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Report

Dual Inhibition of PI3K/Akt Signaling and the DNA Damage Checkpoint in p53-Deficient Cells with Strong Survival Signaling

Implications for Cancer Therapy

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KEY WORDS

G_2 arrest, DNA damage checkpoint, survival signaling, PI3K/Akt kinases, chemoresistance, response modulators

ABBREVIATIONS

AML	acute myeloid leukemia
PBS	phosphate-buffered saline
DMSO	dimethylsulfoxide
SDS	sodium dodecyl sulfate
EDTA	ethylenediaminetetraacetic acid

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ABSTRACT

Natural (intrinsic) resistance of many tumor types to DNA damaging agents is closely associated with their capacity to undergo robust cell cycle arrest in G_2/M. G_2 arrest is regulated by the DNA damage checkpoint and by survival signaling, with a potential role of PI3K/Akt in checkpoint function. In this work, we wanted to clarify if inhibition of multiple checkpoint/survival pathways may confer better efficacy in the potentiation of genotoxic agents compared to inhibition of either pathway alone. We compared the influence of UCN-01, which affects both the DNA damage checkpoint and PI3K/Akt-mediated survival signaling, with the PI3K inhibitors wortmannin and LY294002 in p53-deficient M1 acute myeloid leukemia cells treated with the DNA damaging agent cisplatin. Our results show that direct inhibition of PI3K/Akt in G_2-arrested cells by wortmannin or LY294002 strongly enhanced the cytotoxicity of cisplatin without influencing the G_2 checkpoint. Unexpectedly, dual inhibition of both survival and checkpoint signaling by UCN-01, also increased the cytotoxicity of cisplatin, but to a lesser degree than wortmannin or LY294002. The differences in cytotoxicity were accompanied by differences in cell death pathways: direct inhibition of PI3K/Akt was accompanied by rapid apoptotic cell death during G_2, whereas cells underwent mitotic transit and cell division followed by cell death during G_1 when both checkpoint and survival signaling were inhibited. Our results elucidate a novel function for PI3K/Akt as a survival factor during DNA damage-induced G_2 arrest and could have important pharmacological consequences for the application of response modulators in p53-deficient tumors with strong survival signaling.

INTRODUCTION

Successful treatment of human tumors by DNA damaging agents is limited by both natural (intrinsic) and acquired (drug-induced) resistance. Several studies suggest that the intrinsic resistance in many tumor types, including acute myeloid leukemia (AML), is closely associated with the capacity to undergo robust cell cycle arrest in G_2/M.[1-3] Generally, prolonged cell cycle arrest following DNA damage relies on p53-dependent mechanisms leading to increased expression of cell cycle regulators such as p21 and 14-3-3σ.[4,5] However, a subset of tumor cells with nonfunctional p53 is also able to undergo DNA damage-induced G_2 arrest mediated by the DNA damage checkpoint.[6,7]

Genotoxic stress activates the ATM and ATR nuclear kinases that transduce the signal to the downstream checkpoint kinases Chk1 and Chk2/Cds1, eventually leading to the inactivation of the master mitotic kinase Cdk1 and G_2 arrest (reviewed in ref. 8). Another important mechanism, which is lesser appreciated, allows cells to survive during prolonged cell cycle arrest due to active survival signaling.

There are two major anti-apoptotic signaling pathways that operate during G_2 and mitosis, one associated with the IAP protein, survivin, and another regulated by phosphatidylinositol 3-kinases, including Akt/PKB.[9,10] Expression levels of survivin have a prognostic impact in some tumors, but not in AML patients.[11] In contrast, constitutively active PI3K/Akt signaling has been firmly established as a major determinant of cell growth and survival in many different tumors, including AML, nonsmall cell lung cancer, colorectal cancer, melanomas and glioblastomas.[12-19]

The PI3K/Akt pathway may influence both checkpoint and survival signaling. So far, conflicting data have been reported concerning the role of Akt in the G_2 checkpoint. Akt has been shown to phosphorylate the Wee1 kinase resulting in inhibitory phosphorylation of Cdk1, thus blocking G_2/M progression.[20] In apparent contrast, it has also been reported that activated Akt shortens the G_2/M arrest induced by DNA damage.[21,22]

Another important function of PI3K/Akt signaling is to protect cells during mitosis, since chemical inhibition of PI3K/Akt is accompanied by mitotic catastrophe.[23]

Different strategies have been proposed in order to improve the antitumor activity of genotoxic agents, with emphasis on p53-deficient tumors. In one, the functionality of the DNA damage checkpoint is attenuated by cell cycle abrogators such as the staurosporine-derivative, UCN-01 (reviewed in ref. 24). An alternative strategy is aimed at blocking survival signaling in drug-treated cells. A crucial question is whether inhibition of multiple checkpoint/survival kinases may confer better efficacy in the potentiation of genotoxic agents, compared to targeting of a single pathway, in analogy with receptor tyrosine kinase inhibitors, where targeting of multiple tyrosine kinase pathways seems preferable.

Inhibition of multiple checkpoint/survival kinases may be approached in two conceptually different ways. First, we might chose to target a multi-functional signaling pathway such as PI3K/Akt. Alternatively, we might chose to use a pan-kinase inhibitor able to target both cell cycle and survival signaling. From this perspective, it is interesting that besides inhibition of the Chk1 checkpoint kinase, UCN-01 has also been reported to affect the PI3K/Akt pathway through inhibition of PDK1 kinase.[25] However, it is currently unknown to which degree the Akt-mediated effects contribute to the activity of UCN-01.

In this work, we wanted to clarify the contribution of PI3K/Akt signaling to prolonged DNA damage-induced G_2 arrest in a cellular context where Akt signaling is known to play a major role. We therefore compared the influence of UCN-01, which affects both the DNA damage checkpoint and PI3K/Akt-mediated survival signaling, the classical cell cycle abrogator caffeine, and the PI3K/Akt inhibitors wortmannin and LY294002, in p53-deficient M1 cells treated with the DNA damaging agent cisplatin.

