



**Destabilization of CARP mRNAs by Aloe-Emodin Contributes to Caspase-8-Mediated p53-Independent Apoptosis of Human Carcinoma Cells**

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## Destabilization of CARP mRNAs by Aloe-Emodin Contributes to Caspase-8–Mediated p53-Independent Apoptosis of Human Carcinoma Cells

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4 **RUNNING TITLE:** M.-L. Lin *et al.* ALOE-EMODIN-INDUCED p53-INDEPENDENT  
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7 APOPTOSIS IN HUMAN CARCINOMA CELLS  
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41 **KEY WORDS:** Aloe-emodin; CARP; Caspase-8; Cdk2; Chinese herb; E2F1; ERK;  
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44 mitochondrial death pathway; tBid; Endo G; AIF; ROS.  
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**ABSTRACT**

Using short hairpin RNA against p53, transient ectopic expression of wild-type p53 or mutant p53 (R248W or R175H), and a p53- and p21-dependent luciferase reporter assay, we demonstrated that growth arrest and apoptosis of FaDu (human pharyngeal squamous cell carcinoma), Hep3B (hepatoma), and MG-63 (osteosarcoma) cells induced by aloe-emodin (AE) are p53-independent. Co-immunoprecipitation and small interfering RNA (siRNA) studies demonstrated that AE caused S-phase cell cycle arrest by inducing the formation of cyclin A-Cdk2-p21 complexes through extracellular signal-regulated kinase (ERK) activation. Ectopic expression of Bcl-X<sub>L</sub> and siRNA-mediated Bax attenuation significantly inhibited apoptosis induced by AE. Cyclosporin A or the caspase-8 inhibitor Z-IETD-FMK blocked AE-induced loss of mitochondrial membrane potential and prevented increases in reactive oxygen species and Ca<sup>++</sup>. Z-IETD-FMK inhibited AE-induced apoptosis, Bax expression, Bid cleavage, translocation of tBid to mitochondria, ERK phosphorylation, caspase-9 activation, and the release of cytochrome c, AIF, and endonuclease G from mitochondria. The stability of the mRNAs encoding caspase-8 and -10-associated RING proteins (CARPs) 1 and 2 was affected by AE, whereas CARP 1 or 2 overexpression inhibited caspase-8 activation and apoptosis induced by AE. Collectively, our data indicate AE induces caspase-8-mediated activation of mitochondrial death pathways by decreasing the stability of CARP mRNAs in a p53-independent manner.

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4 The transcriptional regulation and tumor suppressor functions of p53 are commonly lost in  
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7 human cancers. More than ~50% of human cancers show mutations or deletion of the *P53*  
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10 gene, which is associated with a poor response to various types of therapies [Nylander et al.,  
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13 2000]. Identifying drugs that induce p53-independent apoptosis of cancer cells is therefore  
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16 urgently needed.  
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20 Mitochondria are considered the main target for the apoptosis induction that occurs in  
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23 response to a variety of stress stimuli, such as growth factor withdrawal,  $\gamma$ -irradiation, and  
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26 chemotherapeutic drugs. The mitochondrial death pathway is initiated by the disruption of the  
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29 outer mitochondrial membrane, and it involves the release of cytochrome *c*, second  
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32 mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with  
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35 low pI (Smac/DIABLO), apoptosis-inducing factor (AIF), and endonuclease G (Endo G) from  
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38 mitochondria into the cytosol [Tsujiimoto, 2003]. Cytosolic cytochrome *c* can trigger the  
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41 proteolytic processing of pro-caspase-9, initiating the formation of an apoptosome composed  
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44 of Apaf-1, dATP, caspase-9, and cytochrome *c*. Apoptosome formation leads to the activation  
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47 of executioner caspase-3, -6, and -7 [Budihardjo et al., 1999]. Nuclear translocation of AIF  
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50 and Endo G occurs and can induce DNA fragmentation and apoptotic cell death in a  
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53 caspase-independent manner [Penninger and Kroemer, 2003]. The release of Endo G and AIF  
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56 from the mitochondria in response to pro-apoptotic stimuli is, however, caspase-dependent  
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59 [Arnoult et al., 2003]. Members of the Bcl-2 family, such as Bcl-2/Bcl-X<sub>L</sub> and Bax/Bak, can  
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4 both negatively and positively regulate mitochondrial events that occur during apoptotic cell  
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7 death by suppressing cytochrome *c* release [Brunelle and Letai, 2009]. Several studies have  
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10 shown that chemotherapeutic agent-induced apoptosis involves the cleavage of cytosolic Bid  
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13 to truncated Bid (tBid) by caspase-8 [Anto et al., 2002; Kim et al., 2009]. tBid induces  
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16 pro-apoptotic protein Bax oligomerization and the generation of the mitochondrial  
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19 permeability transition pore, which leads to the loss of mitochondrial membrane potential  
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22 ( $\Delta\psi_m$ ) and to cytochrome *c* release [Ott et al., 2009]. Caspase-8 and -10-associated RING  
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25 proteins (CARPs), new members of the inhibitor of apoptotic protein family, have been shown  
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28 to interact specifically with caspase-8 and regulate its processing via ubiquitin-mediated  
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31 proteolysis [McDonald and El-Deiry, 2004]. Transient siRNA-mediated attenuation of CARP  
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34 expression can inhibit tumor cell growth. In addition, overexpression of CARPs in cancer  
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37 cells blocks death ligand-induced apoptosis [McDonald and El-Deiry, 2004].  
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42 Among the mitogen-activated protein kinase (MAPK) family members, extracellular  
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44 signal-regulated kinase (ERK)-mediated signaling is typically associated with mitogen- and  
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47 growth factor-induced cell differentiation, cell proliferation, and survival [Hill and Treisman,  
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50 1995], whereas the p38 MAPK and c-Jun N-terminal kinase (JNK) pathways are involved in  
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53 cell growth inhibition and apoptosis [Xia et al., 1995]. However, increasing evidence suggests  
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56 that the complicated roles of these MAPK pathways exist to transmit distinct cellular effects  
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59 in different cell types [Dhillon et al., 2007]. ERK activation is involved in the  
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4 p53-independent induction of S-phase arrest and p21 expression in DLD-1 (human colon  
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7 cancer) and HL-60 (promyelocytic leukemia) cell lines [Song et al., 2008; Zhu et al., 2004].  
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10 In contrast, p38 MAPK and JNK activities are associated with anti-apoptosis [Chao et al.,  
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13 2004] and cancer invasion [Pan et al., 2009].  
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16 Aloe-emodin (AE) (1,8-dihydroxy-3-(hydroxymethyl) anthraquinone) is isolated from the  
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18 rhizomes of *Rheum palmatum*. AE-induced G<sub>1</sub>-phase cell cycle arrest in human hepatoma  
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20 HepG2 cell lines is associated with p53 and p21 up-regulation [Kuo et al., 2002]. Furthermore,  
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23 JNK activation is involved in AE-induced apoptotic cell death of the human hepatoma HepG2  
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26 cell line [Lu et al., 2007]. The inhibition of ERK activity causes the induction of cell  
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29 differentiation, but not of apoptosis, in AE-treated rat C6 glioma cells [Mijatovic et al., 2005].  
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33 Herein, we demonstrate that the induction of p53-independent apoptosis by AE occurs  
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36 through the caspase-8-mediated activation of the mitochondrial death pathway by decreasing  
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39 the stability of CARP mRNAs and that the increases in p21 and E2F1 expression and S-phase  
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45 arrest are mediated by ERK activation.  
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## MATERIALS AND METHODS

### CHEMICALS

AE, actinomycin D, cyclosporine A (CsA), dantrolene, NP-40, paraformaldehyde, PD98059, propidium iodide (PI), Tris-HCl, 4'-6-diamidino-2-phenylindole (DAPI), and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). AE was dissolved in and diluted with DMSO and then stored at  $-20^{\circ}\text{C}$  as a 100 mM stock. Potassium phosphate was purchased from Merck (Darmstadt, Germany). Lipofectamine 2000 was obtained from Invitrogen (Carlsbad, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), whereas Minimum Essential Medium (MEM), fetal bovine serum (FBS), trypsin-EDTA, glutamine, and puromycin were obtained from Gibco BRL (Grand Island, NY, USA). The caspase-3 activity assay kit was purchased from OncoImmunin (Gaithersburg, MD, USA). Caspase-8 and -9 activity assay kits were obtained from Calbiochem (San Diego, CA, USA). Inhibitors of pan-caspase (Z-VAD-FMK), caspase-3 (Ac-DEVD-CMK), caspase-4 (LEVD-CHO), caspase-8 (Z-IETD-FMK), and caspase-9 (Z-LEHD-FMK) were purchased from Calbiochem (San Diego, CA, USA) and were all dissolved in DMSO. pPuro-p53 shRNA, pPuro-GFP shRNA, pFLAG-CMV2-CARP1, and pFLAG-CMV2-CARP2 vectors were obtained from Addgene (Cambridge, MA, USA). Bax siRNA, Bak siRNA, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bax siRNA and Bak siRNA were dissolved in RNase-free water. The double-stranded siRNA sequences



were as follows: Bax siRNA, 5'-GGUGCCGGAACUGAUCAGA-3'/3'-UCUGAUCAGUUCCGGCACC-5' and 5'-AACAAUGGAGCUGCAGAGGAUGA-3'/3'-UCAUCCUCUGCAGCUCCAUGUU-5'; Bak siRNA, 5'-CCGACGCUAUGACUCAGAGTT-3'/3'-CUCUGAGUCAUAGCGUCGGTT-5'; ERK siRNA, 5'-GCAAUGACCAUAUCUGCUA(dTdT)-3'/3'-(TdTd)CGUUACUGGUAUAGAC-5'. The primer sequences were as follows: CARP1, 5'-AACGCAGAGGATCGGAACC-3' and 5'-TTCCACATCATCAAGGCTTGAC-3'; CARP2, 5'-AGCATGGTTCCACCTACCTCAC-3' and 5'-CCTCTTGATCCTGAGACACATGG-3' [McDonald and El-Deiry, 2004]; p53, 5'-TGCGTGTGGAGTATTTGGATG-3' and 5'-TGACCCTTTTTGGACTTCAG-3';  $\beta$ -actin, 5'-GCTTGACTCAGGATTTAAAACTGGAACGG-3' and 5'-TATTCAACTGGTCTCAAGTCAGTGTACAGG-3'.

