells were treated with indicated concentrations of luteolin for 36 h. Cell death was measured by LDH release assay. Data shown are mean ± SD. B, H460 cells were pretreated with z-VAD (20 μM), Nec-1 (30 μM) or z-VAD plus Nec-1 for 1 h, followed by luteolin (40 μM) treatment for 36 h. Cell death was measured as indicated in A. *, $p<0.01$.C, H460 cells were pretreated with BHA (100 nM) or NAC (500 μM) for 30 min and then treated with luteolin (40 μM) for 30 min. Cells were stained with DHE (5 μM) for 30 min before collecting. The cells were analyzed with a flow cytometer (FACSCalibur; BD Biosciences). Untreated cells with DHE staining were used as a negative control. The histogram overlays show the results of treated cells (gray lines) compared with untreated cells (dark lines). X-axis, fluorescent intensity; Y-axis, cell number. D, H460 cells were pretreated with BHA (100 nM) or NAC (500 μM) for 30 min, followed by luteolin (40 μM) for 36 h. Cell death was measured as indicated in A. *, $p<0.01$.

Figure 2. Luteolin-induced and superoxide-dependent JNK activation is essential for luteolin-induced cytotoxicity in cancer cells. A, H460 and A549 cells were treated with luteolin (40μM) for the indicated times. Phospho-JNK and JNK1 proteins were detected by Western blot. β-tubulin was detected as an input control. B, H460 cells pretreated with SB203580 (10 μM), U0126 (10 μM), IKK inhibitor II (10 μM) and SP600125 (20 μM) for 1 h followed by luteolin (40 uM) treatment for 36 h or left

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untreated. Cell death was measured by a LDH release assay. C, H460 cells were pretreated with BHA (100 nM) or NAC (500 μM) for 30 min, followed by luteolin (40 μM) treatment for the indicated times. Phospho-JNK and JNK1 proteins were detected by western blot. β-tubulin was detected as an input control.

Figure 3. Superoxide-independent MKK7 activation induced by luteolin. A, H460 and A549 cells were treated with luteolin (40 μM) for the indicated times. Phospho-MKK7 was detected by western blot. β-tubulin was detected as an input control. B, H460 cells were pretreated with for BHA (100 nM) or NAC (500 μM) for 30 min, followed by luteolin (40 μM) treatment for the indicated times. Phospho-MKK7 was detected by western blot. β-tubulin was detected as an input control.

Figure 4. Luteolin-induced and superoxide-dependent proteosomal MKP-1 degradation. A, H460 and A549 cells were treated with luteolin (40 μM) for the indicated times. MKP-1 was detected by Western blot. β-tubulin was detected as an input control. B, H460 and A549 cells were treated with luteolin (40 μM) for the indicated times. MKP-1 mRNA was detected by RT-PCR. β-actin was detected as an input control. C, upper, H460 cells were pretreated with cycloheximide (CHX, 10 μg/ml), then treated with luteolin (40 μM) for the indicated times. MKP-1 was detected by Western blot. β-tubulin was detected as an input control. Lower, quantification of the band densities and normalized with that of β-tubulin. D, H460

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cells were pretreated with BHA (100 nM), NAC (500 μM), SP600125 (20 μM), U0126 (10 μM), SB203580 (10 μM), Ro31 (10 nM), Go6796 (10 nM), IKK inhibitor II (10 μM) and MG132 (10 μM) for 1 h, and followed by luteolin (40 μM) treatment for 3 h. MKP-1 was detected by Western blot. β-tubulin was detected as an input control.

Figure 5. Overexpression of a degradation-resistant MKP-1 mutant protects cancer cells from luteolin-induced cell death. A, upper, A549 cells were transfected with pcDNA-MKP-1EE. Twenty-four hours after transfection, cells were treated with luteolin (40 μM) for the indicated times. MKP-1 was detected by Western blot. β-tubulin was detected as an input control. Lower, A549 cells treated with luteolin (40 μM) for the indicated times. MKP-1 was detected by Western blot. β-tubulin was detected as an input control. B, A549 cells were co-transfected with HA-JNK1 and pcDNA-MKP-1EE or empty vector (pcDNA3.1B). Twenty-four hours after transfection, the cells were treated with or without luteolin (40 μM) for the indicated times. Phospho-JNK1, HA-JNK1 and MKP-1EE proteins were detected by Western with anti-phosph-JNK, -HA and -Xpress, respectively. C, A549 cells were cotransfected in duplicates with pRSV-lacz and pcDNA-MKP-1EE or empty vector (pcDNA3.1B). Twenty-four hours after transfection, the cells were treated with or without luteolin (40 μM) for 36 h, then fixed and stained with X-gal. Upper, Cells were photographed under a light microscope (100x). Lower, quantification of cell survival. Blue cells from 9 randomly selected fields were counted. The total cell

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counts in non-treated groups were regarded as 100%. The percentages of in cell counts luteolin-treated group related to the control groups show cell survival rates after luteolin challenge. *, $p<0.01$.

