

# A new insight into the apoptotic effect of nitidine chloride targeting Checkpoint kinase 2 in human cervical cancer *in vitro*

Hye-Jeong Kwon,<sup>1,#</sup> Lee-Han Kim,<sup>2,#</sup> Chi-Hyun Ahn,<sup>1</sup> In-Hyoung Yang,<sup>1</sup> Kyoung-Ok Hong,<sup>1</sup> Seong Doo Hong,<sup>1</sup> Ji-Ae Shin<sup>1,\*</sup> and Sung-Dae Cho<sup>1,\*</sup>

<sup>1</sup>Department of Oral Pathology, School of Dentistry and Dental Research Institute, Seoul National University, 101 Daehak-ro, Jongno-gu, Seoul 03080, Republic of Korea

<sup>2</sup>Department of Microbiology, Institute for Immunology and Immunological Diseases, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

(Received 4 April, 2019; Accepted 14 August, 2019; Published online 8 October, 2019)

**Nitidine chloride (NC), a natural, bioactive, phytochemical alkaloid derived from the roots of *Zanthoxylum nitidum*, has been reported to exhibit anti-tumor activity against various types of cancer. However, the potential therapeutic role of NC in human cervical cancer has not yet been studied. We are the first to report that NC acts as a potential apoptosis-inducing agent for human cervical cancer *in vitro*. NC treatment of human cervical cancer cell lines induced caspase-mediated apoptosis, thereby reducing cell viability. Phospho-kinase proteome profiling using a human phospho-kinase array revealed that NC treatment phosphorylated Checkpoint kinase 2 (Chk2) at Thr68, which activates Chk2 in both cell lines. We also found that NC significantly affected the p53/Bim signaling axis, which was accompanied by mitochondrial membrane depolarization and cytochrome c release from the mitochondria into the cytosol. In addition, NC profoundly increased phosphorylation of the histone variant H2AX at Ser139, a typical marker of DNA damage. Taken together, these results provide *in vitro* evidence that NC can increase Chk2 activation, thereby acting as an attractive cell death inducer for treatment of human cervical cancer.**

**Key Words:** cervical cancer, nitidine chloride, Chk2 activation, apoptosis

Nitidine chloride (NC) is a natural, bioactive, chemical alkaloid isolated from the roots of *Zanthoxylum nitidum*, a traditional herbal medicine. Previous studies have shown that NC inhibits the production of various cytokines by modulating the nuclear factor kappa-B (NF- $\kappa$ B) and mitogen-activated protein kinase signaling pathways in lipopolysaccharide-stimulated RAW 264.7 macrophage cells,<sup>(1)</sup> and that it suppresses receptor activator NF- $\kappa$ B ligand-induced osteoclastogenesis *in vitro* and osteolysis *in vivo*.<sup>(2)</sup> In cancer biology, NC showed potent anti-tumor properties in a wide range of cancers by inhibiting cell proliferation and inducing cell cycle arrest or apoptosis through dephosphorylation of Akt or extracellular signal-regulated kinase.<sup>(3–5)</sup> It has also been found that the inhibitory effect of NC on the signaling pathway of signal transducer and activator of transcription 3 (STAT3) contributes to inhibition of neovascularization and tumorigenesis by vascular endothelial growth factor.<sup>(6,7)</sup> Recently, our team found that the biological action of NC plays a crucial role in inducing apoptosis through inhibition of p-STAT3 or myeloid cell leukemia-1 in oral cancer.<sup>(8,9)</sup> However, despite a variety of studies on the potential biological role of NC, it remains unclear whether it has potential as a chemotherapeutic drug candidate for cervical cancer treatment.

Checkpoint kinase 2 (Chk2), an essential signal transducer upon DNA damage, is mainly phosphorylated and activated by ataxia telangiectasia mutated serine/threonine kinase (ATM) on the priming site Thr68, which leads to phosphorylation of cellular substrates involved in DNA repair and apoptosis.<sup>(10,11)</sup> In addition to the role of Chk2 in DNA damage, Chk2 activation is necessary to maintain chromosomal stability by phosphorylating the tumor suppressor Brca1 during mitosis.<sup>(12)</sup> The loss of *Chk2* by mutation, deletion, or epigenetic silencing has been associated with risk of tumor formation and chemoresistance,<sup>(12–14)</sup> suggesting that Chk2 is a tumor suppressor. Accumulated data suggest that natural compounds can increase Chk2 phosphorylation, resulting in DNA damage-induced apoptosis or G2 phase arrest, apparently through inhibition of topoisomerase II activity.<sup>(15,16)</sup> Several natural compounds were associated with cell cycle arrest via the activated ATM/Chk2 signaling pathway in the presence or absence of p53 activation.<sup>(17,18)</sup> Therefore, it is worthwhile to investigate the correlation between NC and Chk2 in cervical cancer.

In the present study, we clarified the mechanism underlying the apoptotic activity of NC *in vitro* and explored the potential of NC as an anticancer drug candidate for cervical cancer.

## Materials and Methods

**Cell culture and chemical treatment.** Hep-2 and KB cells were obtained from Kyungpook National University (Daegu, Korea) and the American Type Culture Collection (Manassas, VA), respectively. Cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS) for Hep-2 cells or 5% FBS for KB cells and 1% penicillin/streptomycin in a 5% CO<sub>2</sub> incubator at 37°C. When the cells reached 50–60% confluence, they were treated with NC (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1%.

**Measurement of cell viability.** The trypan blue exclusion assay was used to measure the effect of NC on cell viability. Cells were stained with 0.4% trypan blue solution (Gibco, Paisley, UK), and viable cells were counted with a hemocytometer. All experiments were performed three times, with triplicates in each independent experiment.

<sup>#</sup>These authors contributed equally as first authors.

\*To whom correspondence should be addressed.

E-mail: efiwdsc@snu.ac.kr (S-D C); sky21sm@snu.ac.kr (J-A S)

**Live/dead assay.** The cytotoxicity of NC was examined using a Live/Dead & Viability/Cytotoxicity Kit (Life Technologies, Grand Island, NY). Briefly, cells were stained with 2  $\mu\text{M}$  Calcein-AM and 4  $\mu\text{M}$  ethidium homodimer-1 and then incubated for 30 min at room temperature (RT). Live (green fluorescence) and dead (red fluorescence) cells were visualized under a fluorescence microscope (Leica DMI8, Wetzlar, Germany) with appropriate excitation and emission filters.

**4'-6-Diamidino-2-phenylindole staining.** Cells were stained with 4'-6-diamidino-2-phenylindole (DAPI) solution (Sigma-Aldrich) to confirm the presence of nuclear morphological changes associated with apoptotic cells. Briefly, cells were fixed in 100% ethanol overnight at  $-20^{\circ}\text{C}$ , deposited on slides, and stained with DAPI fluorescent dye (2  $\mu\text{g}/\text{ml}$ ). A fluorescence microscope was used to observe the morphological characteristics of apoptotic cells: nuclear condensation and fragmentation.

