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Activation of p38 MAPK by damnacanthal mediates apoptosis in SKHep 1 cells through the DR5/TRAIL and TNFR1/TNF-α and p53 pathways

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ABSTRACT

The effect of the natural compound damnacanthal from *Morinda citrifolia* on SKHep 1 cell growth regulation was investigated. Treatment of SKHep 1 cells with damnacanthal for 24 h indicated a dose-dependent antiproliferative activity. Damnacanthal seems to be selective for tumor cell lines, since there is only minimal toxicity against normal hepatocyte cells (FL83B). This is first demonstration that damnacanthal-mediated apoptosis involves the sustained activation of the p38 MAPK pathway, leading to the transcription of the death receptor family genes encoding DR5/TRAIL and TNF-R1/TNF-α genes as well as the p53-regulated Bax gene. The damnacanthal-mediated expression of DR5/TRAIL and TNF-R1/TNF-α results in caspase 8 activation, leading to Bid cleavage. In turn, activated Bid, acting with p53-regulated Bax, leads to cytochrome c released from mitochondria into the cytoplasm. Combined activation of the death receptors and mitochondrial pathways results in activation of the downstream effecter caspase 3, leading to cleavage of PARP. TRAIL- and TNF-α-mediated damnacanthal-induced apoptosis could be suppressed by treatment with caspase inhibitors as well as soluble death receptors Fc:DR5 and Fc:TNF-R1 chimera. Taken together, this study provided first evidence demonstrating that TRAIL-, TNF-α-, and p53-mediated damnacanthal-induced apoptosis require the activation of p38 MAPK and mitochondrion-mediated caspase-dependent pathways.

1. Introduction

Morinda citrifolia L. (Rubiaceae), commonly known as noni, is a small evergreen tree or shrub that is widely distributed throughout the pacific islands, Southeast Asia, and other tropical and semitropical areas. It is also found along the seashore of the Hengchun Peninsula and adjacent islands in Taiwan (Huang, 1998). In Polynesia, *M. citrifolia* has traditionally been used as a folk medicine for arthritis, antibacterial, antiviral, antifungal, antitumor (Kamiya et al., 2005), antihelmin, analgesic, hypotensive, anti-inflammatory, and immune enhancing effects (Wang et al., 2002).

Apoptosis plays a critical role in developmental modeling, embryonic development, the maintenance of homeostasis, and immune repertoires (Oppenheim, 1991). The deregulation of apoptosis results in diseases, including autoimmune, cancer, and neurodegenerative disorders (Evan and Vousden, 2001). Apoptosis is also the basis for therapies designed to target cancerous cells and limit cytotoxicity that results from drug treatment. Thus, the molecular mechanisms and signaling pathways regulating apoptosis are of great significance. Apoptosis occurs via two pathways: the death receptor pathway, initiated by activation of members of the death receptor superfamily, leading to caspase 8 activation (Nagata, 1999) and the mitochondrial pathway, resulting in the mitochondrial release of cytochrome c and caspase 9 activation (Wang et al., 1996). These two pathways converge upon the activation of caspase 3. Mitochondrial involvement in apoptosis is determined by the balance of antiapoptotic and proapoptotic Bcl-2 family members. Importantly, the extrinsic and intrinsic pathways are linked via the function of the protein Bid (Wang et al., 1996). Previous reports suggested that apoptosis is regulated by the p38 MAPK and JNK cellular stress pathways (Kyriakis and Avruch, 2001). These pathways mediate proliferation and differentiation (Davis, 2000). Evidence in support of p38 MAPK and JNK pathways in regulating apoptosis is derived from studies employing treatments simulating cellular stress. These stresses include the presence of proinflammatory cytokines and drugs, growth factor withdrawal, UV radiation, and overexpression of constitutively active effectors (Berberich et al., 1996; Dickens et al., 1997; Chuang et al., 2000; Hatai et al., 2002).

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In our continuing investigation for cancer chemopreventive constituents from Taiwanese medicinal plants, damnacanthal, an anthraquinone derivative, was isolated from the methyl alcohol extract of the stem wood of *M. citrifolia*. The structure was elucidated by spectroscopic methods. The present study was undertaken to understand the role of activation of the p38 MAPK pathway in damnacanthal-induced apoptosis. In this study, we demonstrate for the first time how the activation of the p38 MAPK pathway mediates apoptosis in response to damnacanthal treatment, and we provide a mechanistic understanding of how damnacanthal mediates apoptosis in human liver adenocarcinoma SKHep 1 cells. We have illustrated that damnacanthal-mediated apoptosis involves the sustained activation of the p38 MAPK pathway, resulting in the transcriptional induction of the DR5/TRAIL, TNF-R1/TNF-α, and the p53-regulated Bax genes. In turn, DR5/TRAIL and TNF-R1/TNF-α expression activates the death receptor pathways and caspase 8, followed by the involvement of the intrinsic mitochondrial apoptotic pathway, which leads to the release of cytochrome *c* and the activation of caspases-9 and -3 and apoptosis.

2. Materials and methods

2.1. Plant materials

The stem wood of *M. citrifolia* was collected in Pingtung County, Taiwan in July, 2004. The plant material was identified by Professor Sheng-Zehn Yang, Curator of Herbarium, National Pingtung University of Science and Technology, where a voucher specimen was deposited.

2.2. Extraction, isolation, and identification of damnacanthal

Air-dried pieces of the stem wood (32 kg) of *M. charantia* were extracted with methanol (3×80 l) at room temperature (7 days each). The methanol extract was evaporated in vacuo to afford a black residue, which was suspended in H_2O (3 l), and then partitioned sequentially, using ethyl acetate and *n*-butanol (3×2 l) as solvent. The ethyl acetate fraction (300 g) was passed through a Si gel column (72×12 cm), using solvent mixtures of *n*-hexane and ethyl acetate with increasing polarity as an eluent. Twenty fractions (Fr) were collected as follows: fraction 1 [4000 ml, *n*-hexane], Fr 2 [3000 ml, *n*-hexane-ethyl acetate (49:1)], Fr 3 [4000 ml, *n*-hexane-ethyl acetate (45:5)], Fr 4 [4000 ml, *n*-hexane-ethyl acetate (40:10)], Fr 5 [4000 ml, *n*-hexane-ethyl acetate (37:13)], Fr 6 [3000 ml, *n*-hexane-ethyl acetate (35:15)], fr. 7 [3000 ml, *n*-hexane-ethyl acetate (33:17)], Fr 8 [4000 ml, *n*hexane-ethyl acetate (30:20)], fr. 9 [3000 ml, *n*-hexane-ethyl acetate (28:22)], Fr 10 [3000 ml, *n*-hexane-ethyl acetate (25:25)], Fr 11 [3000 ml, *n*-hexane-ethyl acetate (24:26)], Fr 12 [4000 ml, *n*-hexane-ethyl acetate (22:28)], Fr 13 [3000 ml, *n*hexane-ethyl acetate (20:30)], Fr 14 [3000 ml, *n*-hexane-ethyl acetate (17:33)], Fr 15 [4000 ml, *n*-hexane-ethyl acetate (15:35)], Fr 16 [4000 ml, *n*-hexane-ethyl acetate (12:38)], Fr 17 [4000 ml, *n*-hexane-ethyl acetate (10:40)], fr. 18 [4000 ml, *n*-hexane-ethyl acetate (5:45)], Fr 19 [3000 ml, *n*-hexane-ethyl acetate (1:49)], and Fr 20 (6000 ml, ethyl acetate). Further purification of the fraction 12 was performed on silica gel column (from dichloromethane:ethyl acetate = 200:1 to 100% ethyl acetate) to give damnacanthal (530 mg).

