The Novel Combination of Chlorpromazine and Pentamidine Exerts Synergistic Antiproliferative Effects through Dual Mitotic Action

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Abstract

Combination therapy has proven successful in treating a wide variety of aggressive human cancers. Historically, combination treatments have been discovered through serendipity or lengthy trials using known anticancer agents with similar indications. We have used combination high-throughput screening to discover the unexpected synergistic combination of an antiparasitic agent, pentamidine, and a phenothiazine antipsychotic, chlorpromazine. This combination, CRx-026, inhibits the growth of tumor cell lines in vitro more effectively than either pentamidine or chlorpromazine alone. Here, we report that CRx-026 exerts its antiproliferative effect through synergistic dual mitotic action. Chlorpromazine is a potent and specific inhibitor of the mitotic kinesin KSP/Eg5 and inhibits tumor cell proliferation through mitotic arrest and accumulation of monopolar spindles. Pentamidine treatment results in chromosomal segregation defects and delayed progression through mitosis, consistent with inhibition of the phosphatase of regenerating liver family of phosphatases. We also show that CRx-026 synergizes in vitro and in vivo with the microtubule-binding agents paclitaxel and vinorelbine. These data support a model where dual action of pentamidine and chlorpromazine in mitosis results in synergistic anti-tumor effects and show the importance of systematic screening for combinations of targeted agents. [Cancer Res 2007;67(23):11359–67]

Introduction

Given the biological complexity of disease and the redundancy of biological systems, combination therapies have become a general approach in the treatment of disease. Indeed, combination therapy has proven to be successful in treating a wide variety of aggressive human cancers, including colon, lung, and breast (1–3). The vast array of gene mutations in human cancers and the prevalence of multiple mutations per tumor explain the effectiveness of combination approaches in cancer (4–6). Many current cancer treatment guidelines rely heavily on combinations of cytotoxic drugs, such as anthracyclines, platinum drugs, and taxanes (7–9), and although new, highly selective targeted agents have recently shown promise, it seems that many will only be effective when used in combination (4, 10, 11). Researchers and clinicians thus face the challenge of identifying the best combinations of drugs and refining their dose and schedule for optimum effectiveness. A systematic approach to searching all possible combinations of drugs can significantly advance this process by revealing both synergies and antagonisms that may be overlooked by the current standard practice of intuition-based combination experiments (12, 13).

Mitosis has long been known as a crucial target for therapeutic intervention in cancer. Classic mitotic spindle poisons, such as Vinca alkaloids and taxanes, have been in clinical practice for decades and continue to be important components of chemotherapy regimens (14). Multiple mechanisms working in concert are required to ensure proper mitotic spindle assembly, dynamics, and regulation throughout mitosis (15). Furthermore, numerous network connections between mitosis and other pathways important for cancer growth and survival, such as DNA damage, checkpoint controls, and cell death, suggest that antimitotic combination therapy holds great promise (16). The next generation of antimitotic drugs targeting nonstructural components of mitosis, such as mitotic kinesins, aurora kinases, and polo-like kinases, is now emerging in several drug development pipelines (16, 17). Mitotic kinesins comprise a family of 11 kinesin proteins responsible for spindle and chromosome movement in mitosis (18). Due to their specific role in dividing cells, mitotic kinesins have emerged as compelling drug targets for therapeutic intervention in cancer (14, 16). One member of this class is the kinesin spindle protein KSP/Eg5, a molecular motor required to establish bipolar spindles in mitosis (19, 20). Several potent and selective inhibitors of KSP/Eg5 are currently under development (16).

