Berberine, a genotoxic alkaloid, induces ATM-Chk1 mediated G2 arrest in prostate cancer cells

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1. Introduction

Berberine, an isoquinoline alkaloid present in many medicinal herbs including Huanglian (Coptis chinensis), is one of the most commonly used herbal medicines. Traditionally used for treatment of gastroenteric discomfort, berberine has also been sought for the therapy of diabetes [1]. There are a growing number of reports documenting the anti-tumor activity of berberine [2–5]. Those studies invariably showed that berberine could inhibit tumor cell growth either by inducing cell cycle arrest and/or apoptosis [5,6]. However, the relative contribution of G1 arrest, G2/M arrest or apoptosis to the inhibition of cancer cell proliferation may vary depending on cell type, berberine dose and treatment duration. For example, in human epidermoid carcinoma A431 cells, berberine only induced G1 arrest and apoptosis, but not G2/M arrest [7]. In osteosarcoma cells, while G1 arrest and apoptosis could be readily induced by berberine at relatively low concentrations (10 and 20 μg/ml), G2/M arrest was induced only at a higher concentration (50 μg/ml) [8]. Dose-independent induction of G2/M arrest by berberine was also documented for nasopharyngeal carcinoma cells HONE1 [9]. In a melanoma cell line, the subcellular localization of berberine also varies depending on the concentrations of berberine applied [10]. At low doses (12.5–50 μM), berberine was observed to distribute in mitochondria and to promote G1 arrest. When it was applied at higher doses (over 50 μM), berberine becomes accumulated in cytoplasm and nucleus and promotes G2 arrest. Interestingly, those melanoma cells did not take the path of apoptosis, even at high concentration (100 μM) [10]. In addition to serving as an inducer of cell cycle arrest and apoptosis, berberine was also shown to inhibit angiogenesis [11] and NF-kB signaling [12]. A recent study showed that berberine is capable of suppressing androgen receptor (AR) signaling through induction of AR protein degradation in prostate cancer cells [13].

The berberine-induced G1 arrest is at least partially mediated by p53-dependent p21 upregulation [7,8,14]. We recently showed that by inflicting DNA double-strand breaks (DSBs), berberine activated the p53-p21 cascade in eradicating G1 checkpoint in osteosarcoma cells [8]. However, it remains unclear what is responsible for the G2/M arrest in berberine-treated cells. Some studies showed that levels of proteins that are associated with G2/M progression, CDK1, Cyclin B1, CDC25c, 14–3–3 and Wee1, were altered in some cancer cells such as human gastric carcinoma SNU-5 cell.

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2. Materials and methods

2.1. Reagents and antibodies

The berberine chloride and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). Berberine chloride was dissolved in DMSO, while MTT was dissolved in phosphate buffered solution (PBS). Stock of caffeine, from Wakò Pure Chemical Inc. (Osaka, Japan), was dissolved in 1640 medium at a final concentration of 40 mM. Ku55933 and Chk2 inhibitor (EMD Chemicals Inc., Darmstadt, Germany), UCN-01 (Sigma–Aldrich) and p53 inhibitor Pifithrin-α (Beyotime, Shanghai, China) were dissolved in DMSO.

Antibodies against p53 (sc-6243) and p21 (sc-6246) were purchased from Santa Cruz Biotechnology Inc. (CA). Antibodies against check point kinase-1 (Chk1) (2360), phospho-Chk1 (Ser345) (2348) and check point kinase-2 (Chk2) (26625) were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-γ-H2AX (Ser139) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY), and anti-GAPDH from Sigma-Aldrich.

2.2. Cell culture and treatments

Murine RM-1 prostate cancer cell line was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Human U2OS osteosarcoma cell line was from the American Type Culture Collection (Manassas, VA), and human DU145 prostate cancer cell line was kindly provided by Dr. Huanging Yuan, Shandong University.

The cells were maintained in a humid atmosphere with 5% CO2 at 37 °C in RPMI medium 1640 (Gibco) (RM-1 cells) or Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) (DU145 cells and U2OS cells) plus 10% fetal bovine serum (GIBCO), 100 µg/ml penicillin and 100 µg/ml streptomycin.