Our results document the capacity of these p53-deficient cells to maintain G_2 arrest for prolonged periods of time, due to a combination of strong cell cycle and survival signaling. Interestingly, PI3K/Akt inhibition by itself had no influence, positive or negative, on the maintenance of G_2 arrest following genotoxic stress. However, this pathway was crucially involved in survival signaling in the DNA-damaged cells during prolonged cell cycle arrest, thus elucidating a novel role for PI3K/Akt. Finally, our results clearly demonstrate that Akt inhibition by itself was more potent than cell cycle abrogation and, more surprisingly, that simultaneous inhibition of the DNA damage checkpoint and PI3K/Akt signaling was no more active than checkpoint abrogation by itself. These results could have important pharmacological consequences for the clinical development of response modulators in p53-deficient tumor cells.

MATERIALS AND METHODS

Drugs, antibodies and chemicals. Cisplatin (Cisplatyl®) was purchased from Laboratoire Rhône Poulenc Rorer (Ivry-sur-Seine, France) while UCN-01 was kindly provided by Dr. Edward A. Sausville (University of Maryland, MD). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), propidium iodide, RNase A, caffeine, wortmannin and LY294002 were purchased from Sigma. Cell culture media, antibiotics and serum were from GIBCO (Paisley, UK). Monoclonal mouse anti-Cdk1/Cdc2, polyclonal rabbit anti-cyclin B1, polyclonal goat anti-actin antibodies were from Santa Cruz (Santa Cruz, CA). Anti-MPM-2 monoclonal mouse antibodies were purchased from DAKO (Carpinteria, CA). Polyclonal rabbit anti-phospho-Ser216 of Cdc25C, monoclonal rabbit anti-activated caspase-3, polyclonal rabbit anti-phospho-Ser374 of Akt, polyclonal rabbit anti-Akt, polyclonal rabbit anti-Bad, polyclonal rabbit anti-phospho-Ser136 of Bad were from Cell Signaling (Beverly, MA) and monoclonal mouse gamma-H2AX was from Upstate Biotechnology (Charlottesville, VA). Horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat IgG antibodies were obtained from Jackson ImmunoResearch Labs (West Grove, PA). FITC-conjugated anti-mouse or anti-rabbit antibodies were from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cell lines. The M1 acute myeloid leukemia (AML) cell line has been described previously.[26-29] Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were grown at 37°C in 5% CO_2/air atmosphere and screened routinely for Mycoplasma by the PCR method with Mycoplasma *Plus* PCR Primer Set (Stratagene, La Jolla, CA).

Cytotoxicity assay. Exponentially growing cells were exposed to the indicated drug concentrations for five days and the viability determined by the MTT assay. Alternatively, cells were exposed to cisplatin for 36 h, washed and incubated for 3–6 h in the absence or presence of response modulators followed by post-incubation in drug-free media for five days. The IC_{50} and IC_{90} values are defined as the drug concentration resulting in 50% and 90% reduction of viable cells, respectively, compared to untreated control cells.

Flow cytometry. The cell cycle distribution was measured by flow cytometry using an FACScan flow cytometer (Becton Dickinson) equipped with an argon laser to give 488 nm light. The cells were fixed in 70% ethanol at -20°C, rehydrated in PBS and stained with propidium iodide (20 μg/ml) and RNAse A (100 μg/ml) at room temperature for 30 min. The percentage of cells in each phase of the cell cycle was calculated by MultiPlus software (Phoenix Flow Systems, San Diego, CA).

For the immunofluorescence studies, cells were fixed in 70% ethanol overnight at -20°C, rehydrated in PBS and permeabilized with 0.1% Triton X-100 for 5 min on ice. For determination of activated caspase-3, cells were pre-fixed in 1% formaldehyde in PBS containing 0.2% picric acid for 15 min at room temperature. After one wash in PBS, the cells were first treated with 1% BSA/PBS to block non-specific staining and were then incubated with primary antibodies at 1:100 dilution for 1 h at room temperature. Following two washes in PBS containing 0.5% BSA and 0.2% Tween-20, cells were incubated with secondary antibodies diluted at 1:50 for 30 min at room temperature. After two additional washings with PBS with 0.5% BSA and 0.2% Tween-20, samples were stained in PBS containing 5 μg/ml propidium iodide and 100 μg/ml RNAse A for 30 min at room temperature and analyzed by flow cytometry. Indirect immunostaining for detection of the MPM-2 epitope was analyzed as described earlier.[29]

Western blot analysis. Cells were lysed in RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 8) containing a protease inhibitor cocktail (Roche Diagnostic, Meylan, France) and phosphatase inhibitors (50 mM sodium fluoride, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate) for 15 min on ice. Lysates were centrifuged at 20,000 g for 10 min at 4°C and supernatants collected. Protein concentrations in cellular lysates were determined by the BCA assay. Equal amounts (50 μg per lane) were loaded in Laemmli buffer and separated by the SDS-PAGE electrophoresis in polyacrylamide gels and transferred

onto PVDF membranes (Amersham Pharmacia Biotech). After transfer, membranes were blocked in 5% non-fat milk in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8) and washed in TBST buffer (TBS buffer containing 0.05% Tween-20). Membranes were incubated with primary antibodies diluted in TBST containing 0.5% bovine serum albumin at 1:100 (anti-cyclin B1, anti-Cdk1) or 1:1000 (anti-MPM-2 and anti-actin) for 1–6 h at room temperature. Alternatively, antibodies were diluted in TBST containing 5% fat-free milk at 1:1000 (anti-phospho-Ser216 Cdc25C and anti-Cdc25C, anti-phospho-Ser374 Akt, anti-Akt, anti-Bad and anti-phospho-Ser-136 Bad) and membranes were incubated overnight at 4°C. After three washes in TBST, membranes were incubated with secondary antibodies diluted at 1:40,000 in TBST for 1 h at room temperature. Results were revealed by the ECL kit (Amersham Pharmacia Biotech).