Mitotic kinases and phosphatases have also recently enjoyed attention as novel targets for therapeutic intervention in cancer (16, 21). Aurora kinases have multiple roles in mitosis, including centrosome function, chromosome segregation, and cytokinesis (22, 23). Recent advances in understanding the roles of mitotic phosphatases such as CDC25 and the phosphatase of regenerating liver (PRL) family of phosphatases have generated interest in these protein families as targets for small-molecule drug development (21, 24). Significantly, these emerging kinase and phosphatase targets play roles at multiple points throughout the process of mitosis, and although inhibition of some causes mitotic arrest, inhibition of others seems to cause loss of checkpoint control resulting in catastrophic cell death (16, 22). Striking the right balance between efficacy and therapeutic window is a key challenge in the development of new antimitotic agents, and importantly, most drugs of this class have shown mechanism-based side effects, such as neutropenia (16). The goal of broadening the therapeutic window for these agents may optimally occur through combining mechanisms for maximum specificity and efficacy.

We have previously reported the use of combination high-throughput screening for the systematic identification of synergistic antiproliferative drug combinations (25). This screening effort resulted in the identification of the unexpected synergistic...
combination of an agent previously understood as an antiparasitic agent, pentamidine, and a phenothiazine previously understood as an antipsychotic, chlorpromazine, hereto referred to as the synergistic combination CRX-026. The components of CRX-026 have been coadministered in human phase I trials in patients with solid tumors who have failed prior chemotherapy and are being confromulated into a novel pharmaceutical preparation for continuous infusion.

Here, we characterize the molecular mechanism of CRX-026 antiproliferative activity. We have determined that chlorpromazine is a potent and specific inhibitor of the mitotic kinesin KSP/Eg5. As predicted for an inhibitor of mitotic kinesins, chlorpromazine treatment results in mitotic arrest and accumulation of cells displaying a monopolar spindle with incompletely separated centrosomes. Studies correlating inhibition of proliferation, the appearance of monopolar spindles, mitotic arrest, and inhibition of kinesin motor activity suggest that the effect of chlorpromazine results from its inhibition of KSP/Eg5 motor activity. We further report that pentamidine, the other component of CRX-026, also plays a role in mitosis. Treatment with pentamidine results in defects in chromosome segregation as evidenced by the appearance of a 5-fold increase in postmitotic DNA bridges. Furthermore, treatment of cells with pentamidine results in a delay in progression through mitosis. Both mitotic phenotypes are consistent with the disruption of the function of PRL phosphatases. Finally, we report that CRX-026 synergizes with microtubule-binding agents in vitro and in vivo to inhibit tumor cell proliferation.

Materials and Methods

Reagents and supplies. HCT116 and A549 were from the American Type Culture Collection and cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C. Pentamidine, chlorpromazine, lufenphenazine, perphenazine, promethazine, and paclitaxel were obtained from Sigma; vinorelbine was obtained from Sequoia Research Products. Compounds were dissolved in DMSO as high concentration stocks before dilution into medium.

Inhibition of proliferation assays. Cells were seeded at 1,500 per well and cultured for 16 to 24 h before addition of compound. After compound addition, inhibition of proliferation was determined using CellTiter-Blue (Promega) according to the manufacturer’s instructions. Inhibition of proliferation was determined by the following formula: %I = [ median untreated – treated well ] / median untreated * 100.

Immunofluorescence microscopy. After compound treatment, cells were washed with PBS and fixed in ice-cold 2% paraformaldehyde for 10 min followed by permeabilization with 0.1% Triton X-100. Cells were blocked in 2% bovine serum albumin (BSA) and 4% normal goat serum in PBS for 1 h at room temperature and incubated with primary antibodies in 1% BSA/PBS overnight at 4°C. Cells were washed with PBS and incubated with conjugated secondary antibodies in 1% BSA/PBS for 1 h at room temperature. Cells were washed thrice with PBS for 5 min each. During the second wash, either 4,6-diamidino-2-phenylindole (DAPI; Sigma) or SYTOX Green (Molecular Probes) was added for visualization of DNA. Fluorescent microscope images were obtained using a Nikon TSi100 inverted microscope and 40X Plan Fluor or 60X Plan Apo objectives. Images were photographed using Spot advanced software. Some images of monopolar spindles were taken using a two-photon scanning confocal microscope and images were processed using NIH ImageJ (freeware developed by Wayne Rasband, National Institute of Mental Health, Rockville, MD). Monoclonal mouse anti-α-tubulin antibody and rabbit anti-γ-tubulin antibody (both from Sigma) were used at 1:1,000 dilution and rabbit anti-α-tubulin antisera (Sigma) was used at 1:200 dilution. Secondary antibodies, Alexa Fluor 488 donkey anti-mouse IgG (green) and Alexa Fluor 555 donkey anti-rabbit IgG (red), both from Molecular Probes, were used at 1:1,000 dilution.

