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CR108, a novel vitamin K3 derivative induces apoptosis and breast tumor inhibition by reactive oxygen species and mitochondrial dysfunction

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ABSTRACT

Vitamin K3 derivatives have been shown to exert anticancer activities. Here we show a novel vitamin K3 derivative (*S*)-2-(2-hydroxy-3-methylbutylthio)naphthalene-1,4-dione, which is named as CR108 that induces apoptosis and tumor inhibition through reactive oxygen species (ROS) and mitochondrial dysfunction in human breast cancer. CR108 is more effective on the breast cancer cell death than other vitamin K3 derivatives. Moreover, CR108 induced apoptosis in both the non-HER-2-overexpressed MCF-7 and HER-2-overexpressed BT-474 breast cancer cells. CR108 caused the loss of mitochondrial membrane potential, cytochrome c released from mitochondria to cytosol, and cleaved PARP proteins for apoptosis induction. CR108 markedly increased ROS levels in breast cancer cells. N-acetylcysteine (NAC), a general ROS scavenger, completely blocked the CR108-induced ROS levels, mitochondrial dysfunction and apoptosis. Interestingly, CR108 increased the phosphorylation of p38 MAP kinase but conversely inhibited the survivin protein expression. NAC treatment prevented the activation of p38 MAP kinase and rescued the survivin protein levels. SB202190, a specific p38 MAP kinase inhibitor, recovered the survivin protein levels and attenuated the cytotoxicity of CR108-treated cells. Furthermore, CR108 inhibited the xenografted human breast tumor growth in nude mice. Together, we demonstrate that CR108 is a novel vitamin K3 derivative that induces apoptosis and tumor inhibition by ROS production and mitochondrial dysfunction and associates with the phosphorylation of p38 MAP kinase and the inhibition of survivin in the human breast cancer.

Introduction

Breast cancer is one of the most-often causes and occurrences that leads to the mortality in women (Alberg and Singh, 2001; Barrett, 2010). The major treatment strategies for breast cancer patients include surgery, radiotherapy and chemotherapy. However, more effective anticancer agents for breast cancer therapy are highly desired. Vitamin K consists of a family of structurally similar fat-soluble 2-methyl-1,4-naphthoquinones, including phylloquinone (vitamin K1), menaquinone (vitamin K2), and menadione (vitamin K3) (Lamson and Plaza, 2003). Phllyoquinine and menaquinone are natural products but menadione is a synthetic analog of vitamin K (Lamson and Plaza, 2003). Vitamin K3 contains potent anticancer effects against various types of carcinoma cells, including breast, hepatic, oral cavity, pharyngeal, mammary and bladder (Lamson and Plaza, 2003).

Vitamin K3 has been shown to cause apoptosis in the human breast cancer cells via a mitochondria-related pathway (Akiyoshi et al., 2009). The main effect of vitamin K3 against cancer was due to oxidative stress via redox-cycling of the quinone to produce reactive oxygen species (ROS). Quinones can undergo either one-electron reduction to produce semiquinone radicals or two-electron reduction for resulting in hydroquinones (Dowd et al., 1995; Gant et al., 1988). Vitamin K3 induced single- and double-strand DNA breaks via ROS generation in breast cancer cells (Nutter et al., 1992). Combination with vitamin K3 and conventional chemotherapeutic agents showed the synergistic effects *in vivo* and *in vitro* (Gold, 1986; Liao et al., 2000; Nutter et al., 1992). Moreover, pretreatment with vitamin K3 before doxorubicin or mitomycin increased cytotoxicity to breast cancer cells (Tetef et al., 1995).

Several novel vitamin K3 derivatives have been shown anticancer activities. For example, vitamin K3 analogs induced selective tumor cytotoxicity in the neuroblastoma cells (Kitano et al., 2012). 2-(2-Hydroxy-ethylsulfanyl)-3-methyl-1,4-naphthoquinone (Cpd 5), a vitamin K3 analog, is a potent growth inhibitor for hepatoma cells (Nishikawa et al., 1995, 1999; Wang et al., 2000). Protein tyrosine phosphatases (PTPases) such as the dual specificity phosphatases CDC25A are potential target proteins for Cpd 5 (Tamura et al., 2000). Besides, 2-(2-hydroxy-ethylsulfanyl)-3-methyl-5-nitro-1,4-naphthoquinone (PD-37) and 2-(2-hydroxy-ethylsulfanyl)-3-methyl-

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5-acetylamino-1,4-naphthoquinone (PD-42) are synthesized vitamin K3 analogs, which have similar anticancer activities to Cpd 5 (Osada et al., 2001).

Survivin, a member of inhibitor of apoptosis proteins (IAP), regulates cell division and apoptosis (Ambrosini et al., 1997; Johnson and Howerth, 2004; Li et al., 1998). It has been shown that survivin is an anti-apoptotic protein by binding with caspases (Verhagen et al., 2000). Survivin is highly expressed in a variety of human cancers but not in normal adult cells (Ambrosini et al., 1997). The high levels of survivin proteins in tumors have been correlated with more aggressive behavior, decreased response to chemotherapeutic agents and shortened survival times (Johnson and Howerth, 2004).

The phosphorylation of p38 mitogen-activated protein (MAP) kinase can be activated by various stresses (Cano and Mahadevan, 1995; Nagata and Todokoro, 1999). It has been reported that hydrogen peroxide, osmotic shock, UV radiation, heat shock, X-ray radiation, and tumor necrosis factor-α can induce the activation of p38 MAP kinase (Raingeaud et al., 1995, 1996; Rouse et al., 1994). p38 MAP kinase is an important signaling mediator by ROS stimulation (Finkel, 1998; Torres and Forman, 2003). Several studies have shown that the activation of p38 MAP kinase induces apoptosis in cancer cells (Chao et al., 2004, 2007; Hsiao et al., 2007; Li and Bertino, 2002).

In this study, a novel vitamin K3 derivative, which is named as CR108, exerts more potency in the cell death than other vitamin K3 derivatives in the human breast cancer cells. CR108 induced apoptosis and tumor inhibition through ROS production and mitochondrial dysfunction. The apoptosis induction of CR108 is regulated by the activation of p38 MAP kinase and survivin inhibition.

Experimental procedures

Chemicals and antibodies

CR108 was synthesized as the supporting information (please see the supplementary information). 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI) and N-acetylcysteine (NAC) were purchased from Sigma Chemical Co. (St. Louis, MO). 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) and SB202190 were purchased from Calbiochem (San Diego, CA). 5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was purchased from AAT Bioquest, Inc. (Sunnyvale, CA). Anti-PARP, anti-phospho-p38 MAP kinase (Thr-180/Tyr-182), anti-cytochrome c, and anti-COX IV antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-bax (N-20), anti-p38 MAP kinase (C-20), anti-survivin (D-8), and anti-actin (I-19) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell lines and cell culture

H184B5F5/M10 was a human normal breast epithelial cell line. The MCF-7 cell line was derived from human breast adenocarcinoma of 69 years adult female. The MDA-MB-231 cell line was derived from human breast adenocarcinoma of 51 years adult female. The MDA-MB-231-luc2-tdTomato breast cancer cell line was created from MDA-MB-231 cells (PerkinElmer, Waltham, MA). BT-474 is a HER-2 (human epidermal growth factor receptor 2) overexpressed cell line that was isolated from a breast invasive ductal carcinoma. H184B5F5/M10 cells were cultured in α-MEM medium (Gibco, Life Technologies, Grand Island, NY). MCF-7 and BT-474 cells were cultured in RPMI-1640 medium (Gibco). MDA-MB-231-luc2-tdTomato cells were cultured in DMEM medium (Gibco). The complete medium was supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and L-glutamine (0.03%, w/v). These cells were maintained at 37 °C and 5% CO_2 in a humidified incubator (310/Thermo, Forma Scientific, Inc., Marietta, OH).

Cell viability assay

The human breast cells were plated in 96-well plates at a density of 1×10^4 cells/well for 16–20 h. Then the cells were treated with or without CR108 for 24 h in RPMI-1640 medium. After drug treatment, the cells were washed with phosphate-buffered saline (PBS), and were replaced fresh complete RPMI-1640 medium for 2 days. Thereafter, the medium was replaced and the cells were incubated with 0.5 mg/ml of MTT reagent in complete RPMI-1640 medium for 4 h. The viable cells converted MTT to formazan that generates a blue–purple color when dissolved in DMSO. The intensity of formazan was measured at 565 nm using a plate reader (VERSAmax, Molecular Dynamics, Sunnyvale, CA). The relative percentage of cell viability was calculated by dividing the absorbance of treatment by that of the control in each experiment.

