Identification of a checkpoint modulator with synthetic lethality to p53 mutants

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The G_2 checkpoint is an indispensable pathway for cancers lacking p53 function, for delaying cell cycle progression, and for completing DNA repair. Therefore, disruption of this pathway is expected to offer selective therapy for these highly prevalent cancers. The aim of this study was to identify an inhibitor of the G_2 checkpoint including the ataxia–telangiectasia-mutated and Rad3-related checkpoint kinase 1 pathway that selectively suppresses the growth of p53-deficient cells. To obtain molecules with a novel mechanism of action, we constructed a high-throughput screening system that detected abrogation of the G_2 checkpoint in X-irradiated HT-29 cells. The screening resulted in identification of a guanidine analog, CBP-93872 that dose dependently inhibited the G_2 checkpoint induced by DNA damage. Interestingly, CBP-93872 directly suppressed the growth of p53-mutated cancer cell lines with wild-type CDKN2A by eliciting G_1 arrest, but not CDKN2A-deleted and/or wild-type p53 lines. CBP-93872 decreased phospho-cdc2 Y15 by inhibiting phosphorylation of Chk1, but did not suppress phospho-Chk2 or the kinase activities of either Chk1 or Chk2 in cellular or cell-free assays. These results suggest that a checkpoint modulator through suppression of Chk1 phosphorylation provides synthetic lethality to p53-deficient cells. *Anti-Cancer Drugs* 22:986–994 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Most cancer cells have genetic alterations in p53 or $p16^{INK4a}$ pathway molecules; thus, these molecules are attractive targets for anticancer therapy. To exploit the p53 mutation, clinical trials are under way using p53 gene therapy, MDM2 inhibitors, CDK2/4/6 inhibitors, and G_2 checkpoint inhibitors. The G_2 checkpoint is an indispensable biological response to DNA damage, especially for p53-mutated cells that have no functional G_1 checkpoint and thus depend solely on the G_2 checkpoint for DNA repair. Therefore, drugs that inhibit the G_2 checkpoint have been explored as promising agents in combination with DNA-damaging anticancer drugs [1].

Among the G_2 checkpoint inhibitors, caffeine has been one of the most frequently investigated agents and increases the cytotoxic effects of radiation on p53-mutated cells [2–4]. Additional G_2 checkpoint inhibitors such as UCN-01 [5], CEP-3891 [6], AZD7762 [7], and isogranulatimide [8,9] are potent checkpoint kinase 1 (Chk1) inhibitors, and many other agents targeting Chk1 are being investigated [10]. On the basis of in-vitro screening, PD0166285 was found to inhibit Wee1 and Myt1 [11,12]. CGK733 is an ataxia–telangiectasia-mutated (ATM) and ataxia–telangiectasia-mutated and Rad3-related (ATR) inhibitor that blocks Chk1 and checkpoint kinase 2 (Chk2) phosphorylation [13,14]. On account of their genomic instability and lack of the G_1 DNA-damage checkpoint, p53-mutated cancers depend strongly on the G_2 checkpoint for their survival, even without DNA-damaging therapy. Thus, it is possible that a G_2 checkpoint inhibitor alone may become a selective anticancer drug for p53-mutated cancers. However, few studies have been conducted to evaluate the possible application of G_2 checkpoint inhibitors as monotherapeutic agents.

Our study aimed to identify small-molecule inhibitors that are directly efficacious toward p53-mutated cells through novel mechanisms of action. We developed a unique screening system using p53-mutated cells with G_2 checkpoint abrogation as its output. Subsequently, growth-inhibition activity and the mechanism of action of the expedient compound were examined.

Materials and methods

Cell lines

Cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and cultured in the growth media described below containing 10% fetal

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bovine serum, except for the BxPC-3 growth medium, which contained 15% fetal calf serum: HT-29 in McCoy's 5A medium containing D-glucose (4.5 g/l); MDA-MB-231 in Leibovitz's L-15 medium containing $NaHCO_3$ (0.7 g/l); T47D in RPMI-1640 containing D-glucose (4.5 g/l), HEPES (10 mmol/l), sodium pyruvate (1 mmol/l), and insulin (0.2 U/ml); A549 in Dulbecco's Modified Eagle's Medium containing D-glucose (4.5 g/l); SW837 in Leibovitz's L-15 Medium; MCF-7 in Eagle's Minimum Essential Medium containing nonessential amino acid (0.1 mmol/l), sodium pyruvate (1 mmol/l), and insulin (0.01 mg/ml); NCI-H460, NCI-H23, NCI-H522, and BxPC-3 in RPMI1640; PC-3 in RPMI-1640 containing L-glutamine (4 mmol/l) and sodium bicarbonate (1.5 g/l); MIA PaCa-2 in Dulbecco's Modified Eagle's Medium containing L-glutamine (4 mmol/l), glucose (4.5 g/l), sodium bicarbonate (1.5 g/l), and 2.5% horse serum; and MRC-5 and WI-38 in Eagle's Minimum Essential Medium.

