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Inhibition of the JAK-STAT3 pathway by andrographolide enhances chemosensitivity of cancer cells to doxorubicin

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ABSTRACT

Andrographolide (Andro), a diterpenoid lactone isolated from a traditional herbal medicine *Andrographis paniculata*, is known to possess potent anti-inflammatory and anticancer properties. In this study, we sought to examine the effect of Andro on signal transducer and activator of transcription 3 (STAT3) pathway and evaluate whether suppression of STAT3 activity by Andro could sensitize cancer cells to a chemotherapeutic drug doxorubicin. First, we demonstrated that Andro is able to significantly suppress both constitutively activated and IL-6-induced STAT3 phosphorylation and subsequent nuclear translocation in cancer cells. Such inhibition is found to be achieved through suppression of Janus-activated kinase (JAK)1/2 and interaction between STAT3 and gp130. For understanding the biological significance of the inhibitory effect of Andro on STAT3, we next investigated the effect of Andro on doxorubicin-induced apoptosis in human cancer cells. In our study the constitutive activation level of STAT3 was found to be correlated to the resistance of cancer cells to doxorubicin-induced apoptosis. Both the short-term MTT assay and the long-term colony formation assay showed that Andro dramatically promoted doxorubicin-induced cell death in cancer cells, indicating that Andro enhances the sensitivity of cancer cells to doxorubicin mainly via STAT3 suppression. These observations thus reveal a novel anticancer function of Andro and suggest a potential therapeutic strategy of using Andro in combination with chemotherapeutic agents for treatment of cancer.

1. Introduction

Signal transducers and activators of transcription (STAT) proteins are a family of seven proteins (STATs 1, 2, 3, 4, 5a, 5b, and 6) that mediate signal transduction from extracellular signals to transcription of target genes. Among the STATs, STAT3 is the most intimately linked to tumorigenesis [1,2]. Numerous studies have shown a constitutive activation of the STAT3 pathway in a variety of human cancers, including breast carcinoma, colon cancer, cervical cancer, prostate cancer, melanoma, multiple myeloma, and leukemia [2,3].

STAT3 and its upstream Janus-activated kinase (JAK) signaling were originally identified as the signaling pathway for interferon (IFN). It mediates the immune responses of various cytokines as well as many growth factors and hormones, and thus participates in inflammation, cell growth and metastasis [4,5]. STAT3 can be activated by a number of cytokines, including interleukin-6 (IL-6), IL-11, ciliary neurotrophic factor, oncostatin M, and leukemia inhibitory factor, which all signal through a shared gp130 signal transducer receptor subunit [2]. For IL-6-induced STAT3 activation, upon IL-6 binding to IL-6R, gp130 is recruited to IL-6R, leading to binding and trans-phosphorylation of JAK. Activated JAK then phosphorylates the tyrosine residues of gp130. The phosphotyrosine side chains of gp130 serve as docking sites for latent transcription factors of the STAT3, resulting in homodimerization or heterodimerization and subsequently nuclear localization and DNA binding of STAT3 [4]. Fully activated STAT3 regulates transcription of specific target genes, including anti-apoptotic proteins (Bcl-xL, Mcl-1) [6,7] and proliferation regulatory proteins (cyclinD1, Myc and survivin) [8–10].

Abbreviations: Andro, andrographolide; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DN, dominant negative; FBS, fetal bovine serum; IFN, interferon; IL, interleukin; JAK, Janus kinase; MTT, 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide N-acetylcysteine; NF-κB, nuclear transcription factor-kappaB; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; STAT, signal transducers and activators of transcription; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis.

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STAT3 signaling pathway has recently been shown to confer resistance to chemotherapy-induced apoptosis in human tumors, due to its involvement in the proliferation, angiogenesis, immune evasion and anti-apoptosis of cancer cells and its aberrant activation in various tumor cells [4,11,12]. Thus, abrogation of STAT3 activation is believed to render tumor cells more susceptible to cancer therapeutic agents.

Andro is a diterpenoid lactone isolated from *Andrographis paniculata*, an anti-inflammation herbal medicine, which has been used for the treatment of various ailments including respiratory infection, bacterial dysentery and fever [13,14]. As the major active component contained in *Andrographis paniculata*, Andro has been shown to be responsible for the anti-inflammatory activity, mainly via its inhibitory effect on nuclear transcription factor-kappaB (NF-κB) [15]. In our previous studies, we demonstrated that Andro possesses potent anticancer property through promoting apoptosis in cancer cells. Andro alone or combined with TRAIL could induce both mitochondria-mediated and death receptor-mediated apoptotic cell death [16,17], supporting the development of Andro as an apoptosis inducer or chemo-sensitizer in combined therapy. However, whether Andro could inactivate other survival signaling pathways to achieve its anti-tumor effect remains unclear. In the present study, we sought to examine the effect of Andro on the JAK-STAT3 pathway and evaluate whether suppression of STAT3 activity by Andro could sensitize cancer cells to a common chemotherapeutic drug doxorubicin.

2. Materials and methods

2.1. Cell culture and reagents

Andro was purchased from Sigma (Cat #365645) as a pure compound, as described in our earlier studies [16,17]. Human cancer cells, HCT116, MDA-MB-231, HepG2 and HeLa, were purchased from ATCC. Human papillary thyroid cancer cells (TPC-1) and human anaplastic thyroid cancer cells (ARO) have been described previously [18]. All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. 4,6-diamidino-2-phenylindole (DAPI), ANTI-FLAG® antibody and other common chemicals were all purchased from Sigma–Aldrich (St. Louis, MO). A JAK specific inhibitor, Pyridone 6, anti-human phospho-(Tyr1007/1008)-JAK2 antibody, and recombinant human IL-6 were purchased from Merck (San Diego, CA). pSTAT3 luciferase vector was obtained from Panomics (Redwood City, CA). Anti-human phospho-(Tyr705)-STAT3, phospho-(Ser727)-STAT3, STAT3, Bcl-xL, caspase-3 and PARP antibodies were from Cell Signaling (Beverly, MA). Anti-human Mcl-1, cyclinD1, phospho-(Tyr1022)-JAK1, gp130 (C20) and tubulin were from Santa Cruz (Santa Cruz, CA).

