Tubulin-Binding 3,5-Bis(styryl)pyrazoles as Lead Compounds for the Treatment of Castration-Resistant Prostate Cancer

Vivian W.Y. Liao, Anuradha Kumari, Rajeshwar Narlawar, Soma Vignarajan, David E. Hibbs, Dulal Panda, and Paul W. Groundwater

Abstract

The microtubule-binding taxanes, docetaxel and cabazitaxel, are administered intravenously for the treatment of castration-resistant prostate cancer (CRPC) as the oral administration of these drugs is largely hampered by their low and highly variable bioavailabilities. Using a simple, rapid, and environmentally friendly microwave-assisted protocol, we have synthesized a number of 3,5-bis(styryl)pyrazoles 2a–I, thus allowing for their screening for antiproliferative activity in the androgen-independent PC3 prostate cancer cell line. Surprisingly, two of these structurally simple 3,5-bis(styryl)pyrazoles (2a and 2c) had concentrations which gave 50% of the maximal inhibition of cell proliferation (GI50) in the low micromolar range in the PC3 cell line and were thus selected for extensive further biologic evaluation (apoptosis and cell cycle analysis, and effects on tubulin and microtubules). Our findings from these studies show that 3,5-bis[(1E)-2(2,6-dichlorophenyl)ethenyl]-1H-pyrazole 2c 1) caused significant effects on the cell cycle in PC3 cells, with the vast majority of treated cells in the G2/M phase (89%); 2) induces cell death in PC3 cells even after the removal of the compound; 3) binds to tubulin [dissociation constant (Kd) 0.4 ± 0.1 μM] and inhibits tubulin polymerization in vitro; 4) had no effect upon the polymerization of the bacterial cell division protein FtsZ (a homolog of tubulin); 5) is competitive with paclitaxel for binding to tubulin but not with vinblastine, crocin, or colchicine; and 6) leads to microtubule depolymerization in PC3 cells. Taken together, these results suggest that 3,5-bis(styryl)pyrazoles warrant further investigation as lead compounds for the treatment of CRPC.

Significance Statement

The taxanes are important components of prostate cancer chemotherapy regimens, but their oral administration is hampered by very low and highly variable oral bioavailabilities resulting from their poor absorption, poor solubility, high first-pass metabolism, and efficient efflux by P-glycoprotein. New chemical entities for the treatment of prostate cancer are thus required, and we report here the synthesis and investigation of the mechanism of action of some bis(styryl)pyrazoles, demonstrating their potential as lead compounds for the treatment of prostate cancer.

Introduction

Globally, prostate cancer is the second most common male cancer in terms of incidence and fifth in terms of mortality, with over 300,000 men dying from the disease every year (Todd et al., 2018). Tumor growth in the early stages of prostate cancer is androgen-dependent, so the typical treatment of early metastatic prostate cancer is androgen deprivation therapy with a gonadotropin-releasing hormone analog such as leuprolide, often in combination with an antiandrogen such as flutamide (Crawford et al., 1989; Garnick, 1997; Taplin and Balk, 2004).

As the disease progresses, however, molecular and cellular changes occur so that cancer becomes androgen-independent/hormone refractory (termed castration-resistant prostate cancer [CRPC]) and unresponsive to current hormone therapy (Feldman and Feldman, 2001; Todd et al., 2018). This form of prostate cancer is aggressive, highly metastatic, and associated with poor prognosis (mean survival time of 18–24 months) (Abouelfadel and Crawford, 2008), and there is no effective treatment (Karantanos et al., 2013).

Paclitaxel has been used in the treatment of prostate cancer, and the semisynthetic taxanes, docetaxel and cabazitaxel, are administered intravenously for the treatment of CRPC. The oral administration of these drugs is hampered by their low and highly variable bioavailabilities (Torne et al., 2010), which are due to their poor absorption (a result of their poor solubilities); efficient efflux by P-glycoprotein, which is abundant in the gastrointestinal tract; and high first-pass metabolism by CYP450s, CYP2C8 and 3A4.

ABBREVIATIONS: AUC, area under the curve; CRPC, castration-resistant prostate cancer; G2/M, concentrations which gave 50% of the maximal inhibition of cell proliferation; HPLC, high-pressure liquid chromatography; Kd, dissociation constant; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; OD, optical density; PDB, Protein Data Bank; PI, propidium iodide; PIPES, 1,4-piperazinediethanesulfonic acid.
We report here the discovery of a 3,5-bis(styryl)pyrazole analog 2l that induces cell death in PC3 cells (Pulukuri et al., 2005; Schmitt et al., 2014) even after the removal of the compound. The compound binds to tubulin (Kᵦ 0.4 ± 0.1 μM) and inhibits tubulin polymerization in vitro (with no effect upon the polymerization of the bacterial homolog Streptococcus pneumoniae FtsZ (spn FtsZ) or on the activity of alkaline phosphatase). Pyrazole 2l is competitive with paclitaxel for binding to tubulin (but not with vinblastine, crocin, or colchicine), and treatment with pyrazole 2l results in microtubule depolymerization in PC3 cells, suggesting the further investigation of these 3,5-bis(styryl)pyrazoles as lead compounds for the treatment of CRPC.

Materials and Methods

General Synthetic Chemistry Procedures. The 3,5-bis(styryl) pyrazoles 2a-l were synthesized from the curcuminoinds 1 (Supplemental Methods) using a CEM Discover SP microwave synthesis system. The spectroscopic and analytical data for pyrazoles 2a (Amolins et al., 2009; Mayadevi et al., 2012), 2b (Amolins et al., 2009), and 2k (Luo et al., 2013) were identical to that reported previously. Melting points were determined on a Stuart Scientific SMP10 apparatus. Infrared spectra were obtained on a FTIR-8400S Shimadzu system using NaCl plates, and values are recorded as wave numbers (cm⁻¹). ¹H and ¹³C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Varian 400-MR magnetic resonance spectrometer, with chemical shifts (δ) reported in parts per million (ppm). Spectra were acquired in solutions lead compounds for the treatment of CRPC.