After cancer cells were cultured overnight, the medium was changed to fresh RPMI medium 1640 or DMEM and cells were exposed to berberine for 24 or 48 h before they were processed for cell growth, apoptosis, cell cycle distribution, immunofluorescence staining and flow cytometric analysis. For caffeine/berberine combination treatment, cells were treated with caffeine (2 mM) 1 h prior to berberine treatment. The ATM inhibitor Ku55933 was used at a final concentration of 10 µM 2 h prior to berberine treatment; and UCN-01 was used at a dose of 300 nM 4 h prior to berberine treatment. Pifithrin-α, the p53 inhibitor, was employed to treat RM-1 cells 2 h prior to berberine treatment at a final concentration of 20 µM.

2.3. MTT assay of cell viability/proliferation

RM-1 cells at 3 × 10^4/well were cultured in quadruplicate in a 96-well plate. Once RM-1 cells were adherent, cells were exposed to different doses of berberine at 5, 10, 20 and 50 µM or vehicle (DMSO) for 24 or 48 h. Then the number of viable RM-1 cells was determined by a MTT-based colorimetric assay and analyzed using a Wallac 1420 Multilabel Counter (PerkinElmer, MA) at 490 nm.

2.4. Flow cytometric analysis of cell cycle distribution

Cells were harvested using trypsin at various time points after berberine treatment, washed once with cold phosphate buffered solution (PBS), and then fixed in 70% cold ethanol at −20°C overnight. The fixed cells were washed with PBS once and then stained with 50 µg/ml propidium iodide and treated with RNase A (100 µg/ml) together for 30 min at 37°C. Cell cycle distribution was determined by using a FACScan flow cytometer (BD FACSCalibur™, San Jose, CA).

2.5. Apoptosis analysis

Cancer cells, treated with different doses of berberine alone or in combination with caffeine or Ku55933 or UCN-01, were harvested and washed once in cold PBS, and then stained with Alexa Fluor® 488 annexin V and propidium iodide (PI) (Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit with Alexa Fluor® 488 annexin V and PI for Flow Cytometry, Invitrogen, Grand Island, NY) and analyzed by flow cytometry using 488 nm excitation. The fraction of apoptotic cells was determined with FCS Express V3 software (De Novo Software, Canada).

2.6. Immunofluorescence staining and flow cytometric analysis of γ-H2AX

Cells grown on coverslips were washed once in PBS, and fixed in Immunostaining Fix Solution (P0098, Beyotime, Shanghai, China) for 20 min at room temperature. Then overslips were washed in PBS three times and cells were permeabilized in PBS containing 0.2% Triton X-100. Primary antibody incubation was performed overnight at 1:500 dilutions for anti-γ-H2AX (Ser139) (P16104, Upstate Biotechnology, Lake Placid, NY) at 4°C after blocking with 8% goat serum overnight at 4°C. Thereafter, the overslips were washed three times in PBS, and incubated with TRITC-conjugated Goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h at room temperature. Cells were counterstained with DAPI for 5 min after being washed three times in PBS and viewed with a fluorescence microscope using a 100× objective.

H2AX Phosphorylation Assay Kit (Flow Cytometry, 17-344, Upstate Biotechnology, USA) was also employed to determine the levels of γ-H2AX. RM-1 cells were labeled with anti-phospho-Histone H2AX (Ser139)-FITC conjugate according to the manufacturer’s instructions, and then were analyzed on a FACSCan flow cytometer (BD FACSCalibur™). Data analysis was performed with FCS Express V3 software.

2.7. Western blotting analysis

RM-1 cells after berberine treatment alone or in combination with other inhibitors were harvested and lysed in RIPA Lysis Buffer (hpg) (P0013B, Beyotime, Shanghai, China). Lysates containing 30–80 µg total protein were subjected to SDS–PAGE followed by transfer to a PVDF membrane using an electrophoretic apparatus (Bio-Rad) for 1 h at 100V. After blocking non-specific binding by incubating membrane in 5% skim milk for no less than 1 h at room temperature, the membrane was incubated in primary antibody diluted in Primary Antibody Dilution Buffer (P0021A, Beyotime, Shanghai, China) at 4°C overnight. The horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson Immunoresearch Inc., West Grove, PA) was employed to incubate the membrane for 1 h at room temperature, and then the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., USA). Bandscan V4.3 (Glyko, Novato, CA) was employed to scan the gray values of target protein bands.