DNA fragmentation. DNA fragmentation was determined by a filter elution assay as described earlier after labeling with [^{14}C]-thymidine (0.05 μCi/ml for 48 h) followed by a 6 h chase in drug-free media.[30] Approximately 5 x 10^5 cells were used per filter.

Chromosome spreads and image analysis. Following drug exposure, cells were washed twice with PBS and chromosome spreads were prepared using Carnoy's fixation (methanol/acetic acid 3:1 v/v). Chromosomes were stained with 0.5 μg/ml propidium iodide for 5 min, washed twice with PBS and then mounted in 90% glycerol and 10 mM Tris-HCl, pH 8, containing 25 mg/ml diazabicyclo[2,2,2]octane (DABCO). All images were collected by a Leica DM/IRBE inverted microscope (Leica Microsystems, Wetzlar, Germany) with APO 100X oil 1.4 numerical aperture objective and a Leica TCS spectral confocal system with a krypton-argon dual-line laser. Images were further digitally processed for contrast enhancement by Adobe Photoshop.

RESULTS

Exposure of M1 cells to the DNA damaging agent, cisplatin, leads to prolonged G_2 arrest. M1 AML cells were exposed to cisplatin (2.5 μM, corresponding to the IC_{90} dose) and the cell cycle distribution was determined by flow cytometry analysis. The results show, that continuous cisplatin exposure was accompanied by prolonged S-phase transit followed by robust cell cycle arrest in G_2/M for at least 24 h (Fig. 1A). Once the cells had reached G_2, further drug exposure had no influence on either cell cycle progression or the final cytotoxicity (data not shown). After 48 h cisplatin-exposure, low levels of apoptotic DNA fragmentation and decreased viability was detected, corresponding to approximately 10% dead cells. Longer drug incubation led to progressively increased DNA fragmentation, accompanied by a decrease in mitochondrial transmembrane potential and appearance of cells with apoptotic features (data not shown). Staining of drug-treated M1 cells with antibodies directed against γ-histone H2AX, a surrogate marker for double-stranded DNA breaks (Fig. 1B), showed that the G_2-arrested cells contained several discrete γ-H2AX foci, consistent with induction of the DNA damage checkpoint.[31]

UCN-01 inhibits survival signaling in M1 cells. Previous studies suggest that UCN-01, otherwise known as a potent checkpoint abrogator, may also inhibit Akt signaling through inhibition of PDK1.[25] Our results confirm these findings in the M1 AML model. As shown in Figure 2A, both UCN-01 and wortmannin, a classical PI3K/Akt inhibitor, were rapidly able to inhibit the activating phosphorylation of Akt at Ser473 in nontreated M1 cells. In contrast,

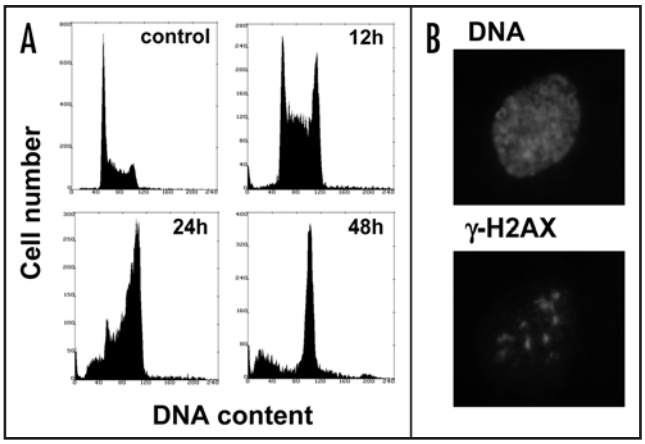

Figure 1. DNA damage following exposure of M1 acute myeloid leukemia cells to cisplatin leads to prolonged G_2 arrest. (A) DNA histograms of M1 cells exposed to 2.5 μM cisplatin for the indicated times. (B) Nuclear morphology and γ-histone H2AX staining of cells treated with 2.5 μM cisplatin for 36 h.

caffeine, a classical checkpoint abrogator, had no detectable influence on the phosphorylation status of Akt, even after 6 h incubation (Fig. 2A).

To determine if UCN-01 also affected survival signaling in cells with DNA damage, M1 cells were treated with cisplatin for 36 h, at which time more than 80% of the cells were arrested in G_2/M. As shown in Figure 2B, post-incubation of G_2-arrested cells with either UCN-01 or wortmannin was able to inhibit the activating phosphorylation of Akt at Ser473, although with different kinetics. Efficient inhibition of Ser473 phosphorylation of Akt was observed already after 1 h in the presence of wortmannin, while longer time was needed for UCN-01.

Prolonged G_2 arrest of drug-treated M1 cells is associated with an active DNA damage checkpoint. To establish the molecular mechanisms underlying the prolonged G_2 arrest of M1 cells following cisplatin-exposure, we characterized three key mitotic regulators known to be crucial for induction of G_2 arrest. These include the Cdc25C dual specificity phosphatase and the two components of the mitotic Cdk1/Cdc2 kinase complex: the Cdk1 catalytic subunit and the regulatory cyclin B1 subunit.

Phosphorylation of Ser216 of Cdc25C is a key step in the induction of transient G_2 arrest following DNA damage. To determine if Ser216 phosphorylation also played a role during prolonged G_2 arrest, cells were treated with cisplatin for 36 h and the presence of phosphorylated Ser216 was determined by a phosphoepitope-specific antibody. The results showed that prolonged G_2 arrest is associated with a strong signal for Ser216 phosphorylated Cdc25C (Fig. 2C, lane 2) in comparison with the untreated control cells, where the Ser216 phosphoepitope was barely perceptible (Fig. 2C, lane 1).

Cdk1 is activated by Cdc25C-mediated dephosphorylation of Thr14 and Tyr15. Western blot analysis of the catalytic Cdk1 subunit showed that the fast-migrating, hypophosphorylated active form of Cdk1 was dominating in untreated control cells (Fig. 2C, lane 1). In clear contrast, after 36 h cisplatin-exposure, the slower migrating, hyperphosphorylated inactive form of Cdk1 was principally present (Fig. 2C, lane 2).

