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# Cell cycle-dependent cytotoxicity and mitotic spindle checkpoint dependency of investigational and approved antimitotic agents

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**The mitotic spindle checkpoint (SPC) is a highly regulated mechanism in eukaryotic cells that ensures the even distribution of the duplicated genome between daughter cells. Malfunction of the SPC or deregulated expression of SPC regulatory proteins is frequently associated with a poor response to chemotherapeutic agents. We investigated various approved and investigational mitosis-specific agents, including spindle poisons, an Eg5 kinesin inhibitor, inhibitors of polo-like kinase 1 (Plk1) or Aurora-B kinase, a benzamide class HDAC inhibitor and compounds identified in a chemical genetics screen for their cell cycle-dependent cytotoxicities and for their activities toward SPC deficient (HT29, Caco-2, T47D) and SPC proficient human cell lines (A2780, HCT116, SW480). Using the RKOp27 cell system that allows inducible cell cycle arrest by the tunable expression of the cdk inhibitor p27Kip1, we found an exquisite proliferation-dependent cytotoxicity for all compounds except the aurora kinase inhibitor VX-680. Cytotoxicity of the antimitotic compounds was in general higher on SPC proficient than on deficient cells. We found two exceptions, a benzamide HDAC inhibitor which was effective on SPC proficient and deficient cells and an investigational compound, BYK72767, with a yet unknown mode of action. The degree of increased mitotic index was no predictor of cytotoxicity of the compounds nor was the phosphorylation of BubR1. However, SPC deficient cell lines were able to tolerate mitotic arrest for far longer times than SPC proficient cells. We conclude that targeting of SPC deficient cancers with novel antimitotic principles remains a challenge but certain drug classes may be equally efficacious regardless of SPC status.**

**Key words:** mitotic spindle checkpoint, mitotic arrest, Polo-like kinase, Aurora, Eg5, HDAC, mitotic targeting, investigational drugs

**Abbreviations:** APC: anaphase promoting complex; Cdc20: cyclin-dependent kinase 20; FCS: fetal calf serum; HDAC, histone deacetylase; IC50: Inhibitory concentration; KSP: kinesin spindle protein; PBS, phosphate-buffered saline; p-gp: p-glycoprotein; Plk1: polo-like kinase 1; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SPC: spindle checkpoint

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Stringent control of mitosis warrants accurate segregation of sister chromatids in dividing cells. Eukaryotic cells have evolved sophisticated mechanisms to monitor faithful progression through mitosis to prevent missegregation of chromosomes resulting in aneuploid, polyploid or binucleate daughter cells. This signal transduction pathway, known as the "spindle assembly checkpoint" (SPC), inhibits the onset of anaphase until all kinetochores are properly attached to spindle microtubules and set under tension during metaphase. Activation of the SPC after nuclear envelope breakdown is associated with the recruitment of SPC proteins to kinetochores that lack either microtubule attachment or kinetochore tension and results in inhibition of the anaphase-promoting-complex (APC/C), an E3 ubiquitin ligase that marks key mitotic proteins for proteasomal degradation.[1] Key substrates of APC/C include securin and cyclin B, whose degradation is required for the onset of anaphase and the exit from mitosis, respectively. The degradation of securin is required at the metaphase to anaphase transition to liberate the active form of separase, a protease cleaving a subunit of the cohesion complex that holds the sister chromatids together.[2,3] Thus, an activated SPC prevents the onset of anaphase through inhibition of proteolysis and the maintenance of the chromatid cohesion. Although little is known about the molecular mechanism of SPC activation, the recruitment of the checkpoint proteins to kinetochores and the activities of the kinases Bub1, BubR1 and Mps1 are essential for activation of the terminal effector protein, Mad2,

which inhibits the ubiquitin ligase activity of the APC/C.[4,1] Despite intensive research, it is still unclear how the lack of microtubule attachment or the lack of kinetochore tension is translated into an active checkpoint signal. The kinetochore-based kinesin CENP-E might act as sensor to monitor the attachment of microtubules to kinetochores and might be involved in initiating the checkpoint signal by activating the BubR1 kinase.[5,6] Failure of the spindle checkpoint (SPC) results in premature separation of sister chromatids even in the presence of misaligned chromosomes giving rise to chromosomal instability (CIN), the perpetual gain or loss of chromosomes or large parts thereof. This is associated with aneuploidy, which is a major hallmark of cancer. In fact, in many tumor cells, the SPC function is compromised, and the checkpoint signal is not sustained.[5]

Progression through mitosis is essential for the proliferation of both normal and tumor cells. Mitotic targeting of cancer cells is one of the most successful chemotherapeutic principles in clinical oncology. This is exemplified by the outstanding success of various classes microtubule interfering agents such as taxanes and vinca alkaloids. Other targeted approaches such as the development of Eg5 kinesin inhibitors or inhibitors of mitotic kinases such as Plk1 or aurora exploit the same essential mechanism of mitotic arrest (reviewed in Ref. [7]).

The involvement of the mitotic SPC in mediating apoptosis induced by spindle poisons has been intensively discussed. Interestingly, it has been shown that apoptosis induced by nocodazole, paclitaxel or kinesin spindle protein (KSP)/Eg5 inhibitors (see below) requires the activation of the SPC as well as the subsequent slippage from mitotic arrest.[8–11] Conversely, besides other known mechanisms of chemoresistance to spindle poisons, such as overexpression of p-glycoprotein (p-gp) or altered tubulin composition or p53 mutation, abnormalities in the SPC might also account for resistance toward antimitotic agents. It has been shown that various cell systems with impaired SPC (by inactivating mutations or deregulated expression) escape from apoptosis on treatment with paclitaxel and other antimitotic drugs. Inactivating mutations in the known SPC genes appear to be rather rare,[12,13] but deregulated expression of SPC genes such as MAD1 or MAD2 might also weaken the SPC function in human cancers.[12,14,15] Moreover, overexpression of Aurora-A has been demonstrated to result in an abrogation of the SPC resulting in resistance toward paclitaxel.[16] Colon carcinomas exhibit a very high incidence of CIN, which might be associated with SPC malfunction thereby possibly explaining a poor efficacy of paclitaxel in this cancer type. As defects in the SPC often result in altered tumor sensitivity to antimitotic agents, this topic is of general outstanding interest in clinical oncology research.

