Andrographolide Sensitizes the Cytotoxicity of Human Colorectal Carcinoma Cells Toward Cisplatin via Enhancing Apoptosis Pathways In Vitro and In Vivo

Hui-Hsuan Lin,∗,† Ming-Der Shi,‡ Hsien-Chun Tseng,§ and Jing-Hsien Chen†,¶,1

TOXICOLOGICAL SCIENCES 139(1), 108–120 2014
doi: 10.1093/toxsci/kfu032
Advance Access publication February 22, 2014

Andrographolide (Andro), a diterpenoid lactone isolated from a traditional herbal medicine Andrographis paniculata, has been shown to suppress the growth and invasion of human colorectal carcinoma (CRC) Lovo cells, and trigger apoptosis in vitro. The potential of Andro as a chemotherapeutic agent in CRC was evaluated by investigating its cytotoxic effects as a single agent or in coadministration with cisplatin (CDDP). Andro potentiated the cytotoxic effect of CDDP in Lovo cells through apoptosis. The molecular mechanism for these favorable cellular responses was further investigated by analyzing the apoptotic profiles, protein levels, and mRNA expression patterns of several key genes after treatments of Andro alone or in combination with CDDP. Molecular results indicated that the effect of Andro alone might be mediated via both intrinsic and extrinsic apoptotic pathways in Lovo cells. The addition of Andro to CDDP induced synergistic apoptosis, which could be corroborated to the formation of platinum-DNA adducts that activate several signal transduction pathways and the ultimate activation of apoptosis. Finally, the combination therapy of Andro with CDDP was evidenced by its synergistic inhibition on the growth of Lovo cells in xenograft tumor studies. The results indicate that Andro, in combination with chemotherapeutics, is likely to represent a potential therapeutic strategy for CRC.

Key words: andrographolide; human colorectal carcinoma; cisplatin; apoptosis; intrinsic and extrinsic apoptotic pathways.

ABBREVIATIONS

Andro andrographolide
CDDP cisplatin [cis-diaminedichloroplatinum (II)]
CRC colorectal carcinoma
DMSO dimethyl sulfoxide
ECL enhanced chemiluminescence
Fas/FasL Fas ligand
LDH lactate dehydrogenase
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI propidium iodide
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS Tris-buffered saline
TNF-α tumor necrosis factor α

Colorectal cancer is one of the most common malignant tumors and is the third cause of cancer deaths worldwide (Jemal et al., 2010). The first-line treatment for colorectal cancer is surgical excision, however, it cannot fully prevent recurrence in most patients. Auxiliary radiotherapy and chemotherapy after surgery can restrain the metastasis and overcome recurrence (Fu et al., 2011). Cisplatin (cis-diaminedichloroplatinum II, CDDP), one of the most widely used metal anticancer drugs, has a well understood mechanism of action and is effective for colorectal cancer treatment (Macdonald and Astrow, 2001). The formation of platinum-DNA adducts that activate several signal transduction pathways and the ultimate activation of apoptosis contributed to the major cytotoxic effect of CDDP (Reed, 1999; Siddik, 2003). However, drug resistance and side effects are still the major limitations to CDDP clinical use (Rabik and Dolan, 2007). Combinations of anticancer drugs with new chemotherapeutic agents are being investigated to explore a significant prognostic benefit and improved clinical response.

Andrographis paniculata (Burm. F.) Nees (Acatheaceae) is an important herb widely used in traditional medicine of Southeastern Asian countries. Its main components are diterpene lactones, and 70% of the plant extract is constituted of andrographolide (Andro) (Zhao et al., 2002). Andro has been identified to have...
many pharmacological properties and has been widely used in clinic treatment for inflammation, diarrhea, fever, cold, and other infectious diseases (Reddy et al., 2005; Shen et al., 2002). Recent studies reported that Andro possesses anticancer and immunomodulatory activities, and therefore has the potential to be applied as a chemotherapeutic agent (Rajagopal et al., 2003; Srinivasa et al., 2006). Studies showed that Andro was able to induce cell-cycle arrest (Cheung et al., 2005; Shi et al., 2008), to inhibit tumor migration and invasion in human cancer cells (Lee et al., 2010b; Shi et al., 2009), and to enhance the secretion of interleukin-2 by cytotoxic T-lymphocytes to inhibit the tumor growth in BALB/c mice (Sheeja and Kuttan, 2007). All these mechanisms contribute to its pharmacological functions. The proapoptotic effect of Andro was found to be carried out by the activation of proapoptotic Bcl-2 family members (Bid and Bax) in human leukemia HL-60 cells and other human cancer cells (Cheung et al., 2005; Zhou et al., 2006), and by the apoptotic change in morphology and the activations of caspase-3 and caspase-8 in human prostate carcinoma PC-3 cells (Kim and Milner, 2005).

