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Carboxyamido-triazole inhibits proliferation of human breast cancer cells via G_2/M cell cycle arrest and apoptosis

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Abstract

Carboxyamido-triazole (CAI), a voltage-independent calcium channel inhibitor, has been shown to be able to induce growth inhibition and apoptosis in cancer cells. In the present study, we demonstrate that CAI significantly inhibits proliferation of cultured MCF-7 human breast cancer cells in a dose-dependent manner with an IC_{50} of ~ 26 μM. Reduced proliferation of MCF-7 cells in the presence of CAI correlated with accumulation of cells in G_2/M phase and induction of apoptosis. A treatment of MCF-7 cells with 30 μM CAI caused a time-dependent decrease in the levels of proteins that regulate G_2/M progression, including Cdk1, Cyclin B1, and Cdc25C. A simultaneous increase in the expression of p21 protein was observed. We also demonstrated a concurrent decrease of the mitochondrial membrane potential ($\Delta\Psi_m$), and down-regulation of anti-apoptotic protein Bcl-2. In conclusion, it seems reasonable to hypothesize that the antitumor effect of CAI in MCF-7 cells is based on G_2/M cell cycle arrest and inducing apoptosis.

Keywords: Carboxyamido-triazole; Human breast cancer; Cell cycle; Apoptosis; Mitochondrial membrane potential

1. Introduction

Carboxyamido-triazole (CAI) is an inhibitor of transmembrane calcium influx and intracellular calcium-requiring signal transduction pathway (Felder et al., 1991; Kohn and Liotta, 1990; Kohn et al., 1992). CAI inhibits the proliferation and invasive characteristics of several tumor cell lines in vitro, including human breast cancer cells (Enfissi et al., 2004; Jacobs et al., 1997; Lambert et al., 1997; Moody et al., 2003; Perabo et al., 2004; Wasilenko et al., 1996, 2001). CAI also demonstrates antiangiogenic activity in the chick chorioallantotic membrane assay, as well as the activity of inhibiting the proliferation of human umbilical vein endothelial cells in vitro (Kohn et al., 1995). The antiproliferative activity of CAI has been proposed to be cytostatic and correlate with calcium-mediated signal transduction pathways (Kohn et al., 1992).

CAI exposure has been shown to hold Madin–Darby bovine kidney cells at the G_1/S restriction point in the cell cycle and no increase in the number of dead cells was found, in a manner consistent with a cytostatic agent rather than a cytotoxic agent (Hupe et al., 1990). However, recent studies have indicated that CAI can inhibit proliferation of cultured cancer cells by inducing apoptosis. Ge et al. (2000) found that CAI induced apoptosis in bovine aortic endothelial cells and human glioma cells in a dose- and time-dependent manner. In another study, Perabo et al. (2004) demonstrated that CAI induced growth inhibition and apoptosis in bladder cancer cells by modulation of Bcl-2, rather than by specific activation of the Fas system. A more recent study has suggested that the combination of CAI and a cox-2 inhibitor LM-1685 may produce cell cycle arrest and apoptosis in cancer cells by creating a combined blockade of mitogenic and antiapoptotic signaling (Winters et al., 2005).

Although the antiproliferative, antiangiogenic, anti-invasive and apoptosis inductive effects of CAI have been well-documented, the cell cycle regulating activity of CAI itself in cancer cells has not been systematically assessed. The cell cycle in eukaryotes is regulated through a precise balance of positive

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and negative regulatory components that exert their effects during distinct stages of the cell cycle. The cyclins, members of the positive acting components, bind to and activate cyclin-dependent kinases (CDKs) (Hunter, 1993; Lukas et al., 2004; Sherr, 1996). In addition, some CDK inhibitors including $p21^{Waf1}$ have been identified. These CDK inhibitors play a key role in controlling cell cycle progression by negatively regulating the CDK activities at appropriate time in the cell cycle (Brugarolas et al., 1995; Elledge and Harper, 1994; Peter and Herskowitz, 1994). As we know, cell-cycle dysregulation is a hallmark of tumor cells and CDKs and CDK inhibitors in these cells are usually sensible targets of chemotherapeutic agents (Senderowicz, 2003). In addition to growth inhibition, apoptosis is also an important determinant of the response of tumors to chemotherapeutic agents (Ghobrial et al., 2005). Accumulating evidences have indicated that a reduction in mitochondrial transmembrane potential ($\Delta\Psi_m$) accompanies early apoptosis in many situations and Bcl-2 family members appear to regulate the commitment to survive or die by controlling the integrity of mitochondrial membrane (Cory and Adams, 2002; Kroemer, 1997; Kroemer et al., 1997; Petit et al., 1997; Yang et al., 1997;).

In the present study, we evaluate the antiproliferative activity of CAI against MCF-7 human breast cancer cells. Our studies indicate that the suppression of MCF-7 cells growth by CAI is associated with cell cycle arrest in G_2/M phase correlated with the down-regulation of Cdk1, cyclin B1 and Cdc25C protein expression and the up-regulation of $p21^{Waf1}$ protein expression. We also provide experimental evidence to indicate that a decrease in Bcl-2 protein level and a reduction in $\Delta\Psi_m$ are involved in CAI-induced apoptosis in MCF-7 cells.

