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Quinacrine Enhances Cisplatin-Induced Cytotoxicity in Four Cancer Cell Lines

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Key Words
Bax · Caspase-3 · cIAP-1 · Cisplatin · Cytotoxicity · Quinacrine

Abstract
Background: Quinacrine has potential as a chemosensitizer when combined with chemotherapy, but its anti-cancer mechanisms remain unclear. The purpose of this study was to explore the capability of quinacrine to enhance the cytotoxic effects of cisplatin and the underlying mechanism involved. ***Methods:*** The potential role of quinacrine in enhancing the effects of cisplatin was investigated in Hela, SCC-VII, SACC-83 and C6 cancer cell lines with different pathologies. The inhibitory effects of quinacrine plus cisplatin on these cell lines were detected using a CCK-8 assay for viability and a TUNEL assay for apoptosis. The molecules involved in apoptotic signal translation, including cIAP-1, Bax, p53 and cleaved caspase-3, were detected by Western blot to investigate the underlying mechanism. ***Results:*** The CCK8 assay showed that quinacrine markedly enhanced the cytotoxicity of cisplatin in a dose-dependant manner in the 4 cancer cell lines. The TUNEL assay showed that treating the 4 cell lines for 24 h with cisplatin plus quinacrine significantly increased the percentage of apoptotic cells compared to treatment with single-agent treatment or untreated controls. Western blot analysis showed that quinacrine plus cisplatin significantly down-regulated cIAP-1 and up-regulated Bax and cleaved caspase-3 expression in Hela and SCC-VII cells compared with single-agent treatment. ***Conclusions:*** Quinacrine has the potential to be used as a chemotherapy adjuvant when combined with cisplatin.

Introduction

Chemotherapy is one of main strategies for cancer treatment. Despite a modest improvement in survival with available chemotherapy treatments, most advanced cancers remain essentially incurable [1]. Cisplatin is one of the most widely used chemotherapeutic drugs [2], and cisplatin-based chemotherapy has been used in the treatment of most cases of advanced solid tumors such as head and neck squamous cell carcinoma, small-cell lung cancer, ovarian carcinoma, breast carcinoma and testicular cancer [2–4]. Cisplatin cytotoxicity and acquired cisplatin resistance, however, can limit its therapeutic potential [2, 5]. Therefore, identifying ways to improve the anticancer effects of cisplatin is a problem in clinic.

Y. Wang and Q. Bi contributed equally to this work.

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Quinacrine is an acridine derivative and is available as quinacrine dihydrochloride. It has multiple properties including antiproliferative and antimutagenic effects [6], antimicrobial properties [7], and inducing sclerosis [8–11]. It has been developed as an antimalarial and antibacterial agent [12], a sclerosis agent in treating malignant pleural effusions [13] and for sterilization in women [8, 9, 14]. The anticancer activity of quinacrine has also been intensively evaluated in many tumors. It has the ability to restore sensitivity to cisplatin in head and neck squamous cell carcinomas with wild-type *TP53* (encoding tumor protein p53) or to paclitaxe in prostate cancer cells [15, 16]. Based on evidence that quinacrine in combination with carmustine leads to a high rate of C6 tumor resolution in rats [17], Briceno et al. [18] treated glioblastoma multiforme patients with chloroquine, a compound analog of quinacrine, in addition to standard therapies and found that chronic administration of chloroquine greatly enhanced the response of glioblastoma multiforme to anti-neoplastic treatment. Survival was significantly improved in patients treated with chloroquine with standard treatment, including resection of lesions, radiotherapy and 4 cycles of chemotherapy.

The anticancer mechanisms of quinacrine, however, remain unclear. Only a few studies have shown that quinacrine can activate p53 and inhibit NF-kB, thus re-establishing cell suicide programs and decreasing cell survival in cancer tissues [15, 19]. In addition, quinacrine has been shown to have the ability to elicit an innate immune response, which could be involved in the elimination of experimental glioma in rat [20].

