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Dynamic Color-Coded Fluorescence Imaging of the Cell-Cycle Phase, Mitosis, and Apoptosis Demonstrates How Caffeine Modulates Cisplatinum Efficacy

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ABSTRACT

Caffeine enhances the effect of certain anticancer drugs, but the mechanism of modulation is poorly understood. In this study, modulation of cisplatinum efficacy induced by caffeine was visualized at the subcellular level by real-time fluorescent-protein imaging. Mitotic and apoptotic changes were observed by imaging 143B human osteosarcoma dual-color cells, in which GFP is expressed in the nucleus and RFP is expressed in the cytoplasm. Modulation of the cell cycle was imaged using time-lapse imaging of HeLa cells expressing a fluorescent ubiquitination-based cell cycle indicator (FUCCI) in the nucleus. Clonogenic assays showed that caffeine increased the inhibition by cisplatinum on cell proliferation. Subcellular imaging demonstrated that cisplatinum decreased mitosis and induced apoptosis in 143B cells. The combination of cisplatinum and caffeine enhanced mitosis and subsequently increased apoptosis. Time-lapse imaging showed that cisplatinum strongly induced cell-cycle arrest in the S/G_2 phase in HeLa-FUCCI cells. Caffeine overcame the cell-cycle arrest induced by cisplatinum, thereby increasing its efficacy, since cisplatinum is ineffective against quiescent cells. The data in this report indicate that caffeine modulates the cell cycle in cancer cells, thereby enhancing efficacy of cell-cycle-dependent anticancer drugs such as cisplatinum. J. Cell. Biochem. 114: 2454–2460, 2013.

KEY WORDS: CISPLATINUM; MODULATION; CELL CYCLE; MITOSIS; APOPTOSIS; CAFFEINE; FUCCI; GFP; RFP; FLUORESCENCE IMAGING; OSTEOSARCOMA

Modulation of cancer chemotherapy such as by the use of leucovorin along with 5-fluorouracil (5-FU) has been an important component of the treatment of colorectal cancer [Chung and Saltz, 2007; Marshall and Gehan, 2007; Ishihara et al., 2010].

Caffeine sensitizes cancer cells to ionizing radiation and other genotoxic agents possibly due to disruption of multiple DNA damage-responsive cell cycle checkpoints [Nishikawa et al., 1995; Valenzuela et al., 2000; He et al., 2003]. Caffeine inhibits ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases, which are regulators of DNA damage-induced cell cycle checkpoints [Sarkaria et al., 1999; Zhou et al., 2000] and thereby prevents cell-cycle arrest after DNA damage and potentiates apoptosis.

Shinji Miwa and Shuya Yano contributed equally to this work.
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We have developed caffeine-potentiated chemotherapy for the treatment of sarcomas [Tsuchiya et al., 1998; Hayashi et al., 2005; Kimura et al., 2009; Miwa et al., 2010]. While conventional multi-agent chemotherapies yield a local tumor response of 63% and a 5-year survival of 67.5% for non-metastatic osteosarcoma [Bacci et al., 2006], the response rate of caffeine-potentiated chemotherapy is over 80% and the 5-year survival rate is 90% for non-metastatic osateosarcoma [Tsuchiya et al., 1998].

Our laboratory pioneered the use of green fluorescent protein (GFP) for live cell in vivo imaging in 01997 [Chishima et al., 1997; Yang et al., 2000; Hoffman, 2005]. Fluorescent proteins enabled us to observe individual cancer cells in fresh unstained tissue or even in live animals. Fluorescent proteins allowed subcellular imaging of mitosis and apoptosis in vivo [Yang et al., 2003; Yamamoto et al., 2004; Hoffman, 2005; Hoffman and Yang, 2006a,b]. Dual-color cells with one color fluorescent proteins in the nucleus and another color fluorescent protein in the cytoplasm can effect visualization of mitosis and apoptosis in living cells including the live animal.

