The Mitotic Spindle Checkpoint Is a Critical Determinant for Topoisomerase-based Chemotherapy*

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Celia Vogel‡, Anne Kienitz‡, Rolf Müller, and Holger Bastians§
From the Institute for Molecular Biology and Tumor Research (IMT), Philipps University Marburg, D-35037 Marburg, Germany

A novel strategy in cancer therapy is the induction of mitotic cell death by the pharmacological abrogation of cell cycle checkpoints. UCN-01 is such a compound that overrides the G2 cell cycle arrest induced by DNA damage and forces cells into a deleterious mitosis. The molecular pathways leading to mitotic cell death are largely unknown although recent evidence indicates that mitotic cell death represents a special case of apoptosis. Here, we demonstrate that the mitotic spindle checkpoint is activated upon chemotherapy treatment with topoisomerase II poisons and UCN-01. Cells that are forced to enter mitosis in the presence of topoisomerase inhibition arrest transiently in a prometaphase like state. By using a novel pharmacological inhibitor of the spindle checkpoint and spindle checkpoint-deficient cells we show that the spindle checkpoint function is required for the mitotic arrest and, most importantly, for efficient induction of mitotic cell death. Thus, our results demonstrate that the mitotic spindle checkpoint is an important determinant for the outcome of a chemotherapy based on the induction of mitotic cell death. Its frequent inactivation in human cancer might contribute to the observed resistance of tumor cells to these chemotherapeutic drugs.

The mitotic spindle assembly checkpoint prevents the onset of anaphase as long as a single chromosome is unattached to the mitotic spindle, thus ensuring proper chromosome alignment and chromosomal stability. Several proteins, including Mad1, Mad2, Bub1, BubR1, Bub3, and Mps1, which are specifically recruited to unbound kinetochores upon checkpoint activation, are required for the spindle checkpoint (1). Spindle-damaging chemotherapeutic drugs like taxanes (e.g. paclitaxel and docetaxel) and various Vinca alkaloids (e.g. vincristine and vinblastine) are frequently used in the clinic and activate the spindle checkpoint leading to a transient mitotic arrest (2). Upon prolonged spindle damage, however, cells undergo apoptosis, which represents the final outcome of chemotherapy. Significantly, spindle checkpoint defects are frequently observed in human cancer including breast, colon, lung, ovarian, and hepatocellular carcinomas (3–7). Interestingly, these defects have recently been associated with resistance to spindle damaging chemotherapeutic drugs (8–10).

Another large group of routinely used chemotherapeutics are various topoisomerase II poisons (e.g. etoposide/VP16, adriamycin/doxorubicin). These agents are thought to cause DNA damage and induce cell cycle arrest followed by the induction of cell death. While the cell cycle arrest in the G2 phase depends on p53, a G2 arrest can also occur in p53-deficient cells (11). A novel strategy for tumor therapy selectively targeting p53-deficient tumor cells is the drug-mediated inactivation of the G2 cell cycle arrest. UCN-01, which has successfully completed phase I clinical trials, is such a compound that abrogates potentially the G2 arrest in p53-deficient tumor cells (12). Treatment with UCN-01 induces mitotic entry in the presence of topoisomerase inhibition and greatly enhances cell death. Therefore, entry into mitosis in the presence of DNA damage appears to be an important determinant for chemotherapy outcome, but the mechanisms of the induction of mitotic cell death are not defined (13–15). Recently, Góñiz et al. (16), a compound similar to UCN-01, was also shown to potently abrogate the G2 arrest, and it was proposed to be a promising G2 cell cycle checkpoint abrogator for clinical studies (16).

EXPERIMENTAL PROCEDURES

Cell Culture—HCT116, HCT116-p53−/− (kindly provided by Dr. Bert Vogelstein, The Johns Hopkins University, Baltimore) (17) and HCT116-MAD2−/− cells (a gift from Dr. Loren Michel and Dr. Robert Benezra, Cornell University, New York) (18) were grown as described (19).

Western Blotting—Cells were lysted and semidry westernblotting was performed as described (19) using the following antibodies: anti-PARP (Pharmingen), anti-actin (Sigma), secondary antibodies conjugated to horseradish peroxidase (Jackson Immunoresearch).

