Potentiation of Kinesin Spindle Protein Inhibitor–Induced Cell Death by Modulation of Mitochondrial and Death Receptor Apoptotic Pathways

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Abstract

Targeting the mitotic motor kinesin kinesin spindle protein (KSP) is a new strategy for cancer therapy. We have examined the molecular events induced by KSP inhibition and explored possible mechanisms of resistance and sensitization of tumor cells to KSP inhibitors. We found that KSP inhibition induced cell death primarily via activation of the mitochondrial death pathway. In HeLa cells, inhibition of KSP by small-molecule inhibitor monastrol resulted in mitotic arrest and rapid caspase activation. BclXL phosphorylation and loss of mitochondrial membrane potential was detected before significant caspase activation, which was required to trigger the subsequent apoptotic pathway. In A549 cells, however, KSP inhibition did not induce mitochondrial damage, significant caspase activity, or cell death. A549 cells aberrantly exited mitosis, following a prolonged drug-induced arrest, and arrested in a G1-like state with 4N DNA content in a p53-dependent manner. Overexpression of BclXL provided a protective mechanism, and its depletion rescued the apoptotic response to monastrol. In addition, Fas receptor was up-regulated in A549 cells in response to monastrol. Treatment with Fas receptor agonists sensitized the cells to monastrol-induced cell death, following exit from mitosis. Thus, activation of the death receptor pathway offered another mechanism to enhance KSP inhibitor–induced apoptosis. This study has elucidated cellular responses induced by KSP inhibitors, and the results provide insights for a more effective cancer treatment with these agents. [Cancer Res 2007;67(1):237–45]

Introduction

Kinesin spindle protein (KSP; Eg5) is one of several microtubule associated motor proteins that are required for proper spindle dynamics (1). KSP is required early in mitosis to separate the centrosomes and form the bipolar mitotic spindle (2). Consequently, KSP inhibitors, such as monastrol (3), prevent centrosome separation and generate a monoastral spindle phenotype (4, 5), triggering mitotic arrest and eventual apoptosis. Monastrol, a selective allosteric inhibitor of KSP ATPase activity, was one of the first small-molecule inhibitors identified in a phenotype-based screen (3). KSP inhibitors have been shown to exhibit antitumor activity and are currently in clinical trials (6). Because KSP localizes to mitotic microtubules and not interphase microtubules (7), KSP inhibitors are more selective to mitotic cells compared with other antimitotic drugs. In addition, because KSP inhibitors do not target microtubules, they provide an opportunity for specificity and improved side effect profile over anti-microtubule drugs, such as Taxol, which exhibits undesired side effects, such as neuropathy. Sustained arrest due to mitotic spindle disruption, such as that mediated by the antimitotic drugs Taxol and monastrol, often triggers apoptosis. Taxol has been shown to initiate apoptosis through multiple mechanisms (8, 9). KSP inhibitors induce mitotic arrest by a mechanism different from Taxol, but whether similar apoptotic pathways are triggered as a consequence of arrest is unclear.

Mechanisms of cell death induced by KSP inhibitors are beginning to be elucidated. Leizerman et al. have shown that monastrol can induce mitochondrial membrane depolarization in two human cell lines (10). The mitochonridia or intrinsic death pathway is one of two major caspase-dependent death pathways activated by anticancer drugs (11, 12). Several studies have correlated the impaired induction of apoptosis in response to chemotherapeutics with overexpression of the mitochondria-associated Bcl-2 family proteins (13, 14). An inhibitor of Bcl-2 family proteins has been shown to have synergistic cytotoxic effects with chemotherapeutics, resulting in regression of solid tumors (15). Posttranslational modification of Bcl-2 by phosphorylation has also been indicated as another level of functional regulation (16). Phosphorylation of Bcl-2 has been detected in Taxol-treated cells and has been suggested as one of the mechanisms by which Taxol induces apoptosis in cancer cells (17, 18). The extrinsic apoptotic pathway mediated by death receptors, such as Fas, tumor necrosis factor (TNF)–related apoptosis-inducing ligand, and TNF receptor 1, is the second major pathway cells use to commit apoptosis (11). Activation of the extrinsic death pathway, mediated by Fas/Fas ligand (FasL; CD95/APO1), has been reported with Taxol in human malignant glioma cells (19). Activation of the Fas receptor pathway has been shown to enhance the anticancer effect of Taxol and other chemotherapeutic agents in human soft tissue sarcoma cells (20). Recently, small-molecule cytotoxic agents that sensitize resistant tumor cells to death receptor ligand stimulation have been identified using a novel high-throughput screen (21).