2.2. Detection of cytotoxicity and apoptosis

The general cytotoxicity of doxorubicin and Andro on different human cancer cells was detected by the MTT test as described previously [19] and the results were presented as the relative cell viability compared with the control group. Cells undergoing apoptosis were evaluated by DAPI staining for morphological changes including chromatin condensation and nuclear shrinkage, as previously reported [20]. Briefly, at the end of designated experiments, the cells were fixed with 70% ethanol at room temperature for 10 min and stained with 0.3 μg/mL DAPI (in PBS) at room temperature for another 10 min and visualized under an inverted fluorescence microscope. The cells with condensed nucleus were visualized and counted under an inverted fluorescent microscope (Nikon ECLIPSE TE2000-S, Nikon Instruments, Tokyo, Japan).

2.3. Colony formation assay

Cancer cells (HCT116, MDA-MB-231, HeLa, and HepG2) were treated as designated for 12 h before reseeded in six-well plates (5000 cells/well), respectively. After 2 weeks, the survival clones were stained by 0.5% crystal violet for 1 h and photos were taken using digital camera.

2.4. Luciferase assay

The transient transfection of pSTAT3 luciferase vector was done in MDA-MB-231 cells using LipofectAMINE PLUS transfection reagent according to the manufacturer's protocols. *Renilla* luciferase vector was used as a transfection control. The luciferase activity was measured in the cellular extracts using a Dual-Luciferase Reporter Assay System (Promega) based on the protocol provided by the manufacturer. Briefly, following the treatments, the cell lysate was collected from each well after the addition of cell lysis reagent. After adding the luciferase assay substrate, the firefly luciferase activity was determined using a luminometer (Promega) and the *Renilla* luciferase activity was then measured by adding the Stop&Glo substrate.

2.5. Preparation of nuclear and cytosolic extracts

Nuclear extracts and cytosolic extracts were prepared by NE-PER® nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's protocol. Briefly, after washed with PBS, cell pellets were collected by centrifugation at 500 × *g* for 3 min, and removed the supernatant. Cell pellets were incubated with cytoplasmic extract reagent I for 10 min then added with ice-cold cytoplasmic extract reagent II. After 5 min centrifugation at maximum speed, supernatant was collected and considered as cytoplasmic extract. The insoluble fraction was resuspended with ice-cold nuclear extract reagent and incubated for a total of 40 min, and centrifuged at maximum speed for 10 min. The supernatant was collected immediately as nuclear extract. Protein concentration was determined using Bio-Rad Protein Assay reagent.

2.6. Transient transfection and RNA interference

HCT116 cells were transfected with Flag-STAT3 vectors (wild type STAT3 and dominant negative STAT3 vectors) as described previously [18], using LipofectAMINE PLUS (Invitrogen) following the manufacturer's instructions. Cells were treated as indicated for 24 h after transfection. For the RNA interference study, synthetic small interfering RNAs (scrambled siRNA and STAT3 siRNA) were from Santa Cruz (Santa Cruz, CA). The cellular delivery of siRNA was carried out by using LipofectAMINE PLUS and optimized with various doses and post-transfection time and evaluated by Western blot experiment in HeLa cells.

2.7. Immunoprecipitation and Western blot

Immunoprecipitation was performed briefly by following the instructions of the manufacturers of the respective antibodies. In brief, the cells were washed three times with cold PBS and lysated in 1 mL RIPA buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.2], 1% Triton X-100, 0.1% SDS, 0.25 mM EDTA [pH 8.0]) containing protease inhibitor cocktails (Roche). Cell lysates containing 3 mg of protein were incubated with appropriate antibody at 4 °C overnight or 50 μl ANTI-FLAG® M1 Monoclonal Antibody-Agarose Affinity Gel (Sigma). Immune complexes were captured by adding 50 μl of protein A/G agarose beads and rotated as 4 °C for 3 h (this step was omitted when ANTI-FLAG® M1 Monoclonal Antibody-Agarose Affinity Gel was used). After five times washing of protein

A/G agarose beads with ice-cold TBS, the immunoprecipitates were fractionated by SDS-PAGE.

For Western blot, equal amount of protein was fractionated on SDS-polyacrylamide gel in the Mini-PROTEAN II system (Bio-Rad, Hercules, CA) and blotted onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk in TBST (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20), the membrane was probed with various antibodies and developed with enhanced chemiluminescence (Pierce, Rockford, IL) using a Kodak Image Station (Kodak, Rochester, NY).

3. Results

3.1. Andro suppresses constitutive STAT3 activation in human cancer cells

STAT3 is an important pro-survival factor in regulation of apoptosis [12]. Here we sought to determine whether Andro is able to suppress STAT3 phosphorylation and activation. First we tested the effect of Andro on the constitutively activated STAT3 in MDA-MB-231 cells, a cell line known to possess high basal level of STAT3 [21]. As shown in Fig. 1A, Andro inhibited the constitutive phosphorylation of STAT3 on both Tyr705 and Ser727 in a dose- and time-dependent manners, without altering the overall STAT3 protein level. Next, to confirm the inhibitory effect of Andro on STAT3 activity, the luciferase reporter assay was carried out using a pSTAT3-TA-Luc vector. After normalization with *Renilla*, the relative luciferase activities in cells treated with different concentrations of Andro were compared with those of the control. As shown in Fig. 1B, Andro, at a low-cytotoxic concentration of 15 μM [16], markedly reduced STAT3 luciferase activity in MDA-MB-231 cells. These data consistently demonstrate that Andro possesses potent inhibitory effect on STAT3 activity.

