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Anti-cancer Effects of JKA97 Are Associated with Its Induction of Cell Apoptosis via a Bax-dependent and p53-independent Pathway*

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p53, one of the most commonly mutated genes in human cancers, is thought to be associated with cancer development. Hence, screening and identifying natural or synthetic compounds with anti-cancer activity via p53-independent pathway is one of the most challenging tasks for scientists in this field. Compound JKA97 (methoxy-1-styryl-9*H*-pyrid-[3,4-*b*]-indole) is a small molecule synthetic anti-cancer agent, with unknown mechanism(s). In this study we have demonstrated that the anti-cancer activity of JKA97 is associated with apoptotic induction via p53-independent mechanisms. We found that co-incubation of human colon cancer HCT116 cells with JKA97 inhibited HCT116 cell anchorage-independent growth *in vitro* and tumorigenicity in nude mice and also induced a cell apoptotic response, both in the cell culture model and in a tumorigenesis nude mouse model. Further studies showed that JKA97-induced apoptosis was dramatically impaired in Bax knock-out ($Bax^{-/-}$) HCT116 cells, whereas the knock-out of p53 or PUMA did not show any inhibitory effects. The p53-independent apoptotic induction by JKA97 was confirmed in other colon cancer and hepatocarcinoma cell lines. In addition, our results showed an induction of Bax translocation and cytochrome *c* release from the mitochondria to the cytosol in HCT116 cells, demonstrating that the compound induces apoptosis through a Bax-initiated mitochondria-dependent pathway. These studies provide a molecular basis for the therapeutic application of JKA97 against human cancers with p53 mutations.

The p53 protein is a transcription factor that enhances the transcriptional expression of several genes involved in the response to genotoxic agents, such as ionizing radiation and certain chemicals, including chemical therapeutic drugs (1). After activation, p53 enables the initiation of cell cycle arrest and DNA damage repair; but when cells harbor irreparable DNA damage, p53 activates cell death programs, and the cells then undergo apoptosis (2). Our recent studies have demonstrated that p53 has a suppressive activity on the cell signaling pathways leading to the activation of AP-1 and NFκB when the cell responds to UV radiation (3). We have also found that the inhibition of AP-1 and NFκB by tumor suppressor p53 is mediated via up-regulation of PTEN (phosphotase and tensin homolog) expression (3). Thus, p53 is a key tumor suppressor against cancer development. However, inactivation of p53 occurs very frequently in various cancers (4). Previous studies have shown that ~50% of human tumors carry inactivating p53 mutations (5, 6).

Cancers of the colon and rectum continue to be the third most common fatal cancers in the United States. They account for 10% of the total cancer deaths among men and women (7). The p53 tumor suppressor pathway is disrupted in most colorectal cancers (8), which is associated with tumor progression. Because conventional treatments such as radiation therapy and chemotherapy are mostly dependent on p53-mediated apoptosis (9, 10), the clinical application of those therapeutic approaches is not satisfactory. Therefore, screening and identification of new colorectal cancer therapeutic agents, which can induce colon cancer cell apoptosis via p53-independent mechanisms, are of great importance and significance. In the present study, we demonstrated that the small molecule synthetic compound JKA97 is able to induce apoptosis in human colon cancer cell HCT116 through p53-independent and Bax-dependent mechanisms and to inhibit tumor growth *in vivo*.

MATERIALS AND METHODS

Chemical Synthesis—The anti-cancer compound methoxy-1-styryl-9*H*-pyrido-[3,4-b]-indole (JKA97)[5] was designed

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[5] The abbreviations used are: JKA97, methoxy-1-styryl-9*H*-pyrido-[3,4-b]-indole; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; PUMA, p53-upregulated modulator of apoptosis; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; PI, propidium iodide; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone;

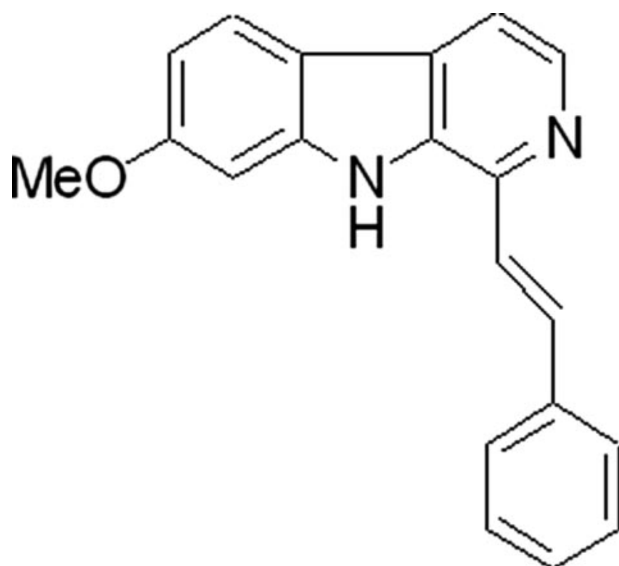

FIGURE 1. **The structure of JKA97.**

based on our previous discovery of natural product analogs as anti-cancer agents. We have been investigating the ß-carboline analogs because there are many reports in the literature showing interesting anti-cancer activity of this class of natural products, especially harmine, recently shown to inhibit cancer cell proliferation and to inhibit several cyclin-dependent kinases such as Cdk1/cyclin B, Cdk2/cyclin A, and Cdk5/p25 (43). Thus, we synthesized and evaluated the anti-cancer activity of benzylidene harmine (JKA97), which hitherto has not been reported to have anti-cancer activity. The structure of JKA97 is shown in Fig. 1. To synthesize this compound, a mixture of methoxy-1-methyl-9*H*-pyrido-[3,4-b]-indole (1 g) and benzaldehyde (15 ml) was refluxed for 4 h. The reaction mixture was cooled to room temperature, and a pale yellow solid was separated. This crude product was filtered and recrystallized from aqueous ethanol (90% v/v) to obtain compound JKA97 as a fluffy yellow solid (64% yield). Physical and structural characterization data obtained for JKA97 showed its purity to be higher than 95%. JKA97 was dissolved in Me_2SO to make a stock concentration at 20 mM for cell culture experiments. The final Me_2SO in the cell culture medium was 0.1% (v/v), and the same amount of Me_2SO was used as a negative control.