Figure 6. A model of luteolin induced cell death in lung cancer cells. While luteolin activates MKK7, ROS-mediated suppression of MKP-1 underlies the main mechanism for luteolin-induced JNK activation and cytotoxicity.

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Fig.1

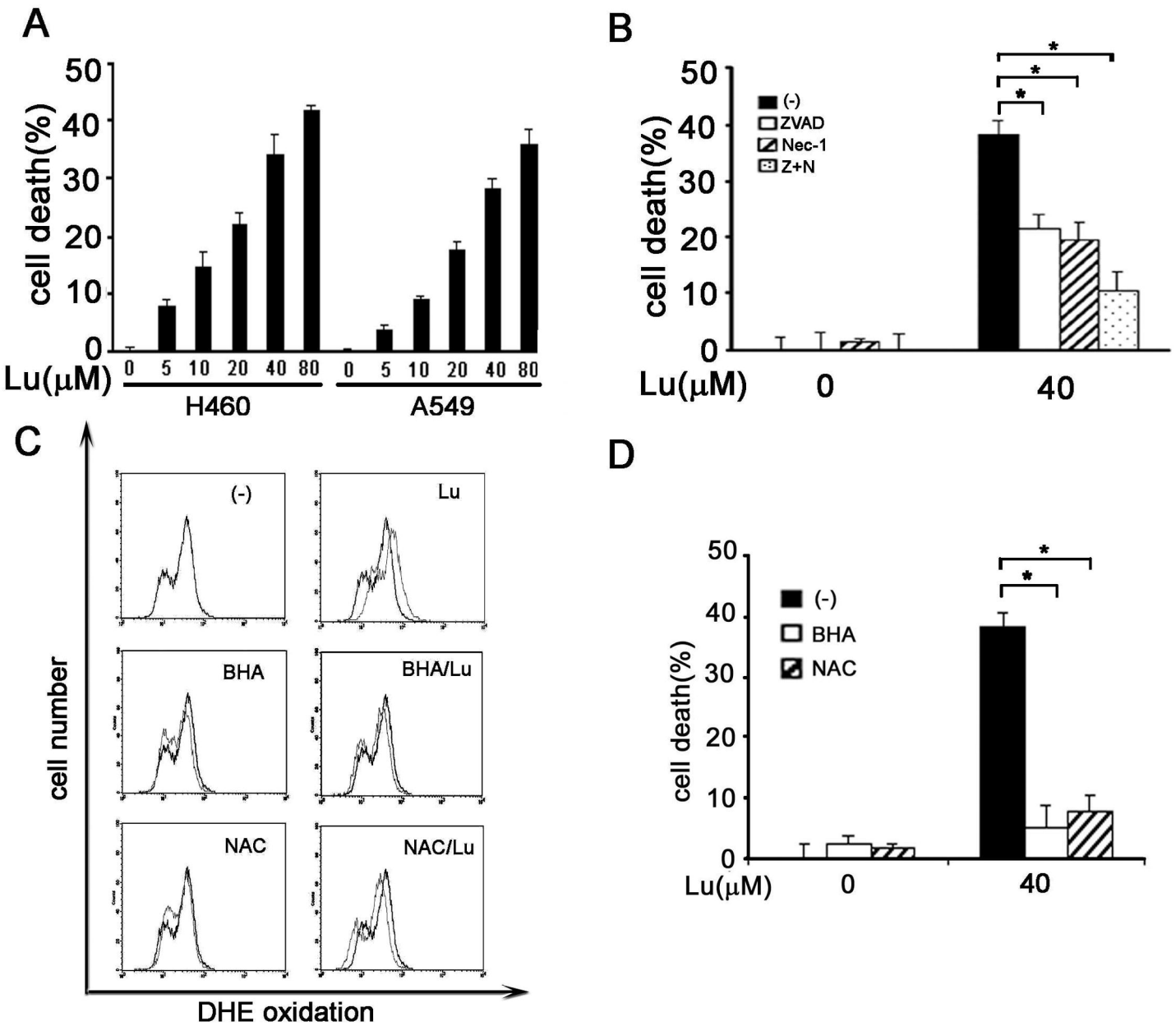