For quantification of monopolar spindles, HCT116 cells were seeded on chamber slides and allowed to recover overnight and then treated with either chlorpromazine, lufenphenazine, promethazine, or vehicle control for 48 h. Cells were fixed and stained as described above using DAPI to visualize DNA and monoclonal mouse anti-α-tubulin antibody with Alexa Fluor 488 donkey anti-mouse IgG (green) secondary antibody to visualize microtubules. The percentage of the mitotic population with monopolar versus bipolar spindle morphologies was determined.

For quantification of DNA bridges, A549 cells were seeded on chamber slides, allowed to recover overnight, and then serum starved for 72 h (to enrich in cells in G1) before the addition of fresh serum containing medium with 8 μmol/L pentamidine or DMSO for an additional 24 to 48 h. DNA was visualized with SYTOX Green and microtubules were visualized with rabbit anti-γ-tubulin antibody and Alexa Fluor 555 donkey anti-rabbit IgG (red). Cells in anaphase through mitotic exit were counted.

Cell-free KSP assay. Kinesin activity was assayed using the Kinesin ATPase End Point Biochem kit (Cytoskeleton, Inc.) according to the manufacturer’s instructions. The following purified motor proteins were used (all from Cytoskeleton): human Eg5 kinesin motor domain protein, human chromokinesin kinesin motor domain protein, and human kinesin heavy chain motor domain protein. Kinesin activity was determined by Pi released based on the standard curve. Inhibition of kinesin activity was calculated by the following formula: average %I = [average untreated – average treated] / average untreated * 100. Data represent four replicates from two separate experiments. A standard curve was performed with all recombinant kinesins to obtain the same specific activity.

Cell cycle analysis and synchronization. A549 cells were plated and serum starved for 72 h to arrest cells in G1. Then, complete medium containing compound was added and cells were incubated for an additional 72 h. HCT116 cells were plated and immediately treated with compound for 72 h. Following compound treatment, cells were stained with propidium iodide using standard methods. Cells were analyzed for DNA by flow cytometry using a Becton Dickinson FACSCalibur and ModFit LT software.

In some experiments, A549 cells were synchronized by the addition of nocodazole (0.2 μg/mL) for 18 h to block cells in prometaphase. Fifteen hours before release, cells were treated with 4 μmol/L pentamidine or DMSO vehicle control. Cells were released from the block in the presence of pentamidine or DMSO, harvested at the indicated time points, and analyzed for cell cycle profile.