Cell Culture—The human colon cancer cell line HCT116 and its Bax-deficient (HCT116-$Bax^{-/-}$), p53-deficient (HCT116-$p53^{-/-}$, SW620), and PUMA-deficient (HCT116-$PUMA^{-/-}$) derivatives were kindly provided by Dr. Bert Vogelstein (The Sidney Kimmel Comprehensive Cancer Center, The Johns Hopkins University Medical Institutions, Baltimore) (11). The human hepatocarcinoma cell line SMMC-7721 and Hep3B were kindly provided by Dr. Lixin Wei (Tumor Immunology and Gene Therapy Center, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China). The HCT116 cell lines were maintained at 37 °C in a 5% CO_2 incubator in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 25 μg/ml gentamicin. SMMC-7721 and Hep3B cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin. The cultures were trypsinized and transferred to new 75-cm^2 culture flasks (Fisher) twice a week. FBS was purchased from Nova-Tech (Grand Island, NE), and the other cell culture reagents were obtained from Sigma.

Reagents and Antibodies—Mitochondria isolation kit was purchased from Pierce. The antibodies against caspase 3, PARP, Bax, Bak, and PUMA were purchased from Cell Signaling Technology (Beverly, MA). The antibody against cytochrome *c* was purchased from BD Biosciences. Anti-β-actin and p53 antibodies were obtained from Sigma. Anti-COX-IV antibody was purchased from Cayman Chemical (Ann Arbor, MI).

HCT116 Cell Anchorage-independent Growth Assay—Soft agar assay was employed to determine the inhibition of JKA97 on HCT116 anchorage-independent growth as described in our previous studies (12). Briefly, 2.5 ml of 0.5% agar in basal modified Eagle's medium supplemented with 10% FBS was layered onto each well of 6-well tissue culture plates. HCT116 (1 ml) cells (1×10^4) were mixed with 2 ml of 0.50% agar-basal modified Eagle's medium supplemented with 10% FBS, and 1 ml of mixture was added into each well on top of the 0.5% agar layer. For the analysis of the inhibitory effect of JKA97 on HCT116 cell anchorage-independent growth, both layers of agar were supplemented with JKA97 compound at concentrations as indicated in figure legend 2. Plates were incubated at 37 °C in 5% CO_2 for 2 weeks, and the number of colonies was scored and presented as colonies/10000 cells (13).

MTS Cell Viability Assay—The CellTiter 96® AQueous non-radioactive cell proliferation assay (Promega) was used to detect cell viability. The kit is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS)) and an electron coupling reagent (phenazine methosulfate (MS)). MTS is bioreduced by cells into a formazan product that is soluble in a tissue culture medium. The conversion of MTS into the aqueous soluble formazan product is accomplished by the dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product, as measured by the amount of 490 nm absorbance, is directly proportional to the number of living cells. HCT116 and its various deficiency cells (1×10^4) were seeded into each well of 96-well plates. The cells were cultured in a CO_2 incubator until 80% confluence. The cells were then treated with either different doses of JKA97 compound or Me_2SO control (0.1% v/v) for 24 h. The medium was then changed and replaced with 100 μl of fresh 10% FBS/Dulbecco's modified Eagle's medium and 20 μl of MTS solution (Promega). Cells were incubated for another 2 h, and 25 μl of 10% SDS was then added to stop the reaction. The optical absorbance was determined using a micro-plate reader (Quant™ Bio-Tek Instruments, Winooski, VT) at a 490 nm wavelength. The results were presented as percentages of inhibition.

Western Blotting—The cells (4×10^5) were cultured in each well of 6-well plates to 80% confluence. The cell culture medium was replaced with 0.1% FBS cell culture medium supplemented with 2 mM L-glutamine and 25 μg of gentamicin and

AP, activating protein; TUNEL, terminal dUTP nick-end labeling.

cultured for 24 h. The cells were exposed to JKA97 (0∼20 μM) for the time periods indicated in figure legends 4–6, then washed once with ice-cold phosphate-buffered saline, and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with primary antibodies as indicated in figure legends 4–6. The protein bands, specifically bound to primary antibodies, were detected by using an anti-rabbit IgG-AP-linked secondary antibody with an ECF Western blotting system (Amersham Biosciences) (12).

Detection of Cell Death with Flow Cytometry in Vitro—To analyze the apoptotic cells by propidium iodide (PI) and Annexin-V staining, the cells were plated in 6-well plates at a density of 4×10^5/well and cultured in 10% FBS serum medium until 80% confluence was reached. After exposure to 10 μM JKA97 for 24 h, the cells were collected and fixed in ice-cold 75% ethanol at −20 °C overnight. The fixed cells were stained in a buffer containing 100 mM sodium citrate, 0.1% Triton X-100, 0.2 mg/ml RNase A, and 50 μg/ml PI at 4 °C for 1 h and then analyzed using an Epics XL FACS (Beckman Coulter, Miami, FL) as described in our previous publication (14).

Detection of the Release of Cytochrome c and Translocation of Bax—HCT116 cells were cultured in 100-mm dishes until 80% confluence was reached and then exposed to JKA97 for 12 h. The cytosolic and mitochondrial proteins were isolated, respectively, according to the protocol provided by the mitochondria isolation kit (Pierce). The protein concentration was determined by a Bio-Rad protein assay kit, and the protein from each fraction was loaded onto a SDS-PAGE. Western blotting was carried out as described in our previous publications with specific antibodies against Bax, Bak, cytochrome *c*, and β-actin (12). The protein bands specifically bound to primary antibodies were detected by using a second antibody IgG-AP-linked secondary antibody and by an ECF Western blotting system (Amersham Biosciences) (12).

Inhibition of HCT116 Cell Tumorigenesis by JKA97 in Nude Mice—Female athymic nude mice (5 weeks old) were purchased from the Chinese Academy of Science Shanghai SLAC Laboratory Animal Co. (SLACCAS, Shanghai, China) and acclimated for 1 week. Mice were used when they reached 6 weeks old (about 20 g). There were 32 mice in total in this experiment. 7.5×10^6 HCT116 cells in 0.2 ml of growth medium without serum were implanted in the flank of each nude mouse. Tumor volumes were measured weekly. Tumor size was measured in two dimensions with calipers and calculated using the formula $(L \times W^2) \times 0.5$, where L is length and W is width (15). After the tumor volume reached around 0.05 cm^3, which occurred at 15 days after cell injection, the mice were grouped randomly into a JKA97 treatment group and a vehicle control group. JKA97 (0.2 mg) in 200 μl of 0.9% saline and ethanol (9:1) was injected intraperitoneally into each mouse in the JKA97 treatment group once a day, 5 days a week, for a total of 5 weeks. Another 16 nude mice were injected with 0.9% saline and ethanol (9:1) as a vehicle control. Tumor sizes were monitored once a week. Tumors were removed from mice at week 7 after HCT116 cell injection, fixed in 10% buffered formalin, and embedded in paraffin. Four-μm sections were dehydrated and stained with hematoxylin and eosin.

