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Potentiation of the growth inhibition activity of 2-({4-[4-(acridin-9-ylamino)phenylthio]phenyl}(2-hydroxyethyl)amino)ethan-1-ol (CK0402) by Herceptin in SKBR-3 human breast cancer cells

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Abstract. The 9-aminoacridine derivative, 2-({4-[4-(acridin-9-ylamino)phenylthio]phenyl}(2-hydroxyethyl)amino)ethan-1-ol (CK0402) was selected as a potential anticancer agent among a series of sulfur-containing 9-aminoacridine analogues. CK0402 is a topoisomerase II inhibitor and has been shown to exert impressive anticancer activities in both *in vitro* and *in vivo* assays. In the present study, we tested the effects of CK0402 in a panel of established human breast cancer cells with varying ER and HER2/*neu* status. The ER(-) and HER2-overexpressing SKBR-3 cells were the most sensitive cells tested in growth inhibition to CK0402 treatment, and the growth inhibition was in a time- and concentration-dependent manner. In addition, CK0402 also induced stronger G_2/M arrest, apoptosis and autophagy in SKBR-3 cells than in ER(+) and HER2(-) MCF-7 cells. To the best of our knowledge, CK0402 is the first 9-aminoacridine analogue to induce autophagy. These findings suggest that CK0402 may be effective against the more aggressive and malignant ER(-) and HER2-overexpressing breast cancer. Towards this end, we further demonstrated that the combination of CK0402 and Herceptin exhibited synergistic/additive cytotoxic effects in SKBR-3 cells using the median-effect/combination-index isobologram methodology (CI value). Our results indicate that the combination of CK0402 and Herceptin may be a potential therapeutic option against the more aggressive ER(-) and HER2-overexpressing breast cancer.

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Introduction

In breast cancer therapy, the anthracyclines (e.g., doxorubicin and daunorubicin) have been widely used either as a single agent or in the majority of combination protocols (1-3). The anticancer effects of these agents are mainly through the inhibition of DNA topoisomerase II (Topo II), which has been targeted by many clinically important anticancer agents including doxorubicin and etoposide (4,5). Although anthracyclines produce significant response rates in breast cancer patients, their use is hampered by cumulative dose-limiting cardiotoxicity (6) and by the development of drug resistance. Therefore, there is an urgent need to develop novel therapeutic strategies including alternatives to anthracyclines and/or their combination with other effective agents for breast cancer treatment.

In an attempt to search for new alternatives to anthracyclines which are less toxic and less prone to elicit resistance, the compound 2-({4-[4-(acridin-9-ylamino)phenylthio]phenyl}(2-hydroxyethyl)amino)ethan-1-ol (CK0402) was selected as a potential anticancer agent among the series of sulfur-containing 9-aminoacridine analogues previously synthesized by our group for treatment against breast cancer (7). CK0402 is a DNA intercalating Topo II inhibitor and has been shown to exert a growth inhibition effect against various types of cancer cell lines; the anticancer activity of CK0402 was also demonstrated *in vivo* in a study in which it significantly increased the lifespan of P388 leukemia-bearing mice (7,8). Unlike anthracyclines, CK0402 is not structurally related to quinone which has been responsible for the generation of free radicals in many active analogues and their mediated toxicity. It was also proposed that the structural modification of 9-aminoacridine analogues as in CK0402 may overcome the multidrug resistance induced by doxorubicin and etoposide (9). Thus, CK0402 has the potential to serve as a safer and more efficient alternative to anthracyclines.

In this study, we examined the cytotoxic effects of CK0402 in a panel of established human breast cancer cell lines with varying levels of the estrogen receptor (ER) and HER2/*neu*, which are the two most common clinically used biomarkers for breast cancer treatment. These cell lines include MCF-7 [ER(+) and HER2(-)], BT-474 [ER(+) and HER2-overexpressing],

T-47D [ER(+) and HER2-expressing], MDA-MB-231 [ER(-) and HER2(-)] and SKBR-3 [ER(-) and HER2-overexpressing]. To elucidate the mechanism underlying the growth inhibition activity of CK0402 in breast cancer cells, the ability of CK0402 to alter cell cycle progression and the nature of the cell death response induced by CK0402 were also examined. Combination strategy has been widely used to enhance the efficacy of chemotherapy. Trastuzumab (Herceptin®), a humanized monoclonal antibody targeting the extracellular domain of the tyrosine kinase receptor HER2, has shown additive/synergistic anticancer activity with chemotherapeutic agents including anthracyclines in HER2-overexpressed metastatic breast cancer (10-12). Overexpression of the HER2/*neu* gene has been found in 20-25% of patients with breast cancer and is correlated with higher stages of malignancy (13). Since SKBR-3 cells are HER2/*neu*-overexpressing, Topo II amplifying cells and sensitive to Herceptin treatment, the SKBR-3 cell line was chosen to investigate the combination effect of CK0402 and Herceptin.