It has been reported that prolonged G_2 arrest may be associated with reduced cyclin B1 levels, at least in some cells.[32,33] This does not appear to be an universal finding, since cyclin B1 levels were

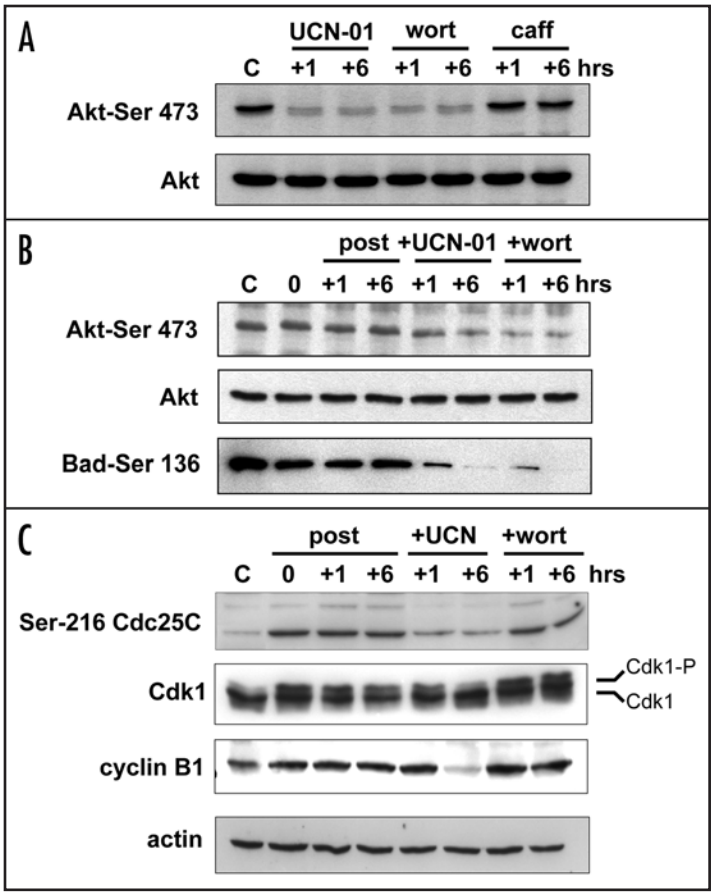

Figure 2. Effect of checkpoint abrogators (UCN-01, caffeine) and wortmannin on Akt and mitotic regulators in untreated and cisplatin-treated M1 cells. (A) Activating phosphorylation of Akt at Ser473 in untreated cells or in the presence of different modulators. Cells were exposed to 100 nM UCN-01, 10 μM wortmannin or 2 mM caffeine for the indicated times. For comparison, blots were also probed with antibodies toward total Akt (row 2). (B) Western blot analysis of the Ser473 phosphorylated, active form of Akt (row 1), total Akt (row 2) or the inactive, Ser136 phosphorylated form of the proapoptotic protein Bad (row 3). Cells were exposed to 2.5 μM cisplatin for 36 h (lane 0) and post-incubated in the absence or presence of 100 nM UCN-01 or 10 μM wortmannin for the indicated times. (C) Western blot analysis of Ser 216-phosphorylated Cdc25C (row 1), Cdk1 (row 2) and cyclin B1 (row 3) or beta-actin (row 4). M1 cells (lane C) were incubated with cisplatin for 36 h (lane 0) followed by post-incubation in drug-free media or in the presence of 100 nM UCN-01 or 10 μM wortmannin for 1 or 6 hours.

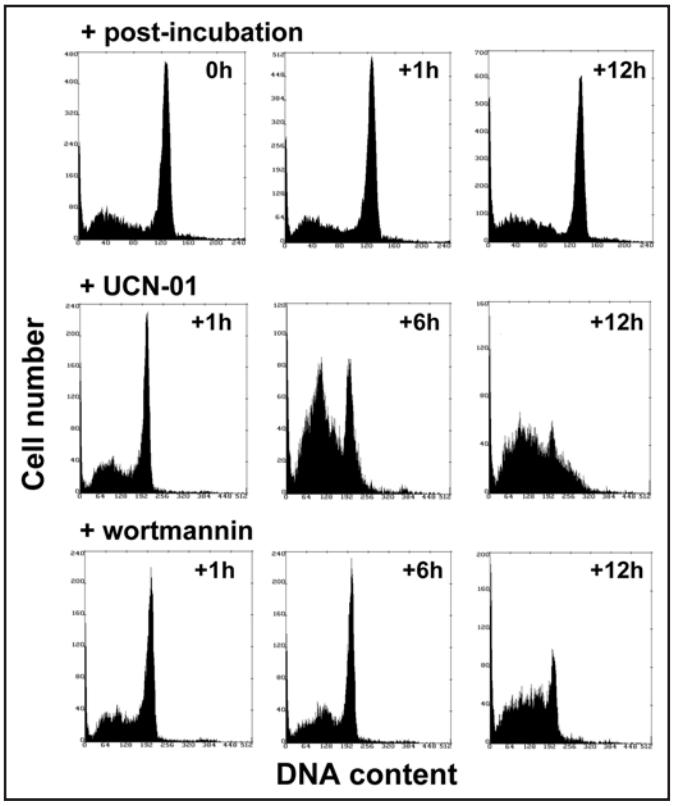

Figure 3. UCN-01, but not wortmannin, induces G_2 exit in long-term arrested M1 cells. Cells were exposed to 2.5 μM cisplatin for 36 h and post-incubated in the presence of 100 nM UCN-01 or 10 μM wortmannin for the indicated times followed by flow cytometry analysis.

increased, rather than decreased in G_2-arrested M1 cells, compared to untreated control cells (Fig. 2C third row, compare lanes 2 to 4 with lane 1).

