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Original Research

Artesunate and chloroquine induce cytotoxic activity on cholangiocarcinoma cells via different cell death mechanisms

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Abstract: Chemotherapy for cholangiocarcinoma (CCA) is not quite successful. In this study, we revisited the possibility of artesunate (ART) and chloroquine (CQ), the antimalarial drugs, as therapeutic agents against CCA. The possible mechanisms of these drugs to exert cytotoxicity on CCA cells were also explored. The effects of ART and CQ on proliferation and death patterns of two CCA cell lines, KKU-214 and its highly metastatic subtype KKU-214L5, were examined using water soluble tetrazolium (WST) assay and time-lapse photometry, respectively. To differentiate and verify the death patterns between necrosis and apoptosis, lactate dehydrogenase (LDH) release, and caspase 3 activity were measured. CellROX™ green reagent staining method was used to assess reactive oxygen species (ROS) production in ART- and CQ-treated cells. ART and CQ significantly inhibited proliferation of CCA cells. Both drugs kill malarial parasites via similar mechanism depending on ROS formation, however, ART induced necrotic cell death and CQ induced apoptotic cell death in CCA cells. ART induced LDH release, whereas CQ activated caspase 3, confirming induction of necrotic and apoptotic cell deaths by ART and CQ, respectively. ART treatment induced higher ROS production than CQ. ART and CQ induce CCA cells death via different death pathways. ART should be suitable for necrosis-sensitive CCA, whereas CQ is more suitable for apoptosis-sensitive CCA.

Key words: Artesunate; Chloroquine; Cholangiocarcinoma; Necrosis; Apoptosis.

Introduction

Cholangiocarcinoma (CCA) is a malignancy of bile duct epithelial cells. The incidence of CCA is extremely high in the Greater Mekong Subregion (GMS), particularly in the Northeast Thailand, whereas it is very low in other parts of the world (1). Pathogenesis of CCA in the GMS is associated with persistent inflammation of the biliary tracts due to chronic liver fluke (*Opisthorchis viverrini*, Ov) infection (2). The problems of CCA are the silent clinical presentation and the difficulty of diagnosis at the early stage. The radical resection is the only effective treatment but almost all CCA-patients come to hospitals with advanced and incurable stage of the disease (3, 4). Thus, large proportions of CCA patients are given palliative treatment, mostly symptomatic treatments as CCA is a chemo-resistant cancer with a high rate of recurrence (5). Although currently patients are prescribed traditional chemotherapy drugs, a new regimen is being looked for. Drug repurposing, a new indication for existing drugs (6, 7), may be one of the strategy to improve clinical outcomes of chemotherapy. The main advantage of drug repurposing is, as the toxicity profile of the drugs have already been known, and the chances of drug failure due to toxicity can be minimized.

Currently, several anti-parasitic drugs are being explored for their anti-cancer activities against several cancers with promising outcomes. The anti-malarial drugs, artemisinin and chloroquine and their derivatives have recently been explored as anti-cancer agents. In this study, we aimed to determine the effects of artesunate (ART) and chloroquine diphosphate (CQ) on CCA cells, both of which are the water-soluble form derivatives of respective prototypes. Artemisinin and its derivatives contain an endoperoxide moiety that reacts with iron to produce reactive oxygen species (ROS) (8) and induce malarial cell death based on the formation of toxic ROS (9). On the other hand, chloroquine is a weak base, which is protonated in acidic compartments of cells (the food vacuole in parasite), thereby increases pH of acid vesicles of parasites to impair their function (10). Anti-malarial mechanism of CQ depends on its ability to accumulate in the food vacuole. Accumulated CQ permeabilizes the food vacuole as well as inhibits the polymerization of heme into hemozoin in the food vacuole (11, 12). Free heme is toxic to induce cell death via formation of oxidative stress (13). Thus, both artesunate ART and CQ induce malarial cell death depending directly or indirectly on oxidative stress. It is, therefore, of interest to see whether or not 1) ART and CQ can be used as an anti-cancer agent on CCA; and 2) ART and CQ can induce cancer cell death via similar mechanisms as they do on malaria parasites.