In a recent report, it was proposed that activation of the spindle checkpoint followed by mitotic slippage is required to trigger KSP inhibitor–induced apoptosis (22). Cells with chromosomal instability, which is associated with mitotic checkpoint defects, have a low mitotic index and exit mitosis prematurely after treatment with mitotic drugs (23, 24). There are conflicting reports of whether impairment of the spindle checkpoint inhibits or sensitizes cells to apoptosis with anti-microtubule drugs (25, 26). Studies from our lab have shown that cells, in which the spindle checkpoint is compromised as a result of BubR1 or Mad2 depletion, undergo...
apoptosis in response to Taxol and monastrol following mitotic cell slippage, suggesting that activation of the spindle checkpoint may not be required for KSP inhibitor–induced apoptosis (27). Some cells can, however, override a functionally activated spindle checkpoint and aberrantly exit mitosis without undergoing cell division. Studies have shown that following a transient mitotic arrest, nocodazole–treated fibroblasts arrest in a G2-like state (28). This phenomenon of adaptation (29), after a failure in cytokinesis in the presence of mitotic drugs, has been reported to depend on p53 (30).

In this study, we have further elucidated the cellular responses following monastrol-induced mitotic arrest. We have examined activation of the intrinsic or mitochondrial apoptotic pathway and the role of mitochondria associated proteins in KSP inhibition–induced cell death. We have also examined the role of the extrinsic or death receptor pathway in modulating the apoptotic response to monastrol.

Materials and Methods

Cell culture and drug treatment. HeLa cells, A549 cells, and A2780 cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively) at 37°C and 5% CO2. A549 and A2780 cells stably expressing p53 small interfering RNA (siRNA) hairpin were maintained in above medium supplemented with 1.5 mg/mL puromycin. Cells were plated at a density of 5 × 105 and treated with either 200 μM monastrol (Tocris Cookson, Inc., Ellisville, MO) or control (0.1% DMSO). Where indicated, caspase inhibitor VI (Calbiochem, San Diego, CA) or DMSO was added to the cells at the time of drug treatment at a final concentration of 100 μM/L. In addition, where indicated, either a control antibody (IgM), 40 ng/mL Fas receptor activating antibody (CH11, BD PharMingen, San Diego, CA) were maintained in DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively) at 37°C and 5% CO2. A549 and A2780 cells stably expressing p53 small interfering RNA (siRNA) hairpin were maintained in above medium supplemented with 1.5 mg/mL puromycin. Cells were plated at a density of 5 × 105 and treated with either 200 μM/L monastrol (Tocris Cookson, Inc., Ellisville, MO) or control (0.1% DMSO). Where indicated, caspase inhibitor VI (Calbiochem, San Diego, CA) or DMSO was added to the cells at the time of drug treatment at a final concentration of 100 μM/L. In addition, where indicated, either a control antibody (IgM), 40 ng/mL Fas receptor activating antibody (CH11, Upstate, Lake Placid, NY), or 5 ng recombinant Fasl. (Upstate) was added to the cells at the time of drug treatment.

Mitotic arrest assay/DNA content analysis. Following drug treatment at various times indicated, attached and floating cells were collected, washed in PBS, and then fixed overnight in ice-cold 70% ethanol. Cells were washed with wash buffer (1% FBS, 0.3 mg/mL saponin in PBS) and incubated with 100 μL of 5 μg/mL anti-MPM-2 antibody (Upstate: diluted in wash buffer) for 1 h on ice. The cells were washed in wash buffer and resuspended in a 1:50 dilution of anti-mouse IgG–FITC antibody (Amersham, Piscataway, NJ) and incubated for 1 h on ice. The cells were then washed again with wash buffer and resuspended in 400 μL propidium iodide/RNase I solution (BD Pharmingen, San Diego, CA). After 1 h on ice, the cells were analyzed by fluorescence-activated cell sorting (FACS) using FACS Calibur (BD Biosciences, San Jose, CA). The percentage of cells stained with MPM-2 antibody was quantified using CellQuest software (BD Biosciences) as mitotic-arrested population. FACS analysis was also used to determine the percentage of cells containing 4N or >4N DNA content.