Here we report that quinacrine had a co-operative effect on cisplatin-induced cytotoxicity in 4 different cancer cell lines. We further investigated the underlying mechanisms from a different perspective beyond p53. We found that cIAP-1 (cellular inhibitor of apoptosis protein-1, a member of the inhibitors of apoptosis proteins) was involved in the underlying mechanism. In mammals the most well documented mechanism for influencing caspase activity is driven by direct interaction with members of the inhibitor of apoptosis proteins (IAPs) [21]. In this study, down-regulation of cIAP-1, up-regulation of Bax and cleaved caspase-3 contributed to the inhibitory effects of the combination of quinacrine and cisplatin.

Materials and Methods

Materials

Quinacrine dihydrochloride was purchased from Sigma (St. Louis, Mo., USA). Cisplatin was purchased from Qilu Pharmaceutical (Shandong, China). A cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Gaithersburg, Md., USA). Growth medium (DMEM and F12K), fetal bovine serum and horse serum were obtained from Invitrogen (Carlsbad, Calif., USA). Antibodies were obtained from the following vendors: cIAP-1 and cleaved caspase-3, Cell Signaling Technology (Beverly, Mass., USA); tumor protein p53, Beijing Biosynthesis Biotech (Beijing, China); Bax and β-actin, Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

Cell Culture

The murine head and neck squamous carcinoma cell line SCC-VII and the human cervical carcinoma cell line Hela were maintained in DMEM containing 10% fetal bovine serum. The rat glioma cell line C6 was maintained in F-12K medium containing 2.5% fetal bovine serum and 15% horse serum. The human salivary adenoid cystic carcinoma cell line SACC-83 was maintained in RPMI 1640 containing 10% fetal bovine serum in a humidified incubator with 5% CO_2. Cells in mid-logarithmic growth (~75% confluence) were used for the following experiments.

Assessment of Cell Growth by CCK8 Viability Assay

Cell viability was measured by conversion of Dojindo's highly water-soluble tetrazolium salt WST-8 to a yellow-colored water-soluble formazan (CCK8 assay). The amount of the formazan dye generated by the activity of mitochondrial dehydrogenases in cells is directly proportional to the number of living cells. This method was applied according to the manufacturer's instructions. Briefly, 1×10^4 cells were plated in 100 μl of growth medium in a 96-well plate. Following an overnight incubation, cells were treated with cisplatin and/or quinacrine at the indicated doses for an additional 24 h. To each well, 10 μl CCK-8-solution was then added, and the cultures were incubated for 3 h at 37°C. Color development was quantified photometrically at 450 nm using an ELx808 Absorbance Microplate Reader (Bio TeK Instruments, Winooski, Vt., USA). Viability is given as a percent of the control value. All of the experiments were performed in triplicate and repeated at least twice.

Detection of Apoptosis by TUNEL Assay

Tumor cells were plated onto 24-well glass chamber slides at 5×10^4 cells/well. Following an overnight incubation, cells were treated with cisplatin and/or quinacrine. Cisplatin was applied at 50 μM for Hela, SCC-VII and SACC-83 cells and at 20 μM for C6 cells. Meanwhile, quinacrine was used at 10 μM for Hela, SCC-VII and SACC-83 cells and 7.5 μM for C6 cells. After a 24-hour incubation, DNA strand breaks were detected by a terminal deoxynucleotidyl transferase (TdT)-mediated nick end-labeling (TUNEL) assay (Dingguo Biotechnology, Beijing, China), according to the manufacturer's instructions. Briefly, slides with tumor cells were washed twice with PBS, fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 5 min, and incubated with recombinant TdT and biotin-11-dUTP for 1 h at 37°C. The slides were then rinsed for 2 min in PBS and covered with alkaline phosphatase converter solution. After a 1-hour incubation, the slides were washed twice with PBS for 2 min, and BM purple substrate (BCIP/NBT) was added. The dark purple color was visible in 15–30 min. Slides were washed and counterstained with nuclear fast red. For each slide, 3–5 random fields at 400× magnification were analyzed. The number of TUNEL-pos-