## ANTIBODIES

Antibodies against cyclin A, cyclin B, cyclin D, Cyclin E, Cdk2, phospho (p)-Cdk2 (Thr14/Thr15), Bak, Bad, Bid, truncated Bid (tBid), NOXA, MCL-1, Bik, BIM, PUMA, Endo G, AIF, p53, p21, E2F1, and proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against ERK, p-ERK (Tyr202/204), Bcl-2, Bcl-X<sub>L</sub>, Bax, and cytochrome *c* were obtained from BD Pharmingen

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4 (San Diego, CA, USA). Antibodies against caspase-3, -8, and tBid were purchased from  
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7 Calbiochem (San Diego, CA, USA). Anti-cytochrome *c* oxidase subunit II (Cox2) was  
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10 obtained from Abcam (Cambridge, MA, USA). Antibodies against  $\gamma$ -tubulin and FLAG-tag  
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13 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Peroxidase-conjugated  
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16 anti-mouse IgG, -goat IgG, and -rabbit IgG secondary antibodies were purchased from  
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19 Jackson ImmunoResearch Laboratory (West Grove, PA, USA).  
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## 26 CELL CULTURE

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29 The human pharyngeal squamous cell carcinoma line FaDu, the human lung cancer cell line  
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32 H1299, the human hepatoma cell line Hep3B, the human osteosarcoma cell line MG-63,  
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35 normal human embryonic lung fibroblasts (WI-38), normal human fetal skin fibroblasts  
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38 (Detroit 551), normal embryonic lung fibroblasts (MRC-5), the normal mouse embryonic  
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41 liver cell line BNL CL.2, and human embryonic kidney 293 cells were obtained from the  
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44 Food Industry Research and Development Institute (Hsinchu, Taiwan). FaDu, RPMI 2650,  
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47 WI-38, Detroit 551, and MRC-5 cell lines were cultured in MEM supplemented with 5% FBS.  
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50 The 293, H1299, Hep3B, and MG-63 cell lines were cultured routinely in DMEM  
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53 supplemented with 5% FBS. All cell lines were grown in 10-cm tissue culture dishes at 37°C  
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56 in a humidified incubator containing 5% CO<sub>2</sub>.  
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4 **ESTABLISHMENT OF CELL CLONES PERMANENTLY EXPRESSING p53 shRNA**  
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7 **OR GFP shRNA**  
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10 To establish cells stably expressing p53 shRNA or GFP shRNA, cells were transfected using  
11 Lipofectamine 2000 with pPuro-p53 shRNA or pPuro-GFP shRNA plasmid. The transfected  
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13 cells were selected and cloned in the presence of 2 µg/ml puromycin. The efficiency of p53  
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20 knockdown was confirmed by western blot analysis with anti-p53 antibody.  
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26 **CELL VIABILITY ASSAY**  
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29 Cells were seeded at  $3 \times 10^4$  cells/well in 24-well tissue culture plates. Cells were grown  
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31 overnight to ~60% confluence and treated with either DMSO (vehicle control) or AE for the  
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33 indicated times. In the controls, DMSO was diluted in culture medium to the same final  
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35 concentration (0.01% vol/vol) as in the medium with AE. At the end of the incubation, treated  
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## MTT ASSAY

The cells were seeded at a density of  $3 \times 10^4$  cells per well into 24-well plates. After 16 h of incubation, cells were grown to ~60% confluence and treated with either vehicle or 60  $\mu$ M AE at 37°C for the indicated times before being harvested. The treated cells were washed once with PBS and incubated with 0.5 mg/ml MTT for 5 h. The resulting formazan precipitate was dissolved in 150  $\mu$ l of DMSO and the optical density (OD) of formazan was determined using an ELISA reader (Thermo Labsystems Multiskan Spectrum, Franklin, MA, USA) at 570 nm.

## RT-PCR

RNA was extracted using Trizol reagent (Invitrogen Life technologies, Carlsbad, CA, USA). First-strand cDNA was made using 1  $\mu$ g of total RNA in the presence of Superscript II reverse transcriptase (Gibco/BRL) and random hexamers. cDNA was PCR-amplified using Vent DNA polymerase (NEB BioLabs) and specific primers corresponding directly to a region in the *P53*, *CARP1*, or *CARP2* gene. Samples were taken from the PCR mixture after 25 or 35 cycles of amplification, separated on a 1.5% agarose gel, and stained with ethidium bromide.

## MEASUREMENT OF DNA FRAGMENTATION

Histone-associated DNA fragments were determined using the Cell Death Detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, Mannheim,

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4 Germany). Briefly, vehicle- or AE-treated cells were incubated in hypertonic buffer for 30  
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7 min at room temperature. After centrifugation, the cell lysates were transferred into an  
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10 anti-histone-coated microplate to bind histone-associated DNA fragments. Plates were  
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12  
13 washed after 1.5 h of incubation, and nonspecific binding sites were saturated with blocking  
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15  
16 buffer. Plates were then incubated with peroxidase-conjugated anti-DNA for 1.5 h at room  
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18  
19 temperature. To determine the amount of retained peroxidase,  
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22 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) was added as a substrate, and a  
23  
24  
25 spectrophotometer (Thermo Labsystems Multiskan Spectrum, Franklin, MA, USA) was used  
26  
27  
28 to measure the absorbance at 405 nm.  
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### 34 35 **p53 AND p21 PROMOTER ASSAY**

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38 p53-knockdown cells were seeded at a concentration of  $5 \times 10^4$  cells per well in 12-well tissue  
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40  
41 culture plates and grown overnight to ~70% confluency. A luciferase reporter driven by three  
42  
43  
44 p53 binding sites or by the p21 promoter and a plasmid expressing wild-type (wt) p53, mutant  
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46  
47 p53 (R248W), or p53 (R175H) were transfected into cells using Lipofectamine 2000. The  
48  
49  
50  $\beta$ -galactosidase expression vector pCH110 was included as an internal control. At 12 h  
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53 post-transfection, cells were treated with vehicle or AE for 24 h. The cell lysates were  
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56 harvested, and the protein expression and luciferase activity were determined using western  
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59 blot analysis and the Dual-Luciferase Reporter Assay kit (Promega; Madison, WI, USA),  
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4 respectively. The value of luciferase activity was normalized to transfection efficiency as  
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7 monitored by  $\beta$ -galactosidase expression.  
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#### 10 11 12 13 **ASSAY FOR CASPASE-3 ACTIVITY**

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15  
16 Caspase-3 activity was measured using the PhiPhiLux G1D2 kit (OncoImmunit, College Park,  
17  
18 MD, USA). Briefly, vehicle- or AE-treated cells were incubated with PhiPhiLux fluorogenic  
19  
20 caspase substrate for 1 h at 37°C, washed three times with PBS, resuspended in 500  $\mu$ l of  
21  
22 ice-cold PBS, and analyzed on a FACSCount flow cytometer.  
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#### 32 **MEASUREMENT OF CELL CYCLE BY FLOW CYTOMETRY**

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35 Cells ( $1 \times 10^5$ ) were trypsinized, washed twice with PBS, and fixed in 80% ethanol. Fixed  
36  
37 cells were washed with PBS, incubated with 100  $\mu$ g/ml RNase for 30 min at 37°C, stained  
38  
39 with PI (50  $\mu$ g/ml), and analyzed on a FACSCount flow cytometer. The percentage of cells  
40  
41 that had undergone apoptosis was assessed as the ratio of the area of fluorescence that was  
42  
43 smaller than the  $G_0$ - $G_1$  peak to the total area of fluorescence.  
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#### 53 **CO-IMMUNOPRECIPITATION ASSAY**

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56 p53 shRNA-expressing stable cells ( $5 \times 10^5$ ) were treated with 60  $\mu$ M of AE for the indicated  
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58 periods. Cells were harvested and lysed in 0.6 ml of cell lysis buffer (50 mM Tris-HCl (pH  
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4 8.0), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 100 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5% (v/v) NP-40, 50 mM NaF, 25  
5  
6  
7 mM leupeptin). Whole-cell extracts were incubated with anti-Cdk2, anti-cyclin A, or normal  
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9  
10 mouse IgG as a negative control at 4°C for 2 h. After incubation, protein A–agarose beads  
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12  
13 (Amersham Biosciences, Piscataway, NJ, USA) were added, and the mixture was incubated  
14  
15  
16 with gentle rocking at 4°C for 2 h. The immunocomplexes were then analyzed by 10%  
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18  
19 SDS-PAGE and immunoblotted with antibodies against cyclin A, Cdk2, p21, PCNA, and  
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22 E2F1.  
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## 29 WESTERN BLOT ANALYSIS

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32 Treated or transfected cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 120 mM  
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34 NaCl, 1 µg/ml aprotinin, 100 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM NaF, 0.5% NP-40). Protein concentration  
35  
36  
37 was determined by the Bradford method (Bio-Rad, Hercules, CA, USA). Proteins were  
38  
39  
40 separated by electrophoresis on a 10% SDS-PAGE gel and then transferred to polyvinylidene  
41  
42  
43 difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were  
44  
45  
46 blocked overnight with PBS containing 3% skim milk and then incubated with primary  
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48  
49 antibody against cyclin A, cyclin B, cyclin D, cyclin E, Cdk2, p-Cdk2 (Thr14/Thr15), E2F1,  
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51  
52 PCNA, p53, p21, ERK, p-ERK (Tyr202/204), Bcl-X<sub>L</sub>, Bcl-2, Bax, Bak, Bid, tBid, caspase-3,  
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54  
55 caspase-8, caspase-9, cytochrome *c*, AIF, Endo G, or Cox2. Proteins were detected with  
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58 horseradish peroxidase–conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat  
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4 antibodies and the Western Blotting Luminol Reagent. To confirm equal protein loading,  
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7  $\gamma$ -tubulin was measured.  
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#### 10 11 12 13 **PLASMID AND siRNA TRANSFECTION** 14

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16 Cells (at 70% confluency in a 12-well plate) were transfected with FLAG epitope-tagged  
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18 Bcl-X<sub>L</sub> or Bcl-2 expression plasmid or with Bax, Bak and ERK (pKD-MAPK1/Erk) siRNAs  
19  
20 using Lipofectamine 2000. The expression of FLAG-Bcl-X<sub>L</sub>, FLAG-Bcl-2, Bax, and Bak in  
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23 transfected cells was assessed by western blotting using antibodies specific against FLAG,  
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26 Bax, or Bak.  
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#### 35 **DETECTION OF CYTOCHROME C, AIF, AND ENDO G** 36

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38 Treated cells were first lysed in isotonic mitochondrial buffer (210 mM mannitol, 70 mM  
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40 sucrose, 1 mM EDTA, 10 mM HEPES (pH 7.5)) and homogenized with 30 to 40 strokes of a  
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42 Dounce homogenizer (Dounce; Bellco Glass Co., Vineland, NJ, USA). Nuclei and unbroken  
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45 cells were removed by centrifugation at 500 × *g* for 5 min at 4°C in a microcentrifuge.  
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48 Supernatants were then centrifuged at 10,000 × *g* for 30 min at 4°C, and the resulting  
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51 supernatants were stored as the cytosolic fraction. Total proteins (20 μg) were separated by  
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54 12% SDS-PAGE and were transferred onto polyvinylidene difluoride membranes, which were  
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57 then incubated with primary antibodies against cytochrome *c*, AIF, and Endo G. Proteins were  
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4 detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit  
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7 antibodies and the Western Blotting Luminol Reagent.  $\gamma$ -Tubulin and Cox2 were used as  
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10 internal controls for sample loading.  
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## 12 13 14 15 16 17 **MEASUREMENT OF MITOCHONDRIAL MEMBRANE POTENTIAL**

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19 Mitochondrial membrane potential ( $\Delta\psi_m$ ) was determined by measuring the retention of the  
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21 dye 3,3'-dihexyloxacarbocyanine (DiOC<sub>6</sub>). Briefly, cells treated with vehicle, AE (60  $\mu$ M),  
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23 AE (60  $\mu$ M) plus CsA (5  $\mu$ M), AE (60  $\mu$ M) plus dantrolene (25  $\mu$ M), or AE (60  $\mu$ M) plus  
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Z-IETD-FMK (40  $\mu$ M) were incubated with 40 nM DiOC<sub>6</sub> for 30 min at 37°C. Cells were  
then pelleted by centrifugation at 160  $\times$  g. Pellets were resuspended and washed twice with  
PBS. The  $\Delta\psi_m$  was determined with a FACSCount flow cytometer.