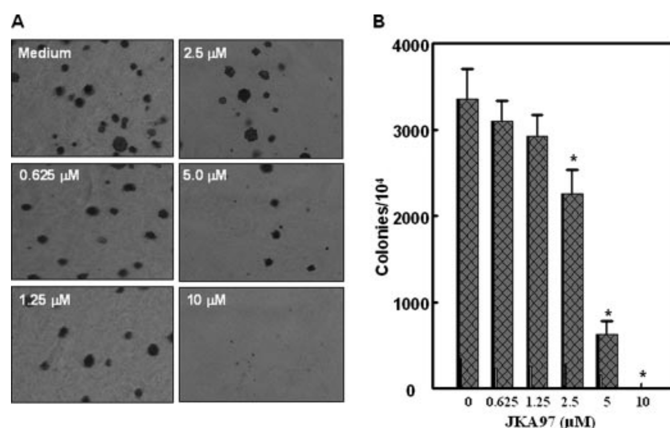

FIGURE 2. **JKA97 inhibits HCT116 cell anchorage-independent growth.** HCT116 cells (1×10^4) were exposed to JKA97 (0–10 μM) in 0.33% agar for 14 days as described under "Materials and Methods." The number of colonies was counted under microscopy at the end of the experiments (*A*). The colonies are expressed as means ± S.E. from six assays. *, $p < 0.05$, significant increase from vehicle control (*B*).

Detection of the Apoptotic Index in Xenografts with TUNEL—TUNEL staining was performed with the *In situ* Cell Death Detection Kit (Roche Applied Science) in tumor tissues according to the manufacturer's instructions. Briefly, slides were incubated with 50 μl of TUNEL reaction mixture containing terminal deoxynucleotidyl transferase in a humidified atmosphere for 1 h at 37 °C in the dark. Rinsed slides were then stained with Hoechst 33258 (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline for nuclear counterstaining and then were washed again with phosphate-buffered saline. Slides were digitized and analyzed under an Olympus BX 71 fluorescence microscope using Ce2001 Cell Explorer software (BioSciTec, Frankfurt, Germany). Semi-quantification was performed for three independent high power fields in each slide.

Statistical Analysis—The significance of the difference between different groups was determined with the Student's *t* test. The differences were considered significant at $p < 0.05$.

RESULTS

JKA97 Inhibited Anchorage-independent Growth of HCT116 Cells—To test the potential application of JKA97 in the treatment of human colon cancers, the inhibitory effect of JKA97 on anchorage-independent growth of human colon cancer cell HCT116 was determined. As shown in Fig. 2, incubation of JKA97 with HCT116 cells in soft agar for 14 days dramatically inhibited anchorage-independent growth of HCT116 cells in a dose-dependent manner, ranging from 2.5 to 10 μM (Fig. 2), which suggests that JKA97 does have an inhibitory activity on human colon cancer cell growth.

JKA97 Inhibited Colon Cancer HCT116 Cell Tumorigenicity in Nude Mice—After a demonstration of the anti-tumor action of JKA97 *in vitro*, we further examined its inhibition of HCT116 cell tumorigenicity *in vivo*. Because of the aforementioned results, HCT116 cells were injected subcutaneously into the flanks of the mice to initiate tumor formation in nude mice. Fourteen days after HCT116 cell injection, tumor size reached around 0.05 cm^3. A total of 32 mice were randomly placed into two groups, including a vehicle control group and a JKA97 treatment group. The mice in the vehicle control group intrap-

TABLE 1
Nude mice weight pre- and post-treatment (mean ± S.D., $n = 16$)

Group	Pretreatment	Post-treatment
Vehicle control	20.91 ± 1.00	24.83 ± 0.90
JKA97 (0.2 mg/kg)	20.86 ± 1.06[a]	24.50 ± 0.96[a]

[a] $p > 0.05$ *vs.* vehicle control group.

eritoneally received a vehicle of saline or ethanol mixture (9:1), whereas JKA97 was intraperitoneally injected into each mouse of the JKA97 treatment group. The treatments were carried out once a day, 5 days a week, for 5 weeks. As shown in Table 1, JKA97 treatment did not show any inhibition of nude mouse growth as compared with the vehicle control group (Table 1). Importantly, JKA97 treatment resulted in a significant reduction of tumor volume at day 35 as compared with that of vehicle control group. Tumor volumes between vehicle group and JKA97 group were almost the same at day 14 before the start of JKA97 treatments (Fig. 3, *A* and *B*). Hematoxylin and eosin staining revealed a significant cell death occurring in the JKA97-treated mice as compared with the control group (Fig. 3*C*). An *in situ* cell TUNEL assay showed that the basal apoptotic index in tumor of the vehicle control group was ~2.53%, and the apoptotic index increased to ~54.56% in the tumors of the JKA97 treatment group (Fig. 3, *D* and *E*). This demonstrated that the inhibition of tumor growth in athymic nude mice by JKA97 treatment is through the induction of cell apoptosis.

JKA97 Induced Cell Apoptotic Responses in HCT116 Cells in Vitro—To elucidate the molecular mechanisms of anti-cancer activity of the JKA97 compound, the apoptotic induction activity of JKA97 on HCT116 cells was determined. The incubation of cells with 10 μM JKA97 for 24 h resulted in more than 29.2% of the HCT116 cells undergoing cell death (Fig. 4, *A* and *B*). Thereafter, the induction of cleavage of caspase 3 and PARP, two indicators of apoptosis (16), was also examined by Western blot. The data showed a clear induction of cleavage of caspase 3 and PARP in JKA97-treated HCT116 cells with doses ranging from 2.5 to 10 μM (Fig. 4*C*), which is consistent with its inhibition of anchorage-independent growth of HCT116 cells in soft agar. Furthermore, the pan-caspase inhibitor (Z-VAD-fmk) and specific caspase 3 inhibitor VII were able to reduce JKA97-induced cell death (Figs. 4, *F* and *G*, and 5*D*), indicating that caspase and caspase 3 activation were involved in the cell apoptotic response to JKA97 exposure. In addition, we also demonstrated the apoptotic induction activity of JKA97 in the hepatocarcinoma cell line SMMC7721 (Figs. 4, *D* and *E*, and 5, *G–J*). It was noted that pan-caspase inhibitor Z-VAD-fmk and caspase 3-specific inhibitor VII could not completely block JKA97-induced cell apoptosis. The explanation for this may be that some caspase-independent pathway is involved.