A fluorescent ubiquitination-based cell cycle indicator (FUCCI) has been developed [Sakaue-Sawano et al., 2008]. The FUCCI probe was generated by fusing monomeric Kusabira Orange2 (mKO2) and monomeric Azami Green (mAG) to the ubiquitination domains of human Cdt1 and Genemin, respectively, which makes cycling cells green fluorescent and G_1/G_0 cells red fluorescent.

In this study, the modulation of the cell cycle by the combination of cisplatinum and caffeine and subsequent enhancement of mitosis followed by apoptosis was imaged in single cells in real time. We thereby demonstrate, at the subcellular level, how caffeine modulates cisplatinum efficacy on cancer cells.

MATERIALS AND METHODS

ESTABLISHMENT OF DUAL-COLOR CANCER CELL LINES

DsRed 2 and H2B-GFP vectors were used to produce dual-color 143B human osteosarcoma cells expressing GFP in the nucleus and RFP in the cytoplasm using previously-published methods [Yamamoto et al., 2004; Hoffman, 2005; Hoffman and Yang, 2006a,b,c; Jiang et al., 2006].

HELA-FUCCI CELLS

The FUCCI cells were generated by fusing mKO2 (monomeric Kusabira Orange2) and mAG (monomeric Azami Green) to the ubiquitination domain of human Cdt1 and Geminin, respectively, using previously

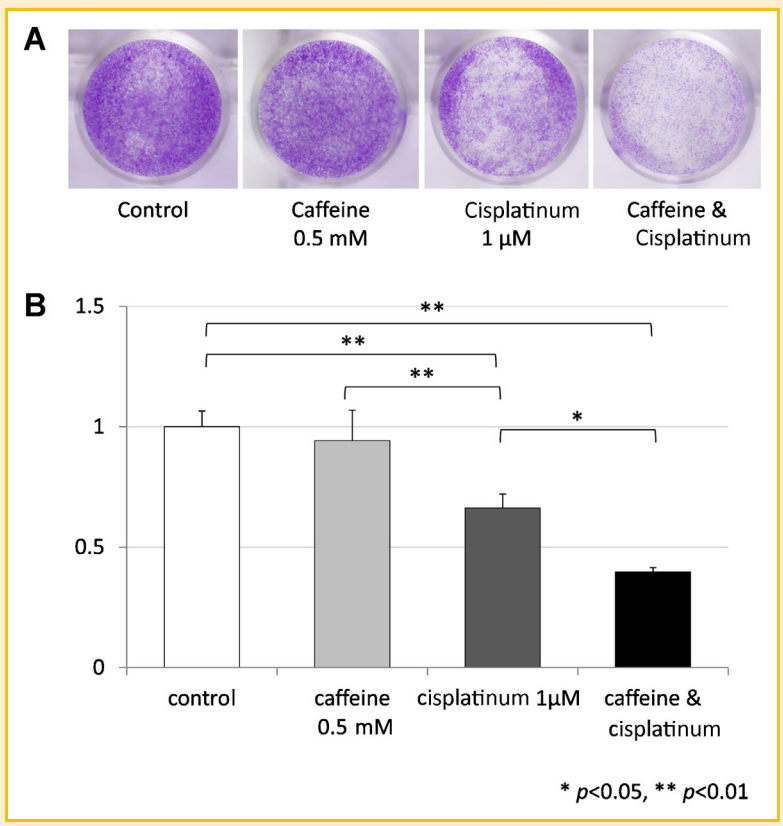

Fig. 1. Efficacy of the combination of cisplatinum and caffeine on proliferation of 143B dual-color cells. 143B dual-color cells, expressing GFP in the nucleus and RFP in the cytoplasm, were cultured in 35 mm dishes (1×10^3 cells/dish) for 24 h. The cells were treated with 1 μM cisplatinum and/or 0.5 mM caffeine. Cisplatinum inhibited the proliferation of the cells (A,B). Although caffeine did not inhibit proliferation of the cells, the combination of cisplatinum and caffeine markedly inhibited proliferation of cells compared to cisplatinum alone ($P < 0.05$). The experimental data are expressed as the mean ± SD. Statistical analysis was performed using the ANOVA test. $^{*}P < 0.05$, $^{**}P < 0.01$.

published methods [Sakaue-Sawano et al., 2008], thereby making cycling cells green fluorescent and G_1/G_0 cells red fluorescent.