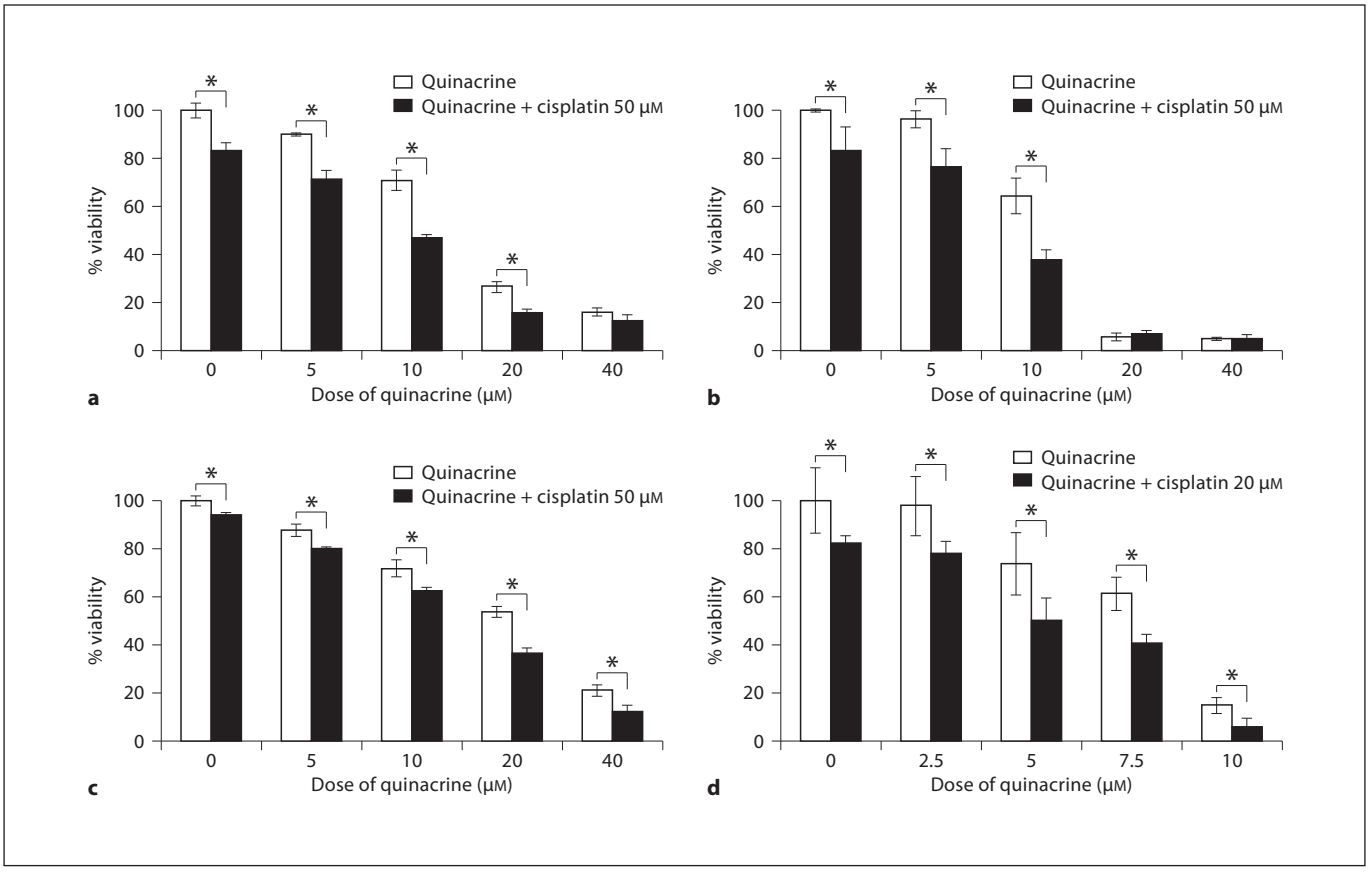


Fig. 1. The effects of a quinacrine and cisplatin combination on cell viability. Cells were treated with quinacrine, cisplatin or both in various combinations for 24 h. Viability was tested using a CCK8 assay. **a** Human cervical carcinoma cell line, Hela. **b** Mouse oral squamous cell carcinoma cell line SCC-VII. **c** Human salivary adenoid cystic carcinoma cell line, SACC-83. **d** Rat glioma cell line, C6. Compared with quinacrine alone and cisplatin alone, the combination of quinacrine and cisplatin at certain indicated doses led to significant growth inhibition in the 4 cancer cell lines. Data are mean ± SD obtained from triplicates of each experiment, and similar results were seen in at least 2 independent experiments. * $p < 0.05$ between the 2 treatments).

itive cells was divided by the total number of cells to determine the percentage of apoptosis. Data are presented as mean ± SD of 2 independent experiments. p values were calculated using a 2-tailed t test.