To explore the effect of SPC functionality on the sensitivity of tumor cells to several antimitotic pharmacologic classes, we investigated various human cancer cell lines described in the literature to be SPC proficient or deficient with regard to their sensitivity for mitosis-targeting drugs. We used microtubule-interfering agents (paclitaxel, vincristine), kinase inhibitors of aurora-B (VX-680[17] and Plk1 (compound 1[18]) and a benzamide pan-selective inhibitor of histone deacetylases (BYK394210). Two compounds (BYK72767 and BYK426115) emanated from a cell-based high throughput screening program to identify novel agents that induce mitotic accumulation without interfering with microtubule dynamics. We investigated their cell cycle-dependent cytotoxic activities and their capabilities to kill SPC proficient or deficient cancer cells. Overall, we observed a very good correlation between SPC deficiency and poor response toward the antimitotic agents. The benzamide HDAC inhibitor, however, was equally efficacious on both, SPC proficient and deficient cells. Moreover, we found no correlation between SPC functionality and the ability to accumulate in mitosis. Investigation of the onset of apoptosis after mitotic arrest revealed that SPC deficient cells are able to tolerate mitotic arrest for far longer times than SPC proficient cells. We conclude that mitotic targeting of SPC deficient cells remains a challenge even with novel targeted mitosis-confined approaches; however, compound classes exist that do display similar activities regardless of SPC status.

## Material and Methods

### Materials

Materials and all other reagents were purchased from Sigma (Deisenhofen, Germany) unless otherwise specified. Paclitaxel (Taxol®) was obtained from Brystol-Myers Squibb (Princeton, NJ, USA). Compounds BYK426115, BYK72767 and BYK394210 emanated from internal programs to identify novel mitosis-specific inducers of apoptosis and novel benzamide HDAC inhibitors, respectively.

### Cells and cell culture

RKO, HT29, Caco-2, HCT15 and HCT116 human colon cancer cells, SW480 human non-small cell lung carcinoma cells and T47D human mamma carcinoma cells and A2780 human ovarian carcinoma cells were obtained from LCG Promochem (Wesel, Germany) and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS). RKOp27 cells[19] were cultured in medium containing 200 μg/ml Zeocin and 500 μg/ml neomycin (G418). RKOp27 cell line verification was conducted by complete cell cycle arrest on addition of ponasterone A into the medium and subsequent cell cycle analysis via flow cytometry.

### Cell viability assay (cytotoxicity)

Metabolic activity correlating with cell proliferation/viability was quantified using the Alamar Blue (Resazurin) assay. Compounds were dissolved in dimethylsulfoxide (DMSO; 20 mM) and subsequently diluted in semilogarithmic steps. These DMSO dilutions were further diluted 1:100 into DMEM containing 10% FCS yielding a final concentration twice as high as the final concentration in the test. Cells were seeded into 96-well flat-bottom plates, in a volume of 200 μl/well. The following cell densities/well were used: 5.000 for proliferating RKOp27 cells; 15.000 for arrested RKOp27 cells;

700 for A2780; 2.500 for Caco-2; 1.000 for HCT116; 3.000 for HCT15; 3.200 for HT29; 2.000 for SW480 and 6.000 for T47D. To determine the compound effect on arrested RKOp27 cells, these cells were seeded in medium containing 10 μM ponasterone A (for the induction of p27Kip1). 24 h after seeding, 10 μl of each compound dilution in DMEM was added per well with 1% DMSO treated cells as a control. After incubation with the compounds for 72 h at 37°C, 10 μl Alamar Blue solution (Invitrogen, Carlsbad, CA, USA) was added and the fluorescence measured (ex 544 nm, em 590 nm). For calculation of cell viability, fluorescence intensity from untreated cells was set as 100% viability, and the fluorescence of treated cells were set in relation to the values of untreated cells. The corresponding inhibitory concentration (IC50) values were determined from the concentration-effect-curves.

### Cell staining

Cells were harvested by trypsinization and an aliquot of $5 \times 10^3$ cells was spun onto glass slides using a cytospin and subsequently fixed and stained with the Hemacolor kit (Merck Darmstadt, Germany). At least three fields with 200 cells each were counted for the determination of mitotic indexes.

### Determination of proliferative indexes

Cells were seeded in 96-well plates (A2780, SW480, HCT116: $0.7 \times 10^3$ cells/well, Caco-2, HT29, T47D $1 \times 10^3$ cells/well and incubated for 96 hr at 37°C. At the indicated time points nuclei were stained with Hoechst 33342 dye (final concentration 0.2 μg/ml), and cells were counted by Cellomics array scan (Pittsburgh, PA, USA). The mask for recognizing cells was adapted for each cell line. The doubling time was calculated as follows: (1) $N_t = N_0 \times e^{\lambda t}$ for exponential growth and (2) $\lambda \times T = \ln 2$ for calculation of the doubling times assuming exponential growth (logarithmic Figure linear, data not shown).

### Determination of mitotic accumulation via phosphorylated histone H3

Cells were harvested by trypsinization, and an aliquot of $1 \times 10^6$ cells was washed once with cold PBS and then fixed with 3% formaldehyde for 10 min at 37°C. After 1 min on ice, methanol was added (final concentration 90%) and cells were stored over night at –20°C. Cells were resuspended in PBS with 0.5% bovine serum albumin (BSA) and incubated with the antiphospho-histone H3 antibody (Ser 10; 1:100) for 60 min at room temperature. Cells were washed twice with BSA (0.5% in PBS) before incubation (30 min, room temperature, in the dark) with the secondary Alexa Flour488 conjugated antibody at a dilution of 1:1000. Cells were washed again before phospho-histone H3 was quantified with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) at an excitation wavelength of 488 nm.