**Western blotting.** Whole cell lysates were extracted with RIPA lysis buffer (EMD Millipore, Billerica, CA) containing phosphatase inhibitor and protease inhibitor cocktail. Protein concentrations of whole cell lysates were measured using a DC Protein Assay Kit (Bio-Rad Laboratories, Madison, WI). After normalization, equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immuno-Blot PVDF membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween20 for 2 h at RT, incubated with specific primary antibody overnight at  $4^{\circ}\text{C}$ , and finally probed with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at RT. Antibodies that detect cleaved caspase 3, cleaved poly(ADP-ribose) polymerase (PARP), p-Chk2 (Thr68), Chk2,  $\gamma\text{H2AX}$ , H2AX, Bim, Bax, and Puma were purchased from Cell Signaling Technology, Inc. (Charlottesville, VA). Actin and  $\alpha$ -tubulin antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). COX4 antibody was purchased from Abcam (Cambridge, UK). Antibodies against cytochrome c and p53 were obtained from BD Biosciences (San Diego, CA) and Calbiochem (San Diego, CA), respectively. Protein bands were immunoreacted with ECL solution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then visualized by an ImageQuant LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ) or X-ray film.

**Human phospho-kinase proteome profiling.** Cells were treated with 10  $\mu\text{M}$  NC for 3 h, and then phospho-kinase proteome profiling was performed using a Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN), which can detect the phosphorylation levels of 43 kinases, according to the manufacturer's protocol. Briefly, the membrane was blocked with array buffer for 1 h at RT. The cell lysate was then added and kept on a rocking platform shaker overnight at  $4^{\circ}\text{C}$ . After washing with 1 $\times$  washing buffer, the membrane was incubated with antibody cocktails at RT for 2 h and then incubated with streptavidin-HRP for 30 min at RT. Capture spots corresponding to the amount of kinase protein bound were detected with 1 ml of Chemi reagent mix.

**Immunofluorescence staining.** Cells were seeded on 4-well culture plates and treated with DMSO or 10  $\mu\text{M}$  NC. After NC treatment, cells were fixed and permeabilized with cytofix/cytoperm solution (BD Bioscience, San Diego, CA) for 1 h at  $4^{\circ}\text{C}$ . Cells were blocked with 1% bovine serum albumin in phosphate buffered saline (PBS) for 1 h at RT and incubated overnight at  $4^{\circ}\text{C}$  with antibodies against p-Chk2 (Thr68) or  $\gamma\text{H2AX}$ . Cells were then exposed to fluorescein-5-isothiocyanate (FITC)-conjugated secondary antibody for 1 h at RT and visualized using a fluorescence microscope equipped with filters suitable for DAPI and FITC dyes.

#### **Preparation of cytosolic and mitochondrial fractions.**

Cytosolic and mitochondrial fractions were isolated using a Mitochondria/Cytosol Fractionation Kit (Abcam). Briefly, cells were washed with ice-cold PBS, and the cell pellet was resuspended in 1 $\times$  cytosol extraction buffer mix containing DTT and

protease inhibitor for 10 min on ice. After centrifugation at 13,000 rpm for 15 min at  $4^{\circ}\text{C}$ , the supernatants containing the cytosolic proteins were collected, and the pellets were resuspended in mitochondrial extraction buffer mix. The supernatant containing mitochondrial proteins was collected from a final centrifugation.

**Mitochondrial membrane potential assay.** Mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) was measured by flow cytometry using a lipophilic fluorescent dye, JC-1 (BD Biosciences). Cells were harvested by trypsinization, washed with PBS, and then pelleted by centrifugation at 3,500 rpm for 5 min. The pellets were resuspended in 1 $\times$  JC-1 working solution and incubated at  $37^{\circ}\text{C}$  for 30 min in the dark. The stained cells were washed with 1 $\times$  assay buffer and collected by centrifugation at 3,500 rpm for 5 min. After removing the supernatant, they were resuspended in 1 $\times$  assay buffer. Cells were then transferred to FACS tubes and analyzed with FACSCalibur (BD Biosciences). At least 10,000 events per sample were counted.

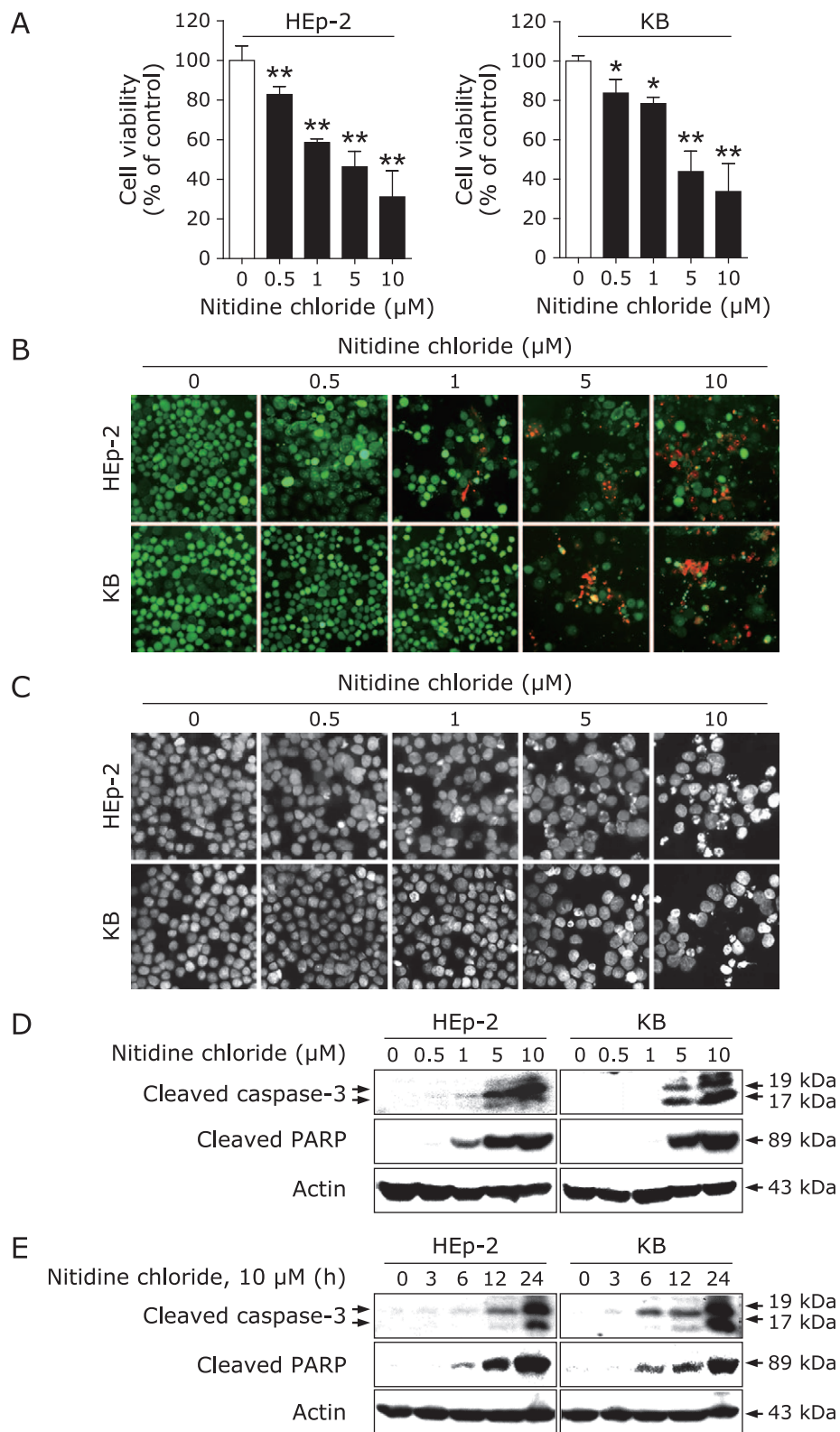
**Statistical analysis.** All statistical analyses of the results were performed using SPSS ver. 22.0 (SPSS Inc. Chicago, IL). One-way ANOVA with Tukey's post hoc test was applied to multiple comparisons to determine the significance of differences between the control and treatment groups. A  $p$  value less than 0.05 was considered statistically significant.