JKA97-induced Cell Apoptosis Was through p53- and PUMA-independent Mechanisms—The tumor suppressor protein p53 is often seen as the "guardian of the genome" because of its key role in the induction of apoptosis (17). Our above results suggested that JKA97 was able to induce apoptosis in HCT116 cells, and considering that HCT116 cells express a wild-type p53, we tested whether the induction of cell death by JKA97 in HCT116 cells was through a p53-dependent pathway. To address this possibility, HCT116 cells with a p53 deficiency (HCT116-p53$^{-/-}$) were employed. The deficiency of p53 protein expression in HCT116-p53$^{-/-}$ cells was demonstrated by Western blotting with specific p53 antibody as compared with parental HCT116 cells (Fig. 5*A*). As indicated by cell morphology and a cell viability assay, there is no significant difference in JKA97-induced apoptosis between HCT116 cells and HCT116-p53$^{-/-}$ cells (Figs. 4*G* and 5, *B–D*). This finding was further confirmed by the PI staining flow cytometry analysis (Fig. 5*E*). Accordingly, there was a marked induction of the cleavage of caspase 3 and PARP in JKA97-treated HCT116-p53$^{-/-}$ cells (Fig. 5*F*). In another human colon cancer cell line with p53 mutations (SW620), JKA97 treatment also showed significant apoptotic activity (Fig. 5, *I* and *J*). We also confirmed the apoptosis induction by JKA97 in both human hepatocarcinoma cell lines, including SMMC-7721 (wild-type p53) and Hep3B (p53 deficient) with caspase 3 cleavage (Fig. 4*H*). p53 protein expression levels in both cell lines were determined by Western blot (Fig. 5*A*). JKA97-induced apoptosis could also be observed at similar levels in both Hep3B and SMMC-7721 cells (Fig. 5, *G–J*). Our results strongly demonstrated that JKA97-induced cell death is through a p53-independent pathway.

PUMA is a mitochondrial protein, which could mediate apoptosis through either p53-dependent or -independent mechanisms based on various experimental systems (18). To determine whether PUMA is implicated in JKA97-induced cell apoptosis we compared apoptosis induction between HCT116 and HCT116-PUMA knock-out (HCT116-PUMA$^{-/-}$) cells. The lack of PUMA expression in HCT116-PUMA$^{-/-}$ cells was confirmed by Western blotting (Fig. 5*A*). There was no difference in JKA97-induced cell apoptosis between HCT116 and HCT116-PUMA$^{-/-}$ cells assessed by flow cytometry analysis, morphology and a TUNEL assay (Figs. 4*G* and 5, *B–E*). The induction of the cleavage of caspase 3 and PARP was similar in HCT116 cells and HCT116-PUMA$^{-/-}$ cells (Fig. 5*F*). These data suggest that PUMA is not involved in apoptosis induction by JKA97 in HCT116 cells.

Bax Was Required for JKA97-induced Cell Apoptosis in HCT116 Cells—Bax is a member of the Bcl-2 family, which plays an important role in promoting the activation of apoptotic signaling cascades (19). To assess the role of Bax in a pro-apoptotic effect of JKA97, we compared the apoptotic responses to JKA97 treatment between HCT116 cells and HCT116-Bax$^{-/-}$ cells. The deficiency of Bax expression in HCT116-Bax$^{-/-}$ cells was verified with Bax-specific antibody (shown in Fig. 5*A*). Exposure of HCT116 cells to 10 μM JKA97 resulted in a significant increase of cell apoptosis at 24 h, but no cell death was observed in HCT116-Bax$^{-/-}$ cells under the same treatment conditions (Fig. 5, *B–D*). This finding was further confirmed by a flow cytometry analysis (Figs. 4*G* and 5*E*). The induction of the cleavage of caspase 3 and PARP was also detected in HCT116 cells but not in HCT116-Bax$^{-/-}$ cells (Fig. 5*F*). These results suggest that Bax is critical for JKA97-induced apoptosis.

The translocation of the pro-apoptotic protein Bax to mitochondria is one of the mechanisms involved in drug-initiated mitochondrial apoptotic cascade (20). We therefore investigated the translocation of Bax in HCT116 cells exposed to JKA97 treatment. As shown in Fig. 6*A*, Bax proteins were com-