### Flow cytometric analysis

Cells were harvested by trypsinization, and an aliquot of $1 \times 10^6$ cells was washed once with cold PBS and fixed with cold 70% ethanol for 24 h at –20°C. The DNA was stained with a solution containing 25 μg/ml propidium iodide and 10 μg/ml RNase A in PBS for 30 min. Cell cycle distribution was analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose) at an excitation wavelength of 488 nm.

### Immunoblot analysis

Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM $Na_3VO_4$ and 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation and the supernatants were collected. Equal amounts of protein were separated by SDS-PAGE and subsequently blotted onto polyvinylidenfluoride membranes for incubation with antibodies used for Western blot analysis as indicated. Specific signals were visualized by use of the ECL chemoluminescence detection kit (GE Healthcare, Braunschweig, Germany).

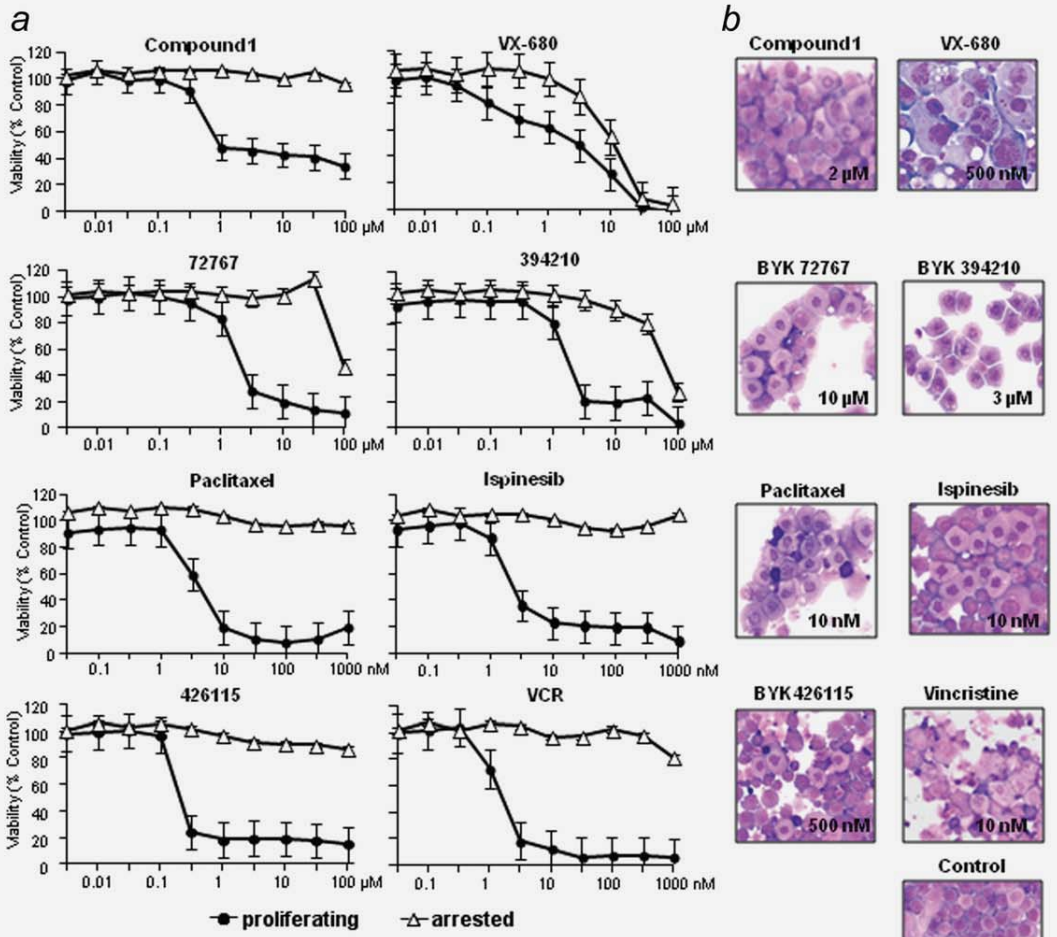
## Results

### Cell cycle-dependent activities of antimitotic substances

To investigate the cell cycle-dependent antimitotic activities of the compounds described, herein, we used the RKOp27 cell system.[19] The parental RKO cells were transfected to express the p27Kip1 cdk inhibitor under an ecdysone-inducible promoter. Inducible expression of p27Kip1 results in a complete cell cycle arrest in the G1 phase 24 hr after induction with ponasterone A. This system has been successfully used to determine cell cycle-dependent cytotoxicity of known and investigational chemotherapeutic agents.[20,21] The results are shown in Figure 1*a*. Almost all investigated compounds displayed a strong cell cycle-dependent activity yet with differing IC50 values. The aurora kinase inhibitor VX-680 displayed only a partial cell cycle-dependent activity with an effect also on the viability of arrested cells at concentrations above 3 μM, which may be due to target-unrelated effects of VX-680. Cell staining at the concentrations indicated in Figure 1*b* revealed that cells were arrested with a highly increased mitotic index except for VX-680, for which a high proportion of multinucleated cells was observed as was expected for inhibitors of aurora kinases.[17] A summary of the IC50 values and modes of action is given in Figure 1*c*.