CLONOGENIC CELL SURVIVAL ASSAY

143B dual-color cells were plated in 35 mm dishes (1×10^4 cells/dish) and incubated at 37°C in a humidified incubator containing 5% CO_2 for 24 h. The cells were treated with cisplatinum (1 μM; Nippon Kayaku. Tokyo, Japan) for 12 h. The cells were further treated with caffeine (0.5 mM; Sigma–Aldrich. St. Louis, MO) for 3 days and incubated in fresh medium for another 3 days. The colonies were fixed with methanol and stained with 1% crystal violet. ImageJ (National Institute of Mental Health, Bethesda, MD) was used to quantify the colonies.

IMAGING CELLULAR RESPONSE TO DNA DAMAGE

To evaluate whether DNA-damage response was induced by cisplatinum and/or caffeine, MiaPaCa2$^{Tet\text{-}On}$ GFP-53BP1 cells [Efimova et al., 2010; Miwa et al., 2013] were generated first by GFP fusion to human 53BP1 which was then cloned into the pLVX-Tight-Puro lentiviral vector (Clontech, Mountain View, CA) [Efimova et al., 2010; Miwa et al., 2013] and then transfected into the MiaPaCa2 Tet-On Advanced cell line (Clontech). The cells were cultured in high-glucose DMEM (Invitrogen, Carlsbad, CA) with 10% Tet system-approved fetal bovine serum (FBS) (Clontech). MiaPaca2 Tet-On Advanced is certified by Clontech as derived from MiaPaCa2 (American Type Culture Collection, Manassas, VA).

One microgram per milliliter doxycycline (Sigma Aldrich) induced expression of GFP-53BP1 in MiaPaCa2$^{Tet\text{-}On}$ cells. MiaPaCa2$^{Tet\text{-}On}$ GFP-53BP1 cells form GFP-53BP1 foci when DNA is damaged [Efimova et al., 2010]. Focus-positive cells were defined as cells which contained five or more foci. The focus index was defined as the percentage of focus-positive cells.

IMAGING

A FluoView FV1000 confocal laser microscope (Olympus Corp., Tokyo, Japan) [Uchugonova et al., 2011] was used to image GFP-53BP1 focus formation. High-resolution images were captured directly on a personal computer (Fujitsu Siemens Computers, Munich, Germany). Images were analyzed with the use of Cell® software (Olympus Biosystems, Planegg, Germany). Mitosis and apoptosis were determined by imaging nuclear-cytoplasmic dynamics using 143B dual-color cells expressing GFP in the nucleus and RFP in the cytoplasm [Yamamoto et al., 2004; Momiyama et al., 2013a,b; Sakaue-Sawano et al., 2008].

To determine the effect of caffeine and cisplatinum on the cell cycle, HeLa-FUCCI cells were used whereby $S/G_2/M$ phase cells were

