

Mechanisms of Mitotic Cell Death Induced by Chemotherapy-Mediated G₂ Checkpoint Abrogation

Celia Vogel, Christian Hager, and Holger Bastians

Institute for Molecular Biology and Tumor Research, Philipps University of Marburg, Marburg, Germany

Abstract

The novel concept of anticancer treatment termed “G₂ checkpoint abrogation” aims to target p53-deficient tumor cells and is currently explored in clinical trials. The anticancer drug UCN-01 is used to abrogate a DNA damage–induced G₂ cell cycle arrest leading to mitotic entry and subsequent cell death, which is poorly defined as “mitotic cell death” or “mitotic catastrophe.” We show here that UCN-01 treatment results in a mitotic arrest that requires an active mitotic spindle checkpoint, involving the function of Mad2, Bub1, BubR1, Mps1, Aurora B, and survivin. During the mitotic arrest, hallmark parameters of the mitochondria-associated apoptosis pathway become activated. Interestingly, this apoptotic response requires the spindle checkpoint protein Mad2, suggesting a proapoptotic function for Mad2. However, although survivin and Aurora B are also required for the mitotic arrest, both proteins are part of an antiapoptotic pathway that restrains the UCN-01–induced apoptosis by promoting hyperphosphorylation of Bcl-2 and by inhibiting the activation of Bax. Consequently, inhibition of the antiapoptotic pathway by genetic ablation of survivin or by pharmacologic inhibitors of Aurora B or cyclin-dependent kinase 1 lead to a significant enhancement of apoptosis and therefore act synergistically with UCN-01. Thus, by defining the mechanism of cell death on G₂ checkpoint abrogation we show a highly improved strategy for an anticancer treatment by the combined use of UCN-01 with abrogators of the survivin/Aurora B–dependent antiapoptotic pathway that retains the selectivity for p53-defective cancer cells. [Cancer Res 2007;67(1):339–45]

Introduction

The introduction of genotoxic damage is a general scheme of anticancer chemotherapy, which activates cell cycle checkpoint signaling pathways arresting the cell cycle and inducing apoptosis when repair cannot be accomplished (1, 2). For instance, DNA-damaging drugs (e.g., topoisomerase inhibitors, cisplatin, etc.) activate the DNA damage checkpoint involving the function of the tumor suppressor p53, which contributes to a cell cycle arrest in G₁ and G₂. In addition, a p53-independent branch of the DNA damage checkpoint is activated that involves the Chk1 kinase and arrests cells solely before mitosis (1). On prolonged cell cycle arrest,

the intrinsic apoptotic pathway is activated, which is associated with the activation of the proapoptotic Bax that mediates the release of cytochrome *c* from mitochondria leading to the subsequent activation of the caspase cascade resulting in cell death (3, 4).

Another large group of chemotherapeutic drugs successfully used in the clinic are the spindle-damaging agents. Various *Vinca* alkaloids (e.g., vincristine, vinblastine, etc.) depolymerize microtubules and prevent the attachment of kinetochores to spindle microtubules resulting in an inhibition of chromosome alignment during mitosis. In contrast, taxanes and epothilones stabilize microtubules and suppress the dynamics of the mitotic spindle resulting also in an inhibition of chromosome alignment. Stabilization of the mitotic spindle still allows the (partial) attachment of chromosomes to the mitotic spindle, but tension across sister kinetochores cannot be generated. A lack of either attachment or tension activates a mitotic signaling pathway known as the mitotic spindle checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned. Thus, treatment with spindle-damaging drugs leads to the activation of the spindle checkpoint and to an arrest during mitosis. Mad1, Mad2, Bub1, BubR1, and Mps1 are essential spindle checkpoint components that are recruited to kinetochores on checkpoint activation (5). In addition, the “chromosomal passenger” proteins survivin and Aurora B are required for spindle checkpoint function in response to the lack of tension (6–9). Thus, two functional arms of the spindle checkpoint can be defined. However, the molecular mechanisms of spindle checkpoint activation are not well understood. During chemotherapy, the spindle checkpoint–mediated mitotic arrest is followed by an unscheduled exit from mitosis due to checkpoint adaptation. Interestingly, checkpoint activation as well as slippage from mitosis seem to be required for efficient induction of apoptosis (10–12). Although the underlying mechanisms are still unclear, it is intriguing that survivin can function as a positive regulator of the checkpoint as well as an antiapoptotic protein, possibly mediating a crosstalk between the spindle checkpoint and apoptosis (13).

Checkpoint defects are frequent in human cancer and can confer resistance toward chemotherapy (12, 14). Among the most frequent lesions in human cancer is the functional loss of p53, which can directly contribute to resistance toward DNA-damaging drugs. However, because p53-deficient cancer cells arrest solely before mitosis on DNA damage, those cells can be selectively targeted for cell death by inactivating the remaining G₂ checkpoint (15). Abrogation of the G₂ checkpoint arrest can be mediated by the kinase inhibitor 7-hydroxystaurosporine, also known as UCN-01, which inhibits the Chk1 kinase, an essential component of the G₂ checkpoint (16–18). Thus, a sequential treatment with DNA-damaging agents and UCN-01 forces cells to enter mitosis in the presence of DNA damage resulting in a potentiation of cell death compared with treatment with DNA-damaging agents alone (19, 20). Although UCN-01 is not entirely specific for Chk1, its potential to abrogate the G₂ checkpoint has been well characterized

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Holger Bastians, Institute for Molecular Biology and Tumor Research, Philipps University of Marburg, Emil-Mannkopff-Strasse 2, D-35037 Marburg, Germany. Phone: 49-6421-2863113; Fax: 49-6421-2865932; E-mail: bastians@imt.uni-marburg.de.

©2007 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-2548

(16–18, 20, 21). However, the mechanisms of cell death induced by sequential treatment with DNA-damaging drugs and UCN-01 are not well understood. In fact, “mitotic cell death,” also often referred to as “mitotic catastrophe” is ill defined and apoptotic and nonapoptotic mechanisms have been discussed to be involved in this particular form of cell death (3, 22). Nevertheless, preclinical results using UCN-01 are promising and, thus, several phase I clinical trials in various cancer entities that combine various DNA-damaging drugs with UCN-01 are under way (23, 24).