## **Results**

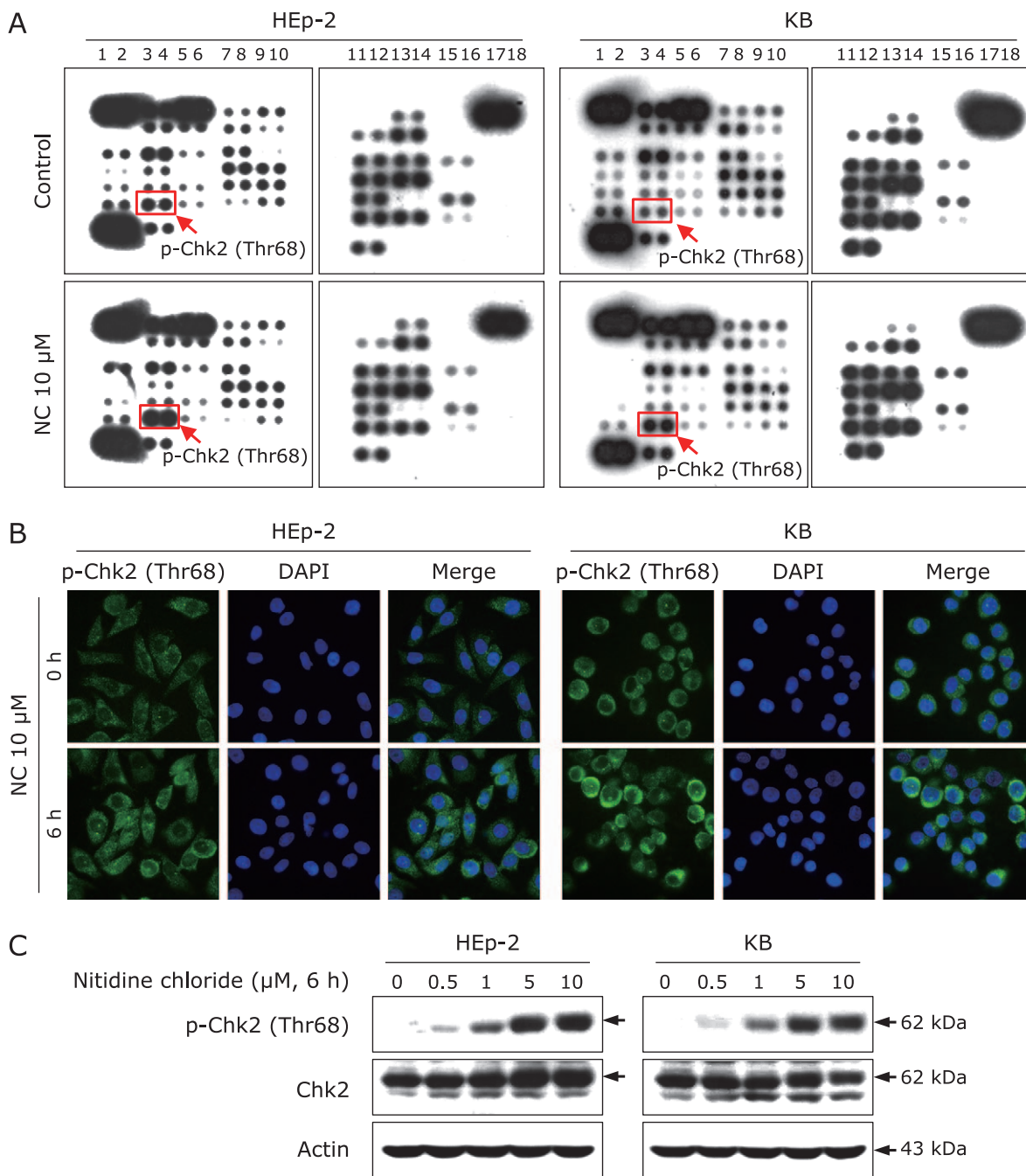
**NC produces cytotoxic effects by inducing caspase-mediated apoptosis in human cervical cancer cell lines.** To investigate the cytotoxic effect of NC on human cervical cancer cell lines, trypan blue exclusion assays were performed in HEP-2 and KB cell lines treated with NC at concentrations of 0.5–10  $\mu\text{M}$  for 24 h. As shown in Fig. 1A, NC significantly reduced the viability of both cell lines, with  $\text{IC}_{50}$  values of 3.9  $\mu\text{M}$  and 4.7  $\mu\text{M}$ , respectively. The live/dead assay showed an increase in cells stained with red fluorescence, suggesting membrane-damaged dead cells (Fig. 1B). To investigate the possible potential toxicity of NC, we also monitored the effect of NC on cell viability of human oral keratinocyte (HOK). As shown in Supplemental Fig. 1\*, NC has less growth-inhibitory effect on HOK compared to two cervical cancer cell lines. To investigate whether the NC-induced cytotoxic effect was due to apoptosis, we looked for characteristics of apoptosis such as chromatin condensation and DNA fragmentation using DAPI, a fluorescent DNA-binding dye. Those results showed an increase in apoptosis in the NC-treated cells compared with DMSO-treated cells (Fig. 1C). We also found that NC increased the cleavage of caspase-3 or PARP, an apoptotic index, in a concentration- and time-dependent manner (Fig. 1D and E). These results suggest that the cytotoxic effect of NC in human cervical cancer cell lines might be related to caspase-mediated apoptosis.

**NC causes Chk2 activation by phosphorylating Thr68 in human cervical cancer cell lines.** To explore the mechanism of NC-induced apoptotic activity, we analyzed the phosphorylation profiles of 43 kinases using a human phospho-kinase array. As shown in Fig. 2A, NC treatment markedly induced phosphorylation of Chk2 at Thr68 compared with the control group. To confirm that result, we performed immunofluorescence staining using a p-Chk2 antibody specific to its activation site, Thr68. We found that the phosphorylation status of Chk2 increased in both cell lines after 6 h of NC treatment (Fig. 2B). Consistent with these results, western blot analysis showed that NC treatment induced high expression of p-Chk2 (Thr68) in a concentration-dependent manner (Fig. 2C). In contrast, we did not find an increase in total Chk2 in NC-treated cells. Thus, phosphorylation of the Thr68 site might be necessary for NC-induced cell death in human cervical cancer cell lines.