NMR spectra were obtained in deuterated chloroform ($CDCl_3$) at a constant temperature controlled and adjusted to around 300 K on a Varian Mercury plus 400 NMR spectrometer, and the residual proton resonance (chloroform) of deuterated chloroform was used as internal shift reference. The 2D NMR spectra were recorded by using standard pulse sequences. EIMS was recorded on Finnigan TSQ-700 mass spectrometer. TLC was performed by using Si gel 60 F254 plates (Merck). Column chromatography was performed on Si gel (230–400 mesh ASTM, Merck). HPLC was performed on a Hitachi L-2130 apparatus equipped with a Hitachi L-2455 photodiode array detector. For semi-preparative HPLC separation, a Lichrosorb Si gel 60 (5 μm) column (250×10 mm) was used.

2.3. Cancer cell lines and normal hepatocyte cells

Various cancer cell lines and normal hepatocyte cells were from American Type Culture Collection (ATCC, The Global Bioresource Center, USA) (Table 1). Six well plates were seeded with 5×10^5 cells. At 70% confluence, different cell lines were grown in specific medium containing10% heat inactivated fetal bovine serum, penicillin G50 (50 units/ml), streptomycin (50 μg/ml), and fungizone (1.25 μg/ml) at 37 °C in a 5% CO_2 incubator.

2.4. Antibodies

Monoclonal antibodies, including tumor necrosis factor apoptosis inducing ligand (TRAIL), death receptor (DR)5, tumor necrosis factor (TNF)-α, TNF-R1, tumor necrosis factor receptor 1-associated death domain protein (TRADD), FasL, Fas and caspase 9, ATF-2, p38 mitogen-activated protein kinase (MAPK), p- p38 MAPK, the extracellular signal-regulated kinase (ERK)1/2, p-ERK1/2, the stress-activated protein kinase/Jun-amino-terminal kinase (JNK/SAPK), and p-JNK) and rabbit polyclonal antibodies (caspase 8, Bid, and caspase 3) were purchased from Cell Signaling. Anti-p53 (ser46) rabbit polyclonal antibody was from R&D. Anti-Bax rabbit polyclonal antibody was purchased from Upstate (Lake Placid, USA). Anti-cytochrome c rabbit polyclonal antibody and anti-human cytochrome c oxidase (Cox-4) mouse monoclonal antibody were from Clontech (Mountain View, CA, USA). Anti-Smac mouse monoclonal antibody, anti-apoptosis-inducting factor (AIF) rabbit polyclonal antibody, and anti-PARP mouse monoclonal antibody were from BD Biosciences (Sparks, MD, USA). Anti-actin mouse monoclonal antibody and anti-Apaf-1 mouse monoclonal antibody were from Chemicon (Temecula, CA, USA). The goat-anti-rabbit and goat-anti-mouse IgG (H + L) HRP conjugate were from KPL and Invitrogen (Carlsbad, USA). Proteins were visualized using the ECL detection system (Roche Applied Sciences, Mannheim, Germany).

2.5. Src and MAPKs inhibitors

In this study, we investigated the role of Src and MAPKs and the involvement of their downstream targets in damnacanthal-induced apoptosis in SKHep 1 cells. Firstly, we blocked Src and MAPKs signalings using Src and MAPKs inhibitors (Davies et al., 2000; Ji et al., 2009). The Src and MAPK inhibitors for signaling molecules were purchased from Calbiochem (San Diego, CA, USA). The working concentrations of the various inhibitors were as follows: 5 μM SU6656 (2-oxo-3-(4,5,6,7-tetrahydro-1H-indol-2-ylmethylene)-2,3-dihydro-1H-indole-5-sulfonic acid dimethylamide), 20 μM PD98059 (2-(29-amino-39-methoxyphenyl)-oxanaphthalen-4-one), 5 μM SB202190 (4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5- (4-pyridyl) 1H-imidazole), and 10 μM SP600125 (anthrapyrazolone) which block Src, ERK1/2, p38 MAPK, JNK/SAPK, respectively (Bain et al., 2003; Lin et al., 2009).

Table 1
Various cell lines used in this study.

Cell lines	ATCC no.	Medium
SKHep 1 (human liver adenocarcinoma)	HTB-52	DMEM
Hep G2 (hepatocellular carcinoma)	HB-8065	EMEM
LNCap (prostate carcinoma)	CRL-1740	RPMI
PC-3 (prostate adenocarcinoma)	CRL-1435	F-12 K
MCF-7 (breast adenocarcinoma)	HTB-22	EMEM
FL83B (hepatocyte cells)	CRL-2390	DMEM

2.6. DNA fragmentation analysis and MTT assay

SKHep1 cells treated with different concentrations of damnacanthal were harvested, washed, and lysed with lysis buffer (50 mM Tris, pH 7.5, 20 mM EDTA, 1% Nonidet p-40). The supernatant was collected and incubated with RNase A at a final concentration of 500 μg/ml for 1 h at 37 °C followed by proteinase K at 500 μg/ml for at least 2 h at 55 °C. The DNA was extracted using the phenol/chloroform and precipitated with ethanol in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and run on a 1.5% agarose gel for DNA fragmentation analysis.

The MTT assay was performed to evaluate the proliferation of viable cells. Briefly, 5000 cells were placed in each well of a 96-well plate with 200 μl medium per well, and after specific treatment, 20 μl (3-(4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) solution was added at a concentration of 5 mg/ml, followed by incubation (37C, 5% CO_2) for 4 h to allow the MTT to be metabolized. The medium was then removed and cells were resuspended in formazan in 200 μl DMSO. The optical density (OD) was read at 560 nm, and was found to be directly correlated with cell quantity, as expected.

2.7. Blocking assay and cell death ELISA

To study the role of Fas, TNF-α, and TRAIL plays in damnacanthal-induced apoptosis pathway, we performed blocking assays with soluble death receptors Fc:Fas, Fc:TNF-R1, and Fc:DR5 (Santa Cruz Biotechnology, USA). To further elucidate the mechanisms involved in damnacanthal-induced apoptosis, we also determined the contribution of each caspase, by employing cell-permeable peptide inhibitors for caspase 8 (Z-IETD-FMK), caspase 9 (Z-LEHD-FMK), and caspase 3 (Z-DEVD- FMK). In this study, SKhep1 cells were pretreated with soluble receptors (20 μg/ml) or caspase inhibitors (20 μM) then treated with damnacanthal (68 μM). The cells were harvested and apoptosis induction was checked by cell death ELISA. Apoptoic cell death was quantitated *in vitro* by using ELISA according to procedures provided by Roche Applied Sciences. Briefly, 10^3 cell lysate is placed into a streptoavidin-coated microtiter plate. A mixture of anti-histone-biotin and anti-DNA-peroxidase are added and incubated. During incubation, the anti-histone antibody binds to the histone components of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin via its biotinylation. Additionally, the anti-DNA-peroxidase reacts with the DNA components of the nucleosomes. Color development was carried out by adding ABTS substrate solution to each sample. The absorbance at 405 nm was measured.

2.8. Western blot assay

The expression and translocation of the death receptors Fas, TNF-R1, and DR5 (Santa Cruz) were analyzed by Western blot assay and confocal microscope. Previous study suggested that activation of Fas receptors by Fas, DR5 by TRAIL, and TNF-R1 receptors by TNF-α can induce caspase 8 dependent cleavage of Bid (Li et al., 1998). We therefore wanted to examine whether damnacanthal treatment induced the activation of caspase 8. Bid is a proapoptotic member of the Bcl-2 protein family. Cleaved Bid can facilitate the release of cytochrome c from the mitochondrion and leads to subsequent apoptosome-mediated activation of caspase 9, caspase 3, and PARP (Luo et al., 1998). We wished to investigate whether Bid was cleaved following damnacanthal treatment, and if this cleavage was dependent on caspase 8 activation.