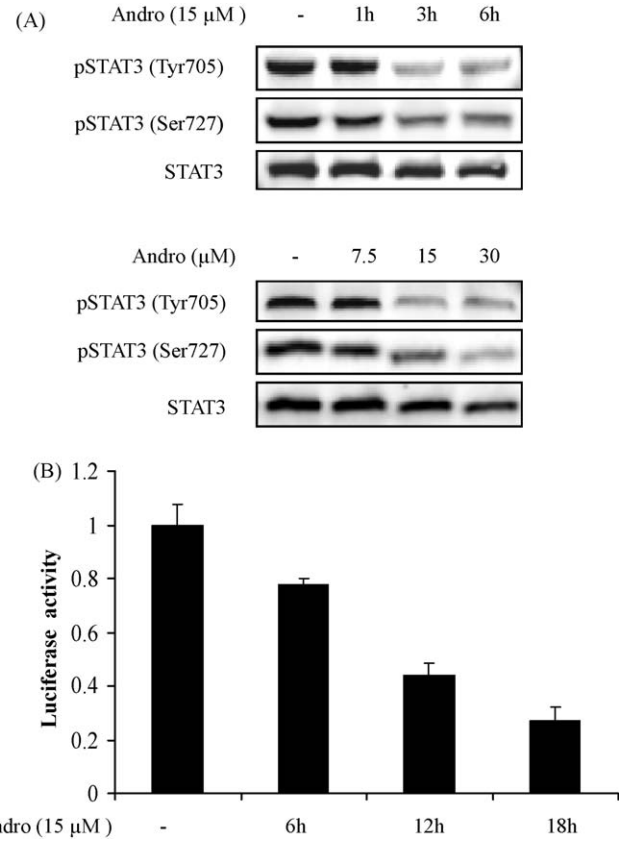

Fig. 1. Andro inhibits constitutive STAT3 activity in MDA-MB-231 cells in dose- and time-dependent manners. (A) Andro inhibits STAT3 phosphorylation in dose- and time-dependent manners. MDA-MB-231 cells were treated either with increasing concentrations of Andro for 3 h, or with 15 μM Andro for the indicated durations. The levels of phosphorylated STAT3 (Tyr705 and Ser727) and overall STAT3 were examined in whole cell lysate by Western blot. (B) Effect of Andro on STAT3 luciferase activity. MDA-MB-231 cells were transiently transfected with a STAT3 luciferase report vector together with *Renilla* luciferase vector as a transfection control. 24 h after transfection, cells were treated with indicated concentrations of Andro for 12 h. Columns, mean of three independent transfection experiments. Andro-treated cells were normalized to DMSO-treated control.

3.2. Andro inhibits IL-6-inducible STAT3 phosphorylation and nuclear translocation in human cancer cells

As IL-6 is an important growth factor and its biological functions are mainly mediated through the STAT3 pathway [22], we examined whether Andro could inhibit IL-6-induced STAT3 activation in human cancer cells. In this context, we used HCT116, a human colorectal cancer cell line which is known to possess low basal level of STAT3 activation [23]. As shown in Fig. 2A, IL-6 stimulation on HCT116 cells was associated with marked increases of phospho-STAT3 levels, which was effectively abolished by Andro treatment. It is known that under resting conditions, STAT3 is retained in the cytoplasm, and it translocates to the nucleus once phosphorylated and activated [4]. Here, we analyzed the effect of Andro on STAT3 nuclear translocation. In the untreated control cells, STAT3 resided predominantly in cytoplasm and phospho-STAT3 was barely detectable (Fig. 2B). Upon IL-6 stimulation, phospho-STAT3 level substantially increased in both cytoplasmic and nuclear fractions, especially the nuclear fraction. Consistently, pretreatment of Andro almost completely abolished the phospho-STAT3 nuclear translocation (Fig. 2B).

3.3. Andro inhibits STAT3 phosphorylation through suppression of JAK1/2

It has been reported that the phosphorylation of STAT3 on Tyr705 is mainly mediated by receptor-associated JAKs [22,24].

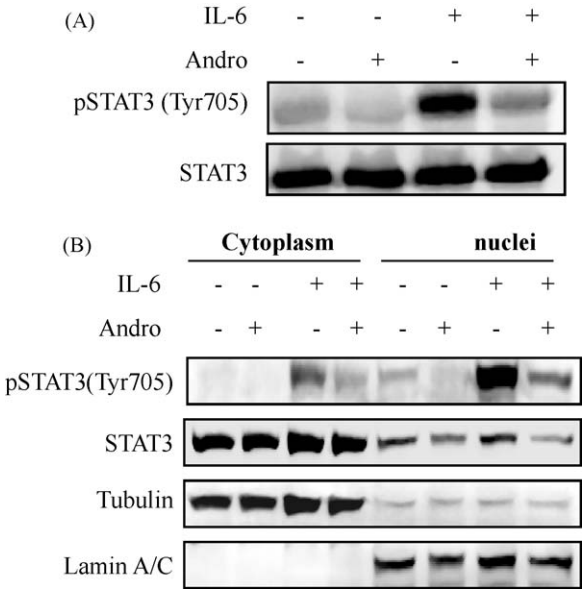

Fig. 2. Andro blocks IL-6-induced STAT3 phosphorylation and nucleus translocation. (A) HCT116 cells were pretreated with Andro (15 μM) for 3 h and then stimulated with IL-6 (20 ng/mL) for 30 min. Whole cell lysates were immunoblotted with the indicated antibodies. (B) Subfraction of cytoplasm and nucleus was conducted according to manufacturer's protocol, as indicated in Section 2. Cytosolic and nuclear lysates were immunoblotted with the indicated antibodies. Tubulin and Lamin A/C were used as loading controls for cytosol and nucleus subfraction, respectively. Data are representatives of three independent experiments.

Therefore, we examined the effect of Andro on JAK activation by checking its phosphorylation status. As shown in Fig. 3A, in MDA-MB-231 cells with constitutive activation of STAT3, there was also high level of tyrosine phosphorylation of JAK1 and JAK2 in the control cells. As expected, Andro markedly suppressed tyrosine phosphorylation of JAK1 and JAK2. The effect of Andro on JAK kinases was found to be similar to that of Pyridone 6, a known pan-JAK inhibitor [25,26].

