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Indole-3-acetic acid/horseradish peroxidase induces apoptosis in TCCSUP human urinary bladder carcinoma cells

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Indole-3-acetic acid (IAA) and horseradish peroxidase (HRP) have emerged as a new strategy for cancer treatment. In the present study, we determined the effects of IAA/HRP treatment on TCCSUP human urinary bladder carcinoma cells. It was found that the IAA/HRP combination decreased cell viability of TCCSUP cells in a time- and dose-dependent manner, whereas IAA or HRP alone showed no such effect. In addition, the decreased cell viability was restored by pretreatment with ascorbic acid. To clarify the mechanism of death of TCCSUP cells by IAA/HRP, we investigated the signal transduction pathways related to the apoptosis. It was found that IAA/HRP activates p38 mitogen-activated protein (MAP) kinase and c-Jun N-terminal kinase (JNK). We further investigated the IAA/HRP-mediated apoptotic pathways and showed that IAA/HRP induces caspase-8 and caspase-9 activation, which results in caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage. To further confirm whether IAA/HRP induces apoptotic cell death, we performed a DNA fragmentation assay after IAA/HRP treatment and found that IAA/HRP-treated cells showed typical apoptotic DNA ladder formation. From these results, we suggest that IAA/HRP induces apoptosis of TCCSUP human urinary bladder carcinoma cells via both death receptor-mediated and mitochondrial apoptotic pathways.

1. Introduction

It is known that bladder cancer is one of the five most common malignant cancers in industrialized countries (Zieger 2008). Because of the high recurrence rates, radical treatment, including surgery, is the standard treatment. However, these treatments result in severe complications, treatment-related morbidity, and a decreased quality of life (Zieger 2008). Thus, to obtain another possible therapy for bladder cancer, the development of novel molecular targeted therapeutics is needed.

It has been suggested that indole-3-acetic acid (IAA), the plant growth hormone, and horseradish peroxidase (HRP) could be used as a novel cancer therapy (Folkes et al. 1998; Greco and Dachs 2001). We also reported the potential use of IAA/HRP for melanoma therapy (Kim et al. 2004). Interestingly, IAA alone was not cytotoxic, but IAA became cytotoxic after reaction with HRP (Folkes and Wardman 2001). It has been reported that IAA activated by HRP produces an abundance of free radicals, including reactive oxygen species (ROS; Canseias et al. 1995; Folkes et al. 1998). We also proposed that hydrogen peroxide plays a major role in IAA/HRP-induced cell death (Kim et al. 2006a). It has been reported that IAA selectively leads to cell death of human T24 bladder carcinoma cells transfected with an HRP-encoding gene (Greco et al. 2002a,b). However, little is known about the molecular mechanisms of IAA/HRP in bladder cancer cells.

It is generally accepted that p38 mitogen-activated protein (MAP) kinase and c-Jun N-terminal kinase (JNK) can be activated by stress stimuli, such as UV, free radicals, heat shock, osmotic shock, and ischemia (Butterfield et al. 1997; Harper and LoGrasso 2001; Xia et al. 1995; Zanke et al. 1996). Thus, it is suggested that p38 MAP kinase and JNK activation are involved in many cellular responses, including apoptosis. Furthermore, it is proposed that ROS is a major mediator in the activation of the p38 and JNK pathways (Du et al. 2001; Haddad and Land 2002; Lee et al. 2002).

Activation of the apoptotic signaling pathways is one of the potential mechanisms for the treatment of cancer (Huang and Oliff 2001). It is known that apoptotic nuclear DNA fragmentation occurs during the apoptotic process (Li et al. 2001). Furthermore, apoptotic processes are mediated by a family of cysteine-dependent aspartate-specific proteases, known as the caspases (Hengartner 2000; Nunez et al. 1998). There are two main apoptotic pathways in mammalian cells. One apoptotic pathway is the death-receptor pathway, which includes activation of caspase-8 triggered by Fas (also known as CD95) or

Abbreviations: DCFH-DA, 2,7-dichlorofluorescin diacetate; HRP, horseradish peroxidase; IAA, indole-3-acetic acid; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species.

tumor necrosis factor receptor 1 (TNFR1). The other pathway is the mitochondrial pathway via the activation of caspase-9. Activation of caspase-8 and caspase-9 converge at the level of caspase-3 activation (Mehmet 2000). One of the major substrates of caspase-3 is poly(ADP-ribose) polymerase (PARP). PARP is a nuclear enzyme that facilitates DNA repair when DNA is damaged by stress stimuli (Nicholson et al. 1995).
In the present study, we determined the effects of the combination of IAA/HRP on the death of TCCSUP human urinary bladder carcinoma cells. It was found that IAA/HRP treatment induces apoptosis in TCCSUP cells. Thus, we further investigated the mechanisms of apoptotic cell death induced by IAA/HRP.