Chromatographic Conditions and Preparation of Standards. The chromatographic method was adapted from Jayaprakash et al. (2002). The mobile phase consisted of varying gradients of acetonitrile (A) and 2% aq. acetic acid solution (B), with a flow rate of 0.75 ml/min. The total run time was 25 minutes, the last 10 minutes equilibrating the column for the next run. The wavelength of detection was the λmax of each compound (2a; 325 nm; 2l 300 nm). The injection volume was 10 μl. Table 1 summarizes the chromatographic conditions used for each of the compounds.

Methanolic standard stock solutions (1 mg/ml) were prepared for each of the analyzed compounds. The stock solutions were diluted in methanol to obtain concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 mg/ml. For less concentrated standard samples of 2a and 2l, a 1 μg/ml standard stock was prepared from the 1 mg/ml stock, and the stock solutions were diluted in methanol to obtain 100, 50, 20, 10, 5, and 2 ng/ml solutions. All standards were freshly prepared from the stock solutions on the day of analysis. The stock solutions were stored at −20°C. The area under the curve (AUC) versus the concentration of each compound standards was plotted and fitted with linear regression analysis using GraphPad Prism to calculate the slope, intercept, and correlation coefficient and thus determine the linearity of the standard curve (Supplemental Tables 1 and 2). For reproducibility, the experiment was repeated three times, on three separate days, with freshly prepared standard solutions for each compound.

Degradation Study. The degradation study was carried out according to the method outlined by Tønnesen and Karlsen (1985). Compounds 2a and 2l were dissolved in methanol (1 mg/ml concentration). The methanolic solution (100 μl) of the compounds was then added to prewarmed PBS (900 μl, 37°C) and placed in the orbital incubator. At time points of 1, 2, 4, 8, 12, 24, 48, and 72 hours, 100 μl of the solution was removed and extracted twice with ethyl acetate (2 × 200 μl). The ethyl acetate extracts were combined and evaporated, and the residues were redissolved in methanol (200 μl) for HPLC analysis. Ten microliters of the original methanol solution were removed, evaporated, and redissolved in methanol (200 μl), and this was taken as t = 0. The AUC is the total absorbance of the compound at that given concentration and was used for quantification. The degradation profiles of each of the compounds are expressed as percentages of compounds remaining in PBS at the sampled time, with the AUC at t = 0 taken as 100%.

The detection and quantification limits for compounds 2a and 2l were calculated according to the method outlined in the guidelines of International Committee on Harmonization (2005) (https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1_Guideline.pdf) using the following formulae:

\[
\text{Detection limit} = 3 \times \text{Sd}/\text{slope}
\]

\[
\text{Quantification limit} = 10 \times \text{slope}.
\]

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>0–15 min (linear gradient)</th>
<th>15–20 min (linear gradient)</th>
<th>20–25 min (isocratic re-equilibration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>30–70</td>
<td>70–30</td>
<td>30</td>
</tr>
<tr>
<td>2l</td>
<td>60–80</td>
<td>80–60</td>
<td>60</td>
</tr>
</tbody>
</table>

A, acetonitrile; B, 2% aq. acetic acid solution.
where \( \sigma \) is the S.D. of errors in regression of the standard curve generated upon linear regression fitting of the average values of three standard curves (\( n = 3 \)).

The detection and quantification limits for 2a and 2l were verified by injecting multiple samples (\( n = 6 \)) at the concentrations of the detection and quantification limits. The accuracy is expressed as the percentage of the S.D. divided by mean values of the six AUC values determined from the injected samples.

**General Experimental Procedure for the In Vitro Testing.** RPMI 1640 medium, PBS, trypsin/EDTA 10× solution, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma Aldrich. Fetal bovine serum (FBS) of Australian origin was purchased from Bovogen. PC3 cells were purchased from American Type Culture Collection (Manasses, VA; CRL-1435). Muse Count & Viability Assay Kit, Muse Annexin V & Dead Cell Assay kit, and Muse Cell Cycle Assay Kit were purchased from Millipore, Australia. A CLARIOstar microplate reader was used to measure the absorbance of MTT formazan at 540 nm. A Muse Cell Analyzer (Merck, Millipore) was used to conduct the annexin V apoptosis and cell cycle analysis assays.

**Antiproliferative Assay.** PC3 cells were grown in RPMI 1640 supplemented with 10% FBS to ~70–80% confluency before seeding. Antiproliferative activity was determined by the MTT colorimetric assay (Mosmann, 1983). The MTT stock solution (12 mM) was prepared by dissolving 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) in PBS (5 mg/ml) filtered through a 0.2 μm syringe filter.

The pyrazoles and methotrexate were dissolved in DMSO in duplicate, with data acquisition of 4000 events, and the experiment was repeated three times on three separate days to give \( n = 3 \).

A set of experiments was performed following the same procedure as above, but after 72 hours of drug treatment, the medium containing the pyrazoles was removed and replaced with fresh medium, and the cells were incubated for a further 72 hours. Cells were then trypsinized, stained with the Muse Annexin V/Dead Cell kit, and analyzed using the Muse Cell Analyzer. The experiment was conducted in duplicate, with data acquisition of 4000 events, and the experiment was repeated three times on three separate days to give \( n = 3 \).

**Cell Cycle Analysis.** PC3 cells were treated in the same way as in the apoptosis assay except, after trypsinization, cells were counted, centrifuged, and fixed with cold ethanol solution 70% (v/v) at 1 × 10^6 cells/ml. The cells were left to fix overnight at ~20°C, then centrifuged, stained with the Muse Cell Cycle kit according to the manufacturer's instructions, and then analyzed using the Muse Cell Analyzer. The experiment was conducted in duplicate with data acquisition of 4000 events, and the experiment was repeated at least four times on four separate days to give \( n = 4 \).