Disruption of apoptotic function is found in many human tumors, which suggests the substantial contribution of apoptosis to the transformation of a normal cell into a tumor cell. Depending on the trigger of the death program, the basic mechanism of apoptosis has been divided into intrinsic and extrinsic pathways. The apoptotic process is subdivided into three phases: initiation, decision, and execution. The initiation phase is started by binding of extrinsic death signals, such as Fas ligand (FasL) or tumor necrosis factor α (TNF-α), to a receptor, which might direct the cell to prepare for suicide (Ashkenazi and Dixit, 1998). The decision phase which is associated with mitochondria alterations and activation of initiator procaspases (procaspase-8) is whereby the cell still has the chance to decide its destiny (Green and Reed, 1998). The intrinsic pathway which is mitochondria dependent and uses Bcl-2 family members as proapoptotic regulators is initiated by death stimuli including oxidative and genotoxic stress, ultraviolet radiation and chemotherapeutics. The execution phase is activated when the cell commits to die. This phase is characterized by the activation of downstream caspases (procaspase-3 and procaspase-9) which carry out the death and clearance program (Susin et al., 1997).

The present study was aimed to identify the underlying mechanism of the enhancing effect of Andro on the drug sensitivity of CRC cell line Lovo toward CDDP, thereby using a lower cytotoxic dose of CDDP without affecting therapeutic effects in coadministration. To further investigate, the differences in the apoptotic profiles after incubation with Andro or CDDP alone and in combination in Lovo cells, the molecular mechanism was studied and focused on cell viability, and expression of apoptosis-related genes and proteins.

**MATERIALS AND METHODS**

**Chemicals.** Purified Andro (Cat. no. 365645), CDDP, Tris-HCl, EDTA, SDS, deoxycholic acid, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA), sodium orthovanadate, leupeptin, nonidet p-40, and aprotinin were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO). Trypsin-EDTA, fetal-calf serum, F12 nutrient mixture, and penicillin and streptomycin mixture (PS) were purchased from Gibco/BRL (Gaithersburg, MD). Protein assay kits were obtained from Bio-Rad Labs. (Hercules, CA).

**Cell culture.** Lovo cells originated from human colorectal carcinoma were maintained as monolayers in F12 medium supplemented with 10% heat-inactivated fetal-calf serum and PS (100 units/ml penicillin and 10 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO2. Cells were seeded at a density of 1 × 10^5 on 10 mm Petri dishes 24 h before treatments. Cells were pretreated with Nok-1 monoclonal antibody (Pharmingen, San Diego, CA) and Bax inhibitor peptide V5 (Tocris, Ellisville, MO) for 30 min before drug treatment in the inhibition experiments.

**Preparation of stock solution.** A stock solution of Andro in dimethyl sulfoxide (DMSO) at a concentration of 100mM was prepared under protection from light, and stored at −20°C before use. For cell treatments, Andro solution was freshly prepared by diluting with medium to the desired concentrations. DMSO (final concentration of 0.2%) was used as solvent control.

**Assessment of cell viability.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the effect of the test drugs on cell viability and to determine the noncytotoxic concentrations as described previously (Alley et al., 1988). Lovo cells seeded at a density of 10^5 cells/ml were incubated with varying concentrations of CDDP (10 and 15μM) and/or Andro (10μM) for 24 h. The medium was then changed, and MTT (0.1 ng/ml) was added for another 4 h incubation. The production of formazan that was directly proportional to the viable cell number was dissolved with isopropanol and measured spectrophotometrically at 563 nm.

**Assessment of cytotoxicity.** To evaluate cytotoxicity, lactate dehydrogenase (LDH) released from cells into the culture medium was measured with an assay kit (Pierce, Rockford, IL) following the instructions of manufacturer. In brief, a mixture composed of 100 μl cell-free supernatant, 250 μl buffer, and 50 μl coenzyme was incubated for 15 min at 37°C, followed by the addition of 2,4-dinitrophenylhydrazine (250 μl) for another 15 min at 37°C in the dark. The reaction was stopped by the addition of 2.5 ml NaOH (0.4M). Three minutes later, 200 μl of each reaction mixture was transferred to a new 96-well plate, and the absorbance at 440 nm was determined along with...
sample blank, standard and standard blank tubes. The activity of LDH was calculated according to the formula: LDH cytotoxicity (%) = [(sample OD − sample blank OD)/(standard OD − standard blank OD)] × 100%. The LDH cytotoxicity of control group was set to 100%.

**Cell cycle analysis.** The cells treated for 24 h were washed twice with PBS solution and centrifuged at 1500 rpm for 5 min at room temperature. One milliliter of 70% methanol was then added to the pellet. After incubation at −20°C for at least 24 h, the cell suspension was added with 1 ml of cold propidium iodide (PI) stain solution (20 μg/ml PI, 20 μg/ml RNase A and 0.1% Triton X-100) and incubated for 15 min in darkness at room temperature before subjecting for analysis by a mini-flow cytometry (GUAVA, Millipore, MA). PI was excited at 488 nm and fluorescence signal was subjected to logarithmic amplification with PI fluorescence (red) being detected above 600 nm. Cell cycle distribution presented as the number of cells under sub-G1 phase were analyzed by CELLQuest Version 3.3 software. The ratio of hypodiploid cells (sub-G1 phase) over total cells was calculated and presented as percentage of apoptosis.