Here, we investigated the mechanism of cell death induced by G₂ checkpoint abrogation. We show that a sequential treatment with Adriamycin and UCN-01 induces a mitotic arrest, which is dependent on the mitotic spindle checkpoint. Subsequently, apoptotic variables are activated in mitotically arrested cells and promoted by a proapoptotic function of the spindle checkpoint component Mad2 but counteracted by a survivin/Aurora B–dependent antiapoptotic pathway. Importantly, pharmacologic abrogation of the mitotic survival pathway using cyclin-dependent kinase 1 (CDK1) or Aurora B kinase inhibitors greatly improves UCN-01–mediated therapy, suggesting a novel strategy for an anticancer treatment.

Materials and Methods

Cell treatments. HeLa cells were maintained in DMEM, 10% FCS, and 1% penicillin/streptomycin at 37°C and treated with 300 nmol/L Adriamycin (Sigma, Sigma, Aldrich Chemie GmbH, Steinheim, Germany) for 24 h followed by a treatment with 100 nmol/L UCN-01 (a kind gift from Developmental Therapeutics Program of the National Cancer Institute, Rockville, MD). Where indicated, cells were treated with 150 nmol/L nocodazole (Sigma), 100 nmol/L Taxol (Sigma), 20 μmol/L roscovitine (Calbiochem, Schwalbach, Germany), 10 μmol/L purvalanol A (Alexis Biochemicals, Grünberg, Germany), or 1 μmol/L ZM447439 (a kind gift from AstraZeneca Pharmaceuticals, Cheshire, United Kingdom; ref. 7).

Fluorescence-activated cell sorting analysis. The mitotic index and the DNA content were determined on a FACScalibur (Becton Dickinson, San Jose, CA) as described before (25).

Antibodies. The following antibodies were used for Western blotting and for immunofluorescence: anti-Bub1, anti-BubR1 (kind gifts from Stephen Taylor, University of Manchester, Manchester, United Kingdom; ref. 26), anti-CREST (Europa Bioproducts, Wicken, Ely, Cambridge, United Kingdom), anti-Mad2 (Covance/Babco, Richmond, CA), anti-hMps1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-survivin (R&D Systems, San Diego, CA), anti-actin (Sigma), anti-poly(ADP-ribose) polymerase (PARP; BD PharMingen, Erebodegem, Belgium), anti-cytochrome *c* (PharMingen), anti-Bak [monoclonal antibody (mAb) TC-102], anti-Bax (mAb 6A7, Calbiochem), anti-γ-tubulin, and anti-α-tubulin (Sigma). Antibodies conjugated to horseradish peroxidase, Alexa Fluor 488, or Red X were from Dianova (Eugene, OR), Molecular Probes (Leiden, the Netherlands), and Jackson Immuno Research (West Grove, PA), respectively.

Apoptosis assays. DNA laddering, the cleavage of PARP, and caspase activity were determined as described (12). All quantitative results shown represent mean values and SDs from at least three independent experiments. Activated Bax was immunoprecipitated using conformation-specific antibodies (mAb 6A7, Calbiochem) and detected on Western blots as described (27). Bak activation was determined using an activation specific antibody (mAb TC-100, Calbiochem) in a fluorescence-activated cell sorting–based assay as described (28).

Transfections. pSUPER plasmids (29) expressing short hairpin RNAs (shRNA) targeting Bub1, BubR1, Mps1, and survivin were cloned. The targeting sequences are provided on request. The pSUPER targeting Mad2 was a kind gift from Rene Medema (University of Utrecht, Utrecht, the Netherlands; ref. 6). The pcDNA-Bcl-2 plasmid was a gift from Martin Eilers (Philipps-University Marburg, Marburg, Germany). Plasmids were transiently transfected into HeLa cells by electroporation using a Bio-Rad (Hercules, CA) electroporator.

Results

G₂ checkpoint abrogation activates a mitotic checkpoint that involves spindle checkpoint and chromosomal passenger proteins. Similar to studies with several p53-deficient human cell lines (17, 20), treatment of p53 compromised HeLa cells with the topoisomerase II inhibitor Adriamycin induced DNA double-strand breaks and arrested cells in G₂ (data not shown). Subsequent treatment with UCN-01 induced a transition from G₂ arrest into mitosis (Supplementary Fig. S1A). Microscopic inspection of mitotic cells revealed a metaphase-like phenotype with up to 70% of cells arrested in mitosis (Supplementary Fig. S1B). The UCN-01–induced mitotic arrest was clearly associated with the activation of the mitotic spindle checkpoint as shown by the specific recruitment of Bub1 and BubR1 to kinetochores (Fig. 1A). To test whether the individual components of the spindle checkpoint are required for the UCN-01–induced mitotic arrest, we expressed shRNAs to down-regulate the spindle checkpoint proteins Bub1, BubR1, Mad2, and Mps1 to similar levels (Fig. 1B). As shown in Fig. 1C, down-regulation of the spindle checkpoint proteins led to an escape from the mitotic arrest after treatment with nocodazole (that depolymerizes microtubules and induces a lack of kinetochore attachment) or Taxol (that stabilizes microtubules and induces a lack of kinetochore tension), whereas control shRNAs showed no effect (Fig. 1B and C). Because the mitotic arrest directly correlates with the spindle checkpoint activation, these results indicate a similar functional impairment of the checkpoint on repression of the individual checkpoint components either after a lack of attachment or on the lack of tension. Importantly, down-regulation of the spindle checkpoint proteins also led to an efficient escape from the mitotic arrest after Adriamycin and UCN-01 treatment (Fig. 1D), indicating that a fully functional spindle checkpoint is required for the UCN-01–induced mitotic arrest.

In addition to the spindle checkpoint proteins, survivin and Aurora B are involved in the spindle checkpoint activation specifically on treatment with Taxol. In agreement with these studies (6–8), both shRNA-mediated repression of survivin (Fig. 1B) and inhibition of the Aurora B kinase activity by the specific inhibitor ZM447439 abolished the mitotic arrest on Taxol but not after nocodazole treatment (Fig. 2A), indicating that both proteins are required for a sustained mitotic arrest in response to a lack of kinetochore tension. Consequently, we investigated if survivin and Aurora B are involved in the UCN-01–mediated mitotic arrest and found a specific requirement for both proteins (Fig. 2B).