**TABLE 2**  

<table>
<thead>
<tr>
<th>Compound</th>
<th>GI(_{50}) values of 3,5-bis(styryl)pyrazoles 2a-l in PC3 cells*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>GI(_{50}) (μM)</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.012 ± 0.008</td>
</tr>
<tr>
<td>2a</td>
<td>0.85 ± 0.34</td>
</tr>
<tr>
<td>2b</td>
<td>39.8 ± 7.8</td>
</tr>
<tr>
<td>2c</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>2d</td>
<td>77.8 ± 6.7</td>
</tr>
<tr>
<td>2e</td>
<td>69.3 ± 9.4</td>
</tr>
<tr>
<td>2f</td>
<td>1 ± 0.10</td>
</tr>
<tr>
<td>2g</td>
<td>4.1 ± 0.14</td>
</tr>
<tr>
<td>2h</td>
<td>26.9 ± 1.7</td>
</tr>
<tr>
<td>2i</td>
<td>39.8 ± 7.8</td>
</tr>
<tr>
<td>2j</td>
<td>24.2 ± 0.5</td>
</tr>
<tr>
<td>2k</td>
<td>28.9 ± 2.5</td>
</tr>
<tr>
<td>2l</td>
<td>2.21 ± 0.33</td>
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*Data represent the mean ± S.D. of three independent replicates (\( n = 3 \)).

Compound showed no growth inhibitory activity in PC3 cells at the highest tested concentration (500 μM).
Experimental Procedure for the Tubulin and Microtubule Studies. The determination of the effects of the pyrazoles on the assembly kinetics of tubulin in vitro, the $K_d$ for the binding of the analogs to tubulin, and effect on microtubules in cells were performed as described previously (Panda et al., 2005).

Tubulin Purification. Tubulin was isolated from goat brain by two cycles of polymerization, and depolymerization as described previously (Panda et al., 2005).

Light Scattering. Tubulin (12 μM) was incubated with different concentrations of 2a and 2l in PEM [50 mM 1,4-piperazinediethanesulfonic acid (PIPES), 1 mM EGTA, and 3 mM MgCl₂] buffer, pH 6.8, on ice for 10 minutes. After 10 minutes of incubation, 10% DMSO and 1 mM GTP was added to the reaction mixture, and polymerization was monitored at 37°C in a spectrophotometer, Spectramax M2e, at 350 nm for 30 minutes. The experiment was repeated three times, and the percentage inhibition of tubulin polymerization was calculated. The $IC_{50}$ for polymerization was determined by fitting the data in dose-response inhibition curve in the GraphPad Prism Software.

Sedimentation Assay. Tubulin (12 μM) was polymerized at 37°C for 30 minutes in the presence and the absence of 2a and 2l as in light scattering. After 30 minutes, the polymerized microtubules were pelleted by centrifuging it at 33,000 g, 30°C for 30 minutes. The supernatant was separated, and the amount of polymerized microtubules was calculated by loading the supernatant and pellet on the SDS-PAGE gel followed by intensity quantification of bands by ImageJ or by quantifying the supernatant protein concentration by Bradford assay.

Determination of Dissociation Constant. Tubulin (2 μM) was incubated with and without different concentrations of 2a or 2l in 25 mM PIPES buffer pH 6.8 for 10 minutes at room temperature. After incubation, the spectra of tryptophan emission were recorded in a spectrofluorometer (FP-6500; JASCO, Tokyo, Japan) by exciting the sample at 295 nm and recording the spectra. The fluorescence intensity at $\lambda_{\text{max}}$ was noted, and it was fitted in the following equation in GraphPad Prism software to determine the $K_d$:

$$\Delta F = \Delta F_{\text{max}} \times \frac{([P_0] + [L_0]) + K_d}{2[P_0]} - \sqrt{([P_0] + [L_0])^2 - 4[P_0][L_0]}$$

where $\Delta F_{\text{max}}$ is the highest fluorescence intensity, $P_0$ is the concentration of protein, and $L_0$ is the concentration of 2a or 2l.

Determination of Tubulin Binding Site of Pyrazole 2l. Competitive inhibition with colchicine: tubulin (5 μM) was incubated without or with 10 and 20 μM of 2l on ice for 10 minutes in 25 mM PIPES buffer pH 6.8 and then incubated with 10 μM colchicine for 45 minutes at 37°C. The fluorescence spectra (370–500 nm) were monitored using a fluorescence spectrophotometer (FP-6500; JASCO) with excitation of the samples at 340 nm.

Competitive Inhibition with Vinblastine and Crocin. First, we examined whether 2l could inhibit the binding of vinblastine to tubulin using BODIPY-FL-Vinblastine, a fluorescent analog of vinblastine (Lin and Chen, 2013). Tubulin (3 μM) was incubated with 200 nM of BODIPY-FL-vinblastine (V12390; Thermo Scientific) on ice for 10 minutes in 25 mM PIPES buffer pH 6.8. The reaction mixture was then incubated without or with 1 and 5 μM of 2l for 10 minutes on ice. The change in fluorescence of BODIPY-FL-Vinblastine was observed by exciting at 488 nm and taking emission spectra from 498 to 540 nm.

Recently, it has been reported that vinblastine and crocin share their binding sites on tubulin (Hire et al.; 2017; Sawant et al., 2019). Therefore, we examined whether 2l could inhibit the binding of crocin to tubulin. Crocin (5 μM) was incubated with 10 μM tubulin for 10 minutes in ice in PEM buffer pH 6.8. The reaction mixture was then incubated with or without 1 and 5 μM of 2l for 10 minutes on ice. The change in fluorescence of BODIPY-FL-Vinblastine was observed in a spectrophluorometer (FP-6500; JASCO) by exciting at 488 nm and taking emission spectra from 498 to 540 nm.