### Activity of antimitotic compounds on SPC proficient and SPC deficient cell lines

We investigated their activities on several cell lines that were already described in the literature to be either SPC proficient (HCT116,[22] A2780,[23] SW480[24] or SPC deficient (HT29, Caco-2, T47D[24,25]). To exclude that overexpression of p-gp accounts for resistance toward the substances described herein (*e.g.*, Caco-2 cells) we tested all substances on HCT15 cells and adriamycin resistant A2780 cells that overexpress p-gp.[26] None of the substances except paclitaxel and vincristine appeared to be a substrate of p-gp (data not shown). The results of the cytotoxicity analyses are shown in Figure 2. In general, there was a good correlation between SPC proficiency (Fig. 2; filled symbols) and sensitivity to the antimitotic compounds. SW480 cells, however, showed only a

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| compound | Mode of Action | IC50 RKO-p27 (µM) | | factor prol./arrested |
|---|---|---|---|---|
| | | proliferating | arrested | |
| Paclitaxel | Tubulin stabilizer | 0.004 | >100 | >1.000 |
| Vincristine | Tubulin destabilizer | 0.0015 | >100 | >1.000 |
| Ispinesib | Eg5 kinesin inhibitor | 0.002 | >100 | >1.000 |
| BYK72767 | Unknown | 2 | 60 | 30 |
| Compound1 | Plk1 inhibitor | 0.7 | >100 | >100 |
| VX-680 | Aurora B inhibitor | 4 | 16 | 4 |
| BYK394210 | HDAC inhibitor | 2 | 60 | 30 |
| BYK426115 | unknown | 0.2 | >100 | >500 |

**Figure 1.** (*a*) Cell cycle-dependent antimitotic activities of the investigated compounds as indicated. Cell viability was determined after 72 h of incubation on proliferating (filled circles) versus arrested (open triangles) RKOp27 cells as described in Materials and Methods. Note the different dilution ranges used in order to reflect full dose-range curves. Error bars indicate SEM of $N = 4$ replicates, $p$ values of IC50 on proliferating cells versus arrested cells are $<0.001$ except for VX-680 ($p < 0.01$) as calculated with the Student's $t$ test. (*b*) Micrographs of methylene blue/eosin stained RKOp27 cells after 24 hr of treatment with the compounds at the indicated concentrations. VX-680 treated cells were incubated for 48 hr. Note the occurrence of mitotic Figures in cells treated with the mitosis-specific compounds and the high level of multinuclearity of cells treated with the aurora kinase inhibitor VX-680. (*c*) Summary of the IC50 values on proliferating versus arrested RKOp27 cells, differentiation factor and substance mode of action. VCR, vincristine.

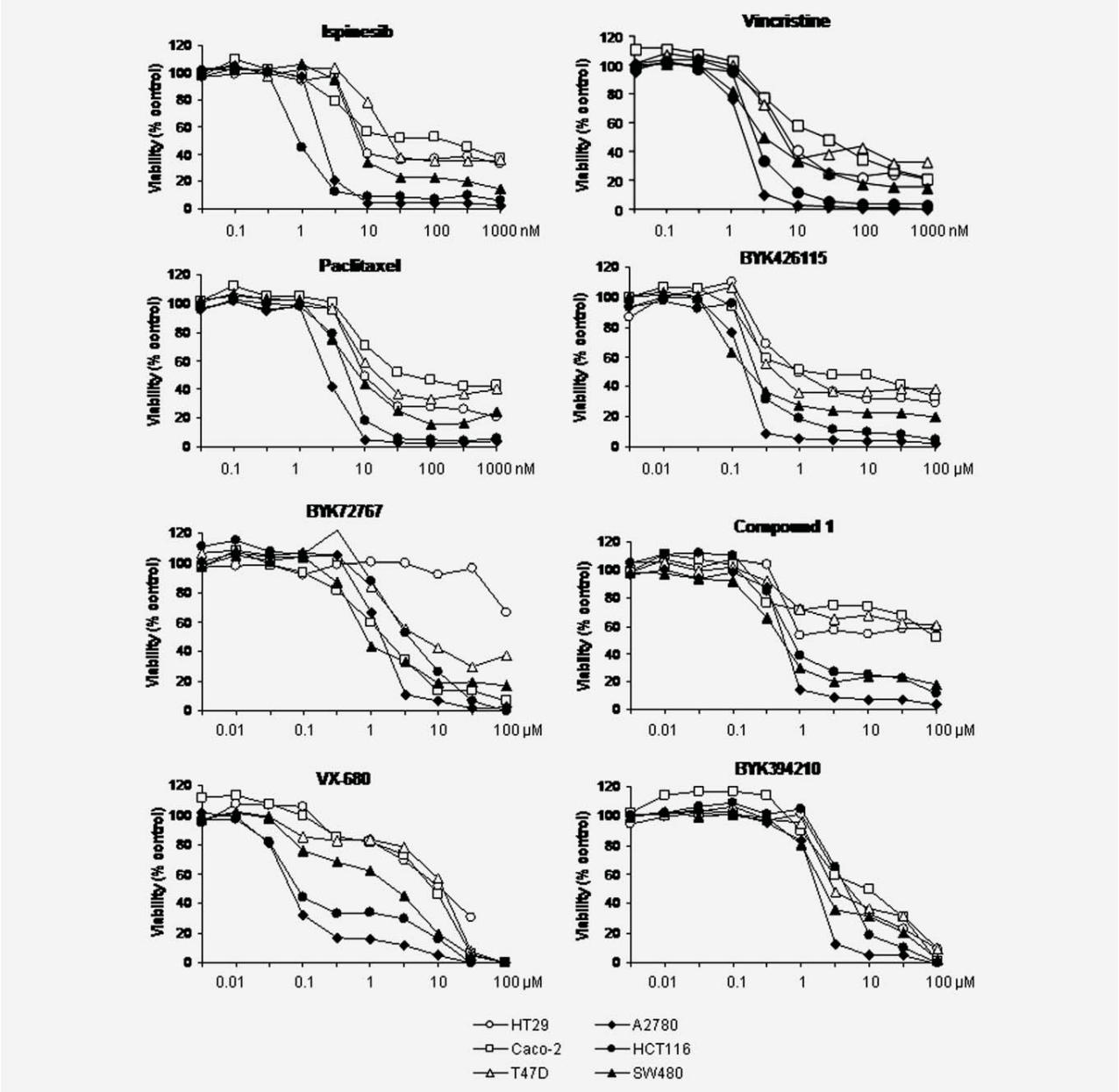


**Figure 2.** Cytotoxic activities of the investigated compounds as indicated for SPC proficient cells (filled symbols) and SPC deficient cells (open symbols). Cell viability was determined after 72 hr of incubation as described in Material and Methods.