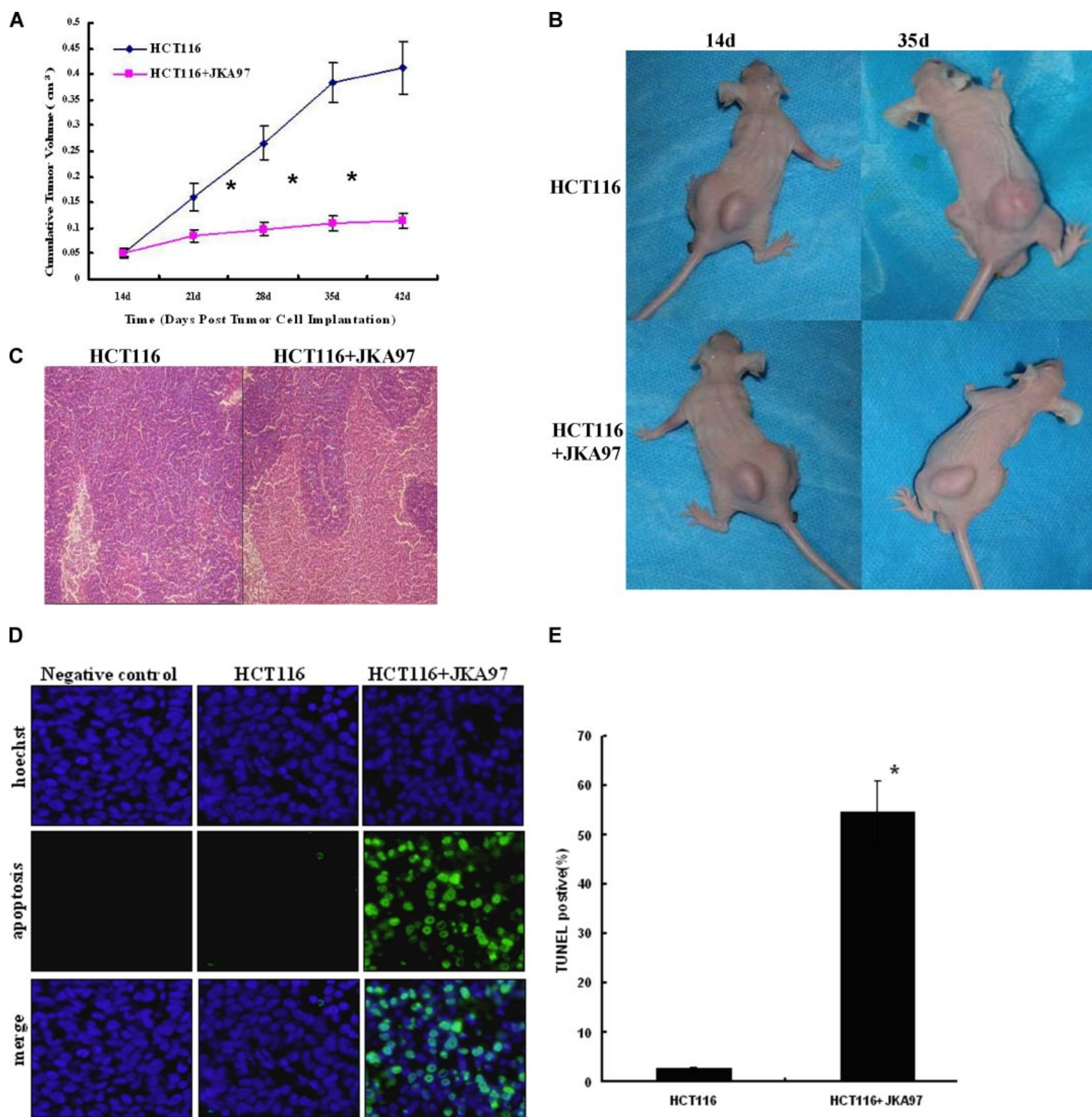

FIGURE 3. **JKA97 Inhibits HCT116 cell tumorigenicity in nude mice.** *A* and *B*, nude mice 6 weeks of age were used. The experiments were conducted with 16 mice in each group. HCT116 cells were injected subcutaneously into the flanks of each mouse to initiate tumor growth for 14 days. The mice were then treated intraperitoneally with either control vehicle or JKA97 once a day, 5 days a week, for a total of 5 weeks as indicated under "Materials and Methods." Tumor size was measured weekly in two dimensions throughout the study. Data are shown as the means ± S.E. (*A*) and external appearance of tumors (*B*). *, $p < 0.05$, significant increase from vehicle control. Tumors were removed from mice at week 7 after HCT116 cell injection, fixed in 10% buffered formalin, and embedded in paraffin. *C*, 4-μm sections were dehydrated and stained with hematoxylin and eosin. The slides were observed under microscope and photographed (original magnification, ×400). *D*, paraffin-embedded tumors xenografts were sectioned (4 μm) and subjected to TUNEL staining as described under "Materials and Methods." Three slides from each tumor sample were used for TUNNEL assay. The slides were then observed under a fluorescent microscope and photographed (original magnification, ×400). The apoptotic cells (*green*) were stained by the TUNEL assay. Cell nuclei (*blue*) were stained with Hoechst. *E*, average percentage of TUNEL positive cells in tumors. Data are shown as the means ± S.E. *, $p < 0.05$, significant increase from vehicle control group.

monly found in the cytosol in the HCT116 cells without JKA97 treatment, whereas Bax translocation from cytosol to mitochondria was observed at 12 h after JKA97 treatment. This Bax translocation appeared to be time-dependent (Fig. 6*B*). These data suggest that JKA97 induces Bax translocation, by means of which Bax may mediate activation of the mitochondrial pathway of apoptosis in HCT116 cells. To determine whether downstream events in mitochondria-mediated cell death cas-