Docking of 2l with Tubulin. The structure of 2l was drawn in PubChem Sketcher (V2.4) and the Protein Data Bank (PDB) coordinates of 2l were generated in PRODRG server (Schüttelkopf and van Aalten, 2004). The Autodock Vina tool was used for docking of 2l on tubulin (Trott and Olson, 2010) as described previously (Rane et al., 2017; Hura et al., 2018). The crystal structure of tubulin (PDB identifier SLYJ) was used for docking 2l with tubulin. The coordinates of all other proteins and molecules (tubulin-tyrosine ligase, CA-4, glycerol, stathmin, etc.) in SLYJ, except one α and one β subunit of...
positive times, and the quantitation is represented as mean ± S.D. of three independent sets in Table 3. PE, phycoerythrin; SSC, side scatter; -Ve, negative; +Ve, positive

tubulin were deleted using PyMOL (DeLano, 2002). The final coordinates used for docking had one α subunit, one β subunit, one GDP, one GTP, one calcium ion, and two magnesium ions. Initially global docking was performed by covering the whole molecule in a grid box of 72 × 102 × 100 Å, with grid spacing of 1 Å. The global docking was performed five times with exhaustiveness of 100 (the number of times the calculation is repeated), and each set of docking produced nine conformations. The greatest number of conformations were found to interact with two sites on the protein (16 conformations at the interface of the α- and β-dimers and 19 conformations at the taxol site) (Jaghoori et al., 2016). These two sites were thus chosen to perform local docking, which was performed in a grid box of 72 × 56 × 58 Å for colchicine and 126 × 86 × 82 Å for epothilone, with a grid spacing of 0.375 Å. The conformation with minimum binding energy was chosen to analyze the interaction with tubulin. Control docking with epothilone was performed in a similar way to validate the docking protocol. The coordinates of epothilone and colchicine were obtained from their PDB structures 4O4I and 1SA0, respectively. The interaction of the docked conformers with tubulin was further analyzed in University of California, San Francisco’s (UCSF) Chimera version 1.11 (Pettersen et al., 2004) to determine the amino acid residues in the binding pocket and the residues within 4 Å of 2l to determine the possible hydrogen bonding interactions.

**Competitive Inhibition with Paclitaxel.** Tubulin (12 μM) was incubated with different concentrations (0.2, 5, 10, 20, and 30 μM) of 2l for 10 minutes on ice in PEM buffer pH 6.8. Different concentrations (2, 5, and 7 μM) of paclitaxel were added to the reaction mixtures for each concentration of 2l and incubated for 10 minutes on ice. The polymerization reaction was monitored immediately after adding 1 mM GTP in Spectramax M2e by taking OD at 350 nm. The percentage inhibition of tubulin polymerization versus concentration of 2l was plotted in a Lineweaver-Burk plot at different concentrations of paclitaxel to determine the nature of inhibition.

**Effects of 2l on the Polymerization of spnFtsZ.** Purified spnFtsZ (10 μM) was polymerized in the presence of 1 mM GTP at 37°C as described previously (Dhaked et al., 2019). The polymerization reaction was monitored in a spectrofluorometer (FP-6500; JASCO) with the wavelength for both excitation and the emission at 400 nm. Polymerization of spnFtsZ was also observed with prior incubation of the protein with 20 μM 2a and 2l in ice for 10 minutes.

**Alkaline Phosphatase Assay.** Two units of alkaline phosphatase was taken in glycine-NaOH buffer pH 10.4 with 1 mM MgCl₂ and 0.1 mM ZnCl₂. Two hundred fifty micromolars of paranitrophenylphosphate was mixed with the reaction mixture in a cuvette. The reaction mixture was immediately put in a spectrophotometer, and the conversion of the yellow colored product was monitored by taking the OD at 410 nm. A similar experiment was performed with both 2a and 2l by incubating 20 μM each of 2a and 2l for 10 minutes with alkaline phosphatase on ice.

**Sulphorhodamine B Assay.** The GI₅₀ in HeLa cells was determined by sulphorhodamine B assay as described previously (Vichai and Kirtikara, 2006). Briefly, HeLa cells were seeded in tissue culture treated 96-well plate (10,000 cells/well). After attachment, the cells were treated with different concentrations of 2a and 2l and incubated for 24 hours followed by fixation with 50% trichloroacetic acid for 1 hour at 4°C. The plates were then washed, dried, and stained with 0.4% sulphorhodamine B for 1 hour at room temperature. After drying of the plates, the dye was dissolved in 10 mM Tris pH 10.5, and the absorbance was measured at 520 nm in Spectramax M2."
inhibition of cell proliferation at each concentration was determined by the formula

\[
\% \text{Inhibition of cell proliferation} = 100 - \left( \frac{N_t - N_0}{N_0 - N_c} \right) \times 100
\]

where \(N_t\) is OD of the wells where \(2a\) or \(2l\) was added, \(N_0\) is the OD of the wells at the time of \(2a\) or \(2l\) addition, and \(N_c\) is the OD of the wells of 0.1% DMSO treatment. This percentage inhibition was then fitted in dose-response inhibition curve in GraphPad Prism software.

Immunofluorescence Microscopy. The coverslips were first coated with poly-L-lysine, and then cells were added onto coverslips (25,000 cells/well) for attachment. After attachment, cells were treated with different concentrations of \(2a\) and \(2l\) and incubated for 24 hours. Cells were then fixed with 4% formaldehyde and permeabilized with 100% chilled methanol. Two percent bovine serum albumin was used for blocking the nonspecific binding of the antibody, and then the anti-\(\alpha\)-tubulin antibody (Sigma) was added to cells and incubated for 3 hours at 37°C. After washing of primary antibody, secondary antibody tagged with fluorescein isothiocyanate (Sigma) was added to the cells and incubated for 1.5 hours. The coverslips were mounted on clean glass slides using the mounting media having 4',6-diamidino-2-phenylindole, purchased from Vector Laboratories, and observed under a fluorescence microscope (CSU-XI; Yokogawa).