The cell cycle abrogator UCN-01, but not the PI3K/Akt inhibitors wortmannin and LY294002, can overcome prolonged G_2 arrest. Since the PI3K/Akt pathway may play an active role in the G_2 to M transition, G_2-arrested M1 cells were post-incubated with a non-toxic dose of UCN-01 (100 nM), wortmannin (10–20 μM) or LY294002 (15 μM).[21,22,34] At these concentrations, UCN-01 is believed to principally inhibit the Chk1 checkpoint kinase, thus favoring the Ser216-dephosphorylated form of Cdc25C, while wortmannin and LY294002 inhibit phosphatidylinositol-3-kinases by different mechanisms.[35-40]

Post-incubation of the G_2-arrested cells in the presence of UCN-01 was accompanied by a dramatic decrease in cellular levels of phosphorylated Ser216 after only 1 h UCN-01 exposure (Fig. 2C, lanes 5–6), in clear contrast to cells post-incubated in drug-free media or in the presence of wortmannin for up to 6 h (Fig. 2C, lanes 3–4 and 7–8). In addition, post-incubation with UCN-01 was associated with dephosphorylation of Tyr15 of Cdk1 (Fig. 2C, row 2, lanes 5–6) while no obvious changes were observed when cells were post-incubated in drug-free media or with wortmannin (Fig. 2C, row 2, lanes 3–4 and 7–8).

Abrogation of both survival signaling and the DNA damage checkpoint by UCN-01 is associated with G_2 exit and mitotic transit. Flow cytometry analysis of drug-treated cells post-incubated with UCN-01 revealed that at least part of the G_2/M arrested cells rapidly divide and reenter G_1. At later incubation times, this was accompanied by the appearance of cells with a sub-G_1 and sub-G_2 DNA content, suggesting induction of apoptotic cell death (Fig. 3). In clear contrast, post-incubation of cisplatin-treated cells with wortmannin or LY294002 led to a progressive decrease of the G_2/M fraction and production of only a sub-G_2 peak representing cells dying out of G_2 or M (Fig. 3 and data not shown).

A hallmark of early mitosis is the appearance of mitosis-specific phosphorylation sites on numerous cellular proteins that are recognized by the monoclonal MPM-2 antibody.[41] To further characterize the influence of UCN-01 and wortmannin on the G_2-arrested cells, cisplatin-treated cells were post-incubated in the absence or presence of the two modulators and analyzed by biparametric flow analysis. The results show that 36 h cisplatin-exposure led to the accumulation of at least 80% of the cells with a 4N DNA content, virtually in the absence of mitotic MPM-2 staining (Fig. 4A).

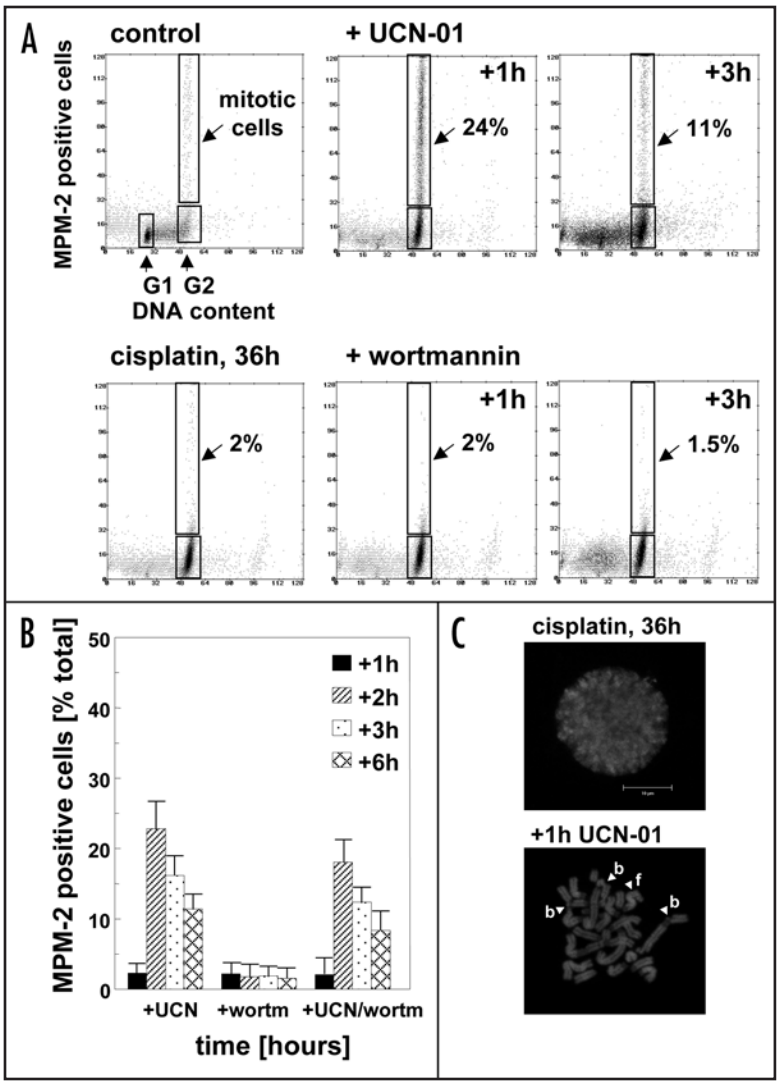

Figure 4. UCN-01 induces mitotic entry of long-term G_2-arrested M1 cells. (A) Biparametric flow cytometry analysis of cells treated with 2.5 μM cisplatin for 36 h followed by post-incubation in the absence or presence of 100 nM UCN-01 or 10 μM wortmannin for the indicated times. The numbers with the arrows indicate the fraction of mitotic MPM-2 positive cells. (B) the fraction of mitotic MPM-2 positive cells after post-incubation with 100 nM UCN-01, 10 μM wortmannin or both together for the indicated times. (C) Nuclear morphology of cells treated with 2.5 μM cisplatin for 36 h and post-incubated in the presence of 100 nM UCN-01 for 1 h. Chromosome lesions such as chromosome breaks (b) and end fusions (f) are indicated with arrows.