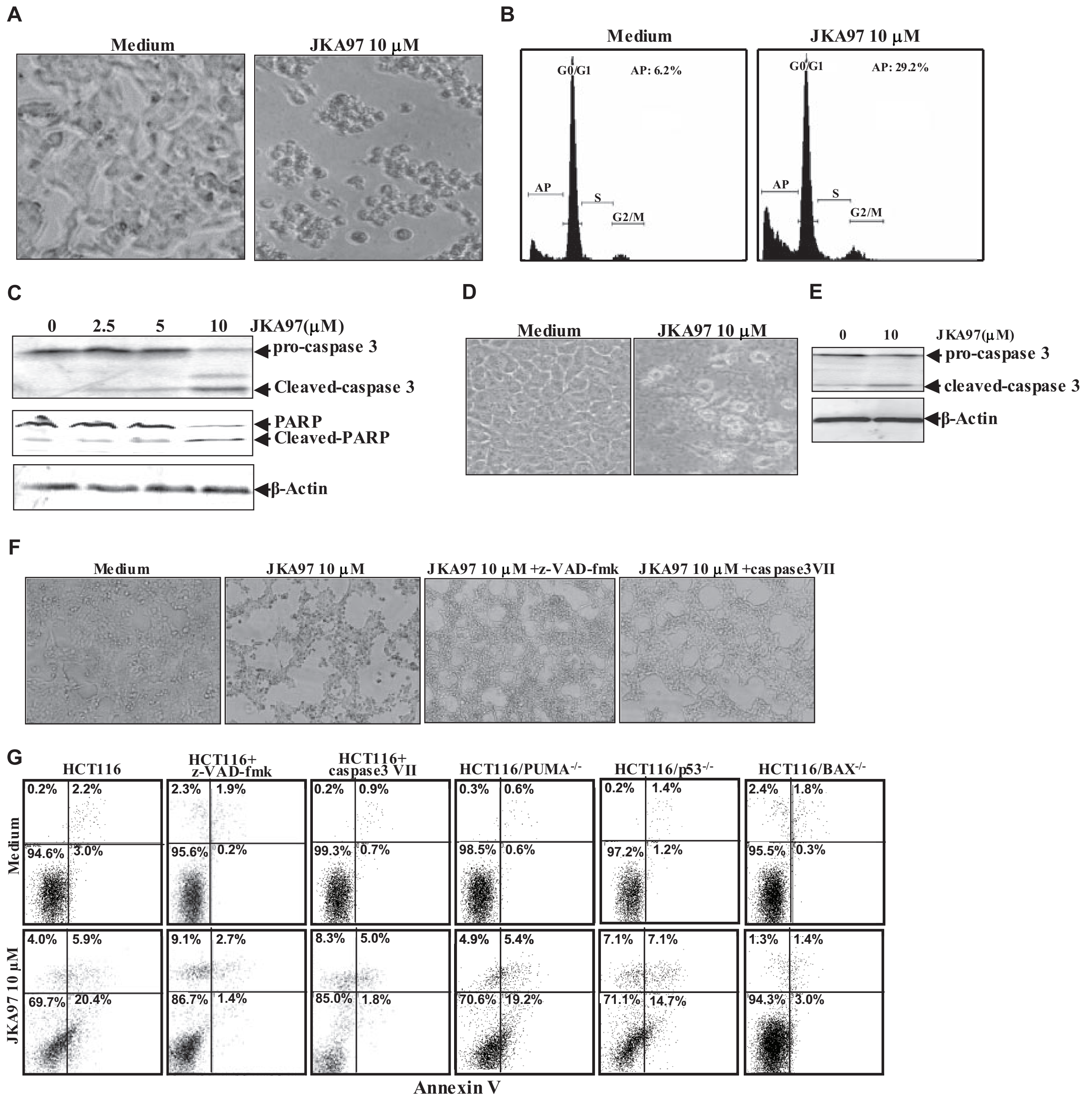

FIGURE 4. **JKA97 induces cell death in human colon cancer HCT116 and human hepatocarcinoma cell line SMMC-7721.** *A*, HCT116 cells (4 × 10^5) were exposed to JKA97 (0 or 10 μM) for 24 h, and the morphological changes were observed under microscope and photographed (original magnification, ×400). *B*, HCT116 cells (4× 10^5) were seeded into each well of 6-well plates and cultured until the cell density reached 70–80% confluence. The cells were then exposed to 0 or 10 μM JKA97 for 24 h. The cell death was determined using PI staining and detected by flow cytometry. *C*, cleavage of caspase 3 and PARP was detected by Western blotting (12 h) after HCT116 cells were exposed to various doses of JKA97. β-Actin was used as the protein loading control. *D*, SMMC-7721 cells (4× 10^5) were exposed to JKA97 (0 or 10 μM) for 24 h, and the morphological changes were observed under an inverting microscope and photographed (original magnification, ×400). *E*, the cleavage of caspase 3 was detected in JKA97-treated SMMC-7721 cells by Western blotting at 12 h after JKA97 exposure. β-Actin was used as the protein loading control. *F*, the HCT116 cells were pretreated with Z-VAD-fmk (25 mM) and caspase 3 inhibitor VII (20 nM) for 30 min and then exposed to 10 μM JKA97 for 24 h; the morphological changes were observed under a microscope and photographed (original magnification, ×400). *G*, JKA97-induced cell death was determined using Annexin-V staining and detected by flow cytometry.

cade are activated in HCT116 cells upon JKA97 treatment, we investigated the release of cytochrome *c* from the mitochondria to the cytosol. Western blotting analysis showed that cytochrome *c* was located mainly in the mitochondria, whereas JKA97 treatment resulted in its release into the cytosolic fraction. Collectively, our results demonstrate that Bax translocation from cytosol to mitochondria induced by JKA97 treatment leads to cytochrome *c* release from mitochondria to the cytosol,