## 41 42 **DETECTION OF REACTIVE OXYGEN SPECIES**

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Overnight-cultured cells ( $1 \times 10^5$  cells) were treated with vehicle, AE (60  $\mu$ M), AE (60  $\mu$ M)  
plus CsA (5  $\mu$ M), AE (60  $\mu$ M) plus dantrolene (25  $\mu$ M), or AE (60  $\mu$ M) plus Z-IETD-FMK  
(40  $\mu$ M) for 1, 3, 6, or 12 h to detect changes in reactive oxygen species (ROS). After  
treatment, cells were harvested and washed twice with PBS. Cells were then resuspended in  
500  $\mu$ l of 2,7-dichlorodihydrofluorescein diacetate (10  $\mu$ M) and incubated for 30 min at 37°C.  
The level of ROS was determined using a FACSCount flow cytometer.

## MEASUREMENT OF CYTOSOLIC Ca<sup>++</sup>

Briefly, cells treated with vehicle, AE (60 μM), AE (60 μM) plus CsA (5 μM), AE (60 μM) plus Z-IETD-FMK (40 μM), or AE (60 μM) plus dantrolene (25 μM) were incubated with 3 μg/ml of Indo-1/AM (Calbiochem, La Jolla, CA, USA) and incubated for 30 min at 37°C. After washing twice with assay buffer, cells were suspended in assay buffer (Calbiochem, La Jolla, CA, USA). The level of Ca<sup>++</sup> was determined using a FACSCount flow cytometer.

## STATISTICAL ANALYSIS OF DATA

Statistical calculations were performed using the unpaired Student's *t*-test and ANOVA.

Statistical significance between vehicle control and experimental groups was set at  $p < 0.05$ .

## RESULTS

### AE INDUCES p53-INDEPENDENT APOPTOTIC CELL DEATH

The first study used the mutant p53 (R248L)-carrying human cell line FaDu and the p53-null-carrying human cancer cell lines H1299, Hep3B, and MG-63. PI staining and flow-cytometric analysis showed that AE significantly reduced FaDu, H1299, Hep3B, and MG-63 cell viability in a dose-dependent manner (Fig. 1A, panel I). Treatment for 36 h with 60  $\mu$ M AE in FaDu, Hep3B, and MG-63 cells resulted in 49.74%, 46.82%, and 53.46% reductions in cell viability, respectively. The  $IC_{50}$  value of AE in these cells was 60  $\mu$ M, which was then used in all subsequent experiments (panel II). In contrast, 60  $\mu$ M AE was less toxic to human normal cell lines (WI-38, Detroit 551, MRC-5 and 293 cells) and normal mouse liver cells [Lin et al., 2010]. To rule out the possibility that mutated p53 (R248L) or endogenous p53 were still functional, we used cells stably expressing a shRNA to knock down p53 and examined the effect of AE on these cells. p53 mRNA and protein levels were reduced in cells expressing the shRNA, demonstrating efficient and stable knockdown (Fig. 1B, panel I). No change in caspase-3 activity or apoptosis was observed in p53 shRNA-treated cells compared to vector or non-specific GFP shRNA control cells (Fig. 1B, panels II and III). The transcriptional activity of p53 is thought to be important for its functions in cell growth regulation, so luciferase activity was measured after treatment with AE in p53-knockdown cells that were co-transfected with a p53-luc plasmid (which contains

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4 three p53 binding sites upstream of the luciferase gene) or a p21-luc plasmid (which contains  
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7 three p21 binding sites upstream of the luciferase gene) and an expression plasmid encoding  
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10 wt, mutant p53 (R248W), or p53 (R175H). The p53 and p21 promoter-driven luciferase  
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13 activities increased markedly in cells co-transfected with wt p53 but not in those  
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16 co-transfected with p53 (R248W) or p53 (R175H), and AE treatment did not reduce this  
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19 increase in activity (Fig. 2 A and B, comparing column 3 with column 4). In contrast, growth  
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22 inhibition was observed in wt p53-, p53 (R248W)-, or p53 (R175H)-expressing cells treated  
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25 with AE (Fig. 2C, columns 4, 6 and 8). The expression and promoter activity of p21, however,  
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28 were increased in AE-treated cells co-transfected with control vector, p53 (R248W), or p53  
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30  
31 (R175H) (Fig. 2C and D, columns 2, 6 and 8). These results clearly indicate that induction of  
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34 both apoptotic cell death and p21 expression by AE occur independently of p53 expression.  
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#### 41 **FORMATION OF CYCLIN A-Cdk2-p21 COMPLEXES IS ASSOCIATED WITH**

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#### 44 **S-PHASE CELL CYCLE ARREST BY AE**

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47 To gain insight into the mechanism responsible for cell growth inhibition by AE, flow  
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50 cytometry was used to determine the effect of AE on the cell cycle profile. With AE treatment,  
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53 more p53 shRNA cells accumulated in S-phase than GFP shRNA control cells (Table 1). A  
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56 significant increase in the number of apoptotic cells (sub-G<sub>1</sub>-phase population) was also  
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59 observed in AE-treated cells expressing either p53 shRNA or non-specific GFP shRNA  
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4 control (Table 1). AE did not affect the level of cyclin B1, cyclin D, or cyclin E, but it did  
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6  
7 increase cyclin A and E2F1 expression as well as the phosphorylation of Cdk2 (Thr14/Thr15)  
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10 (Fig. 3A). A co-immunoprecipitation assay was performed in p53 shRNA cell extracts using  
11  
12  
13 antibodies specific for Cdk2 and cyclin A to characterize the effect of AE on the interaction  
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16 between cyclin A and Cdk2. As expected, AE induced the binding of cyclin A to Cdk2.  
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18  
19 Western blot analysis also showed that p21 was associated with the cyclin A–Cdk2 complex  
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21  
22 after AE treatment (Fig. 3B). However, PCNA and E2F1 were not detected in cyclin A–Cdk2  
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24  
25 complexes. To further investigate whether the increase in cyclin A expression was associated  
26  
27  
28 with AE-induced cell cycle arrest at S-phase, p53 shRNA cells were transfected with cyclin A  
29  
30  
31 siRNA, which inhibited the expression of cyclin A and AE-induced S-phase arrest (panels I  
32  
33  
34 and II, respectively, of Fig. 3C). These results indicate that AE inhibited mutant  
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37 p53–expressing or p53-null cancer cell growth through cell cycle arrest at S-phase, which was  
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40 associated with the formation of cyclin A–Cdk2-p21 complexes.  
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#### **INVOLVEMENT OF ERK ACTIVATION IN THE INDUCTION OF p21 AND E2F1 EXPRESSION, S-PHASE ARREST, AND APOPTOSIS BY AE**

We examined the activation of MAP kinases, which are important regulators of p21 in the p53-independent pathway, to explore the mechanism underlying AE-induced p21 expression. Treatment with AE induced the phosphorylation of ERK, which was greatly reduced by the

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4 ERK inhibitor PD98059 and was attenuated by ERK-specific siRNA (Fig. 4A). Nevertheless,  
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7 AE treatment did not result in the activation of p38 MAPK or JNK (data no shown). The  
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9  
10 induction of p21 and E2F1 expression, phosphorylation of ERK, and growth arrest were  
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12  
13 correlated with ERK activity, because addition of PD98059 or ERK-specific siRNA  
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15  
16 suppressed AE-induced p21 and E2F1 expression, ERK phosphorylation, and S-phase arrest  
17  
18  
19 (Fig. 4A and B (panel I)). AE-induced apoptosis was, however, only partially suppressed by  
20  
21  
22 PD98059 or ERK-specific siRNA (Fig. 4B, panel II). Thus, these results suggest that the  
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25 activation of ERK is required for AE-induced p21 and E2F1 expression and S-phase arrest  
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28 and is involved in the induction of apoptosis.  
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### 35 **INVOLVEMENT OF Bcl-X<sub>L</sub> AND BAX EXPRESION IN AE-INDUCED APOPTOTIC** 36 37 38 **CELL DEATH** 39

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41 We evaluated the expression of anti- and pro-apoptotic Bcl-2 family proteins exposed to AE  
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44 to further define the apoptotic pathway that is associated with AE treatment. Western blot  
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47 analysis showed that Bcl-2 expression did not change, whereas expression of Bcl-X<sub>L</sub> was  
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49  
50 markedly inhibited by AE (Fig. 5A, panel I). Heterodimerization of Bcl-X<sub>L</sub> and Bax appears  
51  
52  
53 to be required for anti-apoptotic activity, and the susceptibility of cells to the induction of  
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56 apoptosis may be regulated by the ratio of Bcl-X<sub>L</sub> to Bax [Sedlak et al., 1995]. Accordingly,  
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58  
59 we determined the level of Bax expression. As expected, AE treatment led to an up-regulation  
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4 of Bax expression (Fig. 5A, panel II). In contrast, Bak and MCL-1 levels were unaffected by  
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7 AE (data no shown). We next examined the effect of AE on the expression of the inactivator  
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10 BH3 and activator BH3 proteins, which regulate the activation of Bax/Bak [Galonek and  
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12  
13 Hardwick, 2006]. The expression of the inactivator BH3 proteins Bad, Bik, and NOXA did  
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15  
16 not change after AE treatment (data no shown). Similarly, no changes were observed in the  
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18  
19 expression of activator BH3 proteins BIM and PUMA; however, an increase in the amount of  
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21  
22 cleaved tBid was detected in AE-treated cells (Fig. 5A, panel II).  
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26 To clarify whether AE induces apoptotic cell death through changes in Bcl-X<sub>L</sub> and Bax  
27  
28 expression, transient ectopic FLAG-tagged Bcl-X<sub>L</sub> or Bcl-2 was expressed in cells, and the  
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31 expression of Bax and Bak was attenuated with siRNAs. Expression levels of Bcl-X<sub>L</sub>, Bcl-2,  
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34 Bax, and Bak were confirmed by western blotting using FLAG-, Bax-, and Bak-specific  
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37 antibodies (panels I and II of Fig. 5B). Compared with control vector-transfected cells,  
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40 ectopic expression of Bcl-X<sub>L</sub> (but not of Bcl-2) significantly suppressed AE-induced  
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43 apoptosis (Fig. 5B, panels III and IV, compare column 4 with column 6). Furthermore,  
44  
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46 siRNA-mediated attenuation of Bax (but not of Bak) abolished PARP cleavage and apoptosis  
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49 induction (Fig. 5C, panels I to III). These results demonstrate that changes in Bcl-X<sub>L</sub> and Bax  
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52 expression are involved in AE-induced apoptotic cell death.  
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## INVOLVEMENT OF CASPASE-8 ACTIVATION IN THE AE-INDUCED LOSS OF