Annexin V–PI staining

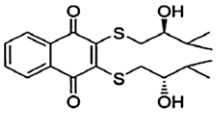
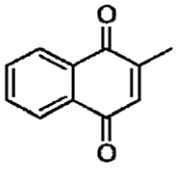
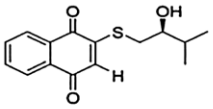
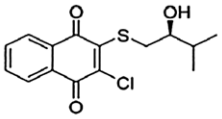
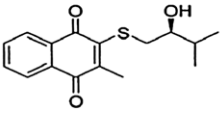
The level of apoptosis induced by CR108 was determined by Annexin V-propidium iodide (PI) analysis. The Annexin V–PI staining kit (BioVision, Mountain View, CA) was used to examine the cells by incubated with fluorescein isothiocyanate (FITC)-conjugated-Annexin V and PI according to the manufacturer's instruction. The cells showed Annexin V^+/PI^- and Annexin V^+/PI^+, which indicated at early and late apoptosis, respectively. The cells were cultured in 60-mm Petri dish at a density of 1×10^6 cells for 16–20 h. After treatment with or without CR108 for 24 h, the cells were washed with PBS. The cells were trypsinized and collected by centrifugation at 1500 rpm for 5 min. Thereafter, the cells were incubated with 500 μl Annexin V–PI labeling solution (containing 5 μl Annexin V-FITC and 5 μl PI in PBS) at room temperature in the dark for 5 min. To avoid cell aggregation, the cell solutions were filtered through a nylon mesh membrane (Becton-Dickinson, San Jose, CA). Finally, the samples were immediately analyzed by a flow cytometer (FACSCalibur, BD Biosciences). The percentage of Annexin V–PI staining cells was quantified from a minimum of 10,000 cells by CellQuest software (BD Biosciences).

Mitochondrial membrane potential assessment

Mitochondrial function was evaluated by the mitochondrial membrane potential ($\Delta\Psi m$). It was monitored using the mitochondrial sensitive probe $DiOC_6$ which accumulated in the mitochondrial matrix driven by the electrochemical gradient. The cells were cultured in 60-mm Petri dish at a density of 1×10^6 cells for 16–20 h. After treatment with or without CR108 for 24 h, the cells were washed with PBS. The cells were trypsinized and collected by centrifugation at 1500 rpm for 5 min. The cell pellets were fixed with ice-cold 70% ethanol overnight at −20 °C. After centrifugation at 1500 rpm for 5 min, the cell pellets were resuspended with 500 nM $DiOC_6$ in PBS, and then incubated at 37 °C for 30 min in the dark. Finally, the cell pellets were collected by centrifugation and resuspended in 1 ml ice-cold PBS. To avoid cell aggregation, the cell solutions were filtered through a nylon mesh membrane. The samples were analyzed by flow cytometer (FACSCalibur, BD Biosciences). The fluorescence intensity of $DiOC_6$ was quantified from a minimum of 10,000 cells by CellQuest software.

In addition to $DiOC_6$, we also used JC-1 to verify the mitochondrial membrane potential following treatment with CR108 in human breast cancer cells. JC-1 is capable of selectively entering mitochondria, and reversibly changes its color from green to orange as mitochondria membrane potentials increase (over values of about 80–100 mV). Briefly, the cells were cultured on coverslips, which were kept in 6-well plates at a density of 2×10^5 per well for 16–20 h. After treatment with or without 6 μM CR108 for 2 h, the cells were washed with PBS and fixed with 4% paraformaldehyde solution for 10 min at 37 °C. The cells were washed with PBS and incubated with 20 μM JC-1 for 30 min

A

VK3 derivatives	Chemical structure	Molecular Weight (g/mol)	VK3 structure
CR107		394.54	
CR108		276.35	
CR109		310.79	
CR110		290.37	

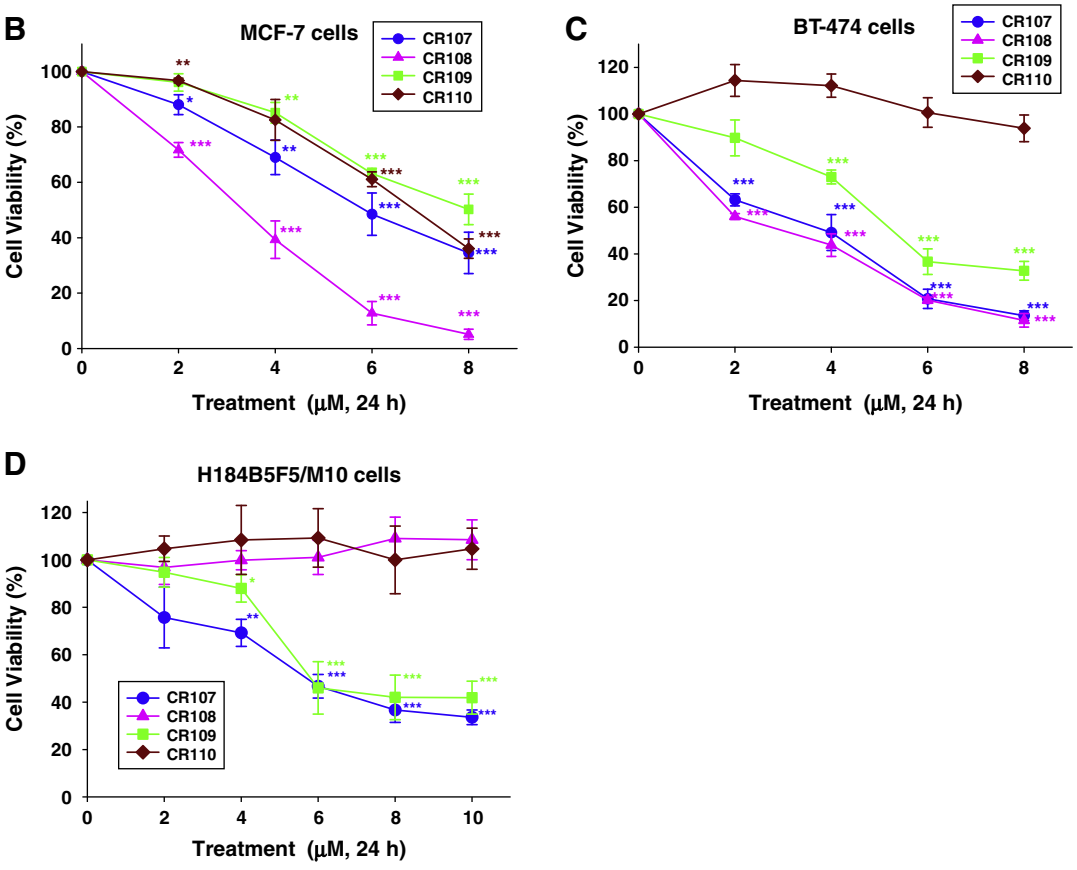


Fig. 1. CR108 is more effective on the cell death than other vitamin K3 derivatives in human breast cancer cells. (A) Chemical structure and molecular weight of vitamin K3 derivatives. (B) The non-Her-2 overexpressed MCF-7 cells were treated with or without 2–8 μM vitamin K derivatives for 24 h. (C) The HER-2-overexpressed BT-474 breast cancer cells were treated with 0–8 μM vitamin K derivatives for 24 h. (D) H184B5F5/M10 were treated with or without vitamin K derivatives (2–10 μM for 24 h). At the end of treatments, the cell viability was analyzed by MTT assays. The results were obtained from three to four experiments and the bar represents the mean ± S.E. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences between the control and vitamin K derivatives treated samples.

at 37 °C in the dark. Then the JC-1 working solution was removed from the cells and then washed with PBS. Finally, the cells were observed under a confocal microscope (Fluoview 300, OLYMPUS, Japan) or a laser scanning confocal microscope system (TCS-SP5-X AOBS, Leica, Germany). The property of JC-1 is due to the reversible formation of JC-1 aggregates upon mitochondria membrane polarization that causes shifts in emitted light from 530 nm (i.e., emission of J-monomeric form) to 590 nm (i.e., emission of J-aggregate form).