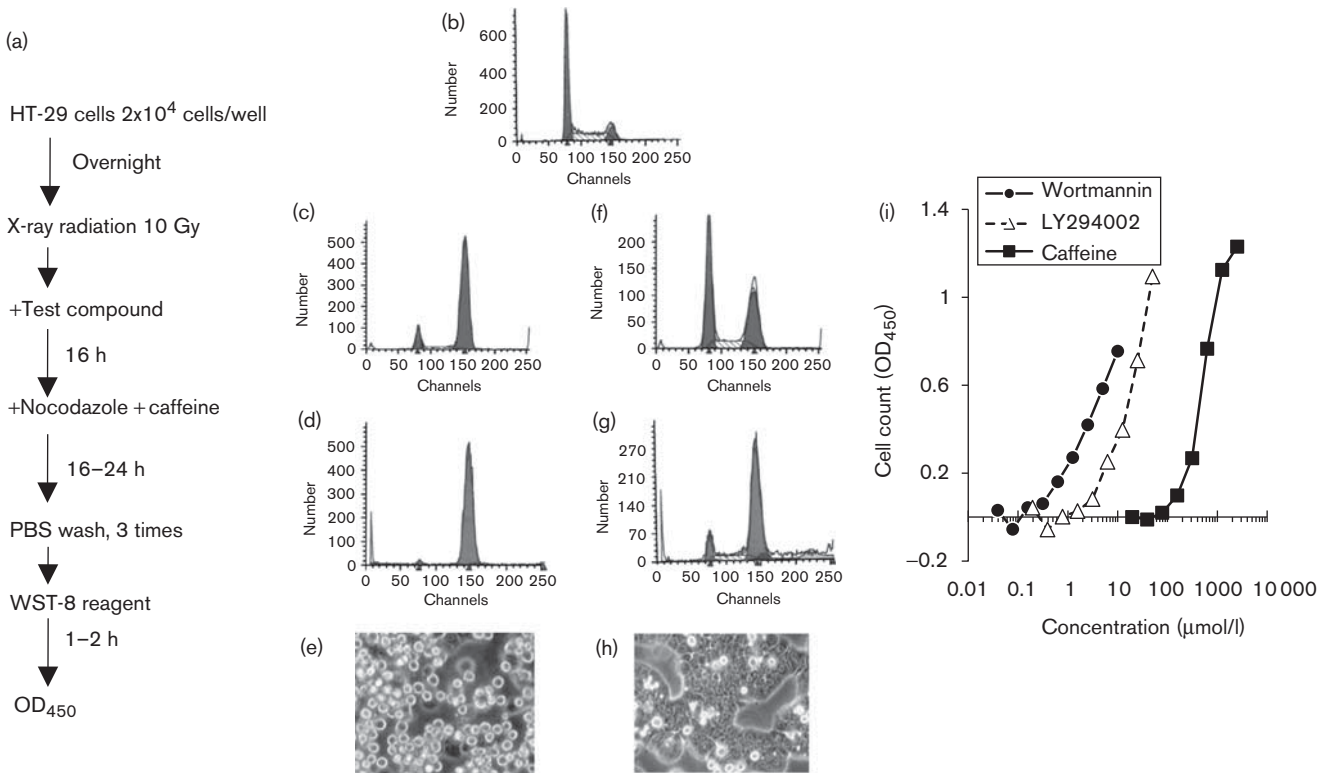
Drugs

Caffeine was purchased from Nacalai Tesque (Kyoto, Japan). Nocodazole, camptothecin, wortmannin, LY-294002, and hydroxyurea were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

High-throughput screening procedure

HT-29 cells were plated at a density of 2×10^4 cells/100 µl onto each well of 96-well plates and then cultured at 37°C overnight until attached to the bottom of the plates. Cells were irradiated with 10-Gy X-rays using an MBR-1520R system (Hitachi Medico Technology, Tokyo, Japan). Ten microliters of the test compounds was added to the wells (final concentration, 10 µmol/l) and was cultured for 16 h. Subsequently, 40 µl of medium containing caffeine (final concentration, 2 mmol/l) and nocodazole (final concentration, 0.1 µg/ml) was added and incubated for a further 16–24 h until the cells detached from the plates. The plates were then washed with phosphate-buffered saline (PBS) three times to remove the detached cells. Finally, 100 µl of culture medium containing 5 µl of Cell Counting Kit-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added, and absorbance at 450 nm was measured after 1-h incubation at 37°C see flow diagram in Fig. 1a).

Fig. 1



Development of cell-based high-throughput assay for G_2 checkpoint abrogation. (a) Flow diagram of the assay. Asynchronized HT-29 cells (b) were X-irradiated and then treated with dimethyl sulfoxide (DMSO) as a negative control (c) or caffeine as positive control (f) for 16 h. To push the G_2-arrested cells to M phase, further incubation with caffeine and nocodazole was conducted. After 16–24 h of incubation, DMSO-treated cells (c) were arrested at M phase (d and e), whereas caffeine-treated cells (f) were arrested at S–G_2 phase (g and h). (i) The assay system was evaluated with known G_2-checkpoint inhibitors. OD, optical density; PBS, phosphate-buffered saline.

Secondary screening

To eliminate agents that independently induced G_1, S, or G_2 arrest, primary target compounds were screened using a secondary screening system as follows. Similar to the primary screening system, HT-29 cells were plated and cultured at 37°C overnight. Cells were irradiated with 10-Gy X-rays, treated with 10 µl of test compounds and nocodazole (final 0.1 µg/ml) and cultured for 16 h. The plates were washed with PBS three times to remove the detached cells. Finally, 100 µl of culture medium containing Cell Counting Kit-8 reagent was added, and absorbance at 450 nm was measured after 1-h incubation at 37°C. Compounds with positive signals were eliminated.

