

# Taxol and Anti-Stathmin Therapy: A Synergistic Combination that Targets the Mitotic Spindle<sup>1</sup>

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## ABSTRACT

**Stathmin is an abundant cytosolic phosphoprotein that plays an important role in the regulation of cellular proliferation. Its major function is to promote depolymerization of the microtubules that make up the mitotic spindle. Taxol is an effective chemotherapeutic agent whose activity is mediated through stabilization of the microtubules of the mitotic spindle. We demonstrate that antisense inhibition of stathmin expression chemosensitizes K562 leukemic cells to the antitumor effects of Taxol and results in a synergistic inhibition of their growth and clonogenic potential. In the presence of stathmin inhibition, exposure to Taxol results in more severe mitotic abnormalities (hypodiploidy and multinucleation). This, in turn, results in increased apoptosis of the aneuploid cells during subsequent cell division cycles. This novel molecular-based therapeutic approach may provide an effective form of cancer therapy that would avoid the severe toxicities associated with the use of multiple chemotherapeutic agents with overlapping toxicity profiles.**

## INTRODUCTION

Stathmin is a major cytosolic phosphoprotein that is expressed at moderately high levels in human solid tumors and at very high levels in leukemias and lymphomas (1–4). It is a major cellular substrate for p34<sup>cdc2</sup> kinase, mitogen-activated protein kinase, and other kinases that are important for cellular proliferation and differentiation (5–8). Recent studies have demonstrated that stathmin promotes depolymerization of microtubules during their dynamic transition between phases of growth and shrinkage (9, 10). The microtubule depolymerizing activity of stathmin is regulated by changes in its state of phosphorylation that occur during progression through the cell cycle (5, 10, 11). These cell cycle-regulated modifications allow it to play a critical role in regulation of the dynamic equilibrium of the microtubules that make up the mitotic spindle (10, 12). Earlier studies have shown that manipulations that increase or decrease the level of stathmin expression interfere with the orderly progression of leukemic cells through the cell cycle (5, 13). Studies from our own laboratory have demonstrated that antisense inhibition of stathmin expression results in a decrease in the rate of proliferation of K562 erythroleukemic cells and accumulation in the G<sub>2</sub>-M phases of the cell cycle (5). We have also demonstrated that stathmin inhibition results in the abrogation of the malignant phenotype of K562 leukemic cells *in vitro* as well as *in vivo* (14). Thus, stathmin may provide an attractive molecular target for disrupting the mitotic apparatus and arresting the growth of malignant cells.

A number of widely used chemotherapeutic drugs exert antitumor effects by interacting with microtubules and interfering with their dynamic equilibrium (15). Taxol belongs to this group of highly active drugs that induce arrest in the G<sub>2</sub>-M phases of the cell cycle (16). The antimitotic effects of Taxol are mediated by stabilizing microtubules

and/or suppressing their dynamic instability (17, 18). This, in turn, interferes with the normal regulation of the mitotic spindle and results in mitotic arrest (17, 18). On exposure to Taxol, some of the mitotically arrested cells undergo apoptosis, whereas others complete the division cycle, giving rise to aneuploid cells that undergo apoptosis during subsequent cell cycles (18–21). Taxol is a highly effective agent against many common solid tumors such as breast, ovarian, and prostate cancers and is less effective against leukemias and lymphomas (16, 22).

One of the important principles of combination chemotherapy is that additive benefits may be derived from exposure to multiple, individually active, chemotherapeutic agents with nonoverlapping toxicity profiles. This principle has recently been expanded to include combinations of chemotherapeutic drugs with antisense strategies that target specific proteins whose expression is necessary for the malignant phenotype (23). Several studies have demonstrated additive effects of combinations of antisense strategies with chemotherapeutic agents (24, 25). However, because the cellular targets of the chemotherapeutic agents in these studies were different from the targets of the antisense therapies, there was no reason to expect *a priori* the effects of these combinations to be synergistic. In contrast, antisense inhibition of stathmin and Taxol exposure target different steps in the same mitotic pathway. Thus, we hypothesized that antisense RNA inhibition of stathmin expression may interact synergistically with Taxol to result in a more potent antitumor effect. The experiments described in this report were designed to test this hypothesis and to analyze the effects of this combination on cell cycle progression and apoptosis of malignant cells.

## MATERIALS AND METHODS

**Reagents.** Taxol, 5-FU,<sup>3</sup> and doxorubicin were purchased from Sigma Chemical Co. All drugs were dissolved in DMSO at 10 mg/ml and stored as stock solutions at –20°C. Hoechst 33342 (Sigma Chemical Co.) was dissolved in water at 1 mg/ml and stored at 4°C in the dark.