In general, cancer chemotherapeutic drugs confer their effects either via inducing apoptotic cell death or necrotic cell death. Although anti-cancer drugs usually accompanied with immunosuppression due to their suppressive effects not only cancer cells but also all rapidly dividing cells (14, 15), necrotic cell death inducing drugs have some potential to avoid immunosuppression due to stimulation of immune cells by cell debris and cellular components released during necrosis (16). Thus, use of immunosuppressive chemotherapeutic drugs after necrosis-inducing drugs should be avoided. Conversely, necrosis-inducing drugs may fit to the treatment of apoptosis-resistant cancers. Thus, for the best effective treatment, the mode of action of chemotherapeutic drugs in terms of their cell death induction pattern should be considered.

In this study, we examined the effects of ART and CQ on cell proliferation, cell death pattern and ROS production of CCA cells. We found that both ART and CQ significantly inhibit proliferation of CCA cells but with different patterns of cell death. CQ induces apoptosis whereas ART induces both necrosis and apoptosis. Cell death patterns should be concerned when these two drugs are considered for designing of combination therapy for CCA treatment.

Materials and Methods

Cell culture

Cholangiocarcinoma cells, KKU-214 and the highly metastatic subtype KKU-214L5 (17) were cultured in the complete medium consisted of Dulbecco modified Eagle medium (DMEM) medium with 10% fetal bovine serum (FBS) and 1% Gibco® antibiotic-antimycotic solution, at 37°C in a humidified atmosphere of 5% CO_2.

Reagents

Artesunate (ART) and chloroquine diphosphate (CQ) were purchased from Sigma-Aldrich (St Louis, MO, USA). The cell counting kit 8 (CCK8) was purchased from Dojindo Molecular Technologies (Rockville, MD, USA). LDH assay kit (product number 88953) was the product from Thermo Scientific (Rockford, IL, USA). ROS determination reagent, CellROX™ green reagent, was purchased from Invitrogen (Eugene, OR, USA) and caspase 3 assay kit (ab39401) was obtained from Abcam (Cambridge, UK).

Colorimetric assay for cell viability

Effects of ART and CQ on cell viability were measured using the CCK8 assay kit. CCA cells in the complete medium were seeded in a 96-well dish and allowed to adhere for 18-24 h. Then, cells were incubated with various concentrations of ART and CQ for 24, 48 and 72 h at 37°C, 5% CO_2. Subsequently, 10 µL of highly water-soluble tetrazolium, WST-8, in the CCK8 kit was added to each well. After 2 h of additional incubation, absorption at 450 nm for each sample was determined with an automatic ELISA plate reader (E11303, EMax Microplate reader, Sunnyvale, CA, USA). The effects of ART and CQ on cell proliferation were expressed as the survival rate (% of untreated control cells

Time-lapse photometric assay

The patterns of cell death induced by ART and CQ were analyzed using a time-lapse photometry system. Cells were seeded in a 24-well dish in the complete medium. After allowing cells to adhere for 18-24 h, various concentrations of ART and CQ were added to the culture. Immediately after addition of ART and CQ, the dish was transferred to an Olympus IX81 microscope (Olympus Corporation, Tokyo, Japan) equipped with a built-in incubator at 5% CO_2 atmosphere. Using the MetaMorph software version 7.6.2.0, the pictures of cells under treatment were captured at every 15 min for 72 h. Then, the captured pictures were analyzed and converted into video clips using the MetaMorph software version 7.6.2.0 (offline). The cell death patterns of ART- and CQ-treated cells were determined.

Lactate dehydrogenase (LDH) assay

Cells in the complete medium were seeded in a 96-well dish and allowed to adhere for 18-24 h. Then, cells were incubated with various concentrations of ART and CQ for 24 h at 37°C, 5% CO_2. Culture supernatant was collected and centrifuged to remove cell debris. LDH activity in the culture supernatant was measured according to the manufacturer's instruction. In brief, 50 µl of culture supernatant was mixed with 50 µl reaction mixture and incubated in dark at room temperature for 30 min. Then 50 µl of stop solution was added and the absorbance at 492 and 620 nm were measured. The effects of ART and CQ on LDH release in the culture supernatant were expressed as the change in percentage of the absorbance of LDH activity in the culture supernatant of drug treated cells compared with that of untreated control cells.