 Xenograft animal studies. Chlorpromazine and pentamidine were prepared in PBS with 10% ethanol. Paclitaxel was prepared using an emulsion of Cremophor EL/ethanol (50/50) and diluted 1:6 in 0.9 mol/L NaCl immediately before injection. Chlorpromazine and pentamidine were given as two separate injections. A549 cells were grown in DMEM and HCT116 cells in McCoy’s 5A medium, each supplemented with 10% FBS, at 37°C. All experiments were carried out using 6- to 8-week-old male or female severe combined immunodeficient Hsd:ICR(CD-1) mice (Harlan). A549 cells were harvested, suspended in DMEM minus serum, and injected s.c. into the right flanks (4 × 106/flank in a 300–500 μL volume). HCT116 cells were harvested, suspended in McCoy’s 5A minus serum, and injected s.c. into the right and left flanks (5 × 106/flank in a 300 μL volume). Tumor volumes were determined by measuring the length (l) and the width (w) and calculating the volume (V = lw2 / 2). Depending on the study, the tumors were between ~400 and 800 mm3 at the time of randomization into treatment groups (n = 8–10 mice per group). Unless otherwise stated, drugs were given daily (Monday to Friday). Paclitaxel was given 3 days per week (Monday, Wednesday, and Friday only). All drugs were given by i.p. injection in a volume of 100 μL/25 g. Animals undergoing combination therapy received two individual injections for a total of 200 μL per mouse. Control animals received 200 μL injections of vehicle only. Reduction in tumor volume relative to control on the last day of treatment was calculated using the following formula: % reduction = (control tumor volume – treated tumor volume) / control tumor volume. Change in body weight on the last day of treatment was calculated using the following formula: % change = (weight at end of treatment – weight at start of treatment) / weight at start of treatment. Percent of surviving animals was calculated at the end of the study. Evaluation of the results included statistical analysis of differences in tumor.
Pentamidine and chlorpromazine combine to inhibit tumor growth. The novel and unexpected combination of pentamidine and chlorpromazine was originally identified in a screen of ~100,000 pairwise combinations of approved drugs for combinations that inhibit the proliferation of the non–small cell lung carcinoma (NSCLC) cell line A549 (25). An isobologram showing the synergistic effect of CRx-026 is shown in Fig. 1A. The synergistic antitumor effect of CRx-026 was further examined in human tumor xenograft models using A549 NSCLC and HCT116 colorectal carcinoma cells (Fig. 1B; Table 1; ref. 25). HCT116 tumors treated with CRx-026 were consistently smaller than vehicle, 5-fluouracil (5-FU), and the individual components throughout the course of the study (Fig. 1B). On the final day of treatment, tumors treated with CRx-026 were reduced by 39% compared with the vehicle control. Tumors treated with 5-FU (25 mg/kg) were 47% smaller than vehicle controls. Mice receiving chlorpromazine or pentamidine alone exhibited nonsignificant decreases in tumor volume. The effect of CRx-026 in reducing tumor volumes was consistent across multiple studies with A549 and HCT116 cells using varied doses and schedules and was generally well tolerated (Table 1).

Chlorpromazine is an inhibitor of the mitotic kinesin KSP/Eg5. During the characterization of CRx-026, we examined mitotic phenotypes associated with chlorpromazine treatment. A549 NSCLC cells were treated with chlorpromazine or vehicle for 48 h followed by staining with an anti-α-tubulin antibody and the DNA-binding dye DAPI (Fig. 2A). Normal, bipolar mitotic spindle morphology was observed in the vehicle-treated samples. Conversely, in the chlorpromazine-treated samples, a striking and aberrant monopolar spindle morphology was observed. The monopolar phenotype was the predominant mitotic morphology observed in chlorpromazine-treated cells (Fig. 2B), with >90% of the chlorpromazine-treated mitotic cells displaying this phenotype.

To determine whether the formation of the monopolar spindle in chlorpromazine-treated cells was a result of failure of centrosome duplication or failure of centrosome separation, we examined the centrosomes by staining with an anti-γ-tubulin antibody (Fig. 2C, h). We consistently observed two distinct foci of γ-tubulin staining that is typical of duplicated centrosomes, indicating that the observed monopolar spindle morphology is not the result of a block in centrosome duplication but instead results from a defect in centrosome separation. Treatment with the small-molecule kinesin inhibitor monastrol is also known to prevent the separation of centrosomes in mitosis and result in the monopolar spindle morphology (26). Monastrol treatment of A549 cells resulted in the accumulation of cells with monopolar spindles morphologically indistinguishable from those observed with chlorpromazine treatment (Fig. 2B).