One to 3 h post-incubation in the presence of 50-100 nM UCN-01 was accompanied by a dramatically increased fraction of MPM-2 positive cells that reached 24% of the total by 1 h. At longer incubation times, the fraction of both MPM-2 positive and negative cells with a 4N DNA content decreased (Fig. 4B, left panel). Similar results were observed in the presence of caffeine, a well-known DNA damage checkpoint abrogator (data not shown). In clear contrast, no marked changes in MPM-2 reactivity were observed, when the G_2-arrested cells were post-incubated in drug-free media or in the presence of wortmannin (Fig. 4A and B) or LY294002 (data not shown). Interestingly, post-incubation of G_2-arrested cells with both UCN-01 and wortmannin was accompanied by the formation of MPM-2 positive mitotic cells with practically the same kinetics as observed in the presence of UCN-01 alone (Fig. 4B). Therefore, at least in this cellular model, wortmannin and LY294002 (and thus PI3K/Akt function) had no detectable influence, positive or negative, on the G_2 DNA damage checkpoint.

UCN-01 exposure of cisplatin-treated M1 cells was accompanied by the formation of cells with metaphase chromosomes, indicating that G_2 checkpoint override was accompanied by mitotic progression (Fig. 4C). Closer examination of the chromosome morphology in metaphase spreads revealed that many of these chromosomes showed abnormal features such as chromosome breaks (b) and end fusions (f) (Fig. 4C, arrows), indicative of abortive or non-completed DNA repair, consistent with the appearance of γ-H2AX foci (Fig. 1B) prior to checkpoint override.

MPM-2 reactivity is known to decrease following metaphase. An other biochemical marker of the metaphase to anaphase transition is proteolysis of cyclin B1.[42] Western blot analysis revealed a reduction in cyclin B1 protein levels when the G_2-arrested cells were post-incubated in the presence of UCN-01 (Fig. 2C, row 3), but not when the cells were post-incubated for the same time in drug-free media or in the presence of wortmannin (Fig. 2C, row 3). Together, these observations suggest that G_2-arrested cells treated with UCN-01, but not wortmannin, are not only able to enter but also transit mitosis. This was further confirmed by cell counting and morphological observations. As shown in Figure 5A (left panel), at least some of the cells post-incubated with UCN-01, were able to undergo cytokinesis, since the cell population contained small viable cells comparable to the size of G_1 cells. At the same time, the cell number increased for cisplatin-treated cells post-incubated with UCN-01 or caffeine, but not for cells post-incubated with wortmannin or in drug-free medium (Fig. 5A, right panel).

Both UCN-01 and Wortmannin induce rapid cell death in cisplatin-treated cells. Post-incubation of cisplatin-treated cells with UCN-01 or wortmannin was accompanied by rapid Ser136 dephosphorylation of Bad, which is associated with activation of this pro-apoptotic mediator (Fig. 2B). Similar results were observed for cells post-incubated with LY294002 (data not shown). Microscopic examinations revealed that some of the cisplatin-treated cells post-incubated with UCN-01 displayed a nuclear morphology typical of cells dying by mitotic catastrophe, with characteristic pulverized chromosomes (Fig. 5A, left panel, bottom). In addition, an increasing number of cells contained fragmented DNA as revealed by the presence of hypodiploid sub-G_1 or sub-G_2 peaks on the flow cytometry histograms (Fig. 3). To quantify this effect, a filter binding assay was used which permits the detection of both small and large DNA fragments formed as a result of apoptotic DNA cleavage. The results show that post-incubation of G_2-arrested cells with UCN-01 is accompanied by induction of DNA fragmentation that became particularly prominent after 6 h post-incubation with UCN-01 (Fig. 5B, left panel). In comparison, G_2-arrested cells post-incubated in drug-free media contained much lower levels of fragmented DNA. Addition of wortmannin to G_2-arrested cells induced an even more rapid increase of DNA fragmentation.

Proteolytic activation of an other apoptotic marker, caspase 3, which is a major executioner caspase, was determined by flow cytometry analysis of the cleaved, active form of caspase 3 in whole cells. Markedly increased caspase 3 activation was observed after 6 and 12 h

