Discovery of novel 2-aryl-4-benzoyl-imidazole (ABI-III) analogues targeting tubulin polymerization as antiproliferative agents

Jianjun Chen,[a] Sunjoo Ahn,[b] Jin Wang,[a] Yan Lu,[a] James T. Dalton,[b] Duane D. Miller,[a,b] Wei Li[a*]

[a]Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN 38163, USA
[b]GTx Inc., Memphis, TN, 38163, USA

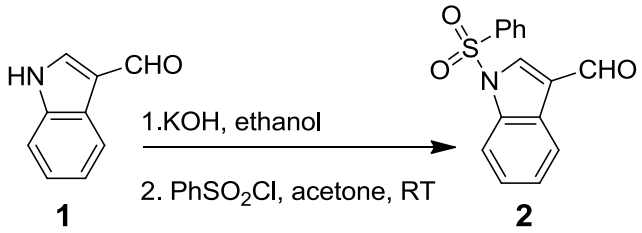
*Corresponding author:
Wei Li, PhD, Tel: (901)-448-7532; Fax: (901)-448-6828; Email: wli@uthsc.edu

Table of contents

Chemical synthesis and characterization by NMR, MS, and HPLC

All reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), Alfa Aesar (Ward Hill, MA), and AK Scientific (Mountain View, CA) and were used without further purification. The solvents for moisture-sensitive reactions were freshly distilled, and the reactions were carried out in an argon atmosphere. Routine thin-layer chromatography (TLC) was performed on aluminum-backed Uniplates (Analtech, Newark, DE). Melting points were measured with the Fisher-Johns melting point apparatus (uncorrected). Nuclear magnetic resonance spectra were obtained on a Varian Inova-500 spectrometer (Santa Clara CA). Chemical shifts are reported as parts per million (ppm) relative to TMS in $CDCl_3$. Mass spectra were collected on a Bruker ESQUIRE electrospray/ion trap instrument in positive and negative ion modes. The purity of the final compounds was examined via reverse phase high performance liquid chromatography RP-HPLC on a Waters 2695 HPLC system equipped with a photodiode array detector (Milford, MA). HPLC condition: 90% methanol at flow rate of 1.0 mL/min using a Luna-PFP 5μM column (250 x 4.6 mm) purchased from Phenomenex (Torrance, CA) at ambient temperature. UV detection was set at 280 nm. Purity of the compounds was established by integration of areas for all peaks detected and is reported for each final compound.

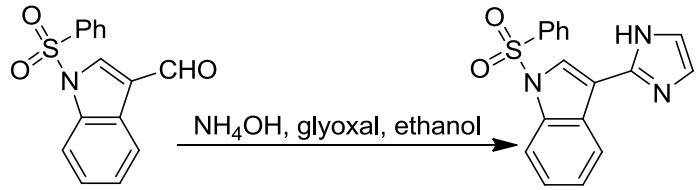
Synthesis of 1-(phenylsulfonyl)-1H-indole-3-carboxaldehyde (2)



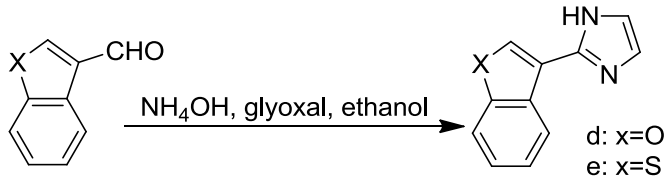
1 **2**

To a solution of indole 3-carboxaldehyde **1** (14.5 g, 100 mmol) in ethanol (500 mL) at room temperature was added potassium hydroxide (6.16 g, 110 mmol), the mixture was stirred till total solubilization.(1) The ethanol was completely removed in vacuum and the residual was dissolved in acetone (250 mL) followed by adding benzenesulfonyl chloride (19.5 g, 110 mmol). The reaction mixture was stirred for half hour. The precipitate was filtered off and the filtrate was concentrated and recrystallized from methanol to give a white solid. Yield: 33%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.17 (s, 1 H), 8.25-8.39 (m, 2 H), 7.97-8.09 (m, 3 H), 7.69 (t, *J*=7.33 Hz, 1 H), 7.59 (t, *J*=7.5 Hz, 2 H), 7.39-7.54 (m, 2 H). MS (ESI) calcd for $C_{15}H_{11}NO_3S$ 285.1, found 286.0 $[M + H]^+$.