Mitotic trap assay

HT29 cells were X-irradiated (10 Gy) and treated with the test compounds in the presence of nocodazole (0.15 µg/ml) for 24 h. The cells were harvested by treatment with trypsin/EDTA, and both attached and detached cells were combined and collected by centrifugation at 1000 g for 2 min at 4°C. The cells were resuspended in 30 µl of PBS and then added to 270 µl of PBS solution containing 3.7% formaldehyde, 1% NP-40, and Hoechst 33258 (10 µg/ml; Sigma-Aldrich) and held on ice for 10 min. More than 200 cells were counted and the M-phase ratio was calculated.

Cell cycle analysis

Synchronized or asynchronized cells were plated and cultured overnight at 5×10^4 cells per 35-mm dish. The cells were then X-irradiated (10 Gy) or treated with camptothecin (10 nmol/l) and were incubated overnight with the test compounds. The cells were harvested by treatment with trypsin/EDTA (Gibco, Carlsbad, California, USA), and both attached and detached cells were combined and collected by centrifugation at 1000 *g* for 2 min at 4°C. DNA staining for cell cycle analysis was conducted using a Cycletest Plus kit (Becton Dickinson, Heidelberg, Germany). The stained cells were analyzed using the LSR flow cytometer (Becton Dickinson). The percentage of cells in each cell cycle phase was calculated using the CellQuest and ModFit LT software packages (Becton Dickinson).

G_1-phase synchronization

HT-29 cells were synchronized at the G_1 phase using the double thymidine block method. In brief, HT-29 cells were seeded in a 35-mm dish and cultured with thymidine (0.5 mol/l) for 16 h. The cells were washed with warmed PBS twice, and culturing was continued using prewarmed culture medium without thymidine for 8 h. The medium was changed to fresh prewarmed medium with thymidine (0.5 mol/l), and the cells were cultured for an additional 16 h. The cells were then washed with warmed PBS twice and used for cell cycle or phosphorylation analysis.

Cell-growth inhibition assay

Cells were plated onto 96-well plates at a density of 2000–5000 cells per well and cultured overnight and then incubated with the test compounds for 72 h. Living cells were detected by incubation with 10 µl of Cell Counting Kit-8 reagent for 1 h at 37°C, and absorbance at 450 nm was measured.

Western blot analysis

HT-29 cells were plated onto six-well plates and cultured overnight. The cells were treated with 10-Gy X-irradiation, hydroxyurea (1 mmol/l), or 30 J/m^2 of UV irradiation (Stratalinker, Stratagene, California, USA) and then incubated with CBP-93872 (20 µmol/l) or caffeine (2 mmol/l) for 16 h. The cells were then washed with PBS and lysed in Cell Lysis Buffer (Cell Signaling Technology Inc., Beverly, Massachusetts, USA) containing PMSF (0.1 mol/l), Phosphatase-Inhibitor Cocktails 1 and 2 (Sigma-Aldrich), and Complete Mini EDTA-Free (Roche Applied Science, Mannheim, Germany). Protein concentrations were determined using a DC Protein Assay Kit (BioRad, Munich, Germany). Equal amounts of protein were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was then incubated with the primary antibodies. Phospho-ATR Ser428 (cat. no. 2853S), ATM D2E2 XP (cat. no. 2873S), phospho-Chk1 Ser317 (cat. no. 2344), phospho-Chk1 Ser345 (cat. no. 2348), phospho-Chk2 Thr68 (cat. no. 2197), Chk1 (cat. no. 2345) and Chk2 (cat. no. 2662), and phospho-Cdc2 Tyr15 (cat. no. 9111) were purchased from Cell Signaling Technology Inc.; phospho-ATM Ser1981 (cat. no. ab81292) and ATR (cat. no. ab2905) from Abcam (Cambridge, Massachusetts, USA); and Phospho-Histone H3 Ser10 (cat. no. 06–570) from Millipore (Billerica, Massachusetts, USA). Primary antibodies were detected with a peroxidase-conjugated anti-rabbit IgG antibody (cat. no. 7074; Cell Signaling Technology Inc.), enhanced with ECL Plus Western Blotting Detection Reagent (GE Healthcare, Piscataway, New Jersey, USA), and scanned using a LAS-4000 Lumino Image Analyzer (Fujifilm, Tokyo, Japan).

Mutation status and gene expression

Somatic mutation data for p53 and CDKN2A were derived from the COSMIC database previously described (*http://www.sanger.ac.uk/cosmic*) [15]. Analysis of high or low CDKN2A gene expression was conducted using the U133A plus GeneChip System (Affymetrix, Santa Clara, California, USA) based on whether the signal for the transcript was above or below 500.

Results

Construction and validation of a cell-based screening assay for G_2 checkpoint inhibition

To identify a G_2 checkpoint inhibitor based on a novel mechanism of action independent of known checkpoint