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4  **$\Delta\psi_m$ , INCREASES IN ROS AND  $Ca^{++}$  LEVELS, ERK PHOSPHORYLATION,**  
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7 **CASPASE-9 ACTIVAION, AND APOPTOTIC DEATH**  
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10 Bcl- $X_L$  is localized to the outer mitochondrial membrane and regulates the  $\Delta\psi_m$  [Breckenridge  
11 and Xue, 2004]. Therefore, we examined whether  $\Delta\psi_m$  loss occurs in AE-treated cells. AE  
12  
13 and Xue, 2004]. Therefore, we examined whether  $\Delta\psi_m$  loss occurs in AE-treated cells. AE  
14  
15 treatment for 1 h caused a rapid decrease in  $\Delta\psi_m$ , and the reduction in  $\Delta\psi_m$  was maintained for  
16  
17 up to 12 h of AE exposure (Fig. 6A, panel I). The alteration of  $\Delta\psi_m$  was completely inhibited  
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19 by CsA and the caspase-8 inhibitor Z-IETD-FMK (Fig. 6A, panel I). We next investigated  
20  
21 whether AE treatment affects the levels of cytosolic ROS and  $Ca^{++}$ . Levels of ROS and  $Ca^{++}$   
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23 in the cytosol, which were determined by flow cytometry, increased in cells after treatment  
24  
25 with AE, and these elevated levels were maintained throughout the 12-h exposure (Fig. 6A,  
26  
27 panels II and III). Co-treatment with Z-IETD-FMK or CsA completely inhibited the increase  
28  
29 in ROS and  $Ca^{++}$  caused by AE (Fig. 6A, panels II and III). The AE-induced increase in  
30  
31 cytosolic  $Ca^{++}$  was, however, unaffected by the addition of dantrolene, an inhibitor of  $Ca^{++}$   
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33 release from the endoplasmic reticulum (Fig. 6A, panel III), indicating that the increase in  
34  
35 cytosolic  $Ca^{++}$  was due to release from mitochondria. Interestingly, apoptosis was also  
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37 prevented by co-treatment with Z-IETD-FMK or with Z-IETD-FMK and PD98059 (Fig. 6A,  
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39 panel IV). Additionally, AE-induced Bax expression, Bid cleavage, translocation of tBid to  
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41 the mitochondria, and ERK phosphorylation were inhibited by co-treatment with  
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43 Z-IETD-FMK (Fig. 6B), suggesting that induction of Bax and cleavage of Bid are dependent  
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4 on the activation of caspase-8. These results suggest that caspase-8 activation is involved in  
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7 the AE-induced loss of  $\Delta\psi_m$ , increase in cytosolic ROS and  $Ca^{++}$  levels, ERK phosphorylation,  
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10 and apoptosis.

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13 To investigate whether caspase-9 activity is involved in the induction of apoptosis, the  
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16 caspase-9-specific inhibitor Z-LEHD-FMK was used. AE-mediated activation of caspase-9  
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19 was inhibited by Z-LEHD-FMK and Z-IETD-FMK but not by treatment with the caspase-4  
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21  
22 inhibitor LEVD-CHO (Fig. 6C, panel I). AE-induced apoptosis was only partially inhibited by  
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25 PD98059, but it was completely inhibited by either Z-IETD-FMK or Z-LEHD-FMK (Fig. 6C,  
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28 panel II). These data indicate that AE-induced apoptosis requires caspase-8-mediated  
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31 activation of the mitochondrial death pathway and ERK signaling.  
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### 38 **INVOLVEMENT OF CASPASE-8 ACTIVATION IN THE AE-INDUCED RELEASE** 39 40 41 **OF CYTOCHROME C, AIF, AND ENDO G FROM MITOCHONDRIA**

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44 Western blot analysis was conducted to investigate whether AE treatment induces the  
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47 mitochondrial release of pro-apoptotic proteins. AE induced the release of cytochrome *c*, AIF,  
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50 and Endo G into the cytosol. The release of cytochrome *c*, AIF, and Endo G was, however,  
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53 completely inhibited by Z-IETD-FMK (Fig. 6D, panels I to III). Therefore, caspase-8 initiates  
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56 the AE-induced release of cytochrome *c*, AIF, and Endo G, in addition to initiating apoptotic  
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59 cell death.  
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7 **DIMINISHING THE STABILITY OF CARP1 AND 2 mRNAs BY AE CONTRIBUTES**  
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10 **TO INDUCTION OF CASPASE-8 ACTIVITY AND CELL DEATH**  
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13 We next tested whether the suppression of CARPs contributes to the activation of caspase-8  
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15 in response to AE, as CARPs have previously been shown to regulate the activity of caspase-8  
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17 [McDonald and El-Deiry, 2004]. Treatment with AE for 1 h reduced the expression of *CARP1*  
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19 and 2 mRNAs, and this reduction in both mRNAs was maintained for up to 12 h of AE  
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21 exposure (Fig. 7A). By using actinomycin D (a transcriptional inhibitor) and cycloheximide (a  
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23 translational inhibitor), we observed that decreased CARP mRNA levels by AE was related to  
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25 reduced stabilization of the mRNAs. To extend the above observations, we tested whether the  
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27 ectopic expression of FLAG-tagged CARP1 or 2 leads to the suppression of caspase-8  
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29 activity and apoptosis. Expression levels of FLAG-CARP1 and -2 were confirmed by western  
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31 blotting using FLAG-specific antibody (Fig. 7B, panel I). As showed in panels II and III of  
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33 Fig. 7B, AE-induced increases of caspase-8 activity and apoptosis were inhibited in the  
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35 CARP1- or CARP2-transfected cells compared with control vector-transfected cells. Because  
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37 the overexpression of CARPs can contribute to the ubiquitin-mediated proteolysis of  
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39 caspase-8 [McDonald and El-Deiry, 2004], we then investigated whether CARP  
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41 overexpression affects the levels of caspase-8 protein. As expected, pro-caspase-8 protein  
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43 levels were reduced when FLAG-CARP1 or FLAG-CARP2 was transfected in cells (Fig. 7B,  
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4 panel I), whereas pro-caspase-10 protein levels were partially affected. In contrast,  
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7 overexpression of FLAG-CARPs did not affect levels of pro-caspase-9 protein. Taken  
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10 together, these data demonstrate that the decrease of CARPs is required for caspase-8  
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13 activation and apoptosis induction in response to AE.  
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## DISCUSSION

In this study, we used the mutant p53 (R248L)-carrying cell line and the p53-null human lung cancer cell lines H1299, Hep3B, and MG-63 to explore the mechanism of p53-independent apoptosis induced by AE. Transient ectopic expression of wt p53, a p53- and p21-dependent luciferase reporter assay, and quantitative analyses of DNA fragmentation and caspase-8 activity in the presence of Z-IETD-FMK confirmed the induction of apoptotic cell death by AE through a caspase-8-dependent but p53-independent pathway. Furthermore, CARP proteins appear to be a crucial regulator activation of caspase-8 in response to AE, since overexpression of CARP 1 and 2 reduced AE-induced caspase-8 activation and apoptosis. AE contains a quinine structure that is predicted to induce ROS production, which may play a role in the induction of cancer cell apoptosis [Lee et al., 2006]. AE displays an affinity for nuclear DNA, and high doses of AE disrupt chromatin structure and DNA template function in susceptible cell lines [Mueller and Stopper, 1999]. The participation of ROS in cancer cell apoptosis that is stimulated by chemotherapeutic agents through the induction of DNA damage has been investigated for several decades [Lau et al., 2008]. Oxidative damage to DNA is a result of the interaction of DNA with ROS. Consistent with previously reported results [Lee et al., 2006], an increase in intracellular ROS levels was observed when cells were induced to undergo apoptosis. ERK has been implicated in DNA damage-induced p53-independent apoptosis, and its activation is regulated by ROS [Wang et al., 1998]. We

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4 found that AE caused an increase in ROS levels that was completely inhibited by co-treatment  
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7 with Z-IETD-FMK or CsA. The phosphorylation of ERK induced by AE was suppressed by  
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10 Z-IETD-FMK. Moreover, PD98058 and ERK siRNA significantly attenuated the AE-induced  
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13 expression of p21 and S-phase arrest, suggesting that ERK activation is correlated with  
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16 AE-mediated p21 induction and S-phase arrest. Our results are in agreement with a previous  
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19 report that activated ERK induces p21 expression in a p53-independent manner and that p21  
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22 up-regulation results in S-phase arrest [Zhu et al., 2004].  
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26 p21 inhibits the phosphorylation of Rb by the cyclin A–Cdk2 complex, which regulates the  
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29 transition of G<sub>1</sub> to S-phase [Harper et al., 1993]. p21 also inhibits DNA synthesis and cell  
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32 growth by binding to PCNA and Cdk2 with its N- and C-terminal regions, respectively  
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35 [Rousseau et al., 1999]. The association of p21 with cyclin A–Cdk2 complexes was detected  
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38 12–48 h after AE treatment. Attenuating the activation of ERK with PD98059 or ERK siRNA  
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41 significantly inhibited the expression of p21 and partially inhibited apoptosis induced by AE.  
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44 Overexpression of p21 inhibits TRAIL death receptor DR4–dependent caspase-8 activation  
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47 [Xu and El-Deiry, 2000]. An analysis using chimeric caspase-8 with the transmembrane and  
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50 extracellular domains of the human CD8 $\alpha$  chain did, however, demonstrate that caspase-8  
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53 oligomerization at the cell membrane is sufficient for its autoactivation and apoptosis  
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56 induction [Martin et al., 1998]. Several chemotherapeutic agents, such as *N,N*-dimethyl  
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59 phytosphingosine and curcumin, induce apoptosis through the cleavage of cytosolic Bid by  
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4 activated caspase-8 [Anto et al., 2002; Kim et al., 2009]. Our results show that Z-IETD-FMK  
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7 inhibited the cleavage and activation of caspase-8 and of apoptotic cell death induced by AE.  
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10 The FaDu cell line has a homozygous deletion of the death receptor DR4 and is resistant to the  
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13 cytotoxic effects of TRAIL [Ozoren et al., 2000]. Moreover, FADD siRNA did not interfere  
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16 with the activation of caspase-8 in FaDu, Hep3B, or MG-63 cells treated with AE (data not  
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19 shown), suggesting that AE-induced caspase-8 activation and apoptosis do not occur through  
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22 the induction of the death receptor. The promoter region of caspase-8 contains an  
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25 E2F1-responsive element, and its transcriptional activity can be regulated by E2F1 [Afshar et  
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28 al., 2006]. Previous findings also show that radiation does not induce E2F1 activity, caspase-8  
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31 activity, or apoptotic cell death when cells express wt p53; however, inhibition of wt p53  
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34 function with HPV-16 E6 induces caspase-8 activity and apoptosis following radiation  
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37 [Afshar et al., 2006]. Taken together, these observations led us to speculate that the  
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40 caspase-8-dependent mitochondrial ROS production is critical for ERK activation and the  
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43 subsequent p21 induction and E2F1 expression. The formation of cyclin A-Cdk2-p21  
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46 complexes induced by activated ERK after treatment with AE was associated with the  
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49 induction of S-phase arrest, and E2F1 upregulation may contribute to caspase-8 activation.  
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54 The release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol in  
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57 response to stress stimuli is a critical step for the activation of caspase-9 in apoptotic cell  
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60 death [Tsujiimoto, 2003]. In cultured dopaminergic PC12 cells, caspase-9 activation via