relatively poor response toward VX-680, BYK426115 and the Eg5 inhibitor ispinesib. SPC deficient cells (Fig. 2, open symbols) generally displayed a higher IC50 toward anti mitotic compounds tested, which, however, was less pronounced as expected. More importantly, a much higher plateau of surviving cells was observed in the SPC deficient cells compared to the proficient ones. An exception was the benzamide pan HDAC inhibitor BYK394210 which appeared to be equally effective regardless SPC status. Similar observations were made with BYK72767 whose mechanism of action is still elusive. BYK72767 was the only substance with lack of activity on HT29 cells. To exclude that decreased sensitivity toward the antimitotic agents is simply due to a slower proliferation rate of the resistant cells, we measured proliferation rates in all cell lines (Supporting Information Fig. 1). Although all sensitive cell lines proliferated at a high rate (14, 15 and 18 hr doubling time for HCT116, SW480 and A2780 cells, respectively), resistant cell lines grew at a slower rate (20, 54 and 68 hr for HT29, T47D and Caco-2 cells, respectively), yet fast enough to allow at least one phase of mitotic progression during the incubation period. HT29 cells proliferated at the fastest rate among the resistant cell lines.

Table 1. Mitotic indexes of SPC proficient (A2780, HCT116, SW480) and deficient (HT29, Caco-2, T47D) cell lines after treatment with antimitotic compounds PTX. paclitaxel; VCR, vincristine

| Cell line | Control | PTX | VCR | VX-680 | BYK 72767 | Cpd. 1 | BYK 426115 | Ispinesib | BYK 394210 |
|---|---|---|---|---|---|---|---|---|---|
| A2780 | 2 | 16 | 33 | <1 | 20 | 50 | 21 | 36 | 13 |
| HCT116 | <1 | 35 | 31 | <1 | 27 | 90 | 59 | 52 | 44 |
| SW480 | 1 | 78 | 52 | <1 | 7 | 68 | 44 | 70 | 40 |
| HT29 | 2 | 96 | 100 | <1 | <1 | 98 | 100 | 96 | 98 |
| Caco-2 | <1 | 36 | 39 | <1 | 32 | 34 | 54 | 41 | 56 |
| T47D | <1 | 35 | 40 | <1 | <1 | 19 | 24 | 49 | 14 |

### Ability of SPC proficient and SPC deficient cell lines to mitotically arrest

To explore whether the differential sensitivities and plateaus observed for SPC proficient and deficient cell lines may be due to their overall ability to arrest in M-phase on treatment with mitosis-targeting substances, we quantified the mitotic indexes of all 6 cell lines on treatment with all antimitotic agents.

Two methods of quantification were used: One method used reactivity with phospho-histone H3 (pH3) antibodies and subsequent quantification by flow cytometry, whereas the other method quantified mitotic figures after fixation and staining with methylene blue/eosin (M/E). Both methods resulted in very similar values with the exception of VX-680-treated cells. Here, no mitotic figures were observed after methylene blue/eosin staining, yet a phosphohistone H3 antibody reactivity was seen in a considerable fraction among various cell lines. The data are consistent with[27] who observed that inhibition of aurora B kinase actually results in enhanced progression though M-phase with the subsequent induction of multinuclearity. Compound concentrations were adapted to the sensitivities of the cells to the respective substances and in general concentrations were applied that yielded IC90 or EC90 values (whatever applicable) from the data shown in Figure 2. The mitotic indexes on M/E staining are depicted in Table 1.

Overall, the data shown in Table 1 are surprising as it was expected that an SPC deficient cell line would have an impaired ability to arrest in M-phase in response to antimitotic agents. HT29 (SPC-deficient) responded with exceptionally high mitotic indexes on treatment with all of the antimitotic compounds applied in this study with the exception of BYK72767. No mechanistic explanation exists with regard to the low sensitivity of HT29 cells toward this substance. SW480 cells (SPC proficient) also generally displayed a high mitotic index in response to any antimitotic treatment tested. The ability to induce a mitotic arrest of HCT116 (SPC proficient) was similar to Caco-2 cells that were described to be SPC deficient. A2780 (SPC proficient) and T47D cells (SPC deficient) also behaved in a comparable fashion with the exception that T47D cells did not respond to BYK72767. Overall, it was striking to see an overall gross heterogeneity of mitotic accumulation in response to the antimitotic treatments over all cell lines and mechanistic principles tested. From the data shown in Table 1 we conclude that the cytotoxic potencies of the antimitotic agents to the cell lines tested (Fig. 2) do not correlate with the degree of mitotic accumulation. Moreover, there is no correlation between SPC status and the degree of mitotic accumulation. Thus, other factors than mere differences in proliferation must account for this observation, otherwise it would not be explainable why T47D cells are able to respond with mitotic arrest toward treatment to an Eg5 inhibitor but only to a much lesser extent than to a Plk1 inhibitor. In addition, Caco-2 cells proliferating at a much lower rate than A2780 cells responded to treatment with a much higher degree of mitotic accumulation than the latter ones (with the exception of the Plk1 inhibitor to which A2780 cells appear to be more prone to mitotic arrest).