2. Materials and methods

2.1. Materials

CAI was synthesized by the Institute of Materia Medica, Chinese Academy of Medical Sciences. Based on the data of elemental analysis, IR spectroscopy, UV–VIS spectroscopy, electrospray mass-spectrometry, thermogravimetry, differential scanning calorimetry, ^{1}H NMR spectroscopy, and solid-state ^{13}C CP MAS NMR spectroscopy, we have confirmed that the product we used is the same compound as the carboxyamido-triazole synthesized by the National Cancer Institute, Bethesda, MD. It was dissolved in dimethylsulfoxide (DMSO) and diluted with the fresh medium to achieve the desired concentrations. Cell culture medium and fetal bovine serum were procured from Gibco (Grand Island, NY). Trypan blue, DMSO, propidium iodide, RNase A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), aprotonin and leupeptin were purchased from Sigma Chemical Co. (St. Louis, MO). Rhodamine123 was from Molecular Probes Inc (Eugene, OR). PMSF was obtained from Amresco (USA). ApoAlert Annexin V/FITC kit was from Clontech (Palo Alto, CA). The antibodies against Cdk1, cyclin B1, p21, Bcl-2, Bax, actin, and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); antibody against Cdc25C was from Cell Signaling Technology (Beverly, MA).

2.2. Cell culture

MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA) and grew in DMEM supplemented with 10% FBS, 50 mg/ml penicillin, 100 mg/ml streptomycin, and L-glutamine at 37 °C in a humidified 95% O_2–5% CO_2 atmosphere.

2.3. Cell survival/proliferation assays

The effect of CAI treatment on survival/proliferation of MCF-7 cells was determined by trypan blue dye exclusion assay or MTT assay. The IC_{50} value was determined from a plot of percentage of survival versus CAI concentrations. For trypan blue dye exclusion assay, 5×10^4 cells were plated in 12-well plates, and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of CAI or control (0.5% DMSO), and the plates were incubated for 24 h and 48 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Both floating and adherent cells were collected and pelleted by centrifugation at 800 rpm for 5 min. The cells were resuspended in 2 ml phosphate buffered saline (PBS), mixed with 2 ml of 0.4% trypan blue solution, and counted using a hemocytometer. MTT assay was performed as described elsewhere. Briefly, cells were seeded at a density of 1×10^4 cells per well in flat-bottomed 96-well microplates. Various concentrations of CAI were added to each well in quadruplicate. After having been treated for the indicated times, cells were incubated with MTT (0.5 mg/ml) for 4 h. The formazan crystals were then solubilized with 200 μl DMSO in the dark. The absorbance at 490 nm was measured by a Benchmark microplate reader (Bio-Rad, CA).

2.4. Assessment of DNA synthesis

Breast cancer cells were plated at 20,000 cells/well in 24-well Corning tissue culture plates. Triplicate samples of cells were treated for 24 h with either vehicle control (0.5% DMSO) or varying concentrations of CAI. After 24-h culture, 0.5 μCi [^{3}H]-thymidine was added, and cultures were continued for 6 h to permit labeling of DNA as an index of cell proliferation. MCF-7 cells were treated with 10% TCA and recovered in 1 N NaOH. Then samples were collected on glass fiber filters and washed to remove soluble radioactivity with a Skatron (Flow Laboratories, Rockville, MD) cell harvester. Incorporation of [^{3}H]-thymidine was determined by liquid scintillation counting. Triplicates were averaged and expressed as counts/min/well.

2.5. Cell cycle analysis

The effect of CAI treatment on cell cycle distribution was determined by flow cytometric analysis of DNA content of nuclei of cells following staining with propidium iodide. MCF-

7 cells (10^6) were seeded in T75 flasks, and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentrations of CAI or control (0.5% DMSO). After incubation for indicated time intervals at 37 °C, cells were washed with PBS and fixed in 70% ethanol for at least 12 h. The cells were then treated with 100 mg/ml RNase A and 50 mg/ml propidium iodide for 30 min. Nuclei (10,000) emitted fluorescence was measured with a Coulter Elite flow cytometer equipped with an argonion laser emitting a 488-nm beam at 15 mW. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined using a MultiCycle program (Phoenix Flow Systems, San Diego, CA).

2.6. Determination of apoptosis

Apoptosis induction in control (DMSO-treated) or CAI-treated MCF-7 cells was assessed by (i) flow cytometry analysis of sub-G_0–G_1 DNA content and (ii) quantification of phosphatidylserine exposure using Annexin V/FITC kit according to the manufacturer's instructions. For analysis of sub-G_0–G_1 DNA content, MCF-7 cells (10^6) were treated as described above. After having been stained with propidium iodide, cells were analyzed on a Coulter Elite flow cytometer. Apoptosis was evaluated by the proportion of cells harboring less than 1 N DNA in the nucleus, and the apoptotic cells appeared as the sub-G_0–G_1 peak on the DNA histogram (10,000 events were counted). For Annexin V assay, cells were exposed to 30 μM CAI or DMSO for different time intervals, and the floating and adherent cells were collected. Pooled cells were washed with the manufacturer supplied binding buffer. Approximately 5×10^5 cells were suspended in 200 μl binding buffer, and then the suspension was mixed with 5 μl Annexin V/FITC and 10 μl propidium iodide. After 15 min of incubation in the dark, cells were analyzed using a Coulter Epics XL flow cytometer.