Flow Cytometry—For FACS analyses DNA was stained using propidium iodide, and the mitotic index was determined by staining for the MPM2 epitope following procedures as described (19). Apoptotic cells were counted as cells containing a sub-G1 DNA content and were verified by staining apoptotic nuclei with DAPI.

Microscopy—Cells were fixed for 10 min in 1% p-formaldehyde/ ()/. The abbreviations used are: PARP, poly(ADP-ribose) polymerase; FACS, fluorescence-activated cell sorter; DAPI, 4',6-diamidino-2-phenylindole; Pipes, 1,4-piperazinediethanesulfonic acid; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

RESULTS

Mitotic Cell Death Is Associated with the Activation of the Spindle Checkpoint—We wished to investigate whether the spindle checkpoint is involved in mitotic cell death induced by chemotherapeutic treatment. Treatment of p53-deficient human colon carcinoma cells (HCT116-p53−/−) with Adriamycin resulted in a cell cycle arrest in G2 before entry into mitosis.
Consistent with previous work (12), addition of UCN-01 abrogated the G2 arrest and increased the proportion of mitotic cells in p53 deficient but not in wild-type cells within a short time interval (Fig. 1A). We noted that cells, which were forced to enter mitosis by treatment with UCN-01, accumulated in mitosis in a time-dependent manner, and this was not significantly further enhanced by treatment with nocodazole (data not shown). This observation raised the possibility that the mitotic spindle checkpoint might be activated when cells enter mitosis upon topoisomerase inhibition before cell death occurs. To test this, we determined the localization of Bub1 and BubR1 in cells treated with adriamycin and UCN-01. Clearly, cells arrested in a prometaphase-like state with condensed and unaligned chromosomes and both, Bub1 and BubR1, were co-localized with CREST at kinetochores, demonstrating an activated spindle checkpoint (Fig. 1B). Thus, cells arrest before anaphase in a spindle checkpoint-dependent manner in response to topoisomerase poison and UCN-01 treatment.

**The Indolocarbazole Compound Gö6976 Is a Potent Inhibitor of the Mitotic Spindle Checkpoint**—In a recent report, Gö6976, an indolocarbazole compound structurally similar to UCN-01, was found to be a similarly potent abrogator of the G2 DNA damage checkpoint (16). Interestingly, we identified Gö6976 in an independent screen for inhibitors of the mitotic spindle checkpoint. Gö6976, but not UCN-01, potently overrides the spindle checkpoint-mediated mitotic arrest in response to nocodazole or taxol (Fig. 2A). Significantly, when applied to asynchronously growing cells, Gö6976 greatly inhibited spindle

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**FIG. 1.** A mitotic arrest in response to topoisomerase poison and UCN-01 treatment is mediated by the spindle assembly checkpoint. *A,* UCN-01 overrides the G2 checkpoint and drives cells into mitosis. HCT116-wild-type and isogenic p53-deficient cells were treated with 750 nM adriamycin for 14 h followed by the addition of 100 nM UCN-01. The mitotic index was determined after 0, 3, 6, and 9 h. Mean values and standard deviations of three independent experiments are shown. *B,* topoisomerase inhibition induces mitotic arrest and activates the spindle checkpoint. HCT116-p53<sup>−/−</sup> cells were treated with adriamycin for 14 h followed by the addition of UCN-01 for 6 h. Co-immunolocalizations of Bub1 and BubR1 with CREST in mitotically arrested cells is demonstrated. The condensed and unaligned mitotic chromosomes were stained with DAPI.

**FIG. 2.** Pharmacological inhibition of the spindle checkpoint reveals a requirement for topoisomerase poison and UCN-01-induced cell death. *A,* Gö6976 overrides the mitotic spindle checkpoint. HCT116 cells were treated with 150 nM nocodazole or 100 nM taxol for 14 h and were subsequently treated with Me<sub>2</sub>SO, Gö6976, or UCN-01. After 2 h treatment the mitotic index was determined. *B,* Gö6976 inactivates the mitotic spindle checkpoint without affecting the cell cycle. Asynchronously growing p53-deficient HCT116 cells were treated with either nocodazole alone or in combination with Gö6976 or UCN-01 for up to 48 h. In 8-h intervals the mitotic index was determined. *C,* treatment with Gö6976, but not with UCN-01, leads to reduced mitotic arrest. HCT-wt and HCT-p53<sup>−/−</sup> cells were treated with adriamycin for 14 h followed by...
checkpoint dependent accumulation of mitotically arrested cells in response to nocodazole without causing disruption of the cell cycle per se (Fig. 2B and data not shown). Thus, both UCN-01 and Go6976 are inhibitors of the G2 DNA damage checkpoint; however, Go6976 is the first potent pharmacological spindle checkpoint inhibitor that can be used as a tool to convert asynchronously growing wild-type cells into spindle checkpoint-compromised cells.