Caspase 3 activity assay

To verify apoptotic cell death, caspase 3 activity in the cell lysate (protein) of ART and CQ-treated and untreated CCA cells was measured according to the manufacturer's procedure. CCA cells were incubated with ART (85 and 170 µM) or CQ (10 and 25 µM) for 24 h. Cells cultured without drug were used as controls. Cell lysate was collected and adjusted to contain 200 µg protein/50 µl. To 50 µl of cell lysate, 50 µl of 2x reaction buffer was added. Then, 5 µl of 4 mM substrate, chromophore p-nitroaniline (DEVD-*p*-NA substrate) was added to yield the final concentration of 200 µM and incubated at 37°C for 2 h. The p-NA light emission after cleavage by caspase 3 was quantified calorimetrically at 405 nm. The caspase 3 activity was expressed as the change in percentage of the absorbance of caspase 3 activity in ART and CQ treated cells compared with that of untreated control cells.

ROS determination

Fluorescence of CellROX™ green staining of ART and CQ treated cells was captured using an inverted microscope. Cells were seeded in a 96-well dish. After allowing cells to adhere for 18-24 h, they were treated with various concentrations of ART (10.125, 21.25, 42.5 µM) and CQ (5, 10, 25 µM) for 24 h. CellROX™ green reagent was added to a final concentration of 5 µM and cells were incubated at 37°C for 30 min. Cells were washed twice with PBS and were fixed with 4%

paraformaldehyde for 15 min. Cells were washed twice again with PBS and pictures were captured using an Olympus IX81 inverted system microscope (Olympus Corporation, Tokyo, Japan). The intensity of green fluorescence of treated and untreated cells was compared.

Statistical analysis

Student's *t* test was used to evaluate the statistical significance of the results at the 95% confidence level, and a p value <0.05 was considered to be statistically significant. The results are presented as mean value ± standard deviation (SD).

Results

ART and CQ exhibited higher cytotoxicity in highly metastatic cells than the lower metastatic CCA cells

The anti-proliferative effects of ART and CQ on KKU-214 and KKU-214L5 cells were measured using WST assay. When cells were exposed to various doses of ART (0 - 340 µM) and CQ (0 - 50 µM) for 24, 48, and 72 h, both CQ and ART inhibited the proliferation of CCA cells in a dose- and time-dependent manner (Figure 1). The IC_{50} of ART for KKU-214 cells was 277.9, 260.6 and 181.3 µM, while those for KKU-214L5 cells were 241.6, 149.3 and 81.5 µM at 24, 48 and 72 h, respectively. The IC_{50} of CQ for KKU-214 cells was 43.5, 31.3 and 23.9 µM and for KKU-214L5 cells was 44.9, 19.1 and 7.6 µM at 24, 48 and 72 h, respectively. Both ART and CQ inhibited proliferation of higher metastatic and aggressive cells, KKU-214L5, more effectively than that of the parental cells, KKU-214, at 48 and 72 h.

ART and CQ induces CCA cell death with different patterns

Two types of cell death process, namely necrosis and apoptosis, have different morphological and phenotypic changes. The cell death patterns induced by ART and CQ treatment were analyzed using a time-lapse photometric assay. The morphology of cells was taken at every 15 min after drug treatments and the data were converted to video clips using MetaMorph software (version 7.6.2.0) for cell death pattern analysis. Under ART treatment, both KKU-214 and KKU-214L5 underwent cell death with increasing their sizes, forming spherical shape and then showing signs of cell membrane damage with leakage of cell content towards cell death (Figure 2A), suggesting their necrotic cell death. The increase in cell size, loss of cellular membrane potential and membrane rupture are the hallmarks of necrotic cell death (18). In contrast to the cell death pattern induced by ART, the cells undergoing death with CQ seemed to reduce their size to form spherical shape, and form blebs on the plasma membrane (Figure 2B). These morphological changes are the characteristic features of apoptotic cell death (19). The videos of morphological changes of the cells undergoing ART and CQ treatment can be referred in the supplementary data.