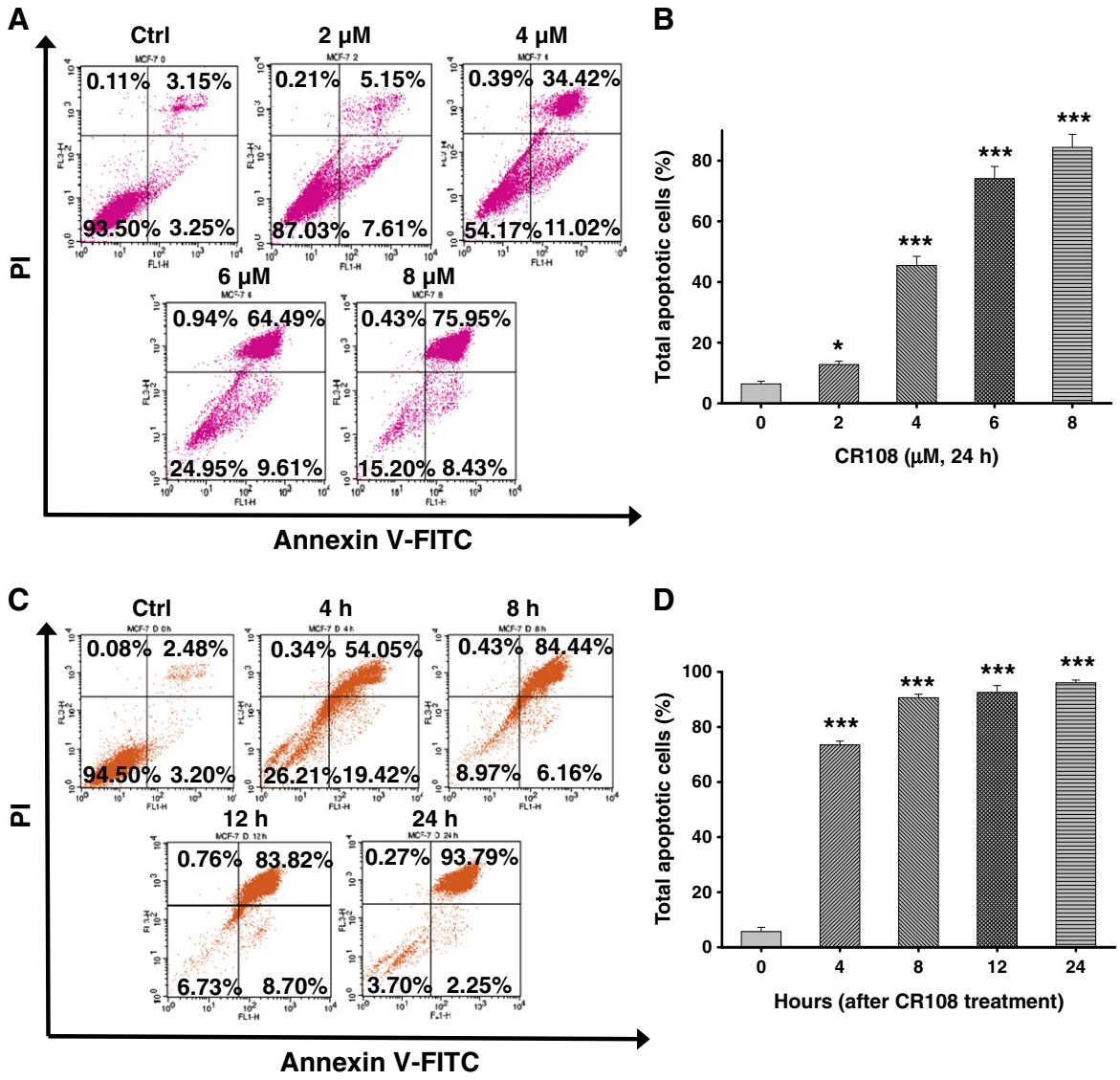


Fig. 2. CR108 induces apoptosis in human breast cancer cells. (A) MCF-7 cells were treated with 0–8 μM CR108 for 24 h. Apoptosis was determined by Annexin V-FITC/PI staining using flow cytometry analysis. Cells staining with Annexin V^+/PI^- are those undergoing early apoptosis (lower right), Annexin V^+/PI^+ stained cells are undergoing late apoptosis (upper right). The results were shown from one of three independent experiments with similar findings. (B) Populations of total apoptotic cells were quantified using CellQuest software. (C) MCF-7 cells were treated with 8 μM CR108 for 0, 4, 8, 12 and 24 h and stained with Annexin V–PI. Cells staining with Annexin V^+/PI^- are those undergoing early apoptosis (lower right), Annexin V^+/PI^+ stained cells are undergoing late apoptosis (upper right). The results were shown from one of four independent experiments with similar findings. (D) Populations of total apoptotic cells were quantified using CellQuest software. The bar represents the mean ± S.E. $^*p < 0.05$, and $^{***}p < 0.001$ indicate significant differences between the control and CR108 treated samples in total apoptosis.

Measurement of ROS production

2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) has been used as a detector of intracellular ROS. H_2DCF-DA can be converted by intracellular esterases to H_2DCF, which oxidized by ROS to highly fluorescent DCF. The cells were cultured in 60-mm Petri dish at a density of 1×10^6 cells for 16–20 h. After treatment with or without CR108 for 24 h, the cells were washed with PBS. The cells were trypsinized and collected by centrifugation at 1500 rpm for 5 min. The cell pellets were resuspended with 5 μM H_2DCF-DA in RPMI-1640 medium, and then incubated at 37 °C for 30 min in the dark. Finally, the cell pellets were collected by centrifugation and resuspended in 1 ml ice-cold PBS. To avoid cell aggregation, the cell solutions were filtered through a nylon mesh membrane. The samples were analyzed by flow cytometer. The fluorescence intensity of DCF was quantified from a minimum of 10,000 cells by CellQuest software. It was also observed under a confocal microscope. Briefly, the cells were cultured on coverslips, which were kept in 6-well plates at a density of 2×10^5 per well for 16–20 h. After treatment with or without 6 μM CR108 for 2 h, the cells were washed with PBS and fixed with 4% paraformaldehyde solution for 10 min at 37 °C. Thereafter, the cells were washed with PBS and incubated with 5 μM H_2DCF-DA for 30 min at 37 °C in the dark. Finally, the H_2DCF-DA working solution was removed from the cells and immediately observed under a confocal microscope.

Separation of mitochondrial and cytosolic fractions

The mitochondrial and cytosolic fractions of cells were performed using Mitochondria/Cytosol Fractionation kit (BioVision Inc., Mountain View, CA). The cells were cultured in 100-mm Petri dish at a density of 1×10^7 cells for 16–20 h. After treatment with or without CR108 for 24 h, the cells were washed with PBS. Thereafter, the cells were trypsinized and collected by centrifugation at 600 ×*g* for 5 min. The cell pellet was resuspended in 10 ml ice-cold PBS and collected by