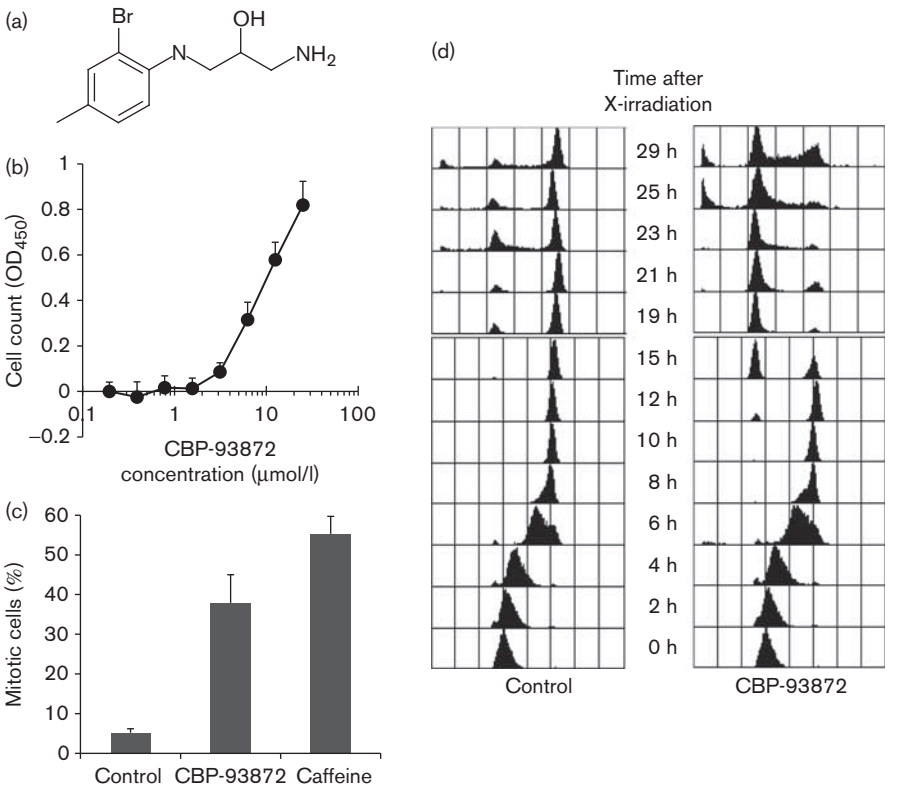
factors such as Chk1, we constructed a high-throughput screening system using the biological responses of p53-deficient cells. Two key attributes of cancer cells were indispensable for our screening system: (a) exhibiting clear G_2 arrest after X-irradiation but later abrogated by treatment with a reagent such as caffeine and (b) detaching from the culture plate in the M-phase after nocodazole treatment, to simplify detection and to increase throughput for screening. Thus, we selected HT-29 cells for the screening assay.

When asynchronized HT-29 cells (Fig. 1b) were X irradiated and incubated with dimethyl sulfoxide (DMSO) for 16 h, G_2 arrest was induced and continued for at least 40 h after irradiation (Fig. 1c). When caffeine and nocodazole were added, the cells were pushed out of G_2 arrest to M arrest in 8–16 h and continued for at least 24 h (Fig. 1d); most of the cells detached from the plate and were undetectable after PBS washing (Fig. 1e). In contrast, when the cells were incubated with caffeine as a representative G_2 checkpoint inhibitor, the G_2 checkpoint was abrogated and the cell cycle proceeded to the G_1 phase (Fig. 1f). After an additional 16–24 h incubation with caffeine and nocodazole, the cells were still arrested at the G_2 phase (Fig. 1g) and adhered to the plate after washing with PBS (Fig. 1h). To validate the screening system, we tested wortmannin and LY294002, known inhibitors of the G_2 checkpoint, and phosphatidylinositol 3-kinase. Detected cell signals increased dose proportionately with both wortmannin (0.3–10 μmol/l), LY294002 (3.1–50 μmol/l), and with caffeine (156–2500 μmol/l; Fig. 1i).

Cell-based screening identified an inhibitor of G_2 checkpoint

High-throughput screening of approximately 100 000 chemical compounds identified 95 primary hits. In the secondary screening step, to eliminate pseudopositives that potently induce cell cycle arrest at G_1, S, or G_2, compounds that enhanced cell attachment, even in the presence of nocodazole, were excluded. Ultimately, we identified CBP-93872 as a target compound (Fig. 2a). CBP-93872 is a guanidine analog that has been identified previously as a β-adrenergic-blocking agent [16]. CBP-93872 showed dose-dependent G_2 checkpoint abrogation activity (Fig. 2b) and also exhibited approximately 70%

Fig. 2



CBP-93872 abrogates the G_2 checkpoint induced by X-irradiation. (a) Chemical structure of CBP-93872, isolated by the screening. (b) CBP-93872 shows dose-dependent activity in the cell-based G_2-checkpoint abrogation assay. Error bars represent standard deviation ($n=3$). (c) G_2-checkpoint abrogation was evaluated using the mitotic trap method; the percentage of mitotic cells compared with that of unirradiated cells is shown. (d) Synchronized cells at G_1 were X-irradiated and then incubated with a fresh thymidine-free medium containing dimethyl sulfoxide or CBP-93872 (20 μmol/l). Cell cycle distributions were analyzed at the indicated time points.

activity of caffeine using a mitotic trap assay that detected M-phase cells produced by G_2 checkpoint inhibition in X-irradiated HT-29 cells (Fig. 2c).

To further clarify the inhibitory activity of CBP-93872 on the G_2 checkpoint, the cell cycle distribution after X-irradiation was examined using HT-29 cells synchronized at G_1 using the double-thymidine block method. Control cells treated with DMSO reached the G_2 phase 10–12 h after irradiation, and G_2 arrest continued for at least 29 h. When irradiated cells were incubated with CBP-93872, G_2 arrest was abrogated and progressed to G_1 phase at 15 h, and then the cells had re-entered the S–G_2 phase by 29 h. An increased number of sub-G_1 cells was observed at 25–29 h (Fig. 2d). Abrogation of the G_2 checkpoint was also observed in other p53-mutated cell lines such as PC-3, prostate cancer, and PAN-7, a pancreatic cancer cell line (data not shown).