2.7. Analysis of mitochondrial membrane potential

Alterations in the $\Delta\Psi_m$ were analyzed by flow cytometry using the mitochondrial membrane potential-sensitive dye rhodamine123 (Johnson et al., 1981). After incubation, cells were harvested, washed twice with PBS, and resuspended in prewarmed Hanks' solution with 0.5 μM rhodamine123. After 20 min of incubation at 37 °C, cell samples were analyzed on a Coulter Elite flow cytometer, which was equipped with an argonion laser emitting a 488-nm beam at 15 mW. Green fluorescence was gated in a scattergram of log SS-log FS in order to include the subpopulation with the highest frequency and homogeneity in the fluorescence measurements. The data were analyzed with the Multigraph software (Phoenix Flow Systems).

2.8. Western blotting

Cells were exposed to 30 μM CAI for different time intervals as indicated. The cells were washed twice with ice-cold PBS, and then lysed on ice with a solution containing 50 mM Tris, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 20 mM β-glycerol phosphate, 50 mM NaF, 1 mM dithiothreitol, 1% Nonidet P-40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 1 mM phenyl methyl sulfonyl fluoride. The cell lysate was cleared by centrifugation at 14,000×*g* for 15 min. Protein content in 14,000×*g* supernatant fraction was determined by the method of Bradford (1976). Lysate containing 40 μg protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred onto polyvinylidene fluoride membrane. After having been blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween-20, the membrane was incubated with the desired primary antibody for 2 h at the following dilutions: Cdk1 (1:200 dilution), cyclin B1 (1:200 dilution), Cdc25C (1:1000 dilution), p21 (1:200 dilution), Bcl-2 (1:200 dilution), Bax (1:200 dilution), and actin (1:400 dilution). Subsequently, the membrane was incubated with appropriate secondary antibody, and the immunoreactive protein bands were visualized using enhanced chemiluminescence kit (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions. All proteins were detected by ECL-based autoradiography as recommended by the manufacturer (Amersham Pharmacia Biotech). Western blots are representative of at least two independent experiments.

2.9. Statistics

All data were reported as mean±S.D. except where indicated. Comparisons among multiple groups were subjected to a one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference post-hoc test. The 95% confidence limits ($P<0.05$) were considered significant.

3. Results

3.1. Effects of CAI on cell proliferation and DNA synthesis

The effect of CAI treatment on proliferation of MCF-7 cells was assessed by trypan blue dye exclusion assay, and the results are shown in Fig. 1A. The treatment of MCF-7 cells for 24–48 h with 5–40 μM of CAI resulted in a dose-dependent decrease in cell survival with an IC_{50} of ~26± 7 μM (Fig. 1A). The antiproliferative activity of CAI was further confirmed by MTT assay, and the results are shown in Fig. 1B. Proliferation of the cells was inhibited significantly upon treatment with CAI in a concentration- and time-dependent manner. The cell density OD490 of MCF-7 cells was significantly reduced at ≥ 10 μM CAI (Fig. 1B). To our surprise, we observed unequal antiproliferative abilities of CAI measured by trypan blue exclusion assay and MTT assay. For example, the viability of MCF-7 cells was reduced by 52.7±1.7%, 59.2±3.1%, or 81.8±3.0% ($n=12$), respectively, upon a 48-h exposure to 20, 30, or 40 μM CAI analyzed with trypan blue exclusion method (Fig. 1A), whereas only 25.8±0.6%, 47.1±2.36%, or 54.3±1.68% ($n=12$) of MCF-7 cells was inhibited under similar conditions of CAI treatment using MTT assay (Fig. 1B). Analysis of