### Analysis of markers of SPC activation

Phosphorylation of BubR1 can serve as a useful indicator of SPC activation, although it has to be emphasized that BubR1 phosphorylation alone may not suffice to indicate the activation of the SPC. We subjected lysates of two SPC proficient cell lines (A2780 and HCT116) and deficient cell lines (HT29 and Caco-2) to Western blot analysis with BubR1 antibodies to investigate an electrophoretic shift in an immunoblot of BubR1 being indicative of phosphorylation. Compound concentrations were adapted to the sensitivities of the cells to the respective substances and in general concentrations were applied that yielded IC90 or EC90 values (whatever applicable) from the data shown in Figure 2. The results are shown in Figure 3. Surprisingly, all cell lines tested, regardless of SPC status, responded with BubR1 phosphorylation and mitotic accumulation on treatment with vincristine, ispinesib, or BYK426115. BubR1 phosphorylation on treatment with the Plk1 inhibitor compound 1 was not observed in either cell line, which is consistent with the observation that BubR1 is as substrate of Plk1.[28] VX-680 induced a phosphorylation of BubR1 only in HT29 cells coinciding with an increased reactivity toward phospho-nucleophosmin, an acknowledged mitotic marker. Despite being cytotoxic to all cell lines tested the benzamide HDAC inhibitor BYK394210 induced a BubR1 phosphorylation only in SPC deficient cells. A tabular summary of the BubR1 phosphorylation is given in

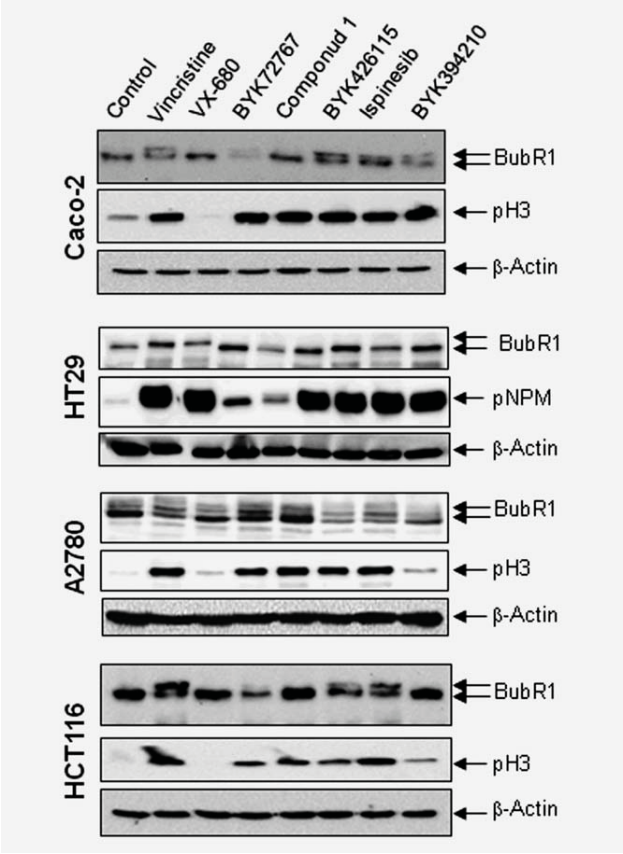


**Figure 3.** Phosphorylation of BubR1 in SPC proficient (HCT116, A780) and deficient cell lines (HT29, Caco-2). The cells were seeded out in 6-well plates and treated for 24 hr with the substances as indicated. Treatment with VX-680 was conducted for 40 hr. Lysates of treated cells were analyzed by western blotting for a decreased electrophoretic mobility of BubR1 (spindle checkpoint activation), phospho-histone H3 (pH3) or phospho-nucleophosmin (pNPM) for mitotic accumulation, and β-actin to check for equal loading. Antibody binding was visualized as described in Materials and Methods. The concentrations used for treatment of the cells are 20 nM for vincristine, 10 nM for ispinesib, 100 nM for VX-680, 10 μM for BYK72767, 2 μM for compound 1, 5 μM for BYK394210 and 1 μM for BYK426115.

Supporting Information Table 1. In summary, there was an overall good correlation between mitotic accumulation and phosphorylation of BubR1, but some striking exceptions such as with compound 1 and the HDAC inhibitor in SPC proficient cell lines were observed.

### Kinetics of apoptosis induction after treatment with antimitotic agents

We hypothesized that SPC deficiency and/or resistance toward antimitotic agents may correlate with a cell's ability to tolerate prolonged periods of mitotic arrest without onset of apoptosis. Therefore the kinetics of induction of apoptosis subsequent to mitotic arrest after treatment with three representative compounds, vincristine, the Plk-1 inhibitor compound 1 and BYK426115 was compared between selected SPC proficient cell lines (A2780 and HCT116) and deficient cells (HT29 and Caco-2). Compound concentrations were adapted to the sensitivities of the cells to the respective substances and in general concentrations were applied that yielded IC90 or EC90 values (whatever applicable) from the data shown in Figure 2. The results are shown in Figure 4: A2780 and HCT116 cells underwent massive apoptosis after 30 hr of treatment with all treatment regimens, and at time points later than 40 hr, there were not sufficient cell populations left over for a reliable quantification of apoptosis. In contrast, HT29 and Caco-2 cells maintained in M-phase even for 56 hr before onset of cell death in a substantial proportion of cells. These data revealed a massive difference in the kinetics of apoptosis induction between SPC proficient and deficient cell lines.

In summary, there was no correlation between SPC functionality and the ability to arrest in response to treatment with inhibitors of mitosis. Moreover, we found no correlation between antimitotic drug sensitivity of a cell line and its capability to mitotically arrest on exposure to the particular substance. However, we observed a strong delay in the onset of apoptosis after sustained arrest in mitosis in SPC deficient cell lines. Flow cytometric analysis were indicative of apoptosis, but we cannot rule out that SPC deficient cells may also died by necrosis.