Thus, on abrogation of the G₂ checkpoint by UCN-01, p53-deficient tumor cells enter mitosis in the presence of DNA damage and activate a mitotic checkpoint that requires the spindle checkpoint proteins as well as the chromosomal passenger proteins leading to a sustained mitotic arrest.

UCN-01–treated mitotic cells undergo apoptosis. Sequential treatment of DNA-damaging drugs and UCN-01 potentiates cell death by inducing mitotic cell death or mitotic catastrophe (Supplementary Fig. S2, refs. 16, 19, 20, 30). We asked whether this form of cell death represents a mitotic form of apoptosis or whether it is rather unrelated to apoptosis as suggested before (3). After Adriamycin and UCN-01 treatment, we separated mitotic from nonmitotic cells and determined the activation of biochemical parameters specific to the intrinsic pathway of apoptosis, which include the release of cytochrome *c* from mitochondria into the cytoplasm (Fig. 3A); the enzymatic activation of caspase-2, caspase-3, and caspase-9 (Fig. 3B); the cleavage of PARP (Fig. 3C); and the internucleosomal fragmentation of chromosomal DNA

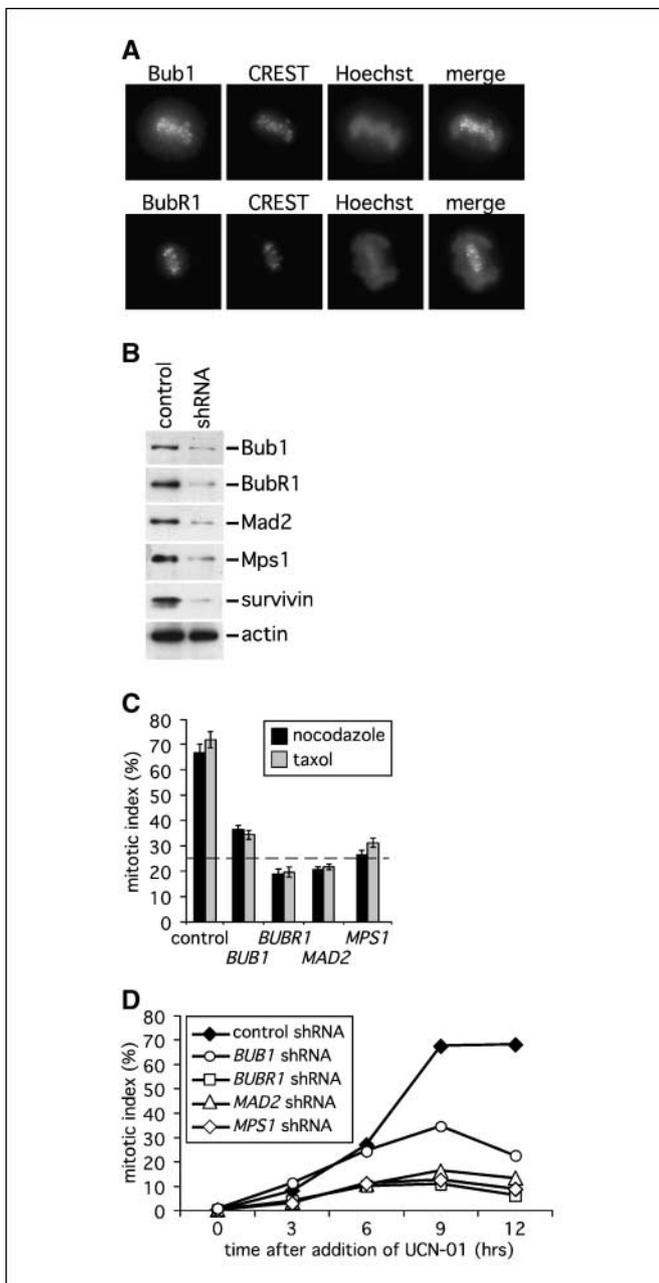


Figure 1. Activation of the mitotic spindle checkpoint in response to a sequential Adriamycin and UCN-01 treatment. *A*, immunofluorescence studies showing kinetochore colocalization of CREST with Bub1 or BubR1 in HeLa cells treated with Adriamycin (24 h) and UCN-01 (6 h). *B*, Western blot showing the shRNA-mediated down-regulation of spindle checkpoint proteins. *C*, mitotic index in response to a 16 h nocodazole or Taxol treatment in cells expressing the indicated shRNAs. *D*, time-dependent mitotic index in response to Adriamycin and UCN-01 treatment in cells expressing the indicated shRNAs.

(Fig. 3D). Clearly, the activation of all specific apoptotic variables was restricted to mitotic cells reflected by a high mitotic index ($74 \pm 5\%$; Fig. 3A). Cells arrested in mitosis by nocodazole (mitotic index, $84 \pm 6\%$) did not show any activation of the apoptotic variables (Fig. 3A), excluding the possibility that mitotically arrested cells show an activation of these apoptotic variables per se. These results show that the forced entry into mitosis by UCN-01 is a prerequisite for the activation of the mitochondria-associated apoptosis machinery.

Mad2 is part of a proapoptotic pathway on UCN-01 treatment. To evaluate the role of the spindle checkpoint for the induction of mitotic apoptosis, we down-regulated Mad2, Bub1, and BubR1 as shown before (Fig. 1), treated the cells sequentially with Adriamycin and UCN-01, quantified the induction of apoptotic figures (Fig. 4A), and detected the cleavage of PARP (Fig. 4B). Surprisingly, although equally required for the mitotic arrest (Fig. 1), down-regulation of Mad2, but not of Bub1 or BubR1, resulted in an efficient escape from mitotic apoptosis. In fact, caspase-3 activity was reduced by $\sim 60\%$ after Mad2 repression and UCN-01 treatment (Fig. 4C). These results suggest a proapoptotic function for Mad2 that might be independent of the checkpoint-mediated mitotic arrest, although we cannot exclude an involvement of additional spindle checkpoint proteins.