Several proteins with proapoptotic functions are localized to the mitochondria. These include cytochrome c, Smac/DIABLO, AIF, and endonuclease G (Liu et al., 1996). In the present study, mitochondrion-free lysates prepared from both mock- and damnacanthal-treated cells at the indicated concentrations were analyzed by Western blot assay for the presence of cytosolic cytochrome c and other mitochondrial molecules (AIF, and Smac/DIABLO). In addition, the levels of p53 and Bax translocation from cytosol to mitochondria were also examined.

Damnacanthal-treated cells were harvested, washed, and lysed in 0.5 ml RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet p-40, 1 mM PMSF). The supernatant and pellet were collected and mixed with Laemmli sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Amersham Pharmacia Biotech, New Territories, Hong Kong) for immunobloting. Blots were probed with the respective primary antibodies (1:3000) and secondary antibodies (1:3000). Proteins were visualized using the ECL detection system (Roche Applied Sciences, Mannheim, Germany).

2.9. Evaluation of mitochondrial membrane potential with a lipophilic cationic dye

For assessment of mitochondrial membrane potential (ΔΨm), damnacanthal-treated cells were stained with a fluorescent lipophilic cationic reagent known as MitoCapture (provided in the Mitochondria BioAssay™ kit, US Biological). This dye was trapped in mitochondria with normal ΔΨm and released from mitochondria with abnormal ΔΨm into the cytosol.

2.10. Statistical analysis

All data were analyzed using independent sample t-test and are expressed as averages of three independent experiments. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Isolation and identification the structure of damnacanthal

This is the first report that the large quantity of damnacanthal was isolated from stem wood of *M. citrifolia*. The complete ^1H and ^{13}C NMR assignments of damnacanthal are presented. The Structure of damnacanthal is shown in Fig. 1. Analytical data are as follows: ^1H NMR (400 MHz, $CDCl_3$): δ 4.11 (3 H, s, 1-OCH_3), 7.66 (1H, s, H-4), 7.76 (1H, ddd, $J = 1.6$, 7.8, and 7.8 Hz, H-7), 7.81 (1H, ddd, $J = 1.6$, 7.8, and 7.8 Hz, H-6), 8.23 (1H, dd, $J = 1.6$ and 7.8 Hz, H-5), 8.27 (1H, dd, $J = 1.6$ and 7.8 Hz, H-5), 10.45 (1H, s, CHO), 12.27 (1H, s, 3-OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 64.7 (OCH_3), 113.1 (C-4), 117.6 (C-9_a), 118.0 (C-4_a), 127.1 (C-5), 127.4 (C-8), 132.5 (C-8_a), 133.7 (C-7), 134.9 (C-6 and 10_a), 141.6 (C-2), 166.6 (C-1 and 3), 180.2 (C-9), 181.9 (C-10), 195.5 (CHO); EI-MS (70 eV) *m/z* (rel.int.) 282 $[M]^+$ (30), 267 (10), 264(12), 254(100), 225 (23), 208 (9), 196 (7), 139 (8), 126 (5).

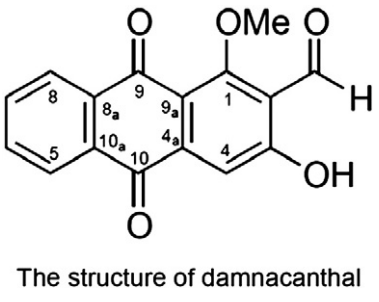

Fig. 1. A 10 mg/ml solution of damnacanthal in deuterated chloroform was used to determine the structure of damnacanthal.

3.2. Effect of damnacanthal on the proliferation of various cancer and normal cell lines

The inhibitory effect of damnacanthal on the proliferation in various cancer and normal cell lines was investigated by MTT assay. As shown in Fig. 2, damnacanthal inhibited the proliferation of various cancer cell lines in a dose-dependent manner. Damnacanthal seems to be selective for tumor cells, since there is only minimal toxicity against normal hepatocyte cells (FL83B).

3.3. Damnacanthal-induced apoptosis in SKHep 1 cells

As shown in Fig. 3A, damnacanthal-treated SKHep 1 cells produced oligonucleosomal DNA ladders in a dose-dependent manner. When SKHep 1 monolayers were treated with damnacanthal, the extensive oligonucleosomal DNA laddering could be detected 34 μM and the intensity of the ladder bands increased with doses (Fig. 3A).

In this study, addition of 20 μM caspase 8 inhibitor of Z-IETD-FMK inhibits damnacanthal-induced apoptosis by approximately 68% at 24 h after treatment (Fig. 3B). Similarly, 20 μM of caspase 9 inhibitor Z-LEHD-FMK inhibits apoptosis by 92% at 24 h after treatment (Fig. 3B). Similar assays performed with the caspase 3 inhibitor (Z-DEVD-FMK) demonstrated a 84% inhibition (Fig. 3B). Our observations identified caspase 8 as the initiator caspase in damnacanthal-induced apoptosis.

3.4. Involvement of p38 MAPK signaling pathway in damnacanthal-induced apoptosis in SKHep 1 cells

Having demonstrated damnacanthal-induced apoptosis in SKHep 1 cells, we next want to clarify whether the MAPK and Src pathways play significant roles in mediating this apoptotic phenomenon. We first blocked MAPK signaling by MAPK inhibitors PD98059, SB203580, and SP600125, and inhibited Src by SU6656, and then we treated the cells with damnacantha and measured the levels of apoptosis. The results revealed that p38 MAPK, but not ERK 1/2 and JNK, were significantly activated by damnacanthal (Fig. 4A; upper panel). When cells treated with SB201290 significantly reversed the apoptosis induction by damnacanthal, cells treated with both ERK1/2 inhibitor (PD98059) and JNK inhibitor (SP600125) were unaffected (Fig. 4B). These results suggest that MAPK p38, but not ERK1/2 and JNK, are involved in

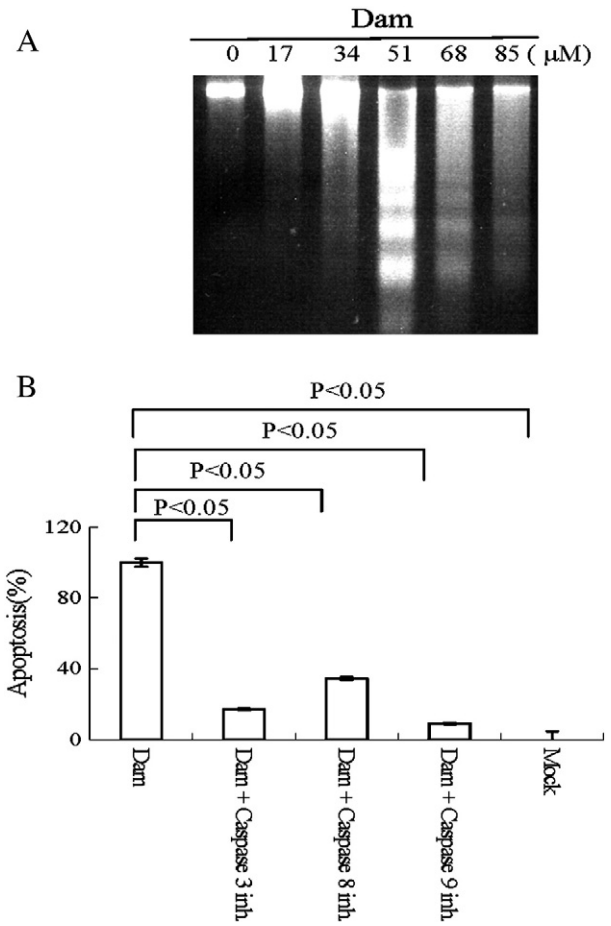

Fig. 3. Damnacanthal-induced apoptosis in SKHep 1 cells and suppression by caspase inhibitors 3, 8, and 9. (A) Damnacanthal-induced apoptosis was analyzed by DNA fragmentation. (B)To identify whether damnacanthal-induced apoptosis is entirely dependent on caspase activation, cells were pretreated with the caspase 3 inhibitor Z-DEVD-FMK, caspase 8 inhibitor Z-IETD-FMK and caspase 9 inhibitor Z-LEHD-FMK and then treatment with damnacanthal (68 μM). Cells were harvested at 24 h after treatment and apoptosis was analyzed by cell death ELISA. The results are the mean ± standard deviation (S.D.) of three separate experiments (*$P<0.05$).