To explore the possibility that chlorpromazine inhibits the activity of one or more mitotic kinesins, we examined the effect of chlorpromazine on the microtubule-dependent hydrolysis of ATP by the mitotic kinesin KSP/Eg5 (Fig. 2C). In this assay, which measures inorganic phosphate generated from the hydrolysis of ATP by kinesin motor proteins (27), chlorpromazine caused a dose-dependent decrease in the ability of KSP/Eg5 to hydrolyze ATP with an IC50 of approximately 5 to 10 μmol/L. Interestingly, this inhibitory effect of chlorpromazine seems to be specific to the kinesin KSP/Eg5 (Fig. 2D). Chlorpromazine did not appreciably inhibit the motor activity of another mitotic kinesin, chromokinesin (28), or a nonmitotic kinesin, the kinesin heavy chain, even at concentrations 10-fold higher than the IC50 for inhibition of KSP/Eg5 activity.

Chlorpromazine inhibits proliferation through inhibition of KSP/Eg5. To explore the possibility that inhibition of the mitotic kinesin KSP/Eg5 is the primary mechanism by which chlorpromazine inhibits tumor cell proliferation in vitro, we sought to correlate the potency of chlorpromazine as an
inhibitor of KSP/Eg5 motor activity with its potency in assays for proliferation and mitotic arrest (Fig. 3A). The dose-response relationship between chlorpromazine inhibition of proliferation of HCT116 human colon carcinoma cells and inhibition of KSP/Eg5 motor activity closely overlaps. Treatment of HCT116 cells also results in a dose-dependent increase in the population of mitotic cells displaying the monopolar spindle phenotype, with an IC50 of 5 to 7 μmol/L, similar to that of the inhibition of KSP/Eg5 motor activity. Interestingly, chlorpromazine treatment also results in a dose-dependent increase in the population of HCT116 cells present in the G2-M phase of the cell cycle, with an IC50 of 10 μmol/L, as would be expected of cells displaying the monopolar spindle phenotype (29). The dose-response relationship of accumulation of cells in G2-M in response to chlorpromazine treatment correlates well with that of chlorpromazine inhibition of KSP/Eg5 motor activity. Thus, chlorpromazine shows a similar potency in four independent assays, indicating that, in our tumor cell proliferation system, the levels of chlorpromazine required for inhibition of the KSP/Eg5 motor activity in a cell-free assay are similar to those required for activity in cell-based assays.

To show that inhibition of KSP/Eg5 motor activity is the primary mechanism by which chlorpromazine exerts its antitumor effects, we compared chlorpromazine and the additional phenothiazine compounds, fluphenazine and promethazine, for their efficacy and potency in inhibition of proliferation, monopolar spindle formation, and inhibition of KSP/Eg5 motor activity (Fig. 3B and C). Chlorpromazine is the most potent inhibitor of KSP/Eg5 motor activity followed closely by fluphenazine. Promethazine is significantly less potent in this assay than either chlorpromazine or fluphenazine (Fig. 3B). Interestingly, the three phenothiazines display the same relative potency in inhibition of proliferation and in causing the monopolar spindle phenotype as they do in the inhibition of KSP/Eg5 motor activity (Fig. 3C). Thus, structural changes that diminish the potency of chlorpromazine toward KSP/Eg5 also diminish its potency toward the other endpoints examined. This correlation of potencies in three different assays suggests that action at KSP/Eg5 is responsible for the observed antiproliferative effect of chlorpromazine.

Pentamidine inhibits mitotic progression. A search of the literature revealed that pentamidine is reported to inhibit the activity of PRL phosphatases (30) and that these phosphatases are reported to play a role in the progression of mitosis and segregation of chromosomes (31). Given our understanding of the effect of chlorpromazine on the mechanics of spindle formation, we hypothesized that the synergistic inhibition of

<table>
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<tr>
<th>Combination dosage</th>
<th>Tumor cell line</th>
<th>Dose regimen</th>
<th>Reduction in tumor volume</th>
<th>CRx-026 (%)</th>
<th>Positive control (%)</th>
<th>Body weight change (%)</th>
<th>Survival (%)</th>
<th>CRx-026 (%)</th>
<th>Positive control (%)</th>
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Abbreviation: ND, not determined.