Survivin is part of an antiapoptotic pathway on UCN-01 treatment. Because survivin acts as a spindle checkpoint component that is required for the UCN-01-induced mitotic arrest (Fig. 2B), we asked whether it also participates in the regulation of mitotic apoptosis. First, we followed the protein level of survivin during the treatment. Survivin accumulated in G₂-arrested cells treated with Adriamycin, which is consistent with the notion of a cell cycle-regulated expression of survivin (31). Moreover, on UCN-01 treatment when cells traverse from the G₂ arrest into mitosis, we found a dramatic increase of survivin protein (Fig. 5A). Interestingly, the mitotic accumulation of survivin was associated with a hyperphosphorylation of the antiapoptotic Bcl-2 protein (Fig. 5A), which has been shown recently to be associated with stabilization and enhancement of the antiapoptotic activity of Bcl-2 (32–34). shRNA-mediated repression of survivin led to a loss of the UCN-01-induced hyperphosphorylation of Bcl-2, suggesting a functional interaction between survivin and a Bcl-2-mediated antiapoptotic activity (Fig. 5A). To test this directly, we assayed caspase-3 activity

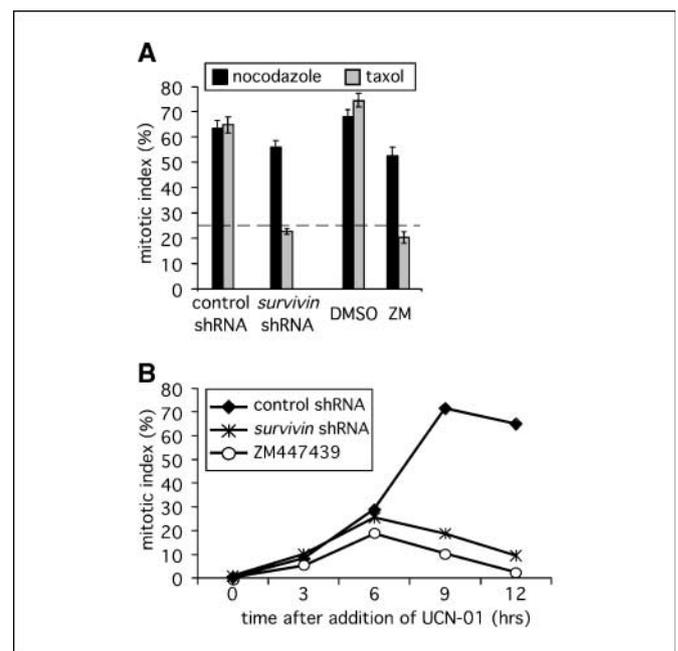


Figure 2. Involvement of chromosomal passenger proteins in the UCN-01-induced mitotic arrest. *A*, mitotic index in response to a 16 h nocodazole or Taxol treatment in cells with reduced expression of survivin or in cells treated with DMSO or the Aurora inhibitor ZM447439 (ZM). *B*, time-dependent mitotic index after Adriamycin and UCN-01 treatment and on inhibition of survivin or Aurora B.

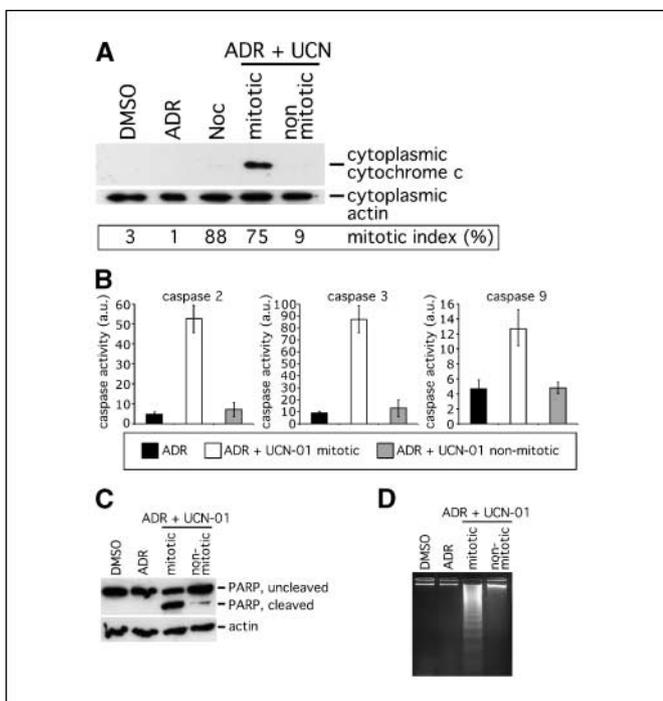


Figure 3. UCN-01-induced entry into mitosis causes mitochondria-associated apoptosis. *A*, mitochondrial cytochrome *c* release. Mitotic and nonmitotic populations of cells treated with Adriamycin (ADR; 24 h) and UCN-01 (UCN; 12 h) were used to detect cytoplasmic cytochrome *c* on Western blots. *B*, activity of caspase-2, caspase-3, and caspase-9. *C*, Western blot detection of PARP cleavage after UCN-01 treatment (24 h). *D*, detection of internucleosomal laddering of chromosomal DNA after UCN-01 treatment (24 h).

on survivin depletion in response to UCN-01 treatment and found that the caspase activity was dramatically increased (Fig. 5B). Intriguingly, whereas apoptosis in response to UCN-01 was not influenced by expression of Bcl-2, the increase in caspase activity on survivin repression was completely suppressed by the expression of Bcl-2 (Fig. 5B). Because Bcl-2 is a potent antagonist for the proapoptotic Bax protein, our findings raise the interesting possibility that UCN-01-induced apoptosis might occur independent of the activation of Bax, which might be suppressed by survivin. Therefore, we investigated the activation status of Bax by using conformation-specific immunoprecipitation assays (27, 28). Indeed, we found that Bax became activated during the G₂ arrest in response to Adriamycin treatment when the survivin level is low but inactivated on UCN-01 treatment when the survivin level is high (Fig. 5C). Importantly, on survivin repression, Bax was found to be reactivated, suggesting that Bax activation might indeed be controlled by survivin. In contrast, down-regulation of Mad2, which was associated with a high survivin level and a low mitotic index (Fig. 5C), did not induce a reactivation of Bax, arguing against the possibility that Bax is solely activated due to the unscheduled exit from mitosis. Unlike Bax, we found no evidence for an activation of its homologue Bak, neither after Adriamycin nor after subsequent UCN-01 treatment (Supplementary Fig. S3).

Together, these results suggest that survivin might be part of a UCN-01-induced antiapoptotic pathway, which is associated with the hyperphosphorylation of Bcl-2 and a mitotic inactivation of Bax (Fig. 6C).