The Spindle Checkpoint Is Required for Mitotic Cell Death Induced by Topoisomerase Poison and UCN-01 Treatment—The identification of Go6976 as an abrogator of the DNA damage-induced G2 arrest and as a potent inhibitor of the spindle checkpoint allowed us to ask whether the spindle checkpoint is involved in topoisomerase poison induced mitotic arrest and cell death. We treated HCT-p53−/− cells with adriamycin and either UCN-01 or Go6976. As expected, both inhibitors, but not adriamycin alone, greatly enhanced the mitotic index within short time intervals. Consistent with the observation that Go6976 acts as a potent inhibitor of the spindle checkpoint, the mitotic arrest in Go6976-treated cells was compromised (Fig. 2C). Most significantly, entry into mitosis was associated with the induction of cell death and this was clearly diminished in cells treated with Go6976 (Fig. 2D). To demonstrate that Go6976 efficiently overrides the G2 checkpoint and drives cells through mitosis without a prolonged mitotic delay or arrest we determined the DNA content after 24 and 48 h of treatment. We found that Go6976 treatment grossly induces endoreduplication and only a small proportion of cells showed a 4n DNA content typical for G2 cell cycle arrest (Fig. 3A). In response to topoisomerase poison treatment MAD2−/− cells are spindle checkpoint-defective as indicated by a dramatically reduced mitotic arrest in response to spindle damage (Fig. 3A). In response to topoisomerase poison treatment MAD2−/− cells are spindle checkpoint-defective as indicated by a dramatically reduced mitotic arrest in response to spindle damage (Fig. 3A).

DISCUSSION

UCN-01, which has successfully completed phase I clinical trials, is a promising anti-cancer drug that is used in combination with DNA-damaging agents to induce mitotic cell death (11–13). Although the induction of mitotic cell death is a major determinant for the chemotherapy outcome, the underlying...
molecular pathways are largely unknown. Most recently, it has been shown that the activation of caspase-2 and caspase-3 and the permeabilization of the mitochondrial membrane are associated with mitotic cell death indicating that it might represent a special case of apoptosis (20). Here, we show that the mitotic cell death induced by topoisomerase poisons and UCN-01 is preceded by a prolonged and spindle checkpoint-mediated mitotic arrest. Moreover, the mitotic spindle checkpoint is required for the induction of mitotic apoptosis suggesting that the spindle checkpoint is required for efficient induction of apoptosis in response to spindle damage. Indeed, spindle checkpoint defects in human tumors have been correlated with resistance to paclitaxel (8–10). From our studies, it is expected that spindle checkpoint-compromised tumors are resistant not only toward spindle-damaging drugs like paclitaxel, vinblastine, and vincristine but also to therapies based on the induction of mitotic cell death-like treatments using UCN-01 in combination with topoisomerase poisons. Thus, the status of the mitotic spindle checkpoint is likely to represent a major determinant for the clinical outcome of these chemotherapeutic treatments. Furthermore, the inactivation of the spindle checkpoint in tumor cells might contribute directly to an acquired resistance to these cancer therapies.

Our findings places the mitotic spindle checkpoint into a central position of the apoptotic cell death pathways induced by spindle-damaging agents and topoisomerase poisons. It is unknown how the spindle checkpoint triggers the apoptotic response, but it is tempting to speculate that molecules such as survivin that is involved in both, spindle checkpoint activation and apoptosis, might mediate the cross-talk between mitotic spindle checkpoint activation and induction of apoptosis (21, 22). It will be of great interest to elucidate how the activation of the spindle checkpoint and the apoptotic signaling are connected to develop novel therapies, which induce spindle checkpoint dependent apoptosis without harming normal cells.

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