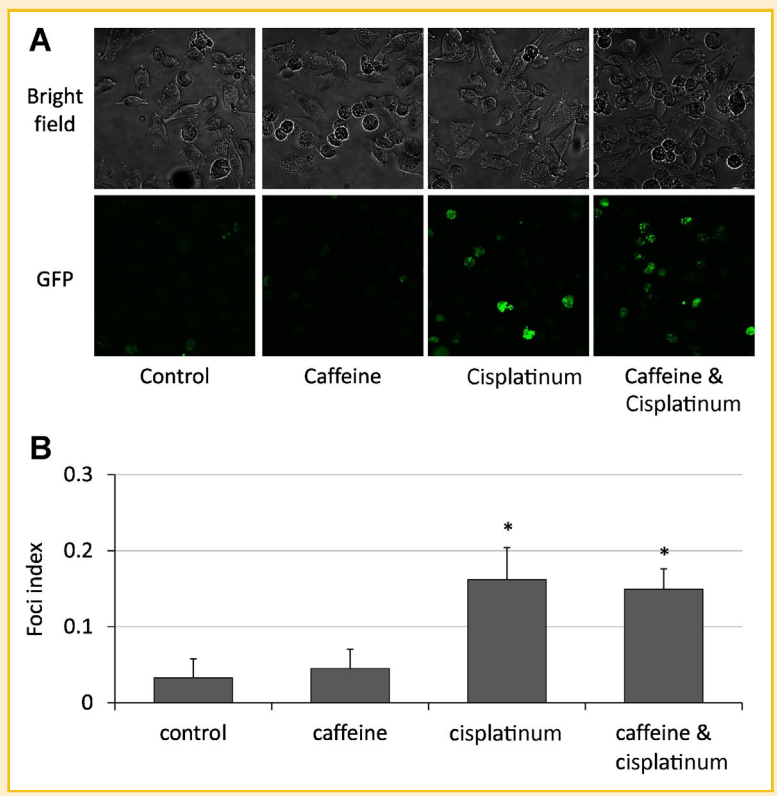

Fig. 2. Cisplatinum induces DNA damage response in MiaPaCa2$^{Tet\text{-}On}$ GFP-53BP1 cells. MiaPaca2$^{Tet\text{-}On}$ GFP-53BP1 cells were incubated in 35 mm dishes with doxycycline (1 μg/ml) for 48 h. The cells were treated with cisplatinum (5 μM) and/or caffeine (1 mM) for 12 h. GFP-53BP1 focus formation was imaged with the FV1000 confocal microscope (A). Focus-positive cells were defined as the cells which had five or more foci. The focus index was defined as the percentage of focus-positive cells. The cisplatinum-treated cells and combination-treated cells had a significantly higher focus index than control or caffeine-treated cells ($P < 0.05$; B). There was no significant difference between control cells and caffeine-treated cells or between the cisplatinum-treated cells and combination-treated cells. The experimental data are expressed as the mean ± SD. Statistical analysis was performed using the ANOVA test. $^*P < 0.05$.

green fluorescent and G_1/G_0 cells were red fluorescent, which were imaged with the IV100 scanning-laser microscope (Olympus) [Yang et al., 2007].

STATISTICAL ANALYSIS

The experimental data are expressed as the mean ± SD. Statistical analysis was performed using the ANOVA test. A P-value of <0.05 indicated a significant difference.

RESULTS

CAFFEINE SENSITIZES 143B DUAL-COLOR CELLS TO CISPLATINUM

To determine the enhancement of caffeine on cisplatinum efficacy, a clonogenic assay was performed. Cisplatinum (1 μM) alone inhibited the proliferation of 143B dual-color cells with an IR of 33.7% (Fig. 1). Caffeine (0.5 mM) did not inhibit proliferation of 143B dual-color cells. The combination of cisplatinum and caffeine markedly inhibited proliferation of the 143B cells with an IR of 60.3% ($P < 0.001$).

CAFFEINE DID NOT HAVE AN EFFECT ON CISPLATINUM DNA-DAMAGE REPAIR RESPONSE

To evaluate the DNA damage repair response to caffeine and cisplatinum, GFP-53BP1 focus formation was imaged in the human pancreatic cancer cell line MiaPaca2$^{Tet\text{-}On}$. Control cells and cells treated with caffeine (1 mM) had only a few GFP-53BP1 foci (Fig. 2A, B). In contrast, cells treated with cisplatinum (5 μM) had a significant increase of focus formation ($P < 0.01$; Fig. 2A,B). Cells treated with the combination of cisplatinum and caffeine also showed a significant increase in focus formation ($P < 0.001$; Fig. 2A,B), but there was no significant difference in focus formation between the