2. Investigations and results

2.1. IAA/HRP combination induces cell death in TCCSUP human urinary bladder carcinoma cells

In this study, we first investigated the effects of IAA alone and of the IAA/HRP combination on cell viability of TCCSUP human urinary bladder carcinoma cells. To determine the

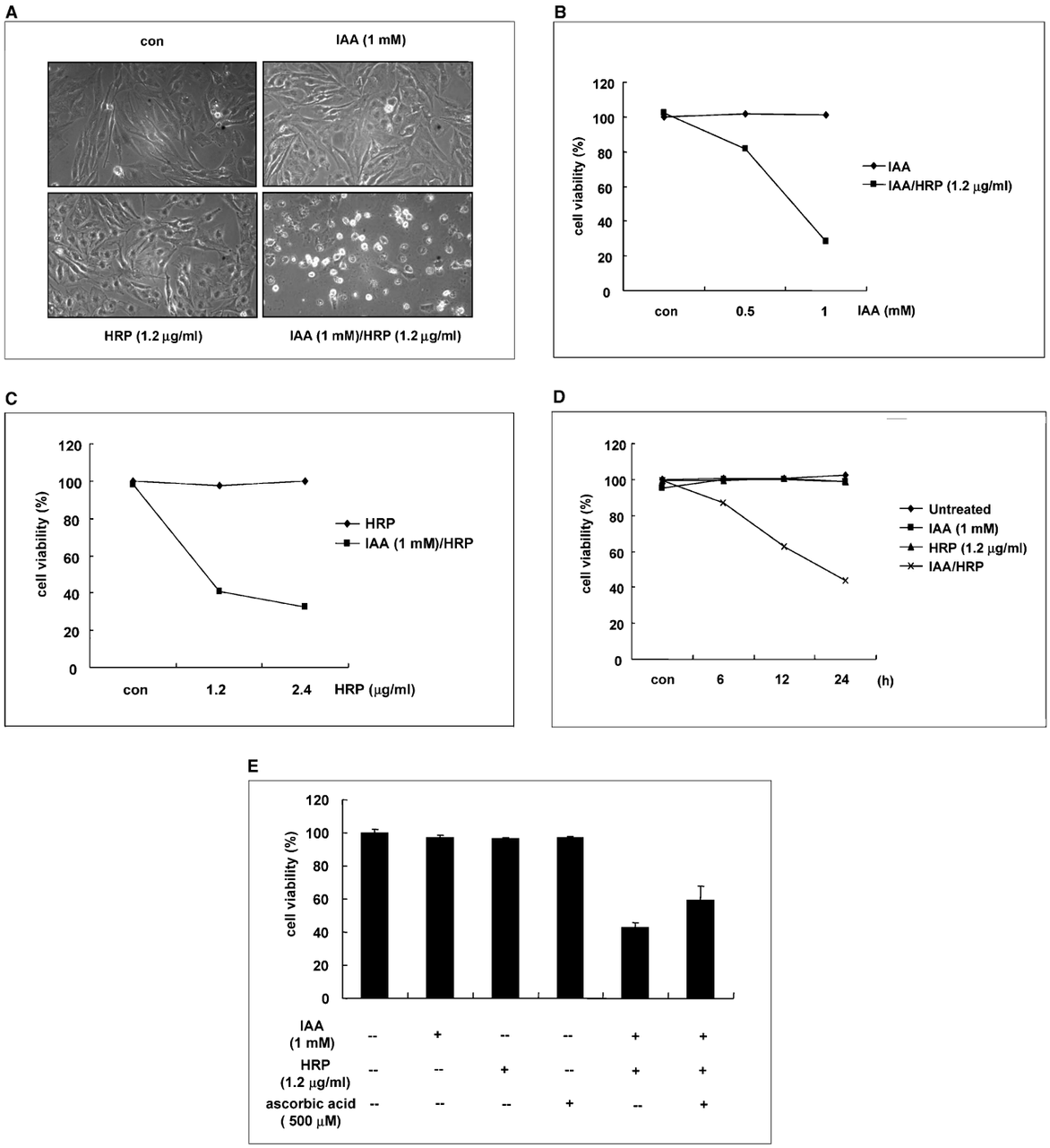

Fig. 1: Cytotoxic effect of IAA/HRP in TCCSUP human urinary bladder carcinoma cells. (A) After serum starvation, TCCSUP cells were treated with 1 mM of IAA in the absence and in the presence of HRP (1.2 μg/ml). Phase contrast photomicrographs were then taken using a digital video camera. (B) Cells were treated with 0.5 or 1 mM of IAA in the absence or presence of HRP (1.2 μg/ml). After 24 h, a crystal violet assay was performed. (C) Cells were treated with 1 mM of IAA in the absence or presence of HRP (1.2 μg/ml or 2.4 μg/ml). After 24 h, a crystal violet assay was performed. (D) Cells were treated with IAA (1 mM) and HRP (1.2 μg/ml). At the indicated time points after treatment, cell viability was measured by a crystal violet assay. (E) Cells were treated with IAA (1 mM) and/or HRP (1.2 μg/ml) with or without ascorbic acid (500 μM). After 24 h, a crystal violet assay was performed. Data represent the means ± S.D. of triplicate assays expressed as percentages of the control

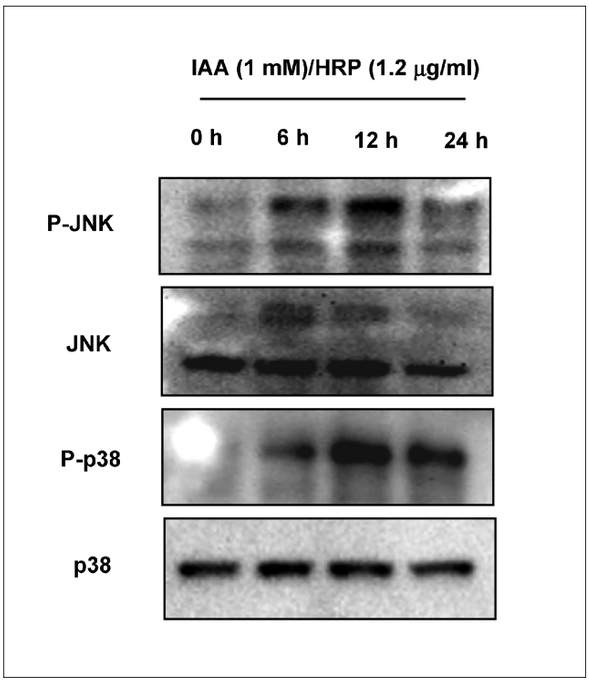