\*See online. <https://doi.org/10.3164/jcfn.19-28>



**Fig. 1.** Effects of NC on cell viability and apoptosis in human cervical cancer cell lines. HEP-2 and KB cells were treated with DMSO or the indicated concentration of NC (0.5, 1, 5, or 10  $\mu$ M) for 24 h. (A) Cell viability was evaluated using a trypan blue exclusion assay. The graphs express the mean  $\pm$  SD of triplicate experiments (\* $p$ <0.01, \*\* $p$ <0.001). (B) Live (green fluorescence) and dead (red fluorescence) cells were observed under a fluorescence microscope. Representative images are displayed (magnification,  $\times$ 200). (C) Cells stained with DAPI solution were observed under a fluorescence microscope. Representative images are displayed (magnification,  $\times$ 400). (D, E) Protein levels of cleaved caspase-3 and PARP were determined by Western blotting. Actin was used as a loading control. See color figure in the on-line version.



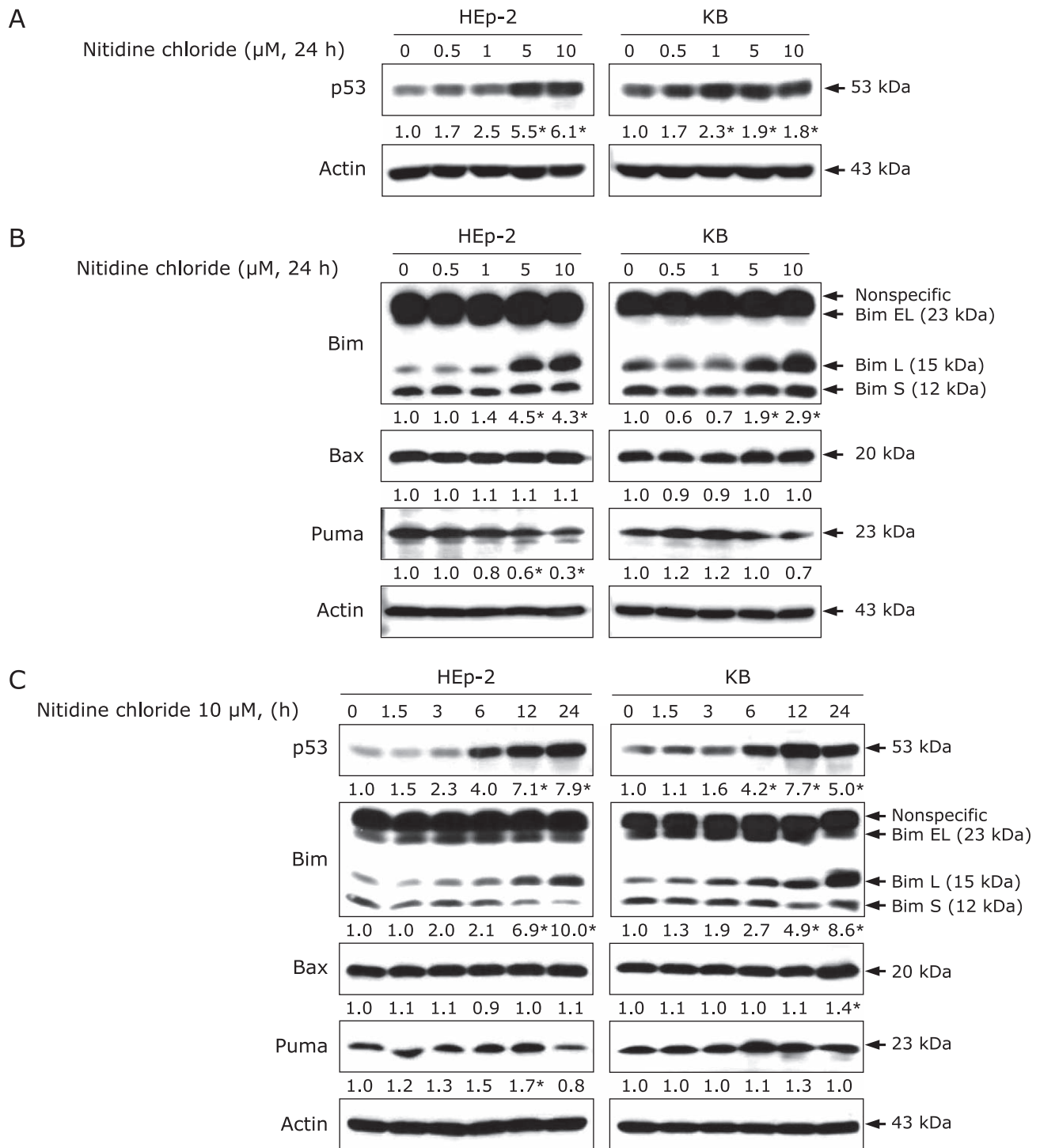
**Fig. 2.** Effect of NC on Chk2 activation in human cervical cancer cell lines. (A) HEp-2 and KB cells were treated with DMSO or 10 μM NC for 3 h, and the cell lysates were analyzed using the human phospho-kinase array. (B) Immunofluorescence staining of p-Chk2 (Thr68) in HEp-2 and KB cells treated with NC for 6 h. Representative images of staining for p-Chk2 (Thr68, green) and counterstaining with DAPI (blue) are shown. The merge panel combines the two images (magnification, ×400). (C) HEp-2 and KB cells were treated with the indicated concentrations of NC for 6 h. Protein levels of p-Chk2 (Thr68) and Chk2 were determined by Western blotting. See color figure in the on-line version.

**NC affects the p53/Bim signaling pathway in human cervical cancer cell lines.** Next, we used western blotting to assess the expression level of p53 to clarify the association between p53 and Chk2-related apoptosis induced by NC. NC treatment resulted in a significant increase in p53 protein expression in both cell lines (Fig. 3A). We investigated three Bcl-2 family proteins (Bim, Bax, and Puma) that are downstream molecules of the p53 protein, and we found an increase in Bim protein expression after NC treatment. The protein expression patterns of Bax and Puma did not increase significantly (Fig. 3B). NC

increased p53 and Bim at similar time points in both cell lines after treatment for 6–24 h, but minimal effects were observed before that (Fig. 3C). Thus, NC might induce p53/Bim signaling pathways that could be involved in NC-induced apoptosis in human cervical cancer cell lines.

**NC leads to loss of  $\Delta\Psi_m$ , which causes cytochrome c release into the cytosol in human cervical cancer cell lines.**

Because our data showed that NC induced the p53/Bim signaling pathway and caspase-3 activation, we assumed that, during NC treatment, the p53/Bim signaling pathway regulates mitochondrial