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4 cytochrome *c* release in response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine results in  
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7 the activation of caspase-3 and -8 and the cleavage of Bid to tBid [Viswanath et al., 2001].  
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10 Our results show that either CsA or Z-IETD-FMK inhibited AE-induced loss of  $\Delta\psi_m$  and  
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12 apoptotic cell death. Co-treatment with Z-IETD-FMK also completely blocked Bid cleavage,  
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14 the translocation of tBid to the mitochondria, Bax induction, caspase-9 activation, and  
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16 the translocation of tBid to the mitochondria, Bax induction, caspase-9 activation, and  
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18 apoptosis. Moreover, experiments using Z-IETD-FMK showed that caspase-8 activity  
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20 appeared to be involved in the AE-induced release of cytochrome *c*, AIF, and Endo G from  
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22 the mitochondria and apoptosis. Our findings suggest that caspase-9 was activated directly by  
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24 caspase-8 through the release of cytochrome *c*.  
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32 CARPs act as ubiquitin protein ligases that bind to and regulate caspase-8 and -10.  
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34 siRNA-mediated attenuation of CARP expression significantly sensitizes cells to  
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36 chemotherapy-induced apoptosis and inhibits colony formation by cancer cells [McDonald  
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38 and El-Deiry, 2004]. In addition, treatment with TNF- $\alpha$ /cycloheximide or TRAIL leads to  
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40 enhanced cleavage of caspase-8 and -10 after treatment with CARP1 or CARP2 siRNA. One  
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42 study using a caspase-3 inhibitor or a combination of caspase-8 and -10 inhibitors and the  
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44 proteasome inhibitor MG132 demonstrated that cleavage of CARPs was caspase-dependent  
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46 during death receptor-mediated apoptosis [McDonald and El-Deiry, 2004]. CARPs are also  
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48 overexpressed in a variety of human cancers and cancer cell lines, suggesting that CARPs  
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50 could contribute to development of tumors [McDonald and El-Deiry, 2004]. However,  
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4 resistance to chemotherapy-induced or death receptor-mediated apoptosis has not been  
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7 observed in cells overexpressing CARP1 or CARP2. AE treatment of FaDu, Hep3B, and  
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10 MG-32 cells led to the rapid decrease of both *CARP1* and *CARP2* mRNAs. Overexpression of  
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13 either CARP1 or CARP2 blocked AE-induced caspase-8 activity and apoptosis as well as  
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16 decreased the levels of pro-caspase-8 protein. Unexpectedly, pro-caspase-10 protein levels  
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19 only slightly decreased in CARP1- or CARP2-overexpressing cells. The sequences of  
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22 caspase-8 and -10 are highly similar [Fernandes-Alnemri et al., 1996]. The involvement of  
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25 caspase-10 in apoptosis signaling by death receptors has also been reported [Wang et al.,  
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28 2001]. However, *in vitro* experiments showed that overexpression of caspase-10 is able to  
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31 induce the process of caspase-3 cleavage and apoptosis [Vincenz and Dixit, 1997].  
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34 Caspase-8-deficient cell lines were shown to be resistant toward Apo2L/TRAIL-induced  
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37 apoptosis, implying that caspase-8 plays a critical role in death receptor-mediated signaling,  
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40 whereas caspase-10 is not important for this function [Bodmer et al., 2000]. Interestingly,  
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43 studies using a series of caspase-10-specific antibodies demonstrated that apoptosis signaling  
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46 by death receptors involves not only caspase-8 but also caspase-10 [Kischkel et al., 2001]. If  
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49 AE treatment induced apoptosis by increasing the activities of both caspase-8 and -10, the  
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52 addition of an inhibitor of either caspase-8 or -10 would be predicted to result in decreased  
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55 apoptosis. We found that apoptotic induction by AE was inhibited by caspase-8 inhibitor, but  
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58 not by caspase-10 inhibitor. Furthermore, caspase-10 cleavage and activation were not  
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4 detected in AE-treated cells (data no shown). These findings indicate that caspase-8 activation  
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7 is an important event in induction of apoptosis by AE. These observations together with our  
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10 findings suggest that the function and regulation of both caspase-8 and -10 are independent of  
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13 apoptosis induction. This raised the possibility that the two proteins have distinct and perhaps  
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16 complementary functions.  
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20 In conclusion, we present a novel p53-independnet mechanism of AE-induced apoptosis of  
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23 cancer cells involving decreased stability of CARP mRNAs and the subsequent induction of  
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26 ERK and caspase-8-mediated mitochondrial death pathways. However, the molecular  
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29 mechanisms of the diminished CARP mRNA stability in AE-treated cells remain to be further  
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4 arrest and p21 overexpression by a small molecule 2[[3-(2,3-dichlorophenoxy)propyl]  
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**FIGURE LEGENDS**

Fig. 1. AE induces apoptotic cell death in a p53-independent manner. (A) The effect of AE on cell viability. FaDu, Hep3B, MG-32, or H1299 cells were plated in 24-well plates and treated with either DMSO (vehicle control) or the indicated concentrations of AE for 36 h (panel I). After treatment, cell viability was determined by flow-cytometric analysis of PI uptake. The values presented are the mean  $\pm$  standard error from three independent experiments.  $*p < 0.05$ , significantly different from vehicle-treated cells. The  $IC_{50}$  value of AE in these cells is indicated (panel II). (B) p53 is not involved in the induction of apoptotic cell death or of caspase-3 activity by AE. Panel I: levels of p53 proteins determined by western blot analysis with p53 antibodies (left) and levels of p53 transcripts determined by RT-PCR with specific primers (right) in cells stably expressing the empty control vector (-), GFP shRNA, or p53 shRNA.  $\beta$ -Actin was used as an internal control for sample loading. Panels II and III: after treatment of the empty control vector (-), GFP shRNA, and p53 shRNA cells with vehicle, AE (60  $\mu$ M), AE (60  $\mu$ M) plus Ac-DEVD-CMK (10  $\mu$ M), or AE (60  $\mu$ M) plus Z-VAD-FMK (15  $\mu$ M) for 36 h, caspase-3 activity and DNA fragmentation were determined using flow cytometry and cell death-detection ELISA, respectively. The values presented in panels I and II are the mean  $\pm$  standard error from three independent experiments.

Fig. 2. p53 activity is not involved in the induction of cell death or of p21 activity. (A) A



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4 luciferase reporter driven by three p53-binding sites was co-transfected with vector alone  
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7 (lanes 1 and 2), wt p53 (lanes 3 and 4), mutant p53 (R248W) (lanes 5 and 6), or mutant p53  
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10 (R175H) (lanes 7 and 8) into stably p53 shRNA-expressing cells. (B) As in (A), except that  
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13 the reporter was driven by the p21 promoter. At 12 h post-transfection, cells were treated with  
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16 vehicle or AE (60  $\mu$ M) for 24 h, and then luciferase activity was determined. (C and D). p53  
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19 shRNA-expressing stable cells were transfected with vector alone (lanes 1 and 2), wt p53  
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22 (lanes 3 and 4), mutant p53 (R248W) (lanes 5 and 6), or mutant p53 (R175H) (lanes 7 and 8)  
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25 for 12 h. Cells were then treated with either vehicle or AE (60  $\mu$ M) for 24 h. Cell viability and  
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28 protein expression were determined using the MTT assay and western blot analysis,  
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30  
31 respectively.  $\gamma$ -Tubulin was used as an internal control for sample loading in panel IV.  
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38 Fig. 3. Involvement of cyclin A-Cdk2-p21-E2F1 complexes in AE-induced S-phase cell  
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41 cycle arrest. (A) Effects of AE on cyclin protein expression and the levels of cyclin A, p21,  
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44 and E2F1 bound to Cdk2. Panel I: after 36 h of treatment of the GFP shRNA- or p53  
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47 shRNA-expressing cells with vehicle or AE (60  $\mu$ M), the levels of cyclin A, cyclin B1, cyclin  
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50 D, cyclin E, Cdk2, p-Cdk2 (Thr14/Thr15), E2F1, and PCNA in the cell lysates were analyzed  
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53 using specific antibodies.  $\gamma$ -Tubulin was used as an internal control for sample loading. Panel  
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56 II: co-immunoprecipitation of cyclin A, Cdk2, and p21 was performed using lysates prepared  
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59 from AE (60  $\mu$ M)-treated p53 shRNA-expressing stable cells at 36 h. The antibody used for  
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4 co-immunoprecipitation is indicated at the top. The proteins in the immunoprecipitated  
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7 complexes were detected using western blotting with specific antibodies. Mouse normal IgG  
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10 was used as a control for antibody specificity. (B) At 12 h after transfection of p53  
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13 shRNA-expressing cells with control or cyclin A siRNA, cells were treated with either  
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16 vehicle or AE (60  $\mu$ M) for 24 h. After WB was used to examine the cyclin A and Cdk2 levels,  
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19 the percentage of cells in S-phase was analyzed by flow-cytometric analysis of PI-stained  
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23 cells.

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28  
29 Fig. 4. Activation of ERK is involved in the induction of p21 and E2F1 expression, S-phase  
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32 arrest, and apoptosis. At 12 h after transfection with ERK siRNA (pKD-MAPK1/Erk) or 2 h  
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34  
35 after treatment with PD98059, cells were treated with either vehicle or AE (60  $\mu$ M) for 24 h.  
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37  
38 (A) The levels of ERK, p-ERK (Tyr202/204), p21, and E2F1 in the cell lysates were then  
39  
40  
41 determined by western blotting using specific antibodies.  $\gamma$ -Tubulin was used as an internal  
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43  
44 control for sample loading. (B) The cell cycle profile and fraction of apoptotic cells (sub-G<sub>1</sub>  
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47 phase population) were determined using flow cytometry. The values presented are the mean  
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50  $\pm$  standard error from three independent experiments.  
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57 Fig. 5. Changes in Bcl-X<sub>L</sub> and Bax expression are involved in AE-induced apoptosis. (A) The  
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60 effect of AE on the expression of Bcl-2 family proteins. The levels of Bcl-X<sub>L</sub>, Bcl-2, Bax, Bak,