Fig. 2: Effects of IAA/HRP on the JNK and the p38 pathways. (A) After serum starvation, TCCSUP cells were treated with IAA (1 mM)/HRP (1.2 μg/ml) for the time indicated. Cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific JNK or p38 MAP kinase. Equal protein loadings were confirmed by reaction with phosphorylation-independent JNK or p38 MAP kinase antibodies, respectively

cytotoxic effect of IAA/HRP on TCCSUP cells, cells were exposed to increasing doses of IAA with or without HRP (1.2 μg/ml) treatment. Twenty-four hours after IAA/HRP treatment, the cells were photographed under a phase contrast microscope. We observed that many cells were detached from the culture dish after IAA/HRP treatment, indicating that the cells died (Fig. 1A). Cell viability was measured using the crystal violet assay 24 h after IAA/HRP treatment. In agreement with the morphologic observation, IAA/HRP caused cell death in a concentration-dependent manner, whereas IAA alone was not cytotoxic at concentrations tested up to 1 mM for 24 h (Fig. 1B). We next treated cells with increasing doses of HRP with or without IAA (1 mM) treatment, and found that IAA/HRP leads to cell death in an HRP dose-dependent fashion (Fig. 1C). In addition, the viability of cells treated with IAA/HRP decreased in a time-dependent manner. From 6 h after IAA (1 mM)/HRP (1.2 μg/ml) treatment, cells showed significantly reduced viability, while IAA or HRP alone did not show any cytotoxic effects (Fig. 1D). We next examined the effects of ascorbic acid, an antioxidant, on IAA/HRP-induced cell death. Our results showed that ascorbic acid restored the cytotoxic effect of IAA/HRP (Fig. 1E). In addition, IAA, HRP, or ascorbic acid alone had no effect on cell viability.