Live-Dead Assay. The percentage of the live and dead cell was determined by propidium iodide (PI) staining. The cells were seeded up to 50% confluency in T25 flasks and then incubated with 4 and 8 \(\mu\)M of \(2a\) and \(2l\) for 24 hours. The cells were trypsinized, pelleted, and resuspended in PBS with a final concentration of cells approximately 1 million per ml. The cells were stained with PI and then analyzed by flow cytometry for PI-positive and PI-negative cells. Ten thousand cells were counted in each case. The experiment was repeated three times.

Statistical Analysis. “±” represents S.D., and \(P\) values were calculated using Student’s \(t\) test.

Results

Synthetic Chemistry. The synthesis of the bis(styryl)pyrazoles \(2a\)–\(l\) takes advantage of our recently described microwave-assisted synthesis of curcuminoids (Groundwater et al., 2017), in which the total reaction time was shortened to 10 minutes and the analytically pure compounds were mostly obtained by the simple recrystallization from ethanol of the crude solid obtained from the acid hydrolysis of the boron-containing intermediate. The ready availability of these synthetic intermediates \(1a\)–\(l\) lead to the preparation of

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Live cells (%</th>
<th>Dead cells (%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95.7 ± 2.2</td>
<td>3.9 ± 1.9</td>
</tr>
<tr>
<td>(2a)</td>
<td>86.7 ± 3.5</td>
<td>12.3 ± 3.7</td>
</tr>
<tr>
<td>(2l)</td>
<td>67.5 ± 9.1</td>
<td>29.4 ± 9.8</td>
</tr>
</tbody>
</table>

\(^a\)Data represent the mean ± S.D. of three independent replicates (\(n = 3\)).
noncurcuminoid analogs such as 3,5-bis(styryl)pyrazole analogs 2a-1 for biologic evaluation, via the cyclization with hydrazine in an acid-catalyzed microwave-assisted condensation, to generate the respective 2a-1 (Scheme 1).

**Antiproliferative Activity of 3,5-Bis(styryl)pyrazoles 2a-1 in PC3 Cells.** The growth inhibitory effect of all the 3,5-bis(styryl)pyrazoles 2a-1 on the PC3 cell line was determined by the MTT assay (Table 2), which was validated by the trypan blue exclusion cell count. Methotrexate was used as a positive control, with a GI50 value (obtained in this study) of 0.012 ± 0.008 μM, which is within the ranges of the reported GI50 values in PC3 cells for methotrexate (0.001–0.1 μM) (Derenne et al., 2010; Marques et al., 2010; Serova et al., 2011). Two compounds (2a and 2l) had GI50 values in the low micromolar range (<2.5 μM) (Supplemental Fig. 1) and were thus selected for further biologic evaluation, as well as degradation and solubility studies.

**Degradation Studies.** We evaluated the stability and solubility of the two analogs selected for extensive biologic testing, 2a and 2l, under simulated physiologic conditions (pH 7.4, 37°C), and the degradation kinetics are summarized in Fig. 1. After 24 hours under the simulated physiologic conditions, 10% of analog 2a remained, whereas 71% of analog 2l remained after 72 hours (Supplemental Figs. 2 and 3).

**The Effect of 3,5-Bis(styryl)pyrazoles 2a and 2l on the Induction of Apoptosis in PC3 Cells.** The two selected analogs (2a and 2l) did not induce apoptosis at their GI50 concentrations, as determined by the annexin V/PI apoptosis assay. PC3 cells were treated at the GI50 of analogs 2a and 2l, with cells treated with DMSO (0.01%) as the vehicle control. The cells were then incubated for 72 hours, stained with annexin V/PI, and analyzed. The representative dot plots depicting the results from this apoptosis assay are shown in Fig. 2; cells treated with and without these compounds maintained more than 90% viability, indicating that they did not undergo apoptosis when treated at the GI50 concentrations. However, the cells died after treatment with higher concentrations of 2a and 2l (4 and 8 μM), as shown by the increasing number of PI-positive cells after PI staining and counting 10,000 cells by flow cytometry (Fig. 3; Table 3). In addition, we quantified the extent of apoptosis in three other cell lines, namely, lung carcinoma (A549), skin melanoma (B16F10), and liver carcinoma (Huh-7), at similar concentrations of 2a and 2l. The compounds induced apoptosis in all three cell lines in a concentration-dependent manner (Supplemental Fig. 4; Supplemental Table 3).

**The Effect of 3,5-Bis(styryl)pyrazoles 2a and 2l on the Cell Cycle of PC3 Cells.** A cell cycle analysis was conducted...
to determine the effects of 3,5-bis(styryl)pyrazoles 2a and 2l on cell division. PC3 cells were treated for 72 hours at the GI50s of these compounds, with cells treated with 0.01% of DMSO used as the vehicle control. The cell cycle analysis showed a significant increase in the percentage of cells in the G2/M phase when treated with the 3,5-bis(styryl)pyrazoles at their GI50 concentrations, in comparison with the control (Fig. 4; Supplemental Table 4).

**Assessment of the Outcome of Mitotic Arrest Induced by 3,5-Bis(styryl)pyrazoles 2a and 2l.** To evaluate the potential of 3,5-bis(styryl)pyrazoles 2a and 2l as effective agents for the treatment of CRPC, we examined whether cell death results from mitotic arrest in PC3 cells. PC3 cells were treated for 72 hours with 3,5-bis(styryl)pyrazoles 2a and 2l (at their GI50s), and the medium containing the compounds was then removed and replaced with fresh medium. The cells were incubated for a further 72 hours before being stained with annexin V/PI and analyzed. The percentage of cell death 72 hours after the removal of 2a and 2l (Supplemental Fig. 5; Table 4) was lower than the mitotic block after 72 hours treatment with 2a and 2l (Supplemental Table 4), suggesting that the effects of the compounds on PC3 cells are reversible.