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Fig. 2

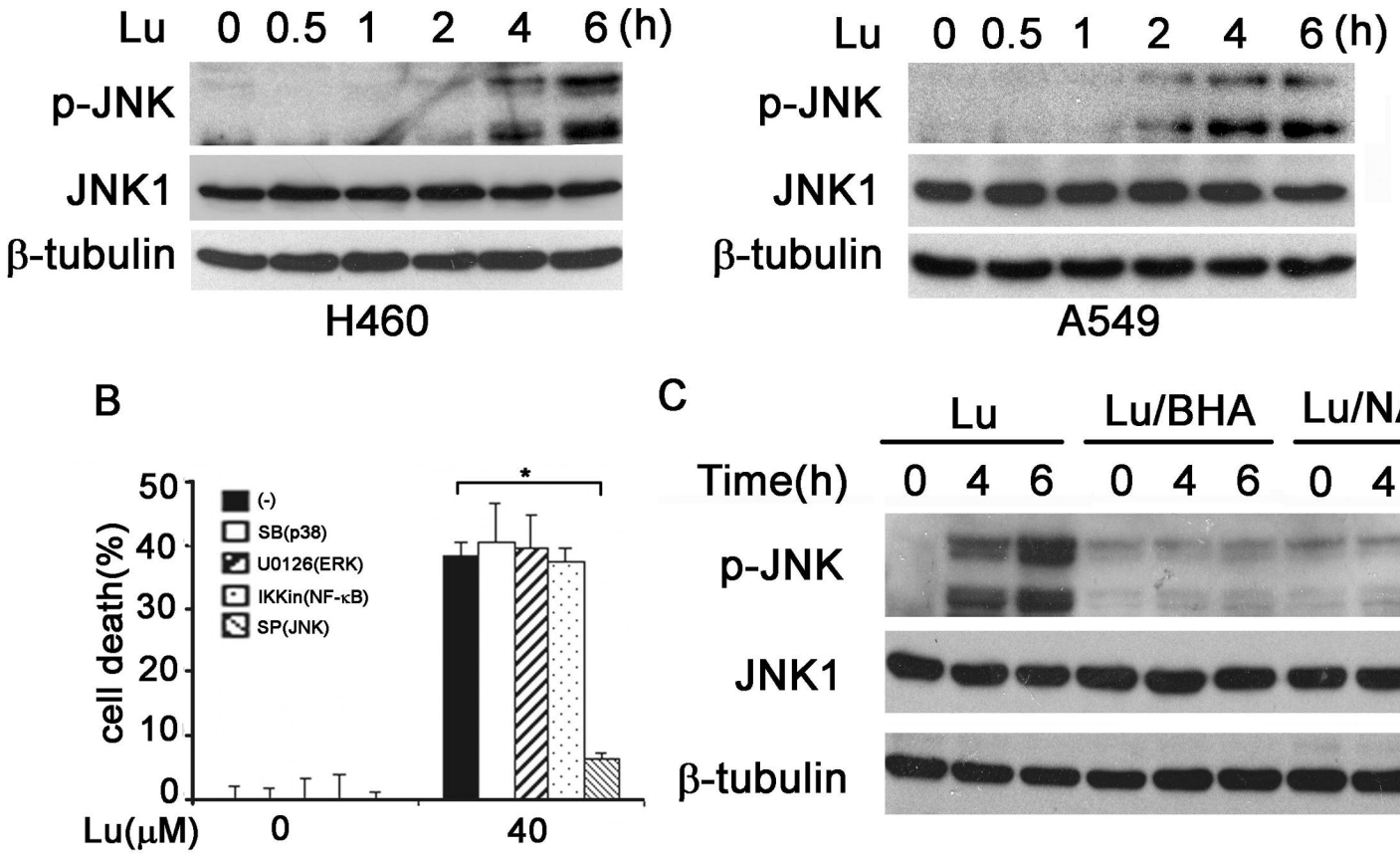

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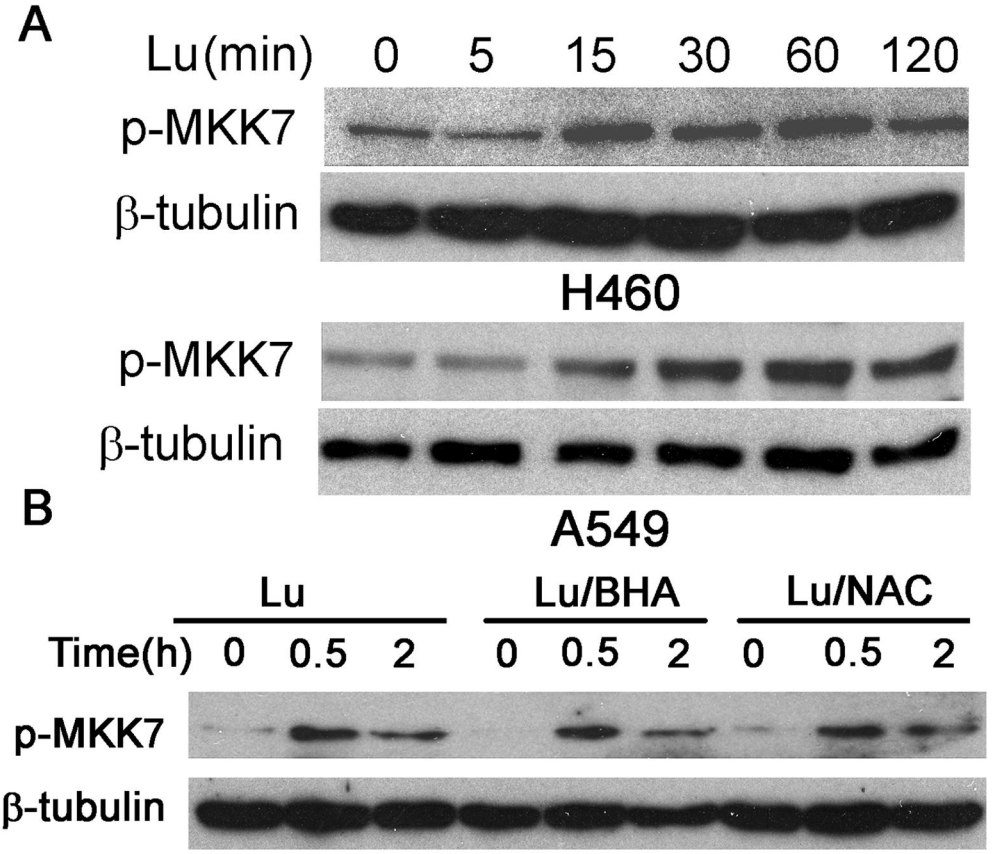
Fig. 3


A
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p-MKK7
β-tubulin
H460
p-MKK7
β-tubulin
B
A549
Lu
Lu/BHA
Lu/NAC
Time(h) 0 0.5 2 0 0.5 2 0 0.5 2
p-MKK7
β-tubulin