2.2. Effects of IAA/HRP on the stress-regulated signaling pathways

In a time course experiment, we next examined the activation of the JNK and p38 MAP kinase signal transduction pathways by IAA/HRP treatment. IAA (1 mM)/HRP (1.2 μg/ml) led to the activation of JNK from 6 h to 12 h (Fig. 2). p38 MAP kinase was also activated by IAA/HRP from 6 h after treatment, but the activation of p38 MAP kinase was prolonged for at least 24 h (Fig. 2).

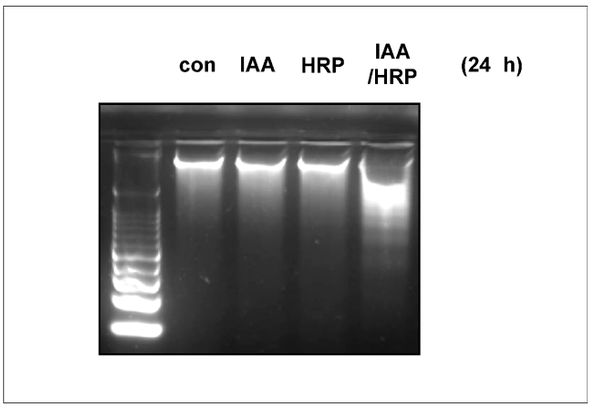

Fig. 3: IAA/HRP treatment caused DNA fragmentation. Agarose gel electrophoresis showing DNA fragmentation of TCCSUP cells treated IAA (1 mM)/HRP (1.2 μg/ml) for 24 h. DNA fragmentation assay was performed, as described in "Experimental"

2.3. IAA/HRP activates caspases, cleaves PARP, and induces apoptosis

Although the crystal violet assay indicated that IAA/HRP induced cell death, it does not discriminate between apoptosis and necrosis. Thus, to verify whether IAA/HRP-induced TCCSUP cell death is due to apoptosis, DNA fragmentation assays were performed. At 24 h after IAA/HRP treatment, a typical DNA ladder pattern of apoptosis was observed (Fig. 3). In contrast, cells treated with IAA or HRP alone did not show the ladder pattern.

It is well-known that caspase activation and PARP cleavage are the major features of apoptosis. Thus, we next investigated the proteolytic processing of caspase-9, -8, and -3, and PARP in response to IAA/HRP by Western blotting. Because caspases become active when they are cleaved into processed fragments, we used caspase-9 and -8 antibodies directed against domains of the precursor forms. As shown in Fig. 4A, the precursor forms of caspase-9 and caspase-8 decreased clearly after 24 h of IAA/HRP treatment, indicating that caspase-9 and -8 were activated. For caspase-3, we used an antibody against its activated form, and increased active caspase-3 was detected 24 h after IAA/HRP treatment. Caspase-3 is known to cleave PARP efficiently. Thus, the proteolytic cleavage of PARP was also detected by Western blotting. Our results showed that the 116-kDa full-length PARP was cleaved to the apoptotic 85-kDa fragment after IAA/HRP treatment (Fig. 4A). We further performed a time-course experiment to determine the nature of the apoptotic pathway induced by IAA/HRP. As shown in Fig. 4C, IAA/HRP cleaved and activated caspase-9, -8, and -3 in a time-dependent manner. Moreover, PARP was found to be cleaved in a time-dependent manner with IAA/HRP treatment. In addition, cells were photographed under a phase contrast microscope. We observed that many cells were detached from the culture dish in a time-dependent fashion after IAA/HRP treatment. Taken together, these results indicate that IAA/HRP results in TCCSUP cell apoptosis (Fig. 4B).