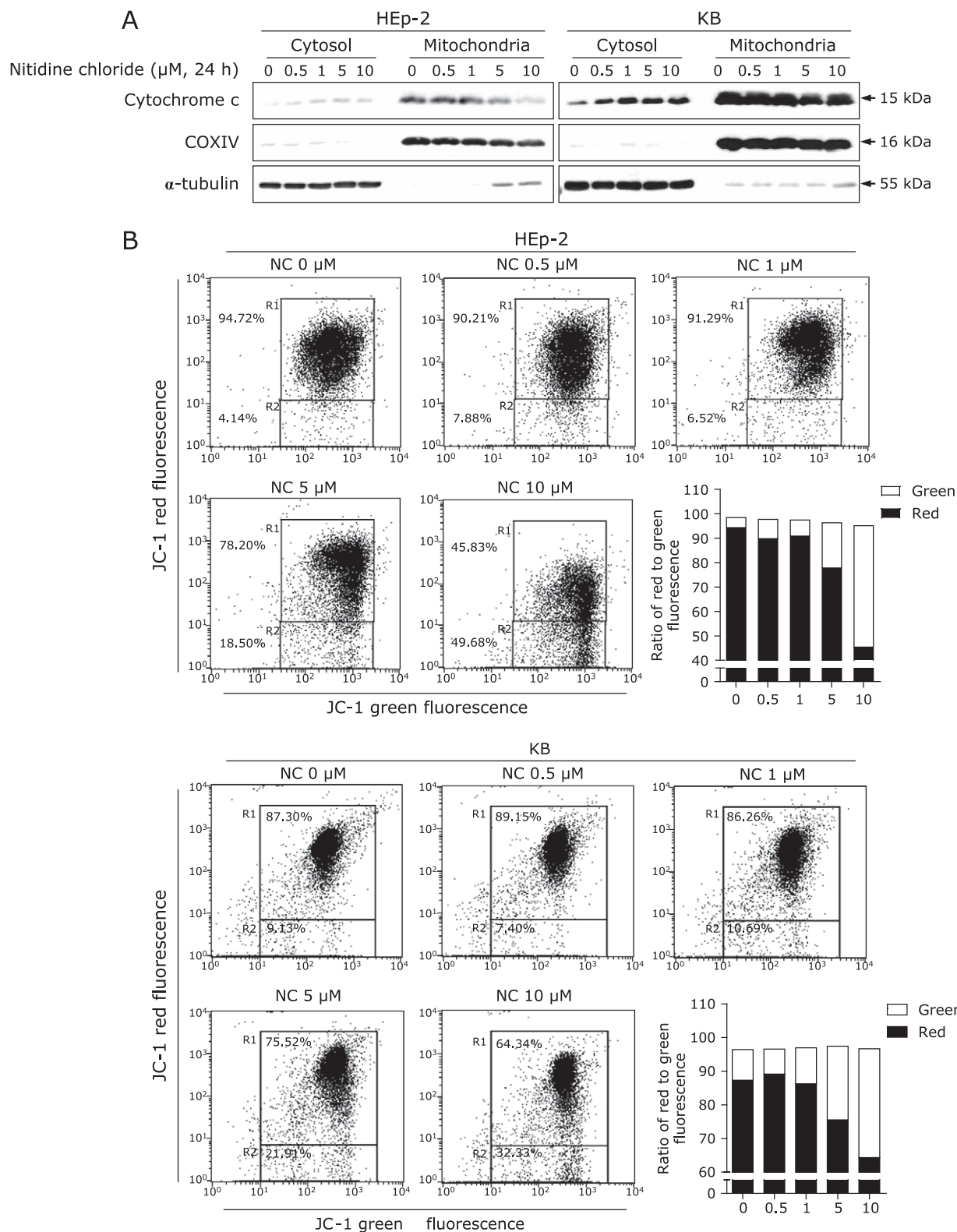


**Fig. 3.** The effects of NC on p53 and its downstream targets in human cervical cancer cell lines. (A, B) HEp-2 and KB cells were treated with DMSO or the indicated concentration of NC for 24 h. Protein levels of p53, Bim, Bax, and Puma were analyzed by western blotting. (C) HEp-2 and KB cells were treated with 10  $\mu\text{M}$  NC for the indicated time points. Levels of p53, Bim, Bax, and Puma proteins were detected by western blotting. Values represent the mean of three independent experiments (\* $p < 0.05$ ).

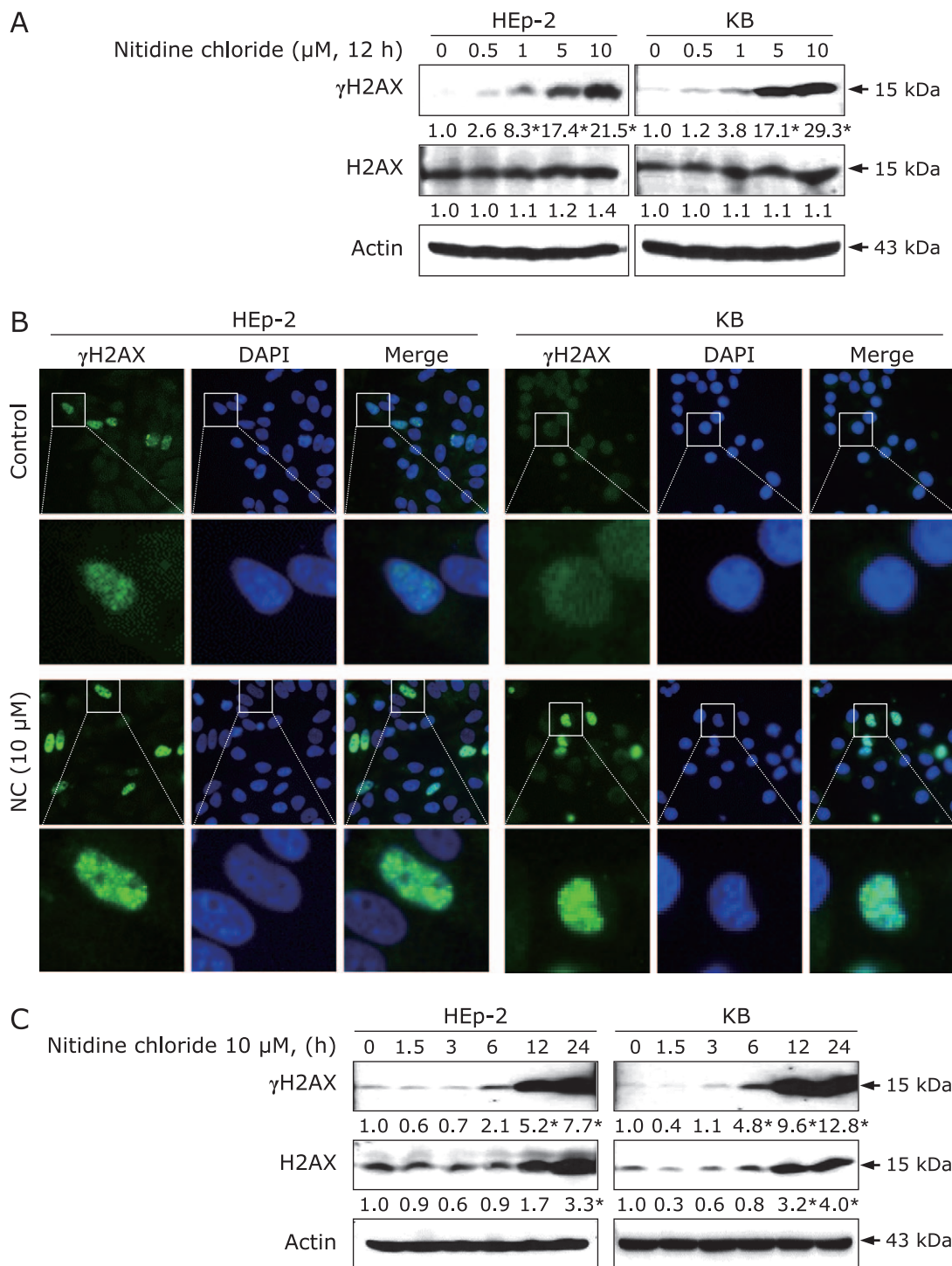
membrane depolarization, which would cause an efflux of cytochrome c from mitochondria. As shown in Fig. 4A, cytochrome c was released from mitochondria into the cytosol in both cell lines in response to NC treatment. We next observed  $\Delta\Psi_m$  using JC-1, a fluorescence-based, mitochondria-specific dye that aggregates in the mitochondria of healthy cells (red fluorescence) and is present

as a monomer in the cytosol in the presence of apoptotic signals (green fluorescence). The results show that the proportion of cells with loss of  $\Delta\Psi_m$  was increased by NC treatment (Fig. 4B), suggesting that NC induced apoptosis through mitochondrial membrane depolarization.