Synthesis of 3-(1H-imidazol-2-yl)-1-(phenylsulfonyl)-1H-indole (3), 2-(benzofuran-3-yl)-1H-imidazole (9d), and 2-(benzo[b]thiophen-3-yl)-1H-imidazole (9e)



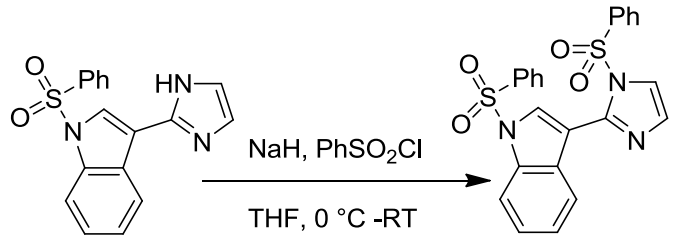
2 **3**



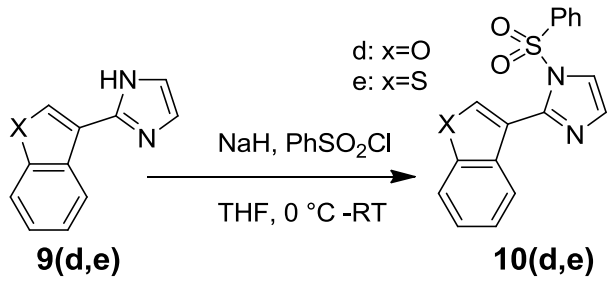
8(d,e) **9(d,e)**

To a solution of 1-(phenylsulfonyl)-1H-indole-3-carboxaldehyde **2** or **8d/e** (28 g, 100 mmol) in ethanol (400 ml) at 0°C was added a solution of 40% oxalaldehyde (glyoxal) in water (22 mL, 110 mmol) and a solution of 29% ammonium hydroxide in water (136 mL, 1000 mmol). (2) After stirring for 2-3 days at room temperature, the reaction mixture was quenched by water and extracted by dichloromethane. The organic layer was removed by vacuum and the residue was subjected to flash column chromatography with hexane/ethyl acetate (4:1-2:1) as eluent to yield the titled compound as a yellow powder. Compound **3:** 15.7% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.33 (d, *J* =2.9 Hz, 2 H), 8.13 (d, *J*=7.8 Hz, 2 H), 7.98-8.04 (m, 1 H), 7.62-7.67 (m, 1 H), 7.55 (d, *J*=7.8 Hz, 2 H), 7.22-7.34 (m, 4 H). MS (ESI) calcd for $C_{17}H_{13}N_3O_2S$ 323.1, found 324.0 $[M + H]^+$. **9d:** 12.2% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.04-8.07 (m, 2 H), 7.55 (d, *J*=8.0 Hz, 1 H), 7.32-7.38 (m, 2 H), 7.21 (s, 2 H). MS (ESI) calcd for $C_{11}H_8N_2O$ 184.1, found 185.1$[M + H]^+$. **9e:** 16.8% yield, ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.54 (s, 1 H), 8.66 (d, *J*=9.6 Hz, 1 H), 7.95 (d, *J*=9.6 Hz, 1 H), 7.73 (s, 1 H), 7.53 (t, *J*=9.6 Hz, 1 H), 7.48 (t, *J*=9.6 Hz, 1 H), 7.29 (br, 2 H). MS (ESI) calcd for $C_{11}H_8N_2S$ 200.0, found 200.9$[M + H]^+$.

Synthesis of 1-(phenylsulfonyl)-3-(1-(phenylsulfonyl)-1H-imidazol-2-yl)-1H-indole (4), 2-(benzofuran-3-yl)-1-(phenylsulfonyl)-1H-imidazole (10d), and 2-(benzo[b]thiophen-3-yl)-1-(phenylsulfonyl)-1H-imidazole (10e)