Caspase activity assay. Following drug treatment attached and floating, cells were collected and resuspended in medium. A carboxyfluorescein caspase detection kit (Biocarta, San Diego, CA) was used according to protocol to measure activation of general caspases. Activated caspases were quantified by FACS analysis using FACS Calibur (Becton Dickinson, San Diego, CA) and CellQuest software.

Western blot analysis. Following drug treatment, attached and floating cells were collected, washed with PBS, centrifuged, and resuspended in lysis buffer [50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, protease inhibitors]. Lysates were clarified by centrifugation and assayed for protein concentration. Membrane and cytosolic preparations, the adherent and floating cells were incubated in homogenizing buffer [150 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA and EGTA, protease inhibitors] on ice for 30 min. Cells were then washed through a 26-gauge needle 10 times and centrifuged at 750 × g for 10 min. The supernatant was centrifuged at 100,000 × g for 1 h at 4°C. Cells pellets were lysed in lysis buffer as indicated above; 30 μg total protein was loaded onto a SDS-PAGE gel, transferred to polyvinylidene difluoride membrane, and blotted with 5% milk in TBS Tween 20 (TBS-T). Primary antibodies used were anti–poly(ADP-ribose) polymerase (anti-PARP; Cell Signaling, Danvers, MA), anti–cyclophilin D (in house), anti–cytochrome c (BD PharMingen), anti-BclXL (Cell Signaling), anti-KSP (BD Biosciences), anti-p53 (Oncogene, Cambridge, MA), anti-p21 (NeoMarkers, Fremont, CA), and anti–β-actin (Sigma, St. Louis, MO) at recommended concentrations. Membranes were washed with TBS-T and incubated with horseradish peroxidase–conjugated anti-rabbit or anti–mouse secondary antibody (Amersham Biosciences, Piscataway, NJ). Chemiluminescence was detected with Pierce Supersignal WestPico.

Mitochondrial membrane permeabilization (Mitotracker) assay. Mitochondrial integrity was assayed with the mitochondrial dye Mitotracker Red ( Molecular Probes, Eugene, OR). Cells were treated with monastrol or DMSO as described above. After indicated times, the cells were incubated with 100 nmol/L/dye for 45 min at 37°C under 5% CO2. Cells were then collected, washed with PBS, resuspended in PBS + 1% FBS, and analyzed by FACS. The dye intensity was measured in the FL1 channel.

siRNA knocking down. siRNA duplexes to knockdown BclXL, p53, and KSP mRNAs were synthesized by Dharmaco, Inc. (Lafayette, CO). BclXL target sequence used was 5′-AGCAUUACCAGGGUUGA-3′. KSP target sequence used was 5′-GAACTAAATTTACCTTG-3′ (31). A luciferase siRNA duplex (5′-CAUUCCUACCCAGAGGAUG-3′) was used as a control. Transient transfection of siRNA duplexes was done as described in Tanudji et al., using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) as the transfection reagent (32). p53 target sequence used was 5′-AGAAGCTTCAGGTGTAACCTA-3′ (33). A p53 siRNA expression cassette with puromycin selective marker or an empty cassette was cloned into pLXSN vector (BD Biosciences) as described (33). A retroviral stock was generated by transfecting GP293 cells with p53 hairpin siRNA construct and VSVG (virus envelope) construct using Opti-MEM transfection reagent. A549 and A2780 cell line stably expressing p53 siRNA was generated by infecting cells with the retrovirus and then selecting several clonal cell lines lacking p53 under puromycin selection. Results shown are representative of at least three individual clones.

Expression analysis by real-time quantitative PCR. RNA was isolated using the RNeasy method, according to the manufacturer's protocol (Qiagen, Valencia, CA). Total RNA (5 μg) was subjected to treatment with DNase (Roche Molecular Biochemicals, Indianapolis, IN). DNase-treated total RNA was reverse-transcribed using Superscript II (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Gene-specific primers were designed using Primer Express (PE Biosystems, Foster City, CA). Two unlabeled primers at 900 nmol/L each were used with 250 nmol/L of FAM-labeled probe (Applied Biosystems, Foster City, CA) in a Taqman real-time quantitative PCR reaction on an ABI 7700 sequence detection system. Ubiquitin levels were measured in a separate reaction and used to normalize the data by the Δ−ΔCt method.