Pharmacologic interference with the survivin-dependent mitotic survival pathway potentiates UCN-01-induced apoptosis. Our results after genetic abrogation of the survivin-dependent

survival pathway led to the idea that pharmacologic inhibition of the antiapoptotic pathway could be used in a clinical setting to enhance the efficacy of UCN-01-mediated therapy. Previous reports have indicated that survivin stability and function might be positively regulated by phosphorylation through CDK1 (13, 35, 36). Further, survivin is part of the chromosomal passenger complex and interacts directly with the Aurora B kinase, which might also contribute to its stability and function (13, 37–40). Therefore, we used potent inhibitors for CDK1 (roscovitine and purvalanol A) and the Aurora kinases (ZM447439) to investigate whether interference with these kinases would act synergistically with UCN-01. Interestingly, CDKs and Aurora kinases are validated targets for anticancer treatment and inhibitors are currently explored in clinical trials (23, 37). Indeed, cotreatment with roscovitine, purvalanol A, or ZM447439 decreased the protein level of survivin and led to the loss of Bcl-2 hyperphosphorylation similar to that seen before on survivin repression (Fig. 6A). Accordingly, the UCN-01-induced caspase activity was greatly enhanced on cotreatment with the inhibitors and, as seen on repression of survivin, this increase was reversed by expression of Bcl-2, whereas treatment with the kinase inhibitors alone had only a minor effect (Fig. 6B).

Together, our results clearly suggest that pharmacologic inhibitors of CDK1 and Aurora kinases abrogate the survivin-dependent antiapoptotic pathway and thus greatly improve the outcome of UCN-01-mediated therapy (Fig. 6C).

Discussion

The Chk1 kinase is essential for the DNA damage-induced G₂ checkpoint and restrains entry into mitosis (18, 21). The development of the Chk1 kinase inhibitor UCN-01, although not entirely

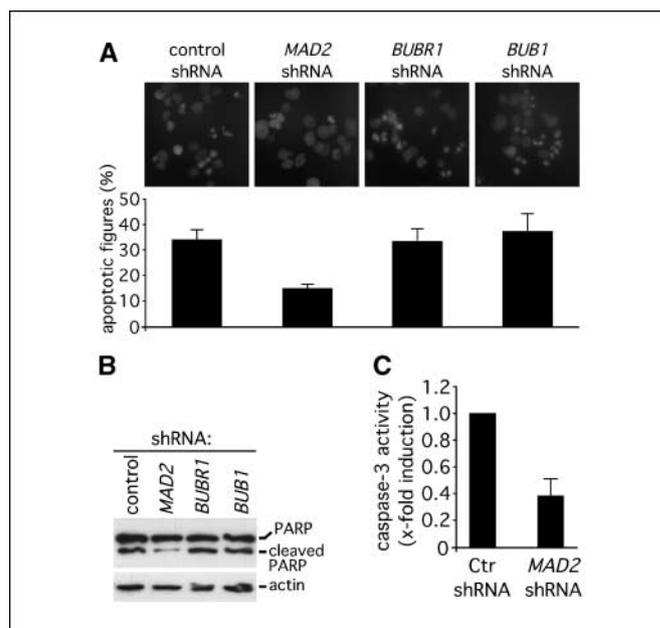


Figure 4. Mad2 is required for UCN-01-induced apoptosis. *A*, quantification of apoptotic figures after treatment with Adriamycin (24 h) and UCN-01 (18 h) in cells expressing the indicated shRNAs. Apoptotic figures (1,500) from three experiments were quantified. *B*, detection of PARP cleavage on treatment with Adriamycin and UCN-01 (24 h). *C*, caspase-3 activity in cells expressing control-specific (Ctr) or Mad2-specific shRNA on treatment with Adriamycin and UCN-01.

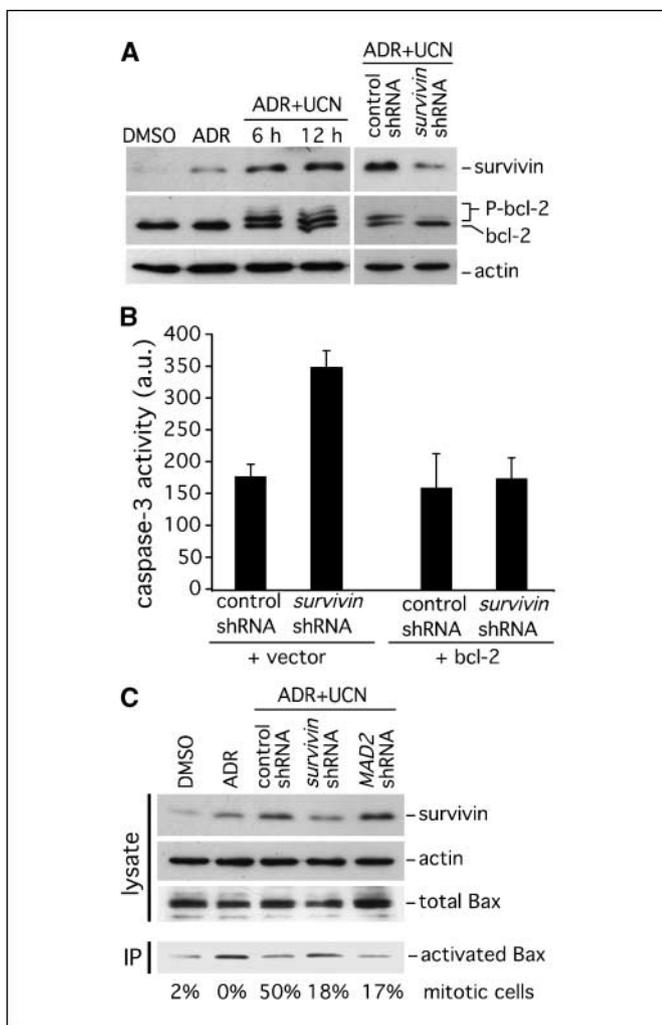


Figure 5. A survivin-dependent antiapoptotic pathway counteracts UCN-01–induced apoptosis. **A**, detection of survivin, Bcl-2, and actin on Western blots. HeLa cells were treated with DMSO, Adriamycin alone, or Adriamycin and UCN-01 for 6 and 12 h or transfected with control or survivin shRNAs and treated with Adriamycin and UCN-01 for 12 h. **B**, caspase-3 activity in HeLa cells expressing control- or survivin-specific shRNAs in the absence or presence of ectopic Bcl-2 expression. Cells were sequentially treated with Adriamycin for 24 h and UCN-01 for 12 h. **C**, Western blots of survivin and activated Bax after transfection with control, survivin, or Mad2 shRNAs and treated with Adriamycin (24 h) and UCN-01 for 6 h. Survivin and Bax proteins were detected in total lysates and activated Bax was isolated by immunoprecipitation using conformation-specific antibodies.