Fig. 4

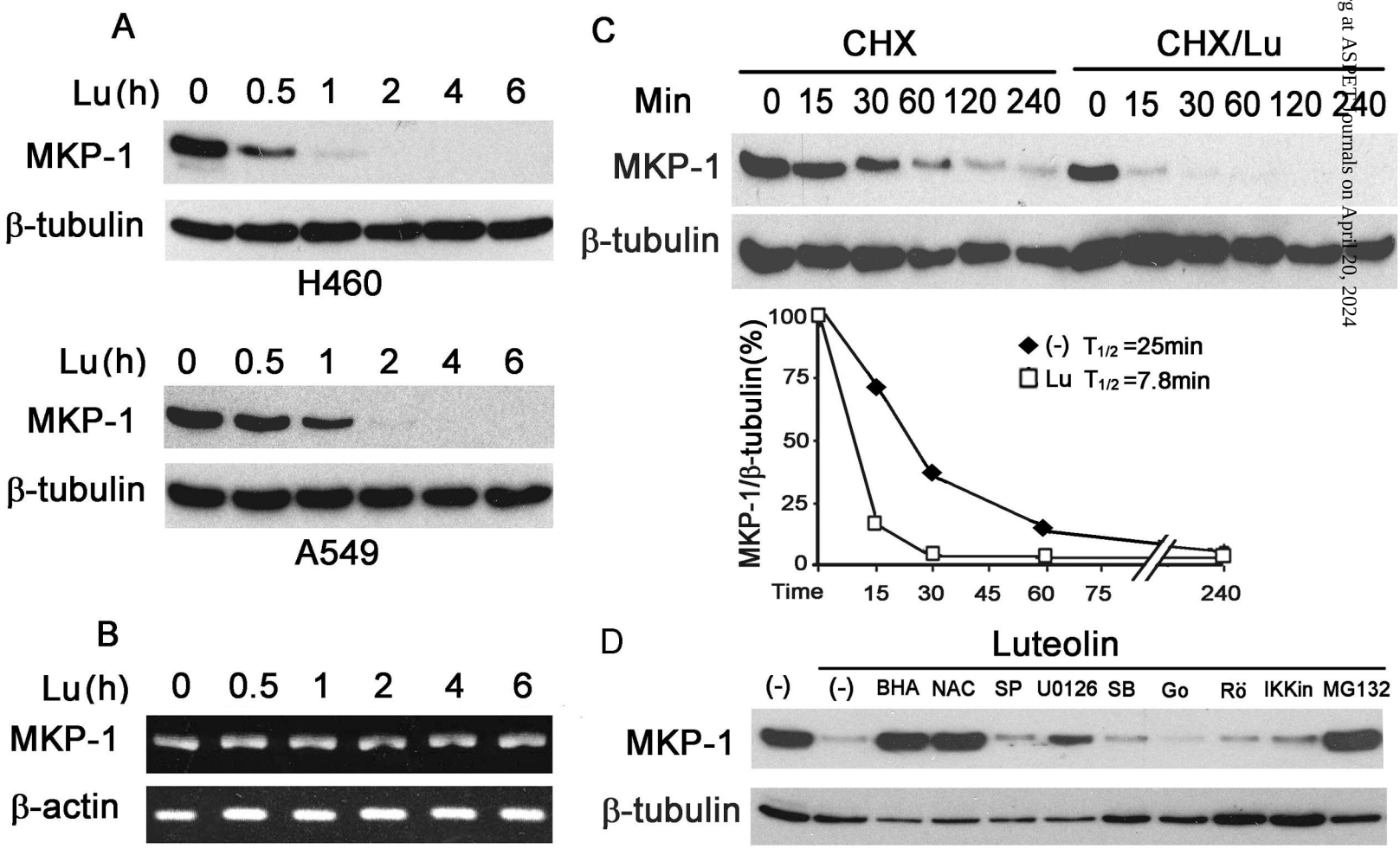