**Determination of the Effect of 3,5-Bis(styryl)pyrazoles 2a and 2l on Tubulin Polymerization.** Since both the compounds, 2a and 2l, caused a mitotic block, a characteristic of most antitubulin agents (Mukhtar et al., 2014), the effect of 2a and 2l on tubulin was investigated. 3,5-Bis(styryl)pyrazole 2l inhibited the polymerization of purified tubulin (Fig. 5), inhibiting both the rate and extent of tubulin assembly, as monitored by light scattering. The extent of inhibition of tubulin polymerization by the compound was also determined by the sedimentation assay (Supplemental Fig. 6). The IC50 of 3,5-bis(styryl)pyrazole 2l for tubulin polymerization was found to be 4.7 ± 1.2 μM by light scattering and 4 ± 1.5 μM by the sedimentation assay.

The effect of analog 2a on the polymerization of purified tubulin was also determined by light scattering and sedimentation assay (Supplemental Fig. 7). Unlike the pyrazole 2l, analog 2a was not able to inhibit the tubulin polymerization in vitro (Supplemental Fig. 7).

**Effect of 3,5-Bis(styryl)pyrazoles 2a and 2l on Microtubules of PC3 Cells.** Next, the effects of 3,5-bis(styryl)pyrazoles 2a and 2l on the microtubules of PC3 cells were examined by fluorescence microscopy. Cells were seeded on poly-L-lysine coated coverslips and treated with 4 and 8 μM of 2a or 2l. Both the compounds caused the depolymerization of the interphase microtubules in PC3 cells (Fig. 6A). As in the control cells, thread-like microtubule structures can be seen, which were well spread in the cytoplasm of the cells. These thread-like structures were not properly visible in cells treated with 2a and 2l (Supplemental Table 4), suggesting that the effects of the compounds on PC3 cells are reversible.

Although both 2a and 2l also strongly depolymerized spindle microtubules and caused the formation of abnormal spindles in PC3 cells (Fig. 6B), the effects of 2l on interphase microtubules were more pronounced (Supplemental Fig. 8A). A normal mitotic cell with spindle microtubules is shown in the Fig. 6B control panel (arrow). In treated cells, abnormal multipolar spindles can be seen, and the number of mitotic cells per field increased significantly after the treatment with 2a and 2l, which also confirms that these compounds cause mitotic block (Fig. 6B; Supplemental Fig. 8B).
DNA is aligned on the metaphase plate (arranged more compactly in a straight line), whereas after treatment the DNA is scattered and unable to align. We also determined the effect of 2a and 2l on the microtubules of HeLa cells. The GI50 of 2a and 2l was determined to be 2.6 ± 1.2 and 2.4 ± 0.5 μM, respectively (Supplemental Fig. 9, A and B). Both 2a and 2l depolymerized interphase and spindle microtubules in HeLa cells (Supplemental Fig. 9, C and D), with the depolymerization of microtubules by 2l being more pronounced than that produced by 2a (Supplemental Fig. 10A). The population of mitotic cells also increased upon treatment with 2a and 2l, suggesting that the compounds cause mitotic block (Supplemental Fig. 10B).

**Kinetics of Microtubule Depolymerization and Cell Death after Treatment of PC3 Cells with 3,5-Bis(styryl)pyrazole 2a.** To examine whether microtubules are the primary targets of 2a and 2l, we compared the kinetics of microtubule disassembly and cell death upon treatment with both compounds. PC3 cells were incubated without or with either 4 and 8 μM of 2a and 2l for 2 and 4 hours. The fluorescence intensity of microtubules was found to be
strongly reduced after both 2- and 4-hour treatment with 2a and 2l (Fig. 7; Supplemental Fig. 11). The fluorescence intensity of microtubules in vehicle treated cells was determined to be 1525 ± 229 (a.u.), dropping to 1054 ± 112 and 913 ± 105 (a.u.) after treatment with 4 μM 2a for 2 and 4 hours, respectively, and 825 ± 61 and 771 ± 54 (a.u.) after treatment with 4 μM 2l for 2 and 4 hours, respectively (Fig. 7; Supplemental Fig. 11). The fluorescence intensity of the microtubules was reduced by 54 and 57% in the presence of 8 μM 2a for 2 and 4 hours, and 41 and 72% in the presence of 8 μM 2l for 2 and 4 hours, respectively (Fig. 7; Supplemental Fig. 11). These results indicate that treatment with both 2a and 2l led to the formation of depolymerized microtubules within a short time period. In contrast, treatment with 4 and 8 μM 2a and 2l, for 2 or 4 hours, did not induce cell death in PC3 cells (Fig. 8; Supplemental Table 5), indicating that the depolymerization of microtubules preceded cell death upon treatment with 2a or 2l and that microtubules are the primary targets of both 2a and 2l in PC3 cells.

**Binding of 3,5-Bis(styryl)pyrazole 2l to Tubulin.** Since 2a and 2l caused microtubule depolymerization, we characterized the binding of 2a and 2l with tubulin; the binding of 2l with tubulin was examined by monitoring the tryptophan fluorescence of tubulin (Bhattacharyya et al., 2010). The fluorescence of tubulin was found to be reduced in the presence of 2l, indicating that the compound binds to tubulin (Fig. 9). The change in the fluorescence intensity of tubulin in the presence of 2l was plotted in a binding isotherm, which yielded a Kd of 0.4 ± 0.1 μM for the binding interaction.