It has been established that the activated JAK1 and JAK2 phosphorylate gp130, which then serves as the docking protein for STAT3 [1,27]. To investigate whether Andro could affect the recruitment of STAT3 to gp130, we transiently transfected Flag-STAT3 into MDA-MB-231 cells, and examined the interaction with the endogenous gp130. As shown in Fig. 3B, the basal level of protein–protein interaction in the untreated control cells probably reflects the constitutive activation of STAT3 observed in this cell. Andro was able to markedly reduce both the basal as well IL-6-stimulated recruitment of STAT3 to gp130. To verify the above findings, we performed reciprocal immunoprecipitation against endogenous gp130 and consistent results were observed (Fig. 3C). On the other hand, the non-receptor tyrosine kinase, src is also one important upstream kinase for STAT3 phosphorylation [24], and Andro was reported to be able to down-regulate v-src protein level in one kind of v-src-transformed cells [28]. However, with all cancer cell lines tested in our experiments, we did not observe the change of v-src protein level upon Andro treatment (data not shown).

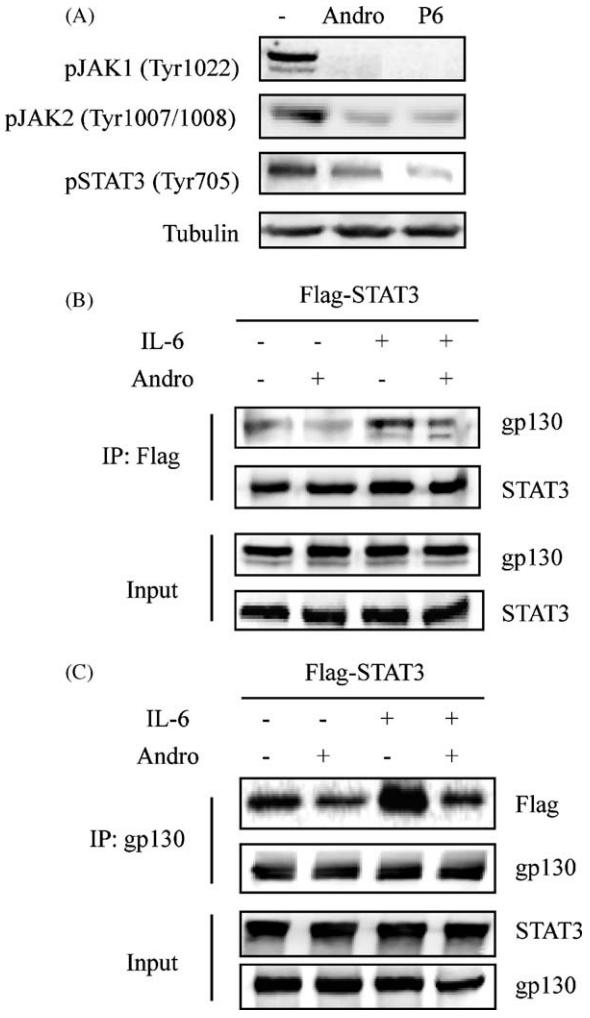

Fig. 3. Andro disrupts the interaction between STAT3 and gp130 via inhibition on JAK1/2. (A) Andro inhibits JAK1/2 phosphorylation. MDA-MB-231 cells were treated with Andro (15 μM) or Pyridone 6 (5 μM) for 3 h, and cell lysates were collected and subjected to Western blot for detecting phospho-JAK1 and phospho-JAK2. Panels (B) and (C), MDA-MB-231 cells were transfected with Flag-STAT3 vector and treated with IL-6 (20 ng/mL), Andro (15 μM) or their combination as indicated on top. Subsequently, 3 mg of cell lysates were immunoprecipitated with ANTI-FLAG® Agarose Affinity Gel (B) or 5 μg of gp130 antibody (C), and the bound proteins were fractionated on SDS-PAGE gel, then analyzed by Western blot with the antibodies indicated on the left. Data are representatives of three independent experiments.

3.4. Constitutively active STAT3 confers resistance to doxorubicin-induced cell death in cancer cells

In this study, we selected a panel of cancer cell lines including HCT116, MDA-MB-231, HepG2 and HeLa to evaluate the constitutive activation of STAT3 in these cell lines. Notably, both the total STAT3 protein level, and more strikingly, the pSTAT3 level were found to be different in these four cell lines (Fig. 4A). Among them, HeLa cells possess the highest level of pSTAT3, while the pSTAT3 level in HCT116 cells was hardly detectable. Due to its well-established anti-apoptotic function, STAT3 has been implicated in the resistance to chemotherapeutic drug treatment [7]. To confirm the effect of STAT3 in chemo-resistance, we tested the relationship between STAT3 activation level and cellular resistance to doxorubicin, a common cancer therapeutic agent. All four cell lines were treated with increasing doses of doxorubicin for 24 h or 48 h, and the cell viability was scored using the MTT assay. As shown in Fig. 4B, these four cell lines show obvious different sensitivities to doxorubicin, especially when treated for 48 h. Among them, HCT116 cells are the most sensitive ones, while HeLa cells are the most resistant to doxorubicin-induced cell death, a pattern closely associated with the STAT3 phosphorylation status. To confirm the important role of STAT3 activation in cellular resistance to doxorubicin-induced cell death, we selected a pair of thyroid cancer cell lines with different activation levels of STAT3: TPC-1 and ARO cells. TPC-1 cells are known to bear the RET/PTC1 rearrangement, which has been reported to promote STAT3 tyrosine phosphorylation [18,29]. As shown in Fig. 4C, the pSTAT3 level in TPC-1 cells was much higher than that in ARO cells, although the expression levels of total STAT3 are same, consistent to the literature [18]. As expected, upon doxorubicin treatment, significant cell death could be detected in ARO cells, but not in TPC-1 cells, which possess constitutively activated STAT3 (Fig. 4D and E).

To further understand the role of STAT3 signaling in doxorubicin-induced cell death, a dominant negative mutant of STAT3 (STAT3-Y705F) was over-expressed into HeLa cells. Over-expression of this mutant form of STAT3 completely abrogated constitutively activated STAT3 (Fig. 5A), and significantly enhanced doxorubicin-induced cell death (Fig. 5B). Furthermore, we manipulated the STAT3 expression by using specific siRNA against STAT3. It is well documented that Mcl-1 and Bcl-xL, two important anti-apoptotic proteins, are transcriptionally regulated by STAT3 [4,6]. Moreover, up-regulation of these proteins is reported to be responsible to cell resistance to doxorubicin [30,31]. As expected, both over-expression of dominant negative STAT3 and knockdown of STAT3 in HeLa cells resulted in reduced expression level of Mcl-1, one important target gene of STAT3 activation (Fig. 5A and C), and consequently sensitized doxorubicin-induced apoptosis (Fig. 5D). These data thus support the notion that suppression of STAT3 would be a legitimate approach to enhance the therapeutic activity of doxorubicin.