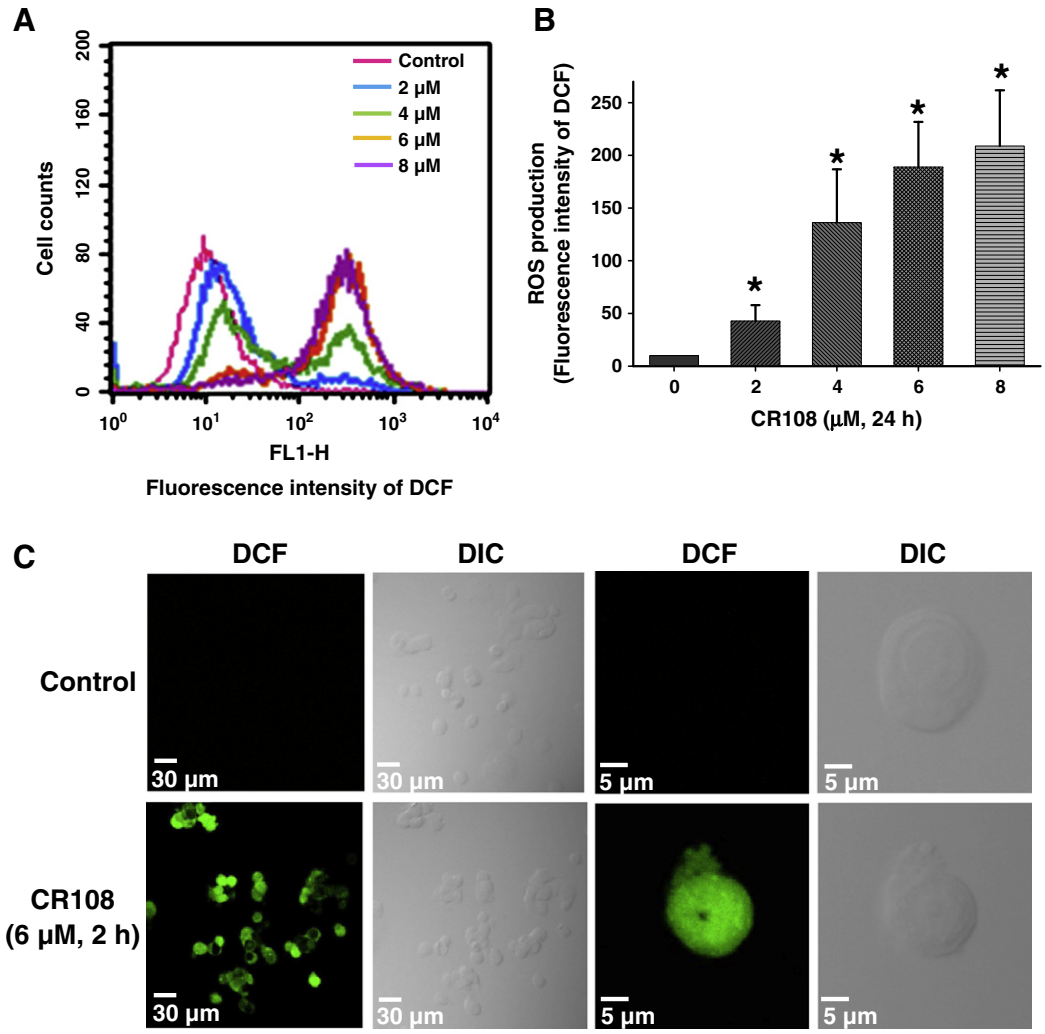


Fig. 3. CR108 increases the ROS levels in human breast cancer cells. (A) MCF-7 cells were treated with 0–8 μM CR108 for 24 h. The ROS levels were stained with 5 μM H_2DCF-DA and analyzed by flow cytometry. (B) The fluorescence intensities of DCF were quantified by CellQuest software. The results were obtained from three experiments and the bar represents the mean ± S.E. $^*p < 0.05$ indicates significant difference between the control and CR108 treated samples. (C) MCF-7 cells were treated with or without 6 μM CR108 for 2 h. At the end of treatment, the cells were stained with H_2DCF-DA and observed under a confocal microscope. Green color indicates the location of DCF fluorescence.

centrifugation at 600 ×*g* for 5 min. The cells were lysed in 500 μl extraction buffer containing dithiothreitol and protease inhibitor cocktail on ice for 10 min. Then the cells were vortexed for 30 min at 4 °C. The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was transferred into a new tube and centrifuged at 13000 rpm for 30 min at 4 °C. The supernatant was carefully removed and collected as the cytosolic fraction. The remaining mitochondrial pellet was resuspended in the mitochondrial extraction buffer. The protein concentrations of the cytosolic and the mitochondrial fraction were determined by the BCA protein assay kit (Pierce, Rockford, IL).

Western blot

Briefly, the cells were cultured in 100-mm Petri dish at a density of 6×10^6 cells for 16–20 h. Then the cells were treated with 0–8 μM CR108 for 24 h. At the end of treatment, the cells were washed with PBS and lysed in the ice-cold cell extract buffer (pH 7.6) containing 0.5 mM DTT, 0.2 mM EDTA, 20 mM HEPES, 2.5 mM $MgCl_2$, 75 mM NaCl, 0.1 mM Na_3VO_4, 50 mM NaF, 0.1% Triton X-100. The protease inhibitors including 1 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 100 μg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride were added to the cell suspension. The protein concentrations were determined by the BCA protein assay kit (Pierce, Rockford, IL). The total cellular protein extracts were prepared. The proteins were separated on 10–12% sodium dodecyl sulfate-polyacrylamide (SDS) gels, and transferred electrophoretically onto polyvinylidene difluoride membranes. The membranes were sequentially hybridized with primary antibody and followed with a horseradish peroxidase-conjugated secondary antibody. The protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer). Western analyses of PARP, Bax, survivin, cytochrome c, phospho-p38 MAP kinase, p38 MAP kinase and COX IV were performed using specific antibodies. To verify equal protein loading and transfer, actin was used as the protein loading control.

Xenografted human breast tumors in nude mice

BALB/cAnN.Cg-*Foxn1nu*/CrlNarl mice (3-week-old male) were obtained from BioLASCO (BioLASCO Co., Ltd., Taipei, Taiwan). 6×10^6 MDA-MB-231-luc2-tdTomato human breast cancer cells were inoculated subcutaneously into the right flank in each mouse. Mice were divided two groups (each group contained 4 mice) and administered either vehicle control (corn oil) or CR108 (10 mg/kg body weight). Solid MDA-MB-231-luc2-tdTomato flank tumors were established for 10 days and then treated with control or CR108 with subcutaneous injection by every interval 4 days for three times. For the assessment of drug effect on tumor inhibition in nude mice, animals were observed by IVIS spectrum imaging system (PerkinElmer) under