Analysis of the Molecules Involved in Signal Transduction by Western Blot

3×10^5 Hela or SCC-VII cells were plated in a 60-mm dish with 3 ml complete medium overnight. The following day, cells were treated with 50 μM cisplatin and/or 10 μM quinacrine for an additional 24 h. Cells were harvested and lysed in RIPA buffer with protease inhibitors. Protein concentration was determined using the BCA Protein Assay, and 30 μg of protein was loaded for each sample. Proteins were separated on an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membranes were blocked in 5% non-fat dry milk for 1 h and probed with antibodies against cIAP-1, cleaved caspase-3, Bax, p53 and β-actin separately at 4°C overnight. After incubation with peroxidase-linked secondary antibodies, immunoreactive proteins were visualized by ECL reagent (Applygen Technology, Beijing, China).

Statistical Analysis

Quantitative data were expressed as mean ± SD and compared using a Student's t test. $p < 0.05$ was considered statistically significant.

Results

Effects of Cisplatin and Quinacrine on Tumor Cell Viability

Using a CCK8 viability assay, we evaluated the growth inhibitory effects of cisplatin, quinacrine or a combination on the 4 cancer cell lines. First, we analyzed cispla-

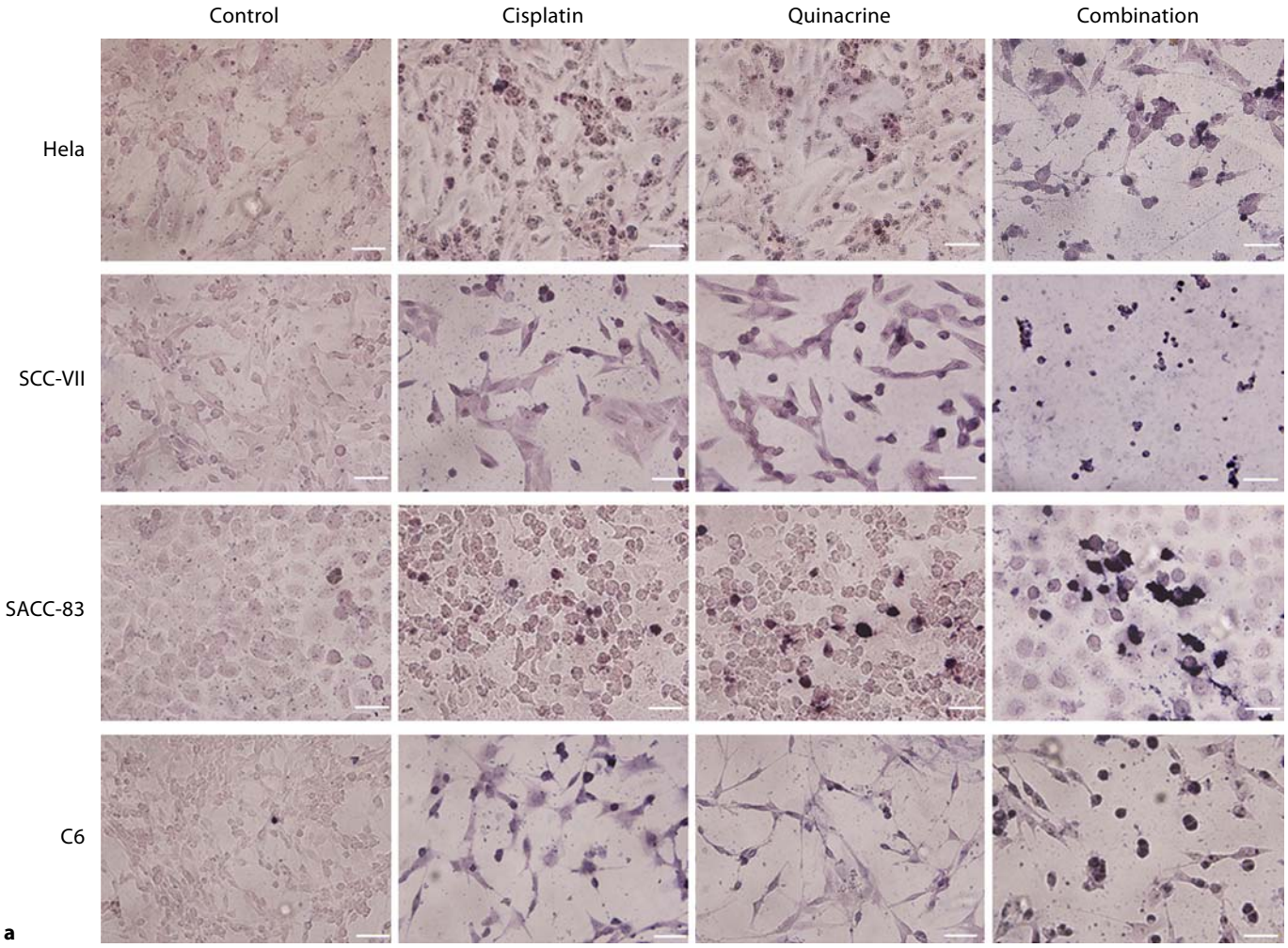
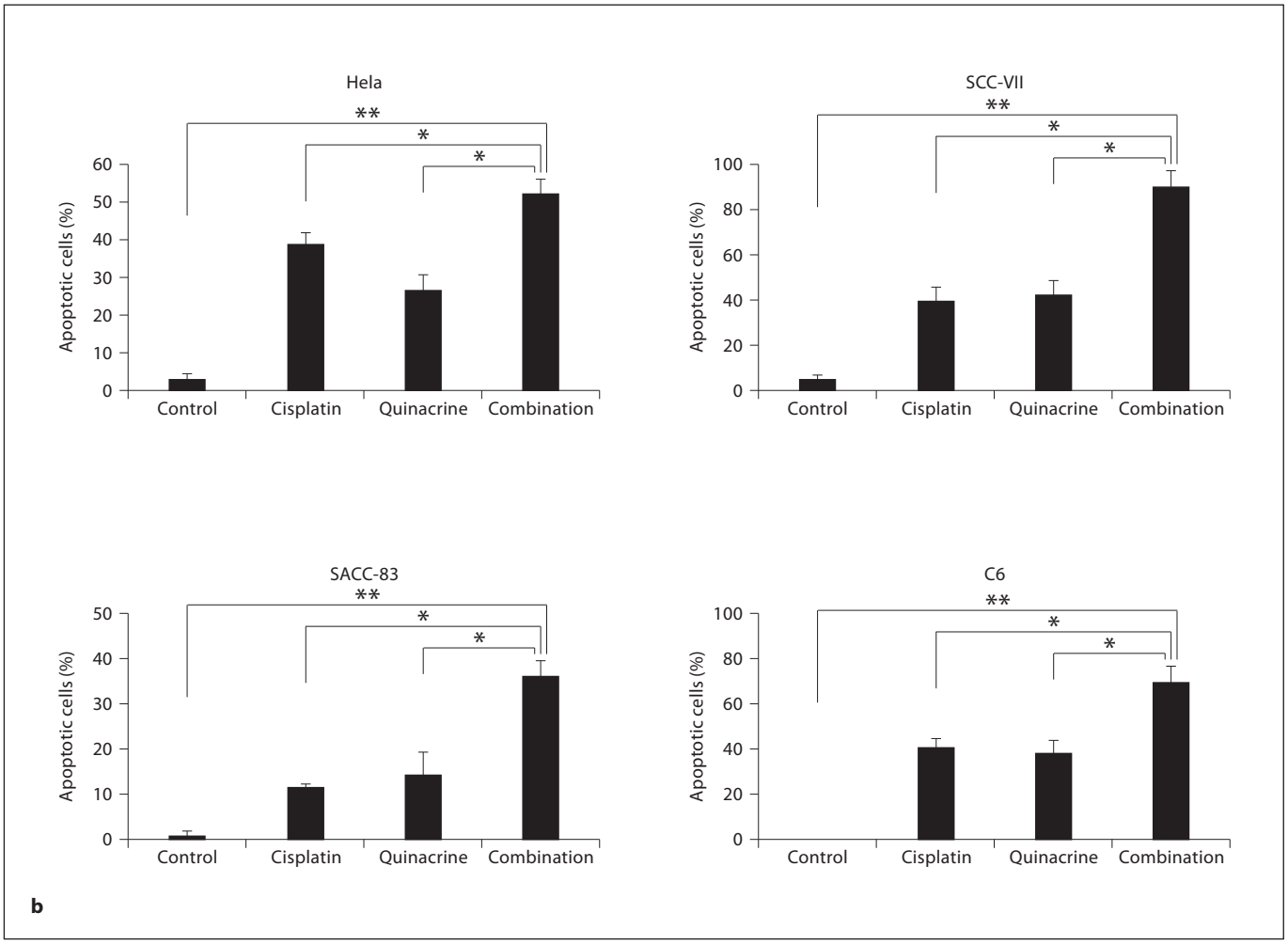