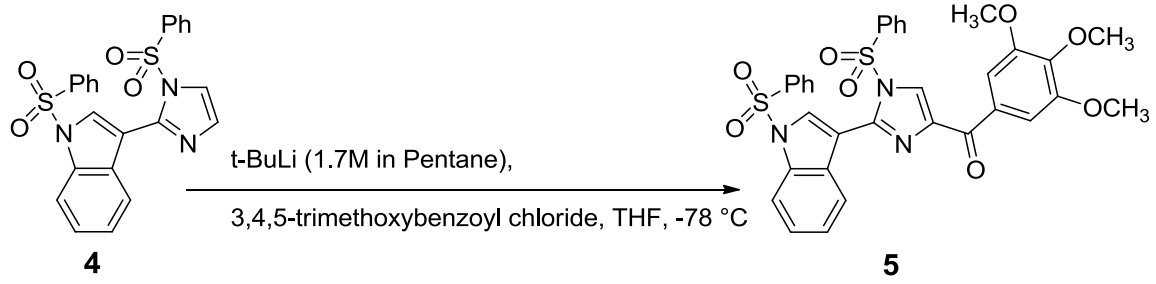


3 **4**



To a solution of 3-(1H-imidazol-2-yl)-1-(phenylsulfonyl)-1H-indole **3** or **9d/e** (6.46 g, 20 mmol) in anhydrous THF (300 ml) at 0 °C was added sodium hydride (60% dispersion in mineral oil, 0.96 g, 24 mmol) and stirred for 20 min. (2) Benzenesulfonyl chloride (4.25 g, 24 mmol) was added and the reaction mixture was stirred overnight. After dilution by 200 ml of saturated $NaHCO_3$ solution (aqueous), the reaction mixture was extracted by ethyl acetate (600 ml). The organic layer was dried over magnesium sulfate and concentrated. The residue was purified by flash column chromatography (hexane: ethyl acetate 5:1) to give a white solid. Compound **4**: 40% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.02-8.08 (m, 4 H), 7.72 (d, *J*=1.5 Hz, 1 H), 7.35-7.60 (m, 8 H), 7.23 (d, *J* = 1.5 Hz, 1 H), 7.10-7.16 (m, 3 H). MS (ESI) calcd for $C_{23}H_{17}N_3O_4S_2$ 463.1, found 486.0 $[M + Na]^+$. **10d:** 50.2% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.19 (s, 1 H), 7.70 (s, 1 H), 7.64 (d, *J*=2.0 Hz, 1 H), 7.55 (d, *J*=7.5 Hz, 2 H), 7.50 (d, *J*=8.5 Hz, 1 H), 7.46 (t, *J*=7.0 Hz, 1 H), 7.24-7.32 (m, 4 H), 7.19 (t, *J*=8.0 Hz, 1 H). MS (ESI) calcd for $C_{17}H_{12}N_2O_3S$ 324.1, found 325.1$[M + H]^+$. **10e:** 55.3% yield. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.87 (d, *J*=10.2 Hz, 1 H), 7.85 (s, 1 H), 7.82 (d, *J*=2.8 Hz, 1 H), 7.30-7.38 (m, 6 H), 7.21 (t, *J*=10.0 Hz, 1 H), 7.15 (t, *J*=10.0 Hz, 2 H). MS (ESI) calcd for $C_{17}H_{12}N_2O_2S_2$ 340.0, found 341.0$[M + H]^+$.

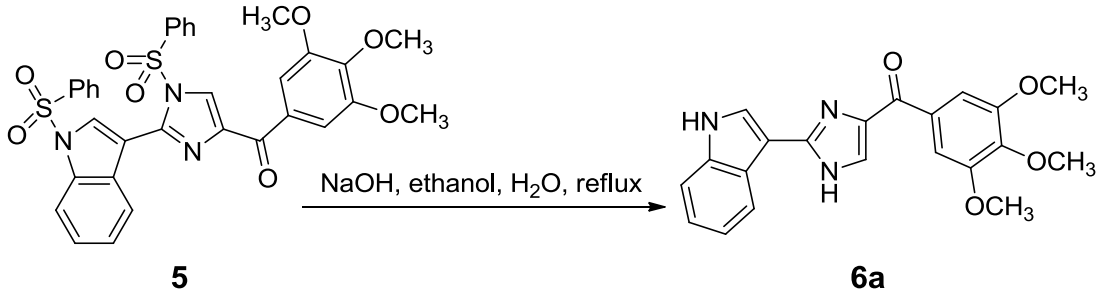
Synthesis of (1-(phenylsulfonyl)-2-(1-(phenylsulfonyl)-1H-indol-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone (5)



To a solution of 1-(phenylsulfonyl)-3-(1-(phenylsulfonyl)-1H-imidazol-2-yl)-1H-indole **4** or **10d/e** (2.32 g, 5.0 mmol) in anhydrous THF (100 ml) at -78°C was added 1.7M tert-butyllithium in pentane (3.5 mL, 6.0 mmol) and stirred for 10 min. A solution of 3,4,5-trimethoxybenzoyl chloride (1.38 g, 6.0 mmol) in THF was added at -78°C and stirred overnight. (2) The reaction mixture was quenched with 100 ml of saturated $NaHCO_3$ solution (aqueous) and extracted by ethyl acetate (300 ml). The organic layer was dried over magnesium sulfate and concentrated. The residue was purified by flash column chromatography (hexane: ethyl acetate 3:1) to give a white solid. Yield: 30%. ^{1}H NMR