**Cell Lines.** The previously described K562 erythroleukemic cell lines that were used in this study were generated by stable transfections using an amplifiable expression vector containing the complete transcription unit for a mutant DHFR that has low affinity for methotrexate (5). The control cells K562(C) were transfected with an expression construct without stathmin cDNA (5). The stathmin-inhibited cells were generated by transfecting the expression construct containing full-length stathmin cDNA in the antisense orientation (5). The stathmin cDNA sequences were under the control of the SV40 promoter. The antisense stathmin cDNA sequences were coamplified with the DHFR sequences by exposing the transfected cells to 1 μM methotrexate (5). The cell lines used in this study consisted of a pool of stably transfected cells rather than unique clonal isolates. The cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO<sub>2</sub> environment. All experiments were initiated using cells in the logarithmic phase of growth.

**Proliferation Assays.** Cells were seeded at a density of 2 × 10<sup>4</sup> cells/ml in the presence or absence of Taxol, 5-FU, or doxorubicin at the indicated concentrations. The cells were stained with trypan blue, and viable cell counts were determined daily over a period of 5 days using a hemocytometer.

<sup>3</sup> The abbreviations used are: 5-FU, 5-fluorouracil; DHFR, dihydrofolate reductase; PI, propidium iodide.

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**In Vitro Clonogenic Assays.** The cells were first grown in the presence of either Taxol, 5-FU, or doxorubicin at the indicated concentrations for a period of 24 h. The cells were then washed in PBS and resuspended in 5 ml of drug-free methylcellulose-based semisolid culture medium (0.9% methylcellulose, 1% BSA, and 0.1 mM  $\beta$ -mercaptoethanol prepared in RPMI 1640 containing 30% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin). The cells were plated at a density of  $1 \times 10^3$  in 6-well tissue culture plates, and the colonies that formed were counted after 8–10 days.

**Flow Cytometric Analyses.** PI staining of fixed whole cells was performed for cell cycle analyses (5, 26). The cells were incubated for 24 h in the presence or absence of 2 nM Taxol and then washed twice in PBS and fixed in 0.5% paraformaldehyde for 30 min. The fixed cells were permeabilized in 0.1% Triton X-100 for 3 min, washed, and resuspended in 1 ml of PI solution (PBS containing 0.05 mg/ml PI and 1 mg/ml RNase). The cells were then incubated at 37°C for 30 min and analyzed within 2 h using a Becton Dickinson FACStar Plus flow cytometer at 488 nm single laser excitation. The cell cycle distribution was determined using Lysis II software.

**Morphological Assessment of Apoptosis.** Cells were grown in the presence or absence of 2 nM Taxol for 24, 48, or 72 h. The cells were washed in PBS and then fixed in 3.7% paraformaldehyde for 15 min. The fixed cells were cytocentrifuged on slides, washed twice in PBS, and permeabilized with 0.5% Triton X-100. The cells were then stained with Hoechst 33342 for 30 min at 37°C, rinsed in PBS, and mounted under coverslips. The nuclear morphology of the cells was analyzed using a Zeiss fluorescence microscope. More than 300 cells were counted to quantify apoptotic nuclei in three different experiments.

## RESULTS AND DISCUSSION

We had previously established several stable K562 cell lines that were transfected with either a control plasmid or a plasmid that expresses antisense stathmin RNA (5). Expression of the antisense gene reduced the level of stathmin mRNA in the transfected cells and inhibited their growth (5). These stable cell lines provided convenient tools for investigation of the consequences of inhibition of stathmin expression on the phenotype of leukemic cells (5). Two of these stable cell lines were used in all experiments described in this report. We will refer to the K562 cell line that was transfected with the control plasmid as K562(C) and the cell line that was transfected with the antisense construct as K562(AS). These cell lines were previously referred to as K562.DHFR and K562.DHFR.p18(-) (1  $\mu$ M MTX), respectively, in our prior publications (5, 14).