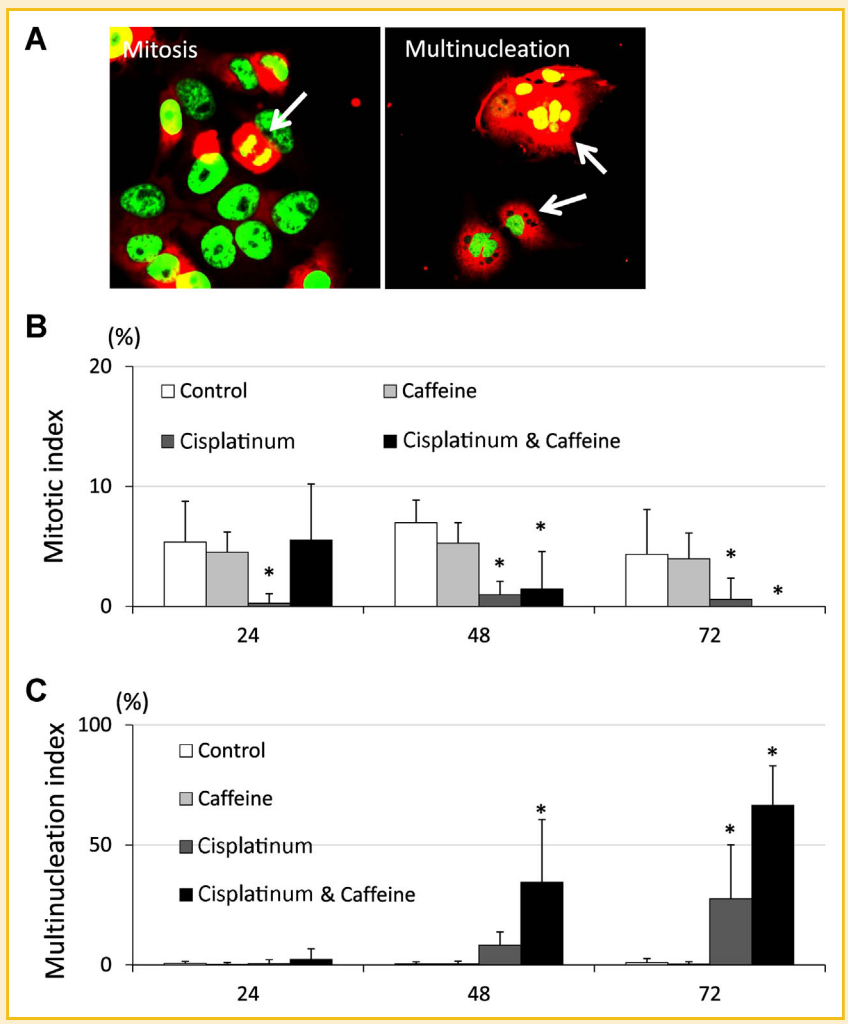

Fig. 3. Effect of the combination of cisplatinum and caffeine on mitosis and apoptosis on 143B dual-color cells. To determine the effect of cisplatinum and caffeine on mitosis and apoptosis, the mitotic index and apoptosis index of cells treated with cisplatinum and/or caffeine were imaged (A). Cells were treated with cisplatinum (1 μM) and/or caffeine (1 mM; B,C). Cisplatinum markedly inhibited the mitotic index, and induced apoptosis at 24–72 h. The combination-treated cells had a higher mitotic index than the cisplatinum-treated cells at 24 h (B). The combination-treated cells also had a higher apoptosis index (multinucleation index) than the cisplatinum-treated cells ($P < 0.05$; C). These results suggest that caffeine enhanced chemosensitivity by overcoming cisplatinum-induced cell-cycle arrest. The experimental data are expressed mean ± SD. Statistical analysis was performed using ANOVA test. *$P < 0.05$, compared with control.

cells treated with cisplatinum alone and the combination of cisplatinum and caffeine (Fig. 2C).