Fig. 2. Analysis of tumor cell apoptosis. Tumor cell apoptosis as determined by the TUNEL assay. The number of apoptotic tumor cells was counted in 3–5 random fields at 400× magnification. Treatment of the 4 cell lines for 24 h with cisplatin plus quinacrine significantly increased the percentage of apoptotic cells compared to cells treated with cisplatin alone, quinacrine alone or untreated controls. **a** Representative photomicrographs of apoptosis detected by the TUNEL assay for each treatment. Original magnification, 400×. Scale bar = 50 μm. **b** Percentage of apoptotic tumor cells. * $p < 0.05$, ** $p < 0.01$ between the 2 treatments.

tin alone with respect to its cytotoxic effects on these cell lines. Cisplatin alone inhibited the growth of all 4 of the cancer cell lines in a dose-dependent manner. Cisplatin at a concentration of more than 100 μM for Hela, SCC-VII and SACC-83 cells, and at a concentration of more than 50 μM for C6 cells, proved to be very toxic (data not shown). Therefore, cisplatin was used at a low concentration (50 μM for Hela, SCC-VII and SACC-83 cells and 20 μM for C6 cells) in further experiments in order to obtain a large window for clear observation of the effects. We then examined the cytotoxic effects of quinacrine combined with cisplatin. As shown in figure 1, quinacrine alone inhibited the growth of all 4 of the cancer cell lines in a dose-dependent manner. Co-incubation of quinacrine with cisplatin showed that quinacrine markedly enhanced cisplatin-induced cytotoxicity in the 4 cancer cell lines. Compared with quinacrine alone and cisplatin alone, the combination of quinacrine and cisplatin led to significant growth inhibition ($p < 0.05$ in certain comparisons for the 4 cancer cell lines, respectively; fig. 1).

Measurement of Apoptosis

The TUNEL assay results are shown in figure 2. Tumor cell apoptosis was relatively low in untreated con-



trols of the 4 cancer cell lines. Cisplatin alone, quinacrine alone and cisplatin plus quinacrine yielded respective percentages of apoptotic cells as follows: 39.05 ± 3.28%, 26.66 ± 4.28% and 52.49 ± 3.92% in Hela cells; 39.97 ± 6.02%, 42.40 ± 6.02% and 90.7 ± 6.71% in SCC-VII cells; 11.30 ± 0.82%, 14.39 ± 4.91% and 32.33 ± 3.61% in SACC-83 cells; and 40.73 ± 4.31%, 38.88 ± 5.02% and 69.65 ± 7.19% in C6 cells. Treatment of the 4 cell lines for 24 h with cisplatin plus quinacrine significantly increased the percentage of apoptotic cells compared to cells treated with cisplatin alone, quinacrine alone or untreated controls ($p < 0.05$ for the combination vs. cisplatin alone or quinacrine alone, $p < 0.01$ for the combination vs. untreated controls in the 4 cancer cell lines, respectively; fig. 2a, b).