**Apoptosis analysis.** Apoptotic cells were analyzed by double stains of Annexin V-FITC which detects translocation of phosphatidylisinositol from the inner to the outer cell membrane during early apoptosis, and 7-AAD which can enter the cell in late apoptosis or necrosis, using untreated cells as control (Madhok et al., 2010). In brief, Lovo cells seeded in six-well culture plates (10⁴ cells/ml) were treated with varying concentrations of CDDP (10 and 15μM) and/or Andro (10μM) for 24 h. The treated and control ells were washed twice with cold PBS and resuspended in 1× binding buffer (BD Bioscience, Franklin Lakes, NJ). Cell suspension (100 μl) was transferred to 5 ml culture tubes, and stained with 5 μl Annexin V-FITC and 10 μl 7-AAD (BD Bioscience) at ambient temperature for 15 min in dark after a gentle vortex. Following this 400 μl 1× binding buffer was added to each tube and analyzed within an hour using a mini-flow cytometry method. For each measurement, at least 20,000 cells were counted, and the data were analyzed by the ExpressPro software.

**Western blot analysis.** Western blotting and the basic methodology for the preparation of cytosolic and mitochondrial fractions of the cell were performed as described previously (Lin et al., 2012). In short, the cell lysates with equal amount of total protein were denatured in sample buffer containing SDS, separated on 8–15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with the indicated primary antibodies for overnight at 4°C after blocking with 5% nonfat dry milk. The antibodies against caspase-3, caspase-9, caspase-8, Fas, FasL, TNF-α, TNFR1, Bcl-2, Bax, t-Bid, Bad, Mcl-1, COX-IV, and cytochrome c were from Santa Cruz, CA, and anti-β-actin was purchased from Sigma Chemical Co. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (goat antimouse IgG or goat anti-rabbit IgG), detected with enhanced chemiluminescence (ECL) reagent (Amersham Life Science) and exposed using FUJIFILM LAS-3000 (Tokyo, Japan). Protein level was determined quantitatively by densitometry using FUJIFILM-Multi Gauge V2.2 software.

**Immunoprecipitation.** Immunoprecipitation assay was carried out as described previously (Chen et al., 2003). Cell lysates (500 μg of protein) prepared as aforementioned was precleared with protein A-Sepharose (Amersham Pharmacia Biotech), and then immunoprecipitated with polyclonal anti-Fas antibody. After harvested with protein A, the immunoprecipitated proteins were analyzed by SDS-PAGE, and detected by blotting with polyclonal anti-Fas or anti-FasL antibodies.

**Real-time quantitative RT-PCR.** Total RNA isolated from cells with a guanidinium chloride procedure were analyzed by real-time quantitative RT-PCR for the RNA levels of different genes using a Bio-Rad iCycler system (Bio-Rad) as described previously (Lin et al., 2011). All results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**In vivo tumor xenograft study.** Athymic nude mice (BALB/c nu/nu mice, 6 weeks old) obtained from the National Laboratory Animal Center, Taiwan, were housed in cages that were maintained at a temperature of 22 ± 2°C and humidity 65 ± 5% in a controlled animal facility with a 12 h light-dark cycle and ad libitum access to water. Lovo cells (2 × 10⁶ in 0.2 ml matrigel) were subcutaneously implanted into the right flank of nude mice for tumor formation. The animals were randomly divided into four groups (12 mice per group) and treated as follows: (1) saline (vehicle); (2) Andro at 10 mg/kg of body weight once daily, po; (3) CDDP at 6 mg/kg of body weight weekly, ip; (4) combination of (2) and (4). The injection regiments and doses for the treatments were based on the published reports (Ji et al., 2011; Matsui et al., 2008). The day of cell implantation was designated as day 0, and tumor growth and tumor appearance were registered daily thereafter. The animals were euthanized, and the primary tumors were removed and measured 40 days later. Tumor tissue extracts were further analyzed by Western blotting for protein expression levels.