In the first experiment, we investigated the effects of exposure of K562 leukemic cells to Taxol in the presence or absence of stathmin inhibition. Fig. 1A illustrates the effects of different concentrations of Taxol on the proliferation of control K562(C) cells and stathmin-inhibited K562(AS) cells. When the cells were exposed to 1 nM Taxol, the rate of proliferation of K562(AS) cells was significantly decreased, whereas the rate of proliferation of K562(C) cells was essentially unchanged (Fig. 1A). In other words, in the absence of stathmin inhibition, 1 nM Taxol had no effect on the rate of proliferation of K562(C) cells, whereas exposure to the same concentration of Taxol resulted in a significant decrease in the rate of proliferation of K562(AS) cells in the presence of stathmin inhibition. Therefore, the effects of the combination of Taxol exposure and stathmin inhibition in these cells are clearly greater than the sum of their individual effects. This defines the interaction between the two therapeutic modalities as a synergistic interaction. Moreover, when the two cell lines were exposed to 2 nM Taxol, the rate of proliferation of the K562(C) cells was only modestly decreased, whereas the K562(AS) cells ceased to divide completely (Fig. 1A).

We also investigated the effects of combinations of two other chemotherapeutic agents, 5-FU and doxorubicin, with stathmin inhibition to determine whether the synergy observed above is specific to agents whose antitumor effects are mediated through the mitotic

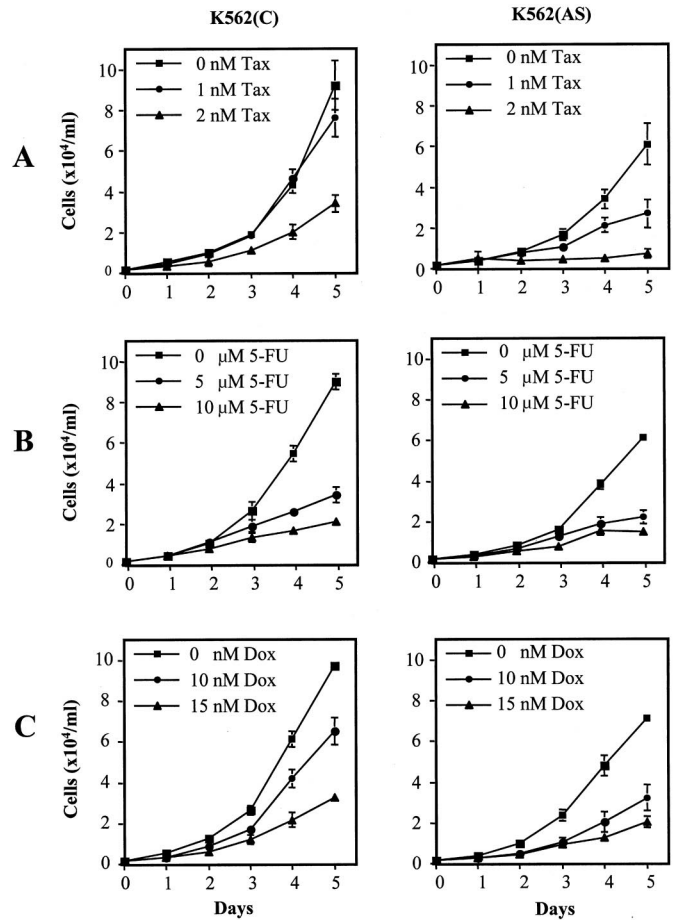


Fig. 1. Effects of combination of chemotherapeutic agents and stathmin inhibition on the proliferation of K562 leukemic cells. Exponentially growing control K562(C) and stathmin-inhibited cells K562(AS) were plated at a density of  $0.2 \times 10^5$  cells/ml in the presence or absence of Taxol (A), 5-FU (B), or doxorubicin (C) at the specified concentrations. Viable cells were counted daily for 5 consecutive days. Error bars, the SD calculated from three different experiments.

spindle. In contrast to Taxol, the antitumor activities of antimetabolites like 5-FU and doxorubicin do not result from direct interactions with microtubules and the mitotic spindle (27, 28). The growth curves shown in Fig. 1, B and C, illustrate the effect of different concentrations of 5-FU and doxorubicin on the rate of proliferation of the control K562(C) cells and the stathmin-inhibited K562(AS) cells. Exposure of K562(C) and K562(AS) cells to increasing concentrations of 5-FU and doxorubicin resulted in similar dose-dependent growth inhibition (Fig. 1, B and C). The stathmin-inhibited K562(AS) cells are not more sensitive to the growth-inhibitory effects of 5-FU and doxorubicin than the control K562(C) cells. This is in marked contrast to the increased sensitivity of K562(AS) cells to Taxol compared with K562(C) cells (Fig. 1A). Thus, the growth-inhibitory effects of combinations of 5-FU or doxorubicin with stathmin inhibition appear to be additive rather than synergistic.