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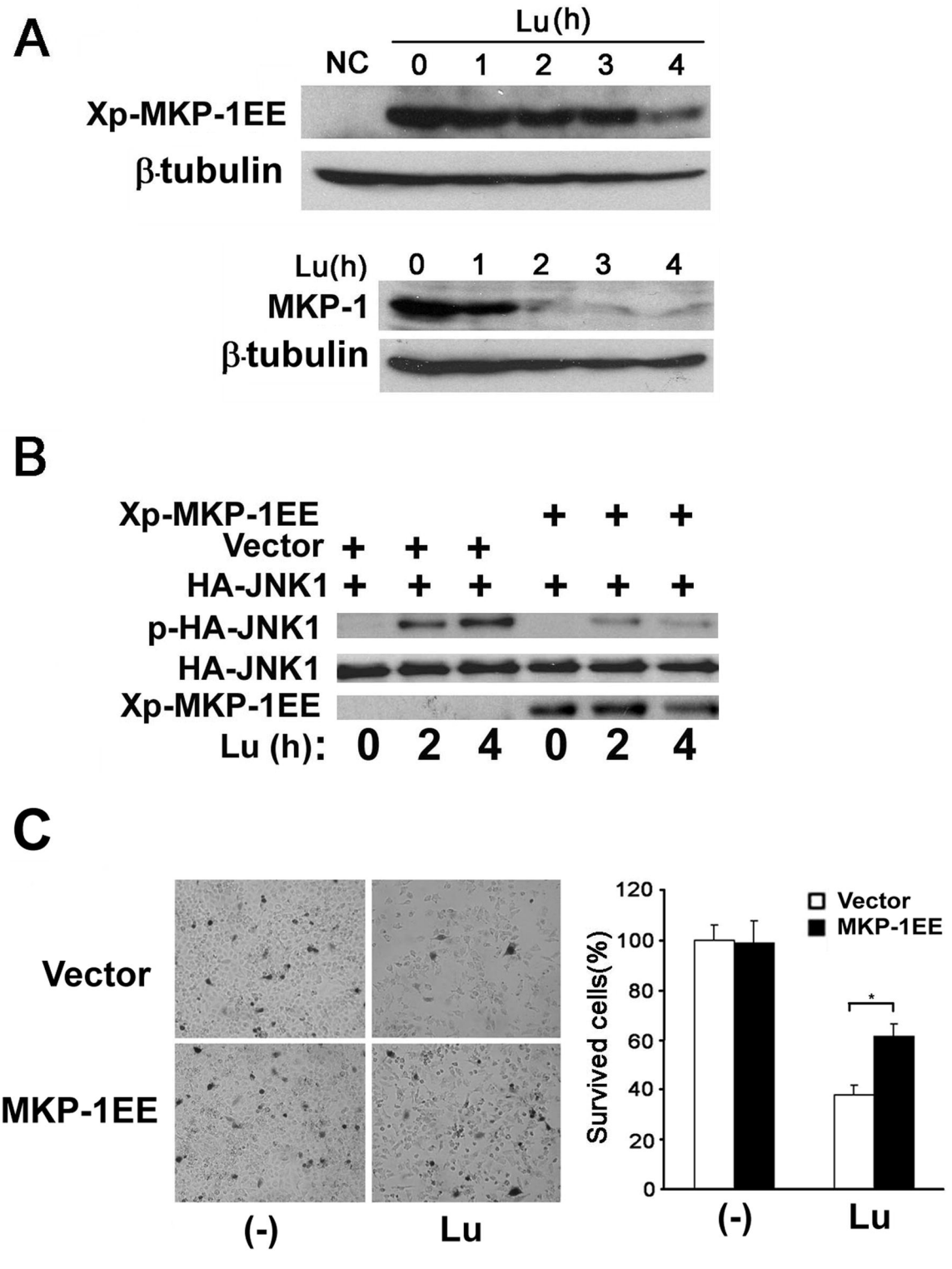
Fig. 5