**Statistical analysis.** Data were presented as means ± SD, and statistical significance was analyzed by Student’s t-test. A value of p < 0.05 was considered statistically significant.
RESULT

Andro Potentiates the Cytotoxic Effect of CDDP in Lovo Cells

In our previous study, Andro at dosages above 10 μM was demonstrated to be a chemotherapeutic agent as evidenced by its ability of inducing cell-cycle arrest in CRC cell line Lovo (Shi et al., 2008). Using MTT assay a preliminary screening was conducted to assess the effect of Andro as a single agent or in combination with CDDP on proliferation of Lovo cells at different time points. The growth of Lovo cells was suppressed dose- and time-dependently by 10 μM of Andro along with various concentrations of CDDP (Fig. 1A). In addition, the proliferation of Lovo cells treated with combinations of Andro and various doses of CDDP was significantly lower than those of Andro or CDDP alone treatments (Fig. 1B). The cotreatment of Andro and CDDP exerted a significant synergistic therapeutic efficacy, especially in the dosage of 10 μM of Andro and 10 μM of CDDP which suppressed 56.5% of cell growth, whereas the same dose of Andro and CDDP alone showed inhibition rates of 35.8 and 15.4%, respectively. Furthermore, the cytotoxic effect of increasing concentrations of CDDP (5, 10, and 15 μM) and 10 μM of Andro was monitored using LDH assay (Fig. 1C). LDH release caused by increasing concentrations of CDDP was not significant after 24 h incubation. Importantly, the increase in cytotoxicity was significantly higher in the cells treated with combinations of Andro and CDDP, when compared with CDDP alone. LDH assay confirmed that the cytotoxic effect was more pronounced when CDDP concentrations were used in combination with Andro. All further mechanistic studies were conducted under the treatment of Andro (10 μM) and CDDP (10 μM), because the combination has the best synergistic inhibition capacity of cell growth.

Andro Enhances CDDP-Induced Apoptosis in Lovo Cells

In order to determine the apoptotic effect of Andro alone and to evaluate the enhancing effect of Andro on the CDDP-induced apoptosis in Lovo cells, flow cytometry was performed to quantify the apoptotic state. The number of hypodiploid cells (apoptotic cells) that are stained less intensely with PI was measured from the peak of sub-G1 region in the flow cytometry (Fig. 2A, up panel). The cells cotreated with Andro and CDDP for 24 h showed a significant accumulation of cells in the sub-G1 phase, 27.80% of total cells, as compared with 4.27% of control cells (Fig. 2A, lower panel). Although both single-agent CDDP and Andro caused apoptosis in Lovo cells, the number of apoptotic cells induced by their combination was significantly higher.

The quantification of apoptotic cells induced by Andro or CDDP was confirmed by flow cytometry using Annexin V and 7-AAD double stains. The results also revealed that the combined treatment increased significantly the portion of apoptotic cells (Fig. 2B). The molecular mechanisms of the apoptotic cell death induced by Andro and/or CDDP treatment were further examined. Caspases are cytosolic proteins that exist normally as inactive precursors with higher molecular weights (about 32, 46, and 55 kDa), and are cleaved proteolytically into lower molecular weights (about 17–23 kDa) upon apoptosis induction (Susin et al., 1997). Among caspase family, Caspase-3 plays a central executioner role of apoptosis. In this study, the active forms of caspase-3 (p20), caspase-8 (p23) and caspase-9 (p20) of Lovo cells cotreated with Andro and CDDP were increased significantly (about 3.22-, 2.25- and 1.68-fold of the control level, respectively), whereas the active form of caspase-3 were only increased slightly to 1.28- and 1.36-fold of the control in the cells treated with single agent Andro or CDDP, respectively (Fig. 2C). We observed that the bands of active (cleaved) caspases increased as the procaspases diminished concurrently in the Lovo cells cotreated with Andro/CDDP for 24 h.

Andro/CDDP Combination Enhances the Intrinsic Apoptotic Pathway

Two known alternative pathways are known initiate apoptosis. One is the intrinsic pathway that is characterized by cytochrome c release and Bcl-2 family proteins modulation, and the other is extrinsic pathway that involves caspase-8 and caspase-10 activations (Zhang et al., 2010). To identify which pathway is responsible for the effect of Andro, the expressions of cytochrome c and Bcl-2 family proteins, including Bcl-2, Bad, Bax, t-Bid and Mcl-1 were determined by Western blotting. Figure 3A shows that the mitochondrial protein levels of Bcl-2 were suppressed by about 80% after cotreatment with Andro/CDDP for 24 h, but Mcl-1 and Bad remained unaffected. At the same time, the translocation of Bax and t-Bid (p15) to the mitochondria increased to 3.75-fold and 1.87-fold of those of control, respectively (Fig. 3A, lines 3 and 4). The control cells and those treated with CDDP alone did not express Bax, whereas the addition of Andro to CDDP induced the protein level (Fig. 3A). Concurrently, an increase of the release of cytochrome c from mitochondria to cytosol was observed after the cotreatment (Fig. 3B), indicating activation of the intrinsic pathway.