Cell surface expression analysis by flow cytometry. Following drug treatment, attached and floating cells were collected and washed with 1× PBS + 1% FBS. Cells were then incubated with either control antibody (IgG) or anti–Fas receptor antibody (ZB4, Upstate) at 500 ng/mL for 1 h at 4°C. Cells were washed 2× with 1× PBS + 1% FBS and incubated with anti–mouse FITC for 1 h at 4°C. Cells were washed and resuspended in 1× PBS + 1% FBS, and the fluorescence intensity was determined by FACS analysis.

Results

Monastrol induced mitotic arrest and mitochondrial damage followed by caspase activation in mitotically arrested HeLa cells. Microtubule poisons, such as vinblastine and Taxol, induce a mitotic block in cells, due to the activation of the spindle checkpoint (29), resulting in apoptotic cell death (34, 35). We first examined the kinetics of monastrol-induced arrest and cell death in asynchronously growing HeLa cells. HeLa cells treated with monastrol for 16 h arrested in mitosis as determined by the accumulation of phosphorylated MPM-2 signal, a marker for the mitotic stage of the cell cycle (Fig. L1). Monastrol induced a dose-dependent arrest of HeLa and other cell lines in mitosis, with maximum arrest observed with 200 μmol/L concentration by
16 h that was comparable with mitotic arrest induced with 50 nmol/L Taxol (data not shown; ref. 27). There was a rapid increase in caspase activity followed by an accumulation of cells in the sub-G1 compartment (Fig. 1A). Maximum mitotic arrest and activation of caspases was detected within 16 h, and the decrease in arrested cells by 24 h was due to significant induction of cell death (increase in caspase activity and sub-G1 cells). Within 36 h of monastrol treatment, the majority of cells underwent apoptosis (data not shown). Cleavage of PARP, a marker for cells undergoing apoptosis, in monastrol-treated cells confirmed the induction of apoptosis in these cells (Fig. 1C). Inhibition of caspase activity by caspase inhibitor (Z-VAD-fmk) not only reversed monastrol-induced cell death (sub-G1 population) but also prolonged the arrest of cells in mitosis (Fig. 1B), suggesting that activation of caspases occurred in mitotically arrested cells. PARP cleavage observed in cells treated with monastrol was also inhibited in cells pretreated with the caspase inhibitor (Fig. 1C). Monastrol-treated cells showed increased cyclin B levels (Fig. 1C) and a decreased cdc2-Tyr15 phosphorylation levels (data not shown), both of which are markers for the mitotic phase of the cell cycle. The absence of cyclin E (Fig. 1C), a marker for G1 phase of the cell cycle, showed that HeLa cells treated with monastrol did not exit mitosis following arrest in the presence or absence of caspase inhibitor. This was consistent with time lapse analysis of cells treated with monastrol, showing that following mitotic arrest, the monoaster cells underwent apoptosis without mitotic exit (27). Together, these results indicate that in HeLa cells, caspase activation and cell death are triggered in mitosis as a result of drug-induced mitotic arrest.
Because the release of cytochrome c from mitochondria plays a key role in the activation of caspases, we examined whether monastrol induced mitochondrial damage with a subsequent release of cytochrome c into the cytoplasm. Mitochondrial damage was determined by a decrease in the intensity of mitochondrial dye as a result of loss of mitochondrial membrane potential. Monastrol-treated cells showed decreased intensity of mitochondrial dye (Fig. 1D, M2) with time. The loss of mitochondrial membrane potential correlated with the release of cytochrome c into the cytoplasm (Fig. 1D, bottom). Following monastrol treatment, cytochrome c was detected in the cytoplasmic compartment by 16 h. Pretreatment with caspase inhibitor (Z-VAD-fmk) partially blocked drug-induced mitochondrial damage, indicating some requirement for the activation of initiator caspases upstream of mitochondrial damage (data not shown).