($CDCl_3$, 500 MHz) δ 8.09 (d, *J*=10 Hz, 1 H), 8.04 (d, *J*=10 Hz, 2 H),7.91 (s, 1 H), 7.76 (d, *J*=5 Hz, 2 H),7.65 (t, *J*=10 Hz, 1 H), 7.55-7.58 (m, 5 H), 7.40 (s, 2 H), 7.33-7.36 (m, 3 H), 7.25 (t, *J*=10 Hz, 1 H),4.05 (s, 3 H), 4.03 (s, 6 H). MS (ESI) calcd for $C_{33}H_{27}N_3O_8$ 657.0, found 680.1 $[M + Na]^+$.

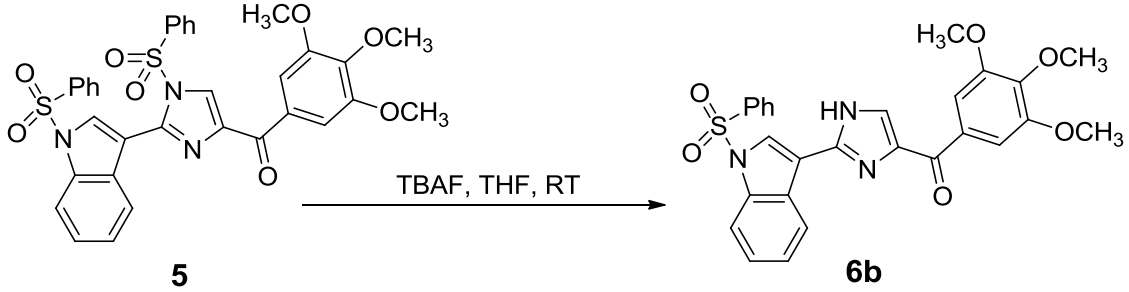
Synthesis of 2-(1H-indol-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone (6a)



5 **6a**

To a solution of (1-(phenylsulfonyl)-2-(1-(phenylsulfonyl)-1H-indol-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone **5** (0.66 g, 1 mmol) in ethanol (40 ml) and water (4 ml) was added sodium hydroxide (0.4 g, 10 mmol) and stirred overnight under refluxing condition in darkness. The reaction mixture was diluted by 50 ml of water and extracted by ethyl acetate (200 ml). The organic layer was dried over magnesium sulfate and concentrated. The residue was purified by flash column chromatography (hexane: ethyl acetate 1:1) to give a yellow solid. Yield: 60%. Mp 210-212°C. ^{1}H NMR (CD_3OD, 500MHz) δ 8.31 (d, *J*=6.5 Hz, 1 H), 7.99 (s, 1 H), 7.90 (s, 1 H), 7.48-7.52 (m, 3 H), 7.24-7.28 (m, 2 H), 4.00 (s, 6 H), 3.93 (s, 3 H). MS (ESI) calcd for $C_{21}H_{19}N_3O_4$ 377.1, found 400.1 $[M + Na]^+$. HPLC: t_R 4.33 min, purity >99%.

(2-(1-(phenylsulfonyl)-1H-indol-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl) methanone (6b)

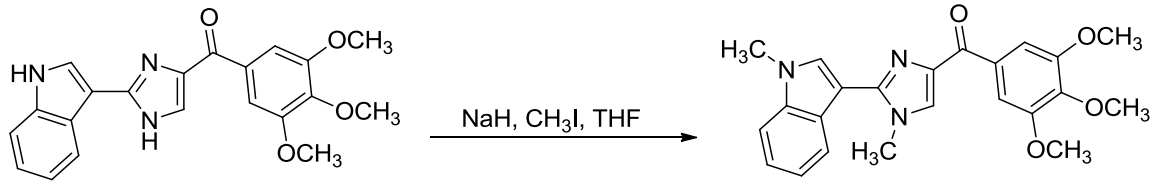


5 **6b**

To a solution of compound **5** (66 mg) in THF (1.0 ml) was added 1.0M tetrabutyl ammonium fluoride (0.4 mL, 0.4 mmol) and stirred overnight. The reaction mixture was diluted by 20 ml of saturated $NaHCO_3$ solution (aqueous) and extracted by ethyl acetate

(20 ml). The organic layer was dried over magnesium sulfate and concentrated. The residue was purified by flash column chromatography (hexane:ethyl acetate, 2:1) to give a pale white solid. Yield: 45%. Mp 110-112°C. ^{1}H NMR ($CDCl_3$, 500MHz) δ 8.40-8.42 (m, 2 H), 8.09 (d, *J*=8.0 Hz, 1 H), 7.93-7.98 (m, 4 H), 7.59 (t, *J*=7.5 Hz, 1 H), 7.41-7.49 (m, 5 H), 4.01 (s, 3 H), 3.97 (s, 6 H). MS (ESI) calcd for $C_{27}H_{23}N_3O_6S$ 517.1, found 540.0 $[M + Na]^+$. HPLC: t_R 6.81 min, purity 96.3%.