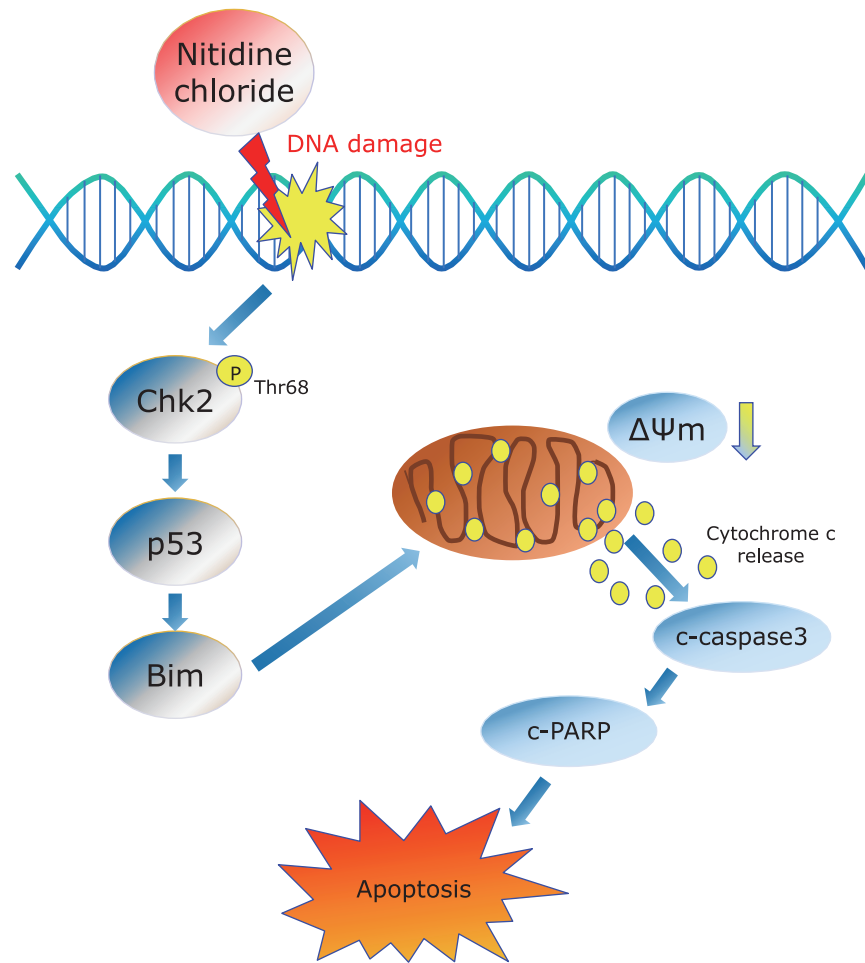
**Fig. 4.** The effects of NC on  $\Delta\Psi_m$  and cytochrome c release in human cervical cancer cell lines. (A) Cytochrome c release into the cytosol was assessed using cytosolic and mitochondrial fractions. COX IV and  $\alpha$ -tubulin were used as specific markers for mitochondria and cytosol, respectively. The experiments were performed twice. (B) The effect of NC on  $\Delta\Psi_m$  was evaluated by JC-1 staining. A representative R1 (upper region)/R2 (lower region) profile with red/green fluorescence is shown. The upper region represents healthy cells with normal  $\Delta\Psi_m$ , and the lower region represents apoptotic cells with depolarized  $\Delta\Psi_m$ . Bar graphs show the ratio of red to green fluorescence. The experiments were performed twice. See color figure in the on-line version.



**Fig. 5.** Effect of NC on  $\gamma\text{H2AX}$  accumulation in human cervical cancer cell lines. HEp-2 and KB cells were treated with DMSO or the indicated concentration of NC for 12 h. (A) The expression levels of  $\gamma\text{H2AX}$  and H2AX were detected using western blotting. Values represent the mean of three independent experiments ( $*p < 0.05$ ). (B) Immunofluorescence staining of  $\gamma\text{H2AX}$  in HEp-2 and KB cells. Representative images of the staining for  $\gamma\text{H2AX}$  (green) and counterstaining with DAPI (blue) are shown. The merge panel combines the two images (magnification,  $\times 400$ ). Enlarged views of the white line box areas are shown (lower row) for better visualization of  $\gamma\text{H2AX}$  foci. (C) HEp-2 and KB cells were treated with 10  $\mu\text{M}$  NC for the indicated times. Levels of  $\gamma\text{H2AX}$  and H2AX were detected by western blotting. Values represent the mean of three independent experiments ( $*p < 0.05$ ). See color figure in the on-line version.

**NC enhances the accumulation of  $\gamma\text{H2AX}$  in human cervical cancer cell lines.** Because the DNA damage response can lead to induction of apoptosis, we investigated the possibility that NC elicits the phosphorylation of histone variant H2AX at Ser139

(called  $\gamma\text{H2AX}$ ), a typical marker used to examine DNA damage. As shown in Fig. 5A, NC significantly increased  $\gamma\text{H2AX}$  in a concentration-dependent manner, but we found no significant changes in total H2AX level. To further assess the formation of



**Fig. 6.** A proposed working model of the molecular mechanisms underlying the pro-apoptotic action of NC through Chk2 activation in human cervical cancer cell lines. NC-induced apoptosis is mediated by Chk2 activation, which promotes p53/Bim signaling that leads to depolarization of mitochondrial membrane potential.

$\gamma$ H2AX in response to DNA damage after NC treatment, we quantified  $\gamma$ H2AX foci in the nucleus and found that NC increased the number of  $\gamma$ H2AX foci in the nucleus (Fig. 5B). We also observed that  $\gamma$ H2AX accumulated in a time-dependent manner after NC treatment (Fig. 5C). Therefore, the apoptotic effect of NC in human cervical cancer cell lines might somehow be a consequence of the accumulation of  $\gamma$ H2AX. A summary of the working model by which NC has pro-apoptotic action through Chk2 activation in human cervical cancer cell lines is illustrated in Fig. 6.