2.8. siRNA transfection of RM-1 cells

One day before transfection, RM-1 cells were plated at 60 nm culture dishes at a lower density so that they would be 30–50% confluent at the time of transfection.

200 pmol siRNA (Sigma–Aldrich) was transiently transfected into murine RM-1 cells in RPMI medium 1640 without serum and antibiotics using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. The siRNA-treated RM-1 cells were treated with berberine for cell cycle analysis 24 h after transfection and Western blotting analysis was performed to determine the knockdown efficiency.

2.9. Statistical analysis

Data analysis was performed using the 2-tailed student’s t-test (SPSS 13.0 software, SPSS Inc., Chicago, IL) with significant differences when p < 0.05. All data were expressed as the mean ± SEM, and differences were considered statistically significant when the p value <0.05.

3. Results

3.1. Berberine reduced the proliferation of murine prostate cancer cells and induces G1-phase and G2/M-phase arrest

We first evaluated the viability of berberine-treated murine prostate cancer RM-1 cells by using MTT assay. The RM-1 cells were treated with 5, 10, 20 and 50 µM berberine or vehicle (DMSO) for 24 and 48 h. As shown in Fig. 1A, berberine reduced the viability of RM-1 cells in a time- and dose-dependent manner. The treatment of RM-1 cells with berberine (5–50 µM) resulted in a significant reduction in cell viability.

To determine whether or not berberine exerted its inhibitory effect via the induction of cell cycle arrest, we examined the cell cycle progression after treatment with berberine. As shown in Fig. 1B, treatment of RM-1 cells with berberine for 24 h resulted in a significantly higher percentage of cells in the G1 phase, 5 µM (38.82 ± 1.36%), 10 µM (44.96 ± 3.27%), 20 µM (49.11 ± 0.54%) and
50 μM (51.69 ± 1.52%), compared with control (26.85 ± 1.39%), with a concomitant reduction in the number of cells in the S phase. Similar results were obtained on RM-1 cells treated with berberine for 48 h, 5 μM (39.22 ± 5.06%), 10 μM (40.87 ± 3.86%), 20 μM (48.23 ± 4.21%) and 50 μM (50.19 ± 3.49%), compared with control group (31.83 ± 1.83%) (Fig. 1C). As expected, Western Blotting analysis showed the activation of the p53-p21 cascade (Fig. 1D), as was previously reported for osteosarcoma cells by our group [8]. However, the activation of p53-p21 cascade did not follow a linear pattern with increasing concentrations of berberine. The levels of p53 and p21 were lower at 50 μM than in the 5–20 μM dose range. This pattern was unexpected, and appeared to be specific to RM-1 cells, because similarly treated U2OS showed a dose-dependent increase in p53, as in our previous report [8]. Nevertheless, the lesser induction of p53-p21 cascade at higher concentrations than at lower concentrations suggested that G1 checkpoint becomes less important in restraining cell cycle progression in RM-1 cells.

Notably, as shown in Fig. 1B and C, berberine at 50 μM also induced a significant accumulation of cells in the G2/M phase in RM-1 cells, 23.68 ± 3.29% at 24 h, 27.21 ± 3.02% at 48 h, compared with control, 15.14 ± 0.65%. These data suggested that the berberine can induce cell cycle arrest at both G1 and G2/M stages.

3.2. Berberine induces apoptosis of murine prostate cancer cells

We further investigated whether berberine could also induce apoptosis. RM-1 cells were treated with various doses of berberine, and the apoptotic cells were assessed by employing the Alexa Fluor® 488 annexin V and propidium iodide (PI) (Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit). Early apoptotic cells are shown in the lower right quadrant of the scatter plot and necrotic cells in the upper right quadrants (Fig. 2A). Berberine-induced apoptosis of RM-1 cells was increased in a time- and dose-dependent manner, 0 μM (2.53 ± 0.19%), 10 μM (4.31 ± 0.57%), 20 μM (5.46 ± 0.6%) and 50 μM (17.08 ± 0.9%) after treatment with berberine for 24 h, and 0 μM (1.84 ± 0.52%), 10 μM (9.09 ± 0.94%), 20 μM (19.18 ± 2.0%) and 50 μM (30.47 ± 1.79%) for 48 h (Fig. 2B). Thus, the anti-proliferative effect of berberine was also mediated...
by induction of apoptosis. This finding is consistent with previ-
ous reports of berberine inducing apoptosis in prostate cancer cells
[14,18,19]. The number of necrotic cells after berberine treatment
also increased in a time- and dose-dependent manner as shown in
Fig. 2C.