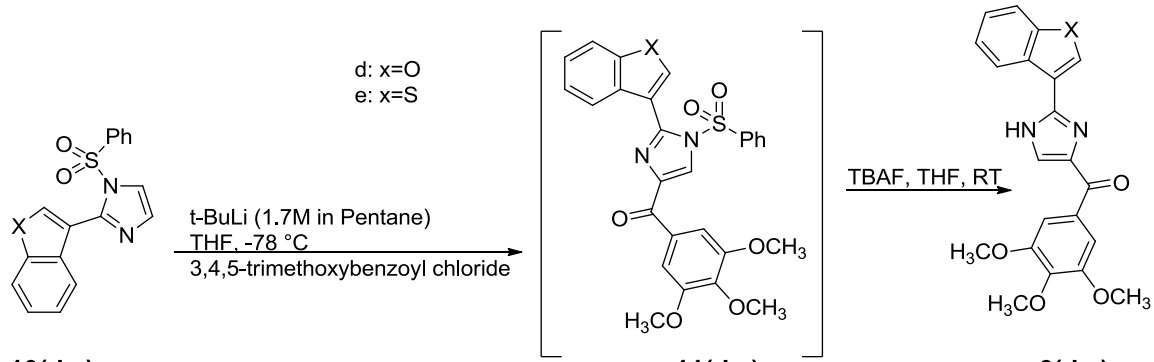
(1-methyl-2-(1-methyl-1H-indol-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl) methanone (**6c**)



6a **6c**

To a solution of **6a**(75 mg, 0.2 mmol) in anhydrous THF (20 ml) at 0 °C was added sodium hydride (60% dispersion in mineral oil, 20 mg, 0.5 mmol) and stirred for 20 min. Methyl iodide (70 mg, 0.5 mmol) was added, and the reaction mixture was stirred 1h. After dilution by 20 ml of saturated $NaHCO_3$ solution (aqueous), the reaction mixture was extracted by ethyl acetate (60 ml). The organic layer was dried over magnesium sulfate and concentrated. The residue was recrystallized from water and methanol to give a white solid. 75% yield. Mp 164-166 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.30 (d, *J*=7.5 Hz, 1 H), 8.01 (s, 1 H), 7.87 (s, 1 H), 7.41 (t, *J*=8.5 Hz, 1 H), 7.39 (s, 1 H), 7.35 (t, *J*=7.0 Hz, 1 H), 7.23 (t, *J*=7.0 Hz, 1 H), 3.98 (s, 6 H), 3.95 (s, 3 H), 3.91 (s, 3 H), 3.89 (s, 3 H). MS (ESI) calcd for $C_{23}H_{23}N_3O_4$ 405.2, found 406.4 $[M + H]^+$. HPLC: t_R 4.80 min, purity >99%.

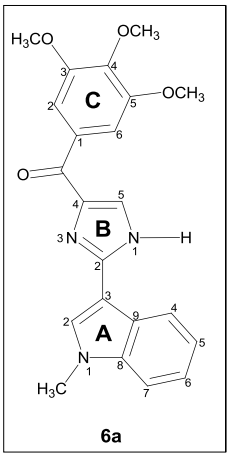
(2-(benzofuran-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone (6d), and (2-(benzo[b]thiophen-3-yl)-1H-imidazol-4-yl)(3,4,5-trimethoxyphenyl)methanone (6e)