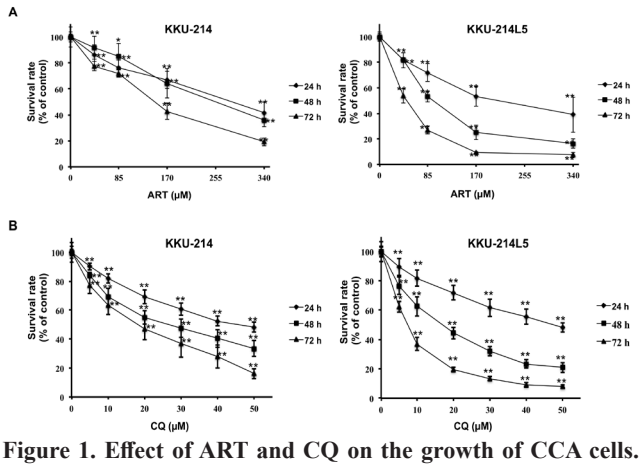

Figure 1. Effect of ART and CQ on the growth of CCA cells. CCA cells KKU-214 and KKU-214L5 cells were treated with various concentrations of (A) ART and (B) CQ for 24, 48, and 72 h, respectively. Cell proliferation was measured using WST assay. Both ART and CQ inhibited proliferation of CCA cells in a dose- and time-dependent manner. The points indicate the average of three independent experiments, and the bars represent the SD. *p < 0.05 vs control, **p < 0.01 vs. control.

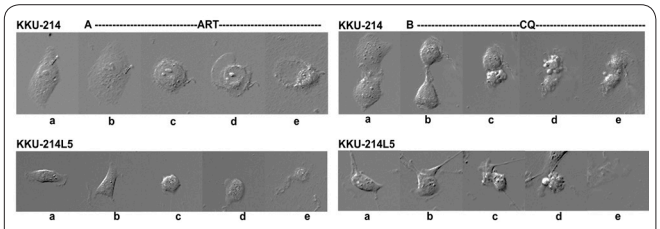

Figure 2. Cell death pattern of CCA cells treated with ART and CQ. CCA cells KKU-214 and KKU-214L5 cells were treated with various concentrations of ART and CQ for 72 h and the pictures of cells were taken every 15 min. The cells undergoing death were traced and cell death pattern was determined. (A) The stages of the cell undergoing death with ART treatment: (a) at the start, 0 h, (b) increase in cell size, (c) undergoing spherical shape, (d) membrane damage and leakage of cell content, and (e) cell death, 72 h. (B) The stages of the cell undergoing death with CQ treatment: (a) at the start, 0 h, (b) shrinkage in cell size, (c) formation of spherical shape, (d) plasma membrane blebbing, and (e) cell death, 72 h. The pictures presented in the figure are representatives taken from cells undergoing death when treated with ART 170 µM and CQ 50 µM. (see videos in supplementary data).

LDH release confirmed ART induced necrotic death of CCA cells

The cell death pattern of CCA cells treated with ART seems to be necrosis while that of CQ seems to be apoptosis. During necrosis cell membrane is damaged and cytoplasmic contents including LDH leak out of cells, leading to presence of LDH activity in culture supernatant, which is absent in apoptotic cell death (20, 21). Thus, to verify whether necrosis or apoptosis process is involved in ART- and CQ-induced cell death, LDH activity in the culture supernatant was measured after ART and CQ treatment using LDH assay kit. The results show that there was a dose-dependent increase in LDH activity in the culture supernatant of ART-treated KKU-214 and KKU-214L5 cells compared with that of untreated cells (Figure 3). On the other hand, CQ at the doses of 25 and 50 µM, which are the doses equivalent to ART doses of 170 and 340 µM that inhibit CCA cell proliferation at 24 h (Figure 1), do not show significant increase in LDH activity in the culture supernatant (Figure 3). These results confirm that ART induced necrotic death pattern.

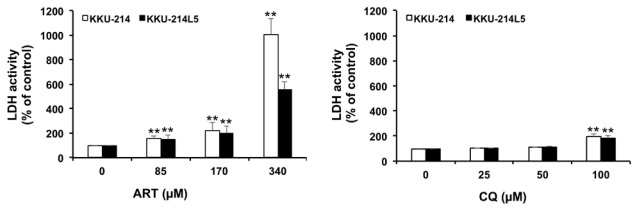

Figure 3. Effect of ART and CQ treatment on LDH release of CCA cells. CCA cells KKU-214 and KKU-214L5 cells were treated with various concentrations of ART and CQ for 24 h. Culture supernatant was collected and measured for LDH activity. There was a dose-dependent increase in LDH activity in the culture supernatant from ART treated cells as compared with those from untreated cells in both KKU-214 and KKU-214L5 cells. The increase was minimally observed in the culture supernatant from CQ treated cells. The points indicate the average of three independent experiments, and the bars represent the SD. *$p < 0.05$ vs. control, **$p < 0.01$ vs. control.