In A549 cells, monastrol induced a sustained mitotic arrest, but cells exited mitosis to a G1-like state without activation of the mitochondrial death pathway. Monastrol induced a dose-dependent and sustained mitotic arrest, up to 24 h of drug treatment, in non–small cell lung carcinoma A549 cells (data not shown; Fig. 2A). An increase in the MPM-2–positive cells with 16- and 24-h drug treatment corresponded with the accumulation of cells with 4N DNA content. However, no increase in caspase activity, sub-G1 population (Fig. 2A), or PARP cleavage (Fig. 2C) was detected with 24-h drug treatment. Time course analysis (Fig. 2A) revealed that after 48 h of treatment with monastrol, caspase activity was only slightly increased; however, there was no accumulation of sub-G1 cells. In addition, no PARP cleavage was detected with up to 96 h of treatment with monastrol (Fig. 2C). Instead, as indicated by the loss of MPM-2 signal, monastrol-treated cells exited mitosis and arrested in a post-mitotic G1 state with 4N DNA content (Fig. 2A). This post-mitotic G1 phase arrest was p53 dependent because in the absence of p53, monastrol-treated cells exhibited endoreduplication with little further increases in caspase activity or cell death, resulting in aneuploidy cells with 8N DNA content (Supplementary Fig. S1). p53 and its target p21 were up-regulated in A549 cells in response to monastrol (data not shown). The aberrant mitotic exit of cells with 4N DNA content to G1 state after 48 h of monastrol treatment was confirmed by increased cyclin E levels and decreased cyclin B levels (Fig. 2C) and cdc2-Tyr15 dephosphorylation (data not shown). Some PARP cleavage was, however, detected in these cells after 48 h of treatment with Taxol, at which time cells have exited mitosis (Fig. 2C). The low level of sub-G1 cells observed with monastrol could be reversed by pretreatment of the cells with a caspase inhibitor (Fig. 2B). Under these conditions, the percentage of cells with 4N DNA content increased without a corresponding increase in MPM-2–positive cells. These results indicated that unlike HeLa cells, A549 cells did not accumulate in mitosis in the absence of caspase activation. Thus, A549 cells exited mitosis without cell division following drug-induced arrest irrespective of caspase activation. We next analyzed mitochondrial membrane damage in these cells in response to monastrol and found that monastrol failed to induce any mitochondrial damage. No decrease in Mitotracker dye intensity was detected with 24 and 48 h of monastrol treatment (Fig. 2D). These results suggest that

![Figure 2](cancerres.aacrjournals.org)
monastrol is unable to activate the mitochondrial death pathway and trigger cell death in A549 cells.

**Effect of KSP inhibition on the antiapoptotic protein BclXL.**

We next investigated the effect of monastrol on several mitochondrial-associated proteins that could mediate the response to KSP inhibition. Quantitative reverse transcription-PCR (RT-PCR) expression analysis revealed that the mitochondria-associated antiapoptotic protein BclXL was overexpressed in A549 cells compared with HeLa cells. There was little change in BclXL mRNA or protein levels in monastrol-treated cells compared with DMSO-treated cells (Fig. 3A). We observed that in HeLa cells, monastrol induced phosphorylation of BclXL by 16 h as indicated by band shift (Fig. 3B). BclXL phosphorylation was also detected in A2780 ovarian carcinoma cells in response to monastrol. A2780 cells exhibited a strong mitotic arrest phenotype in response to monastrol at 16 h. Increased caspase activity by 24 h with significant cell death was detected with 48-h drug treatment (Supplementary Fig. S2). A2780 cells, however, were also susceptible to mitotic slippage and exhibited p53-dependent endoreplication (Supplementary Fig. S2) similar to A549 cells. However, no BclXL phosphorylation was detected in A549 cells in response to monastrol (Fig. 3B). All these cell lines showed little or no expression of antiapoptotic protein Bcl-2, as determined by either quantitative RT-PCR expression analysis or by Western blot analysis (data not shown). Phosphorylation of BclXL was also detected in HeLa cells depleted in KSP protein by siRNA (Fig. 3C). HeLa cells treated with KSP siRNA duplex showed depletion of KSP protein after 40 h, at which time BclXL protein band shift was detected, suggesting that BclXL phosphorylation was a consequence of KSP inhibition-induced mitotic arrest. The appearance of BclXL band shift in drug-treated and KSP-depleted cells coincides with the activation of the apoptotic pathway. These results show that KSP inhibition induces phosphorylation of BclXL, thereby activating the mitochondrial death pathway following arrest of cells in mitosis.