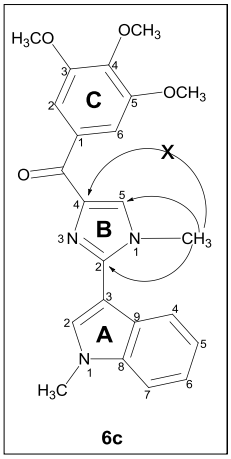
10(d,e) **11(d,e)** **6(d,e)**

To a solution of compounds **10d/e** (2.32 g, 5.0 mmol) in anhydrous THF (100 ml) at -78°C was added 1.7M tert-butyllithium in pentane (3.5 mL, 6.0 mmol) and stirred for 10 min. A solution of 3,4,5-trimethoxybenzoyl chloride (1.38 g, 6.0 mmol) in THF was added at -78°C and stirred overnight. (2) The reaction mixture was quenched with 100 ml of saturated $NaHCO_3$ solution (aqueous) and extracted by ethyl acetate (300 ml). The organic layer was dried over magnesium sulfate and concentrated. The residue was used for next step by adding 10 mL of 1.0M tetrabutyl ammonium fluoride and stirred overnight. The reaction mixture was diluted by 200 ml of saturated $NaHCO_3$ solution (aqueous) and extracted by ethyl acetate (200 ml). The organic layer was dried over magnesium sulfate and concentrated. The residue was purified by flash column chromatography (hexane: ethyl acetate 3:1) to give a white solid. **6d:** 4.7% yield. Mp 208-210 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.77 (s, 1 H), 8.12 (d, *J*=7.6 Hz, 1 H), 7.90 (s, 1 H), 7.632-7.65 (m, 1 H), 7.44-7.49 (m, 2 H), 7.29 (s, 2 H), 3.99 (s, 3 H), 3.93 (s, 6 H). MS (ESI) calcd for $C_{21}H_{18}N_2O_5$ 378.1, found 377.1[M - H]$^-$. HPLC1: t_R 5.18 min, purity 98.8%. **6e:** 3.2% yield. Mp 185-187 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 10.62 (s, 1 H), 8.74 (d, *J*=5.0 Hz, 1 H), 8.06 (s, 1 H), 7.92-7.95 (m, 2 H), 7.48-7.54 (m, 2 H), 7.29 (s, 2 H), 3.99 (s, 3 H), 3.97 (s, 6 H). MS (ESI) calcd for $C_{21}H_{18}N_2O_4S$ 394.1, found 392.8[M - H]$^-$. HPLC: t_R 5.38 min, purity 95.6%.

Structure elucidation of **6a** and **6c**.