Analysis of cIAP-1, Bax, p53 and Cleaved Caspase-3 Expression by Western Blot

To investigate the underlying mechanism involved in quinacrine-enhanced cisplatin-induced cytotoxicity, we chose Hela and SCC-VII as our models and analyzed the effects of treatment on the expression of proteins that play a critical role in cancer cell apoptosis. In Hela cells, cIAP-1 was slightly inhibited by either cisplatin alone or quinacrine alone and was clearly inhibited by the 2 agents in combination. Meanwhile, Bax was clearly induced by either cisplatin alone or quinacrine alone and was strongly induced by a combination treatment. p53 was markedly induced by either cisplatin alone or quinacrine alone, but this effect was not enhanced by a combination treatment. Another important apoptosis-executioner protein, caspase-3, was further investigated. Neither cisplatin alone nor quinacrine alone induced

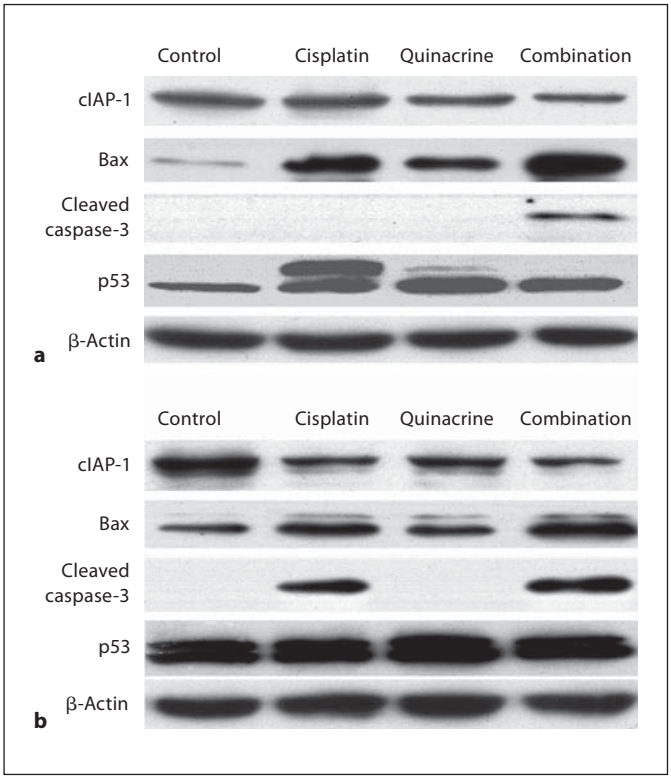


Fig. 3. Effect of quinacrine plus cisplatin on cIAP-1, Bax, p53 and cleaved caspase-3 expression in Hela cells (**a**) and SCC-VII cells (**b**). Hela and SCC-VII cells were treated with either 50 μM cisplatin, 10 μM quinacrine or both for 24 h, followed by cell lysate preparation and western blot analysis of cIAP-1, Bax, p53 and cleaved caspase-3. Quinacrine plus cisplatin significantly down-regulated cIAP-1 and unregulated Bax and cleaved caspase-3 expression in Hela and SCC-VII cells compared with single-agent treatments and untreated controls. Meanwhile, p53 was markedly induced by either cisplatin alone or quinacrine alone in Hela cells and was slightly induced by quinacrine alone in SCC-VII cells, but this effect was not reinforced by quinacrine plus cisplatin combination treatment in either Hela or SCC-VII cells. β-actin served as a loading control.

cleaved caspase-3 expression, whereas the combination markedly up-regulated the expression of cleaved caspase-3 (fig. 3a). In SCC-VII cells, cIAP-1 was clearly inhibited by either cisplatin alone or quinacrine alone and was strongly inhibited by the 2 agents in combination. Meanwhile, Bax was clearly induced by either cisplatin alone or quinacrine alone and was strongly induced by a combination treatment. p53 was slightly induced by quinacrine alone, but there was no significant difference between untreated controls, cisplatin alone and a combination treatment. Cleaved caspase-3 was not induced by quinacrine alone, while it was induced by cisplatin alone and markedly induced by a combination treatment (fig. 3b).