## Discussion

Cervical cancer is the fourth most frequently diagnosed female cancer worldwide, with 570,000 new cases occurring in 2018, accounting for 6.6% of all female cancer cases.<sup>(19)</sup> Concurrent chemo-radiation therapy is the current standard of care for patients with locally advanced cervical cancer, and targeted therapies using tyrosine kinase inhibitors have been consistently attempted to manage metastatic or recurrent patients with low response rates.<sup>(20)</sup> Even though this disease is generally preventable, the overall prognosis for women with metastatic or recurrent types is poor.<sup>(21)</sup> Here, we shed light on the *in vitro* anti-cancer activity of NC for treatment of human cervical cancer. NC inhibited cell viability and induced apoptosis in two types of cervical cancer

cell lines, Hep-2 and KB (Fig. 1). This result suggests that NC, a naturally derived substance, exhibits anticancer activity in cervical cancer by causing apoptosis.

We used human phospho-kinase proteome profiling to identify the specific protein kinases that play a role in the apoptotic action of NC in human cervical cancer cell lines. The results revealed that phosphorylation of Chk2 at Thr68 is an important regulator of caspase-mediated apoptosis during NC treatment (Fig. 2). The serine/threonine kinase Chk2 is a key component that regulates an appropriate cellular response to DNA damage by phosphorylating substrates involved in DNA repair, cell cycle regulation, apoptosis, and p53 signaling.<sup>(11)</sup> In particular, p53 appears to be dependent on Chk2.<sup>(22)</sup> In the present study, we confirmed that p53 accumulation increased rapidly beginning 6 h after NC treatment (Fig. 3). A previous report indicated that p53 is also phosphorylated through Chk2 activation at several individual residues of the N-terminal or C-terminal domain, which is partially required for p53 acetylation in response to DNA damage.<sup>(23)</sup> Other cumulative data have shown that Chk2 activation leads to p53 stabilization and accumulation through mitigation of p53 degradation by MDM2/MDMX, eventually allowing p53 to bind to numerous target gene promoters.<sup>(24–26)</sup> Thus, p-Chk2 (Thr68) stimulated by NC treatment could contribute to p53 accumulation. p53 can act as a transcription factor that regulates the proapoptotic Bcl-2 family of genes, including Bim, Bax, and Puma.<sup>(27–30)</sup> Therefore, we further



investigated whether p53 accumulation leads to mitochondria-dependent apoptosis in cervical cancer cell lines. NC treatment significantly altered the expression level of Bim (Fig. 3). Consistent with our data, Han *et al.*<sup>(31)</sup> also demonstrated that the induced expression of p53 was highly associated with an increased level of Bim, resulting in the release of Bim from sequestration by anti-apoptotic Bcl-2 family proteins. Thus, NC could regulate the p53/Bim signaling axis in its apoptotic activity. Previously, several studies have reported that NC can act as a potent STAT3 signaling inhibitor.<sup>(6,7)</sup> Based on the results of human phosphokinase proteome profiling, NC did not affect STAT3 signaling in the KB cell line meaning that it was not commonly related to STAT3 signaling during NC-mediated apoptosis in human cervical cancer cell lines. However, further studies are needed in the future to determine the reason why NC has differential responses on STAT3 signaling in both cell lines because NC still decreases p-STAT3 (Y705) only in the HE-p2 cell line.

Mitochondria, energy-producing organelles, play a central role in the intrinsic apoptosis pathway. Mitochondrial dysfunction such as mitochondrial membrane depolarization triggers the release of an apoptogenic factor (for example, cytochrome c) that participates in caspase-dependent apoptosis.<sup>(32)</sup> Bcl-2 family proteins can cause mitochondrial outer membrane permeabilization to induce apoptosis.<sup>(33)</sup> Because we found that NC modulates the p53/Bim signaling axis, we investigated whether NC can affect mitochondrial membrane permeabilization. As expected, NC promoted mitochondrial membrane depolarization and subsequent release of cytochrome c into the cytosol (Fig. 4). Taken together, our results demonstrate the involvement of Bim in the mitochondrial response to p53 during NC-mediated apoptosis in human cervical cancer cell lines.

In response to a DNA double-strand break (DSB), the phosphorylation of histone variant H2AX at the Ser139 residue ( $\gamma$ H2AX) occurs.<sup>(34)</sup> Naturally derived compounds play a crucial role in inducing DNA damage through either increased DSBs or reduced DNA repair mechanisms. Safranal causes H2AX phosphorylation in hepatocellular carcinoma cell lines through inhibition of tyrosyl-DNA phosphodiesterase 1, a main contributor to the DNA repair machinery.<sup>(35)</sup> The new epipolythiodioxopiperazine derivative G226 inhibits the activity of DNA topoisomerase II and concurrently elevates the expression of  $\gamma$ H2AX, contributing to its cytotoxic effect on human cancer cell lines.<sup>(36)</sup>

## References

- 1 Wang Z, Jiang W, Zhang Z, Qian M, Du B. Nitidine chloride inhibits LPS-induced inflammatory cytokines production via MAPK and NF-kappaB pathway in RAW 264.7 cells. *J Ethnopharmacol* 2012; **144**: 145–150.
- 2 Liu Q, Wang T, Zhou L, *et al.* Nitidine chloride prevents OVX-induced bone loss via suppressing NFATc1-mediated osteoclast differentiation. *Sci Rep* 2016; **6**: 36662.
- 3 Li P, Yan S, Dong X, *et al.* Cell cycle arrest and apoptosis induction activity of nitidine chloride on acute myeloid leukemia cells. *Med Chem* 2018; **14**: 60–66.
- 4 Zhai H, Hu S, Liu T, *et al.* Nitidine chloride inhibits proliferation and induces apoptosis in colorectal cancer cells by suppressing the ERK signaling pathway. *Mol Med Rep* 2016; **13**: 2536–2542.
- 5 Fang Z, Tang Y, Jiao W, *et al.* Nitidine chloride induces apoptosis and inhibits tumor cell proliferation via suppressing ERK signaling pathway in renal cancer. *Food Chem Toxicol* 2014; **66**: 210–216.
- 6 Chen J, Wang J, Lin L, *et al.* Inhibition of STAT3 signaling pathway by nitidine chloride suppressed the angiogenesis and growth of human gastric cancer. *Mol Cancer Ther* 2012; **11**: 277–287.
- 7 Liao J, Xu T, Zheng JX, *et al.* Nitidine chloride inhibits hepatocellular carcinoma cell growth *in vivo* through the suppression of the JAK1/STAT3 signaling pathway. *Int J Mol Med* 2013; **32**: 79–84.
- 8 Kim LH, Khadka S, Shin JA, *et al.* Nitidine chloride acts as an apoptosis inducer in human oral cancer cells and a nude mouse xenograft model via inhibition of STAT3. *Oncotarget* 2017; **8**: 91306–91315.

In our previous study, we demonstrated that the apoptotic effect of oridonin in human oral cancer cell lines requires induction of  $\gamma$ H2AX.<sup>(37)</sup> As in those previous studies, we here found that  $\gamma$ H2AX level was greatly increased by NC treatment, and that  $\gamma$ H2AX was recruited to DNA break sites to form nuclear foci, suggesting that NC treatment could induce a DNA damage response in human cervical cancer cell lines (Fig. 5). DNA topoisomerases are essential nuclear enzymes that control topological DNA errors in replication, transcription, and recombination. They are affected by topoisomerase inhibitors, which induce DNA damage, cell cycle arrest, or apoptosis.<sup>(38)</sup> Recently, it was reported that topoisomerases could be targets of NC in cancer treatment, suggesting its function as a topoisomerase inhibitor.<sup>(39)</sup> Thus, we checked the mRNA levels of *topoisomerase 1* and *2*. The results showed that NC inhibit them only in KB cells, but not in HEp-2 cells (Supplemental Fig. 2\*). These results cautiously suggest that  $\gamma$ H2AX induction by NC might be caused by inhibition of topoisomerases in a cell context-dependent manner.