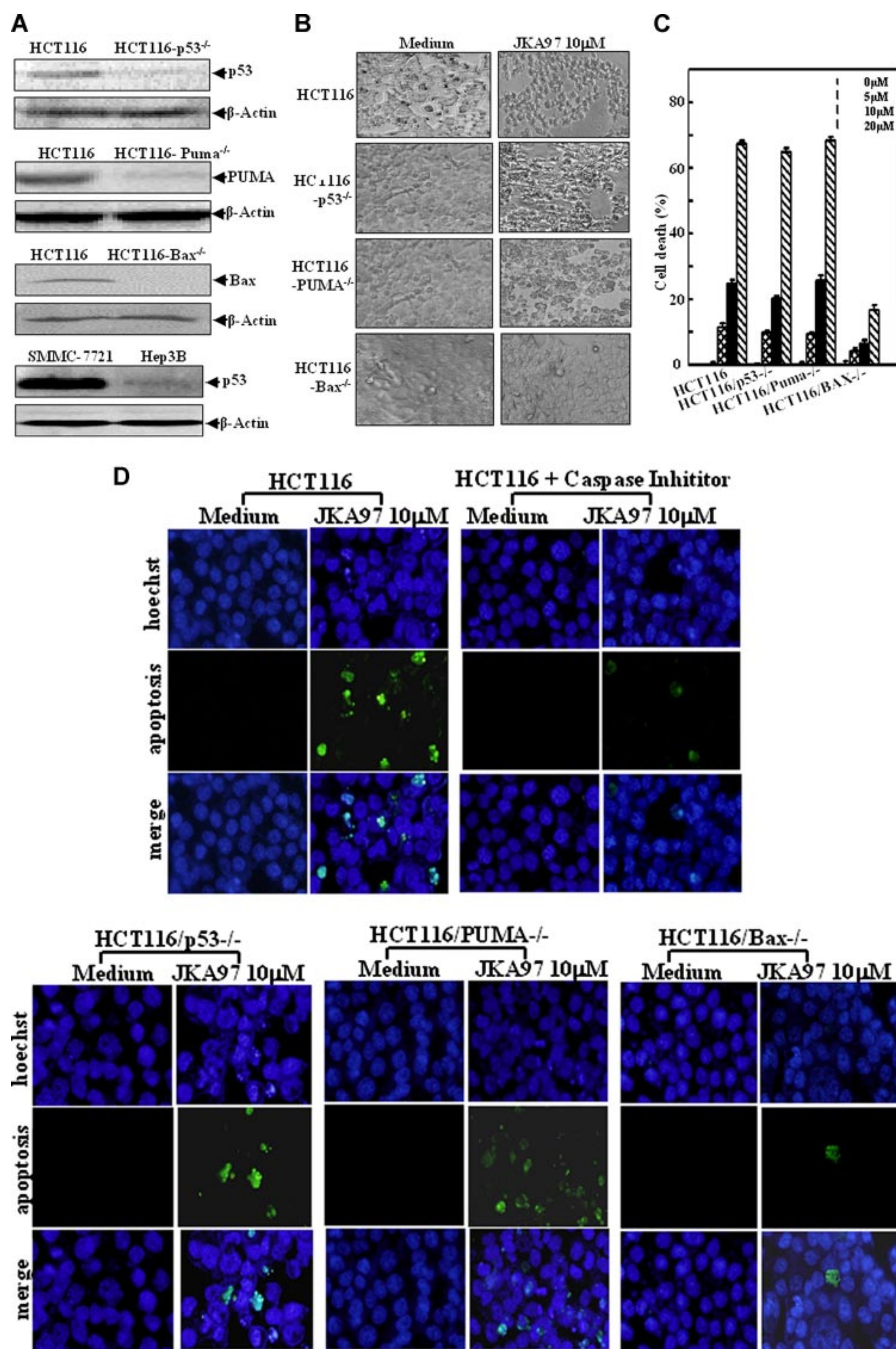

FIGURE 5. **JKA97-induced HCT116 cell apoptosis was through Bax-dependent and p53- and PUMA-independent mechanisms.** *A*, HCT116 (wild type), HCT116-p53$^{-/-}$, HCT116-PUMA$^{-/-}$, HCT116-Bax$^{-/-}$, SMMC7721, and Hep3B cells were analyzed for phenotype identification by Western blotting with specific antibodies as indicated. β-Actin was used as the protein loading control. *B*, HCT116, HCT116-p53$^{-/-}$, HCT116-PUMA$^{-/-}$, and HCT116-Bax$^{-/-}$ cells were plated in 96-well plates (1 × 10^4 cells/well) in McCoy's 5A medium supplemented with 10% fetal bovine serum. 24 h later, the medium was replaced with 0.1% serum, and the cells were exposed to either vehicle control or 10 μM JKA97 for 24 h. The morphological changes were observed under microscope and photographed (original magnification, ×400). *C–E*, HCT116, HCT116-p53$^{-/-}$, HCT116-PUMA$^{-/-}$, and HCT116-Bax$^{-/-}$ cells were exposed to vehicle control or 10 μM JKA97 for 24 h. Cell death was determined by MTS assay (*C*), TUNEL assay (*D*), and a PI-staining flow cytometry assay (*E*). *F*, caspase 3 and PARP activation and cleavage were detected by Western blotting (12 h). β-Actin was used as the protein loading control. *G*, SMMC-7721 and Hep3B cells were plated in 6-well plates and cultured until the cell density reached 70–80% confluence. The cells were exposed to vehicle control or 10 μM JKA97 in 0.1% FBS medium for 24 h. The morphological changes were observed under a microscope and photographed (original magnification, ×400). *H*, JKA97-induced apoptosis was detected by determination of cleavage of caspase 3 at 12 h after SMMC-7721 and Hep3B cells were exposed to 10 μM JKA97. *I* and *J*, JKA97-treated cell apoptosis was determined with Annexin-V staining (*I*) and a TUNEL assay (*J*) in SMMC-7721, Hep3B, and SW620 cells.

which may in turn cause apoptotic pathway activation. In addition, we noted that JKA97 treatment was able to induce Bak translocation to the mitochondria, whereas it did not show any effect on AIF (apoptosis-inducing factor) translocation (Fig. 6*B*). The biological significance of Bak translocation is currently being investigated in our laboratory.

DISCUSSION

Cancer is a disorder of deregulated cell proliferation and/or cell survival of genome-damaged cells (21). Inhibiting cell proliferation and increasing apoptosis in tumors are effective ways to prevent tumor growth and eliminate cancers. It is well known that apoptosis induction by conventional chemotherapeutic agents is mediated mostly through p53-dependent pathways. However there are ∼50% of human tumors that have p53 mutations and inactivation. Therefore, applications of those therapeutic reagents are limited. The goal of this study was to investigate the potential anticancer activity of JKA97 through a p53-independent pathway and to elucidate the molecular basis of JKA97-induced apoptosis in human colon cancer cells. We found that JKA97 treatment could inhibit anchorage-independent growth and induce apoptotic responses in HCT 116 cells. We also found that JKA97 could inhibit colon cancer cell growth in nude mice by the induction of cell apoptosis. By utilization of p53 null, PUMA-null, and Bax-null HCT116 cells, we determined that apoptotic induction by JKA97 is through Bax-dependent, p53- and PUMA-independent mechanisms. Furthermore, this JKA97-induced apoptotic response is mediated by a mitochondrial pathway through its induction of Bax translocation, which in turn leads to cytochrome *c* release and subsequently to caspase 3 activation and apoptosis.

More than 60% of the currently used cancer chemotherapeutic agents are small molecule compounds or natural products (22). Small molecule compounds