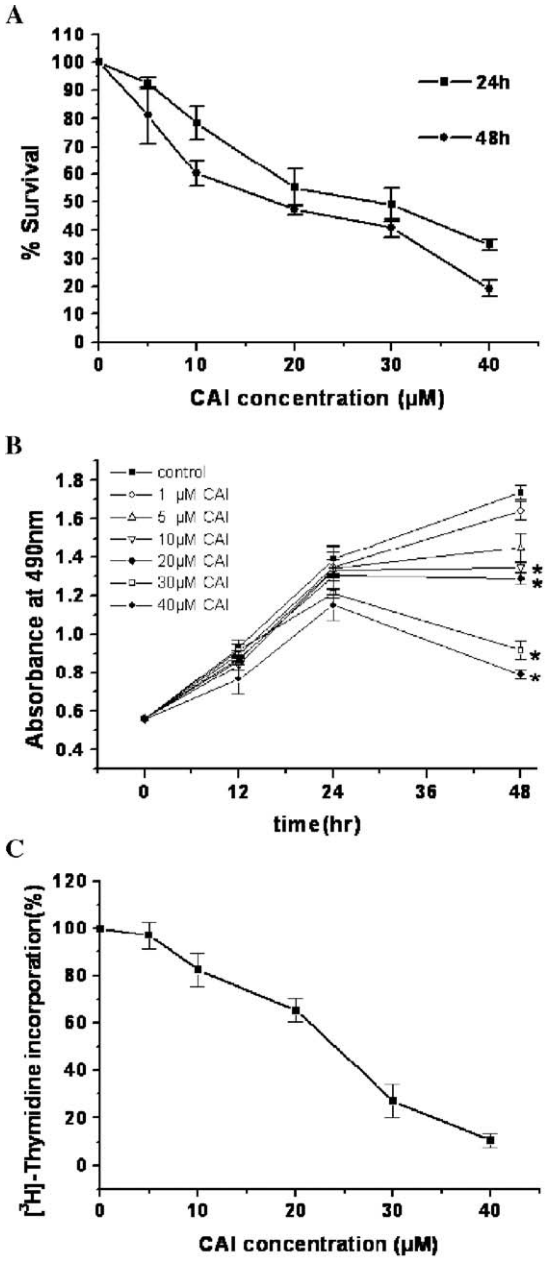

Fig. 1. (A) Effect of CAI treatment on proliferation of MCF-7 cells determined by trypan blue dye exclusion assay. MCF-7 cells were treated with 0–40 μM CAI, after specified time intervals, cells were harvested by trypsinization, and counted. The number of living cells was plotted versus concentrations of CAI. (B) Growth curves of MCF-7 cells exposed to various concentrations of CAI. MCF-7 cells were treated with DMSO (control) or CAI for indicated periods, MTT dye reduction method was used to assess the viability, represented by absorbance at 490 nm. The plot is an average of quadruplicate points for each treatment and representative of three independent experiments; bars, S.D. *$P<0.05$ ($n=12$). (C) Effect of CAI on DNA synthesis in MCF-7 breast cancer cells. The reported values are an average of triplicate samples; bars, S.D.

[^{3}H]-thymidine incorporation revealed that CAI caused a dose-dependent decrease in DNA synthesis over 24 h in MCF-7 cells. CAI in concentrations of 10, 20, and 30 μM decreased [^{3}H]-thymidine incorporation into MCF-7 cells by 18.8±7.1%, 34.6±4.8%, and 73.0±6.9% ($n=6$), respectively (Fig. 1C).

3.2. Cell cycle distribution and expression of Cdk1, cyclin B1 and Cdc25C, as well as cell cycle inhibitory proteins p21^{Waf1}

To gain insights into the mechanism of antiproliferative activity of CAI, its effect on cell cycle distribution was analyzed and the result of a typical experiment is shown in Fig. 2A. As determined by flow cytometry, exposure of MCF-7 cells to 10 or 30 μM CAI for increasing time intervals produced a concentration- and time-dependent G_2/M phase cell cycle arrest, which resulted in a clear increase of the percentage of cells in the G_2/M phase compared with control cells. For example, after treatment at the presence or absence of CAI for 24 h, the cell proportion in G_2/M phase was either 1.7- or 2.7-folds higher, respectively, in MCF-7 cultures exposed to 10 or 30 μM CAI compared with control that was accompanied by a 13.4% or 20.7% ($n=3$) decrease in G_0/G_1 phase cells, respectively (Fig. 2A, middle).

The progression through the various phases of the cell cycle is promoted by cyclins and Cdks and inhibited by Cdk inhibitors such as p21^{Waf1} and p27^{Kip1} (Elledge and Harper, 1994; Hunter, 1993; Lukas et al., 2004; Peter and Herskowitz, 1994). To elucidate the mechanism for G_2/M arrest in CAI-treated cells, we assessed its effect on expression of proteins that are pivotal for G_2/M transition, including Cdk1, cyclin B1, Cdc25C and p21 (Stein et al., 1998; Stewart et al., 2003). Representative Western blots for the time course response effect of 30 μM CAI treatment on those G_2/M regulating proteins expression in MCF-7 cells are depicted in Fig. 2B. When cells were treated with 30 μM CAI, a marked time-dependent decrease of Cdk1, cyclin B1 and Cdc25C expression was observed. Conversely, the expression of p21^{Waf1} increased in a time-dependent manner. Thus, the changes of time-dependent expressions of the cyclins, CDKs, and the Cdk inhibitor corresponded with the effects on cell cycle progression. These data provided strong evidence for cell cycle arrest induced by CAI and, in turn, the inhibition of cell growth. However, the reduced cell growth could also be related to the apoptotic cell death. Hence, we investigated whether CAI could induce apoptosis in MCF-7 cells.