Materials and methods

Chemicals, reagents and cell lines. CK0402 was synthesized and purified as described previously (7). Herceptin was kindly provided by Genentech Inc. (South San Francisco, CA). Dimethyl sulfoxide (DMSO, cell culture grade), chloroquine, sulforhodamine B and propidium iodide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM)/F12, Iscove's modified Dulbecco's medium (IMDM), penicillin/streptomycin, phosphate-buffered saline (PBS), RNase A and trypsin-EDTA were purchased from Gibco-Invitrogen. Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland, CA). MCF-7, BT-474, T-47D, MDA-MB-231 and SKBR-3 cells were obtained from the American Type Culture Collection (Manassas, VA).

Cell culture and treatment. MCF-7 and MDA-MB-231 cells were maintained in IMDM/F12 (1:1 mixture) medium; SKBR-3, T47-D and BT-474 cells were maintained in DMEM/F12 (1:1 mixture). All the media were supplemented with 10% FBS and penicillin/streptomycin (50 μg/ml). Cells were grown from frozen stock and maintained at 37˚C in a humidified atmosphere with 5% CO_2. Drug treatment involved continuous exposure to the compound. For all cell culture experiments, cells were allowed to seed at least 24 h before treatment.

Assessment of viable cell number. Cell viability was determined by trypan blue exclusion at various time points after the initiation of drug treatment. Cells were harvested by trypsinization, stained with 0.4% trypan blue dye and counted using phase contrast microscopy on a hemacytometer. Cells that excluded trypan blue dye were considered to be viable.

Cell proliferation assay and multiple drug effect analyses. Cells (approximately 3,000-5,000/well) were seeded and grown in a 96-well plate for at least 24 h before treatment. CK0402 was dissolved in DMSO, yielding a final DMSO concentration ≤0.1% (v/v) in the medium. When cells were treated with the combination of CK0402 and Herceptin, Herceptin was added 3 h prior to the addition of CK0402. At the end of the incubation, cultures were fixed with 50 μl of 50% cold trichloroacetic acid and incubated at 4˚C for 1 h. The plates were washed five times with water and then air-dried. The fixed cells were stained for 30 min with 100 μl of 0.4% sulforhodamine B solution in 1% acetic acid. At the end of staining, the plate was washed five times with 1% acetic acid to remove unbound dye. The bound dye was dissolved with 10 mM Tris buffer, and the absorbance of the resulting solution was measured at 570 nm to quantify the number of surviving cells. All treatments were in triplicate and performed at least three times. The drug concentration that produced a 50% reduction (LC_{50}) in cell survival was determined by the median-effect plot. Combined effects of CK0402 and Herceptin in SKBR-3 cells were analyzed by the multiple drug effect analysis of Chou and Talalay (14). LC_{50} and the combination index (CI) were calculated by the program CompuSyn (CompuSyn, Paramus, NJ). CI values were derived from variables of the median effect plots, and statistical tests (Student's t-test) were applied to determine whether the mean CI values at multiple effect levels were significantly different from CI=1. In this method, synergy is defined as CI values significantly <1.0, additivity as CI values =1.0 and antagonism as CI values significantly >1.0.

Flow cytometry of cell cycle analysis. MCF-7 and SKBR-3 cells were grown to exponential phase and treated with the indicated concentration of CK0402 or DMSO. At the end of the incubation, cells were harvested, fixed in ice-cold 70% ethanol and stored at -20˚C. The fixed cells were washed with phosphate-buffered saline, treated with RNase A (3 U/ml) at 37˚C for 30 min, and stained with propidium iodide (50 μg/ml) for 5 min. DNA content for 250,000 cells per analysis was monitored with a Becton-Dickinson FACScan flow cytometer, and Modfit software (LT version 2.0) was used for the analysis of cell cycle status.