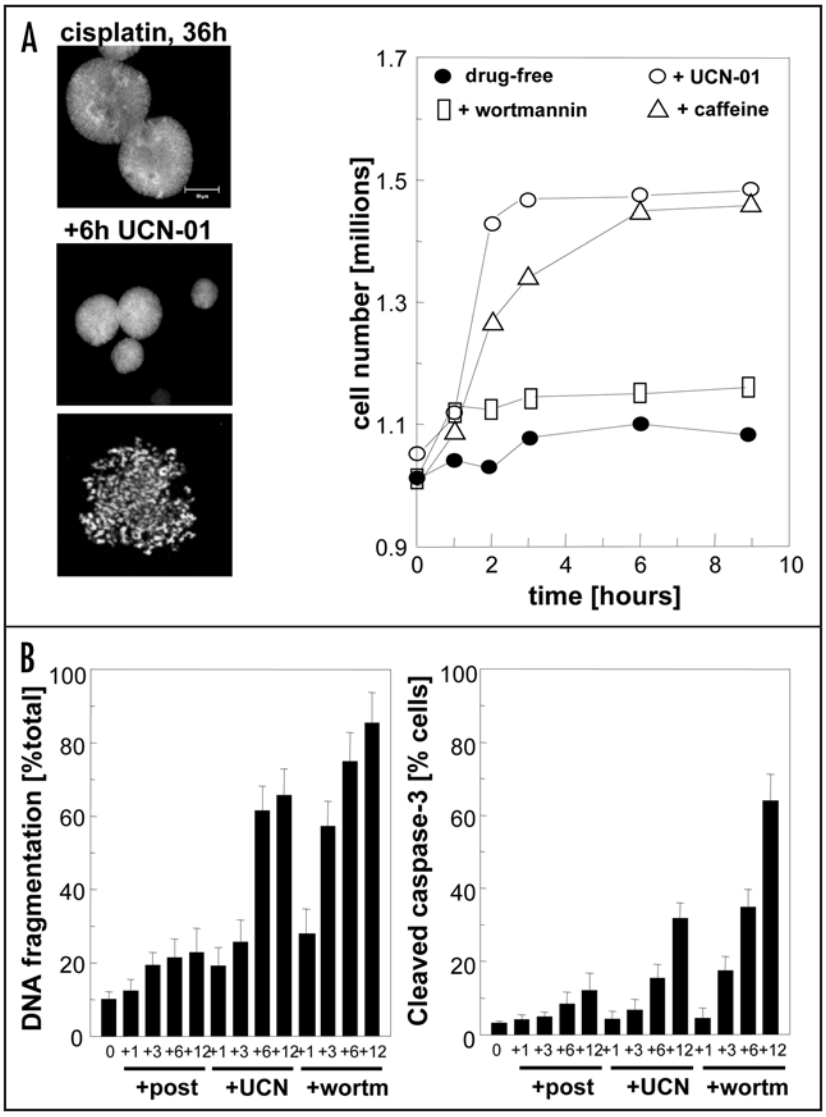

Figure 5. UCN-01 and wortmannin induce rapid apoptosis in long-term G_2-arrested M1 cells. (A) Changes in nuclear morphology (left panel) and cell number (right panel) of cells treated with cisplatin and post-incubated in drug-free medium or in the presence of 100 nM UCN-01, 10 μM wortmannin or 2 mM caffeine for the indicated times. (B) Apoptotic execution in M1 cells as determined by DNA fragmentation (left) or caspase-3 cleavage (right). Cells were exposed to 2.5 μM cisplatin for 36 h (lane 2) and post-incubated in the absence or presence of 100 nM UCN-01 or 10 μM wortmannin for the indicated times. Bars, standard deviation.

post-incubation with UCN-01 in comparison to cells post-incubated in drug-free media. Post-incubation with wortmannin was also accompanied by caspase 3 activation, which occurred more rapidly and to a greater extent than in the presence of UCN-01 (Fig. 5B, right panel), in agreement with the findings for DNA fragmentation (Fig. 5B, left panel).

Abrogation of the DNA damage checkpoint and/or PI3K/Akt signaling is accompanied by increased sensitivity to cisplatin. To characterize the influence of UCN-01 on the sensitivity to cisplatin, cells were exposed to different doses of cisplatin for 36 h, followed by 3 h post-incubation in the presence or absence of an otherwise non-toxic dose of UCN-01, and subsequent post-incubation in drug-free media for five days. The results showed, that DNA checkpoint override was accompanied by about two-fold increased sensitivity to cisplatin, compared to cisplatin alone (Fig. 6). In comparison, post-incubation with wortmannin or LY294002 was associated with about four-fold increased cytotoxicity. The activity of the checkpoint abrogator caffeine was comparable with UCN-01, sensitizing M1 cells about two-fold to cisplatin. The IC_{50} values obtained for cisplatin in the presence or absence or UCN-01, wortmannin, LY294002 or caffeine are statistically different ($p < 0.001$) as determined by Students t-test.

DISCUSSION

Modulation of the cellular response to DNA damaging agents by checkpoint abrogators or inhibitors of survival signaling is an active area of research, since it is believed that interference with these two types of signaling may enhance the therapeutic efficacy, in particular toward tumors with non-functional p53. A crucial question is whether inhibition of multiple checkpoint/survival pathways is preferential compared to inhibition of a single pathway.

The PI3K/Akt pathway plays a major role in several human tumor types which, at least in part, is linked to its activity as a survival factor. In addition, it has been proposed that PI3K/Akt contributes to checkpoint function during DNA damage-induced G_2 arrest, although it is not clear if PI3K/Akt enforces the checkpoint function or weakens it.[20-22,34] To clarify the roles of PI3K/Akt following DNA damage, we have characterized the mechanisms underlying prolonged G_2 arrest in a cellular context where Akt signaling is known to play a major role. For this, the influence of UCN-01, which affects both the DNA damage checkpoint and PI3K/Akt-mediated survival signaling, was compared with wortmannin and LY294002, inhibitors of the PI3K/Akt pathway, in cells treated with the DNA damaging agent cisplatin.[25,37] For comparison, we also included caffeine, a well-known DNA damage checkpoint abrogator, with no effect on the PI3K/Akt survival signaling (this study).

Our results show that UCN-01 was able to rapidly induce mitotic entry and cell division in G_2-arrested M1 cells following cisplatin treatment, as previously reported for other p53-deficient tumor cell lines.[7] Optimal effects of UCN-01 were observed between 50 and 100 nM, where all cells entered mitosis within 3 hr. However, a less synchronous effect of UCN-01 could be observed at doses as low as 10 nM (data not shown). These results are consistent with the potent effect of UCN-01 toward the Chk1 checkpoint kinase, a key player in the G_2 DNA damage checkpoint.[43-45]

The mechanisms underlying G_2 arrest in p53-deficient cells are only partly understood. The results presented here suggest that p53-independent mechanisms may not only be able to induce the DNA-damage G_2 checkpoint, but also to maintain it for prolonged periods of time, at least in cells with strong survival signaling. However, the prolonged G_2 arrest can be overcome rapidly by both UCN-01 and caffeine, suggesting an essential role for the ATM/ATR-Chk1-Cdc25B/C axis not only for the induction, but also in the maintenance of the DNA damage checkpoint.