3.3. Berberine induces DNA double-strand breaks in prostate
cancer cells

DNA damage is a common cause of cell cycle arrest and apo-
piesis. We previously showed that berberine induced DNA DSBs
in osteosarcoma cells, thus acting as a genotoxican [8]. To test
whether berberine would similarly cause DNA damage in prostate
cancer cells, we determined the formation of γ-H2AX foci, which
rapidly form at sites of DNA DSBs, in berberine-treated prostate
cancer cells. Indeed, berberine treatment led to an increased forma-
tion of γ-H2AX foci as determined by immunofluorescence staining
(Fig. 3A). Flow cytometry also showed a dose-dependent increase
in the percentage of γ-H2AX positive cells after berberine treat-
ment (Fig. 3B). These results suggested that RM-1 cells treated
with berberine were indeed experiencing considerable genotoxic stress.

3.4. G2/M arrest induced by berberine treatment can be
abrogated by caffeine

Both ATM (ataxia telangiectasia mutated) and ATR (ATM- and
Rad3-related) play a central role in coordinating the DNA dam-
age response, including cell cycle checkpoint control, DNA repair
and apoptosis [20]. We therefore applied caffeine, an inhibitor
of ATM/ATR [21], together with berberine to RM-1 cells to test
whether G2/M arrest in RM-1 cells caused by berberine treatment
was mediated by ATM/ATR-dependent pathways.

Flow cytometry analysis indicated that G2/M arrest caused by
berberine treatment could be abrogated when RM-1 cells were
pretreated with 2 mM caffeine for 1 h (Fig. 4A and B). Caffeine
attenuated the accumulation of RM-1 cells in G2/M phase, reduc-
ing the percentage from 30.51±2.71% to 16.09±2.54% at 24 h,
from 28.67±2.64% to 16.98±1.94% at 48 h. To test whether cafe-
feine could also abrogate G2/M arrest in berberine-treated human
prostate cancer cells, we similarly treated human DU145 prostate
cancer cells and found that G2/M arrest caused by berberine treat-
ment for 48 h also disappeared in cells pretreated with 2 mM
caffeine for 1 h (Fig. 4C). Furthermore, the berberine-induced G2/M
arrest after incubation for 48 h in osteosarcoma U2OS cells, which
was reported previously by our workgroup [8], was also abrogated
by caffeine pretreatment for 1 h (Fig. 4D). Together, these results
indicated that G2/M arrest induced by berberine treatment might
be dependent on ATM/ATR signaling pathways.

3.5. Berberine-induced G2/M arrest was mediated by ATM-Chk1
signaling

Several previous studies showed that Chk1, which lies down-
stream of ATM/ATR, plays an essential role in the activation of G2/M
checkpoint by promoting the degradation of CDC25A [22–27]. We
therefore measured the activation of Chk1 in berberine-treated
RM-1 cells. We found that berberine treatment indeed resulted in
an increase in phosphorylation of Chk1 (Ser345) (Fig. 5A). Consis-
tent with a previous study showing that genotoxic stress resulted
in the degradation of Chk1 [28], we also noted that the total level of
Chk1 was also reduced by berberine treatment (Fig. 5A), which sug-
gested that berberine-induced phosphorylation of Chk1 at Ser345
might trigger the degradation of Chk1 in RM-1 cells.