3.3. Induction of apoptosis by CAI in MCF-7 cells

The apoptosis-inducing effect of CAI was investigated by: (i) flow cytometric analysis of cells with sub-diploid DNA content (Fig. 3A); and (ii) quantification of phosphatidylserine exposure with Annexin V/FITC and propidium iodide double staining kit (Fig. 3B). A concentration- and time-dependent apoptosis-inducing effect of CAI is shown in Fig. 3A. Treatment of MCF-7 cells with 10 or 30 μM CAI for indicated time intervals remarkably increased the percentage of cells with sub-diploid DNA content, which is the apoptotic fraction. Proportion of the sub-G_1 peak was negligible in control cells over indicated time intervals; however, after incubation with 10 or 30 μM CAI, 7.45% or 18.8% ($n=3$) of the cells underwent apoptosis, respectively, at 24 h and the percentage of apoptotic cells increased to 15.7% or 30.3% ($n=3$), respectively, at 48 h (Fig. 3A, medium row, bottom row). Annexin V is a calcium-

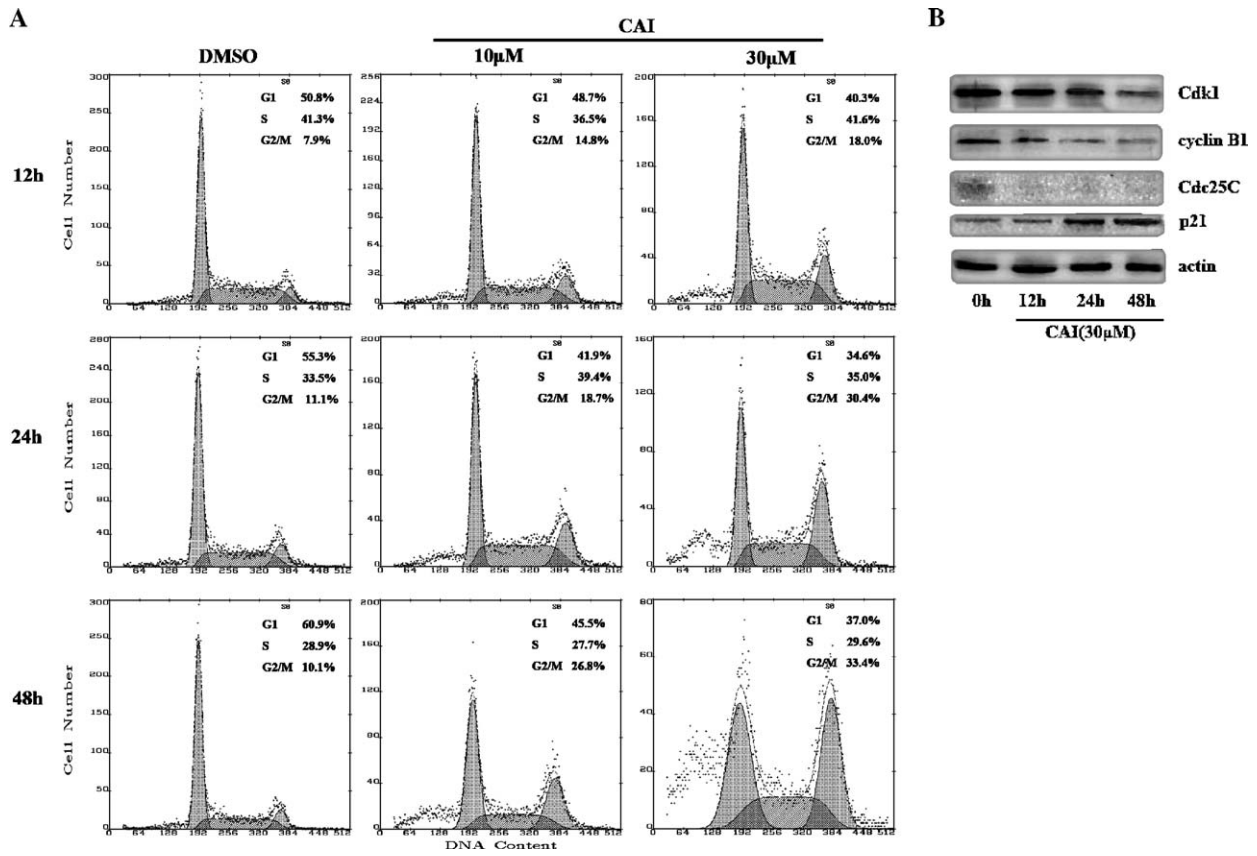

Fig. 2. (A) Effect of CAI on DNA content of MCF-7 cells. MCF-7 cells were treated with DMSO (control) or desired concentrations of CAI (10 and 30 μM) for indicated time intervals, respectively. Cells were then stained with propidium iodide, and nuclei were analyzed for DNA content by flow cytometry with a Coulter Elite Laser. A total of 10,000 nuclei were analyzed from each sample, and the percentages of cells within G_1, S, and G_2/M were determined as described in Materials and methods. Representative profiles are shown for each condition, and the numbers in the upper right corner of the profiles are an average of triplicate samples. (B) Effect of CAI on the expression of cell cycle-related proteins in MCF-7 cells. After incubation with vehicle (DMSO) or 30 μM CAI for the indicated times, cells were lysed and equal amounts of total proteins were resolved on SDS-polyacrylamide gels. Western blot analysis was performed using specific antibodies against Cdk1, cyclin B1, Cdc25C, and p21. Blots were re-probed with anti-actin antibody to normalize each lane for protein content.