In this study, we observed neither positive nor negative effects of the PI3K/Akt pathway on the established G_2 arrest. It is possible that

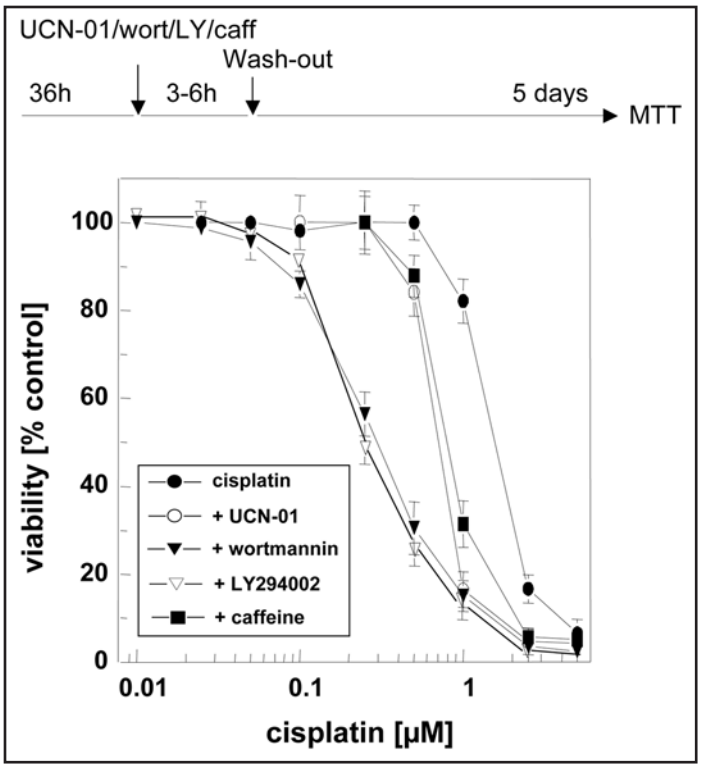

Figure 6. Cytotoxic activity of cisplatin in the absence or presence of response modulators. M1 cells were treated with cisplatin for 36 h, rinsed and post-incubated in the absence or presence of 100 nM UCN-01 for 3 h, 10 μM wortmannin, 15 μM LY294002 for 3 h or 2 mM caffeine for 6 h, followed by post-incubation in drug-free medium for five days and the determination of viable cells by the MTT assay. Bars, standard deviation.

the role of PI3K/Akt in the DNA damage checkpoint depends on cellular context and requires cross-talk with other signaling pathways, which may differ between tumor cells. Alternatively, Akt activity may depend on the mechanisms leading to activation of the DNA damage checkpoint. Accordingly, overexpression of activated Akt in glioblastoma cells has been shown to inhibit activation of checkpoint kinase Chk2 following temozolomide treatment.[34]

Recent studies show an important function for PI3K/Akt in protection of mitotic cells from cell death.[23] The results presented here expand the protective role of PI3K/Akt to DNA damage-induced G_2 arrest, most likely through inactivation of the proapoptotic protein Bad by Ser126 phosphorylation. Inhibition of the survival signaling by wortmannin or LY294002 was accompanied by rapid induction of apopototic cell death in G_2. Interestingly, UCN-01 was also able to suppress Bad phosphorylation, although with slower kinetics, which may explain why the damaged cells had sufficient time for mitotic transit and cell division. These results elucidate a novel function for PI3K/Akt as a survival factor during DNA damage-induced G_2 arrest.

An important pharmacological question is why wortmannin and LY294002, that only inhibit the PI3K/Akt pathway, were more potent modulators of cisplatin-induced cytotoxicity compared to UCN-01 which inhibits both checkpoint function and survival signaling. One possible explanation is that UCN-01 might be less potent/ slower than wortmannin and LY294002 because it is an indirect inhibitor of the PI3K/Akt signaling pathway through PDK1 inhibition, which would provide the cisplatin-treated cells sufficient time to progress through mitosis and divide before a sufficient pool of Akt became inactivated.[25] This possibility is supported by our data, which show that the kinetics of decreased Akt Ser473 phosphorylation, as well as of decreased Bad Ser126 phosphorylation, is slower for UCN-01 than for wortmannin and LY294002 (Fig. 2B and data not shown).

It is possible that G_2 checkpoint abrogation may have several, partially opposing, effects. On one hand, the cells will enter mitosis with damaged genomes, which makes them more prone to undergo mitotic catastrophe.[24] In agreement, both UCN-01 and caffeine increased the cytotoxic activity of cisplatin. Our results suggest that the DNA-damaged cells were able to transit and exit mitosis only to die in G_1. One explanation why apoptosis first occurs in G_1 might be linked to the relative strength of survival signaling. The expression of Akt is cell cycle regulated with minimal levels observed during G_1, similar to survivin-regulated survival signaling, making the damage cells more vulnerable during G_1.[9,46] On the other hand, the two PI3K/Akt inhibitors which induced apoptotic cell death during G_2 were more efficient than the two compounds which induced mitotic transit. The explanation for this might be that the absence of functional p53 in the M1 cells would allow a fraction of the DNA-damaged cells to escape G_1 and reenter the cell cycle. This hypothesis is in agreement with our current understanding of cell fate following mitotic catastrophe (reviewed in refs. 47 and 48).

Several current studies aim to evaluate the efficacy of pan-kinase inhibitors targeting multiple signaling pathways. In the case of receptor tyrosine kinases, this approach seems successful, resulting in improved clinical activity. In contrast, a recent attempt to inhibit different checkpoint kinases individually, or in combination, indicated that Chk1 inhibition alone was sufficient to sensitize cancer cells to widely used chemotherapeutic agents including doxorubicin, camptothecin and 5-fluorouracil. Unexpectedly, downregulation of additional targets, such as Chk2 or MK2 not only failed to improve efficacy but actually diminished the efficacy achieved with Chk1 inhibition alone.[45] In the current study, we show that efficient inhibition of the PI3K/Akt pathway alone is sufficient to sensitize p53-deficient cancer cells to cisplatin, while additional cell cycle abrogation at the best failed to improve efficacy.

In conclusion, we here show that UCN-01 is able to overcome prolonged G_2 arrest as well as of inhibiting the activating phosphorylation of Akt in both undamaged and DNA-damaged cells. In contrast, wortmannin and LY294002 rapidly inhibited survial signaling without any detectable influence on checkpoint function. In spite of the dual activities of UCN-01, it was less efficient in enhancing the cytotoxic effect of cisplatin than wortmannin and LY294002, possibly because the loss of p53 function in these cells allows a fraction of the DNA-damaged cells to exit G_1 and reenter the cell cycle. These studies elucidate a novel function for PI3K/Akt as a survival factor in G_2-arrested cells and provide guidelines for the development of response modulators in p53-deficient tumor cells.

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