UCN-01, an inhibitor of Chk1 kinase activity [29], can abrogate
the G2 checkpoint in cells experiencing DNA damage [30]. We
therefore pretreated RM-1 cells with UCN-01 prior to berberine
treatment to test whether it could abrogate the berberine-induced
G2 arrest. As shown in Fig. 5B, the G2/M arrest induced by

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Fig. 3. DNA double-strand breaks, as measured by γ-H2AX, were induced by berberine treatment. (A) Detection of γ-H2AX by immunofluorescence. Cells were treated with the indicated concentrations of berberine for 24 h, stained with mouse anti-γ-H2AX (Ser139) antibody and goat anti-mouse TRITC-conjugated secondary antibody (red), then counterstained with DAPI (blue). (B) Detection of γ-H2AX (Ser139) by flow cytometry using H2A.X Phosphorylation Assay Kit. Cells were treated with the indicated concentrations of berberine for 24 h. Berb, berberine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 4. Berberine-induced G2/M arrest was abrogated by caffeine. Cells were cultured as described in Materials and methods, and treated with DMSO alone (berberine (0 μM)) or indicated doses of berberine and/or caffeine (2 mM) for 24 h (A) or 48 h (B–D). Caffeine was added 1 h before berberine treatment. Cells were harvested and analyzed for cell cycle distribution as detailed in Materials and methods. Data are presented as the mean ± SEM of triplicate samples at least. Berb, berberine; Caff, caffeine. *p < 0.05 and #p < 0.01 when compared with control group.
Berberine-induced G2/M arrest was Chk1-dependent. (A) Berberine treatment resulted in an increase in phosphorylation of Chk1 (Ser345). G2/M arrest was abrogated after pretreatment with 300 nM UCN-01 for 4 h before berberine treatment compared with berberine treatment alone (B, F, G). Both UCN-01 and caffeine pretreatment reduced the level of berberine-induced phosphorylation of Chk1 significantly in RM-1 cells (C and D). (E) Western Blotting analysis of Chk1 protein expression after transfection of Chk1-siRNA or Control-siRNA (top). RM-1 cells were transiently transfected with Chk1-siRNA or Control-siRNA and then treated with 50 μM berberine for 48 h. Silencing of Chk1 protein expression attenuated G2/M arrest (lower). Berb, Berberine; Caff, Caffeine. Data are presented as the mean ± SEM. *p < 0.05 and #p < 0.01 when compared with control group. NS, not significant.
berberine (50 μM) treatment for 24 h was indeed absent after pretreatment with 300 nM UCN-01 for 4 h, and the percentage of RM-1 cells in G2/M phase decreased from 25.85 ± 0.50% to 17.73 ± 0.65%. Furthermore, Western Blotting analysis showed that pretreatment with UCN-01 for 4 h significantly reduced the level of berberine-induced phosphorylation of Chk1 in RM-1 cells (Fig. 5C). Pretreatment with caffeine for 1 h also reduced berberine-induced phosphorylation of Chk1 (Fig. 5D).

To corroborate the results obtained with UCN-01 treatment, we further tested the role of Chk1 in the activation of G2 checkpoint by RNA interference (RNAi) of Chk1 in RM-1 cells. As shown in Fig. 5E (top), Chk1 was efficiently knocked down in Chk1 siRNA-treated RM-1 cells. As expected, berberine-induced G2 arrest was significantly attenuated in Chk1 siRNA-treated RM-1 cells (Fig. 5E, bottom).

Similar results were obtained with human DU145 and U2OS cells (Fig. 5F and G). Treatment of RM-1 cells with Chk2 inhibitor or by Chk2 RNAi, on the other hand, did not significantly attenuate the G2/M arrest caused by berberine (Supplementary data). Chk1 is activated by ATR when cells encounter replication stress or UV [23]. Activation of Chk1 in response to DSBs caused by ionizing radiation requires the function of ATM [31–33]. The fact that berberine caused DSBs, yet did not cause S phase arrest, suggested that the Chk1 activation caused by berberine treatment might be mediated by ATM. Interestingly, it was reported that curcumin, also a natural product, is capable of inducing G2/M checkpoint in pancreatic cancer cells via the ATM-Chk1 cascade [25]. We therefore employed KU55933, a specific ATM inhibitor [34], to test whether ATM lies upstream of Chk1 in establishing G2 checkpoint in berberine-treated cells. As shown in Fig. 6A, the G2/M arrest induced by berberine (50 μM) treatment for 24 h was indeed abrogated after pretreatment with 10 μM KU55933 for 2 h, and the percentage of RM-1 cells in G2/M phase decreased from 25.85 ± 0.50% to 18.91 ± 0.20%. Pretreatment of DU145 and U2OS cells with 10 μM KU55933 for 2 h prior to berberine treatment produced similar results (Fig. 6B and C). In consistent with the role of ATM in mediating Chk1 activation, the phosphorylation of Chk1 was attenuated in RM-1 cells pretreated with KU55933 (Fig. 6D). Together, these results indicated that berberine-induced G2/M arrest was ATM/Chk1-dependent.