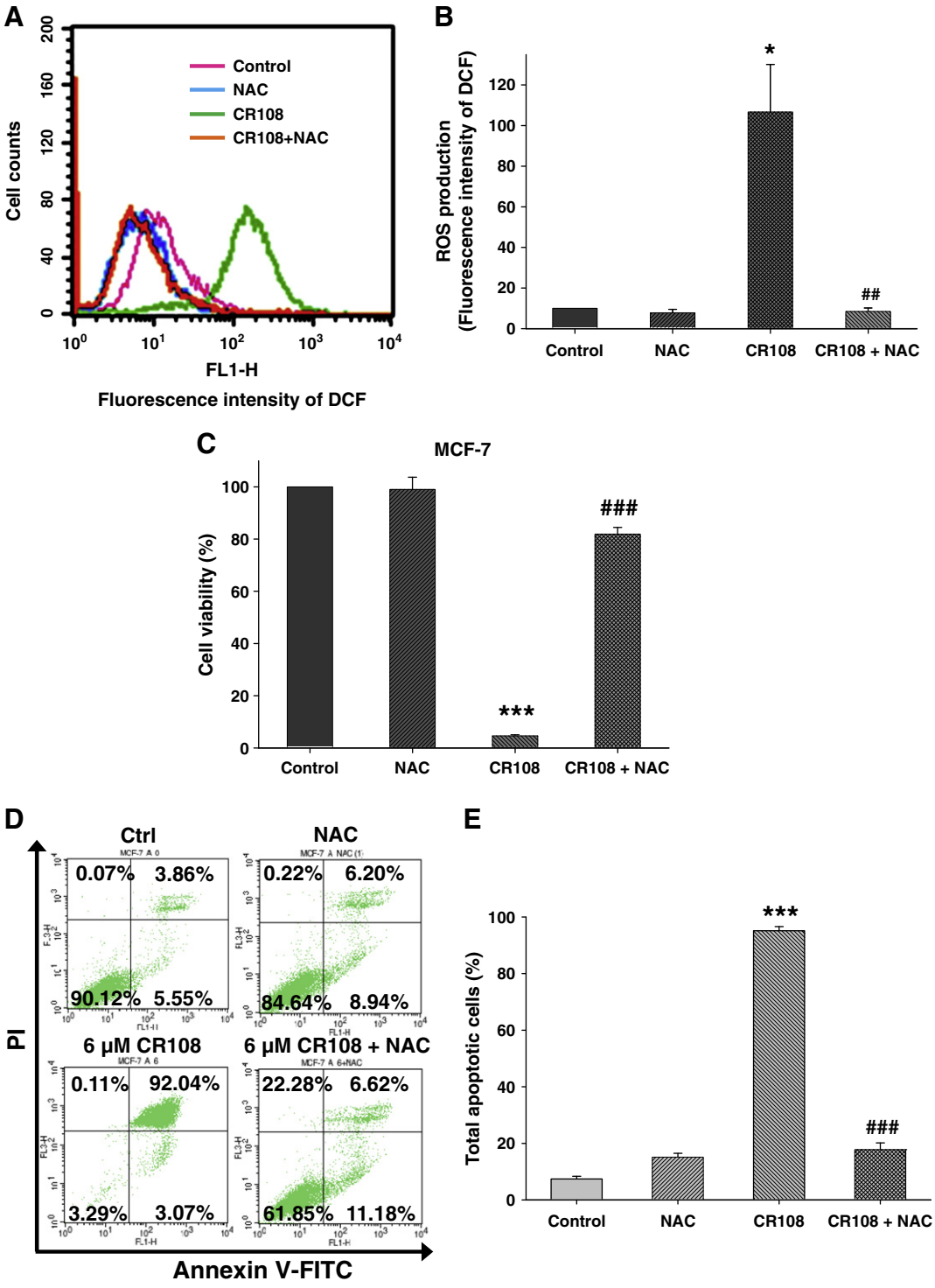


Fig. 4. NAC blocks the levels of ROS and apoptosis in the CR108-treated cells. (A) MCF-7 cells were co-treated with or without 5 mM NAC and CR108 (6 μM for 24 h). ROS levels were stained with H_2DCF-DA and analyzed by flow cytometry. (B) The fluorescence intensities of DCF were quantified by CellQuest software. The results were obtained from three experiments and the bar represents the mean ± S.E. $^{*}p < 0.05$ indicates significant difference between the control and CR108 treated samples. $\#\#p < 0.01$ indicates significant difference between CR108 alone and co-treatment with NAC. (C) MCF-7 cells were co-treated with or without 5 mM NAC and 6 μM CR108 for 24 h. The cell viability was assessed by MTT assay. The results were obtained from four experiments and the bar represents the mean ± S.E. $^{***}p < 0.001$ indicates significant difference between untreated and CR108 treated samples. $\#\#\#p < 0.001$ indicates significant difference between CR108 alone and co-treatment with NAC. (D) MCF-7 cells were co-treated with or without 5 mM NAC and 6 μM CR108 for 24 h. Apoptosis was determined by Annexin V–PI staining using flow cytometry analysis. Cells staining with Annexin V^+/PI^- are those undergoing early apoptosis (lower right), Annexin V^+/PI^+ stained cells are undergoing late apoptosis (upper right). The results were shown from one of three independent experiments with similar findings. (E) Populations of total apoptotic cells were quantified using CellQuest software. The bar represents the mean ± S.E. $^{***}p < 0.001$ indicates significant difference between untreated and CR108 treated samples. $\#\#\#p < 0.001$ indicates significant difference between CR108 alone and co-treatment with NAC.

isoflurane anesthesia. Sample fluorescence images were then taken by using excitation at 535 nm and collecting the emission at 600 nm. Photographic and fluorescence images were taken at constant exposure time. The acquired images and the fluorescence intensity of breast tumors were analyzed by Xenogen Living Image® software, Version 4.0 (PerkinElmer).

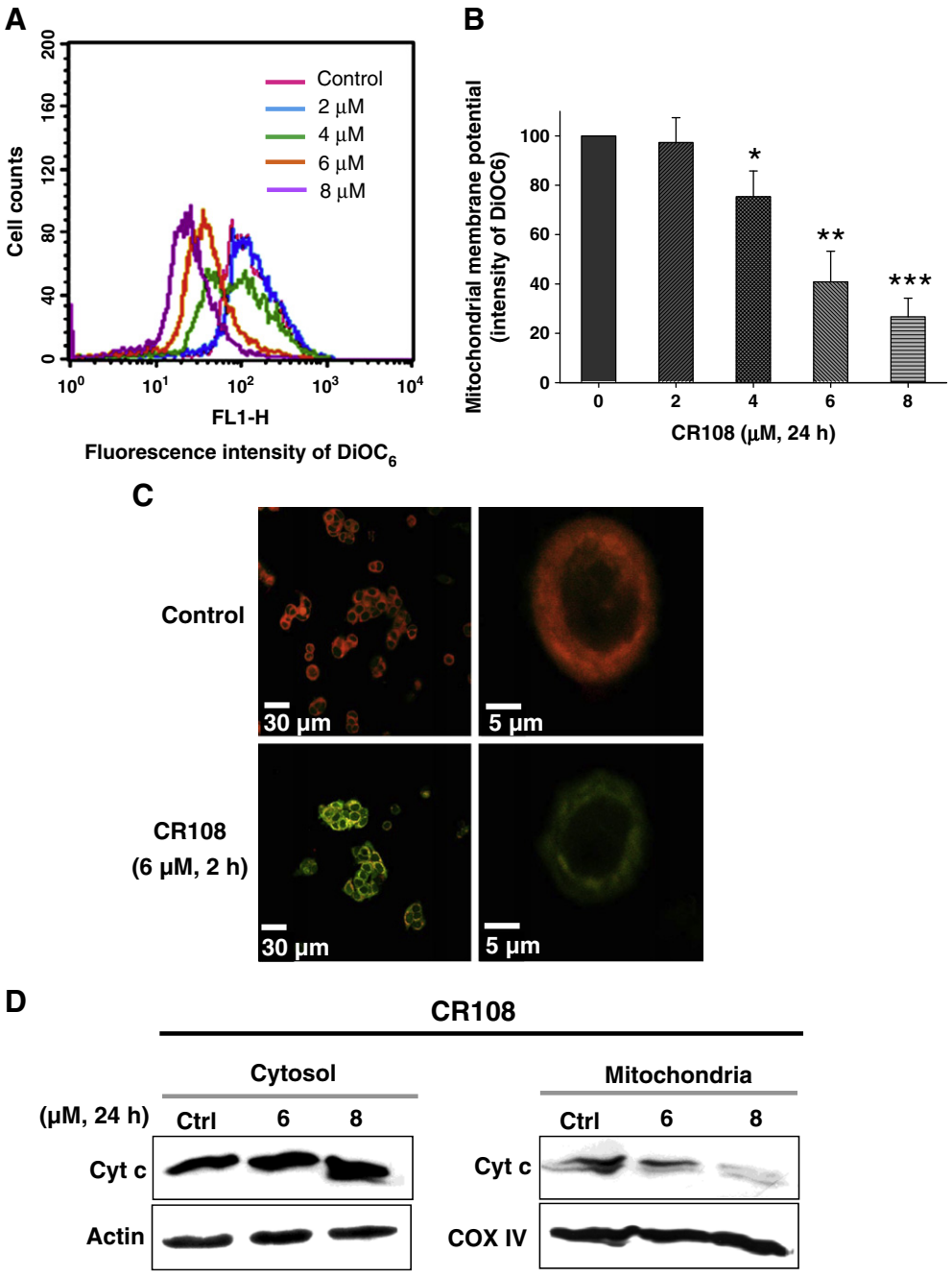


Fig. 5. CR108 induces the loss of mitochondrial membrane potential and cytochrome c release in human breast cancer cells. (A) MCF-7 cells were treated with 0–8 μM CR108 for 24 h. The mitochondrial membrane potential was analyzed by staining with 500 nM $DiOC_6$ and flow cytometry. (B) The fluorescence intensities of $DiOC_6$ were quantified by CellQuest software. The results were obtained from three experiments and the bar represents the mean ± S.E. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ indicate significant differences between the control and CR108 treated samples. (C) MCF-7 cells were treated with CR108 (6 μM for 2 h). The cell were stained with JC-1 and observed under a confocal microscope. Orange fluorescence of J-aggregates indicates the cell areas with high mitochondrial membrane potential. Green fluorescence of J-monomers indicates prevalent in the cell areas with low mitochondrial membrane potential. (D) MCF-7 cells were treated with or without CR108. The separation of mitochondrial and cytosolic fractions was performed by Mitochondria/Cytosol Fractionation Kit. The total protein extracts were subjected to Western analysis using specific antibodies for cytochrome c, COX IV, and actin. Representative Western blot data were shown from one of three separate experiments with similar findings.

Statistical analysis

Each experiment was repeated at least three times. Data were analyzed by Student's *t*-test. In a comparison of multiple groups, data were analyzed by two-way ANOVA with Bonferroni post-tests. A *p* value of <0.05 was considered statistically significant in the experiments.