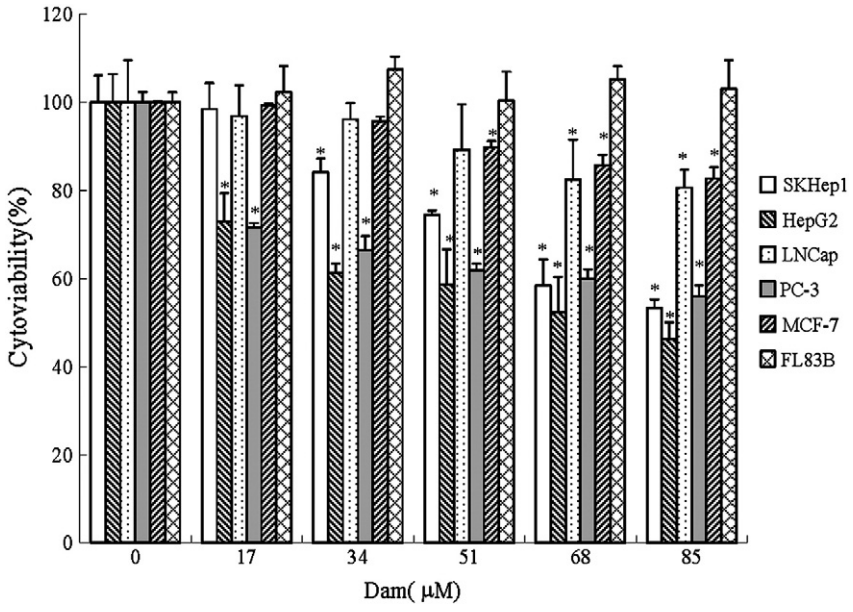

Fig. 2. Effect of damnacanthal on the proliferation of different cell lines. Cells were seeded in 96-well culture plates. Various concentrations of damnacanthal were added to plates and were incubated for 48 h. After the end of incubation, viable cells were determined by MTT assay. The damnacanthal-treated cells were compared with untreated cells. The results are the mean ± standard deviation (S.D.) of three separate experiments (*$P<0.05$).

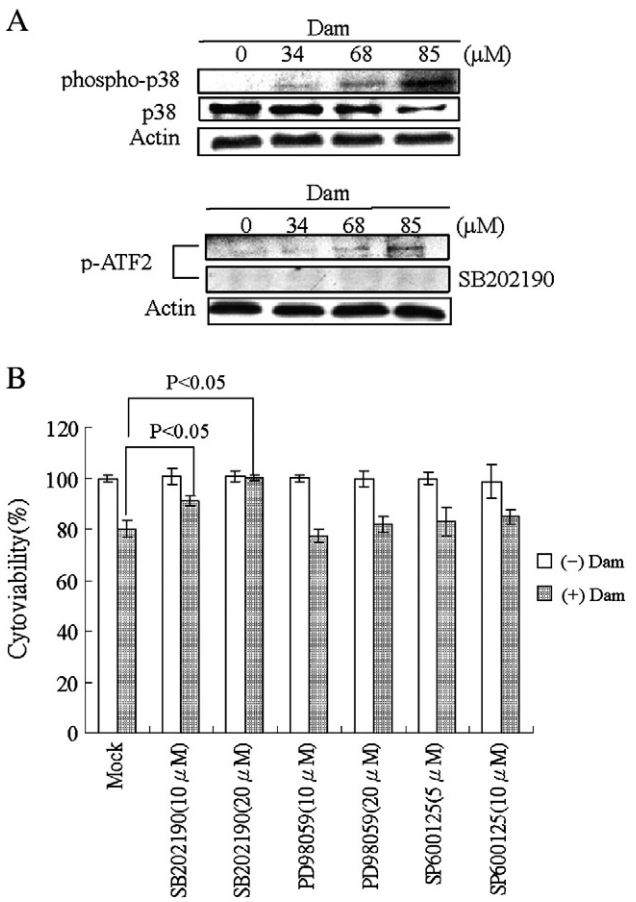

Fig. 4. Activation of p38 MAPK signaling pathway in damnacanthal-treated SKHep 1 cells. (A) The damnacanthal up-regulated the level of phosphorylated p38 MAPK and ATF-2 in SKHep 1 cells in a dose-dependent manner. This experiment was repeated three times, and representative blots are shown. (B) p38 MAPK inhibitor SB202190, but not ERK1/2 inhibitor PD98059 and JNK/SAPK inhibitor SP600125, reversed the apoptosis induction by damnacanthal. The results are the mean ± standard deviation (S.D.) of three separate experiments (*$P<0.05$).

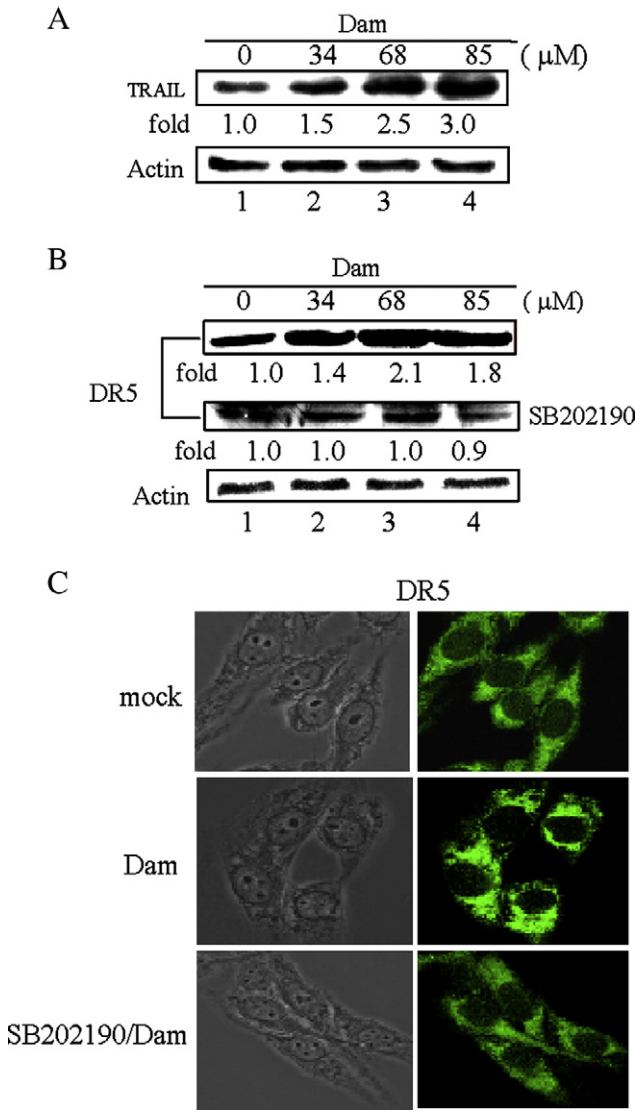

Fig. 5. Damnacanthal-induced apoptosis mediated by TRAIL. (A–B) To determine whether TRAIL and death receptor DR5 was up-regulated during damnacanthal-induced apoptosis, SKHep 1 cells were treated with indicated concentrations of damnacanthal. At 24 h after treatment, the cells were harvested and TRAIL and death receptor DR5 expression were confirmed by Western blot analysis. (C) Aggregation of death receptor DR5 on surface of damnacanthal-treated cells was also observed by confocal microscope. The expression of death receptor DR5 on surface can be suppressed by p38 MAPK inhibitor SB202190. The intensity of each protein was calculated using Photocapt (Vilber Lourmat).