3. Discussion

Despite the potential use of IAA/HRP in cancer therapy (Folkes and Wardman 2001; Greco et al. 2002a), the mechanism underlying the IAA/HRP-induced cytotoxic effect in bladder cancer has received little attention. Thus, we chose TCCSUP high grade bladder carcinoma cells to investigate the possible applications of IAA/HRP in human bladder cancer. In the present study, the crystal violet assay showed that the viability of TCCSUP

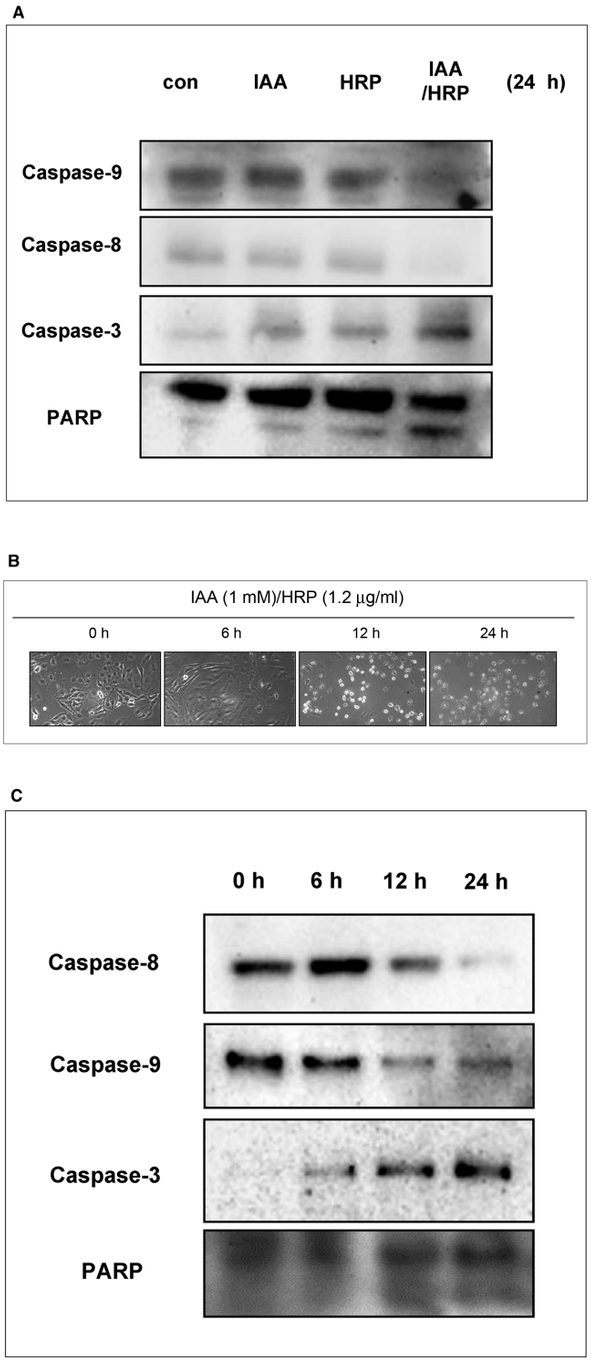

Fig. 4: IAA/HRP induced apoptosis via caspase activation and PARP cleavage. (A) After serum starvation, TCCSUP cells were treated with IAA (1 mM)/HRP (1.2 μg/ml). Western blot analysis of caspase-9, -8, and -3, and PARP was performed, as described in "Materials and Methods." (B) Cells were treated with IAA (1 mM) and HRP (1.2 μg/ml). At the indicated time points after treatment, phase contrast photomicrographs were then taken using a digital video camera. (C) And then, Western blot analysis of caspase-9, -8, and -3, and PARP was performed

cells decreased with IAA/HRP treatment in a concentration-dependent manner. Furthermore, DNA fragmentation assay showed that IAA/HRP induced apoptosis of TCCSUP cells. In agreement with our results, it has also been reported that IAA induces apoptosis of human T24 bladder carcinoma cells transiently transfected with HRP cDNA (Greco et al. 2002a).

Because of the importance of stress-regulated kinase pathways in apoptosis, we first examined the effects of IAA/HRP on the p38 MAP kinase and JNK pathway. It was found that IAA/HRP leads to the activation of p38 and JNK. Recently, we reported that hydrogen peroxide is one of the major mediators of IAA/HRP-induced apoptosis (Kim et al. 2006a). In addition, it is known that ROS, including hydrogen peroxide, may be responsible for JNK and p38 activation (Haddad and Land 2002; Katiyar et al. 2001; Lee et al. 2002). Thus, we propose that hydrogen peroxide induced by IAA/HRP is related to the IAA/HRP-induced activation of JNK and p38.