Apoptotic cell death detection. A cell death detection enzyme-linked immunosorbent assay kit (Roche Diagnostics, Indianapolis, IN) was used to quantitatively determine cytoplasmic histone-associated DNA oligonucleosome fragments associated with apoptotic cell death, according to the manufacturer's manual. Briefly, after cells were lysed and incubated for 30 min at room temperature, 20 μl of supernatant was transferred into the streptavidin-coated microtiter plate, and 80 μl of the immunoreagent was added to each well. After incubation at room temperature for 2 h, the solution was decanted, and each well was rinsed three times with incubation buffer. Color development was carried out by adding 100 μl of ABTS solution, and absorbency was measured at 405 nm in a microtiter plate reader against ABTS solution as a blank.

Western blot analysis. Cells treated with various doses of CK0402 at various incubation times were harvested by scraping and then washed with PBS. Cellular proteins were isolated with cell lysis buffer purchased from Cell Signaling Technology (Beverly, MA). Equal amount of protein (40 μg) was taken, boiled for 5 min, electrophoresed on a 10% SDS-PAGE at 100 V for 110 min, then electro-transferred to PVDF membranes. Antibody against LC-3 was purchased from

Table I. LC_{50} of CK0402 in a panel of breast cancer cells.

Cell lines	LC_{50} (μM)[a]
MCF-7	0.65±0.21
MDA-MB-231	0.44±0.02
BT-474	3.07±1.72
T-47D	0.97±0.18
SKBR-3	0.29±0.03

[a]Results were quantitated from three independent experiments, and growth inhibition was determined by the SRB assay. Data are presented as means ± SD.

NanoTools (Munich, Germany). Antibody against caspase-3, cleaved caspase-3 and caspase-7 were obtained from Cell Signaling Technology. Mouse monoclonal antibody against α-tubulin was purchased from Sigma-Aldrich Chemical Co. After extensive washing, specific bands were detected using Immobilon Western Chemiluminescent substrate (Millipore, MA). Secondary anti-mouse or anti-rabbit IgG, conjugated with horseradish peroxidase (HRP), were purchased from Cell Signaling Technology.

Results

Growth inhibition activity of CK0402 in human breast cancer cells. The effects of CK0402 on human breast cancer cells were evaluated in a panel of established human breast cancer cell lines which express varied levels of ER and HER2. Initial studies were conducted to examine the time-dependent growth inhibition effects of CK0402 in MCF-7 [ER(+) and HER2(-)] and SKBR-3 cells [ER(-) and HER2-overexpressing]. Fig. 1A and B shows the time-dependent growth inhibition effect of CK0402 in MCF-7 and SKBR-3 cells, respectively. Although no direct cell killing effect of CK0402 was observed within the 48 h of drug exposure, the growth inhibition effect was consistently observed after 72 h in both cell lines. Based on our observations, we chose to determine the LC_{50} of CK0402 in the selected cell lines after 6 days of continuous drug exposure. Subsequently, sulforhodamine B protein assay was used to estimate cell viability, and LC_{50} was calculated. With this well-established methodology, the growth inhibitory activity of CK0402 was demonstrated in all of the cell lines, and values of LC_{50} were determined (0.29-3.07 μM) (Table I). Of the cell lines tested, the ER(-) and HER2-overexpressing SKBR-3 cell line was the most sensitive (LC_{50}=0.29), while the ER(+) and HER2-overexpressing BT-474 cell line was the most insensitive to CK0402 treatment. Although both SKBR-3 and MDA-MB-231 cells are ER(-) and appeared to be more sensitive to CK0402 than ER(+) MCF-7, T-47D and BT-474 cells, correlations between hormone receptor status and sensitivity to CK0402 would be speculative and could not be established in this study (15).

Cell cycle analysis. To investigate the mechanisms underlying the growth inhibition activity of CK0402, we examined the cell cycle distribution in both MCF-7 [ER(+) and HER2(-)]