damnacanthal-induced apoptosis in SKHep 1 cells. Further, inhibition of Src with an inhibitor (SU6656) did not alter p38 MAPK and p53 activation (data not shown). JNK and p38 MAPK have been shown to phosphorylate a number of transcription factors such as c-Jun and ATF-2. c-Jun is phosphorylated specifically by JNK and ATF-2 can be phosphorylated by both JNK and p38 MAPK (Davis, 2000; Kyriakis and Avruch, 2001). In this study, the ATF-2 which is downstream targets of p38 MAPK was found to be activated in damnacanthal-treated cells (Fig. 4A; lower panel) while c-Jun activation was not detected (data not shown). Our results demonstrate that damnacanthal induced ATF-2 phosphorylation which is normally activated in response to signals that converge on stress-activated protein kinas p38 MAPK.

3.5. Signaling through the DR5 and TNF-R1 death receptors is required for damnacanthal-induced apoptosis in SKHep 1 cells

In the present study, levels of TRAIL and TNF-αbut not FasL increased with doses of damnacanthal (Figs. 5A and 6A). Since activation of TRAIL and TNF-α signaling are often accompanied by the translocation of DR5 and TNF-R1 to the cell surface from intracellular stores, surface expression of DR5 and TNF-R1 proteins were analyzed by Western blot and confocal microscope. The expression levels of DR5, TNF-R1, and TRADD proteins that increased with treatment doses of damnacanthal were detected by Western blot assay and could be masked in cells treated with SB202190 (Figs. 5B and 6B, D). It was also found that damnacanthal treatment upregulated DR5 and TNF-R1 expression on the surface of SKHep 1 cells (Figs. 5C and 6C). Further, the translocation of DR5 and TNF-R1 to surface of SKHep 1 cells could be blocked in cells treated with SB202190 (Figs. 5C and 6C).

Having shown that both TRAIL and TNF-α were upregulated we next further determined whether TRAIL:DR5 and TNF-α:TNF-R1 binding were required for damnacanthal-induced apoptosis. Soluble death receptors Fc:DR5 and Fc:TNF-R1 significantly blocked damnacanthal-induced apoptosis (Fig. 7), suggesting that both TNF-α:TNF-R1and TRAIL:DR5 binding are required for damnacanthal induced apoptosis. Take together, our results suggests that damnacanthal-induced apoptosis is mediated by both TRAIL and TNF-αsignaling pathways.

3.6. Caspase 8 activation is required for damnacanthal-induced apoptosis in SKHep 1 cells

As shown in Fig. 8B, damnacanthal treatment induces the activation of caspase 8 as evidenced by disappearance of the full-length

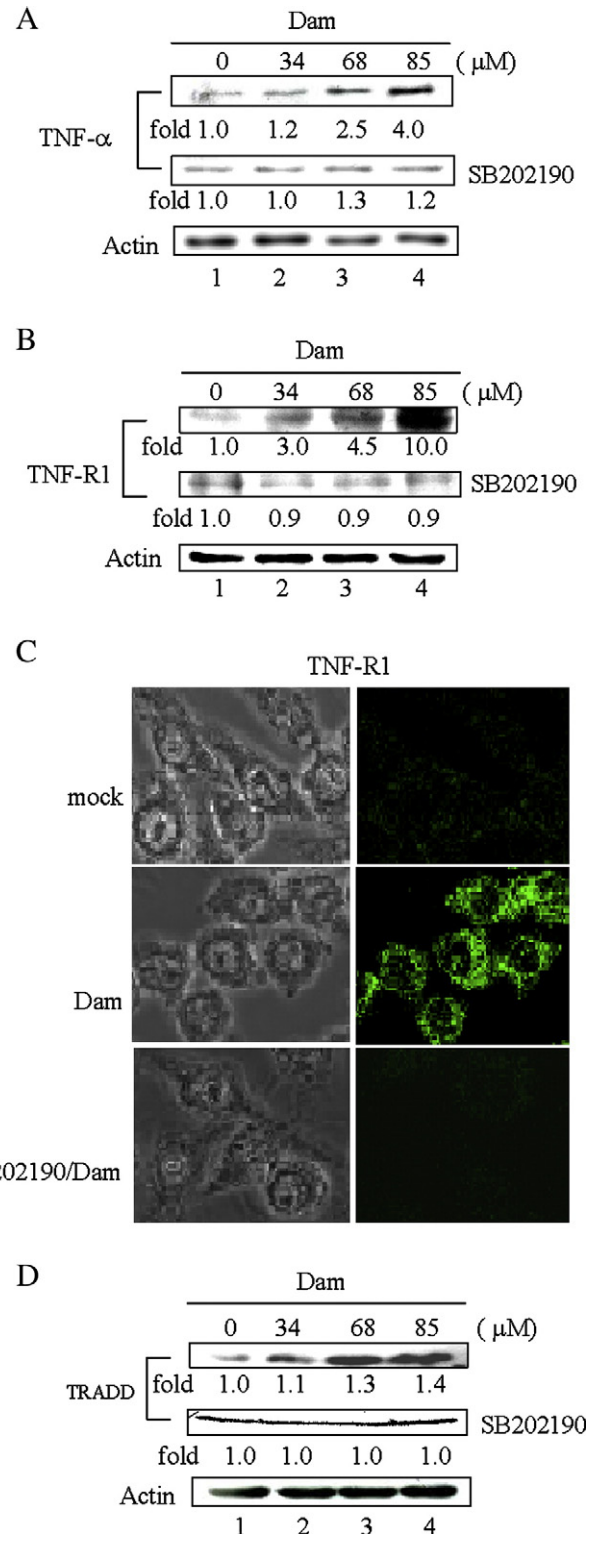

Fig. 6. Damnacanthal-induced apoptosis mediated by TNF-α. To determine whether TNF-α and death receptor TNF-R1 was up-regulated during damnacanthal-induced apoptosis, SKHep 1 cells were treated with indicated concentrations of damnacanthal. At 24 h after treatment, the cells were harvested and TNF-α (A) and death receptor TNF-R1 (B), and TRADD (D) expression were confirmed by Western blot analysis. (C) Death receptor TNF-R1 surface expression was observed by confocal microscope and can be suppressed by p38 MAPK inhibitor SB202190. This experiment was repeated three times, and representative blots are shown. The intensity of each protein was calculated using Photocapt.

proenzyme. Additional evidence in support of above conclusion is derived from the kinetics of Bid cleavage, a known substrate of caspase 8. It was observed that Bid cleavage occurred (Fig. 8B) accompanied by