Caspase activation plays an important role in apoptosis (Hengartner 2000; Nunez et al. 1998). In mammalian cells, the death-receptor pathway and the mitochondrial pathway are deeply involved in the apoptotic process. In the death-receptor pathway, Fas ligand or TNF-α binds to cell surface death receptors, which results in the activation of caspase-8 (Srinivasan et al. 1998). In the mitochondrial pathway, a variety of stimuli induce the release of cytochrome c, which binds to Apaf-1 and activates caspase-9 (Soengas et al. 1999). In the present study, we showed that IAA/HRP activates both caspase-8 and caspase-9. Our results indicate that IAA/HRP activates both the mitochondrial and death-receptor pathways. These two pathways converge at the activation of caspase-3 (Nunez et al. 1998; Stennicke et al. 1998), which leads to the cleavage of PARP (Nicholson et al. 1995). In agreement with these studies, we also demonstrated caspase-3 activation and PARP cleavage after IAA/HRP treatment. As mentioned, hydrogen peroxide is a major mediator of IAA/HRP (Kim et al. 2006a). Furthermore, it has been reported that urothelial cell death is mediated via hydrogen peroxide-induced oxidative stress (Coyle et al. 2008). Thus, hydrogen peroxide by IAA/HRP may be responsible for IAA/HRP-induced TCCSUP cell apoptosis.

In summary, the present study demonstrated that IAA/HRP induces apoptosis in human TCCSUP bladder carcinoma cells, and that both death receptor-mediated and mitochondrial apoptotic pathways are involved in IAA/HRP-induced apoptosis. We suggest that this combined treatment may provide a new therapeutic option to overcome bladder cancer.

4. Experimental

4.1. Materials

IAA, HRP, ascorbic acid, and DCFH-DA (2,7-dichlorofluorescin diacetate) were obtained from Sigma (St. Louis, MO, USA). Antibodies that recognize phospho-specific JNK, total JNK, phospho-specific p38, and total p38 were obtained from Cell Signaling (Danvers, MA, USA). Antibodies against caspase-9 (sc-8355), caspase-8 (sc-7890), caspase-3 (sc-7272), and actin (I-19) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and anti-PARP antibody was obtained from BD Pharmingen (San Diego, CA, USA).

4.2. Cell cultures

TCCSUP human urinary bladder carcinoma cells were obtained from the ATCC (Rockville, MD, USA). The cells were grown in minimum essential medium (MEM) supplemented with 10% FBS, 50 μg/ml of streptomycin, and 50 μg/ml of penicillin at 37 °C in 5% CO_2.

4.3. Crystal violet assay

Cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed using a DCM300 digital camera for a microscope (Scopetek, Inc., Hangzhou, China), which was supported by ScopePhoto software (Scopetek, Inc.). Cell viability was assessed using crystal violet staining assays (Kim et al. 2006b). After treating with the test substances for 24 h, the culture medium was removed. Cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature and then rinsed 4 times. The crystal violet retained by the adherent cells was extracted with 95% ethanol, and the absorbance was determined in

lysates at 590 nm using an ELISA reader (VERSAMax; Molecular Devices, Sunnyvale, CA, USA).

4.4. Western blot analysis

Cells were grown in 60 mm culture dishes, starved of serum for 24 h, and treated with the test substances at the time points indicated. Cell lysates were prepared in M-PER mammalian protein reagent (Pierce, Rockford, IL, USA) containing a complete protease inhibitor mixture (Roche, Mannheim, Germany). Samples were separated on 12% SDS-polyacrylamide gels and were transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% dried milk in PBS containing 0.4% Tween 20. The blots were incubated with the appropriate primary antibodies at a dilution of 1:1000. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with HRP and chemiluminescent substrate (Pierce). The images of the blotted membranes were obtained using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

4.5. Detection of DNA fragmentation

After serum starvation for 24 h, TCCSUP cells were treated with IAA and HRP, as described above. Twenty-four hours later, the cells were harvested. Then, DNAs were isolated using genomic DNA purification kits, according to the manufacturer's recommendations (Axygen Bioscience, San Francisco, CA, USA). Ten micrograms of DNA from each sample were separated by 1.9% agarose gel electrophoresis and visualized by ethidium bromide staining.

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