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4 Bid, and tBid in cell lysates prepared after 36 h of treatment with vehicle or AE (60  $\mu$ M) were  
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6 analyzed using specific antibodies (panels I and II).  $\gamma$ -Tubulin was used as an internal control  
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8 for sample loading. (B) Ectopic expression of Bcl-X<sub>L</sub> suppresses AE-induced apoptosis. At 12  
9  
10 h after transfection with a FLAG-tagged Bcl-X<sub>L</sub> or Bcl-2 expression construct, cells were  
11  
12 treated with vehicle or AE (60  $\mu$ M) for 24 h. Panel I: Expression levels of Bcl-X<sub>L</sub> in lysates  
13  
14 prepared from cells transfected with vector alone, FLAG-Bcl-X<sub>L</sub>, or FLAG-Bcl-2.  
15  
16 FLAG-Bcl-X<sub>L</sub>, FLAG-Bcl-2, Bcl-X<sub>L</sub>, and Bcl-2 were detected with the antibodies shown. (C)  
17  
18 Bax siRNA suppresses AE-induced apoptosis and PARP cleavage. At 12 h after transfection  
19  
20 with control siRNA, Bax siRNA, or Bak siRNA, cells were treated with vehicle or AE (60  $\mu$ M)  
21  
22 for 24 h. Panel I: the levels of Bax, Bak, and PARP in the cell lysates were determined by  
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24 western blotting using specific antibodies.  $\gamma$ -Tubulin was used as an internal control for  
25  
26 sample loading. Panels II and III (B and C): DNA fragmentation and the fraction of apoptotic  
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28 cells (sub-G<sub>1</sub> phase population) were determined using cell death–detection ELISA and flow  
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30 cytometry, respectively. The values presented are the mean  $\pm$  standard error from three  
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32 independent experiments.  
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54 Fig. 6. Caspase-8 activation is involved in AE-induced  $\Delta\psi_m$  loss, increases in cytosolic ROS  
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56 and Ca<sup>++</sup> levels, ERK phosphorylation, caspase-9 activation, apoptosis, and the release of  
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58 cytochrome *c*, AIF, and Endo G from mitochondria. (A) AE-induced changes in the levels of  
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4  $\Delta\psi_m$ ,  $Ca^{++}$ , and ROS. At the indicated times, cells treated with vehicle, AE (60  $\mu$ M), AE (60  
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7  $\mu$ M) plus CsA (5  $\mu$ M), AE (60  $\mu$ M) plus Z-IETD-FMK (40  $\mu$ M), AE (60  $\mu$ M) plus dantrolene  
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10 (25  $\mu$ M), AE (60  $\mu$ M) plus PD98059 (15  $\mu$ M), or AE (60  $\mu$ M) plus PD98059 (15  $\mu$ M) and  
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13 Z-IETD-FMK (40  $\mu$ M) were harvested for the measurement of  $\Delta\psi_m$ , ROS,  $Ca^{++}$ , and DNA  
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16 fragmentation. Panel I: the decrease in DiOC<sub>6</sub> fluorescence was measured by flow cytometry.  
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19 Panels II and III: the generation of ROS and the cytosolic level of  $Ca^{++}$  were monitored by  
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22 measuring increased fluorescence of Indo-1 and 2,7-dichlorodihydrofluorescein diacetate  
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25 (DCF), respectively, by flow cytometry. Panel IV: DNA fragmentation was determined using  
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28 cell death–detection ELISA. (B) Z-IETD-FMK suppresses AE-induced Bax activation, Bid  
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31 cleavage, translocation of tBid to the mitochondria, and ERK phosphorylation. The levels of  
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34 caspase-8, Bax, Bid, tBid, ERK, and p-ERK (Tyr202/204) in whole-cell lysates (panel I) or  
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37 tBid in mitochondrial extracts (panel II) were detected after 24 h of treatment with vehicle,  
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40 AE (60  $\mu$ M), or AE (60  $\mu$ M) plus Z-IETD-FMK (40  $\mu$ M).  $\gamma$ -Tubulin and Cox2 were measured  
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43 as internal controls for whole cells and mitochondria, respectively. (C) Caspase-8 is involved  
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46 in the induction of caspase-9 and -3 activities. Cells were treated with vehicle, AE (60  $\mu$ M),  
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49 AE (60  $\mu$ M) plus Z-LEHD-FMK (30  $\mu$ M), AE (60  $\mu$ M) plus LEVD-CHO (40  $\mu$ M), AE plus  
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51  
52 Z-IETD-FMK (40  $\mu$ M), or AE (60  $\mu$ M) plus PD98059 (15  $\mu$ M) for 36 h. Panels I and II:  
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55 caspase-9 activity and DNA fragmentation were determined using flow cytometry and cell  
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58 death–detection ELISA, respectively. The values presented are the mean  $\pm$  standard error  
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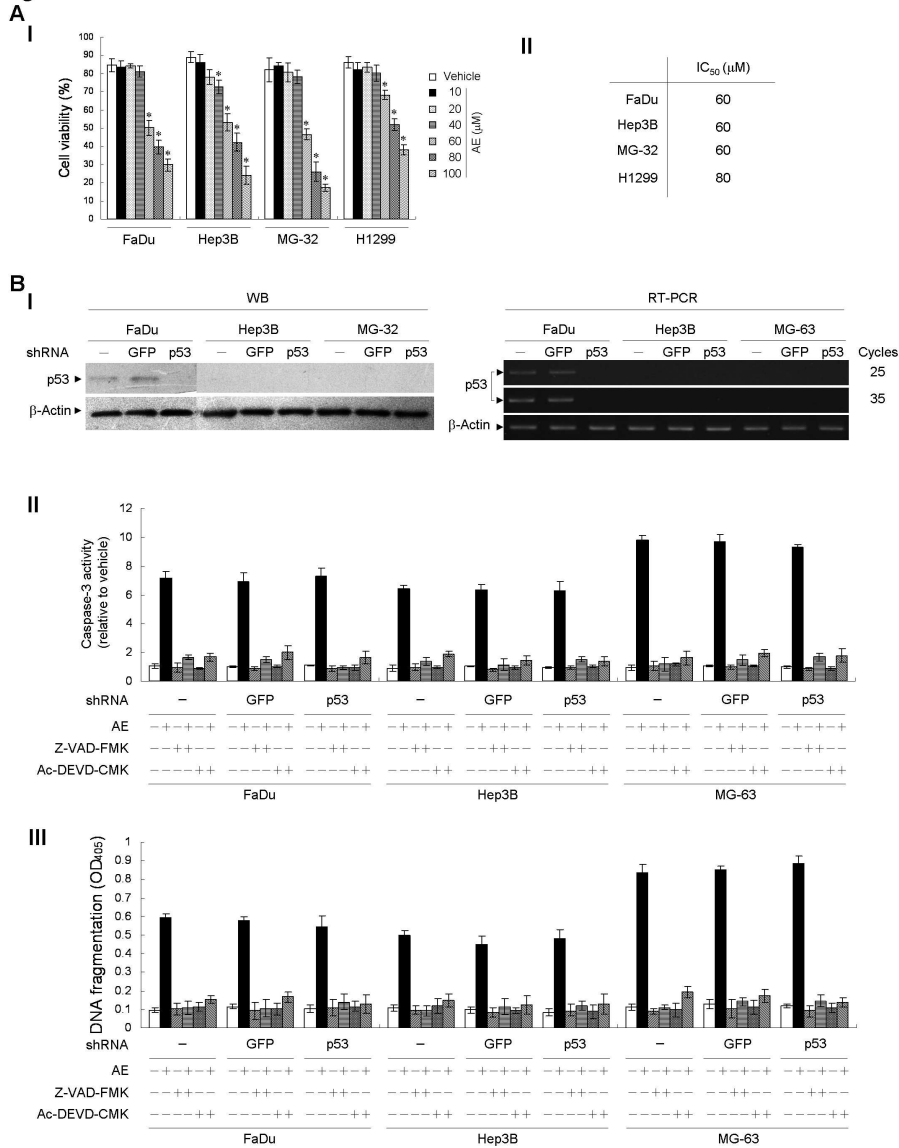
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4 from three independent experiments. (D) Caspase-8 activation is involved in the release of  
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7 cytochrome *c*, AIF, and Endo G from mitochondria in AE-treated cells. Cells were treated  
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10 with vehicle, AE (60  $\mu$ M), or AE (60  $\mu$ M) plus Z-IETD-FMK (40  $\mu$ M) for 36 h, and the levels  
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12  
13 of cytochrome *c* (panel I), AIF (panel II), and Endo G (panel III) in the cytosol and  
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16 mitochondria were determined by western blotting using specific antibodies.  $\gamma$ -Tubulin and  
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19 Cox2 were determined as internal controls for whole cells and mitochondria, respectively.  
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26 Fig. 7. AE induces caspase-8 activation and apoptosis by decreasing the stability of *CARP1*  
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28 and *CARP2* mRNAs. (A) RT-PCR reveals that the stability of *CARP1* and *CARP2* mRNAs  
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31 was reduced in AE-treated cells. The cells were pre-incubated with 0.5  $\mu$ g/ml of actinomycin  
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34 D (AcD) or 2  $\mu$ g/ml of cycloheximide (CHX) for 1 h, cells were then treated with vehicle or  
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37 AE (60  $\mu$ M) for the indicated periods. Total RNAs were reverse-transcribed and  
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40 PCR-amplified using *CARP1*- or *CARP2*-specific primers.  $\beta$ -Actin was used as an internal  
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43 control for sample loading. The values above the figures represent relative densities of the  
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46 bands normalized to  $\beta$ -Actin. (B) Ectopic expression of *CARP1* or *CARP2* suppresses  
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49 AE-induced caspase-8 activation and apoptosis. Panel I: Expression levels of Bcl-X<sub>L</sub> in  
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52 lysates prepared from cells transfected with vector alone, FLAG-CARP1, or FLAG-CARP2.  
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55 FLAG-CARP1, FLAG-CARP2, pro-caspase-8, and pro-caspase-9 were detected with the  
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58 antibodies shown. Panels II and III: At 12 h after transfection with vector alone,  
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4 FLAG-CARP1, or FLAG-CARP2, cells were treated with vehicle, AE (60  $\mu$ M) or AE (60  $\mu$ M)  
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7 plus Z-IETD-FMK (40  $\mu$ M) for 24 h. Caspase-8 activity and DNA fragmentation were  
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10 determined using flow cytometry and cell death–detection ELISA, respectively. The values  
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13 presented are the mean  $\pm$  standard error from three independent experiments.  
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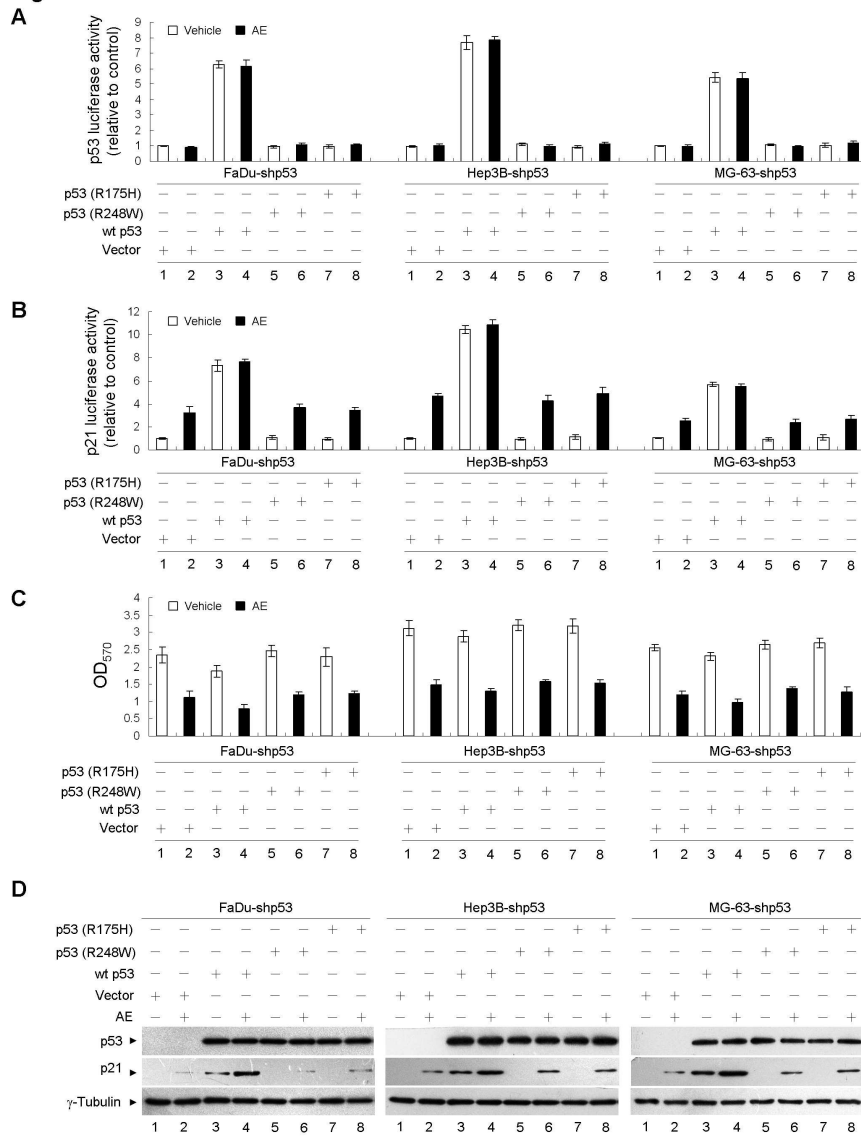
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Figure 1