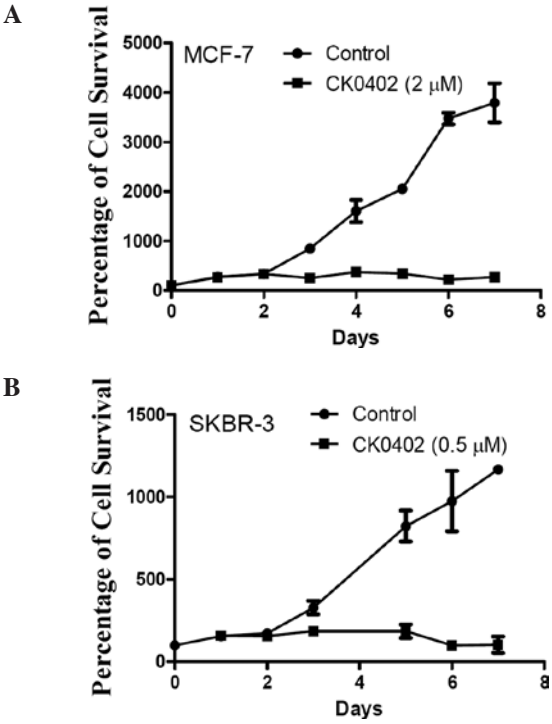

Figure 1. The effect of CK0402 on cell survival in breast cancer cell lines. The time-dependent effect of CK0402 is shown in SKBR-3 (A) and MCF-7 (B) cells; cell survival was determined by the SRB assay. Results were quantitated from three independent experiments. Data are presented as means ± SD.

and SKBR-3 [ER(-) and HER2-overexpressing] cells treated with CK0402. We found that after continuous exposure of cells to 1 and 10 μM of CK0402 for 24, 48 and 72 h, CK0402 induced concentration- and time-dependent G_2/M arrest with increased G_2/S ratio in MCF-7 cells (Fig. 2A). However, the same treatment in SKBR-3 cells induced a transient S phase accumulation at 24 h, but cells progressed to accumulate in the G_2/M phase by 72 h with a massive loss of cells in the G_1 phase (Fig. 2B). The lack of S phase cell accumulation in MCF-7 cells was also confirmed by conducting experiments at early time points (data not shown). Our results showed that the induction of G_2/M arrest by CK0402 was weaker in MCF-7 cells than in SKBR-3 cells; in addition, G_2/M arrest in MCF-7 cells was not accompanied by a massive reduction of cells in the G_1 phase. In fact, a substantial fraction of MCF-7 cells remained in the G_1 phase even after 6 days of exposure to CK0402 (data not shown).

Induction of apoptosis and autophagy. To characterize the cell death response induced by CK0402, analysis of apoptosis was performed in both MCF-7 and SKBR-3 cells using an enzyme-linked immunosorbent ELISA kit, which quantitatively determines the cytoplasmic histone-associated DNA oligonucleosome fragments associated with apoptotic cell death. An initial study was conducted at 24, 48 and 72 h in MCF-7 cells treated with various concentrations of CK0402. However, an apoptotic effect was not observed at 24 and 48 h (data not shown). At 72 h, a concentration-dependent apoptosis induced by CK0402 in MCF-7 cells (doses 5, 10 and 20 μM) was observed, but the apoptotic effect in MCF-7 cells was not as potent as that observed in SKBR-3 cells (doses 0.31, 0.63