We performed other experiments to determine whether the antitumor activities of stathmin inhibition and Taxol exposure would also be synergistic when analyzed by *in vitro* clonogenic assays. *In vitro* clonogenic assays correlate very well with *in vivo* assays of tumorigenicity in nude mice (29, 30). Fig. 2A illustrates the effects of Taxol on the relative clonogenicity of the control K562(C) and the stathmin-inhibited K562(AS) cells. Clonogenicity of K562(C) cells was reduced to 54% of baseline after exposure to Taxol alone. Similarly, antisense inhibition of stathmin alone decreased the clonogenicity of K562(AS) cells to 56% of baseline. However, when K562(AS) cells

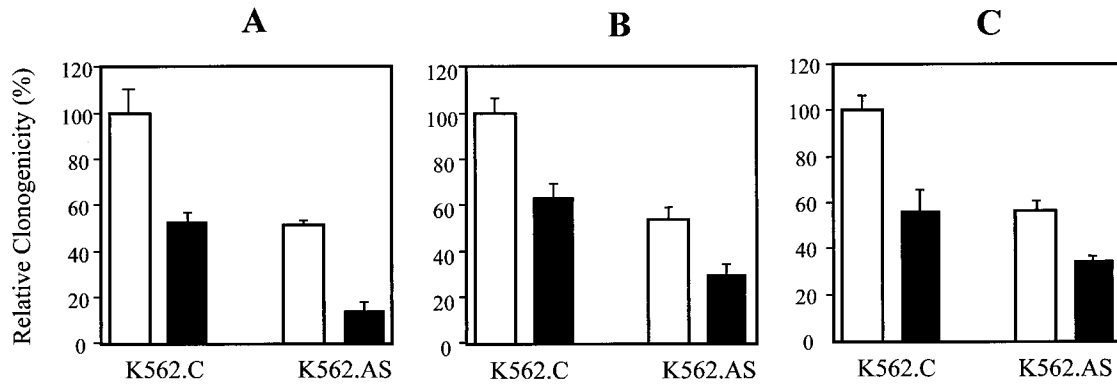


Fig. 2. Effects of combinations of stathmin inhibition and chemotherapeutic agents on the clonogenic potential of K562 leukemic cells. A, effect of combination of stathmin inhibition and Taxol exposure on the relative clonogenicity of control K562(C) and stathmin-inhibited K562(AS) cells. B, effect of combination of stathmin inhibition and 5-FU on the relative clonogenicity of K562(C) and K562(AS) cells. C, effect of combination of stathmin inhibition and doxorubicin on the relative clonogenicity of K562(C) and K562(AS) cells. □, the relative clonogenicity of K562(C) and K562(AS) cells in the absence of the drugs. ■, the relative clonogenicity of K562(C) and K562(AS) cells in presence of either Taxol (2 nM), 5-FU (30  $\mu$ M), or doxorubicin (10 nM). Error bars, the SD calculated from three experiments.

were exposed to Taxol in the presence of stathmin inhibition, clonogenicity was drastically reduced to 13% of baseline (Fig. 2A). We also examined the effects of 5-FU and doxorubicin on the clonogenic potential of K562(C) and K562(AS) cells (Fig. 2, B and C). Clonogenicity of K562(C) cells was reduced to 63% of baseline after exposure to 5-FU alone. Similarly, antisense inhibition of stathmin alone decreased the clonogenicity of K562(AS) cells to 56% of baseline (Fig. 2B). Moreover, when K562(AS) cells were exposed to 5-FU in the presence of stathmin inhibition, their clonogenicity was reduced to 30% of baseline (Fig. 2B). Similarly, clonogenicity of K562(C) cells was reduced to 57% of baseline after exposure to doxorubicin alone, whereas antisense inhibition of stathmin alone decreased the clonogenicity of K562(AS) cells to 56% of baseline (Fig. 2C). However, when K562(AS) cells were exposed to doxorubicin in combination with stathmin inhibition, their clonogenicity was reduced to 35% of baseline (Fig. 2C). We also compared the effects of vinblastine, another microtubule-interfering drug, on the growth of control and stathmin-inhibited cells using similar *in vitro* assays. Interestingly, in contrast to Taxol, exposure to vinblastine caused growth inhibition of control K562(C) cells but had no significant effect on stathmin-inhibited K562(AS) cells (data not shown). Thus, stathmin inhibition seems to protect cells from the effects of vinblastine instead of sensitizing them. This is not surprising because Taxol and vinblastine have opposite effects on the dynamics of the mitotic spindle.