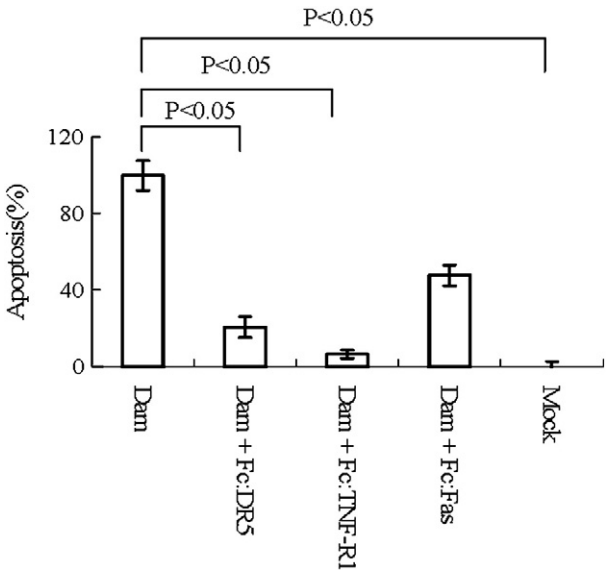

Fig. 7. Soluble death receptors Fc:DR5 and Fc:TNF-R1 significantly blocked damnacanthal-induced apoptosis. SKHep 1 cells were pretreated with soluble receptors (20 μg/ml) then treatment with damnacanthal (68 μM). The cells were harvested at 24 h after treatment, and apoptosis induction was examined by cell death ELISA. The results are the mean ± standard deviation (S.D.) of three separate experiments (*$P<0.05$).

major activation of caspase 8. Caspase 8-mediated Bid cleavage could be blocked by the treatment with MAPK p38 inhibitor SB20219 (Fig. 8B) and caspase 8 inhibitor (data not shown), suggesting that p38 MAPK is required for damnacanthal-induced apoptosis. Damnacanthal-induced apoptosis was also suppressed by caspase 8 inhibitor (Fig. 3B).

3.7. Damnacanthal-mediated apoptosis is required to trigger the release of cytochrome c from mitochondria to cytoplasm and Bax translocation from cytosol to mitochondria

Furthermore, activated Bid translocates to mitochondria and induces cytochrome c release, thus mediating the activation of caspase 9. In the present study, the presence of cytosolic cytochrome c was observed while other mitochondrial molecules (AIF, and Smac/DIABLO) were not detected in the cytoplasm of damnacanthal-treated cells (Fig. 8C). Further, damnacanthal-induced cytochrome c release from mitochondria to cytoplasm in cells could be masked by p38 MAPK inhibitor SB20219 (Fig. 8C). Anti-sera directed against the mitochondrial integral membrane protein cytochrome c oxidase (Cox-4) were also employed to ensure the samples were free of mitochondrial contamination. AIF is a mitochondrial protein that can translocate to the nucleus, leading to nuclear apoptosis. AIF localization in damnacanthal-treated cells using mitochondrion-free lysates was also examined. The nuclear extracts from both mock- and damnacanthal-treated cells were examined to ensure that AIF had not translocated to the nucleus. Nuclearly localized AIF following damnacanthal treatment was not detected. These results demonstrate that the mitochondrial proapoptotic proteins cytochrome c but not AIF and Smac/DIABLO are released in damnacanthal-treated cells.

In addition, the increase levels of p53 and Bax translocation from cytosol to mitochondria were also observed in our case (Fig. 8 A and C). The level of phosphorylated p53 was also reduced by treatment with p38 MAPK inhibitor SB20219 (Fig. 8A). Taken together, our results suggest that p53 is also activated and involved in damnacanthal-induced apoptosis.

3.8. Damnacanthal triggers the dissipation of the membrane potential

Since our data indicated the release of cytochrome c from the mitochondria to the cytosol of damnacanthal-treated cells, the

mitochondrial apoptotic events that are downstream to caspase 9 activation in damnacanthal-treated cells were investigated. The state of the mitochondrial membrane potential was examined by staining mock- and damnacanthal-treated cells with Mitocapture according to the manufacturer's instructions. The collapse of ΔΨm is a common feature of apoptosis. The collapse of ΔΨm may occur directly during activation of the mitochondrial pathway, leading to the activation of caspases. Alternatively, the collapse of ΔΨm may result from cross talk following activation of the extrinsic pathway. The sample was visualized using a confocal microscope. We differentiated normal cells with a high mitochondrial membrane potential from those with low ΔΨm by their fluorescent color. Fig. 8D shows mock-treated cells stained with red fluorescence, indicating a normal mitochondrial membrane potential, where as damnacanthal-treated cells show a green fluorescent signal characteristic of loss mitochondrial membrane potential.

3.9. Activation of caspases 9 and 3 as well as cleavage of PARP Occur in damnacanthal-treated cells

Having shown that cytochrome c is released from the mitochondria of damnacanthal-treated cells, we next wanted to examine whether Apaf-1 and caspase 9 was activated in damnacanthal-treated cells. Apaf-1 was up-regulated following the cytochrome c release to cytoplasm (Fig. 8E). As shown in Fig. 8E, damnacantha induced the activation of caspase 9 as evidenced by the presence of cleaved caspase 9. No procaspase-9 cleavage occurred in mock-treated cells.

Having shown that damnacanthal-treated cells were associated with activation of mitochondria-associated initiator caspases, we next want to determine which effector caspases were subsequently activated. The cleavage of caspase-3 and proteolytic cleavage of PARP in damnacanthal-treated cells was examined. PARP, a 116-kDa enzyme implicated in DNA single-strand damage repair, has been shown to be cleaved into two specific fragments (85 and 23 kDa) during the onset of apoptosis (Los et al., 2002). Fig. 8F shows the cleavage of PARP at damnacanthal-treated cells but not in the mock-treated cells. We further wanted to know whether the cleavage of PARP correlated with the activation of caspase 3, a cysteine protease known to use PARP as a substrate *in vitro* (Los et al., 2002). As shown in Fig. 8F, damnacanthal induced the activation of caspase 3 as evidenced by disappearance of the full-length proenzyme and the cleavage could be blocked by p38 MAPK inhibitor SB202190, further confirming that damnacanthal-induced apoptosis was executed by p38 MAPK- and caspase-dependent pathways.

4. Discussion

Damnacanthal was first isolated from noni fruit, a traditional Tahaitian fruit commonly used as a folk medicine by Polynesians for over 2000 years (Wang et al., 2002). It has also been demonstrated that damnacanthal reverts the morphology of K-ras transformed cells to normal one (Hiramatsu et al., 1993) and that it is a potent and selective inhibitor of $p56^{lck}$ tyrosine kinase activity (Faltynek et al., 1995). Our current data provide a novel in-depth view of damnacanthal-mediated apoptosis in SKHep 1 cells. The principal findings of this work suggest activation of p38 MAPK by damnacanthal-mediated apoptosis via induction of TNF-R1/TNF-α, DR5/TRAIL, and p53 expression. A number of anticancer agents, including taxol, daidzein, bufalin, and emodin have been isolated from natural products and used for treatment of cancer (Bacus et al., 2001). The present problems of cancer treatment are not only how to effectively increase anticancer activities but also how to reduce side effects. It is found that damnacanthal isolated from stem wood of noni fruit seems to be selective for tumor cells and possesses minimal toxicity against normal cells.