**Antiapoptotic protein BclXL plays a key role in resistance to monastrol-induced death.** We further investigated the resistance of A549 cells to monastrol-induced apoptosis by examining the role of BclXL in the response to monastrol. A549 cells lacking BclXL were generated using siRNA depletion. Cells were transfected with either luciferase siRNA or BclXL siRNA duplexes, and cell lysates were analyzed 48 and 72 h after transfection. Transfection with a BclXL siRNA duplex resulted in significant depletion of BclXL protein (Fig. 4A) and the knockdown lasted up to 96 h after transfection. Twenty-four hours after transfection with siRNA, the cells were treated with monastrol for up to 72 h to analyze their mitotic arrest, caspase activity, and cell death profile. Interestingly, in the absence of BclXL, monastrol induced a marked increase in caspase activity and cell death with time that was not detected in luciferase-transfected cells treated with monastrol (Fig. 4B). No increase in caspase activity or cell death was detected in BclXL-depleted cells treated with DMSO (data not shown), suggesting that BclXL depletion alone does not trigger apoptosis. Thus, BclXL depletion sensitized cells to mitotic drug-induced apoptosis.

We then tested the ability of monastrol to induce mitochondrial damage in these cells (Fig. 4C). BclXL-depleted cells treated with monastrol showed significant mitochondrial damage, as indicated by a decrease in the intensity of the mitochondrial dye (M2), within 24 h. No mitochondrial damage was observed in luciferase-transfected cells treated with monastrol. In addition, no mitochondrial damage was detected in BclXL-depleted cells treated with DMSO. Thus, BclXL depletion can rescue the apoptotic response to monastrol in A549 cells. These results suggest that the overexpression of BclXL and/or its inability to be phosphorylated in A549 cells in response to monastrol-induced mitotic arrest prevents activation of the mitochondrial death pathway.

**Fas receptor agonists synergize with antimitotic drugs.** We next examined whether monastrol could modulate expression of other proteins associated with the apoptotic machinery of the cell. Quantitative RT-PCR analysis revealed that the Fas receptor mRNA and other proteins associated with the apoptotic machinery of the cell were up-regulated in monastrol-treated A549 cells (Fig. 5A). Increase in the cell surface expression of Fas receptor, with time, was also detected in response to monastrol in A549 and A2780 cells (Fig. 5B). Depletion of p53 in both the cell lines inhibited the up-regulation of Fas receptor mRNA and cell surface protein expression (Fig. 5A and B), suggesting that drug-induced Fas

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Figure 3. A, BclXL mRNA levels normalized to ubiquitin in 16 h DMSO- and monastrol-treated HeLa and A549 cells as determined by quantitative RT-PCR analysis. B, BclXL protein expression in cytoplasmic (C) and membrane (M) preparations of HeLa and A549 cells treated with DMSO (−) and 200 µmol/L monastrol for 16 h as determined by Western Blot analysis (top). BclXL protein expression in total cell lysates from 16 and 24 h, DMSO (−) and 200 µmol/L monastrol-treated A2780 cells (bottom). C, HeLa cells were transfected with either luciferase siRNA or KSP siRNA duplex. Twenty-four hours and 40 h after transfection, cells lysates were immunoblotted with either anti-BclXL antibody or anti-KSP antibody.
luciferasesiRNA–transfected cells, indicating damaged mitochondrial membrane. Activation of the Fas receptor by Fas receptor–activating antibody significantly enhanced caspase activation and cell death (sub-G₁ population) in monastrol-treated cells but not DMSO-treated cells (Fig. 5C). Activation of the Fas receptor by recombinant FasL also significantly enhanced monastrol-induced caspase activity that was inhibited by anti–FasL–neutralizing antibody (Fig. 5D). Similarly, p53-dependent Fas receptor up-regulation and Fas agonist–induced enhanced cell death was also observed with Taxol (data not shown). Thus, Fas receptor agonists (Fas receptor–activating antibody or recombinant FasL) can enhance cell death in combination with the antimitotic drugs but do not induce cell death by themselves. In addition, no increase in caspase activity and cell death was detected when cells were treated with monastrol in combination with anti–Fas receptor antibody for 16 h (Supplementary Fig. S3), at which time cells are still in drug-induced mitotic arrest. This suggests that Fas receptor agonists potentiate monastrol-induced cell death following mitotic exit in cells susceptible to mitotic slippage. In addition, anti–Fas receptor antibody–induced caspase activity and cell death was inhibited in p53-depleted cells treated with monastrol compared with cells wild type for p53 (Fig. 5C). These results suggest that KSP inhibitor–induced up-regulation of Fas receptor sensitizes the post-mitotic G₁ phase arrested cells to Fas receptor agonist–mediated death that is p53 dependent.