dependent phospholipid-binding protein with a high affinity for phosphatidylserine, which is a negatively charged membrane phospholipid located on the inner (cytoplasmic) surface of the plasma membrane of living cells. However, phosphatidylserine is translocated to the outer side of the plasma membrane in cells undergoing apoptosis (Martin et al., 1995). Simultaneous staining with Annexin V/FITC and propidium iodide can distinguish among intact cells, early apoptotic cells, late apoptotic cells and necrotic cells (Engeland et al., 1998). As shown in Fig. 3B, CAI treatment increased the percentage of apoptotic cells in a time-dependent manner in MCF-7 cells. In comparison with DMSO-treated control cells where roughly 5.22% cells were positive for Annexin V, 30 μM CAI treatment increased the percentage of apoptotic cells to 13.6%, 20.37% or 38.32% (n=3), respectively, at 12h, 24 h or 48 h. These results indicate that induction of apoptosis or necrosis may be another antiproliferative mechanism of CAI in MCF-7 cells.

3.4. Effect of CAI on Bcl-2 family members and mitochondrial membrane potential analysis

The protein expression levels of Bcl-2 and Bax in MCF-7 cells treated with 30 μM CAI for 12–48 h was analyzed by Western blot. No change in Bax protein after CAI treatment was detected; however, expression level of Bcl-2 protein decreased considerably upon CAI treatment, resulting in a greater ratio of Bax over Bcl-2 in CAI-treated cells than in DMSO-treated cells (Fig. 4A). It has been suggested that a high ratio of proapoptotic proteins (e.g., Bax) to antiapoptotic proteins (e.g., Bcl-2 and Bcl-XL) can cause the collapse of $\Delta\Psi_m$ (Cory and Adams, 2002; Yang et al., 1997). To determine whether the apoptosis-inducing effect of CAI is related to a reduction of $\Delta\Psi_m$, mitochondrial depolarization was monitored by the fluorescence of rhodamine123, a potential-sensitive lipophilic cationic dye, with a flow cytometer. Cells were treated with 10 or 30 μM CAI; after 24 h, the entire cell population exhibited a shift to weaker fluorescence (Fig. 4B). The $\Delta\Psi_m$ was reduced by ~32.7% or ~59% upon a 24-h exposure to 10 or 30 μM CAI (Fig. 4C), suggesting that apoptosis induced by CAI is tightly related to or depended on the loss of $\Delta\Psi_m$.

4. Discussion

CAI, an inhibitor of transmembrane calcium influx and intracellular calcium-requiring signal transduction pathway, was shown to have antiproliferative, antiangiogenic, anti-