Andro/CDDP Combination Enhances the Extrinsic Apoptotic Pathway

The expressions of tumor necrosis factor (TNF) family proteins, including FasL and TNF-α, and their receptors Fas (CD95) and TNFR (Ashkenazi and Dixit, 1998) of Lovo cells treated with Andro or and CDDP were determined to evaluate the synergistic effects of Andro and CDDP on the extrinsic apoptosis pathway of apoptosis. Western blotting analysis showed that the protein levels of FasL and Fas increased to about 1.62-fold and 1.51-fold of control levels, respectively 24 h after the combined treatment, whereas the cellular levels of both TNF-α and TNFR1 were little influenced (Fig. 4A). The formation of Fas/FasL complex as analyzed by immunoprecipitation was significantly enhanced in the cells with cotreatment as compared with that of one modality alone (Fig. 4B).
FIG. 1. Effects of Andro in combination of various doses of CDDP on Lovo cell viability. Lovo cells were treated with 10μM of Andro in the presence or absence of various concentrations (1–20μM) of CDDP for 24 and 48 h. Cell viability was analyzed by MTT assay. The quantitative data of dose- and time-dependent effects (A), or growth inhibitory effects (B) are presented as mean ± SD (n = 3) from three independent experiments. *p < 0.05, **p < 0.01 compared with the control. (C) LDH release assay of media from Lovo cells treated with various concentrations (1–20μM) of CDDP in the presence or absence of 10μM of Andro for 24 h was performed. The quantitative data are presented as mean ± SD (n = 3) from three independent experiments. *p < 0.05, **p < 0.01 compared with CDDP alone.

In addition, quantitative real-time PCR analysis was conducted to test the effect of Andro or/and CDDP on the mRNA levels of the extrinsic and intrinsic apoptotic signal factors. As shown in Figure 5A, although Andro alone could induce the mRNA levels of FasL and Fas, the combined treatment of Andro and CDDP increased strongly their expressions. On the other hand, those of the CDDP alone treated cells remained unchanged as compared with the control. Similar results were observed for t-Bid (Fig. 5B) and active caspase-8 (Fig. 5C). The mRNA level of Bcl-2, on the contrary, was reduced by the treatment of CDDP alone or the combination of Andro and CDDP for 24 h. Andro alone had little effect on Bcl-2 (Fig. 5B). Therefore, it suggested that Andro enhanced the CDDP-induced cytotoxicity mainly via promoting extrinsic Fas/FasL-dependent apoptotic pathway. Cotreatment of Lovo cells with Andro and CDDP also resulted in increases of the mRNA levels of Bax and active caspase-9/caspase-3 (Figs. 5B and C), which is consistent with the observation that Andro enhanced the sensitivity of Lovo cells to the CDDP-induced apoptosis. The result of real-time quantitative RT-PCR demonstrated coincided increases of the mRNA levels of FasL, Fas, t-Bid, and active caspase-8/caspase-9/caspase-3 to their protein levels in the cotreated cells (Fig. 5), indicating that Andro and CDDP in combination might regulate these factors’ expressions at the transcriptional level.
FIG. 2. Effects of Andro or/and CDDP on apoptosis in Lovo cells. (A) Lovo cells were treated with vehicle (control), 10μM of Andro, 10μM of CDDP, or 10μM of Andro and 10μM of CDDP in combination for 24 h. The DNA content was analyzed using fluorescence flow cytometry. The position of the sub-G1 peak (M1), integrated by apoptotic cells, and the G0/G1 (M2), S (M3) and G2/M (M4) peaks are indicated. Quantitative assessment of the cell number percentage in the sub-G1 phase was indicated by PI, and represents as mean ± SD (n = 3) of three independent experiments ± SD. *p < 0.05, **p < 0.01 compared with the control. (B) Quantification of apoptosis using flow cytometry, as assessed by Annexin V staining. Numbers within each panel receptively represent early and late apoptotic cells following treatment. Results are representative of at least three independent experiments. (C) Immunoblot analysis of the expressions of caspase members (caspase-3, caspase-8 and caspase-9) in Lovo cells treated with Andro or/and CDDP. β-Actin served as an internal control. The quantitative data are presented as mean ± SD (n = 3) from three independent experiments. *p < 0.05, **p < 0.01 compared with the control.
FIG. 3. Effects of Andro or/and CDDP on the intrinsic apoptotic pathway in Lovo cells. (A) Effects of Andro or/and CDDP on the translocation of Bcl-2 family proteins. Mitochondrial fractions of Lovo cells treated with vehicle (control), 10 μM of Andro, 10 μM of CDDP, or 10 μM of Andro and 10 μM of CDDP in combination for 24 h were analyzed by SDS-PAGE and subsequently immunoblotted with antibodies against Bcl-2, Mcl-1, Bax, t-Bid, Bad, and β-actin that served as an internal control. (B) Effects of Andro or/and CDDP on the release of cytochrome c. The expressions of cytochrome c (Cyt c) in cytosol and mitochondria were analyzed by Western blotting. To check the selectivity of proteins from subcellular fractionation, β-actin and COX-IV were used as marker proteins representing the cytosolic and mitochondrial fractions, respectively. The quantitative data are presented as mean ± SD (n = 3) from three independent experiments. *p < 0.05, **p < 0.01 compared with the control.