We used the combination index method of Chou and Talalay (31) to determine whether the observed interactions between stathmin inhibition and the three different chemotherapeutic agents are additive or synergistic. According to Chou and Talalay (31), if an interaction is additive, the sum of the effects of two inhibitors should equal the product of their fractional activities [*i.e.*,  $f(u)_{1,2} = f(u)_1 \times f(u)_2$ ] where  $f(u)_1$  = the fraction unaffected by drug 1,  $f(u)_2$  = the fraction unaffected by drug 2, and  $f(u)_{1,2}$  = the fraction unaffected by drugs 1 and 2]. This formula is valid for drugs that act independently on a certain target. In contrast, if an interaction is synergistic, the inhibitory effects that result from exposure to the combination of two agents would be greater than the product of their fractional activities. Table 1 compares the predicted effects of the different combinations on clonogenicity (assuming the interactions are additive) with the experimentally observed effects. When Taxol is combined with antistathmin therapy, the experimentally observed degree of inhibition of clonogenicity is more profound than the predicted effect. In contrast, the experimental effects observed when antistathmin therapy was combined with 5-FU or doxorubicin were similar to the predicted

Table 1. Analysis of the effects of combination therapy on clonogenicity of K562 leukemic cells

The table presents a comparison of the predicted clonogenicity (based on an assumption that the activities are additive) and the experimentally observed clonogenicity after combination therapy.

Combination	Predicted clonogenicity (relative to a baseline clonogenicity of 1X)	Observed clonogenicity (relative to a baseline clonogenicity of 1X)
Stathmin inhibition + TAX <sup>a</sup>	$0.56 \times 0.54 = 0.30X$	0.13X
Stathmin inhibition + 5-FU	$0.56 \times 0.63 = 0.35X$	0.30X
Stathmin inhibition + DOX	$0.56 \times 0.57 = 0.32X$	0.35X

<sup>a</sup> TAX, Taxol; DOX, doxorubicin.

effects (Table 1). Thus, the combination of Taxol with stathmin inhibition results in a synergistic decrease in the clonogenic potential, whereas the combinations of 5-FU or doxorubicin with stathmin inhibition result in an additive decrease in the clonogenic potential of K562 leukemic cells.

We then investigated the mechanism of the synergistic interaction between stathmin inhibition and Taxol exposure. We first analyzed the effect of Taxol on the cell cycle profile of K562 cells in the presence or absence of stathmin inhibition. After 24 h of Taxol exposure, 13% of the control K562(C) cells accumulated in the hypodiploid region of the DNA histogram (Fig. 3). The hypodiploid fraction was significantly larger (27%) when cells were exposed to

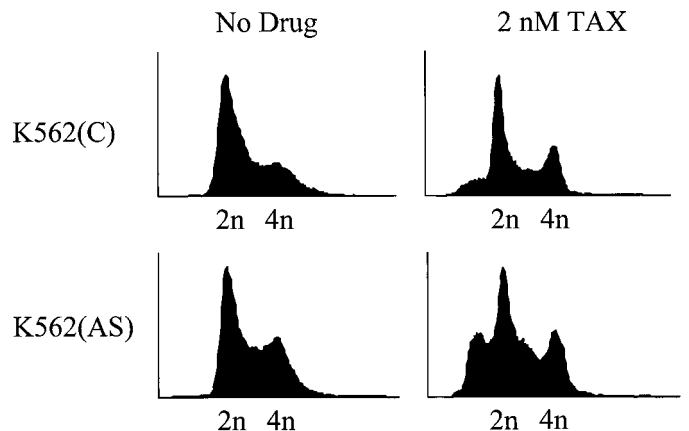


Fig. 3. Effects of Taxol on the cell cycle profile of K562 cells in the presence or absence of stathmin inhibition. Control K562(C) and stathmin-inhibited K562(AS) cells were grown in the absence (0 nM) or presence of Taxol (2 nM) for 24 h. The cells were then harvested, and DNA content was analyzed by flow cytometry.