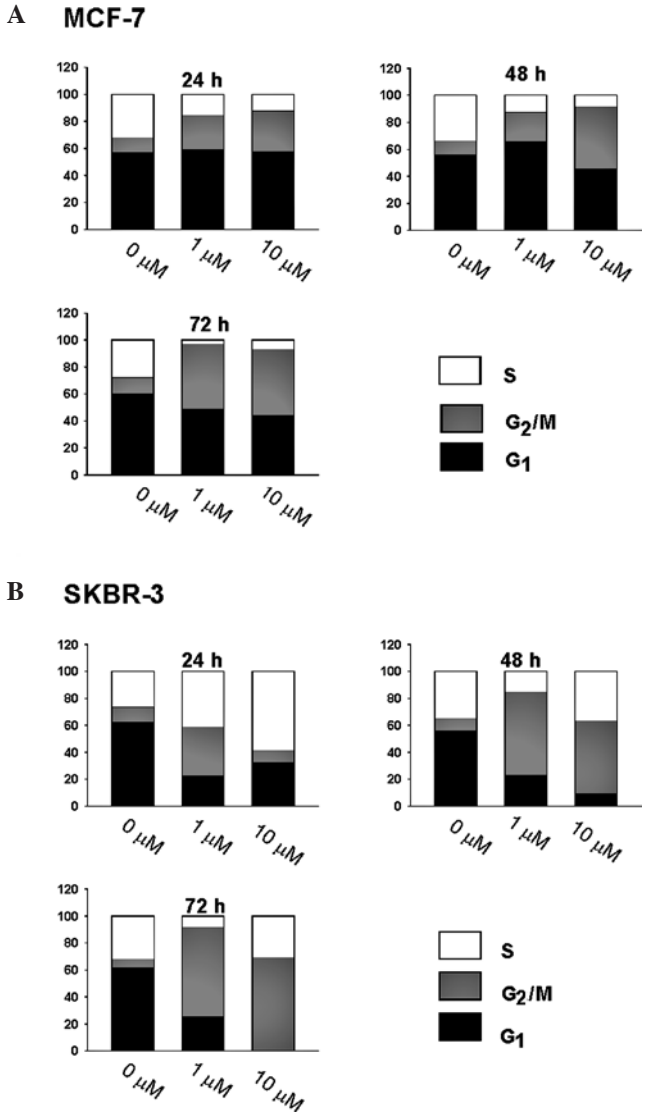

Figure 2. Effect of CK0402 on cell cycle progression of breast cancer SKBR-3 (A) and MCF-7 (B) cells. Cells were stained with propidium iodide and analyzed by flow cytometry. The results are representative data from three independent experiments.

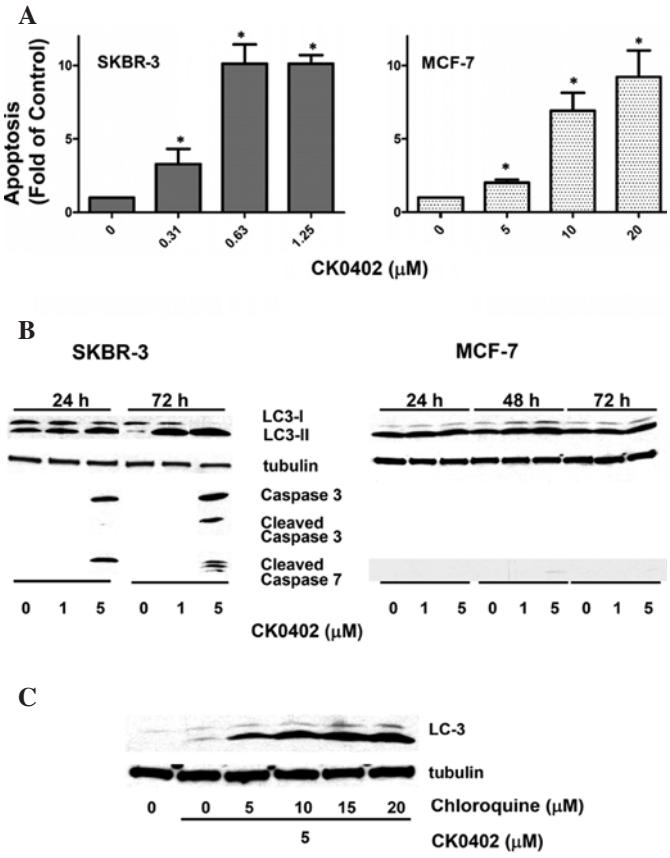

Figure 3. Induction of apoptosis and apoptosis by CK0402 in breast cancer cells analyzed by ELISA or Western blot analysis. (A) Both SKBR-3 and MCF-7 cells were incubated with CK0402 for 72 h and apoptitic cell cell-death was determined by ELISA. Results were quantitated from three independent experiments. Data are presented as means ± SD. *, statistically significant (P<0.01) as compared with untreated controls. (B) Representative illustrations of Western blot analysis of cell lysates obtained from SKBR-3 and MCF-7 cells treated with CK0402. (C) A representative illustration of Western blot analysis of the dose response effect of chloroquine on SKBR-3 cells treated with fixed amount of CK0402 (5 μM).

and 1.25 μM) (Fig. 3A). To further investigate the apoptotic events involved in CK0402-induced apoptosis, we examined the levels of caspase proteins in SKBR-3 and MCF-7 cells by Western blot analysis. In the case of SKBR-3 cells, which exhibited a strong apoptosis response in the ELISA assay at a dose as low as 0.63 μM, activation of caspase-3/7 was only observed at a dose of 5 μM (Fig. 3B), but not at a dose of 1 μM. In MCF-7, which is a caspase-3 deficient cell line, no caspase-7 activation was detected at 24 and 72 h. These results suggest that other caspase-independent pathways may be involved in the cell death process induced by CK0402.