MODULATION OF MITOSIS AND APOPTOSIS BY CAFFEINE IN COMBINATION WITH CISPLATINUM

To investigate the effect of caffeine (1 mM) and cisplatinum (1 μM) on mitotic activity and apoptosis, mitosis and apoptosis were imaged in 143B dual-color cells expressing GFP in the nucleus and RFP in the cytoplasm (Fig. 3A) [Yamamoto et al., 2004; Hoffman and Yang, 2006a,b,c; Jiang et al., 2006]. The mitotic indices of the control group at 24, 48, and 72 h were $5.4 \pm 3.4\%$, $7.0 \pm 1.9\%$, and $4.3 \pm 3.7\%$, respectively (Fig. 3B). The mitotic indices of the caffeine-treated cells at 24, 48, and 72 h were $4.5 \pm 1.7\%$, $5.3 \pm 1.7\%$, and $4.0 \pm 2.1\%$, respectively. There was no significant difference between the control group and the caffeine group. The mitotic indices of cisplatinum-treated cells at 24, 48, and 72 h were $0.3 \pm 0.8\%$, $0.9 \pm 1.2\%$, and $0.6 \pm 1.8\%$, respectively and significantly lower than that of the control cells ($P < 0.05$). The mitotic indices of the combination caffeine-cisplatinum-treated cells at 24, 48, and 72 h were $5.5 \pm 4.7\%$, $1.4 \pm 3.1\%$, and 0%, respectively. The mitotic index of caffeine-cisplatinum-treated cells at 24 h was significantly higher than the cisplatinum-alone treated cells ($P < 0.05$).

There were a few apoptotic cells in the control cells and caffeine-treated cells (Fig. 3C). The apoptosis index of the cisplatinum-treated cells at 72 h was significantly higher than that of the control cells ($P < 0.05$). The apoptosis index of the combination-treated cells at 48 and 72 h was significantly higher than the control cells ($P < 0.05$) as well as the cisplatinum-only-treated cells at 72 h ($P < 0.05$).

CELL-CYCLE MODULATION INDUCED BY CAFFEINE IN COMBINATION WITH CISPLATINUM

To determine the effect of caffeine and cisplatinum on the cell cycle, HeLa-FUCCI cells were used (Fig. 4). The control cells had 56.4–64.0% $S/G_2/M$ phase cells and 27.3–37.4% G_1 phase cells. The caffeine-