Table 2 Effects of duration of exposure to 2 nM Taxol on apoptosis in the presence or absence of stathmin inhibition

Cell line	% Apoptotic cells
K562 (C) (0 h)	1.0 ± 0.4
K562 (C) (24 h)	4.2 ± 1.7
K562 (C) (72 h)	7.2 ± 2.4
K562 (AS) (0 h)	1.2 ± 0.4
K562 (AS) (24 h)	4.1 ± 1.1
K562 (AS) (72 h)	20.0 ± 4.4

Taxol in the presence of stathmin inhibition (Fig. 3). Because the appearance of a hypodiploid fraction in a DNA histogram is suggestive of apoptosis (32), we used Hoechst fluorescence staining to quantify the effects of Taxol and stathmin inhibition on apoptosis of leukemic cells. In this assay, nuclei of normal cells are characterized by homogeneous staining of chromatin, whereas apoptotic cells are characterized by irregular, bright aggregates of condensed chromatin or chromatin condensed on the inner aspect of the nuclear envelope (33). After 24 h of exposure to Taxol, both cell lines showed a mild degree of apoptosis (4.2% and 4.1%, respectively; Table 2). However, after 72 h of exposure to Taxol, the fraction of apoptotic cells increased to  $7.2 \pm 2.4\%$  in the absence of stathmin inhibition and to  $20 \pm 4.4\%$  in the presence of stathmin inhibition (Table 2). In addition, morphological assessment after a 24-h exposure to 2 nM Taxol revealed a larger number of big multinucleated cells in stathmin-inhibited K562(AS) cells (39.3%) than in the control K562(C) cells (13.3%). Both cell lines appeared to have a similar increase in the number of very small cells (or microcells) after exposure to Taxol.

Thus, when both K562(C) and K562(AS) cells are exposed to 2 nM Taxol for 24 h, they do not show evidence of significant mitotic arrest in the DNA histogram analysis (Fig. 3) or increased apoptosis by morphology (Table 2). This suggests that after a relatively short exposure to Taxol (24 h) at low concentrations (2 nM), the majority of the cells are able to complete their mitotic division without undergoing apoptosis. However, under these conditions, a larger fraction of the K562(AS) cells undergo aberrant mitosis compared with the control K562(C) cells. The abnormal mitosis was manifested morphologically as multinucleated cells and/or as microcells. Microcells characteristically have reduced DNA content and would appear in the hypodiploid region of the DNA histogram. Multinucleated cells may also appear in the hypodiploid region of the DNA histogram because the cell cycle analysis was performed on lysed cells with disaggregated nuclei. In contrast, when the cells were exposed to 2 nM Taxol for a longer period (72 h), the apoptotic fraction increased moderately in the K562(C) cells (7.2%) and markedly in the K562(AS) cells (20%). Abnormal mitotic exit resulting in hypodiploidy followed by apoptotic death in subsequent cell cycles is a well-described phenomenon after exposure to microtubule-interfering drugs (18–21). Thus, the experiments described above demonstrate that the hypodiploid fraction detected at 24 h and the apoptotic fraction detected at 72 h are significantly greater after Taxol exposure in K562(AS) cells than in the K562(C) cells. In unpublished experiments, we observed that stathmin inhibition alone results in marked abnormalities of the mitotic spindle.<sup>4</sup> It is conceivable that the mitotic spindle abnormalities of the K562(AS) cells would make the cells more susceptible to the mitotic-interfering effects of Taxol, giving rise to a larger fraction of hypodiploid cells early on that undergo apoptotic cell death during subsequent cell cycles. We suggest that this may be the mechanism by which stathmin inhibition synergizes with Taxol to exert more potent antiproliferative and antitumor effects.

In conclusion, the studies we describe in this report represent a

novel approach to cancer therapy based on the combination of a chemotherapeutic agent with antisense therapy that targets different steps in the same mitotic pathway. Our experiments demonstrate a synergistic interaction between inhibition of stathmin expression and exposure to Taxol. Whereas stathmin inhibition chemosensitizes leukemic cells to other agents like 5-FU and doxorubicin, the combinations of antistathmin therapy with these agents have additive rather than synergistic activities. Because leukemic cells are not particularly sensitive to the effects of Taxol, the combination of Taxol and antistathmin therapy may be more effective in tumors that are inherently sensitive to Taxol such as breast cancer and prostate cancers. There are obvious advantages of a synergistic combination over an additive combination. A synergistic combination could provide a more potent therapeutic effect at lower drug concentrations that are less likely to result in severe toxicity. The development of this novel therapeutic approach was made possible by recent improvements in our molecular understanding of the role of stathmin in the assembly of the mitotic spindle. If the *in vitro* findings described in this report are confirmed in an animal model *in vivo*, it may be possible to extrapolate this novel therapeutic approach to the treatment of human cancers.

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## Taxol and Anti-Stathmin Therapy: A Synergistic Combination that Targets the Mitotic Spindle

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