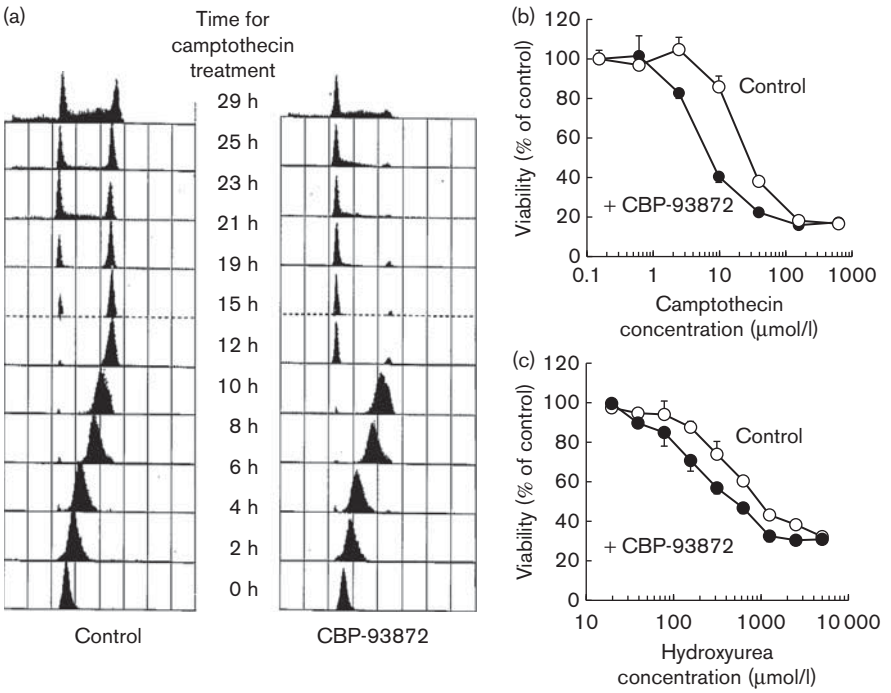
CBP-93872 inhibits the G_2 checkpoint induced by camptothecin

We further examined whether CBP-93872 sensitizes HT-29 cells to camptothecin, which induces a single-strand break in DNA by forming a ternary complex with topoisomerase I and DNA. Synchronized HT-29 cells were treated with camptothecin (10 nmol/l) and harvested at appropriate time points, and the cell cycle distributions were examined. Although 10 nmol/l of camptothecin induced clear G_2 arrest in DMSO-treated cells at 15 h, CBP-93872 completely inhibited the arrest (Fig. 3a). When combined with camptothecin or hydroxyurea, which inhibits DNA replication by suppressing ribonucleotide reductase, CBP-93872 exhibited enhanced cellular cytotoxicity against HT-29 cells as reported for other G_2 checkpoint inhibitors (Fig. 3b and c). These results indicate that CBP-93872 has the ability to abrogate the G_2 checkpoint induced by DNA-damaging agent, in addition to X-irradiation, and that CBP-93872 enhances its cytotoxic activity in p53-deficient cells.

CBP-93872 specifically inhibits the growth of p53-deficient cells with wild-type CDKN2A

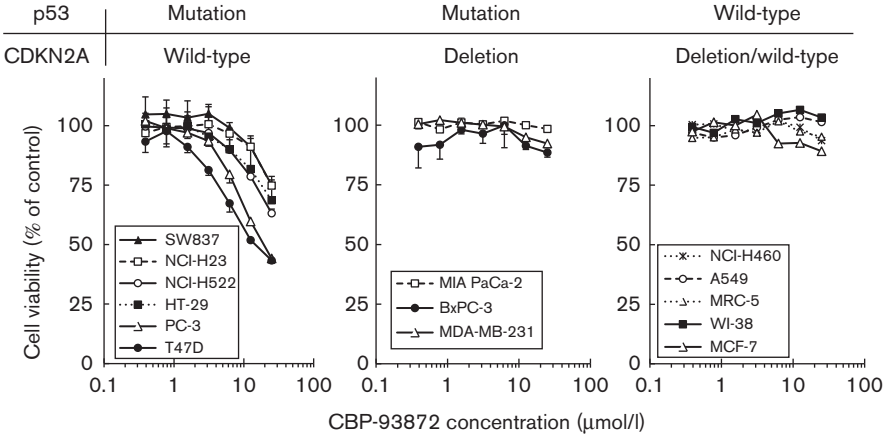
In p53-mutated cells, the DNA repair function is diminished, and constitutive activation of checkpoint controls must occur for these cells to survive. Thus, it was considered possible that CBP-93872 alone might exhibit strong cell growth inhibition. We tested the effect of CBP-93872 alone on cell-growth inhibition in 12 cancer cell lines and two normal lung fibroblasts with different p53 statuses. As expected, six of the nine cell lines with p53 mutations were sensitive to CBP-93872. However, three of the p53-deficient cell lines were resistant to CBP-93872 treatment. Although researching gene alterations related to G_1 progression in these cell lines, such as Rb or cyclin

Fig. 3



CBP-93872 inhibits the X-ray-induced G_2 checkpoint in synchronized HT-29 cells. (a) Synchronized cells at G_1 were incubated with a fresh thymidine-free medium containing dimethyl sulfoxide or CBP-93872 (20 μmol/l) together with camptothecin. Cell cycle distributions were analyzed at the indicated time points. Cytotoxicity of CBP-93872 in combination with camptothecin (b) or hydroxyurea (c). Cell viability was assessed using WST-8 assay as described in 'Materials and methods.' Error bars represent standard deviation ($n=3$).