A
Lu(h)
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β-tubulin
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Xp-MKP-1EE + + +
Vector + + +
HA-JNK1 + + + + + +
p-HA-JNK1
HA-JNK1
Xp-MKP-1EE
Lu (h): 0 2 4 0 2 4
C
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MKP-1EE
(-) Lu
Survived cells(%)
120
100
80
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40
20
0
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MKP-1EE
*
(-) Lu

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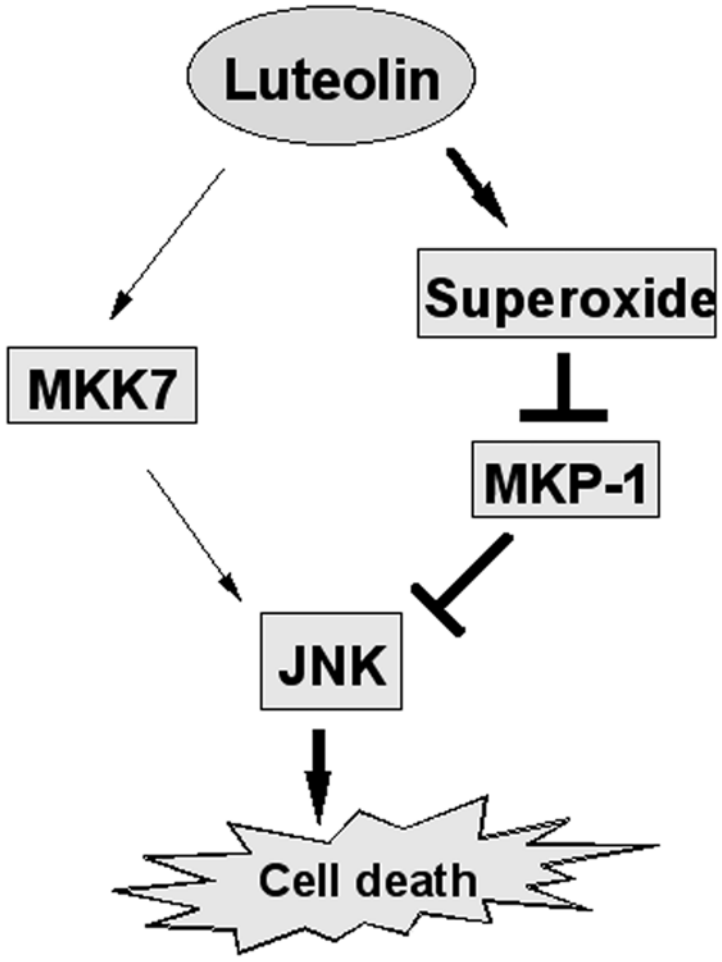
Fig-6



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