Results

CR108 is more sensitive on the cell death than other vitamin K3 derivatives in human breast cancer cells

Fig. 1A shows the chemical structures of CR108 and other vitamin K3 derivatives (CR107, CR109 and CR110). Treatment with various vitamin

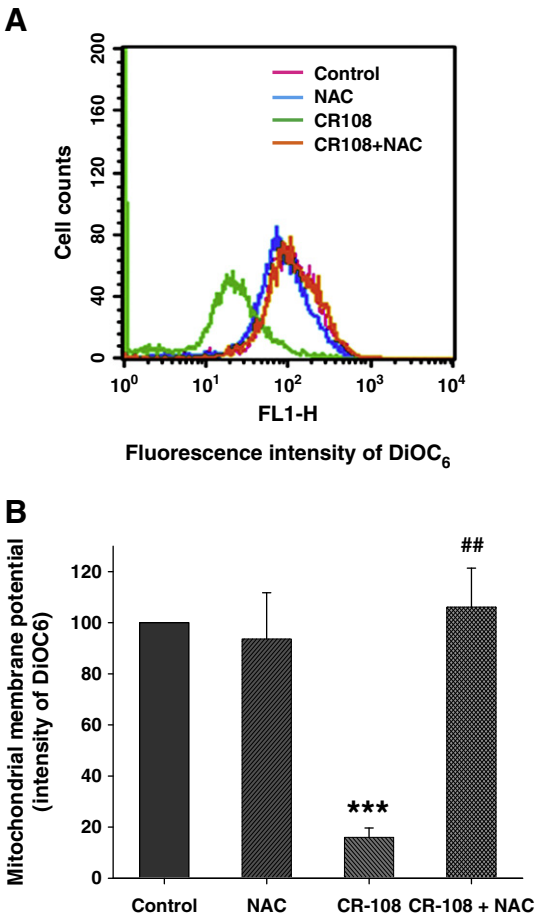


Fig. 6. NAC blocks the CR108-induced mitochondrial damage in human breast cancer cells. (A) MCF-7 cells were co-treated with or without 5 mM NAC and 6 μM CR108 for 24 h. At the end of treatments, the cells were stained with 500 nM $DiOC_6$ and analyzed by flow cytometry. (B) The fluorescence intensities of $DiOC_6$ were quantified by CellQuest software. The results were obtained from three experiments and the bar represents the mean ± S.E. $^{***}p < 0.001$ indicates significant difference between the control and CR108 treated samples. $\#\#p < 0.01$ indicates significant difference between CR108 alone and co-treatment with NAC.

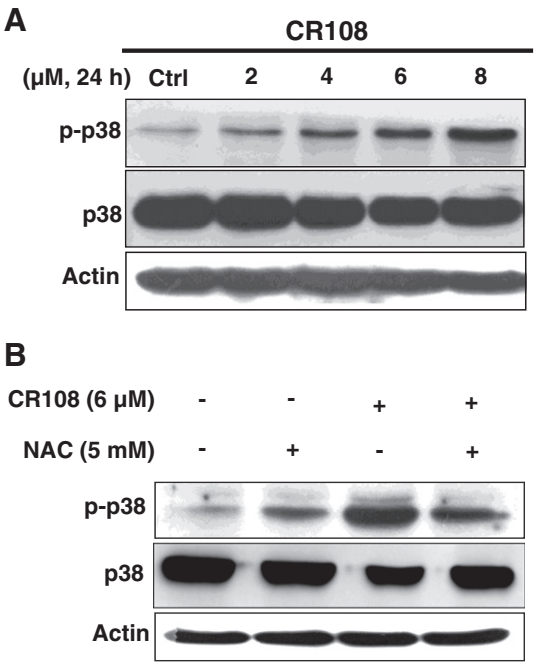


Fig. 7. CR108 induces the protein phosphorylation of p38 MAP kinase in human breast cancer cells. (A) MCF-7 cells were treated with 0–8 μM CR108 for 24 h. The total protein extracts were subjected to Western analysis using specific antibodies for phospho-p38 MAP kinase, p38 MAP kinase and actin. (B) MCF-7 cells were co-treated with or without 5 mM NAC and 6 μM CR108 for 24 h. The total protein extracts were subjected to Western analysis using specific antibodies for phospho-p38 MAP kinase, p38 MAP kinase and actin. Representative Western blot data were shown from one of three separate experiments with similar findings.

K derivatives (2–8 μM for 24 h) significantly reduced the cell viability via a concentration-dependent manner in the non-HER-2-overexpressed MCF-7 cancer cells (Fig. 1B). CR108 exhibited the most potent on the cytotoxicity among vitamin K derivatives. In addition, CR108 induced cell death in the HER-2-overexpressed BT-474 breast cancer cells (Fig. 1C). The IC_{50} value of CR108 was around 2.8 μM in both MCF-7 and BT-474 cells. Interestingly, CR108 (2–10 μM for 24 h) did not induce the cytotoxic effects in the H184B5F5/M10 human normal breast epithelial cells (Fig. 1D). Nonetheless, CR107 and CR109 could reduce cell viability in these cells. We also found that CR110 did not significantly induce the cytotoxicity in normal breast epithelial cells; however, CR108 was more potent on the reduction of cell viability than CR110 in human breast cancer cells. Accordingly, we selected CR108 to examine the anticancer abilities and possible mechanisms in this study.

CR108 induces apoptosis in human breast cancer cells

The apoptotic levels induced by CR108 were analyzed by Annexin V–PI staining. Fig. 2A and C show that the cells staining with Annexin V^+/PI^- are those undergoing early apoptosis (lower right), and Annexin V^+/PI^+ stained cells are undergoing late apoptosis (upper right). The percentages of total apoptosis populations were quantified. The total apoptotic levels were increased following treatment with CR108 (2–8 μM for 24 h) via a concentration-dependent manner in MCF-7 cells (Fig. 2A and B). Furthermore, treatment with 6 μM CR108 for 4–24 h increased the total apoptotic levels in a time-dependent manner (Fig. 2C and D).

N-acetylcysteine inhibits the CR108-induced ROS, cytotoxicity and apoptosis in human breast cancer cells

Treatment with CR108 significantly increased the intracellular ROS levels in a concentration-dependent manner in MCF-7 cells (Fig. 3A and B). The green fluorescence of DCF was markedly elevated after treatment with 6 μM CR108 for 2 h in MCF-7 cells (Fig. 3C). Co-treatment with 5 mM NAC for 24 h completely blocked the CR108-induced ROS levels (Fig. 4A and B). NAC alone did not alter the fluorescence intensity of DCF (Fig. 4A and B). To further examine the role of ROS in the CR108-induced cytotoxicity in breast cancer cells, NAC was used for blocking the CR108-induced ROS generation and apoptosis. Co-treatment with NAC reduced ~80% cell death in the CR108-treated cells (Fig. 4C). Besides, NAC decreased the CR108-induced apoptosis (Fig. 4D). Co-treatment with NAC significantly decreased the average 77.31% of total apoptosis in the CR108-treated cells (Fig. 4E).

CR108 induces mitochondrial dysfunction and cytochrome c release

We have evaluated the effect of CR108 on the mitochondrial membrane potential ($\Delta\Psi m$) by staining with fluorescence probes of $DiOC_6$ or JC-1. CR108 significantly induced the loss of mitochondrial membrane potential by the decrease of $DiOC_6$ fluorescence intensities in MCF-7 cells (Fig. 5A and B). Exposure of MCF-7 cells to 6 μM CR108 for 2 h induced remarkable changes in the loss of mitochondrial membrane potential as evident from the disappearance of orange fluorescence of J-aggregate forms and the increase of green fluorescence