Fig. 4



CBP-93872-mediated cell-growth inhibition depends on p53 and CDKN2A gene statuses. Cells were treated with CBP-93872 for 3 days and cell viability was assessed using WST-8 assay as described in 'Materials and methods'. Proliferation curves are shown with respect to TP53 or CDKN2A statuses: mutated TP53 and wild-type CDKN2A (left), mutated TP53 and deleted CDKN2A (middle), and wild-type TP53 and deleted CDKN2A (right).

D1, we found that two of the p53 mutants had different CDKN2A statuses, wild-type or deletion, which could potentially result in differing responses. Interestingly, sensitive cell lines had mutated p53 and wild-type CDKN2A, and resistant cell lines had mutated p53 and deleted/mutated CDKN2A. Cells with wild-type p53 including normal lung fibroblasts also showed the resistant phenotype, regardless of CDKN2A status (Fig. 4). These results indicate that p53 and CDKN2A gene status may be determinants for the overall cytotoxicity of CBP-93872.

Lack of G_1 arrest contributes to the growth of $p16^{INK4a}$ null cells

Gene expression of $p16^{INK4a}$, a transcript produced from CDKN2A locus, correlated with the mutation status of CDKN2A: SW837, NCI-H23, HCI-H522, PC-3, T47D, and HT-29 have the wild-type gene and showed high mRNA expression (> 500), whereas MIA PaCa-2, BxPC-3, MDA-MB-231, NCI-H460, A549, and MCF-7 have the deleted gene and showed low signals (< 500) in our Gene Chip analysis. To elucidate how $p16^{INK4a}$ affects the growth-suppression activity of CBP-93872, cell cycle distributions for three breast cancer cell lines, representative of different p53 gene statuses, were examined after treatment with CBP-93872. The results indicated that CBP-93872 resulted in potent G_1 arrest in the T47D (p53-mutated and $p16^{INK4a}$ normal) cell line, but not in the MDA-MB-231 (p53 mutated and $p16^{INK4a}$ null) or MCF-7 (p53 wild type and $p16^{INK4a}$ null) cell lines (Fig. 5). In addition, the selectivity of cell cycle arrest by CBP-93872 was quite different from that of caffeine, which equally inhibited the growth of the three breast cancer cell lines regardless of p53 or $p16^{INK4a}$ status, suggesting that CPB-93872 may have a different mode of action than caffeine, an ATM and ATR inhibitor.

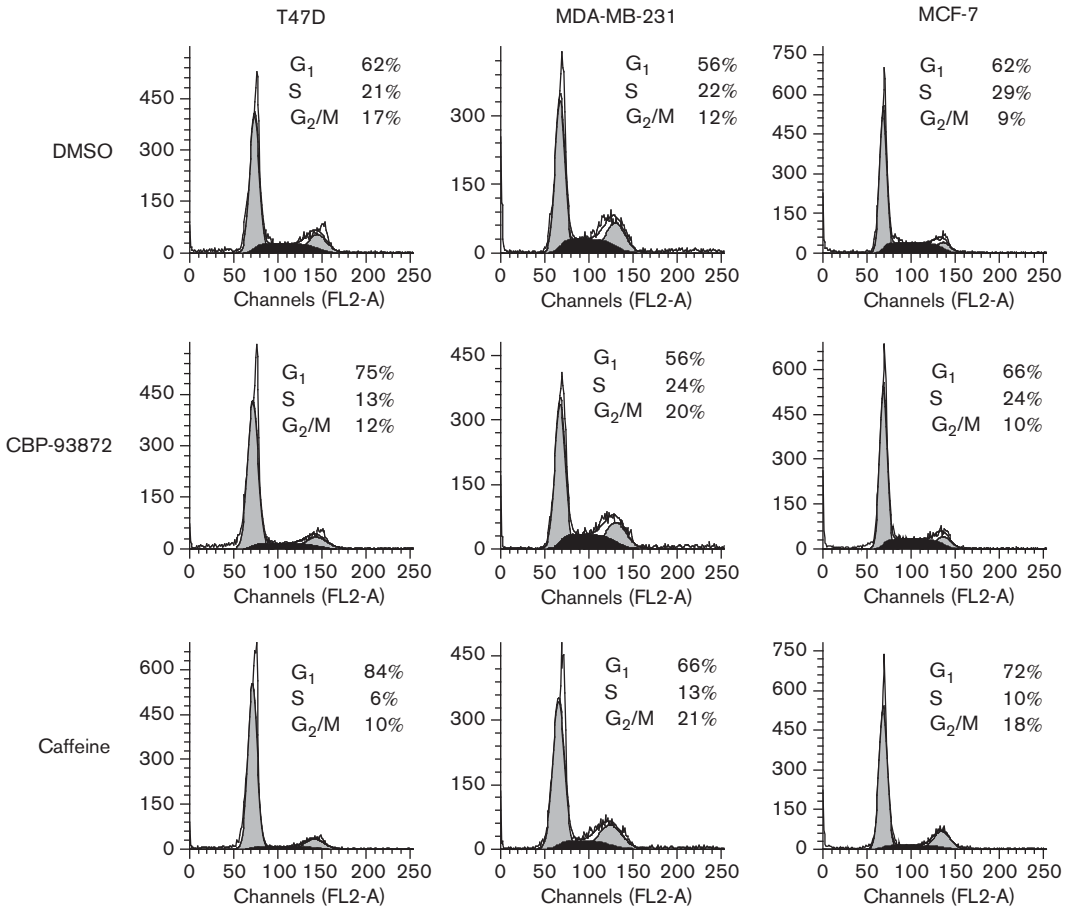
CBP-93872 causes activation of cdc2 by inhibiting phosphorylation of Chk1