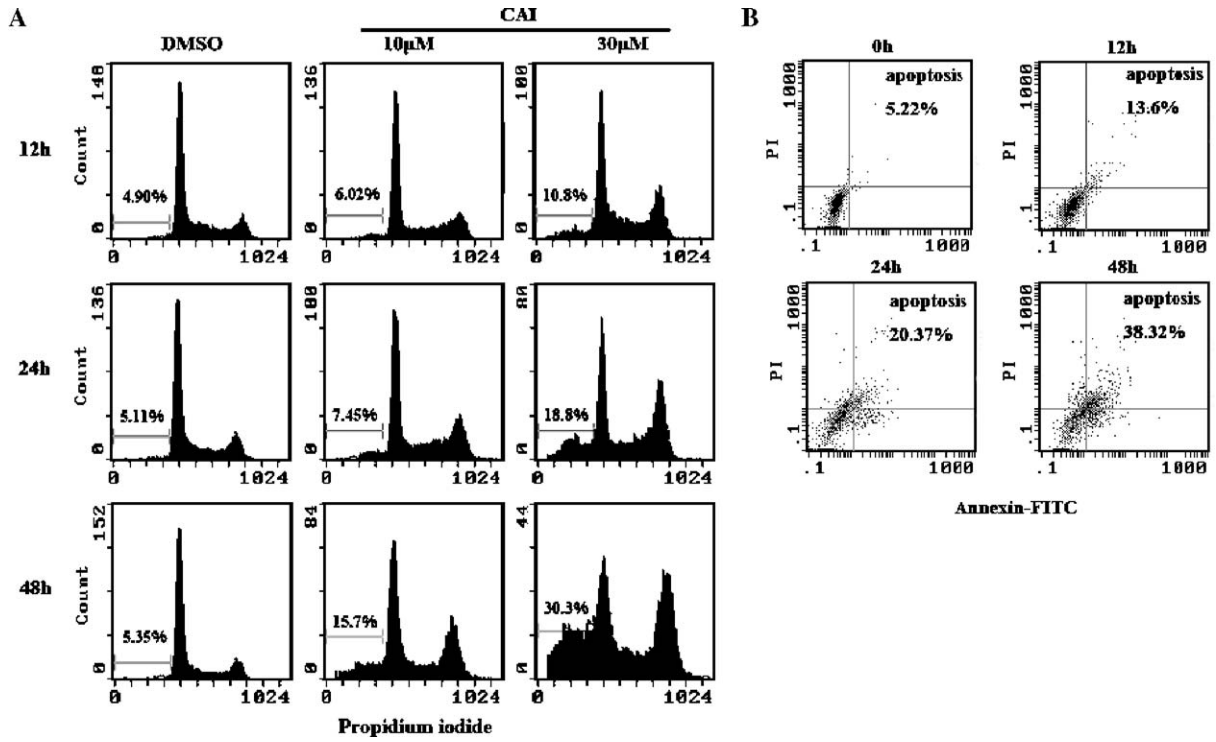

Fig. 3. Effect of CAI treatment on apoptosis induction assessed by flow cytometric analysis. (A) MCF-7 cells were treated with DMSO (control) or desired concentrations of CAI (10 and 30 μM) for indicated time intervals, respectively. Cells were then stained with propidium iodide, followed by detection of apoptotic cells by flow cytometry with a Coulter Elite Laser. The percentage of cells of the sub-diploid population is indicated in every diagram. Results shown are representative of three separate experiments. (B) MCF-7 cells were treated with 30 μM CAI for indicated time intervals. Cells were then double-stained with annexin V-FITC and propidium iodide, followed by detection of apoptotic cells by flow cytometry with a Coulter Elite Laser. One representative experiment of three similar repeats is shown. The data indicate the percentage of annexin V-positive cells (apoptosis).

invasive and apoptosis inductive properties in several tumor cell lines, including a panel of breast cancer cell lines (Lambert et al., 1997). We have further investigated the mechanism through which CAI inhibits the growth of human breast cancer MCF-7 cells, establishing that the antiproliferative activity of CAI against human breast cancer MCF-7 cells was due to its ability to arrest cells in G_2/M phase and induce apoptosis with the reduction of $\Delta\Psi_m$. Lambert et al. (1997) have reported that survival of MCF-7 cells was reduced upon a 5-day exposure to CAI with an IC_{50} of 16.5±4.2 μM. We confirmed the antiproliferative activity of CAI by the same MTT assay and the trypan blue exclusion assay. Also we have found that CAI inhibited DNA synthesis in cultured MCF-7 cells in a dose-dependent manner. And with that we began to gain insights into the effect of CAI on cell cycle distribution. Eukaryotic cell cycle progression involves sequential activation of CDKs, whose activation is dependent upon their association with cyclins (Hunter, 1993; Lukas et al., 2004; Sherr, 1996). For G_2/M phase transition control, a complex formed by the association of Cdk1 (also known as p34Cdc2) and cyclin B1 appears to play a major role (Lukas et al., 2004). Whereas phosphorylation of Thr161 of Cdk1 is required for the complete activation of the complex, reversible phosphorylations at Thr14 and Tyr15 suppress activity of Cdk1/cyclin B1 kinase complex. At the onset of mitosis, the Cdc25 phosphatase activates mitosis-promoting factor by dephosphorylating Cdk1 on Tyr15 and Thr14 (Lukas et al., 2004; Stewart et al., 2003). In addition, the role of p21, an important CDK inhibitor, in the regulation of cell cycle progress has been well documented. P21 arrests the cell cycle through binding and inactivating the CDK system (Brugarolas et al., 1995; Elledge and Harper, 1994; Peter and Herskowitz, 1994; Stein et al., 1998). A p21 mutation, which specifically abrogates its binding to CDKs, was identified in a primary breast tumor (Balbín et al., 1996). We found that, over 10 μM, CAI exerts a cytotoxic effect characterized by a strong inhibition of cell proliferation associated with G_2/M cell cycle arrest. In addition, we have shown that CAI treatment causes a significant reduction in the expression of Cdk1, cyclin B1, and Cdc25C, accompanied by a marked increase in the level of p21 protein in MCF-7 cells. Thus, it is reasonable to postulate that CAI treatment may cause cell cycle arrest by reducing the activity of Cdk1/cyclin B kinase complex due to down-regulation of multiple G_2/M regulating proteins. It was noted that CAI exposure has been reported to hold Madin–Darby bovine kidney cells at the G_1/S restriction point in the cell cycle (Hupe et al., 1990), suggesting that CAI induces cell cycle arrest depending on cell types. Therefore, the mechanism of CAI is not the same in all cell types.

Accumulating evidences have indicated that chemotherapeutic agents induce tumor regression through inhibition of proliferation and/or activation of apoptosis. Here, we demonstrated that CAI elicits apoptotic cell death as characterized by increased percentage of cells with sub-diploid DNA content, phosphatidylserine exposure, accompanied by Bcl-2 down-