*End of study (16 d after last treatment).
†End of first treatment phase.
‡Sample size too small for accurate calculation.
$P < 0.05$ versus vehicle control.
||All animals dead by end of study.
*End of second treatment phase.
followed by treatment with pentamidine and release from mitosis, we arrested A549 cells in prometaphase with nocodazole, ultimately resulting in aberrant or failed mitosis. Treatment results in defects in proper chromosome segregation, displaying DNA bridges. These results indicate that pentamidine acts on mitotic cells. Conversely, in pentamidine-treated cells, there was a 2.6-fold increase in the frequency of postmetaphase mitotic cells that had reentered G1 by propidium iodide staining and fluorescence-activated cell sorting analysis. Cells treated with pentamidine returned to G1 with slower kinetics than vehicle-treated cells. Vehicle-treated cells took on average 125 min for 50% of the population to enter G1. Notably, cells treated with pentamidine required on average 172 min for 50% of the population to enter G1, ~47 min longer than vehicle-treated cells. These results are very similar to the ~50-min delay in progression through mitosis reported to be caused by overexpression of a catalytically inactive mutant of PRL-1 (31), indicating that pentamidine treatment results in a significant delay in the progression of cells through mitosis and/or cytokinesis, possibly via inhibition of the mitotic function of the PRL phosphatase.

CRx-026 synergizes with microtubule-binding agents. An agent with a multitarget effect in mitosis would be expected to synergize with other antimitotic agents, such as the microtubule-binding agents paclitaxel and vinorelbine. To examine this hypothesis, we screened multiple ratios of CRx-026 against a panel of 48 known antineoplastic agents in multiple tumor cell lines. In this screen, we observed the strongest synergies between CRx-026 and the microtubule-binding classes taxanes and Vinca.

Figure 2. Chlorpromazine inhibits the mitotic kinesin KSP/Eg5. A, CRx-026 treatment results in monopolar spindle formation. A549 cells were treated with DMSO (a–c) or 16 μmol/L chlorpromazine (d–f) for 48 h and spindle morphology was visualized through anti-α-tubulin staining and confocal (a–f) or inverted (g and h) microscopy. Centrosomes (h) were visualized though γ-tubulin staining. a and d, Alexa Fluor 488 (green)-stained microtubules; b and e, DAPI (blue)-stained DNA; c, f, and g, overlays of Alexa Fluor 488 and DAPI. h, arrows, Alexa Fluor 555 (red)-stained centrosomes. B, quantitation of monopolar spindle formation. A549 cells were treated with DMSO, 16 μmol/L chlorpromazine, or 68 μmol/L monastrol and spindle morphology was visualized as described in A. The number of cells with monopolar or bipolar spindle morphology was counted and expressed as a percentage of the total mitotic cells counted (vehicle, n = 24; chlorpromazine (Chlor), n = 106; monastrol (Mon), n = 96). C, chlorpromazine inhibits KSP/Eg5 ATPase activity. The ATPase activity of KSP/Eg5 was measured in the presence of varying concentrations of chlorpromazine using the Cytoskeleton Motor Werks assay kit according to the manufacturer’s instructions. Activity is expressed as average percent inhibition of kinesin motor protein ATPase activity compared with untreated control. A total of four replicates from two independent experiments are shown. D, chlorpromazine is selective for mitotic kinesins. The ATPase activity of KSP/Eg5 (n = 6), chromokinesin (Chrom; n = 6), and kinesin heavy chain (KHC; n = 5) was measured in the presence of 16 μmol/L chlorpromazine as described in C.
alkaloids. A fixed ratio of 1:2 chlorpromazine/pentamidine combined with paclitaxel in an A549 proliferation inhibition assay in vitro shows significant synergy as indicated by potency shifting in an isobolographic analysis (Fig. 5A, left). In a murine