Effects of a FasL Blocking Antibody or a Bax Inhibitor Peptide on Andro/CDDP Combination-Induced Apoptosis

The use of FasL blocking antibody (Nok-1) which has been shown to be an effective blocker for FasL-induced apoptosis (Villunger et al., 1997) provided additional evidence for the role of Fas/FasL, the best characterized death receptor/death ligand, in Andro/CDDP-induced apoptosis. The cells treated with both Andro and CDDP had increased expressions of FasL, Bax, and cytosolic cytochrome c as compared with the untreated or Nok-1-treated cells. Nok-1 treatment alone did not show any effect. However, the pretreatment of Nok-1 significantly suppressed the expressions of FasL and cytosolic cytochrome c induced by Andro and CDDP cotreatment (Fig. 6A, lines 1 and 3). Because the expressions of active caspases-8 (p23) and caspase-3 (p20) were increased in the Lovo cells cotreated with Andro/CDDP for 24 h (Fig. 2C), an attempt was carried out to determine the dependency of Andro/CDDP combination-mediated caspases activation on Fas/FasL pathway. The results of Figure 6A (lines 4 and 5) revealed that using Nok-1 cells to block the action of FasL repressed the combination-induced cleavage of caspase-8/caspase-3. Nok-1 alone or control cells showed no cleavage products. Taken tighter, it indicated that Fas/FasL pathway mediated the activation of the caspases that subsequently executes apoptosis in Lovo cells by the combined use of Andro and CDDP.

V5, a membrane-permeable Bax inhibitor peptide (Sawada et al., 2003), was used to resolve whether the observed increase of Bax protein level in the mitochondria was responsible for the release of cytochrome c and the cleavage of caspase-3 that were suggested to be involved in the Andro/CDDP combination-
induced intrinsic apoptotic pathway. The induced levels of the intrinsic pathway related proteins, including Bax, cytosolic cytochrome c, and active caspase-3, by the coexposure of Andro and CDDP were almost completely reversed by the pretreatment of V5 (Fig. 6A, lines 2, 3, and 5), which alone had no influence on these proteins.

The effects of both highly specific inhibitors, Nok-1 and V5, on the Andro/CDDP combination-induced apoptotic cell death were determined by FACSscan analysis in order to identify the involved pathway in Lovo cells. Lovo cells pretreated with Nok-1 and V5 before a 24-h coexposure of Andro and CDDP displayed significant decreases, about 49.6 and 87.2% respectively, in the sub-G1 phase as compared with that of the combination (from 32.0 to 18.60% and 8.45%) (Fig. 6B). This result implies that the synergistic activation of both intrinsic and extrinsic pathways by Andro and CDDP might play an essential role in inducing apoptotic cell death in Lovo.

**Andro Enhances Effectively CDDP-Induced Cytotoxicity in In Vivo Mouse Model**

To evaluate the clinical application of Andro for CRC, the effects of Andro alone or in combination with CDDP on the *in vivo* tumor growth of Lovo cells was tested in a subcutaneous xenograft model. The cotreatment of Andro and CDDP did not show any adverse effect on the body weight in comparison with Andro or CDDP alone during the experimental period (data not shown), indicating the safety of this combination. The data of Fig. 7A showed that Andro or CDDP only partially delayed the steady tumor growth, and that their combination on the other hand suppressed almost completely. Furthermore, Western blotting analysis of tumor samples demonstrated that the expressions of FasL and Bax were highly promoted by the combination comparing to Andro or CDDP alone. The involvement of active caspase-3 in the Andro/CDDP-induced apoptosis in the cell model was confirmed in the animal model showing enhanced expression in the tumors obtained from the cotreated...
FIG. 5. Effects of Andro or/and CDDP on the mRNA levels of the extrinsic and intrinsic apoptotic signal factors in Lovo cells. Real-time quantitative RT-PCR analysis of mRNA levels of FasL, Fas (A), t-Bid, Bax, Bcl-2 (B), caspase-8, caspase-9 and caspase-3 (C) in cells treated with vehicle (control), 10μM of Andro, 10μM of CDDP, or 10μM of Andro and 10μM of CDDP in combination for 24 h. The quantitative data are presented as mean ± SD (n = 3) from three independent experiments. *p < 0.05, **p < 0.01 compared with the control.

FIG. 6. Effects of a FasL-blocking antibody (Nok-1) or a Bax inhibitor peptide (V5) on the combined treatment-induced signaling cascades and apoptosis. (A) Lovo cells were pretreated with Nok-1 or V5 for 30 min, and then treated with 10μM of Andro and 10μM of CDDP in combination for 24 h. The expressions of FasL, Bax, Cyt c (cytosol), active caspase-8 and active caspase-3 were analyzed by Western blotting. β-actin was served as an internal control. The quantitative data are presented as mean ± SD (n = 3) from three independent experiments. *p < 0.05, **p < 0.01 compared with the control. #p < 0.05, ##p < 0.01 compared with the Andro/CDDP-cotreated group. (B) Under the same treatment condition, apoptosis was analyzed by flow cytometry. Quantitative assessment of the cell number percentage in the sub-G1 phase, as indicated by PI, and represents as mean ± SD (n = 3) from three independent experiments. *p < 0.05, **p < 0.01 compared with the control. #p < 0.05, ##p < 0.01 compared with the Andro/CDDP-cotreated group.
mice (Fig. 7B). These results indicated that Andro could enhance the cytotoxicity induced by CDDP both in vivo and in vitro via strengthening the apoptosis pathway.