3.6. Abrogation of G2/M arrest by inhibiting ATM enhanced apoptosis induced by berberine

Continuing cell cycling in presence of DNA damage may lead to apoptosis or catastrophe, or accumulation of mutations if the cells can survive. Thus, cell cycle checkpoints may promote survival of cells experiencing genotoxic stress. We therefore tested whether berberine-induced apoptosis could be enhanced when the G2/M checkpoint is abolished. The results showed that when compared with the group treated with berberine alone for 24 h, apoptosis of RM-1 cells was increased significantly when cells were pretreated with 2 mM caffeine for 1 h. As shown in Fig. 7A and B, the percentage of early apoptotic RM-1 cells shifted from 16.50 ± 0.53% induced by 50 μM berberine treatment for 24 h, to 23.55 ± 0.55% when RM-1 cells were pretreated with 2 mM caffeine for 1 h prior to berberine treatment. Similarly, apoptosis of RM-1 cells was enhanced when they were pretreated with 10 μM KU55933 for 2 h before berberine treatment (Fig. 7C). However, inhibition of Chk1, by applying UCN-01 at 300 nM, had little effect.
on the apoptosis induced by berberine treatment, although it efficiently abrogated the berberine-induced G2/M checkpoint (shown above). It was previously reported that inhibition of Chk1 only radiosensitized p53-deficient cells to apoptosis [26,35,36]. Therefore, the lack of further induction of apoptosis by UCN-01 may be due to the functional p53 in RM-1 cells. To test this, we pretreated RM-1 cells with p53 inhibitor Pifithrin-α for 2 h before berberine treatment. As shown in Fig. 7C, berberine-induced apoptosis was greatly enhanced by UCN-01 pretreatment for 4 h when p53 was inhibited, suggesting that abrogation of G2/M arrest by Chk1 inhibitor sensitized the cells to berberine only when p53 function was compromised.

4. Discussion

We showed that acting as a genotoxicant that causes DSBs, berberine induced apoptosis of RM-1 cells in a dose-dependent and time-dependent manner. The inhibitory effect of berberine on RM-1 cells was also attributable to cell cycle arrest. We showed that while G1 arrest was induced when berberine was applied in the low dose range, G2/M arrest took over when concentration of berberine was increased. As well documented in previous studies, G1 arrest was associated with an upregulation in the p53-p21 cascade. Importantly, this study for the first time established that the G2/M arrest induced by berberine was mediated by an ATM-Chk1 signaling pathway. Interestingly, G2/M arrest in RM-1 cells was established at the expense of p53-p21 activation. We further showed that inhibiting ATM, by caffeine or KU55933, can abolish G2 checkpoint and promote apoptosis. However, while Chk1 inhibition abrogated G2 checkpoint, it did not sensitize RM-1 cells to berberine-induced apoptosis, suggesting that other ATM targets, such as p53, may contribute to cell survival when Chk1 is not functional. Indeed, when p53 function was compromised, berberine-treated cancer cells exhibited a greater sensitivity to Chk1 inhibitor. Our results suggest that a better understanding of the complex scenario in which cancer cells respond to berberine may enable more efficient cancer cell-specific killing when berberine is correctly combined with other chemotherapeutic agents.

ATM is usually activated in response to ionizing radiation and oxidative stress and relays its effect via Chk2 [37–40]. ATR, on the other hand, senses UV damage and replication stress and phosphorylates Chk1 [41]. However, ATM was also shown to be required for Chk1 activation in some circumstances [25,31,33]. Phosphorylation of H2AX and Chk1, and G2/M arrest, in pancreatic cancer cells treated with curcumin was dependent on ATM [25]. ATM can also regulate ATR activation in S and G2 cell cycle phase in response to DNA DSBs, which need to be processed for RPA coating and subsequent ATR activation [31,33]. Our results provided another example of ATM-Chk1 signaling pathway in mediating G2/M arrest, though it remains to be determined whether ATR also mediated the activation of Chk1 in berberine-treated cells.