3.5. Andro enhances doxorubicin-induced apoptosis in human cancer cells

As shown earlier, Andro is capable of suppressing both constitutive and IL-6-stimulated STAT3 activation (Figs. 1–3), and STAT3 is implicated in doxorubicin-induced cell death (Fig. 4),

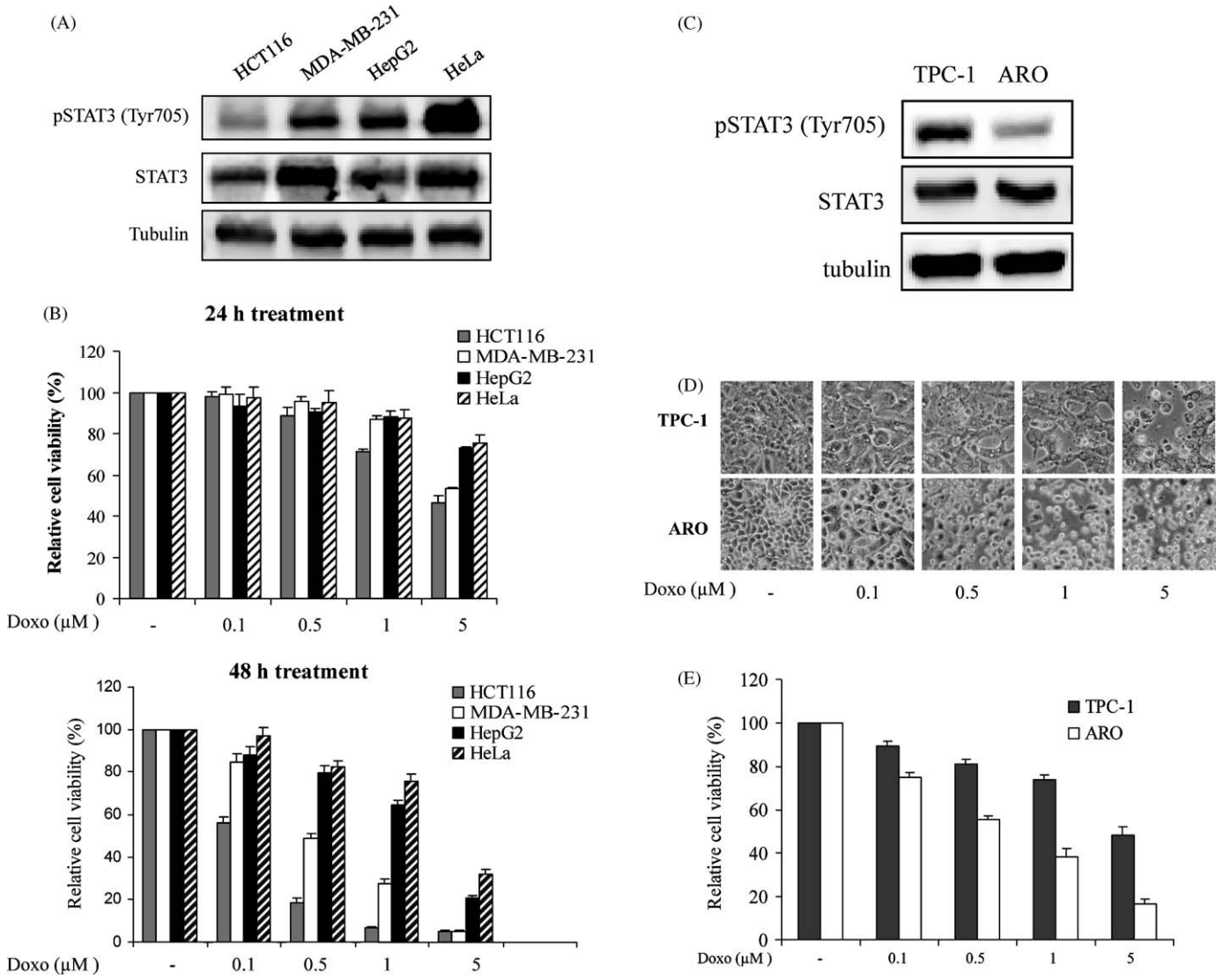

Fig. 4. Constitutively active STAT3 plays a crucial role in chemo-resistance of tumor cells to doxorubicin. (A) Western blot was used to detect the pSTAT3 and STAT3 levels in HCT116, MDA-MB-231, HepG2 and HeLa cells. (B) HCT116, MDA-MB-231, HepG2 and HeLa cells were treated with indicated concentrations of doxorubicin for 24 h or 48 h, and cell viability was evaluated by MTT assay. Data were presented as mean ± SD from three independent experiments. (C) Western blot was used to detect the pSTAT3 and STAT3 levels in TPC-1 and ARO cells. (D) TPC-1 and ARO cells were treated with indicated concentrations of doxorubicin for 48 h, and cell death was indicated with representative cell images which were photographed using a normal light microscope (magnification, 200×). (E) TPC-1 and ARO cells were treated as indicated in panel (D), and cell viability was evaluated by MTT assay. Data were presented as mean ± SD from three independent experiments.

we thus hypothesize here that Andro would lower the apoptotic threshold and increase chemotherapeutic activity of doxorubicin via suppression of STAT3. As shown in Fig. 6A, when the cells were pretreated with Andro for 2 h following by a low-cytotoxic concentration of doxorubicin for 24 h, the cell viability of all these cell lines was reduced dramatically, comparing to the Andro alone and doxorubicin alone treatment. One interesting observation in this part of our study is that in the presence of Andro, the different cancer cells with different STAT3 activation levels responded to the combined treatment of Andro and doxorubicin in similar patterns, suggesting that the suppressed STAT3 activity by Andro abolishes chemo-resistance of cancer cells to doxorubicin. In addition, to test the long-term effect of Andro and doxorubicin on cancer cell growth, the colony formation assay was conducted in HCT116 cells. As shown in Fig. 6B, although Andro alone or doxorubicin alone could reduce the number of colony formation to a certain extent, combined treatment with Andro and doxorubicin almost completely suppressed cell growth and colony formation.