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Figure 2

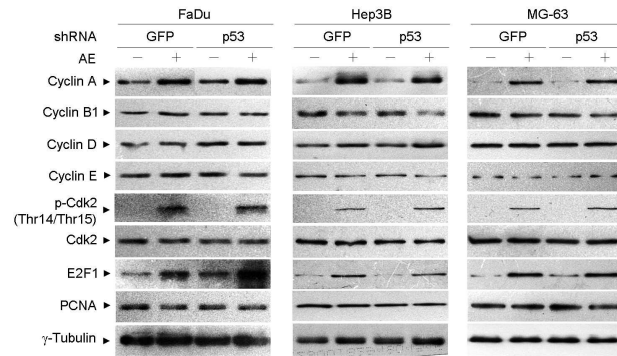


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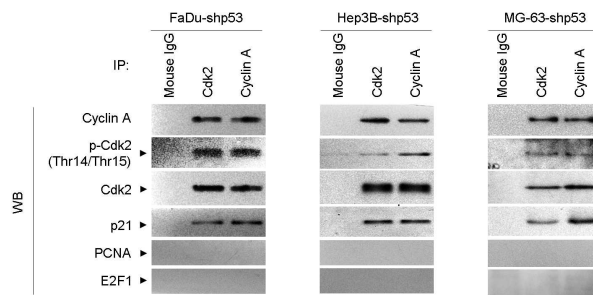


Figure 3

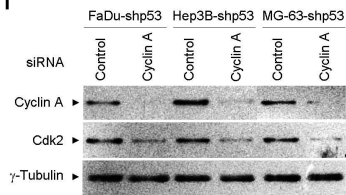
A<sub>I</sub>



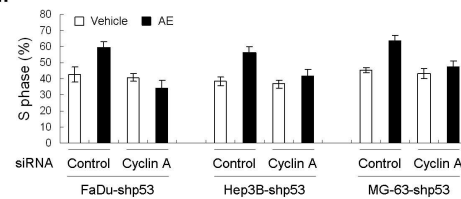
II



B<sub>I</sub>

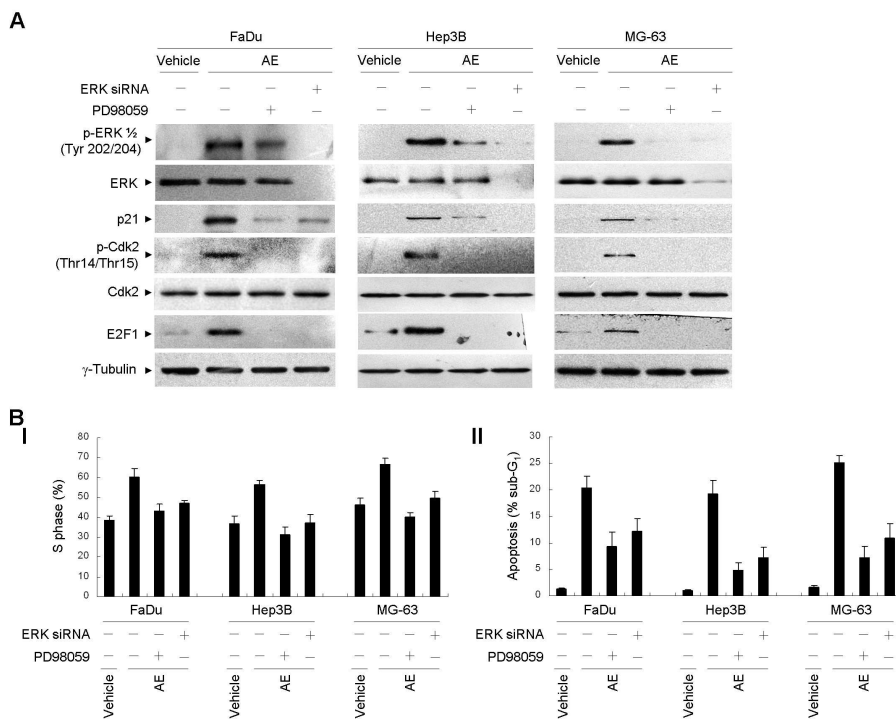


II



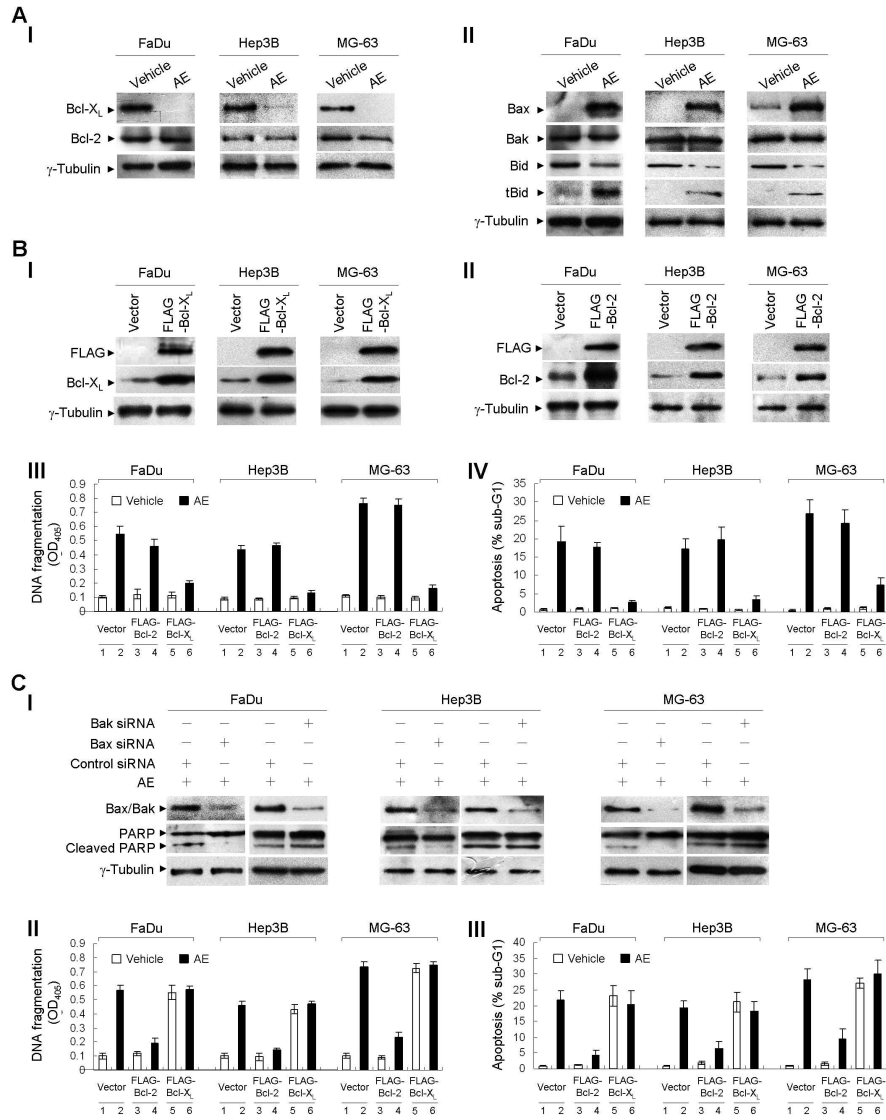
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Figure 4



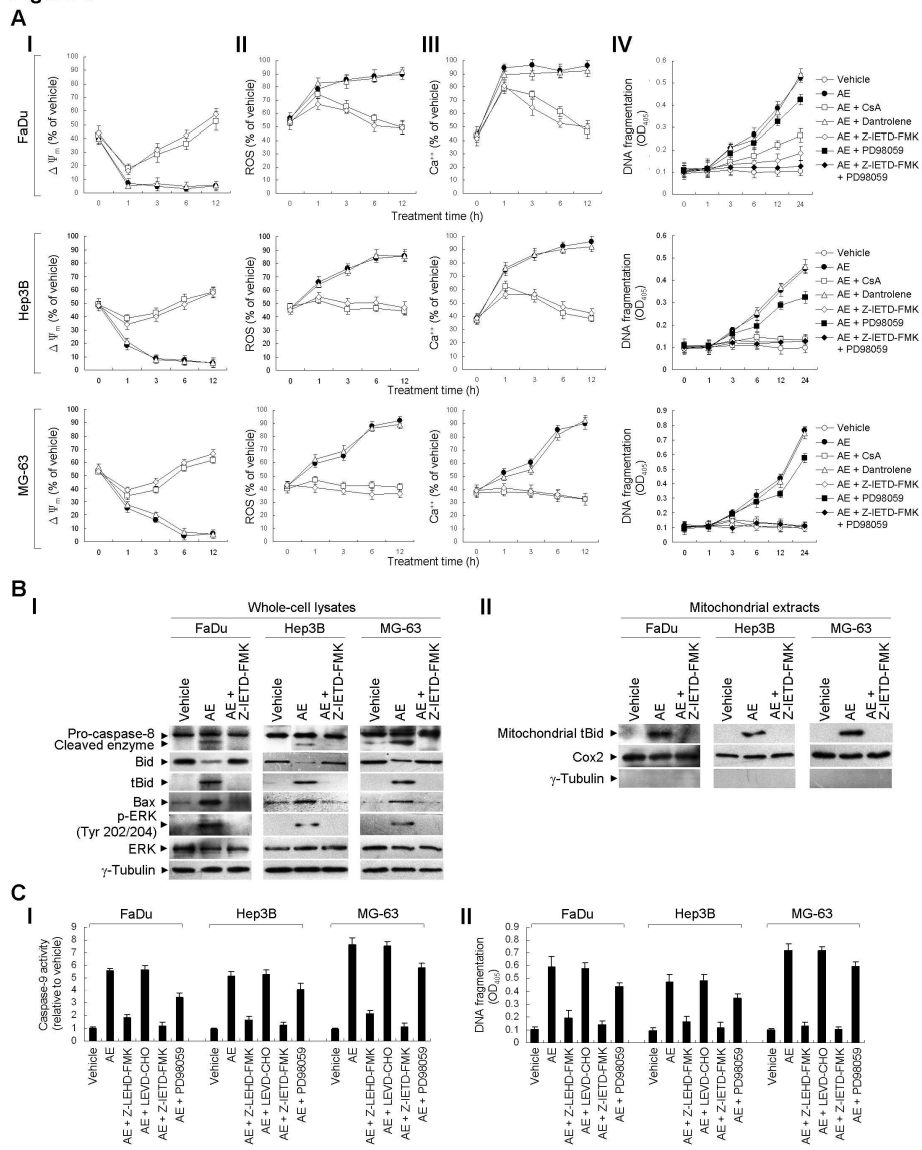
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Figure 5