Caspase 3 activity suggested ART and CQ indicated apoptotic death in CCA cells

Caspases are cysteine-aspartic proteases that are activated during apoptosis and they execute the cleavage of protein, thereby executing cell death. Caspases are activated through a cascade of different caspases. Caspase 3 is an executer caspase in the apoptotic pathway (22) and its activation verifies apoptosis process. The morphological changes (Figure 2) and LDH release assay (Figure 3) after ART and CQ treatment strongly suggested that ART induces necrotic cell death whereas CQ induces apoptotic cell death. We then examined the caspase 3 activity in the cell lysate of ART and CQ-treated cells for the further verification of apoptotic cell death. There was significant dose-dependent increase in caspase 3 activity in both CCA cell lines, KKU-214 and KKU-214L5, after ART and CQ treatment (Figure 4), showing that both drugs induce apoptotic cell death of CCA cell lines.

ART and CQ increased ROS production in CCA cells

As the anti-malarial drugs, ART and CQ both induce cell death of malarial parasites directly or indirectly via oxidative stress. Thus, it is of interest to see the effects of ART and CQ on ROS production in CCA cells. For this purpose, CCA cells treated with various doses of ART and CQ were stained with CellROX™ green reagent

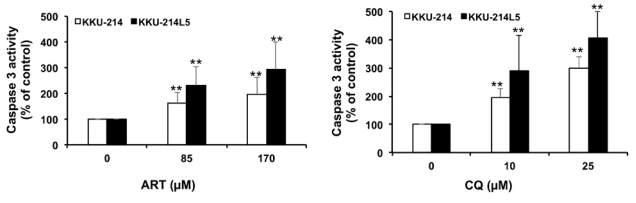

Figure 4. Effect of ART and CQ treatment on caspase 3 activity of CCA cells. Cell lysates were collected and the caspase 3 activity was measured. There was a dose-dependent increase in caspase 3 activity in cell lysates from ART and CQ treated cells as compared with those from the untreated cells in both KKU-214 and KKU-214L5 cells. The increase in caspase activity is higher in CQ treated cells than in ART treated cells. The points indicate the average of three independent experiments, and the bars represent the SD. *$p < 0.05$ vs. control, **$p < 0.01$ vs. control

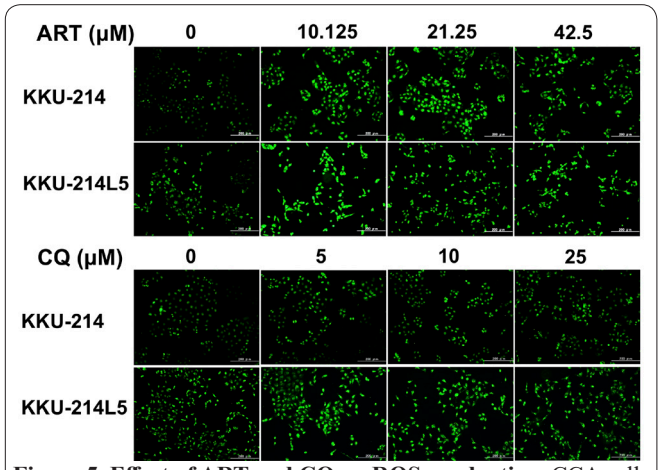

Figure 5. Effect of ART and CQ on ROS production. CCA cells KKU-214 and KKU-214L5 cells were treated with various concentrations of CQ and ART for 24 h and stained with CellROX™ green reagent. The stained cells were captured under a fluorescent microscopy. ROS production was increased in CCA cells treated with (A) ART and (B) CQ. The pictures are one representation from three independent experiments with 10x magnification. Bar = 200 μm.

and the green fluorescence was captured as pictures (Figure 5). Both ART and CQ treatments augmented the intensity of green fluorescence with the predominance in ART-treated cells than in CQ-treated cells.