### Discussion

Disruption of the mitotic spindle has been a successful strategy for cancer therapeutics. Inhibition of the mitotic motor kinesin protein KSP/Eg5 is a novel method to target the mitotic spindle. Inhibition of KSP with small-molecule inhibitors, such as monastrol, or KSP depletion by siRNA induced arrest of cells in mitosis. The mitotic arrest phenotype was observed in HeLa cells (Fig. 1), A549 cells (Fig. 2), and A2780 cells (Supplementary Fig. S2), suggesting that these cells activated the mitotic spindle checkpoint (36, 37). In HeLa cells, monastrol induced rapid activation of caspasas in mitosis as a consequence of mitotic arrest (Fig. 1). Inhibition of caspase activity not only inhibited cell death but also prolonged arrest of the cells in mitosis (Fig. 1). Expression of cyclin B and the absence of cyclin E accumulation, in the presence or absence of caspase inhibitor, indicated that HeLa cells did not exit mitosis. A549 cells, on the other hand, underwent mitotic slippage following prolonged KSP inhibition–induced mitotic arrest. However, no significant caspase activation or cell death was detected either during the arrest or following mitotic slippage. Instead, these cells arrested in a post-mitotic G₁-like phase without undergoing cell division (Fig. 2). Thus, inhibition of caspase activity affected the kinetics of cell death but not the kinetics of mitotic exit. Similarly, A2780 cells also underwent mitotic slippage after monastrol-induced mitotic arrest but, unlike A549 cells, showed increased caspase activity and cell death (Supplementary Fig. S2). Together, these results suggest that exit from mitosis does not correlate with induction of apoptosis in response to monastrol.

Aberrant mitotic exit without cytokinesis has been shown to activate a p53-mediated tetraploidy checkpoint (38). Indeed, monastrol induced up-regulation of p53 in A549 cells similar to anti-microtubule drugs. Depletion of p53 protein in A549 and A2780 cells resulted in endoreduplication (Supplementary Figs. S1 and S2). The predominant phenotype of endoreduplication was observed only after 48 h of drug treatment, at which time cells have undergone mitotic slippage into G₂ phase. However, endoreduplication was accompanied with little increase in A549 cell death,
suggesting their inability to undergo cell death in response to monastrol. In addition, HeLa cells, which are inherently p53 deficient (expressing human papillomavirus E6) and which when depleted in the checkpoint proteins BubR1 and Mad2, also aberrantly exit mitosis and undergo endoreduplication. Unlike A549 cells, however, checkpoint-deficient HeLa cells eventually undergo apoptosis (27). Thus, based on our results, there seems to be no coupling of the induction of apoptosis to mitotic slippage as proposed by Tao et al. (22). Rather, failure to undergo cell death suggests an inability to activate the apoptotic machinery.

Activation of the caspase cascade characterizes cells undergoing apoptosis (39) and can be triggered by the mitochondrial or death receptor apoptotic pathway (40). Our results indicate that KSP inhibition primarily activates the mitochondrial death pathway, and that BclXL, the antiapoptotic Bcl-2 family protein, is a key player in monastrol-induced apoptosis. Although the mechanisms responsible for mitochondrial membrane permeabilization are unclear, the Bcl-2 family proteins have emerged as key regulators (41). BclXL, and not Bcl-2, is predominantly expressed in HeLa, A549, and A2780 cells (data not shown). BclXL mRNA, as well as protein, was expressed at significantly higher levels in A549 cells compared with HeLa cells (Fig. 3). The enforced overexpression of Bcl-2 into most cell types has been shown to confer resistance to cell death signals (42). Studies in patients with several types of leukemia’s, non-Hodgkin’s lymphomas, multiple myeloma, and prostate cancer support the hypothesis that high-level expression of Bcl-2 family proteins confers a clinically important chemoresistant phenotype (43). Thus, an imbalance in the ratios of