specific, has allowed to establish the therapeutic concept of G₂ checkpoint abrogation that potentiates tumor cell killing selectively in p53-deficient tumor cells (16, 17, 19, 20, 24). Consequently, several phase I clinical trials currently explore this concept by combining various DNA-damaging drugs with UCN-01 (e.g., ref. 24). However, the mechanisms and requirements of cell death on G₂ checkpoint abrogation remained largely unknown. We have addressed these questions and established the following points: (a) UCN-01–mediated G₂ checkpoint abrogation induces entry into mitosis, which is associated with a metaphase-like arrest that requires the mitotic spindle checkpoint. (b) The spindle checkpoint protein Mad2 is required for the induction of apoptosis in response to UCN-01 treatment. (c) The chromosomal passenger protein survivin, which is required for the mitotic arrest, is part of a prosurvival pathway induced by UCN-01. (d) Genetic ablation of

survivin or pharmacologic abrogation of the prosurvival pathway by compounds targeting either CDK1 or Aurora B kinase results in a significant enhancement of apoptosis in response to UCN-01 treatment.

Based on these results, we suggest a novel and highly improved strategy of chemotherapy combining DNA-damaging drugs, UCN-01, and inhibitors of CDK1 or Aurora B kinase to significantly enhance the efficacy of G₂ checkpoint abrogation without neglecting the selectivity for p53-deficient tumor cells.

At first glance, it seems odd that some checkpoint proteins (e.g., Mad2) promote apoptosis, whereas others (e.g., survivin and Aurora B) inhibit cell death, but our data suggest that these

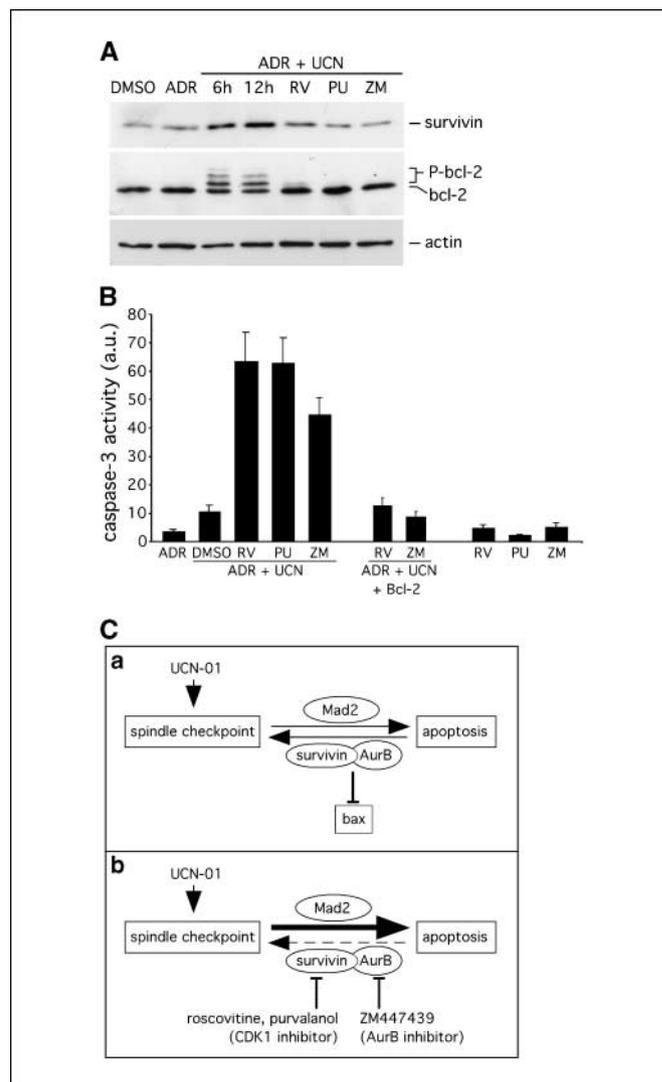


Figure 6. Synergistic effects between CDK1 and Aurora B inhibitors with UCN-01. **A**, Western blot detection of survivin, Bcl-2, and actin in lysates from HeLa cells after cotreatment with Adriamycin, UCN-01, and roscovitine (RV), purvalanol A (PU), or ZM447439. Cells were first treated with UCN-01 for 6 h to allow entry into mitosis before treatment with the CDK inhibitors for additional 6 h. ZM447439 was added 1 h before UCN-01. **B**, caspase-3 activity in cells treated as in (A) in the absence or presence of ectopic Bcl-2 expression or with the kinase inhibitors alone. **C**, model for the role of proapoptotic and antiapoptotic pathways regulating UCN-01–induced apoptosis: a, a Mad2-dependent proapoptotic pathway is counteracted by a survivin/Aurora B–dependent antiapoptotic pathway; b, pharmacologic or genetic abrogation of survivin or Aurora B enhances the efficacy of UCN-01–induced apoptosis.

proteins might have dual functions in regulating the mitotic checkpoint and apoptotic pathways, whereas other checkpoint proteins, including Bub1, BubR1, and Mps1, are restricted to checkpoint functions during mitosis. Thus, the right balance between death and survival signals during mitosis might be mediated by specific subsets of spindle checkpoint proteins and could explain why mitotic cells are especially vulnerable to chemotherapeutic drugs that perturb this fine-tuned balance.

Interestingly, on Taxol or kinesin inhibitor treatment that activates the mitotic spindle checkpoint, the subsequent induction of apoptosis is dependent on both the activation of the spindle checkpoint and a subsequent slippage from the mitotic arrest (11, 12). Because mitotic slippage is associated with the down-regulation of survivin (41) and the inactivation of Aurora B (42), it is expected that exit from mitosis leads to an inactivation of the antiapoptotic pathway of survivin/Aurora B and, thus, to the induction of apoptosis in the postmitotic G₁ phase due to the proapoptotic activity of Mad2.