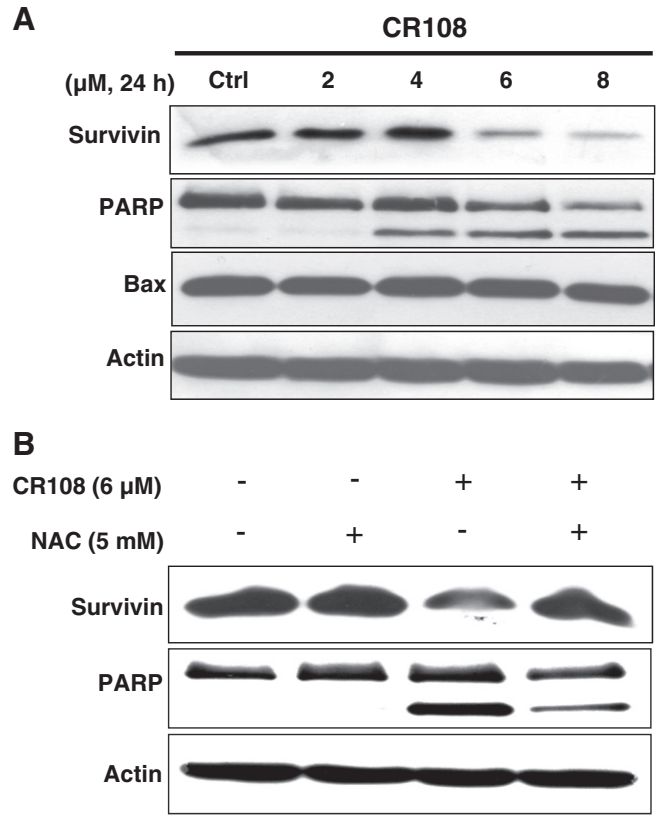


Fig. 8. CR108 inhibits the survivin protein expression in human breast cancer cells. (A) MCF-7 cells were treated with 0–8 μM CR108 for 24 h. The total protein extracts were subjected to Western blot using specific antibodies for survivin, PARP, Bax and actin. (B) MCF-7 cells were co-treated with or without 5 mM NAC and 6 μM CR108 for 24 h. The total protein extracts were subjected to Western blot using specific antibodies for survivin, PARP and actin. Representative Western blot data were shown from one of three separate experiments with similar findings.

of J-aggregate forms in the CR108-treated cells (Fig. 5C). Moreover, CR108 (6–8 μM for 24 h) decreased the amount of cytochrome c in the mitochondrial fractions and conversely elevated in the cytosolic fractions (Fig. 5D). Actin was used as a loading control in the cytosolic fractions. COX IV protein was used as a loading control for the mitochondrial fractions. To investigate whether ROS induction by CR108 mediated mitochondrial damage, the cells were co-treatment with NAC and CR108 and analyzed by mitochondrial membrane potential. Co-treatment with 5 mM NAC for 24 h significantly restored the mitochondrial membrane potential in the CR108-treated cells (Fig. 6A and B).

CR108 induces the phosphorylation of p38 MAP kinase and inhibits survivin protein expression

Treatment with 2–8 μM CR108 for 24 h increased the protein phosphorylation of p38 MAP kinase in MCF-7 cells (Fig. 7A). CR108 did not alter total protein level of p38 MAP kinase. Co-treatment with NAC attenuated the protein level of phospho-p38 MAP kinase in the CR108-treated cells (Fig. 7B). Moreover, CR108 inhibited the survivin protein expression and increased the cleaved form of PARP (89 kDa) proteins (Fig. 8A). However, CR108 did not alter the apoptotic protein levels of Bax. Actin was used as a loading control protein that was not altered by CR108. In contrast, NAC restored the survivin protein level and reduced the PARP protein cleavage in the CR108-treated cells (Fig. 8B). To further examine the role of p38 MAP kinase on the regulation of survivin and apoptosis, a p38 MAP kinase inhibitor SB202190 was combined treatment with CR108. SB202190 significantly inhibited the CR108-induced cell death in breast cancer cells (Fig. 9A). Moreover, SB202190 restored the survivin protein levels in the CR108-treated cells (Fig. 9B).

CR108 inhibits the tumor size of xenografted human breast tumors in nude mice

To verify the breast tumor inhibition by CR108, the MDA-MB-231-luc2-tdTomato breast cancer cells were subcutaneously inoculated into nude mice, and the fluorescence intensities of human breast tumors were observed by IVIS spectrum imaging system (Fig. 10A). The fluorescence intensities of human breast tumors in nude mice were compared between vehicle (corn oil) and 10 mg/kg CR108 (Fig. 10A). The fluorescence intensities of tumors in three groups were markedly reduced by treatment with CR108 at 20 days observation (Fig. 10A). After quantification, CR108 significantly reduced the fluorescence intensities of tumors by comparison with vehicle (Fig. 10B). In addition, the tumor weight from scarified mice was significantly reduced by treatment with CR108 (data not shown).

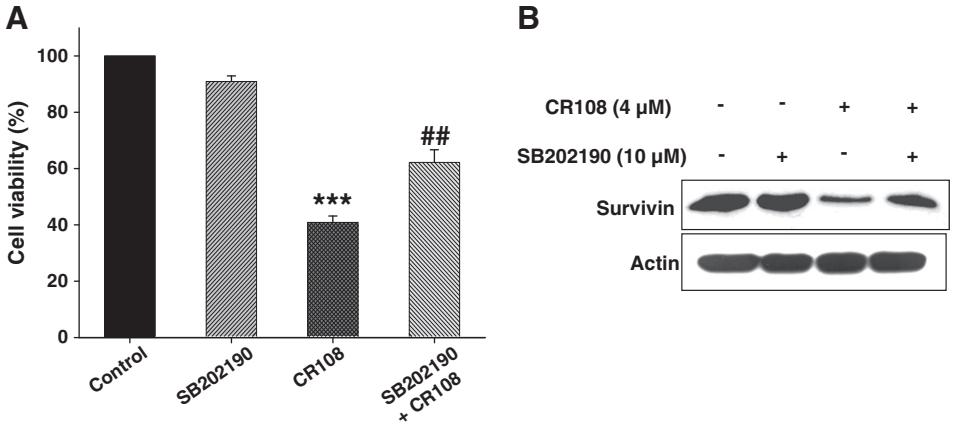


Fig. 9. SB202190 reduces the CR108-induced cytotoxicity and survivin inhibition in human breast cancer cells. (A) MCF-7 cells were co-treated with or without 10 μM SB202190 and 4 μM CR108 for 24 h. Cell viability was assessed by MTT assay. The results were obtained from three experiments and the bar represents the mean ± S.E. ***$p < 0.001$ indicates significant difference between untreated and CR108 treated samples. ##$p < 0.01$ indicates significant difference between CR108 alone and co-treatment with SB202190. (D) MCF-7 cells were co-treated with or without 10 μM SB202190 and 4 μM CR108 for 24 h. The total protein extracts were subjected to Western analysis using specific antibodies for survivin and actin. Representative Western blot data were shown from one of three separate experiments with similar findings.