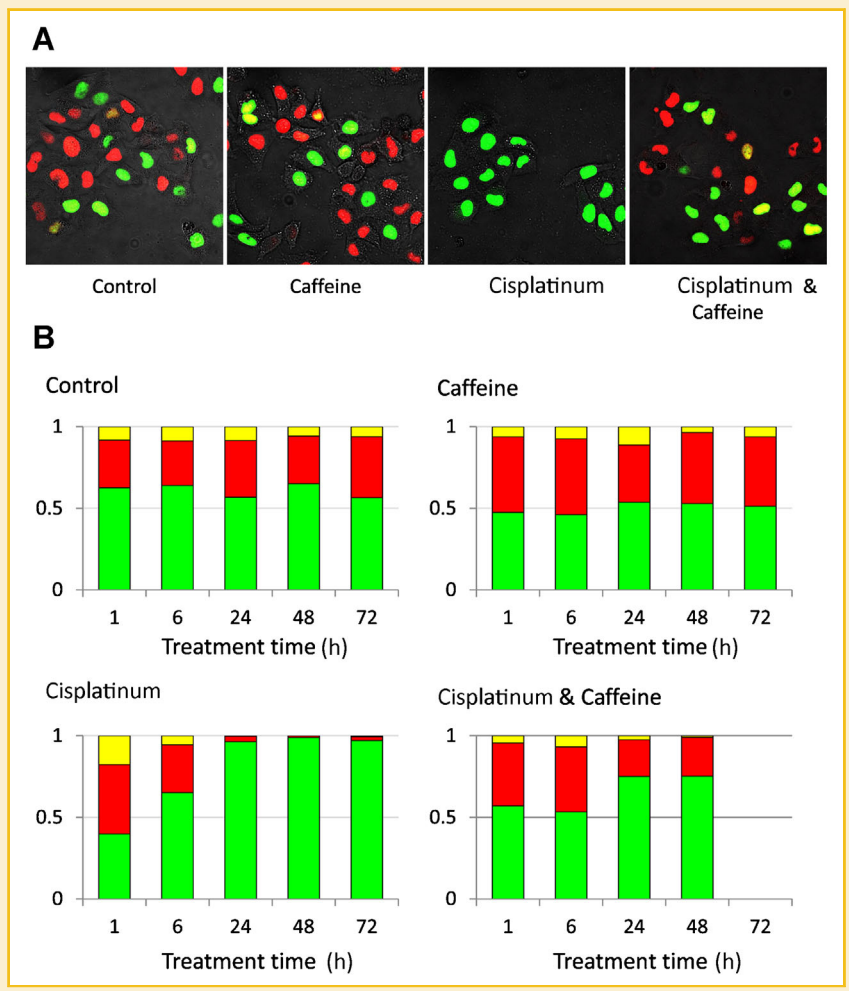

Fig. 4. Cell-cycle modulation induced by cisplatinum and caffeine. To determine the effect of caffeine and cisplatinum on the cell cycle, HeLa cells were imaged with FUCCI. In the control cells, the percentage of $S/G_2/M$ cells and G_1 cells was 56.5–65.0% and 27.3–37.4%, respectively. In the caffeine-treated cells (1 mM), the percentage of $S/G_2/M$ cells and G_1 cells was 46.2–53.6% and 35.2–46.3%, respectively. In the cisplatinum-treated cells (2 μM), the percentage of $S/G_2/M$ phase cells gradually increased and G_1 phase cells decreased to less than 5%. The combination caffeine and cisplatinum-treated cells had an increased percentage to more than 20% G_1 cells at 24 and 48 h compared to cells treated with cisplatinum alone ($P < 0.01$). These results indicate that caffeine overcame the cell cycle block induced by cisplatinum. Green: $S/G_2/\mu$ phase. Red: G_1 phase. Yellow: Early S phase.

treated cells had 46.2–53.6% $S/G_2/M$ phase cells and 35.1–46.3% G_1 phase cells, which was not significantly different than control cells. The cisplatinum-treated cells had an increased percentage of $S/G_2/M$ phase cells and decreased percentage of G_1 phase cells with less than 5% G_1-phase cells at 24–72 h. This result indicates that cisplatinum induced cell-cycle arrest at $S/G_2/M$ phase within 24 h. In contrast, the combination-treated cells had more than 20% G_1-phase cells at 24–48 h, indicating that caffeine overcame the cell-cycle arrest induced by cisplatinum.

TIME-LAPSE IMAGING OF CELL-CYCLE MODULATION BY CAFFEINE IN COMBINATION WITH CISPLATINUM

To confirm that cell-cycle modulation by caffeine increases cisplatinum efficacy, HeLa-FUCCI cells were time-lapse imaged for 72 h after treatment with caffeine and/or cisplatinum (Fig. 5). Control cells showed rapid progression through the cell cycle, resulting in an increased cell number (Fig. 5 and Supplemental Video 1). Cells treated with caffeine also showed rapid progression through the cell cycle (Fig. 5 and Supplemental Video 2). In contrast, the cells treated with cisplatinum had cell-cycle arrest in $S/G_2/M$ and a decrease in cell number (Fig. 5 and Supplemental Video 3). Furthermore, the cells treated with the combination of caffeine and cisplatinum had slow progression through the cell cycle including mitosis (Fig. 5 and Supplemental Video 4). However, most cells subsequently became apoptotic and the total number of cells decreased. These results demonstrate that cisplatinum induced cell-cycle arrest at $S/G_2/M$ and caffeine overcame the cell-cycle arrest with subsequent apoptosis in a large fraction of the cells.