Discussion

Two anti-malarial drugs, ART and CQ, are the representative repurposing drugs for chemotherapy for several cancers. The aim of this study was to determine the possibility of using these two anti-malarial drugs for CCA treatment. To elucidate their mode of action on CCA cells, Ov-associated CCA cell line, KKU-214, and its highly metastatic derivative, KKU-214L5, cell lines were used. The results indicated that both ART and CQ inhibited proliferation of CCA cells in a dose- and time-dependent manner. The IC_{50} of ART in this study is similar to that of artemisinin and its derivatives in the previous reports on CCA (CL-6) cells (23) and osteosarcoma cells (24). CL-6 cell line was established from non-Ov-associated CCA, whereas two cell lines used in this study are originated from Ov-associated CCA. It is well known that the molecular profiles involved in the carcinogenesis of Ov-associated CCA endemic in Thailand and adjacent GMS are different from those for non-Ov-associated CCA (25, 26). Thus, ART can be repurposed for chemotherapy against both Ov-associated and non-Ov-associated CCA. Regarding CQ, its IC_{50} for inhibition of CCA cell proliferation is comparable to those of other cancer cells (27-30). CQ is known to inhibit metastatic activity of CCA-cells under hypoxic condition (31). Our results added the information that CQ also can inhibit proliferation of highly metastatic CCA-cells under normoxic condition. CQ can be a good candidate for CCA treatment under heterogeneous aerobic condition.

In the present study, cytotoxic effects of ART and CQ are more prominent against highly metastatic KKU-214L5 cells than that against the parental KKU-214 cells (Figure 1). Since the proliferation rate of KKU-214L5 and KKU-214 cells were comparable (17), the greater

inhibition due to rapidly dividing cells can be ruled out. Higher efficacy of ART and CQ against metastatic CCA cell line than its parental cell line may indicate the advantage of these drugs for CCA treatment, because CCA patients are mostly present at the metastatic stage.

Our results highlighted also that ART and CQ induce different pattern of cell death of two CCA cell lines. Cytomorphological study revealed that ART treatment preferentially induced necrotic cell death (18) (Figure 2A), whereas CQ treatment induced apoptotic cell death (19) (Figure 2B). This difference was confirmed further by LDH release assay in that ART induced dose-dependent increase in LDH activity in the culture supernatant of the treated cells, whereas CQ treatment induced minimum LDH release (Figure 3). On the other hand, caspase 3 activity in cell lysates, an indicator for apoptotic cell death, was increased in CCA cells treated with both ART and CQ, and it was higher in CQ-treated than in ART-treated cells (Figure 4). Taken together, our findings indicate that CQ induce apoptotic cell death of CCA cells whereas ART may induce mixed types of cell death of CCA cells with the predominance of necrotic cell death. ART is known to induce necrosis of schwannoma cells (32) and gastric cancer cells (33). In addition, artemisinin and its derivatives can induce other pattern of cell death, for example, apoptosis in leukemic cells (34) and ferroptosis in pancreatic cancer cells (35). Depending on the cancer cell types ART may confer the different cytotoxic effects via different intracellular pathways. As for the mode of action of CQ, it is known to induce apoptosis of various cancer types such as cancers of breast (27, 29), colon (28) and lung (30), and also lymphoma (36). Since CQ clearly induced apoptosis of CCA cells in this study, CQ is considered as the common appoptotic cell death inducer for the wide range of cancer types.

In the present study, when ROS production of CCA cells were visualized with CellROX™ green staining, both ART and CQ augmented ROS production in CCA cells with higher increase by ART than CQ treatment (Figure 5). The different magnitude of ROS production may reflect the different cell death pattern induced by ART and CQ.

In conclusion, our results clearly show that two antimalarial drugs, ART and CQ, can suppress CCA cell growth via induction of different cell death patterns; CQ preferentially induces apoptotic cell death, whereas ART induces both apoptotic and necrotic cell death in CCA cells. Since the susceptibility of cancer cells to necrosis-inducing or apoptosis-inducing drugs is variable depending on the origin and cell types of each cancer, different mode of action of ART or CQ may provide wider choice of chemotherapeutic drugs for CCA treatment.

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Interest conflict

The authors have no conflicts of interest to declare.

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