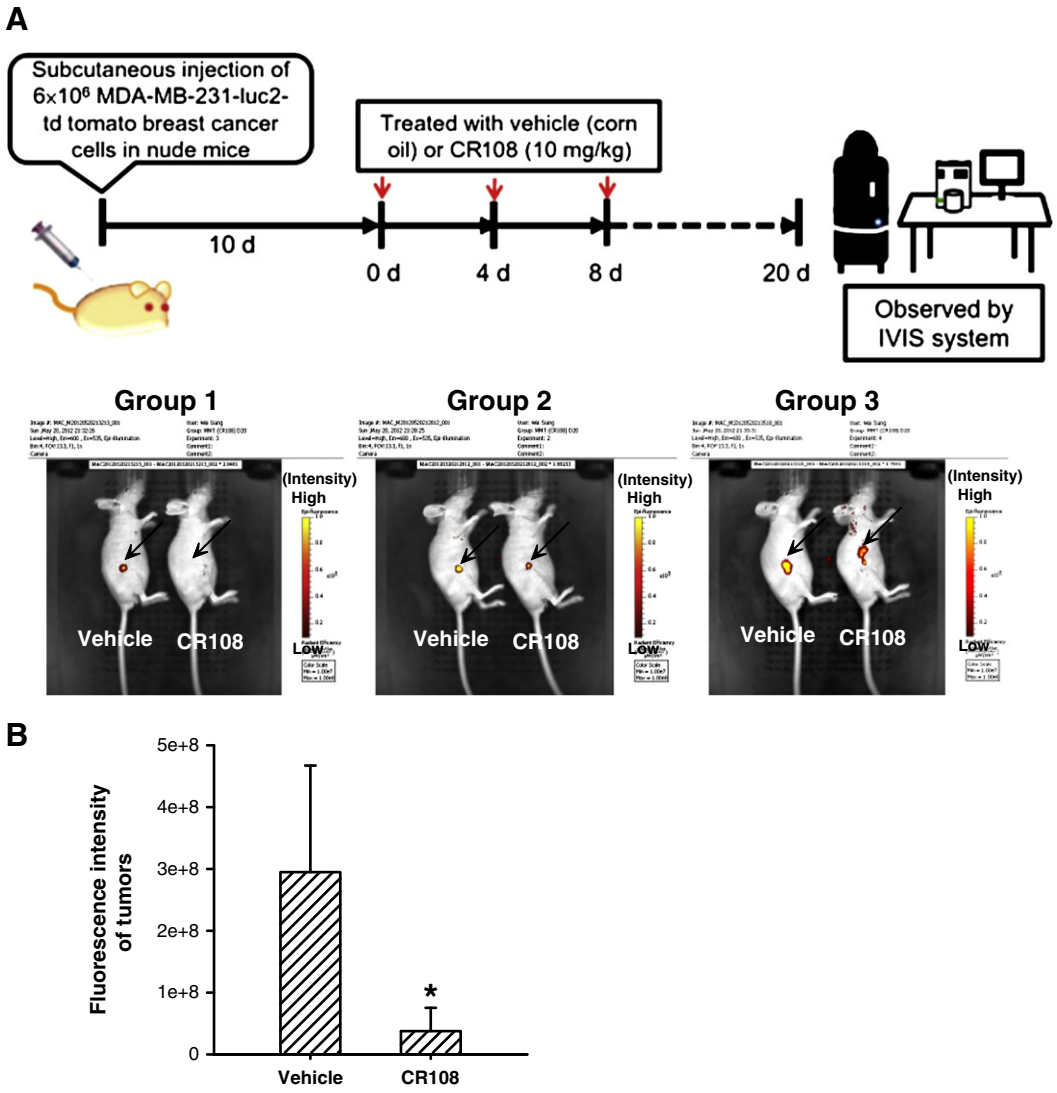


Fig. 10. CR108 inhibits xenografted human breast tumors in nude mice. (A) The three-week-old nude mice were subcutaneously injected with 6×10^6 MDA-MB-231-luc2-tdTomato human breast cancer cells. After 10 days inoculation, the mice bearing human breast tumors were treated with vehicle (corn oil) or 10 mg/kg of CR108 as indicated time. The fluorescence intensities of MDA-MB-231-luc2-tdTomato cells were observed by IVIS spectrum imaging system. The pictures were obtained by treatment with vehicle or CR108 after 20 days. Results were from three groups of mice. (B) The fluorescence intensities of tumors in mice were quantified at 20 days after CR108 administration. The bar represents the mean ± S.E. $^*p < 0.01$ indicates significant difference between vehicle and CR108-treated groups.

Discussion

Breast cancer is one of the most common cancers, which causes mortality in women. Drug combinations and new cancer drugs development are highly desired for breast cancer patients. Anticancer agents have shown to induce the cancer cell death by ROS generation (Davis et al., 2001; Hug et al., 1997; Kim et al., 2009; Wang et al., 2004). Clinical cancer drugs such as doxorubicin (DOX), tamoxifen and bleomycin can induce the cytotoxicity and the abrogation of proliferative signals through ROS production in cancer cells (Davis et al., 2001; Hug et al., 1997; Wang et al., 2004). ROS mediated the mitochondrial dysfunction, cytochrome c release and caspase-3 activation in the DOX-induced apoptosis (Mizutani et al., 2005). In this study, we show a vitamin K3 derivative (CR108), which is more potent on the induction of cell death than other vitamin K3 derivatives in human breast cancer cells. ROS production by CR108 mediated mitochondrial dysfunction and apoptosis. Depletion of ROS by NAC completely blocked the CR108-induced mitochondrial damage and apoptosis. CR108 markedly blocked tumor growth in xenografted human breast tumors in nude mice. Moreover, CR108 did not induce cytotoxicity in human normal breast epithelial cells. Development of vitamin K3 derivatives without significant toxicity for human treatment is highly desired. Accordingly, CR108 is a novel vitamin K3 derivative, which may develop for cancer therapy in human breast cancer.

Vitamin K3 has been shown to exert anticancer activities (Akiyoshi et al., 2009; Gold, 1986; Lamson and Plaza, 2003; Liao et al., 2000; Nutter et al., 1992). Cpd 5, a vitamin K analog, inhibits cell growth in the doxorubicin-resistant and -sensitive breast cancer cells (Kar and Carr, 2000). Vitamin K3 has been shown to cause the cytotoxicity at an IC_{50} value of 14.2 μM in the MCF-7 breast cancer cells (Akiyoshi et al., 2009). Cpd 5 inhibited the cell growth of MCF-7 cells with an IC_{50} of approximately 15 μM (Kar and Carr, 2000). We found that CR108 caused the cytotoxicity at an IC_{50} value of 2.8 μM in MCF-7 cells. Thus, CR108 is more potent on the induction of cell death than other vitamin K3 derivatives in breast cancer cells.

Anticancer agents exert anticancer activities through the induction of apoptotic pathways or the blockade of survival pathways in cancer cells (Chao et al., 2004, 2007; Kuo et al., 2004). Survivin proteins are highly expressed in various human cancer cells that exert anti-apoptosis and prevent cell death (Ambrosini et al., 1997; Deveraux and Reed, 1999). The blockade of survivin provides an important

strategy for cancer therapy. Interestingly, CR108 reduced the survivin protein expression in human breast cancer cells. Meantime, CR108 elevated the protein levels of cleaved PARP. PARP is a DNA repair protein that has been shown to be cleaved when cells undergo apoptosis (Miller, 1997). The blockage of ROS production by NAC restored the survivin proteins and attenuated the PARP protein cleavage in the CR108-treated cells. These findings indicate that CR108-induced ROS production mediates the survivin inhibition and apoptosis induction in the human breast cancer cells.

The p38 MAP kinase pathway has been shown to regulate apoptosis. Anticancer drugs induce the protein phosphorylation of p38 MAP kinase to lead apoptosis in cancer cells (Chao et al., 2004, 2007; Hsiao et al., 2007; Li and Bertino, 2002). p38 MAP kinase is an important signaling mediator for ROS (Chao et al., 2004; Torres and Forman, 2003). The phosphorylation of p38 MAP kinase has shown to down-regulate survivin expression (Chao et al., 2004, 2007; Liu et al., 2010). We found that CR108 induced the phosphorylation of p38 MAP kinase in human breast cancer cells. Furthermore, NAC could block the activation of p38 MAP kinase in the CR108-treated cells. SB202190 has been shown to restore the survivin protein levels and reduces cancer cell death (Liu et al., 2010). Also, SB202190 recovered the survivin levels and attenuated the cell death in the CR108-treated cells. These findings demonstrate that the generation of ROS by CR108 mediates the phosphorylation of p38 MAP kinase to down-regulate survivin expression.

Mitochondrial pathway plays a crucial role in the intrinsic induction of apoptosis (Liu et al., 1996). Abnormal ROS production can cause the loss of mitochondrial membrane potential and mitochondrial damage (Chen et al., 2010; Park et al., 2003; Sakon et al., 2003). The loss of mitochondrial membrane potential has been indicated mitochondrial dysfunction and subsequent apoptotic events (Green and Reed, 1998). CR108 dramatically increased the ROS levels and decreased the mitochondrial membrane potential in breast cancer cells. In contrast, the loss of mitochondrial membrane potential by CR108 can be blocked by NAC. Moreover, CR108 induced cytochrome c release from mitochondria into cytosol. Once cytochrome c releases to cytosol, it interacts with Apaf-1 and leading to activate caspase cascade (Kluck et al., 1997; Yang et al., 1997). Thus, CR108 may potentiate ROS production to mediate mitochondrial dysfunction and apoptosis.

HER-2, also known as ErbB-2, is a membrane tyrosine kinase and oncogene (Coussens et al., 1985). Approximately 20–30% of breast cancers have an amplification of the *HER-2/neu* gene or protein overexpression (Davoli et al., 2010; Rubin and Yarden, 2001). Overexpression of HER-2 in breast cancers is associated with increased disease recurrence and worse prognosis (Sotiriou and Pusztai, 2009) and more resistant in cancer drug therapy (Callahan and Hurvitz, 2011). Interestingly, CR108 reduced the cell viability in both the non-HER-2 overexpressed MCF-7 and the HER-2 overexpressed BT-474 breast cancer cells. Thus, CR108 is a potential vitamin K3 analog for developing on therapeutics in the HER-2-overexpressed breast cancers.

In summary, we show a novel vitamin K derivative CR108 that has potential for breast cancer therapy. CR108 induces apoptosis through ROS production and mitochondrial damage pathway that are associated with the p38 MAP kinase activation and survivin inhibition in human breast cancer.

Conflict of interest

None declared.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2013.10.007.

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