**Figure 5.** p53-dependent upregulation of Fas receptor in monastrol-treated A549 cells. Fas receptor agonists enhance monastrol-induced caspase activation and cell death. A, Fas receptor mRNA levels determined by quantitative RT-PCR in wild-type (WT) and p53-depleted cell lines treated with DMSO and 200 μmol/L monastrol for indicated times. B, Fas receptor protein cell surface expression levels in wild-type and p53-depleted A549 and A2780 cells, treated with 200 μmol/L monastrol for indicated times, were analyzed by FACS using anti–Fas receptor antibody (CH11). C, wild-type and p53-depleted A549 cells were treated with DMSO or 200 μmol/L monastrol in the presence of control antibody (IgM) or anti–Fas receptor–activating antibody (CH11, 40 ng/mL). After 48 h, cells were collected, and mitotic arrest, caspase activation, cell death, and DNA content were determined by FACS as described in Materials and Methods. D, A549 cells were treated with DMSO or 200 μmol/L monastrol in the presence of control antibody (IgG) or an anti–FasL–neutralizing antibody (BR17, 60 ng/mL). After 40 h, cells were incubated with or without recombinant FasL (5 ng) for 8 h and then assayed for caspase activity by FACS analysis.
antiapoptotic and proapoptotic Bcl-2 family members that favors survival can render tumor cells more resistant to cell death stimuli. Overexpression of Bcl-2 family proteins alone, however, may not be enough to render cells resistant to apoptosis. Expression levels of Bcl-2 family proteins in tumor samples of patients with acute lymphoblastic leukemia did not always correlate with outcomes or resistance to chemotherapeutic drugs (44). Posttranslational modification, such as phosphorylation, has been implicated as another level of functional regulation of the Bcl-2 family proteins (45). Anti-microtubule drug–induced phosphorylation of Bcl-2 and BclXL is thought to modulate the ant apoptotic function of Bcl-2 proteins and thus contribute to apoptosis (46, 47). BclXL was phosphorylated in monastrol-treated HeLa and A2780 cells and in KSP-depleted HeLa cells (Fig. 3), suggesting that BclXL phosphorylation is a consequence of antimitotic drug-induced arrest. Phosphorylation of BclXL in response to monastrol was not observed in A549 cells (Fig. 3). However, BclXL depletion in A549 cells dramatically restored the apoptotic response to monastrol (Fig. 4). Mitochondrial membrane permeabilization and cell death were induced within 16 h of monastrol treatment, at which time cells are still in mitotic arrest. Thus, phosphorylation of BclXL in response to antimitotic drug-induced arrest may play a role in inhibiting the ant apoptotic function of BclXL. Neutralization of BclXL by siRNA depletion of the protein did not induce cell death by itself but sensitized cells to mitotic drug-induced death (data not shown; Fig. 4). This is consistent with recent data that showed that a small-molecule inhibitor of Bcl-2 family proteins enhanced the apoptotic effects of chemotherapeutics and radiation in non–small cell lung cancer (15).

Several studies have shown that chemotherapeutic agents can modulate expression of genes that could be targets for combination therapy (48, 49). Combination of topoisomerase inhibitor CPT-11 with a cytotoxic antibody to the cell surface antigen whose expression is induced by CPT-11 resulted in a synergistic tumor regression as opposed to either agent alone (50). Our data indicated that Fas receptor expression was significantly up-regulated in response to monastrol in A549 and A2780 cells in a p53-dependent manner (Fig. 5). No increase in FasL levels was detected in response to monastrol (data not shown). This is consistent with reports that Fas receptor expression is increased and dependent on p53, following cytotoxic drug treatment (51). Activation of the Fas receptor induced cell death in monastrol-treated A549 cells with significant increase in caspase activity and cell death observed after cells exited mitosis (Fig. 5). Fas receptor agonists enhanced the sensitivity of A549 cells to monastrol but did not induce cell death alone. Thus, it seems that cells susceptible to mitotic slippage that are wild type for p53 can be sensitized to KSP inhibitors and antimicrotubule drugs, such as Taxol, by activating the extrinsic death receptor–mediated apoptotic pathway.

A successful anticancer therapeutic strategy requires not only a better understanding of how apoptosis is triggered but also a need to overcome inherent resistance of many transformed cells to apoptosis. Our studies, with continuous or transient treatment and release, with potent high-affinity KSP inhibitors showed identical results to monastrol, which is a relatively weak KSP inhibitor.1 Potent KSP inhibitors that target the same site as monastrol have shown good efficacy in tumor models (6). Thus, based on our studies with monastrol and other potent small-molecule KSP inhibitors, we propose that inhibition of KSP activates the intrinsic death pathway during arrest of cells in mitosis. KSP inhibitor–induced apoptosis can be induced by simultaneous inhibition of BclXL in cells resistant to mitochondrial death. In cells susceptible to slippage following drug-induced arrest, apoptosis can be triggered or enhanced by activation of the extrinsic death pathway via stimulation of drug-induced Fas receptor expression (Fig. 6).

This study furthers our understanding of the biology of KSP inhibition and can help in selecting tumor types or potential drug combinations for a more effective cancer treatment.

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References


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