## Discussion

The relationship between SPC functionality and cellular sensitivity to antimitotic drugs is an issue of outstanding scientific and clinical interest. Identifying novel drugs with high antitumor activity irrespective of SPC status remains an ambitious goal in oncology drug discovery. Researchers have developed various experimental systems to interfere with SPC functionality, *e.g.*, repression of essential SPC components. Functional interference with the SPC results in great heterogeneity with regard to modulation of tumor cell sensitivity toward antimitotic agents (reviewed in Ref. [29]).

Instead of generating SPC-engineered cell lines which may not reflect clinical reality, we based our investigations on a set of human cancer cell lines that have already been validated in the scientific literature to be SPC proficient (A2780, HCT116, SW480) or deficient (HT29, Caco-2, T47D). To avoid the risk that other factors primarily account for poor response to antimitotic chemotherapy than SPC functionality, we excluded the utilization of primary human cancer isolates that would require thorough molecular characterization before inclusion in our studies.

We analyzed both, their ability to mitotically arrest in response to various approved and experimental mitosis-specific agents and their sensitivity to the cytotoxicities of these compounds. The substances used herein include approved chemotherapeutic spindle poisons (paclitaxel, vincristine), antimitotic substances that are or have been in clinical trials (the Eg5 inhibitor ispinesib and the aurora inhibitor VX-680)

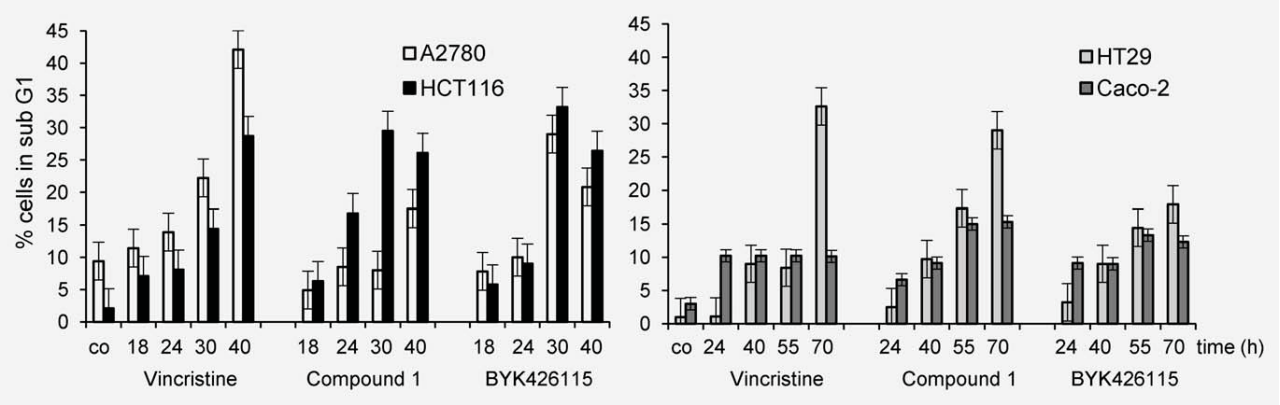


**Figure 4.** Kinetics of apoptosis induction in A2780, HCT116 (left panel), Caco-2 and HT29 cells (right panel) after treatment with vincristine (200 nM), Compound 1 (3 μM), and BYK 426115 (1 μM). At the indicated time points cells were harvested and subjected to cell cycle analysis as described in Material and Methods. The fraction of apoptotic cells was determined as the percentage of cells of the total fraction with a DNA content <2N (G1 phase).

and various investigational substances including an inhibitor of Plk1 kinase (compound 1), a benzamide class HDAC inhibitor (BYK394210) and two compounds identified from a cell based high throughput screen for novel antimitotic agents (BYK72767 and BYK426115). The inclusion of investigational, substances (not optimized for drug-likeness) offered the opportunity to determine whether there is a uniform response toward any antimitotic principle, regardless of the target addressed and an equal activity on cancer cells irrespective of spindle status. Our results show that this is not the case and that especially the response to the investigational antimitotic compound BYK72767 was very heterogeneous. Initial substance characterization confirmed their mitosis-confined, cell cycle-dependent cytotoxic mode of action. The aurora inhibitor VX-680 induced polyploidy instead of a mitotic phenotype consistent with earlier observations[17] and suggesting DNA replication without cytokinesis. Moreover, the cytotoxic activity of VX-680 appears to be only partially cell cycle-dependent (Fig. 1) either due to unspecific action of VX-680 apart from inhibiting aurora kinases or due to a mitosis-unrelated activity of aurora B. Subsequent analysis of the sensitivity of the cell lines used herein with regard to the cytotoxic activities of the substances used identified generally more sensitive (A2780, HCT116, SW480) and less sensitive (HT29, Caco-2, T47D) cell lines, which correlated very well with SPC proficiency and deficiency described for these cell lines. It has to be noted that other factors, especially p53 mutational status (which is far more frequently found in SPC deficient cell lines than in proficient cell lines) can have a significant influence on the cytotoxic activities of the investigational compounds. Although p53 mutations are described for all SPC deficient cell lines used herein, a p53 mutation among the set of SPC proficient cell lines is only known for SW480.

Exceptions to the preferential cytotoxicity toward SPC proficient cell lines are the benzamide HDAC inhibitor BYK394210 and BYK72767. Very little discrimination toward SPC proficient and deficient cells lines was observed for both of these compounds. Especially the broad activity of the HDAC inhibitor of the benzamide class indicates the feasibility of developing novel agents with high antimitotic activity regardless of SPC status. The exact molecular mode of action as well as the target of BYK72767 is still subject of investigation. It has to be noted that differences in sensitivities may not only result in a rightward shift of the IC50, but also in significantly increased plateaus of surviving cell fractions (Fig. 2).