To explore the molecular mechanism by which CBP-93872 inhibited cell growth through the G_2 checkpoint, we examined changes in the phosphorylation status of the downstream kinase cdc2 using X-irradiated HT-29 cells and found that phosphorylation of the 15th tyrosine of cdc2 (cdc2 Y15) was reduced by CBP-93872 treatment. Consequently, phosphohistone H3 was promoted, indicating that CBP-93872 functions through activation of the cdc2 kinase (Fig. 6a). We further examined the activations of major cell cycle-regulating kinases *in vitro* and *in vivo*. Fig. 6b shows that CBP-93872 clearly inhibited phosphorylation of Chk1-Ser317 and -Ser345 induced by X-irradiation or UV-irradiation, but did not affect the phosphorylation status of Chk2, ATM, or ATR. We then examined inhibition of the Chk1, Chk2, Wee1Hu, and Wee1B kinases by using a recombinant enzyme assay [17–19]; however, CBP-93872 did not inhibit these kinases at 50 μmol/l (data not shown), indicating that at least one of the targets of CBP-93872 could be ATR or related molecules [20]. To further evaluate whether CBP-93872 inhibits other protein kinases, CBP-93872 was tested using an Ambit profiling panel [21]; however, no inhibition was observed for 442 kinases, including the Aurora kinase, CDKs, Chk1, Chk2, PI3K family kinases, SRC, Wee1 (Wee1Hu), Wee2 (Wee1B), and mitogen-activated protein kinase family (data not shown). These results indicate that CBP-93872 abrogates the G_2 checkpoint by inhibiting phosphorylation of Chk1, resulting in the activation of cdc2.

Discussion

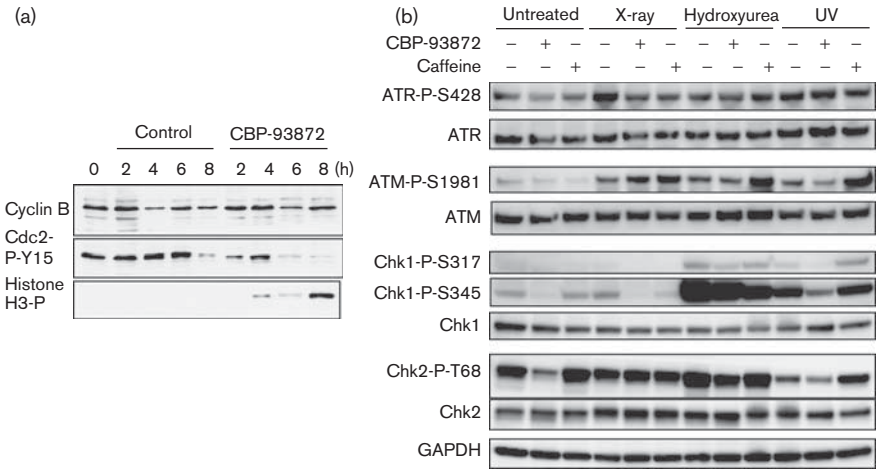
The results of this study indicate that a modulator of the G_2 checkpoint offers selective cytotoxicity to p53-deficient cancer cells with wild-type CDKN2A.

Fig. 5



CBP-93872 treatment induces G_1 arrest in p53-mutated and CDKN2A-normal cells. Cell cycle distributions were shown for T47D, MDA-MB-231, and MCF-7 cells treated with dimethyl sulfoxide, CBP-93872 (20 μmol/l) or caffeine (2000 μmol/l) for 48 h.

Fig. 6



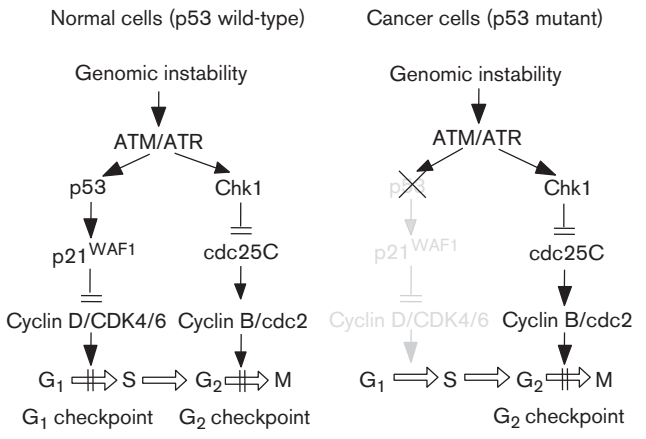
CBP-93872 suppresses Chk1 phosphorylation and decreases the phosphorylated form of cdc2. (a) Changes in the phosphorylation status of cdc2-Y15 and histone-H3 in synchronized HT-29 cells treated with CBP-93872. (b) The phosphorylation status of major G_2 checkpoint kinases were evaluated using asynchronous HT-29 cells after treatment with X-ray, hydroxyurea, or UV. ATM, ataxia–telangiectasia mutated; ATR, ataxia–telangiectasia-mutated and Rad3-related.

A probable target of the inhibitor is ATR with direct or indirect interaction to suppress its activity, and this suggests a new strategy for overcoming p53-disrupted cancers.