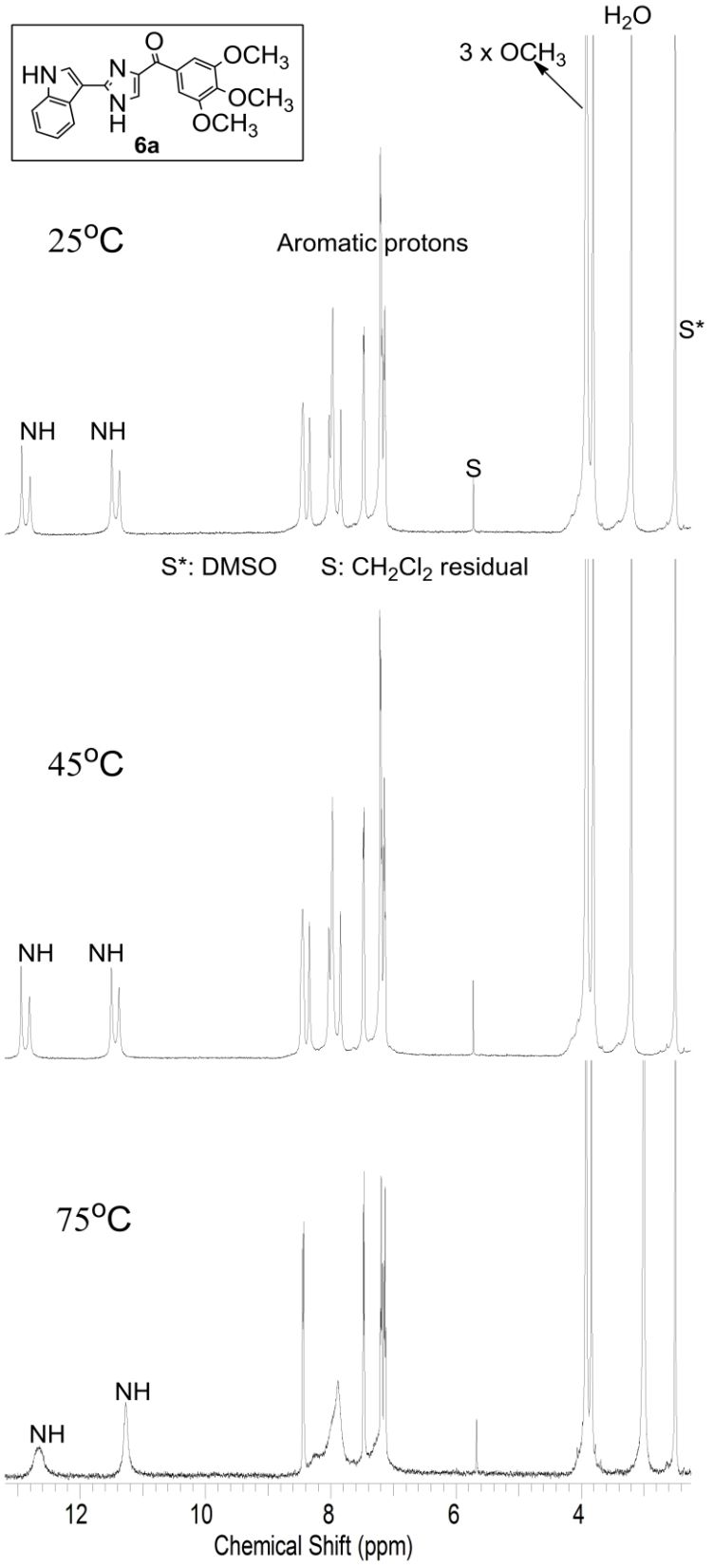
6a



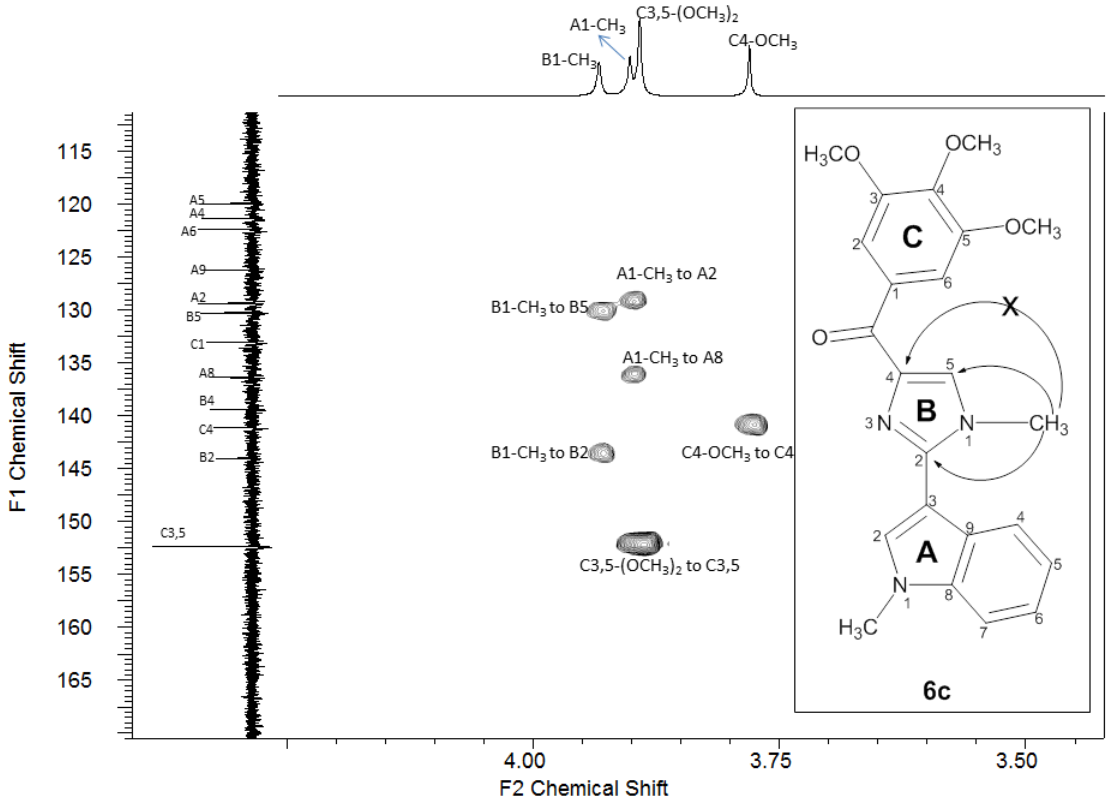
6c

To confirm the existence of two tautomers, we ran temperature-variable ^{1}H NMR experiments for compound **6a** in DMSO (**spectra in S9**). In room temperature we can clearly detect the two tautomers with a ratio of 7:5 based on the splitting of NH peaks. At a higher temperature of 45 °C, the two tautomers still exist. As the temperature is raised to 75 °C, only one tautomer with greater stability is detected (single NH peaks). We assume that the more stable tautomer is with the hydrogen on the N of imidazole 1-position (N1), not the N adjacent (3-position, N3) to the acylated carbon due to steric clash of the hydrogen to the acyl group on imidazole position-4. To confirm this, we ran 2D NMR experiments using compound **6c** which has a methyl group on the N1 position of the imidazole ring. We selected compound **6c** for