Several previous studies have confirmed the critical apoptosis-inducing function of the MAPK and Src tyrosine kinase pathways (Chuang et al., 2000). JNK, p38 and ERK1/2 are well characterized subgroups of a large MAPK family, but functionally different (Chuang et al., 2000). In general, the ERK cascade is activated for proliferation and survival by growth factors (Lewis et al., 1998), while p38 MAPK and JNK pathways are usually stimulated by apoptosis and genotoxic agents (Chuang et al., 2000). This investigation was initiated to determine whether MAPK pathways play a role in damnacanthal-induced apoptosis. In the current study, damnacanthal triggers an apoptosis process that transiently induces a series of early response genes of p38 MAPK and ATF-2. Interleukin (IL)-1α, IL-1β, IL-6, IL-8, IL-12 and TNF-α have been shown to mediate the apoptotic response,

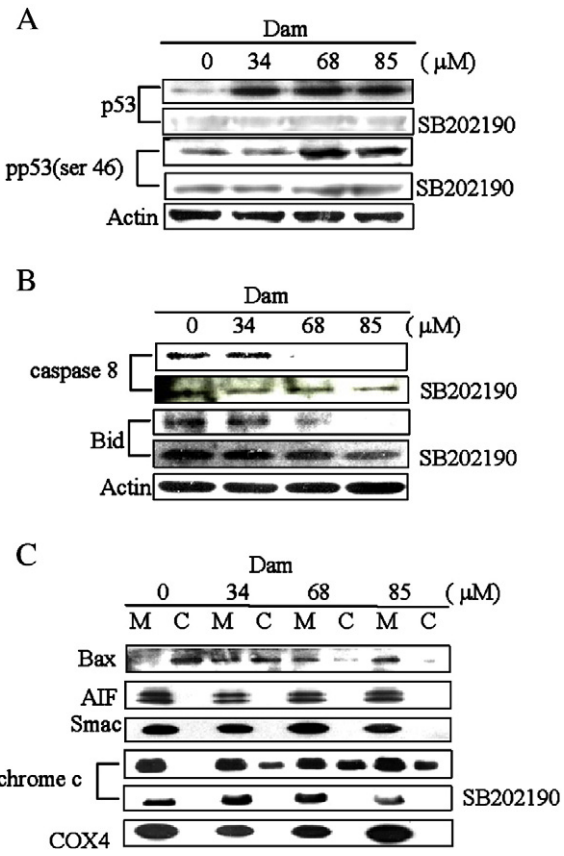

Fig. 8. Activation of p53, mitochondrial, and caspase-dependent pathways are required for damnacanthal-induced apoptosis in SKHep 1 cells. Cells treated with indicated concentrations of damnacanthal were harvested 24 h after treatment. Caspase activation was confirmed by Western blot analysis. (A) The increase level of phosphorylated p53 was observed and reduced by treatment with p38 MAPK inhibitor SB20219. (B)Activation of caspase 8 and cleavage of Bid following damnacanthal treatment. The activation of caspase 8 was evidenced by the disappearance of the full-length proenzyme (procaspase 8). The reductions in the level of procaspase 8 decreased with dosage. The activation of caspase 8 and Bid was inhibited by p38 MAPK inhibitor SB202190. (C) Cytochrome c released from mitochondria to cytoplasm and Bax from cytoplasm to mitochondria was detected. Cytochrome c released from mitochondria to cytoplasm was prevented in cells treated with p38 MAPK inhibitor SB202190. Both AIF and Smac/DIABLO were not detected in the cytoplasm of damnacanthal-treated cells. (D) The mitochondrial membrane potential changes during damnacanthal-treated cells were detected. The mitochondrial function 24 h post treatment was evaluated using a MitoCapture reagent, which contains a lipophilic cationic dye. The latter fluoresces differently in healthy cells (where it aggregates into a fluorescent red polymer within mitochondria) and apoptotic cells (where it remains a fluorescent green monomer in cytoplasm because it cannot aggregate in mitochondria). (E) Apaf-1 was up-regulated and levels increased with the doses. Caspase 9 was activated and the level increased with doses. (F) The reductions in the level of procaspase 3 decreased with dose of damnacanthal. The activation of caspase 3 was inhibited by p38 MAPK inhibitor SB202190. PARP has been cleaved into two specific fragments (85 and 23 kDa) and the levels increased with dose of damnacanthal. The large fragment (85 kDa) is indicated by the arrow. This experiment was repeated three times, and representative blots are shown.

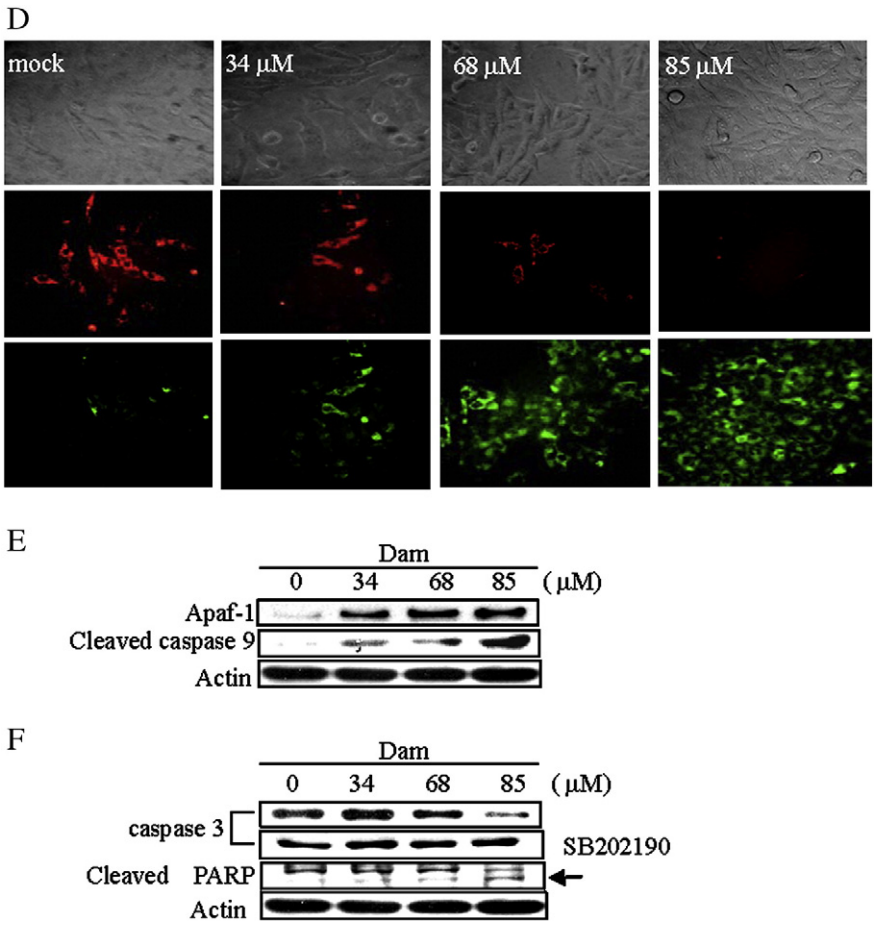

Fig. 8 (*continued*).

and their activation can be mediated by p38 MAPK (Kalra and Kumar, 2004). The p38 MAPK inhibitor significantly protected cells from damnacanthal-induced apoptosis. In contrast, inhibition of Src, JNK, ERK1/2 had no effect on damnacanthal-mediated apoptosis. Damnacanthal increases TNF-α, TRAIL, and p53 transcription, translation and secretion, as well as TNF-R1 and DR5 receptor expression on surface. Inhibition of p38 MAPK also reduced damnacanthal-induced TNF-α, TRAIL, and p53 up-regulation. Taken together all data suggest that the p38 MAPK pathway play important roles in the mechanism of damnacanthal-induced apoptosis.

Previous studies suggested that p38 MAPK inhibitor SB203580 can attenuate hypoxia-induced apoptosis in osteoblast cells (Chae et al., 2001) and that p38 MAPK activity is detected in human liver tumor samples (Iyoda et al., 2003), and the loss of p38 MAPK activation is association with increased tumorigenesis in mutant cells (Brancho et al., 2003). Considering the fact that the suppression of apoptosis often leads to the development of cancer (Kyriakis and Avruch, 2001). The elucidation of the mechanisms of p38 MAPK, TNF-α, TRAIL, and p53-regulated caspase cascade in damnacanthal-mediated apoptosis in cancer cell lines will provide knowledge applicable in the treatment of cancer.