In conclusion, the present *in vitro* study indicates that NC plays an apoptotic role through Chk2 activation in human cervical cancer. These findings will help us to develop a new therapeutic option for patients with human cervical cancer.

## Acknowledgments

This study was funded by the Basic Science Research Program through the National Research Foundation of Korea (NRF) sponsored by the Ministry of Science, ICT, & Future Planning (2017R1D1A1B03029124 and 2017R1A2B2003491).

## Abbreviations

ATM	ataxia telangiectasia mutated
Chk2	Checkpoint kinase 2
$\Delta\Psi_m$	mitochondrial membrane potential
NC	nitidine chloride
NF- $\kappa$ B	nuclear factor kappa-B

## Conflict of Interest

No potential conflicts of interest were disclosed.

- 9 Yang IH, Jung W, Kim LH, *et al.* Nitidine chloride represses Mcl-1 protein via lysosomal degradation in oral squamous cell carcinoma. *J Oral Pathol Med* 2018; **47**: 823–829.
- 10 Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* 2003; **3**: 421–429.
- 11 Zannini L, Delia D, Buscemi G. CHK2 kinase in the DNA damage response and beyond. *J Mol Cell Biol* 2014; **6**: 442–457.
- 12 Stolz A, Ertych N, Bastians H. Tumor suppressor CHK2: regulator of DNA damage response and mediator of chromosomal stability. *Clin Cancer Res* 2011; **17**: 401–405.
- 13 Gogineni VR, Nalla AK, Gupta R, Dinh DH, Klopfenstein JD, Rao JS. Chk2-mediated G2/M cell cycle arrest maintains radiation resistance in malignant meningioma cells. *Cancer Lett* 2011; **313**: 64–75.
- 14 Craig AL, Hupp TR. The regulation of CHK2 in human cancer. *Oncogene* 2004; **23**: 8411–8418.
- 15 Liberio MS, Sadowski MC, Davis RA, *et al.* The ascidian natural product eusynstyelamide B is a novel topoisomerase II poison that induces DNA damage and growth arrest in prostate and breast cancer cells. *Oncotarget* 2015; **6**: 43944–43963.
- 16 Chien CM, Yang JC, Wu PH, *et al.* Phytochemical naphtho[1,2-b]furan-4,5-dione induced topoisomerase II-mediated DNA damage response in human non-small-cell lung cancer. *Phytomedicine* 2018; **54**: 109–119.
- 17 Nagasaka M, Hashimoto R, Inoue Y, *et al.* Anti-tumorigenic activity of chrysin from *Oroxylum indicum* via non-genotoxic p53 activation through the

\*See online. <https://doi.org/10.3164/jcfn.19-28>

- ATM-Chk2 pathway. *Molecules* 2018; **23**: E1394.
- 18 Tsai TC, Huang HP, Chang KT, Wang CJ, Chang YC. Anthocyanins from roselle extract arrest cell cycle G2/M phase transition via ATM/Chk pathway in p53-deficient leukemia HL-60 cells. *Environ Toxicol* 2017; **32**: 1290–1304.
  - 19 Cervical Cancer, World Health Organization. <https://www.who.int/cancer/prevention/diagnosis-screening/cervical-cancer/en/> Accessed 1 Apr 2019.
  - 20 Kumar L, Harish P, Malik PS, Khurana S. Chemotherapy and targeted therapy in the management of cervical cancer. *Curr Probl Cancer* 2018; **42**: 120–128.
  - 21 Cohen PA, Jhingran A, Oaknin A, Denny L. Cervical cancer. *Lancet* 2019; **393**: 169–182.
  - 22 Hirao A, Cheung A, Duncan G, *et al.* Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. *Mol Cell Biol* 2002; **22**: 6521–6532.
  - 23 Ou YH, Chung PH, Sun TP, Shieh SY. p53 C-terminal phosphorylation by CHK1 and CHK2 participates in the regulation of DNA-damage-induced C-terminal acetylation. *Mol Biol Cell* 2005; **16**: 1684–1695.
  - 24 Chehab NH, Malikzay A, Appel M, Halazonetis TD. Chk2/hCds1 functions as a DNA damage checkpoint in G<sub>1</sub> by stabilizing p53. *Genes Dev* 2000; **14**: 278–288.
  - 25 Chen L, Gilkes DM, Pan Y, Lane WS, Chen J. ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. *EMBO J* 2005; **24**: 3411–3422.
  - 26 Shadfan M, Lopez-Pajares V, Yuan ZM. MDM2 and MDMX: alone and together in regulation of p53. *Transl Cancer Res* 2012; **1**: 88–89.
  - 27 Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002; **2**: 594–604.
  - 28 Oda E, Ohki R, Murasawa H, *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000; **288**: 1053–1058.
  - 29 Yu J, Zhang L, Hwang PM, Kinzler KW, Vogelstein B. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* 2001; **7**: 673–682.
  - 30 Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* 2001; **7**: 683–694.
  - 31 Han J, Goldstein LA, Hou W, Gastman BR, Rabinowich H. Regulation of mitochondrial apoptotic events by p53-mediated disruption of complexes between antiapoptotic Bcl-2 members and Bim. *J Biol Chem* 2010; **285**: 22473–22483.
  - 32 Wang C, Youle RJ. The role of mitochondria in apoptosis. *Annu Rev Genet* 2009; **43**: 95–118.
  - 33 Yip KW, Reed JC. Bcl-2 family proteins and cancer. *Oncogene* 2008; **27**: 6398–6406.
  - 34 Podhorecka M, Skladanowski A, Bozko P. H2AX phosphorylation: its role in DNA damage response and cancer therapy. *J Nucleic Acids* 2010; **2010**: pii: 920161.
  - 35 Al-Hrout A, Chaiboonchoe A, Khraiweh B, *et al.* Safranal induces DNA double-strand breakage and ER-stress-mediated cell death in hepatocellular carcinoma cells. *Sci Rep* 2018; **8**: 16951.
  - 36 He PX, Zhang J, Che YS, He QJ, Chen Y, Ding J. G226, a new epipolythiodioxopiperazine derivative, triggers DNA damage and apoptosis in human cancer cells *in vitro* via ROS generation. *Acta Pharmacol Sin* 2014; **35**: 1546–1555.
  - 37 Yang IH, Shin JA, Lee KE, Kim J, Cho NP, Cho SD. Oridonin induces apoptosis in human oral cancer cells via phosphorylation of histone H2AX. *Eur J Oral Sci* 2017; **125**: 438–443.
  - 38 Jain CK, Majumder HK, Roychoudhury S. Natural compounds as anticancer agents targeting DNA topoisomerases. *Curr Genomics* 2017; **18**: 75–92.
  - 39 Liu LM, Xiong DD, Lin P, Yang H, Dang YW, Chen G. DNA topoisomerase 1 and 2A function as oncogenes in liver cancer and may be direct targets of nitidine chloride. *Int J Oncol* 2018; **53**: 1897–1912.