**DISCUSSION**

Colorectal cancer, one of the leading causes of cancer death in the world, could be cured with surgery if found early. Advanced-stage colorectal cancer often becomes fatal be-
cause of recurrence, even in patients who receive combination chemotherapy (Chung and Saltz, 2007). Platinum drugs such as oxaliplatin and CDDP are important chemotherapy agents against several cancers (e.g., colorectal, lung cancer, testicular, head and neck, ovarian, and cervical), particularly in combination therapy (Gramont et al., 2000; Labianca et al., 1988). However, the side effects (hepatotoxicity, nephrotoxicity, and intrinsic/ acquired resistance) caused by CDDP have limited its clinical application (Weiij et al., 1997). The antitumor effects of CDDP are via the formation of DNA adducts and crosslinks that lead to S-phase delay and G2/M phase arrest in cell cycle and cell apoptosis (Yang and Wang, 1996). Because tumor cells are able to repair damage, evade apoptosis, and resume original high rate of proliferation at low doses of CDDP, this type of chemotherapy is only effective at high doses (Sorenson and Eastman, 1988). Using new targeted anticancer drugs along with CDDP for combination therapy to overcome several side effects and the development of resistance of CDDP are under investigation.

Andro, a diterpenoid lactone, has a broad range of pharmacological effects, such as anti-inflammatory, antiviral, immunomodulatory, antioxidant and hepatoprotective activities (Calabrese et al., 2000; Iruretagoyena et al., 2005; Shen et al., 2002; Trivedi et al., 2007). Recently, the following main findings support the anticancer function of Andro: (1) Andro induces cell cycle arrest and growth inhibition (Jada et al., 2008; Shi et al., 2008); (2) Andro causes apoptotic cell death in various human cancer cells (Cheung et al., 2005; Zhou et al., 2006); (3) Andro sensitizes human cancer cells to the cytotoxicity of other cancer drugs such as TNF-related apoptosis-inducing ligand (TRAIL), 5-fluorouracil and doxorubicin (Yang et al., 2009; Zhou et al., 2008, 2010); and (4) Andro suppresses invasion and migration of cancer cell, thus may be applied in antigrowth and apoptosis (Lee et al., 2010b; Shi et al., 2009). The drug activity of Andro is concentration dependent and cell type specific. It is able to suppress apoptosis at certain concentrations in many cell types (Burgos et al., 2005; Lee et al., 2010a), but could also induce apoptosis at high concentrations (Cheung et al., 2005; Zhou et al., 2006). Many reports have demonstrated that Andro induces effectively cell cycle arrest at the G0/G1 stage in several types of cancer cells (Cheung et al., 2005; Shi et al., 2008), and at the G2/M phase in human hepatoma HepG2 cells (Li et al., 2007). The results of recent study also show that Andro leads cervical cancer HeLa cells to apoptosis, even though it arrests cell cycle at the G2/M in several hepatocellular cancer cell lines (Cheung et al., 2012). Andro has also been reported to enhance chemosensitivity of human CRC cell line HCT116 to doxorubicin via inhibiting the pathway of signal transducer and activator of transcription 3 (STAT3) (Zhou et al., 2010). The studies of combination therapy have indicated that the differences in experimental design, specifically in the concentration and incubation time of the combined treatment of these agents, may explain the apparent contradictory data in the literatures showing the antigrowth and apoptotic effects or mechanisms of Andro as a single agent and in combination with various cancer therapeutic agents (Yang et al., 2009; Zhou et al., 2008, 2010). Taken together, these findings proposed that Andro might be an inhibitor with multiple targets and performs its functions cell type-dependently. These studies along with the existing information regarding the biological effect of Andro support that Andro has a strong potency to sensitize the cytotoxicity of cancer therapeutics in human cancer cells. However, very little is known about the effect of Andro on the efficacy of cancer drugs in animal model. The results of present study provide the first in vivo evidence demonstrating the enhanced chemosensitivity of CDDP by Andro in CRC cells. The detailed apoptotic signaling pathway involved in the combined action of Andro and CDDP in CRC cells Lovo in vitro and in vivo is also included in this study.