The binding affinity of 2a to tubulin was also determined (Supplemental Fig. 12), and it was found that it binds to tubulin with a much weaker affinity (Kd, 4.6 ± 1.1 μM) than 2l (Kd, 0.4 ± 0.1 μM), a possible explanation for the weak effect of 2a on the polymerization of purified tubulin and interphase cellular microtubules.

**Determination of Binding Site of 3,5-Bis(styryl)pyrazole 2l on Tubulin.** Pyrazole 2l binds to tubulin with higher affinity than 2a, and we have determined the binding site of 2l on tubulin. Tubulin has three well defined binding sites for small molecules: the taxane, vinca alkaloid, and colchicine sites (Supplemental Fig. 13). Compounds that bind to the vinca alkaloid and colchicine sites generally destabilize microtubules, so we first checked the binding of 2l to these binding sites.

The fluorescence intensity of colchicine and BODIPY-FL vinblastine increases upon binding to tubulin, whereas the absorbance of crocin, a vinca domain binder, increases upon binding to tubulin. If 2l inhibits the binding of colchicine to tubulin, the fluorescence of colchicine-tubulin complex should be reduced in the presence of 2l. However, the prior incubation of 2l with tubulin did not reduce the fluorescence of colchicine-tubulin complex, indicating that 2l does not bind to the colchicine site on tubulin (Supplemental Fig. 14A). To check the binding of 2l at the vinblastine site, we used BODIPY-FL vinblastine and crocin. There was no change in the fluorescence of the BODIPY-FL-vinblastine-tubulin complex or the absorbance of the crocin-tubulin complex, respectively, upon prior incubation with 2l, indicating that 2l does not bind to the vinblastine site on tubulin (Supplemental Fig. 14, B and C).

Consequently, to determine the putative binding site, we performed molecular docking with the tubulin crystal structure (PDB identifier 5LYJ) as described previously (Rane et al., 2017; Hura et al., 2018). We found that most conformations of 2l were interacting with two distinct sites on tubulin; the paclitaxel binding site on β-tubulin (Fig. 10) and the interface of the α- and β-tubulin heterodimer (Supplemental Fig. 15), near the colchicine site. To further validate the docking, we docked epothilone, a paclitaxel site binder, with tubulin. The root mean square deviation of the docked conformation of epothilone to the epothilone conformation present in complex with tubulin (PDB identifier 4041) was found to be 1 Å, confirming that the docking protocols were appropriate (Fig. 11) (Hura et al., 2018). Since competitive binding data with colchicine showed that 2l does not bind to the colchicine site (Supplemental Fig. 14A), we further analyzed the docking data only for paclitaxel site.

We found that 2l possibly forms 2 hydrogen bonds in the paclitaxel binding site, with the peptide backbone of T273 (2.2 Å) and P271 (2.6 Å) (Fig. 12). Further analysis of the docking gave the binding energy of 2l with tubulin, which was found to be −8.4 kcal/mol, whereas that of epothilone was found to be −9.1 kcal/mol. Taxol also forms a similar hydrogen bond with the peptide backbone of T273 (3.0 Å).
In addition, paclitaxel also forms other hydrogen bonds, with R358 (3.3 Å), Q278 (3.4 Å), H226 (2.6 Å), and the peptide backbone of R358 (3.1 Å) (Snyder et al., 2001; Yadava et al., 2015).

We also analyzed the binding pocket of both epothilone and 2l on beta-tubulin and found that there were 12 residues that are common to both 2l and epothilone (Table 5), showing that 2l and epothilone share the same binding pocket. Since the taxol binding pocket has hydrophobic residues in the H7 helix, M loop, and β strand of S7, S9-10, (Ranade et al., 2016), it is possible that 2l also makes some hydrophobic interactions within the binding pocket.

To elucidate the binding site of 2l on tubulin, we performed a competitive inhibition experiment of 2l with paclitaxel. The effects of 2l on the polymerization of tubulin in the presence of different concentrations of paclitaxel were determined. 2l exerted a stronger inhibitory effect on tubulin polymerization in the presence of low concentrations of paclitaxel. The percentage inhibition at each concentration of taxol was plotted in the Michaelis-Menten equation (Fig. 13A). The resulting curves showed a typical competitive inhibition pattern, where $V_{\text{max}}$ (maximum percentage of inhibition) remains unchanged ($P < 0.05$). Furthermore, the Lineweaver-Burk plot indicated that the nature of inhibition is competitive (Fig. 13B), as the plot shows that the affinity of the substrate 2l changes with changes in the paclitaxel concentration, as suggested by a decrease in the x-intercept with increasing concentration of paclitaxel.

**Determination of the Effect of 3,5-Bis(styryl)pyrazoles 2a and 2l on spnFtsZ.** 3,5-Bis(styryl)pyrazoles, 2a and 2l, inhibited neither the polymerization of spnFtsZ (a bacterial homolog of tubulin) nor the enzymatic activity of alkaline phosphatase (Fig. 14) suggesting that the compounds are not Pan-assay interference compounds.

![Fig. 10. Docking of 2l on tubulin.](image1)

![Fig. 11. Docking of epothilone with tubulin.](image2)
A range of 3,5-bis(styryl)pyrazole analogs 2a-l have been prepared using a very simple environmentally friendly microwave-assisted protocol, allowing the rapid and clean generation of analogs in minutes without the need for multiple chromatographic steps for purification. All analogs were screened for antiproliferative activity in the PC3 (androgen-independent) prostate cancer cell line, and two analogs (2a and 2l) with GI\textsubscript{50} values in the low micromolar range were selected for further biologic evaluation. 3,5-Bis(styryl)pyrazole 2a was the most potent compound in the study of the antiproliferative activity in PC3 cells (GI\textsubscript{50} 0.85 ± 0.34 \textmu M), whereas the novel analog 2l was also selected for further biologic evaluation (Supplemental Fig. 1); it has been shown in other studies that analog 2a has GI\textsubscript{50} values of 5.6 \textmu M (in PC3 cells) (Fuchs et al., 2009) and 4.19 and 0.25 \textmu M in breast cancer cell lines, MCF-7 and SKBR3, respectively (Amolins et al., 2009). The GI\textsubscript{50} of 2a and 2l against human cervical cancer (HeLa) cells was determined to be 2.6 ± 1.2 and 2.4 ± 0.5 \textmu M, respectively (Supplemental Fig. 9).