In contrast to spindle-damaging drugs, abrogation of the G₂ checkpoint by UCN-01 leads to the induction of a mitotic form of apoptosis without an apparent requirement for mitotic slippage, which might be the result of a strong Mad2-mediated proapoptotic signal that cannot completely be repressed by the antiapoptotic pathway. However, the rate of UCN-01-induced apoptosis is relatively low and can greatly be enhanced by genetic ablation of survivin or by treatment with pharmacologic inhibitors of CDK1 and Aurora B kinase, which results in decreased survivin levels.

Although an antiapoptotic function of survivin is well established and direct interactions of survivin with caspases have been shown (13), it is still controversial how survivin acts as an inhibitor of apoptosis. Intriguingly, on G₂ checkpoint abrogation, we found that the survivin level, but not the mitotic arrest per se, is tightly associated with the hyperphosphorylation of the antiapoptotic protein Bcl-2 and with a mitotic inactivation of the proapoptotic Bax, suggesting that survivin might act upstream of the mitochondria. In this model, hyperphosphorylation of Bcl-2 associated with an inactivation of Bax would protect cells with high survivin levels from apoptosis. Indeed, it has been shown recently that phosphorylated Bcl-2 shows a higher protein stability and an enhanced antiapoptotic activity (32–34). Moreover, on kinesin inhibitor treatment, Bax is activated only after cells have exited mitosis (11), which coincides with a reduced level of survivin (41) and the initiation of apoptosis.

During mitosis, survivin interacts with inner centromere protein, borealin, and Aurora B within the “chromosomal passenger

complex” and can possibly act as a regulatory subunit of the Aurora kinase (43). It is therefore possible that survivin mediates its antiapoptotic function during mitosis through regulation of the Aurora B kinase. Our results have shown a very similar enhancement of apoptosis either after ablation of survivin or on inhibition of Aurora B kinase activity. In future studies, it will be important to dissect the individual functions of survivin and Aurora B with respect to their antiapoptotic role during mitosis.

Most importantly, our results imply that Mad2 and survivin/Aurora B are central effectors of chemotherapies that target mitosis. Indeed, down-regulation of Mad2 and overexpression of survivin or Aurora B are associated with human cancer and resistance toward chemotherapy (12, 13, 30, 37, 44). Although it is currently unknown whether low Mad2 levels and high survivin or Aurora B levels occur mutually exclusive or concomitantly in human tumors, the expression of Mad2 and survivin/Aurora B represents premier markers to select an appropriate chemotherapeutic strategy.

Moreover, due to their deregulated expression and activity, survivin and Aurora kinases are attractive targets for chemotherapy. In fact, drugs that interfere with the expression of survivin are currently developed and undergo clinical trials [e.g., SPC3042 (Santaris Pharma, Hørsholm, Denmark), YM155 (Astellas Pharma, Tokyo, Japan), and LY21818308 (Isis/Eli Lilly, Carlsbad, CA); ref. 45]. In addition, small-molecule inhibitors for CDK1 (e.g., roscovitine derivatives) that can modulate the mitotic survivin protein (present study; refs. 35, 36) as well as potent pharmacologic inhibitors for the Aurora kinases have been developed and are currently explored in clinical trials (23, 37, 46). A combination of these novel compounds with UCN-01 and possibly with taxanes and kinesin inhibitors is expected to abrogate the survivin/Aurora B-dependent antiapoptotic pathway without interfering with the Mad2-dependent proapoptotic pathway and thus potentiates drug-induced apoptosis. Therefore, this novel strategy is expected to greatly enhance the efficacy of these therapies and, accordingly, future clinical trials combining these drugs are highly desirable.

Acknowledgments

Received 7/11/2006; revised 9/14/2006; accepted 10/23/2006.

Grant support: Deutsche Forschungsgemeinschaft grant (H. Bastians).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Stephen Taylor, Rene Medema, and Martin Eilers for antibodies and plasmids; Robert J. Schultz (Developmental Therapeutics Program of the National Cancer Institute) for the gift of UCN-01; Claire Crafter (AstraZeneca Pharmaceuticals) for the Aurora inhibitor ZM447439; Heike Krebber for the comments on the manuscript; and Rolf Müller for the support.

References

- Kastan BM, Bartek J. Cell-cycle checkpoints and cancer. *Nature* 2004;432:316–23.
- Eastman A. Cell cycle checkpoints and their impact on anticancer therapeutic strategies. *J Cell Biochem* 2004; 91:223–31.
- Okada H, Mak WT. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer* 2004;4: 592–603.
- Wie CM, Zong XW, Cheng H, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001;292:727–30.
- Bharadwaj R, Yu H. The spindle checkpoint, aneuploidy, and cancer. *Oncogene* 2004;23:2016–27.
- Lens MS, Wolthuis MR, Klompmaaker R, et al. Survivin is required for a sustained spindle checkpoint arrest in response to lack of tension. *EMBO J* 2003;22:2934–47.
- Ditchfield C, Johnson LV, Tighe A, et al. Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *J Cell Biol* 2003;161:267–80.
- Hauf S, Cole WR, LaTerra S, et al. The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J Cell Biol* 2003; 161:281–94.
- Carvalho A, Carmena M, Sambade C, Earnshaw CW, Wheatley PS. Survivin is required for stable checkpoint activation in Taxol-treated HeLa cells. *J Cell Sci* 2003; 116:2987–98.
- Weaver AB, Cleveland WD. Decoding the links between mitosis, cancer, and chemotherapy: the mitotic checkpoint, adaptation, and cell death. *Cancer Cell* 2005; 8:7–12.
- Tao W, South JV, Zhang Y, et al. Induction of apoptosis by an inhibitor of the mitotic kinesin KSP requires both activation of the spindle assembly checkpoint and mitotic slippage. *Cancer Cell* 2005;8:49–59.
- Kienitz A, Vogel C, Morales I, Muller R, Bastians H. Partial downregulation of MAD1 causes spindle checkpoint inactivation and aneuploidy, but does not confer resistance towards Taxol. *Oncogene* 2005;24:4301–10.
- Wheatley PS, McNeish AI. Survivin: a protein with dual roles in mitosis and apoptosis. *Int Rev Cytol* 2005; 247:35–88.
- Lee S, Schmitt AC. Chemotherapy response and resistance. *Curr Opin Genet Dev* 2003;13:90–6.