It is known that therapeutic stresses activate pathways that regulate both apoptosis and autophagy. We next examined the effect of CK0402 on the induction of autophagy in MCF-7 and SKBR-3 cells. Autophagy was evaluated by detecting microtubule-associated protein 1 light chain 3-II (LC3-II) expression using Western blot analysis. LC3 is a known protein that specifically associates with autophagosomes. The antibody-based detection of LC3 expression is the 'gold standard' for molecularly defined detection of autophagy, and the processing from LC3-I to LC3-II is required for the formation of autophagosome (16,17). Our results showed that treatment with CK0402 in SKBR-3 cells induced LC3 activation and cleavage in a dose- and time-dependent manner (Fig. 3B). However, CK0402 exhibited a weak effect on LC3 activation in MCF-7 cells. The induction of autophagy by CK0402 in SKBR-3 cells was further confirmed by the addition of chloroquine, which enhanced the accumulation of LC3-II in a dose-dependent manner (Fig. 3C). Although the role of autophagy in the cytotoxic effect of CK0402 remains to be identified, these results clearly showed that CK0402 induced autophagy in breast cancer SKBR-3 cells.

Multiple drug effect analysis. HER2-overexpressing SKBR-3 cells were the most sensitive cells to CK0402 treatment and were selected to evaluate the combination effect of CK0402 and the HER2-targeted Herceptin. Parallel experiments were also conducted with HER2(-) MCF-7 cells as a negative control. CI values for the combination of CK0402 and Herceptin at drug ratios (CK0402/Herceptin) from 1 to 200 at the LC_{50} are summarized in Table II. Results showed that in

Table II. Combined effects of CK0402 and Herceptin at various molar ratios in SKBR-3 cells.

CK0402/Herceptin molar ratio	Combination index (CI) (mean ± SD)[a]	P-value[b]	Interaction
1	1.08±0.09	0.13000	Addition
10	0.47±0.02	<0.00010	Synergy
100	0.69±0.05	<0.00010	Synergy
200	0.91±0.02	0.00026	Synergy

[a]The calculated values for the combination index (CI) were obtained at LC_{50}. Data are presented as means ± SD. CI<1 represents synergism, CI=1 indicates an additive effect and CI>1 represents antagonism. [b]Determined by the Student's t-test.

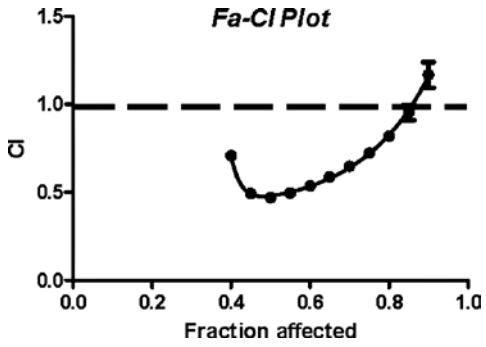

Figure 4. Growth inhibition of SKBR-3 cells by the combination of CK0402 and Herceptin. A fraction affected (Fa) vs. combination index (CI) plot (CK0402/Herceptin, 10) was generated using the method of Chou and Talalay (14) with the commercial software package CompuSyn. Results were quantitated from three independent experiments. Data are presented as means ± SD. CI<1 represents synergism, CI=1 indicates additive effect and CI>1 represents antagonism.

SKBR-3 cells, CK0402 exhibited synergistic interaction (CI<1; P<0.001) with Herceptin at the drug ratios ranging from 10 to 200, while an additive interaction was found at a drug ratio equal to 1. No synergistic or additive effect was observed in MCF-7 cells (data not shown). The lowest CI value (0.47) was observed at a drug ratio 10; therefore, a Fa-CI plot (Fig. 4) of the fraction of affected cells (Fa) vs. CI was constructed at the drug ratio 10. This plot indicated synergism at the range from LC_{40} to LC_{85} (P<0.007), while it indicates an additive effect at LC_{90}.