Apoptosis may occur by two fundamental pathways: (i) the death receptor or extrinsic pathway; and (ii) the mitochondrial or intrinsic pathway. In the current study, there is considerable cross talk between death-receptor and mitochondrial apoptotic pathways in apoptosis induced by damnacanthal in SKHep 1 cells. Recent studies have revealed that caspases play a critical role in executing apoptosis. In order to gain further insight into the mechanism of the signaling cascade, we performed an experiment to examine the molecular sequence of events in damnacanthal-induced apoptosis. We show that damnacanthal-mediated death receptor-dependent activation of caspase 8 leads to cleavage of Bid, resulting in the release of cytochrome c as well as loss of mitochondrial membrane potential. Indeed, it has been suggested that cells can be grouped based upon the degree of mitochondrial involvement in death receptor-mediated apoptosis (Scaffidi et al., 1998). The cytochrome c-dependent activation of caspase 9 activation is thought to be of central importance in this process. Although several reports suggest that Smac/DIABLO release rather than caspase 9 activation may be critical for the mitochondrial pathway in both TRAIL-induced and FasL-induced apoptosis (Zhang et al., 2001), here we demonstrated that caspase 9 was activated by cross talk with the caspase 8 pathway in apoptosis induced by damnacanthal in SKHep 1 cells, resulting in the cytochrome c-dependent activation of caspase 9 and the collapse of $\Delta\Psi m$. As our data shown in the present study, apoptosis is initiated by death receptor pathways but requires mitochondrial amplification producing a biphasic pattern of caspase 8, Bid, caspases -9 and -3 activation. Cleaved PARP is a sign of developing apoptosis, since it is involved in DNA repair and responds to environmental stimuli (Los et al., 2002). The inhibition of caspase-8 and -9, and their downstream effector, by p38 MAPK inhibitors implies that p38 MAPK signaling is a critical pathway in damnacanthal-induced apoptosis and that p38 MAPK is a pro-apoptosis protein that is essential for damnacanthal-induced apoptosis.

In order to investigate which signaling molecules regulate p53 activation, we blocked MAPKs signaling using MAPKs inhibitors (Davies et al., 2000; Ji et al., 2009). Upon inhibition of p38 MAPK, the expression levels and phosphorylation of p53 at serine 46 was significantly inhibited. These results suggested that the intrinsic kinase activation of p38 MAPK and ATF-2 is required for damnacanthal-induced p53 phosphorylation and upon damnacanthal treatment. Previous reports

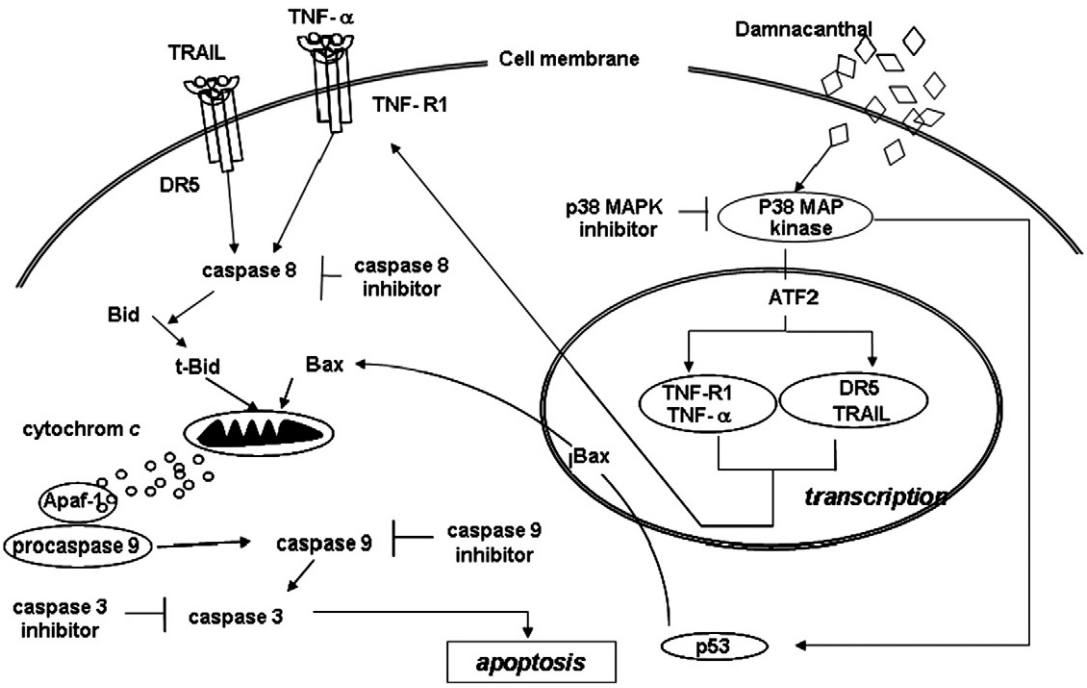

Fig. 9. A proposed model for damnacanthal-induced apoptosis. Damnacanthal-mediated apoptosis involves the sustained activation of the p38 MAPK pathway, resulting in the transcription of the death receptor family genes encoding DR5/TRAIL andTNF-R1/TNF-α genes and the p53-regulated Bax gene. In turn, DR5/TRAIL andTNF-R1/TNF-α expression activates the death receptor pathway and caspase 8. Activation of caspase 8 further leads to the cleavage of Bid. In turn, activated Bid, acting with damnacanthal-induced Bax, mediates the intrinsic mitochondrial apoptotic pathway, which in turn leads to the release of cytochrome c and interaction with Apaf-1 forming a apoptosome subsequently activation of caspase 9. The activation of caspase 9 further activates the caspase 3, leading to apoptosis.

have also indicated there are ATF-2 binding elements within p53 promoter regions (Kirch et al., 1999). Therefore, whether ATF-2 binds to the p53 promoter upon damnacanthal treatment needs to be addressed in the future. In addition to MAP kinases, other protein kinases that participate in DNA damage response, including ATR, ATM and DNA-PK, as well as chk1 and chk2 may modulate p53 activity. Although multiple pathways contribute to the modulation of p53, we have identified p38 MAPK being one of the upstream molecules of p53. Therefore, we propose that p38 MAPK-p53 signaling is one of key pathways in mediating damnacanthal-induced apoptosis.

In addition, the role of the p38 MAPK pathway in transcription of the p53-regulated Bax gene likely involves p53 phosphorylation (Bulavin et al., 1999). The increased damnacanthal-dependent p53 protein levels and the increased damnacanthal -dependent Ser46 phosphorylation, agree with the damnacantha-dependent transcriptional induction of Bax. More-extensive analyses of damnacanthal-dependent modifications of p53 are in progress to link p38 MAPK activity to p53 function in damnacanthal-mediated apoptosis. Although modulation of MAPK and p53 signaling is common, we have established connections between well-known pro-apoptotic molecules in the damnacanthal-induced apoptosis.

In summary, the current data presented herein suggest several aspects of the mechanism of damnacanthal-induced apoptosis in SKHep 1 cells. A model depicting the pathways of damnacanthal-induced apoptosis in SKHep 1cells is shown in Fig. 9. Taken together, all data suggested that: (i) an p38 MAPK-dependent pathway is involved in the activation of TNF-α/TNF-R1 and TRAIL/DR5 signaling; (ii) MAPK p38 is involved in damnacanthal-induced p53 phosphorylation and Bax translocation; and (iii) damnacanthal-induced apoptosis requires death receptor-, mitochondria-, and caspase-dependent cell death pathways.

Conflict of interest

The authors declare no competing financial interests.

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