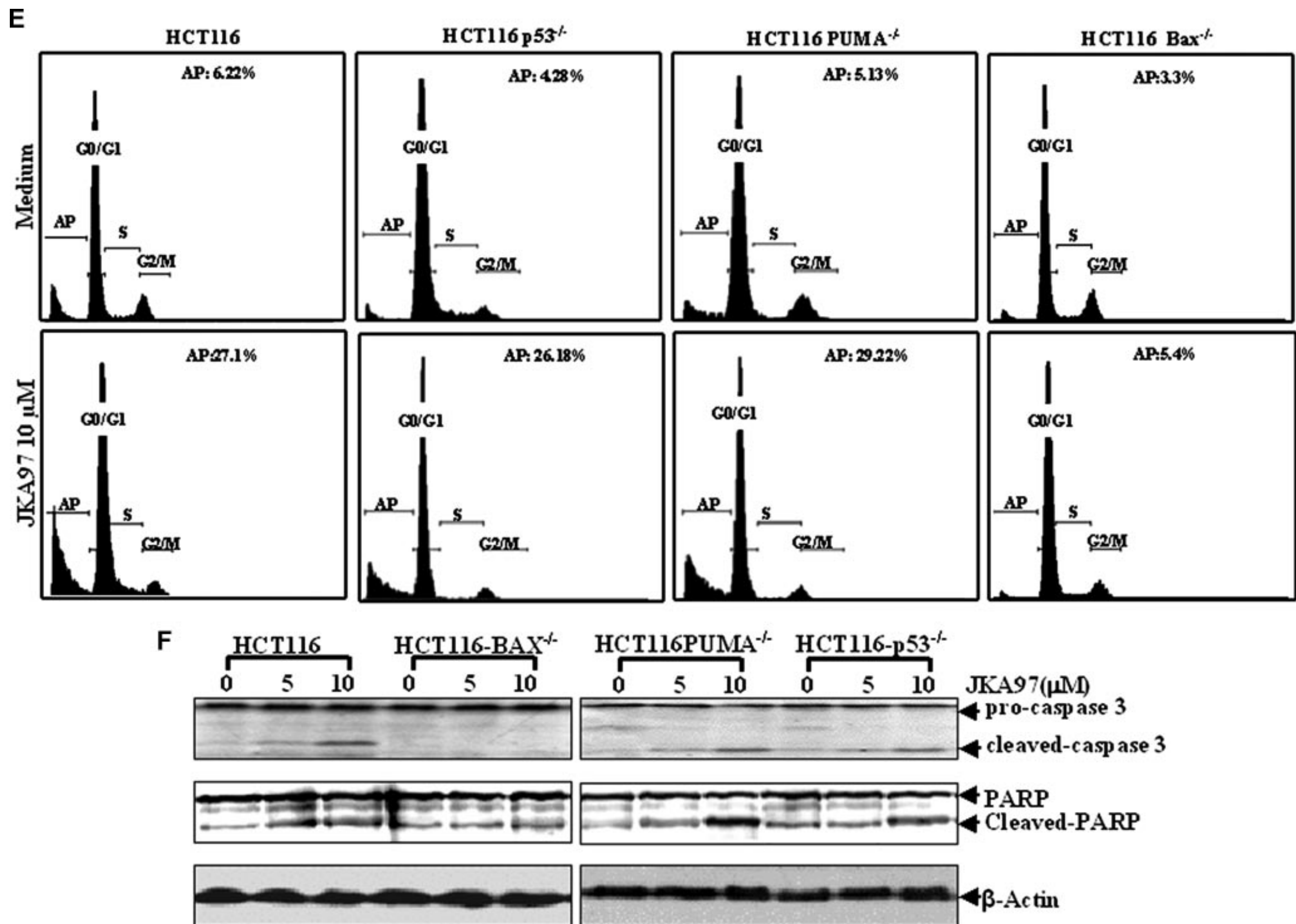

FIGURE 5—*continued*

always play a major role in anti-cancer medicine and are major players in anti-tumor drug discovery. The mechanisms of small molecules are to regulate angiogenesis, apoptosis, cell cycle, macromolecule synthesis, mitochondrial respiration, mitosis, multidrug efflux, and signal transduction (23). Most cancer therapeutic approaches, such as radiation and chemotherapy, inhibit tumors by triggering cancer cell apoptosis (24). The apoptotic machinery can be targeted for therapeutic benefits by using apoptotic inducers or modulators through the targeting of protein kinases, phosphatases, and transcription factors. JKA97 is a synthesized anti-cancer compound with unknown mechanisms. All results from our investigations demonstrate that JKA97 enables HCT116 cell to undergo apoptosis at the dosages of 2.5–10 μM by triggering apoptotic responses.

The apoptotic induction by chemical anti-cancer agents may be mediated by multiple mechanisms. p53 is a well known transcription factor that can modulate the apoptotic process and subsequently is involved in anti-cancer activity of most anti-cancer agents (25). Because more than 50% of human tumors have mutations in the p53 gene, it is important to understand whether apoptotic induction by JKA97 is p53-dependent or -independent. PUMA, as a downstream target of p53, is induced at the transcriptional level in response to DNA damage and plays an essential role in apoptotic induction by a variety of stimuli in several tissues and cell types (18). Thus, we evaluated the role of p53 and PUMA in JKA97-induced apoptosis in the current study. Our studies have demonstrated that cell death upon JKA97 treatment is through p53- and PUMA-independent pathways, because the knock-out of either p53 or PUMA in HCT116 cells did not show any inhibitory effects on JKA97-induced apoptosis.

There are two alternative pathways to initiate apoptosis. One is the extrinsic pathway, which acts through death receptors on cell surfaces, the other is the intrinsic pathway, which acts through mitochondria (26–30). Mitochondria play a critical role in the regulation of various apoptotic processes including drug-induced apoptosis (31, 32). The mitochondrial death pathway is controlled by members of the Bcl-2 family, including the anti-apoptotic Bcl-2 and Bcl-XL proteins and the pro-apoptotic Bax and Bid proteins. Bcl-2 can suppress cell death induced by a variety of stress agents. Bax resides in the cytosol and plays an important role in promoting the activation of apoptotic signaling cascades (19). To identify the role of Bax in JKA97-induced apoptosis, we compared apoptosis induction between wild-type HCT116 and HCT116 with Bax-deficiency ($Bax^{-/-}$) upon JKA97 treatment. Our results showed that JKA97-induced apoptosis was impaired in HCT116-$Bax^{-/-}$ cells, suggesting that Bax is critical for apoptosis induction upon JKA97 treatment. Previous studies indicate that Bax is localized in the cytoplasm, and its translocation between the cytoplasmic and mitochondrial membrane is an important biochemical

FIGURE 5—*continued*

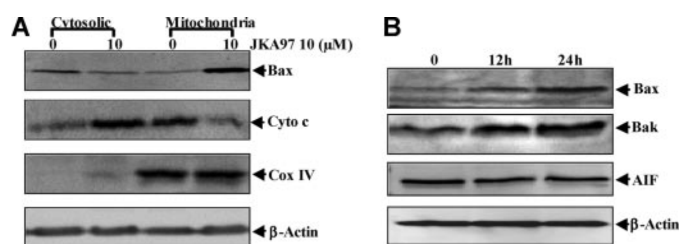

FIGURE 6. **Determination of protein translocation between cytosol and mitochondria in HCT116 cells exposed to JKA97.** *A*, HCT116 cells grown in 150-mm culture dishes were exposed to vehicle control or 10 μM JKA97. The cytosolic and mitochondrial proteins were isolated, respectively, according to the protocol provided by the mitochondria isolation kit. Equal amounts of cytosolic and mitochondrial proteins isolated from HCT116 cells were separated on 12% SDS-polyacrylamide gels and then subjected to Western blotting analysis for various antibodies as indicated. β-Actin was used as the protein loading control. *B*, HCT116 cells were exposed to vehicle control or 10 μM JKA97 for 12 or 24 h, and mitochondrial protein were isolated. Bax, Bak, and AIF (apoptosis-inducing factor) were detected by Western blotting. β-Actin was used as the protein loading control.

event for apoptosis induction (33). Accumulating evidence also shows that some apoptosis-inducing agents cause the translocation of Bax from the cytoplasm to mitochondria in order to initiate mitochondria-dependent apoptosis (34–39). It is reported that overexpression of Bax results in the release of cytochrome *c* from mitochondria to the cytosol (40), and the direct incubation of Bax protein with isolated mitochondria also induces cytochrome *c* release (41). On the other hand, the simple increase of intracellular Bax without its translocation from cytosol to mitochondria does not show a release of cytochrome *c* from the mitochondria to the cytosol (42). The results from our study demonstrate that JKA97 treatment induces Bax translocation from the cytosol to the mitochondria in HCT116 cells and causes the release of cytochrome *c* from the mitochondria to the cytosol. All of these biological effects are blocked in HCT116-Bax$^{-/-}$ cells, indicating that the apoptotic effect of JKA97 is through a Bax-initiated mitochondrial pathway.

In summary, our studies demonstrate that JKA97 triggers an apoptotic response in HCT116 cells through a Bax-cytochrome *c*-dependent pathway. This apoptosis induction may be associated with the anti-cancer activity of JKA97. Because this apoptosis induction is neither p53- nor PUMA-dependent, JKA97 may have a potential use in therapeutic applications for patients with p53 mutation in cancer cells.

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