As the G_1 checkpoint does not function in p53-disrupted tumors and DNA repair depends only on the G_2 checkpoint, targeting the G_2 checkpoint enables p53-specific therapy. Thus, G_2 checkpoint inhibitors have been developed as sensitizers combined with chemotherapeutic agents. In addition, the genomic instability of p53-mutated cells results in constitutive activation of checkpoint pathways, and therefore a G_2 checkpoint inhibitor alone may exhibit potent cell-growth inhibition (Fig. 7). In fact, G_2 checkpoint inhibitors, such as UCN-01, have been reported to exhibit cell growth inhibition without being combined with other anticancer drugs [22–24]. However, they have seldom been reported to show selectivity for p53-deficient cells in single use.

In contrast, in this study, CBP-93872 showed selective cytotoxicity to p53 mutants, even in the absence of genotoxic agents; however, it was less effective for cancers or fibroblast cells with wild-type p53 (Fig. 4). These properties are quite different from other known G_2 checkpoint inhibitors targeting ATM/ATR, such as caffeine (ATM and ATR inhibitor), CGK733 (ATM and ATR inhibitor), and KU5593 (ATM inhibitor), which exhibit stronger inhibition of cell proliferation in MCF-7 (wild-type p53) cells than in T47-D (p53 mutant) cells [14]. Interestingly, CBP-93872 was more effective in p53 mutants with wild-type CDKN2A, but less effective in cells with CDKN2A deletion. Moreover, CBP-93872 induces G_1 arrest in wild-type CDKN2A cells, even when p53 is not functional. Human CDKN2A locus encodes two distinct proteins, $p16^{INK4a}$ and $p14^{ARF}$, that act as tumor suppressors [25–27]. $p16^{INK4a}$ suppresses G_1 progression directly by binding to CDK4/6. $p14^{ARF}$ also contributes to p53-independent G_1/S retarding by binding with E2F1 to inhibit its transcriptional activity [28], interacting with small ubiquitin-like modifier-conjugating enzyme Ubc9 to enhance the sumoylation of E2F-1 and HIF-1α [29] and by forming a complex with $p120^{E4F}$ to cause G_2 arrest dependently or independently of p53 [30]. Although the precise mechanism should be further elucidated, it is likely that CBP-93872 enhances G_1 arrest by promoting CDKN2A protein function or expression and consequently exhibits selectivity for CDKN2A wild-type cancer cells.

Fig. 7



Concept of G_2-checkpoint inhibition for synthetic lethality to p53-deficient cells. ATM, ataxia–telangiectasia mutated; ATR, ataxia–telangiectasia-mutated and Rad3-related.

Thus, CDKN2A gene status seems to be a logical molecular determinant for CBP-93872 cytotoxicity; however, several contrasting studies of molecular determinants for the efficacy of other G_2 checkpoint inhibitors have been reported. Chen *et al.* [24] reported that UCN-01 was effective in MCF-7 cells, but not effective in T47D cells, and they concluded that UCN-01-mediated G_1 arrest is pRb dependent, but p53 independent. Mack *et al.* [23] reported that the effectiveness of UCN-01 depends on retinoblastoma status in nonsmall cell lung carcinoma, and that both p53-mutated and CDKN2A-mutated cells were sensitive to UCN-01. Compared with these results, the cellular spectrum of CBP-93872 is quite different, suggesting that this compound has a target molecule other than Chk1, the target kinase of UCN-01.

The molecular mechanism by which CBP-93872 inhibits cell proliferation of p53-deficient cells requires further investigation; however, among the major target molecules of known G_2 checkpoint inhibitors, Chk1, Chk2, Wee1, Myt1, ATM, and ATR, our results suggest that the most likely target of CBP-93872 is ATR or related molecules, interacting directly or indirectly to diminish its activity. Although caffeine inhibits both ATM and ATR equally *in vitro* [31–33], it induces G_1 arrest and suppressed cell growth regardless of p53 status (Fig. 5). In contrast, CBP-93872 was more selective to ATR than to ATM in the cellular kinase assay (Fig. 6b). Although both CBP-93872 and caffeine suppress ATR activity, their specificity for cell growth is not identical, suggesting that the selectivity of CBP-93872 for ATR could contribute to selective growth suppression of p53 deficient but not wild-type cancers.

This study also demonstrates that cell-based screening is a powerful tool for identifying a modifier without specifying target molecules. Although the G_2 checkpoint signaling pathway has been robustly investigated, we were able to identify an inhibitor that might lead to a novel target, indicating usability of a cell-based assay with the G_2 checkpoint as the readout. We also demonstrated that CBP-93872 inhibited phosphorylation of Chk1 at both Ser^{317} and Ser^{345}, and these phosphorylations could be another possible indicator for cell-based high-throughput screening using specific antibodies [34]. In addition, Roberge *et al.* [8] reported that a structurally unique inhibitor for Chk1 was identified by cell-based screening

for the G_2 checkpoint. Thus, cell-based screening could be a strategy for identifying new inhibitors/molecules, even for well-known pathways.

In conclusion, we discovered a small molecule that inhibits the G_2 checkpoint by suppression of Chk1 phosphorylation. Although ATR or a related molecule is a likely candidate so far, the entire mechanism of action of the target molecules should be completely elucidated. By using this compound, we demonstrated that G_2 checkpoint inhibition has a direct effect on the cell growth of p53-deficient cells having CDKN2A gene alteration caused by deletion. Thus, we suggest that an inhibitor of phosphorylation of Chk1, such as an ATR inhibitor, is a candidate anticancer drug for these genotypic backgrounds. These results also indicate that with appropriate selection of patients G_2 checkpoint-abrogating agents, including Chk1 inhibitors, may provide better clinical outcomes.

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Conflicts of interest

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