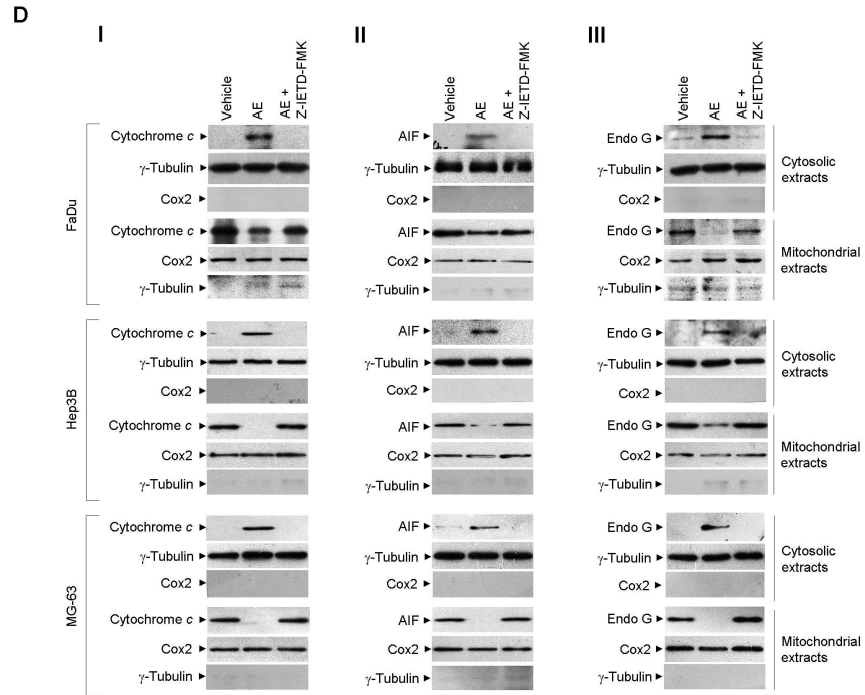


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Figure 6

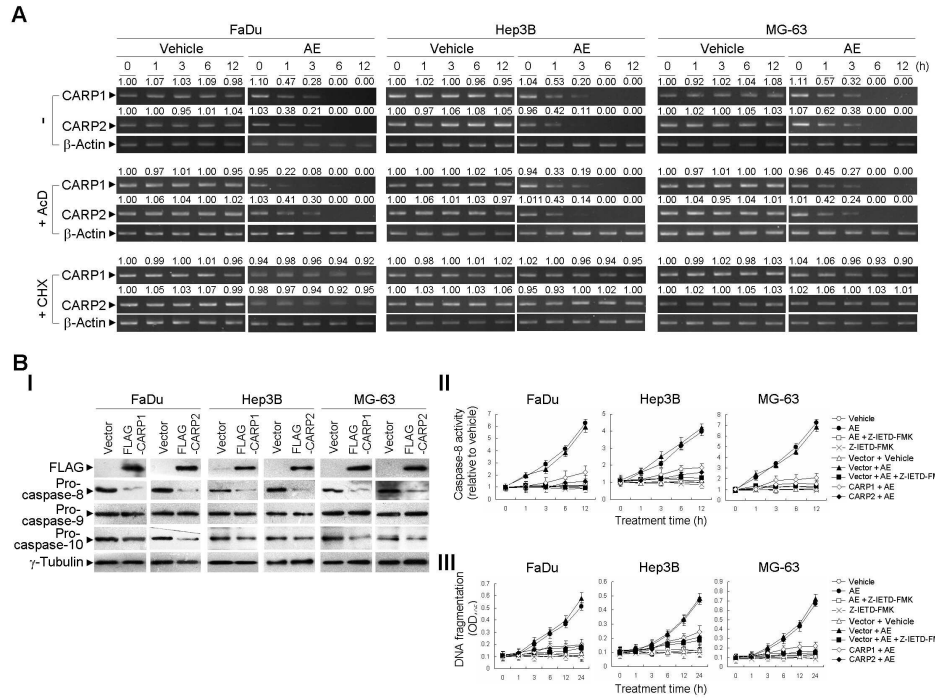


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Figure 7



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Table 1 - Effect of AE on the cell cycle progression of p53-deficient tumor cell lines

Time	Cell line		Cell cycle phase (% of cells)							
			G <sub>1</sub>		S		G <sub>2</sub> /M		Sub-G <sub>1</sub>	
			Vehicle	AE	Vehicle	AE	Vehicle	AE	Vehicle	AE
24 h	FaDu	shGFP	44.2 ± 3.1	25.8 ± 5.2*	46.8 ± 4.2	59.2 ± 2.8*	8.8 ± 3.9	14.4 ± 3.2	0.6 ± 0.1	12.4 ± 2.3
		shp53	43.9 ± 4.3	25.0 ± 3.1*	45.6 ± 2.2	61.8 ± 3.9*	9.9 ± 1.5	13.5 ± 2.2	0.6 ± 0.2	14.7 ± 3.2
	Hep3B	shGFP	48.8 ± 4.2	18.1 ± 4.3*	43.3 ± 3.9	67.8 ± 6.1*	7.8 ± 2.5	14.2 ± 5.0	0.3 ± 0.1	10.2 ± 2.1
		shp53	49.1 ± 5.7	19.4 ± 2.1*	41.2 ± 5.1	65.9 ± 3.8*	9.7 ± 3.5	15.2 ± 3.1	0.3 ± 0.1	12.7 ± 2.6
	MG-63	shGFP	40.7 ± 3.9	20.4 ± 3.8*	44.2 ± 2.1	61.3 ± 5.1*	15.1 ± 2.4	18.3 ± 2.2	0.3 ± 0.1	20.2 ± 1.9
		shp53	41.9 ± 5.3	24.4 ± 2.8*	44.6 ± 3.1	58.7 ± 2.2*	13.4 ± 2.9	16.9 ± 3.7	0.4 ± 0.1	19.6 ± 3.4
48 h	FaDu	shGFP	62.3 ± 4.2	29.1 ± 4.8*	32.6 ± 2.5	62.4 ± 2.6*	5.4 ± 2.2	7.9 ± 3.7	0.9 ± 0.3	30.2 ± 4.2
		shp53	63.4 ± 5.2	27.6 ± 3.1*	31.6 ± 3.1	64.7 ± 4.7*	4.9 ± 2.7	8.6 ± 3.2	0.7 ± 0.2	28.9 ± 2.5
	Hep3B	shGFP	59.3 ± 2.8	11.8 ± 4.3*	26.8 ± 2.2	76.8 ± 4.3*	13.5 ± 2.1	11.7 ± 5.2	1.1 ± 0.3	17.5 ± 3.0
		shp53	56.9 ± 5.1	12.3 ± 2.9*	30.6 ± 4.1	78.6 ± 3.6*	12.6 ± 4.1	9.4 ± 2.9	0.9 ± 0.2	19.2 ± 3.7
	MG-63	shGFP	68.4 ± 2.2	18.4 ± 2.9*	23.6 ± 3.8	72.2 ± 3.9*	8.0 ± 1.1	9.4 ± 1.1	0.9 ± 0.2	39.3 ± 3.8
		shp53	70.1 ± 5.7	16.5 ± 4.1*	24.1 ± 4.1	75.2 ± 2.7*	5.8 ± 3.5	8.3 ± 2.8	1.3 ± 0.5	37.1 ± 4.6

The values presented are the mean ± standard errors from three independent experiments. \* $p < 0.05$ , significantly different from vehicle-treated cells.

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4 Executive Editor, Journal of Cellular Biochemistry

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6 Dear Dr. C. Fred Fox,  
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9

10 Thank you very much for your letter of January 11, 2011. We appreciate very much  
11 your helpful suggestions for the revision of our manuscript (#JCB-10-0480) entitled  
12 “Destabilization of CARP mRNAs by aloe-emodin contributes to caspase-8-mediated  
13 p53-independent apoptosis of human carcinoma cells” by Meng-Liang Lin et al. The  
14 manuscript has been revised according to the instructions given by both you and the  
15 reviewer. The changes made during the revision are as following:  
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27 Comment #1: *The only substantive criticism raised was directed at Table 1, in which*  
28 *the values shown certainly exceed the number of significant figures shown in the*  
29 *data. Please resubmit your manuscript with Table 1 modified to eliminate values at*  
30 *the 1/100 percent level, e.g., 44.2 +/- 3.1 instead of 44.2 +/- 3.12. Please also proof*  
31 *the manuscript one final time since it will be published on line without text editing*  
32 *and I detected a few spelling errors, e.g., data no shown instead of data not shown.*  
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41 **[Answer]**

- 42  
43 1. According to the reviewer’s comment, we have changed new statistical data on  
44 the Table 1 that the present study was performed to analyze the effect of AE on the  
45 cell cycle progression of p53-deficient tumor cell lines.  
46  
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50 2. Corrections were made according to the reviewer’s suggestion. The original  
51 description text has been changed to the following:  
52  
53  
54 (1) MATERIALS AND METHODS section. Page 12, lines 13-15 of the first  
55 paragraph: Underlined portion has been added.  
56  
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59 A luciferase reporter driven by three p53 binding sites or by the p21 promoter  
60 and a plasmid expressing wild-type (wt) p53, mutant p53 (R248W), or p53



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4 (R175H) were transfected into cells using Lipofectamine 2000.  
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- 6 (2) RESULTS section. Page 21, lines 1-2 of the first paragraph: Underlined portion  
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8 has been changed.  
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11 Nevertheless, AE treatment did not result in the activation of p38 MAPK or  
12  
13 JNK (data no shown).  
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- 15 (3) RESULTS section. Page 22, lines 4-5 of the first paragraph: Underlined portion  
16  
17 has been changed.  
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19  
20 The expression of the inactivator BH3 proteins Bad, Bik, and NOXA did not  
21  
22 change after AE treatment (data no shown).  
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- 24 (4) DISCUSSION section. Page 29, lines 4-7 of the first paragraph: Underlined  
25  
26 portion has been changed.  
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29 Moreover, FADD siRNA did not interfere with the activation of caspase-8 in  
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31 FaDu, Hep3B, or MG-63 cells treated with AE (data no shown), suggesting that  
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33 AE-induced caspase-8 activation and apoptosis do not occur through the induction  
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35 of the death receptor.  
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- 38 (5) DISCUSSION section. Page 32, line 1 of the first paragraph: Underlined portion  
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40 has been changed.  
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43 Furthermore, caspase-10 cleavage and activation were not detected in  
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45 AE-treated cells (data no shown).  
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6 We believed that the reviewers' questions have been thoroughly addressed and that  
7  
8 the manuscript has been carefully revised according to your instructions. We would  
9  
10 like to learn your opinions on the appropriateness of the revised manuscript for being  
11  
12 published in Journal of Cellular Biochemistry. If there is anything that I could be of  
13  
14 further assistance, please do not feel hesitated to inform me. Thank you in advance for  
15  
16 your help. I am looking forward to hearing from you.  
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36 Best wishes,  
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