Discussion

In the present study, we investigated the antiproliferative activity of CK0402 in a panel of established human breast cancer cells including MCF-7, T-47D, BT-474, MDA-MB-231 and SKBR-3, as well as its combined effect with Herceptin in SKBR-3 cells. CK0402 is a 9-aminoacridine analogue which has been shown to inhibit Topo IIα-catalyzed decatenation reaction (8). There are two highly related Topo II enzymes coded by different genes: Topo IIα (170 kDa) and Topo IIβ (180 kDa) (18). Studies have shown that, in general, there is no significant variation in the expression of Topo IIβ protein levels in breast cancer cell lines; however, relatively large variations in Topo IIα protein levels are observed (19). The expression of Topo IIα protein levels in these cells was reported as follows: SKBR-3 > MCF-7 > MDA-MB-231 > T-47D > BT-474 (19,20). The order of LC_{50} of these breast cancer cells to CK0402 was determined as SKBR-3 < MDA-MB-231 < MCF-7 < T-47D < BT-474. Despite the controversy between Topo IIα expression and response to Topo II inhibitors (21,22), our results support that Topo IIα expression was the most possible factor determining the cellular sensitivity to CK0402 in our panel of breast cancer cells. BT-474 cells, which were found to exert considerable resistance to various types of chemotherapeutic agents including Topo II inhibitors in human breast cancer cells (19), expressed the lowest level of Topo IIα as well as the most resistance to CK0402 among the tested cells. The two most sensitive cell lines tested were SKBR-3 and MDA-MB-231 which are both ER(-); the former is HER2 overexpressed and the latter is not.

The inhibition of Topo II by CK0402 may cause DNA damage, which may activate pathways that lead to cell cycle arrest or apoptosis. The former may be a major cellular response to DNA damage preceding repair or death. G_2/M arrest is a common response after treatment with DNA damaging agents such as Topo II inhibitors (e.g., doxorubicin, amsacrine and DACA) (23). The fact that CK0402 induced prolonged G_2/M arrest with delayed growth inhibition in both MCF-7 and SKBR-3 cells suggests that these slowly dying cells may initiate cell death only after the accumulation of cells in the G_2/M phase, and differences in the extent of G_2/M arrest may be critical determinants of cellular sensitivity to CK0402.

DNA damage induced by chemotherapeutics may cause various types of cell death in tumor cells, most likely depending on the mechanism of drug action, the dosing regimen used and the type of tumor cells. Although apoptosis has been commonly regarded as the prevailing mechanism of cell death induced by chemotherapeutics, it has recently been acknowledged that multiple modes of cell death combine to generate the overall tumor response (24). Autophagy is involved in a variety of cellular functions, including development, response to nutrient deprivation and cell death (25). Most of the cellular systems in which autophagy contributes to cell death have involved defects in the apoptosis signaling pathways, suggesting that these two forms of cell death can act as backup mechanisms for each other under conditions where cell death is imperative. However, tumor cells may undergo, separately or simultaneously, both apoptosis and autophagy in response to certain chemotherapeutics (26,27). In this study, the delayed cell death response to CK0402 in MCF-7 cells was characterized by prolonged cell arrest without apparent

apoptosis or autophagy. In SKBR-3 cells, although apoptosis was observed, the roles of autophagy in the mechanism of cell death induced by CK0402 need to be further investigated. To the best of our knowledge, this study is the first to demonstrate that 9-aminoacridine compounds induce autophagy.

The development of anti-HER2 therapies such as Herceptin (28) has markedly improved the therapeutic outcomes of adjuvant chemotherapy for patients with breast cancers that overexpress HER-2 (12). The combination of a Topo II inhibitor with Herceptin in breast cancer therapy was initially supported by *in vitro* studies using Herceptin in combination with doxorubicin which demonstrated an additive effect, and with etoposide which showed a synergistic effect. Recent clinical studies have shown that patients with co-amplification of genes encoded for Topo IIα and HER2 received a therapeutic advantage from the anthracycline-Herceptin combination regimens (10,11). The combination of Herceptin with drugs targeting Topo II was further justified based on the findings that genes encoded for Topo IIα and HER2 are both located on chromosome 17q and are frequently co-amplified or deleted (10,11). In the present study, data from the multiple drug effect analysis methodology demonstrated that the combination of CK0402 and Herceptin exerted a maximal synergistic effect at a molar ratio equal to 10, and the effect was decreased as the molar ratio was decreased to 1 or increased to 100 and 200 (Table II). Our results demonstrate that CK0402 possesses a broad spectrum of activity in human breast cancer cells, and the combination of CK0402 and Herceptin exerts a synergistic/additive effect in HER2-overexpressing and Topo IIα-amplified SKBR-3 cells. In conclusion, the synergistic or additive interaction between CK0402 and Herceptin in SKBR-3 cells suggests that such a combination may be a potential alternative to anthracyclines in combination with Herceptin against the more aggressive ER(-) and HER2-overexpressing breast cancer.

Acknowledgements

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