Why does berberine induce G2/M arrest only when it is applied at a higher concentration? This is probably related to the amount of DSBs inflicted by berberine. The activation of G2/M checkpoints probably requires a higher level of DNA damage than that of G1 arrest. It appears that the higher the concentration of berberine, the more DNA damage the cells incur (Fig. 3). Therefore, it is possible that only when the amount of DNA damage reaches certain threshold will the ATM-Chk1 pathway be activated. Whereas the induction of G2/M arrest coincided with the
subidence of p53 and p21 in RM-1 cells, p53 activation is not necessarily incompatible with G2/M arrest, because G2/M arrest in U2OS cells can be induced by berberine when p53 level remains high.

We observed that ATM inhibitors, caffeine or KU55933, promote berberine-induced apoptosis in murine prostate cancer cells. Some recent studies showed that whether or not abrogation of G2/M checkpoint promotes apoptosis may depend on p53 status. While the induction of ATM sensitizes tumors to genotoxic chemotherapy when p53 is deficient or non-functional, it protects tumors from being killed in the presence of functional p53 [35,36]. It should be noted that while p53–p21 cascade in RM-1 cells could be activated by berberine treatment in the low dose range, the levels of both proteins subsided at 24 h after exposure to berberine at higher concentration (Fig. 1D). Thus, at the point when G2/M was induced, p53 was no longer functioning, rendering cancer cells more sensitive to cell killing by ATM inhibition. However, just because the induction of G2/M arrest coincided with the subsidence of p53 and p21 in RM-1 cells does not necessarily mean that p53 activation is incompatible with G2/M arrest, because G2/M arrest in U2OS cells can be induced by berberine when p53 level remains high. Importantly, although the p53 level in RM-1 cells was remarkably reduced when the cells were exposed to the high dose range of berberine, UCN-01 did not enhance berberine-induced apoptosis in RM-1 cells as it would do to p53-deficient cells. Inhibition of Chk1 by UCN-01 promoted the apoptosis caused by berberine treatment only when p53 function was compromised.

Our results also showed that caffeine or UCN-01 treatment alone could impair cell cycle progression, as reflected by the reduction of cell population at S and G2 phase. These treatments, lasting for 24 h or 48 h, may have disrupted the functions of Chk1 and Chk2 that are required for normal cell cycle progression. It is also possible that they may have produced some secondary effects that were independent of their function as ATM or Chk1 inhibitor. Whatever effect they may have when acting alone, they could efficiently overcome the G2/M arrest caused by berberine when used in combination, most likely due to their respective inhibitory effect on ATM and Chk1.

Our findings indicate that the three outcomes caused by berberine treatment, G1/G0 arrest, G2/M arrest and apoptosis, may be mutually exclusive and that one fate may predominate over the others depending on the amount of berberine the cells are exposed to, the duration of berberine treatment, and the intrinsic nature of the cells. It appears that the relative capacity of each of the signaling pathways involved in DNA damage response may contribute to such intrinsic nature. As a consequence, the same treatment may cause different outcomes in different cell lines due to variation in the signaling pathways in response to DNA damage. Whereas the induction of G2/M arrest by berberine is independent of p53, abrogation of G2/M checkpoint can accelerate berberine-induced apoptosis only when p53 is nonfunctional.

It should be pointed that all the results were obtained from experiments performed in vitro. While it is desirable to test whether an abrogation of G2/M checkpoint would enhance the therapeutic effect of berberine in vivo, the following factors make it formidable to conduct. First, the G2/M checkpoint was induced by berberine only at the concentration of 50 μM, which may be hard to deliver in vivo. Second, berberine also inhibits tumor growth by other mechanisms. Angiogenesis [42], NF-κB signaling pathway [12,43], and the androgen receptor signaling pathway [13] were all known to be inhibited by berberine. It would be difficult to differentiate the effect of G2/M abrogation from the other mechanisms.

Nevertheless, berberine and caffeine may serve as a potent combination in cancer chemoprevention and chemotherapy in some situations.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrfmmm.2012.04.005.

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