15. Kawabe T. G₂ checkpoint abrogators as anticancer drugs. *Mol Cancer Ther* 2004;3:513-9.
16. Wang Q, Fan S, Eastman A, Worland JP, Sausville AE, O'Connor MP. UCN-01: a potent abrogator of G₂ checkpoint function in cancer cells with disrupted p53. *J Natl Cancer Inst* 1996;88:956-65.
17. Graves RP, Yu L, Schwarz KJ, et al. The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. *J Biol Chem* 2000;275:5600-5.
18. Lam HM, Liu Q, Elledge JS, Rosen MJ. Chk1 is haploinsufficient for multiple functions critical to tumor suppression. *Cancer Cell* 2004;6:45-59.
19. Bunch TR, Eastman A. Enhancement of cisplatin-induced cytotoxicity by 7-hydroxystaurosporine (UCN-01), a new G₂-checkpoint inhibitor. *Clin Cancer Res* 1996;2:791-7.
20. Tse NA, Schwartz KG. Potentiation of cytotoxicity of topoisomerase I poison by concurrent and sequential treatment with the checkpoint inhibitor UCN-01 involves disparate mechanisms resulting in either p53-independent clonogenic suppression or p53-dependent mitotic catastrophe. *Cancer Res* 2004;64:6635-44.
21. Zachos G, Rainey DM, Gillespie AD. Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects. *EMBO J* 2003;22:713-23.
22. Castedo M, Perfettini LJ, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 2004;23:2825-37.
23. Blagden S, Bono de J. Drugging cell cycle kinases in cancer therapy. *Curr Drug Targets* 2005;6:325-35.
24. Kortmanský J, Shah AM, Kaubisch A, et al. Phase I trial of the cyclin-dependent kinase inhibitor and protein kinase C inhibitor 7-hydroxystaurosporine in combination with fluorouracil in patients with advanced solid tumors. *J Clin Oncol* 2005;23:1875-84.
25. Vogel C, Kienitz A, Hofmann I, Muller R, Bastians H. Crosstalk of the mitotic spindle assembly checkpoint with p53 to prevent polyploidy. *Oncogene* 2004;23:6845-53.
26. Taylor SS, Hussein D, Wang Y, Elderkin S, Morrow JC. Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. *J Cell Sci* 2001;114:4385-95.
27. Yamaguchi H, Chen J, Bhalla K, Wang GH. Regulation of Bax activation and apoptotic response to microtubule-damaging agents by p53 transcription-dependent and -independent pathways. *J Biol Chem* 2004;279:39431-7.
28. Panaretakis T, Pokrovskaja K, Shoshan CM, Grandner D. Activation of Bak, Bax, and BH3-only proteins in the apoptotic response to doxorubicin. *J Biol Chem* 2002;277:44317-26.
29. Brummelkamp RT, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002;296:550-3.
30. Vogel C, Kienitz A, Muller R, Bastians H. The mitotic spindle checkpoint is a critical determinant for topoisomerase-based chemotherapy. *J Biol Chem* 2005;280:4025-8.
31. Li F, Altieri CD. Transcriptional analysis of human survivin gene expression. *Biochem J* 1999;344 Pt 2:305-11.
32. Brichese L, Barboule N, Heliez C, Valette A. Bcl-2 phosphorylation and proteasome-dependent degradation induced by paclitaxel treatment: consequences on sensitivity of isolated mitochondria to Bid. *Exp Cell Res* 2002;278:101-11.
33. Deng X, Gao F, Flagg T, May SW, Jr. Mono- and multisite phosphorylation enhances Bcl2's antiapoptotic function and inhibition of cell cycle entry functions. *Proc Natl Acad Sci U S A* 2004;101:153-8.
34. Breitschopf K, Haendeler J, Malchow P, Zeiher MA, Dimmeler S. Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway. *Mol Cell Biol* 2000;20:1886-96.
35. O'Connor SD, Grossman D, Plescia J, et al. Regulation of apoptosis at cell division by p34cdc2 phosphorylation of survivin. *Proc Natl Acad Sci U S A* 2000;97:13103-7.
36. O'Connor SD, Wall RN, Porter CA, Altieri CD. A p34(cdc2) survival checkpoint in cancer. *Cancer Cell* 2002;2:43-54.
37. Keen N, Taylor SS. Aurora-kinase inhibitors as anticancer agents. *Nat Rev Cancer* 2004;4:927-36.
38. Wheatley PS, Henzing JA, Dodson H, Khaled W, Earnshaw CW. Aurora-B phosphorylation *in vitro* identifies a residue of survivin that is essential for its localization and binding to inner centromere protein (INCENP) *in vivo*. *J Biol Chem* 2004;279:5655-60.
39. Honda R, Korner R, Nigg AE. Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. *Mol Biol Cell* 2003;14:3325-41.
40. Bolton AM, Lan W, Powers ES, McClelland LM, Kuang J, Stukenberg TP. Aurora B kinase exists in a complex with survivin and INCENP and its kinase activity is stimulated by survivin binding and phosphorylation. *Mol Biol Cell* 2002;13:3064-77.
41. Li F, Ambrosini G, Chu YE, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 1998;396:580-4.
42. Stewart S, Fang G. Destruction box-dependent degradation of aurora B is mediated by the anaphase-promoting complex/cyclosome and Cdh1. *Cancer Res* 2005;65:8730-5.
43. Vader G, Medema HR, Lens MS. The chromosomal passenger complex: guiding Aurora-B through mitosis. *J Cell Biol* 2006;173:833-7.
44. Wang X, Jin YD, Ng WR, et al. Significance of MAD2 expression to mitotic checkpoint control in ovarian cancer cells. *Cancer Res* 2002;62:1662-8.
45. Hampton T. Novel targeted cancer drugs highlighted. *JAMA* 2006;296:270.
46. Harrington AE, Bebbington D, Moore J, et al. VX-680, a potent and selective small-molecule inhibitor of the Aurora kinases, suppresses tumor growth *in vivo*. *Nat Med* 2004;10:262-7.