Figure 4. Pentamidine inhibits mitotic progression. A, treatment with pentamidine results in mitotic defects. A549 cells were treated with 8 μmol/L pentamidine for 48 h and mitotic morphology was visualized through anti-α-tubulin and DNA staining and microscopy. a and d, DNA (SYTOX Green); b and e, α-tubulin (Alexa Fluor 555); c and f, overlays of DNA and α-tubulin. B, quantitation of anaphase defects. A549 cells were treated with DMSO or 8 μmol/L pentamidine and nuclear morphology was visualized as described in A. The number of cells with DNA bridges or lagging chromosome morphologies was counted and expressed as a percentage of the total anaphase cells counted. C, pentamidine causes a delay in progression from mitosis to G1. A549 cells were synchronized with nocodazole as described in Materials and Methods. Cells were treated with 8 μmol/L pentamidine or DMSO during the last 15 h of synchronization and released from nocodazole block into a fresh pentamidine stock. Samples were harvested at the indicated times after release from nocodazole and cell cycle analysis was performed. The percentage of cells with 4N DNA content at each time point is presented. Pentamidine-treated cells show a statistically significant difference from DMSO-treated cells at 2 h after nocodazole release (P < 0.001).
xenograft model using A549 cells, tumors treated with a combination of CRx-026 and paclitaxel showed significantly lower tumor volumes than those treated with either paclitaxel or CRx-026 alone (Fig. 5A, right). A fixed ratio of 1:2 chlorpromazine/pentamidine in combination with vinorelbine also showed significant synergy in HCT116 cells in an in vitro proliferation assay (Fig. 5B, left). In a murine xenograft model using HCT116 cells, tumors treated with a combination of CRx-026 and vinorelbine showed significantly reduced tumor volumes compared with tumors treated with either CRx-026 or vinorelbine alone. Thus, in two independent cellular and in vivo efficacy models, CRx-026 synergized with microtubule-binding agents paclitaxel and vinorelbine.

**Discussion**

CRx-026 was previously identified as the unexpected synergistic combination of the antiparasitic agent pentamidine and the phenothiazine antipsychotic chlorpromazine (25). We have now characterized the molecular mechanism of CRx-026 antiproliferative activity showing that it acts through synergistic dual mitotic action.

**Chlorpromazine is an inhibitor of the mitotic kinesin KSP/Eg5.** Several different biological activities have been previously ascribed to chlorpromazine. As an antipsychotic, chlorpromazine is generally believed to act through the antagonism of dopamine receptors. Interestingly, it has been shown that some cancer cell lines do contain dopamine D2 receptors (32). However, the mechanism of action of chlorpromazine for preventing the growth of cancer cell lines in vitro is unlikely to result from D2 antagonism because additional compounds known to specifically antagonize dopamine receptors showed no activity in tumor cell line antiproliferative assays (25). Chlorpromazine has also been reported to antagonize calmodulin (33), and calmodulin activity has been reported to be required for the duplication of centrosomes during S phase (34). It is unlikely that calmodulin inhibition is invoked at chlorpromazine concentrations where we see inhibition of proliferation and monopolar spindle formation...
because we observe proper centrosome duplication under these treatment conditions (Fig. 2A).

We have shown here that chlorpromazine is a specific inhibitor of the mitotic kinesin KSP/Eg5, results that constitute the first published report that chlorpromazine is an inhibitor of a mitotic kinesin. This discovery both highlights interesting new hints of biology invoked by chlorpromazine and also cautions careful consideration when using small-molecule probes to biological targets. Several different small-molecule inhibitors of KSP/Eg5 have been reported. These include monastrol, terpindoles, quinazolones, and other compounds of note from the patent literature (18). Monastrol is an allosteric inhibitor of the kinesin that acts by binding at a site distinct from the ATP-binding site (35). The exact molecular mechanism by which chlorpromazine inhibits the ATPase activity of KSP/Eg5 is unknown and is currently under exploration.

**Pentamidine has a role in mitotic progression.** We report that pentamidine, the other component of CRx-026, also plays a role in mitosis. Treatment with pentamidine results in defects in chromosome segregation as evidenced by the appearance of a 5-fold increase in postmitotic DNA bridges. Furthermore, treatment of cells with pentamidine results in a delay in progression from mitosis to G1. Previous reports have suggested that pentamidine may act to inhibit the activity of the PRL phosphatases (30). Other reports have also indicated that mutations in PRL phosphatases result in mitotic defects, such as DNA bridges and delayed progression through mitosis (31). Our data now suggest a link between the inhibitory effects of pentamidine and the mitotic defects of PRL phosphatases.