To demonstrate that Andro is able to increase the therapeutic activity of CDDP by lowering the apoptotic threshold concentration, MTT assay was conducted to evaluate the combination effect of Andro and CDDP on cell growth in Lovo cells. Figure 1 presented that the cell viability was reduced considerably when the cells were treated with Andro (10μM) together with various low-cytotoxic concentrations of CDDP for 24 h as compared with those exposed to Andro alone and CDDP alone. As the cytotoxicity of CDDP is primarily carried out by inducing apoptosis (Reed, 1999; Siddik, 2003), we therefore inspected the enhancing effect of Andro on the CDDP-induced apoptotic cell death. The results confirmed that Lovo cells underwent severe apoptotic cell death in response to the combined exposure of Andro and CDDP, as evidenced by (1) cell shrinkage and membrane blebbing, the typical apoptotic morphological changes, detected by inverted microscopy (data not shown), (2) an increase in the number of hypodiploid cells measured from the sub-G1 peak of the flow cytometry (Fig. 2A), (3) the translocation of phosphatidylinositol from the inner layer to the outer layer of the cell membrane during early apoptosis, assessed by Annexin V-FITC staining of membrane phosphatidylserine (Fig. 2B), and (4) cleavage of caspase-3 and its upstream regulators caspase-8 and caspase-9 (Fig. 2C). These observations support that the enhancing effect of Andro on the cytotoxicity of CDDP could contribute to the induction of apoptosis in Lovo cells.

Mitochondria play an essential role in many apoptotic responses by releasing apoptogenic factors, such as cytochrome c, that in turn coordinate caspases activation. The release of apoptogenic factors from the mitochondria is mediated through the members of Bcl-2 family which can be sorted into two groups, one is proapoptotic members including Bax, t-Bid or Bad that triggers the release of the proteins and the other is antiapoptotic members including Bcl-2 or Mcl-1 that inhibits their efflux (Green and Reed, 1998). The coadministration of Andro and CDDP significantly enhanced the levels of Bax and t-Bid and concurrently reduced those of Bcl-2 and Mcl-1 (Fig. 3A). Further evidence that suggests the intrinsic apoptotic pathway is the underlying mechanism of the combined used of Andro and...
CDDP is the increased cytosolic level of cytochrome c which plays a key role in the mitochondria-dependent apoptosis (Fig. 3B). Using V5 we have confirmed that Bax signal is involved in the apoptotic cell death initiated by the combined treatment in Lovo cells (Figs. 4A and B), indicating that an increased translocation of Bax to the mitochondria is responsible for the release of cytochrome c from the mitochondria to the cytosol that subsequently activated downstream apoptotic factors.

The extrinsic pathway of apoptosis is activated by surface receptors, but not by the modification of intracellular pools of proteins (Ashkenazi and Dixit, 1998). Among the well characterized members of the death receptor family, the engagement of Fas by a Fas ligand lead to the formation of death-inducing signaling complex that subsequently activates caspase-8 and permits acute execution of apoptosis (Ashkenazi and Dixit, 1998). The addition of Andro to CDDP induced the protein and mRNA levels of Fas and FasL and thus activated the FasL/Fas signaling in Lovo cells, whereas CDDP alone did not affect the expression of both proteins (Figs. 4 and 5). The cells pretreated with Nok-1 monoclonal antibody to block the action of FasL were resistant to the cytotoxicity of Andro/CDDP combined treatment by reversing the activation of caspase-8 and caspase-3, and partially the transaction of Bax and the cytosolic release of cytochrome c (Fig. 6). As discussed above, the apoptosis induced by Andro/CDDP combination may involve two pathways: one is the upregulation of caspase-8-dependent Bid cleavage mediated by FasL that resulted in Bax transactivation, and the other is the direct activation of Bax. Therefore, the apoptosis-inducing property of the Fas/FasL system is a promising antineoplastic strategy.

Based on these results, a schematic presentation was proposed to lay out the possible mechanisms for the synergistic cytotoxic effect of Andro/CDDP combination, showing that both extrinsic (Fas-mediated activation of caspase-8/t-Bid) and intrinsic (Bax/cytochrome c-mediated activation of caspase-9) apoptotic pathways were involved in the apoptotic response in Lovo cells (Fig. 7C). The activation of both death pathways by the cotreatment implies that a superimposed death signaling was triggered to induce cell apoptosis. The mouse xenograft model showed a complete suppression of tumor growth by the combined Andro/CDDP therapy without any side effects (Fig. 7A).

Although Andro and CDDP used as single-agent might activate different apoptotic pathway, the combination induced synergistically apoptotic signals in Lovo cells both in vitro and in vivo (Figs. 7B and C). This study offers, at least in part, a molecular basis for the further application of Andro and CDDP combination in the treatment of colorectal cancer as a novel and pharmacologically safe chemotherapeutic strategy.

**FUNDING**

National Science Council, Taiwan (NSC 97-2313-B-273-002); Yongkang Veterans Hospital, Tainan, Taiwan (VHYK-9924).

**REFERENCES**


Sorensen, C. M., and Eastman, A. (1988). Influence of cis-
diaminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells. Cancer Res. 48, 6703–6707.