As doxorubicin-mediated cytotoxicity is mainly executed by inducing apoptotic cell death [32], here we examined whether Andro could enhance the apoptotic cell death in doxorubicin-treated cells. As shown in Fig. 6C, upon the combined treatment of Andro and doxorubicin, HeLa cells underwent dramatic apoptotic cell death, evidenced by (i) chromatin condensation, a typical apoptotic morphological change detected by DAPI staining, (ii) cleavage of caspase-3 and its downstream substrate PARP (Fig. 6D), and (iii) the protective effect of Z-VAD-FMK, a pan-caspase inhibitor for both parameters (Fig. 6C and D). Similar results were also observed on HepG2, HCT116 and MDA-MB-231 cells (data not shown). Therefore, it is believed that Andro enhances doxorubicin-induced cytotoxicity mainly via promoting caspase activation and apoptotic pathway. In order to elucidate the underlying mechanism responsible for the sensitization effect induced by Andro, we detected several downstream proteins of STAT3 signaling, including Bcl-xL, Mcl-1 and cyclinD1. As expected, Andro treatment markedly decreased the activation of pSTAT3, followed by the concomitant reduction of Bcl-xL, Mcl-1 and cyclinD1 at later time

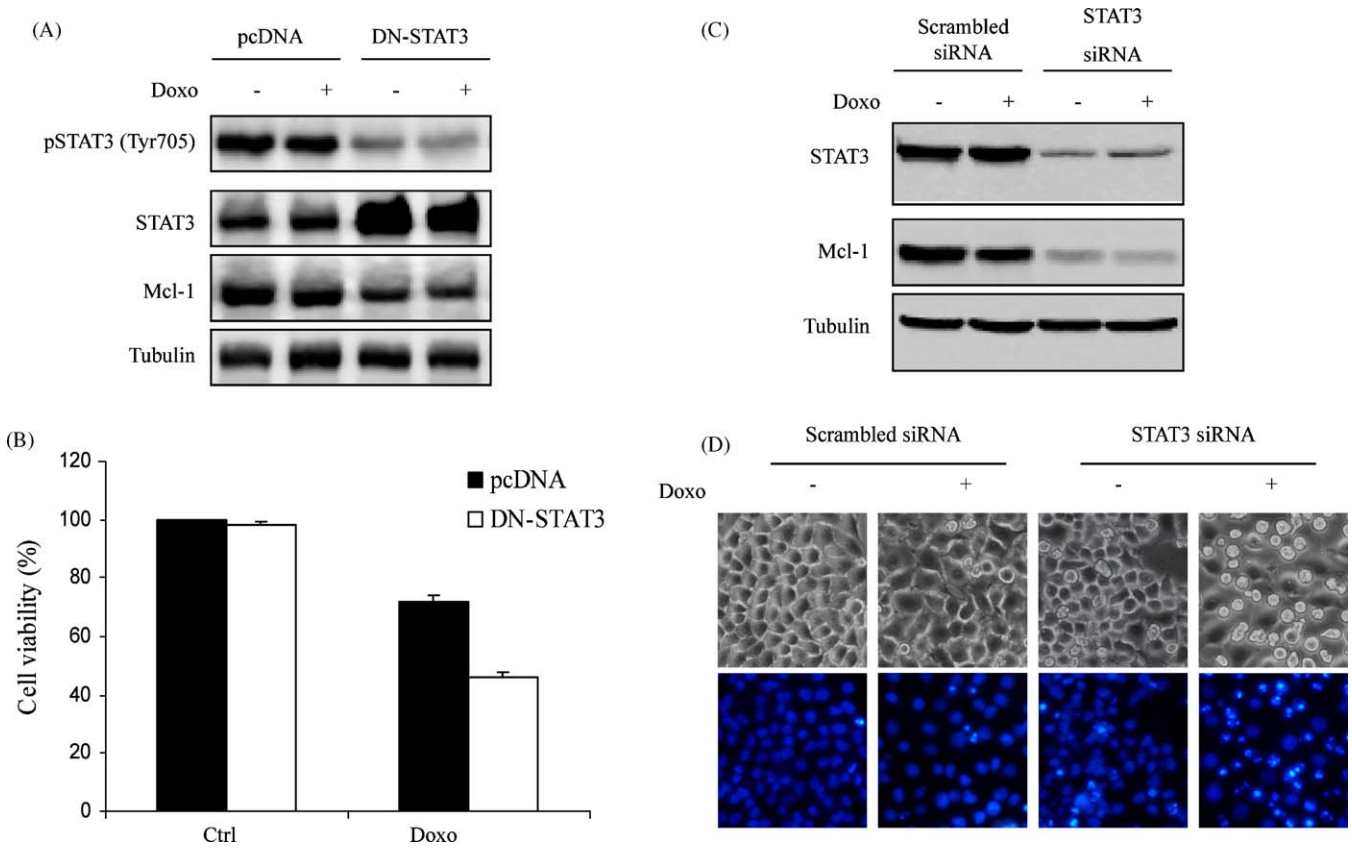

Fig. 5. Constitutively active STAT3 confers resistance to doxorubicin-induced cell death in cancer cells. (A) HeLa cells were transiently transfected with STAT3-Y705F or pcDNA which was used as transfection control, then Western blot was used to detect STAT3, pSTAT3, Mcl-1 and tubulin. (B) After transfection with pcDNA or STAT3-Y705F, HeLa cells were treated with doxorubicin (0.5 μM) for 24 h, and cell viability was evaluated by MTT assay and represented by relative cell viability compared with untreated control group. Data were presented as mean ± SD from three independent experiments. (C) HeLa cells were transfected with scrambled siRNA or STAT3 siRNA for 24 h. Then cells were treated with doxorubicin (0.5 μM) for 24 h. At the end of treatment, cells were collected for detection of STAT3, Mcl-1 and tubulin by Western blot. (D) After STAT3 knockdown, HeLa cells were treated with doxorubicin for 24 h. Cells were assessed using DAPI staining and images were representatives of three independent experiments (magnification, 200×).

point (Fig. 6E), which supports the observed sensitization effect of Andro on doxorubicin-induced apoptosis.