PRL phosphatases are an attractive target for drug discovery (24). Numerous reports show that this enzyme plays diverse roles in proliferation, cell cycle regulation, and metastasis. Wang et al. (31) have reported that overexpression of different mutations in PRL-1 displays differential mitotic phenotypes. Overexpression of a mutant PRL-1 lacking a residue critical for the farnesylation of PRL-1 results in the formation of DNA bridges but not a delay in mitotic transition. Conversely, overexpression of a mutant defective in catalytic activity results in a mitotic delay but not the formation of DNA bridges. We observe that pentamidine treatment of cells results in both mitotic bridges and a statistically significant delay in progression from mitosis to G1. This suggests that pentamidine inhibition of PRL function is more than simply inhibition of catalytic activity and may inhibit PRL ability to be farnesylated, possibly through an allosteric interaction or conformational effect.

Figure 6. Model for CRx-026 multitarget mechanism. Chlorpromazine inhibits KSP resulting in failed centrosome separation and monopolar spindle formation. Pentamidine inhibits PRL phosphatase resulting in defective chromosome segregation and mitotic delay. The combined action of CRx-026 results in synergistic inhibition of tumor cell proliferation. Cells that are able to overcome one mitotic defect are captured by the second. This combined action results in DNA damage, cell cycle arrest, and cell death.

**CRx-026 inhibits proliferation through dual mitotic action.** We observe that the combination of pentamidine and chlorpromazine results in synergistic inhibition of tumor cell growth in vitro and in vivo. This interaction occurs robustly and broadly across multiple doses and ratios of the two drugs. Taken together with the molecular mechanism results, these data support a dual mitotic role for CRx-026 (Fig. 6). According to our model, when cells treated with CRx-026 transition from prophase to metaphase, the inhibition of KSP/Eg5 by chlorpromazine prevents centrosome separation, arresting cells with aberrant monopolar spindle formation and ultimately resulting in mitotic cell death for some cells in the population. Cells that are able to overcome this block and reach anaphase are then further delayed in mitotic progression by the action of pentamidine at PRL phosphatases resulting in delayed anaphase transition, defective chromosome segregation, and postmitotic DNA bridges. Cells that reach this point and exit mitosis with DNA bridges would arrest in G1 with damaged DNA and then be subject to the cellular DNA damage response pathways. This could include cell cycle arrest, DNA repair, and apoptosis.
model of dual action in mitosis is consistent with our observations that CRx-026 and its parts synergize in vitro. The dual action of CRx-026 sensitizes tumor cells that are already defective in cell cycle regulation to mitotic damage followed by cell cycle arrest and cell death while sparing normal cells with more robust cell cycle regulation. Indeed, we have observed that CRx-026 more potently inhibits the proliferation of lung tumor cells than corresponding normal lung cells (25). In addition, the absence of toxic side effects normally associated with agents affecting cell cycle progression supports some form of differential cell cycle sensitivity of tumor cells to CRx-026 (Table 1; ref. 16).

Importantly, the more general discovery that inhibition of mitotic kinesins and PRL phosphatases synergizes to inhibit tumor cell growth may lead to the development of additional multtargeted therapies to treat cancer. Although combining pentamidine and chlorpromazine to access this synergistic effect is a pragmatic and efficient way to enter proof-of-concept studies for mechanistic validation and may provide therapeutic benefit, ultimately the best way to harness this mechanistic synergy may be the use of other chemistries. Several mitotic kinesins and PRL phosphatase inhibitors are advancing through preclinical and clinical pipelines in the drug discovery industry. We predict that combinations of these drugs would also synergize to create a potent antitumor effect. The results reported here show the importance of systematic screening for combinations of targeted agents that synergize to produce antitumor effects.

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