The p-gp expressing cell lines HCT15 (data not shown) and adriamycin resistant A2780 were used as a control to exclude an effect of p-gp overexpression. Greater than tenfold differences to the average sensitivity of SPC proficient cell lines have been observed in both cell lines only for paclitaxel and vincristine, suggesting that the other compounds tested are no or only poor substrates of p-gp.

Determination of the mitotic index after treatment with the various compounds surprisingly did not show a correlation among the extent of drug sensitivity (Fig. 2), mitotic accumulation (Table 1) and SPC functionality. Several markers exist for the activation of the mitotic SPC: While phosphorylation of BubR1 is generally observed on SPC activation,[30] it has to be noted that phosphorylation of BubR1 alone may not be a reliable marker of SPC activation and that analysis of other markers may be necessary. Recruitment of SPC proteins such as Mad1, Mad2, Mad3/BubR1, Bub1, Bub3 and Mps1 to unattached kinetochores may serve as such additional markers.[31] We observed and overall good but not always consistent correlation between phosphorylation of BubR1 and mitotic arrest. BubR1 is a substrate of Plk1 and its phosphorylation by Plk1 is not required for the activation of the SPC.[28] Consequently, phosphorylation of BubR1 is suppressed in response to treatment with the Plk1 inhibitor. The role of Plk1 in the activation of the SPC is well described,[32–34] and it is known that the Plk1 inhibitor compound 1 per se does not induce BubR1 phosphorylation.[35] Therefore it remains elusive why SPC deficient cell lines tested herein responded only poorly to treatment with the Plk1 inhibitor compound 1.

The necessity of SPC activation and mitotic slippage for the induction of apoptosis has been described.[11] Conversely, SPC deficient cells do not arrest in response to such antimitotic compounds.[36] In the light of this definition our results are surprising since, *e.g.*, SPC deficient HT29 cells displayed the highest mitotic index on drug treatment of all cell lines investigated. Conversely, A2780 cells showed rather low mitotic indexes despite generally high sensitivity toward antimitotic agents. While other publications primarily focused on spindle poisons or only single investigational substances such as Eg5 kinesin inhibitors to explore SPC activation, our study included a wide variety of different antimitotic principles and a panel of various cell lines of different pathologic origin. Since the general ability to arrest in mitosis obviously is not a good indicator of sensitivity toward mitosis-specific agents, we investigated the kinetics of apoptosis onset after drug treatment. Intriguingly, cell lines described as sensitive in this study and SPC proficient in the literature displayed a fast onset of apoptosis between 24 and 30 hr after initiation of drug treatment. Considering the asynchronous growth of the cultures and the time required for one cell cycle progression, apoptosis must be initiated rather early after mitotic arrest. This might explain the relatively low mitotic index observed in some of these cell lines, *e.g.*, A2780: Once cells have arrested in mitosis, they initiate the apoptotic program and are not detected by the methods quantifying mitosis anymore. This hypothesis is supported by the fact that at later time points in the kinetic studies, far less cells have remained for flow cytometry. In contrast, HT29 cells that were more resistant in this study and SPC deficient in the literature, accumulated to a very high degree in mitosis. Kinetic analyses showed that they sustained this state for time periods $>$50 hr after initiation of drug treatment. Considering asynchronous growth and cell cycle kinetics, mitotic arrest may be tolerated for much longer time periods ($\gg$20 hr) before the onset of apoptosis. Our results are in agreement with data by[37] who found that cells resistant to mitosis-arresting compounds display a robust block in mitosis. However, the data by[37] only refer to a single cell line and their analyses do not go beyond treatment periods of 24 hr. Therefore we suggest that part of the characterization of SPC deficiency should include investigation of the time periods cells can sustain a mitotic arrest on treatment before they undergo apoptosis. We included several cell lines described to be SPC proficient or deficient in our study in order to provide a solid basis for our observations. However, considering the heterogeneity, *e.g.*, in pathologic origin or additional genetic alterations immanent to these cell lines, caution has to be taken with an overall conclusion of general principles. Indeed, SW480 although described to be SPC proficient, resembled in their drug sensitivities and their degree of mitotic accumulation more SPC deficient cell lines. Another variable is the mechanistic mode of action of the antimitotic drugs applied herein. While paclitaxel and vincristine are well described drugs, much less is known about the necessity of SPC functionality for the mode of action of Eg5 inhibitors, Plk1 inhibitors or the substances we chose from our cell-based screen. The observations we describe here are in good agreement with the hypothesis of Blagosklonny, which suggests that the balance between proapoptotic and antiapoptotic proteins may be causative for a cell's fate after mitotic arrest[38]: As there is no transcription during mitosis, the proportion of antiapoptotic and proapoptotic proteins such as IAP, bad, bax, Mcl-1, Bcl-2, Bcl-Xl or survivin changes due to their different half-life. Further investigation should explore whether in SPC-deficient cell lines the degradation of antiapoptotic proteins might be faster thereby preventing an efficient onset of the apoptotic cascade after mitotic arrest. In the context of this work, early induction of apoptosis could prevent a high mitotic index in SPC-proficient cell lines. Conversely, a high mitotic index as for instance seen in HT29 cells thus might result from mechanisms preventing apoptosis. An improved understanding of mechanisms rendering tumor cells more sensitive toward antimitotic agents is imperative for the enhancement of antimitotic therapies in the clinic. Further experiments are necessary to investigate the kinetics of turnover of these proteins in sensitive and resistant cell lines. Our results with novel investigational compounds show that identifying mechanistically unique antimitotic agents with equal sensitivity on SPC proficient and deficient cell lines remains a challenge but certain drug classes such as antimitotic benzamide HDAC inhibitors may be equally efficacious regardless of SPC status.

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