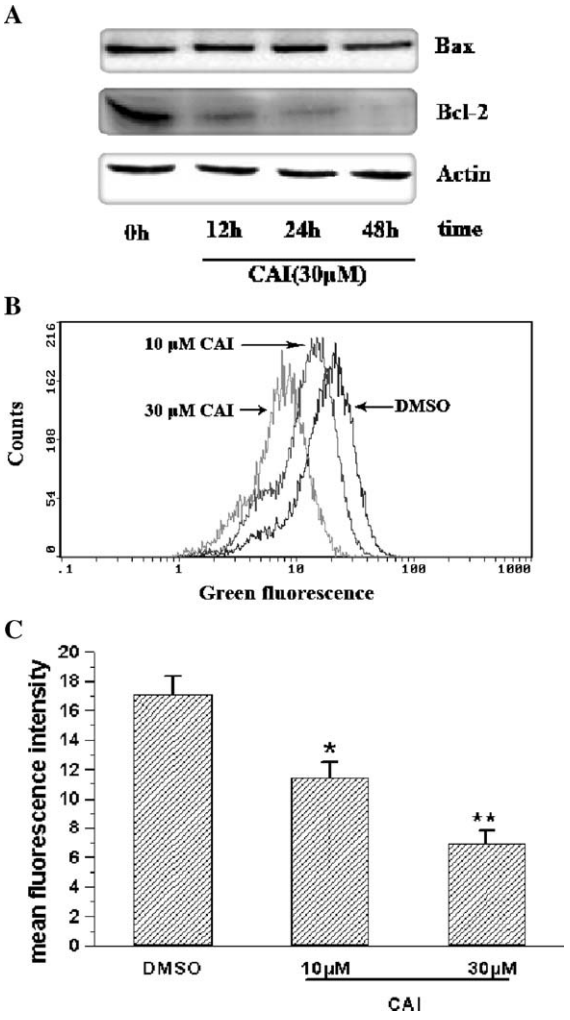

Fig. 4. (A) Western blot analysis of Bcl-2 family members. After incubation with vehicle (DMSO) or 30 μM CAI for the indicated times, MCF-7 cells were lysed and equal amounts of total proteins were resolved on SDS-polyacrylamide gels. Western blot analysis was performed using specific antibodies against Bcl-2 and Bax proteins. Blots were re-probed for actin to normalize each lane for protein content. (B and C) Effect of CAI on $\Delta\Psi_m$ in MCF-7 cells. Cells were treated with DMSO (control) or desired concentrations of CAI (10 and 30 μM) for 24 h, stained with mitochondrial-potential sensitive dye, rhodamine123, and analyzed by flow cytometry. (B) CAI-induced leftward shift in the fluorescent distribution of rhodamine123, indicating mitochondrial depolarization. (C) Average mitochondrial depolarization in the presence of DMSO, 10 and 30 μM CAI. *$P<0.05$, **$P<0.01$ ($n=3$).

regulation and dissipation of $\Delta\Psi_m$. The family of Bcl-2-related proteins regulates susceptibility to apoptosis. Antiapoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-XL, act to prevent or delay cell death, while proapoptotic members, including Bax and Bcl-XS, promote apoptosis (Reed, 1997). Moreover, it has been largely shown that Bcl-2 is inactivated by phosphorylation when cells are arrested in G_2/M (Chadebech et al., 1999). Our data showed that the expression of Bcl-2 proteins decreased after 12, 24, and 48 h of CAI treatment. In contrast, negligible change was noted in the expression of Bax protein. It has been suggested that a high ratio of proapoptotic proteins to antiapoptotic proteins can cause the collapse of $\Delta\Psi_m$, resulting in apoptosis (Kroemer et al., 1997; Yang et al., 1997). We have shown that within 24 h of exposure, CAI clearly induced the loss of $\Delta\Psi_m$ in MCF-7 cells (Fig. 4B,C), and a recent paper demonstrated that 10 μM CAI significantly decreased $\Delta\Psi_m$ within 5 min in HEK-293 cells (Mignen et al., 2005). Although we have not got enough data yet to confirm that the $\Delta\Psi_m$ in MCF-7 cells was altered by CAI in an instant manner, it is tempting to speculate that the depolarizing effect of CAI on mitochondria is direct and persistent. The disruption of $\Delta\Psi_m$ is usually associated with the opening of the mitochondrial permeability transition pore and with the subsequent release of apoptotic factors. Considering a report demonstrating that, due to a functional deletion within exon 3 of the CASP-3 gene, MCF-7 cells lack caspase-3 (Janicke et al., 1998), we could not confirm whether $\Delta\Psi_m$ disruption in MCF-7 cells leads to apoptosis through a "classic" pathway involved in the release of cytochrome *c* and caspase activation.

Our data confirm the previously published results on the antiproliferative effect of carboxyamido-triazole on breast cancer cells; furthermore, CAI may also induce cell cycle arrest at the G_2/M phase in MCF-7 cells. The modulation of key cell cycle regulatory molecules, such as Cdk1, cyclin B, and Cdc25C, contributes remarkably to the cell cycle progression arrest and cell growth inhibition induced by CAI. In addition, the dramatic increase of $p21^{Waf1}$ level may also contribute to the ultimate suppression of MCF-7 cells when exposed to CAI. Our data also provide strong evidence for the induction of apoptotic cell death of CAI, which may be attributable to the down-regulation of Bcl-2 that alters the Bax/Bcl-2 ratio in favor of proapoptosis, as well as the dissipation of $\Delta\Psi_m$ in MCF-7 cells. In conclusion, our results provide molecular evidence for the first time to our knowledge on how CAI may induce cell growth inhibition and apoptosis in breast cancer cells. Hence, our results suggest that CAI, which can be administered p.o., may ultimately prove useful as a potential preventive and/or therapeutic agent in human breast cancer.

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