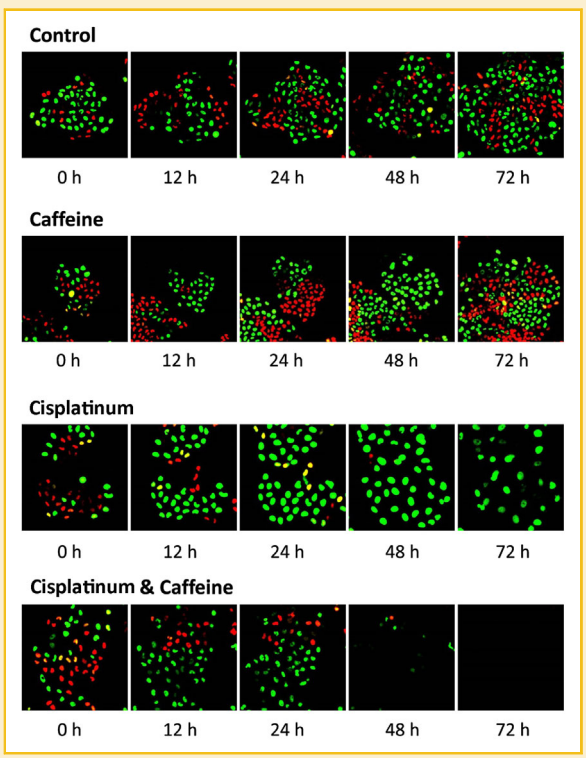

Fig. 5. Time-lapse imaging of HeLa-FUCCI cells treated with cisplatinum and/or caffeine. HeLa-FUCCI cells were treated with caffeine (1 mM) and/or cisplatinum (5 μM), and the cells were observed for 72 h with the IV100 Scanning Laser Imaging System (Olympus, Tokyo, Japan). Control cells and caffeine-treated cells had rapid progression through the cell cycle, resulting in an increased number of cells. In contrast, cisplatinum-treated cells had cell-cycle arrest ($S/G_2/M$ phase) and a slight decrease in cell number. The caffeine and cisplatinum combination-treated cells showed progression throughout the cell-cycle and thereby a greater number of G_1 cells, but the total number of cells was markedly decreased.

DISCUSSION

We have previously reported that caffeine enhances chemosensitivity in malignant bone and soft tissue tumors [Tsuchiya et al., 1998; Hayashi et al., 2005; Takeuchi et al., 2007; Kimura et al., 2009; Miwa et al., 2010]. Several molecular mechanisms of caffeine modulation have been reported [Efferth et al., 1995; Shinomiya et al., 1997; Sarkaria et al., 1999; He et al., 2003; Miwa et al., 2011, 2012]. However, the mechanism of caffeine modulation of cancer chemotherapy drugs is poorly understood.

Imaging, in the present report, enabled visualization of mitosis, apoptosis, and cell-cycle phase in single live cancer cells, and demonstrated that caffeine overcomes the cisplatinum-induced S/G_2 cell-cycle arrest with subsequent increased apoptosis. Cell-cycle arrest is a survival mechanism in chemotherapy-treated cells, and progression of the cell cycle induced by caffeine resensitizes cancer cells to chemotherapy. The imaging technology described in the present report will greatly increase our understanding of chemotherapy modulation at the cellular level.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Video S1. Time lapse imaging of untreated HeLa-FUCCI cells. The cells rapidly progressed through the cell cycle and increased in number.

Video S2. Time lapse imaging of HeLa-FUCCI cells treated with 1 mM caffeine. The cells rapidly progressed through the cell cycle and increased in number.

Video S3. Time lapse imaging of HeLa-FUCCI cells treated with 5 μM cisplatinum. The cells arrested at S/G_2.

Video S4. Time lapse imaging of HeLa-FUCCI cells treated with the combination of 1 mM caffeine and 5 μM cisplatinum. The cells slowly progressed through the cell cycle. A large fraction of the cells underwent apoptosis and the cell number decreased.