Discussion

In this study, we investigated the anti-carcinogenic potential of quinacrine in 4 cancer cell lines. The CCK8 and TUNEL assays revealed that quinacrine could not only have the ability to inhibit tumor cell growth and induce tumor cell apoptosis, but also be used as a chemotherapy adjuvant to sensitize different types of tumor cells to cisplatin. These results are consistent with previous studies showing that quinacrine could enhance the cytotoxicity of chemotherapeutic drugs in head and neck squamous cell carcinoma [15], glioblastoma multiforme and glioma [17]. This indicates that there may be a universal role for quinacrine as a chemotherapy adjuvant in tumor treatment.

Normally, apoptosis is one of main reasons contributing to tumor cell death in response to environmental and developmental signals. The key to understanding apoptosis is the activation and function of a set of proteases known as the caspases [22–24]. The functions of caspases are modulated by other sets of proteins, including the IAPs, bcl-2 family proteins, tumor protein p53, and so on [23–25]. Here we selected cIAP-1 (one of the IAPs), Bax (one of the Bcl-2 family proteins), p53 (an important tumor suppressor), and the apoptosis executioner caspase-3 (one of the caspases) for our analysis. cIAP-1 is an apoptosis inhibitory protein known to directly inhibit the activity of caspase-3, -7 and -9. Down-regulation of cIAP-1 results in tumor cell apoptosis [19]. p53 is important in multicellular organisms where it regulates the cell cycle and thus functions as a tumor suppressor involved in preventing cancer. Up-regulation of wild-type p53 leads to tumor cell apoptosis [26]. Bax is a pro-apoptotic protein that resides in the cytosol and translocates to mitochondria upon induction of apoptosis [27]. In the cytoplasm, cytochrome *c* forms a complex with an apoptotic protease, which finally activates executioner caspases 3, 6 and 7. The executioner caspases can cleave DNA and proteins and lead to cell death [28].

In the present study, we chose Hela and SCC-VII cells to explore the underlying mechanism involved in quinacrine and cisplatin combination therapy. In these 2 samples, we found that the combination of quinacrine and cisplatin clearly down-regulated cIAP-1 expression and markedly up-regulated the pro-apoptotic protein Bax

and the executioner protein-cleaved caspase-3. In the SCC-VII samples, down-regulation of cIAP-1 and up-regulation of Bax were induced by cisplatin, which led to increases in the amounts of active caspase-3. Quinacrine alone also down-regulated cIAP-1 and up-regulated Bax, but did not affect active caspase-3 expression. Furthermore, it slightly enhanced cisplatin-induced cytotoxicity in the combination group. This finding was also evident in Hela samples. We found that quinacrine plus cisplatin markedly enhanced the down-regulation of cIAP-1 and up-regulation of Bax compared with cisplatin alone in Hela cells. Meanwhile, the combination group showed stronger cytotoxicity, manifested by the activation of caspase-3, than cisplatin alone, which was confirmed by the CCK8 and TUNEL assays. Taken together, the Western blot results suggest that the tumor cells in the combination group were more susceptible to apoptosis than those in the cisplatin alone group. In addition, p53 was clearly (or slightly) induced by quinacrine alone in Hela (or SCC-VII) cells. This result is consistent with previous studies [29, 30], but the effect was not reinforced by quinacrine plus cisplatin combination treatment in both Hela and SCC-VII cells. This indicates that p53 did not contribute to the enhanced cytotoxicity of quinacrine plus cisplatin in Hela and SCC-VII cells.

In conclusion, quinacrine has the potential to enhance cisplatin-induced cytotoxicity in 4 cancer cell lines. cIAP-1, Bax and caspase-3 were involved in the underlying mechanism, while down-regulation of cIAP-1 and up-regulation of Bax and cleaved caspase-3 both contributed to this effect. This preliminary study provides evidence for the use of quinacrine as a chemotherapy adjuvant and suggests possible molecular mechanisms that may help explain the combination effect of quinacrine and cisplatin, perhaps providing an improved strategy in the treatment of certain cancers with cisplatin. The underlying mechanisms still remain largely unknown, however, and further studies are necessary.

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