In a stability study, we found that after 72 hours only 10% of derivative 2a remained, in comparison with the 71% of analog 2l that remained. The instability of 2a at pH 7.4 may be due to the presence of the para-hydroxy group; a detailed study of the mechanism of degradation of 1a under physiologic conditions suggested that the major degradation pathway is auto-oxidation due to the presence of this group (Gordon et al., 2015). As 2a is a derivative of 1a, it too could undergo similar auto-oxidation, leading to rapid degradation. Although the GI\textsubscript{50} of 2a is less than that of 2l, 2l might thus be a better lead than 2a. Neither of these analogs induced apoptosis at their GI\textsubscript{50} concentration, but, as can be seen from the cell cycle histograms in Fig. 4, both caused significant effects on the cell cycle, with the vast majority of treated cells in the G2/M phase (89.6% and 89.3%, respectively), suggesting that these analogs inhibit PC3 cell growth as a result of G2/M arrest. With no cell division, as a result of G2/M arrest, the percentage of cells in the G0/G1 phase decreases, and this, in turn, reduces the number of cells in the S phase.

We next examined whether cell death results from mitotic arrest in PC3 cells. The results showed that the tested compounds caused greater cell death in PC3 cells than the

### TABLE 5
Residues within 4 Å of the binding pocket of 2l, epothilone, and paclitaxel

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Common residues</th>
<th>Uncommon residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epothilone</td>
<td>β-tubulin residues D223, H226, L227, A230, F269, P271, T273, L360,</td>
<td>β-tubulin residues L214, L272, S274, R275, R281, L283, Y280, Q278, Q279, G276</td>
</tr>
<tr>
<td>2l</td>
<td></td>
<td>β-tubulin residues L214, L216, L272, S274, R275, P357, R358</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>V22, D25, S233, R358, Q278</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 12. Residues within 4 Å of 2l (red sticks) on beta-tubulin (blue ribbon) showing two possible hydrogen bonds.

Fig. 13. Determination of the nature of inhibition of 2l by paclitaxel. (A) Percentage inhibition data fitted in Michaelis-Menten equation. (B) Lineweaver-Burk plot for 3 (○), 5 (●), and 7 (▲) \textmu M paclitaxel concentration showing that the nature of inhibition is competitive. The experiment was repeated three times, and error bars represent S.D.
control (Table 3, Table 4; Supplemental Fig. 5). Analog 2a had the least effect on PC3 cells 72 hours after removal, despite showing G2/M arrest in the cell cycle analysis, indicating that cells treated with this analog may either have divided or remained in a senescing state. Treatment with analog 21 led to the greatest total percentage of dead PC3 cells. Analysis of the cell cycle in cells treated with analog 21 showed that this compound resulted in the greatest percentage of cells in the G2/M phase, indicating that the arrested cells undergo significant mitotic death.

There are three possible main outcomes from drug-mediated mitotic arrest upon drug removal (Yamada and Gorbisky, 2006): the cells divide (they may recover, leading to normal cell division or divide with abnormalities in the genome); the cells may remain in a senescent state, in which they are metabolically active but do not undergo further cell division; or cell death may be triggered, either by necrosis or apoptosis.

3,5-Bis(styryl)pyrazole 21 inhibited the polymerization of purified tubulin (Fig. 5), inhibiting both the rate and extent of tubulin assembly, as monitored by light scattering and sedimentation assay. Extensive testing of the effects of these pyrazoles on tubulin and microtubules showed that 21 binds to tubulin tightly with a $K_d$ of 0.4 ± 0.1 µM and inhibits the assembly of purified tubulin with no effect on the assembly of FtsZ, a bacterial homolog of tubulin that shows the specific binding of 21 to tubulin only. Treatment with both pyrazoles 2a and 21 caused depolymerization of the microtubules in PC3 and HeLa cells. We also found that, although 21 depolymerizes microtubules, it shares its binding site on tubulin with paclitaxel. Paclitaxel is a known microtubule polymerizing agent, and paclitaxel site binders generally stabilize microtubules; however, few tubulin-binding small molecules, such as estramustine (Laing et al., 1997) and griseofulvin (Rathinasamy et al., 2010), are reported to share their binding sites with paclitaxel but to destabilize microtubules. In summary, we have identified a potential lead that could be the basis for future optimization in the search for novel agents for the treatment of CRPC. Both pyrazoles exert similar effects on HeLa cells (Supplemental Fig. 9), $GI_{50}$ of 2.6 ± 1.2 µM (2a) and 2.4 ± 0.5 µM (21), through their interactions with tubulin and the depolymerization of microtubules in cells.

**Acknowledgments**

We thank Drs. Donna Lai and Sheng Hua at the Molecular Biology Facility, Bosch Institute for their technical support. We also thank Central Facility and Center for Research in Nanotechnology and Science (CRNTS), Indian Institute of Technology Bombay for kindly providing a spinning disc confocal microscope and fluorescence-activated cell sorting facility.

**Authorship Contributions**

Participated in research design: Liao, Kumari, Narlawar, Vignarajan, Hibbs, Panda, Groundwater.

Conducted experiments: Liao, Kumari, Narlawar.

Contributed new reagents or tools: Liao, Kumari, Narlawar.

Performed data analysis: Liao, Kumari, Vignarajan, Hibbs, Panda, Groundwater.

Wrote or contributed to the writing of the manuscript: Liao, Kumari, Hibbs, Panda, Groundwater.

**References**