Taken together, the above results indicate that Andro possesses significant inhibitory effect on JAK-STAT3 pathway and this effect accounts for the sensitization effect of Andro on doxorubicin-induced apoptosis.

4. Discussion

In our previous studies, Andro has been demonstrated to induce apoptosis in cancer cells and to enhance TRAIL-induced apoptosis [16,17]. Here we reveal a novel anticancer potential of Andro: Andro is capable of sensitizing doxorubicin-induced apoptotic cell death via suppression of the JAK-STAT3 pathway.

Constitutive activation of STAT3 has been implicated in tumorigenesis by promoting proliferation and inhibiting apoptosis [4,11,12]. On the basis of the importance of STAT3 in tumor progression and survival, STAT3 recently has been considered as a molecular target for cancer chemotherapeutics. Studies with genetic approaches including antisense and RNA interference (siRNA) have demonstrated that inhibition of STAT3 signaling suppresses tumor growth and induces apoptosis *in vitro* and *in vivo* [33]. Moreover, pharmacological agents including Pyridone 6 and AG490 have also been demonstrated to be potential anticancer drugs due to their inhibitory effects on STAT3 signaling [25,34].

Andro was previously reported to possess powerful anti-inflammatory activity through the inhibition of NF-κB [15], an important transcription factor playing major roles in inflammatory pathways as well as tumorigenesis [35,36]. Interestingly, some inhibitors of NF-κB pathway, such as phytochemicals including curcumin, resveratrol, ursolic acid, capsaicin, and plumbagin, could also suppress the STAT3 pathway [37–40], suggesting an inherent link between these two pathways. But to date, there is no report of Andro on STAT3 signaling pathway.

In this study, we first confirmed the inhibitory effect of Andro on STAT3 activation by demonstrating: (i) Andro suppressed constitutive STAT3 tyrosine phosphorylation in time- and dose-dependent manners (Fig. 1) and (ii) Andro abrogated IL-6-induced phosphorylation and nuclear translocation of STAT3 in tumor cells (Fig. 2). STAT3 is typically maintained in the cytoplasm as an inactive monomer. In response to some inflammatory stimuli, such as ligation of IL-6 to its receptor, heterodimerization of the gp130 leading to JAK1/2 phosphorylation, which induces STAT3 phosphorylation and subsequent translocation to the nucleus, therefore initiating a transcriptional program [4,41]. As documented previously, the inhibition of the STAT3 phosphorylation is majorly achieved via the inhibition of upstream STAT3-activating receptor tyrosine kinases such as JAK1 and JAK2 [42,43]. In this study, the effect of Andro on STAT3 activity was found to be resulted from the suppression of JAK1 and JAK2 phosphorylation, two key tyrosine kinases known for STAT3 phosphorylation [25], and this inhibitory effect was comparable to that of pan-JAK inhibitor P6 (Fig. 3A). Furthermore, Andro disrupted the association between gp130 and STAT3 (Fig. 3B), most probably due to reduced JAK1/2 phosphorylation [1,27]. All these results clearly suggest that Andro's inhibitory effect on STAT3 activation is mainly executed via

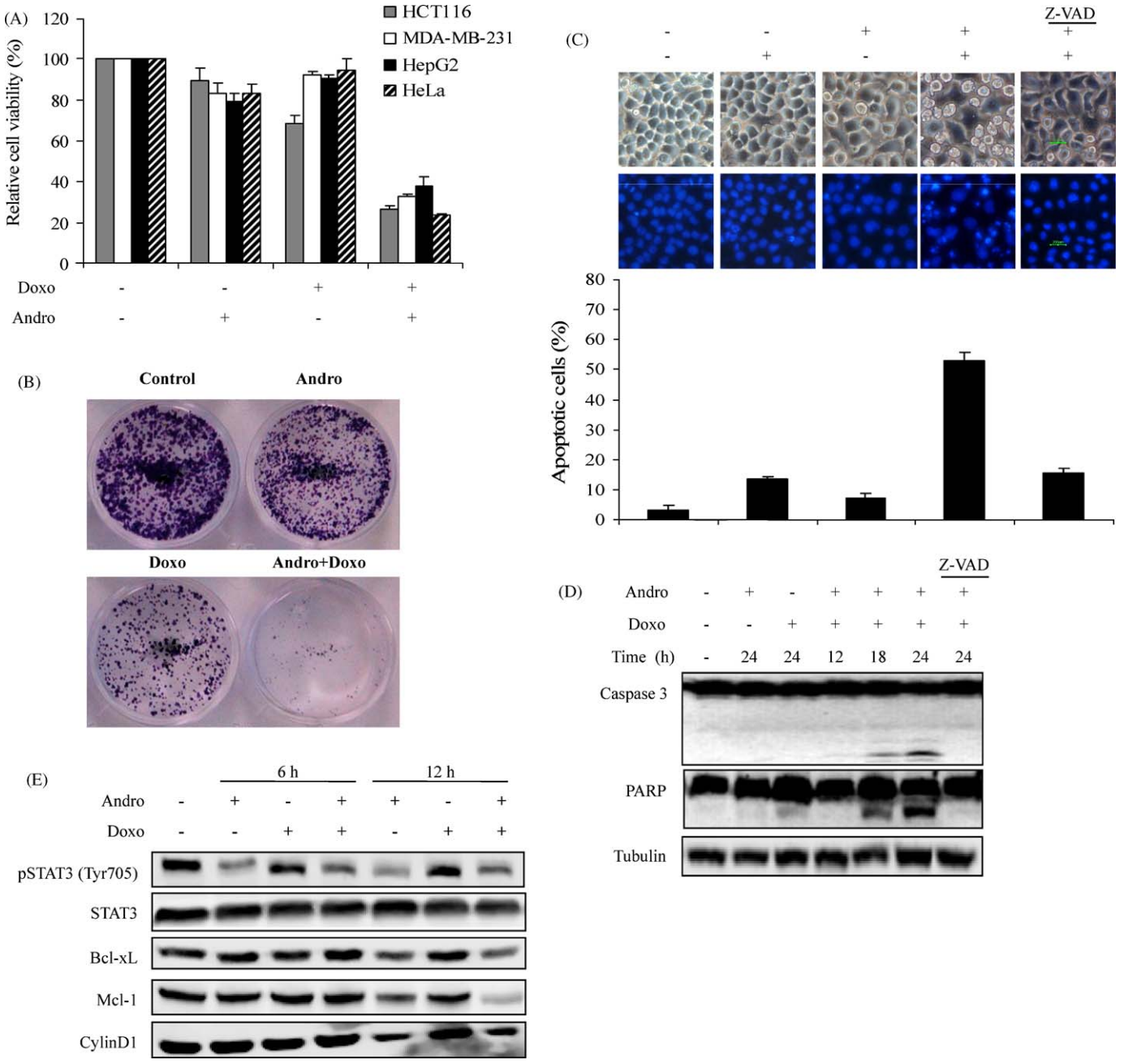

Fig. 6. Andro enhances doxorubicin-induced cytotoxicity in cancer cells. (A) HCT116, MDA-MB-231, HepG2 and HeLa cells were pretreated with Andro (15 μM) for 2 h, followed by treatment with a subtoxic concentration of doxorubicin (0.5 μM) for another 24 h. The cell viability was evaluated by MTT assay and expressed by relative cell viability compared with untreated control group, and data were presented as mean ± SD from three independent experiments. (B) Cells were pretreated with Andro (15 μM) for 2 h, followed by treatment with doxorubicin (0.5 μM) for another 12 h before reseeded in six-well plates (5000 cells/well), respectively. After 3 weeks, the survival colonies were stained by 0.5% crystal violet for 1 h and representative images of HCT116 cells were photographed using digital camera. (C) HCT116, MDA-MB-231, HepG2 and HeLa were pretreated with the indicated concentration of Andro for 2 h, followed by treatment with a subtoxic concentration of doxorubicin (0.5 μM) for another 24 h with or without the pretreatment of Z-VAD-FMK (25 μM). Representative images of HeLa cells with various treatments were photographed using a normal light microscope and an inverted fluorescence microscope (magnification, 200×). The percentage of apoptosis was determined using DAPI staining and data were presented as mean ± SD from three independent experiments (lower panel). (D) HeLa cells were treated as indicated in panel (C) for various periods. Cell lysates were collected and subjected to Western blot for detecting the cleavage of caspase-3 and PARP, and tubulin was used as a loading control. Data are representatives of three independent experiments. (E) MDA-MB-231 cells were treated with Andro (15 μM), doxorubicin (0.5 μM) or the combination of Andro and doxorubicin for indicated periods. Western blot was used to detect the expression levels of phospho-STAT3, Bcl-xL, Mcl-1 and cyclinD1. Data are representatives of three independent experiments.

inhibition of upstream STAT3-activating tyrosine kinase JAK1/2. Such effect of Andro is indeed similar to a number of other natural products that have been reported to inhibit JAK/STAT3 pathway, such as curcumin, ursolic acid, and capsaicin [39,40,44]. At present, the exact mechanisms by which JAK1/2 phosphorylation is abrogated by Andro are still elusive and remain to be further investigated.

Doxorubicin is one of the most commonly used anticancer drugs, but its application has been limited by the presence of cellular resistance [45]. Recent studies on human cancer have established STAT3 as a major regulator of cell proliferation and survival to protect the cells from various therapeutic agents including doxorubicin [34,46,47]. Numerous reports have shown convincing data that the inhibition of STAT3 could sensitize tumor

cells to doxorubicin-induced cell death [34,48–50], which prompted us to analyze the correlation between STAT3 activity and sensitivity of cancer cell lines to doxorubicin, and whether Andro could enhance the chemosensitivity of cancer cells to doxorubicin.

In this study, STAT3 was found to express differently in four cancer cell lines. Interestingly, higher basal activity of STAT3 was found to confer higher resistance to doxorubicin-induced cytotoxicity in HeLa cells, while HCT116 cells harbored with lower basal level of STAT3 were more sensitive to doxorubicin-induced cell death, this result was further confirmed by using a pair of thyroid cancer cell lines harboring different activation levels of STAT3 (Fig. 4). More interestingly, when activated STAT3 was blocked by ectopic expression of a dominant negative mutant form of STAT3 or RNA interference of STAT3, a significant decrease of cell viability and enhanced apoptotic cell death were detected in HeLa cells with doxorubicin treatment (Fig. 5). In this regard, constitutively activated STAT3 represents the major determinant of doxorubicin sensitivity in certain cancer cells. Therefore, here we postulate that Andro may enhance chemosensitivity of tumor cells to doxorubicin through inhibition of STAT3 activity. As expected, combination of Andro and doxorubicin apparently induced cell death and suppressed the cell proliferation in a number of cancer cell lines (Fig. 6A and B).

An important downstream effect of STAT3 activation is the STAT3-dependent regulation of several anti-apoptotic genes including Bcl-xL and Mcl-1 [6,7]. The anti-apoptotic Bcl-2 proteins play important role in preventing tumor cells from apoptosis induction. Treatment with Andro induced apoptosis (Fig. 6C and D) with corresponding decrease in STAT3 activation, accompanied by the reduction of Bcl-xL and Mcl-1, two transcriptional targets of STAT3 (Fig. 6E). The down-regulation of the expression of Bcl-xL and Mcl-1 is likely to provide a link between STAT3 suppression and ability of Andro to enhance apoptosis in tumor cells. Additionally, in view of the reported role of STAT3 in repressing p53 expression and function [51], it remains to be further tested whether inhibition of constitutive STAT3 activity by Andro could partially explain the p53 accumulation in Andro-treated cells, as shown in our previous report [17].

In conclusion, data from this study collectively suggest that Andro could be a promising anticancer agent via its potent inhibitory effect on JAK-STAT3 pathway. Inhibition of STAT3 activity by Andro enhanced chemosensitivity of tumor cells to doxorubicin, suggesting a potential therapeutic strategy using Andro in combination with conventional chemotherapeutic agents for treatment of cancer.

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