the 2D NMR experiment because it is easier to detect the HMBC correlations from the imidazole N1-methyl (more protons) of **6c** than that of the N1-proton of **6a**. Since **6a** is the precursor of **6c**, it is expected that the methyl will react with the more stable tautomer of **6a** to form **6c**. In other words, **6a** and **6c** should have the same regiochemistry. As indicated by HMBC (**spectrum in S10**), the N1-methyl showed expected correlations to C2 and C5 of the imidazole, but not to the C4, while the methyl would have correlation to C4 if it were on the N3-position. Therefore, we unambiguously established the structure to be the one with N-substituents (methyl, proton, or phenylsulfonyl) on N1 position and the acylation on C4 position of the imidazole ring.

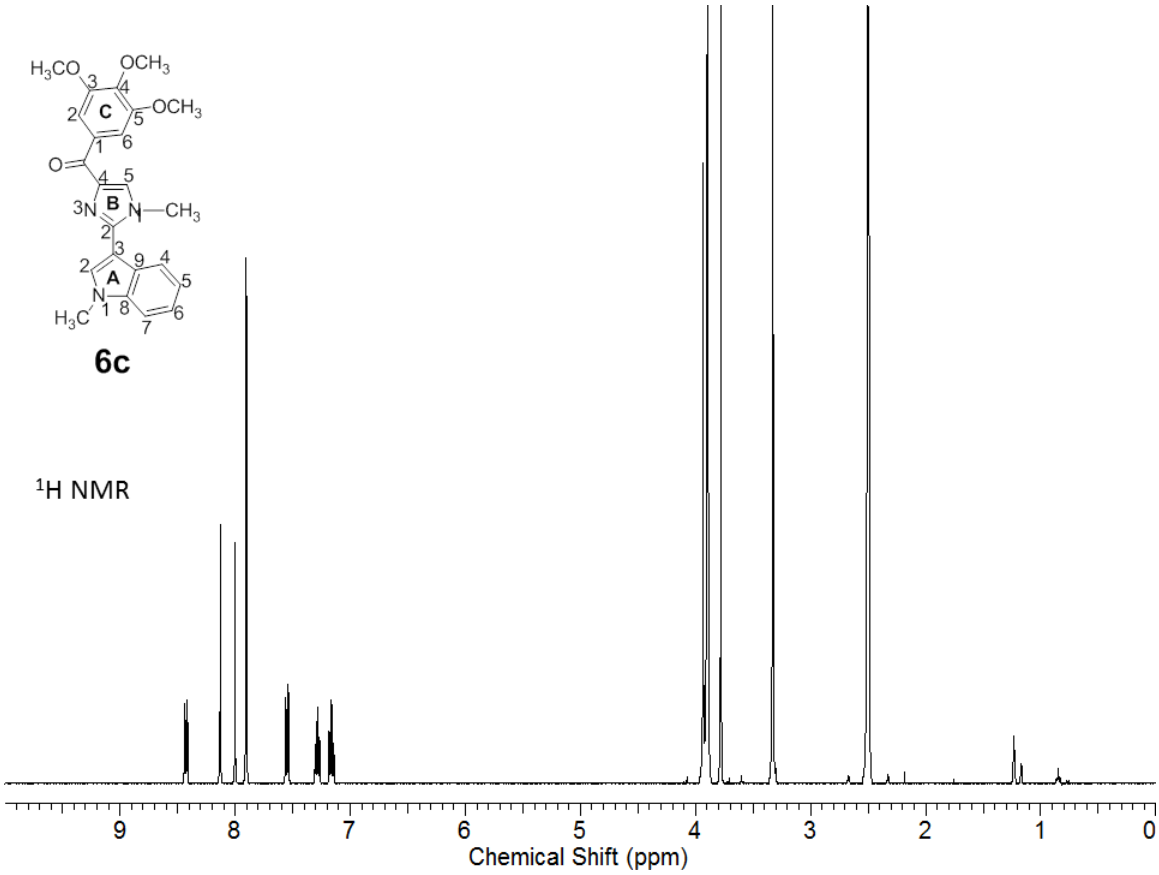
S9: Temperature variable ^{1}H NMR spectra for compound **6a** at 25°C, 45°C, and 75°C



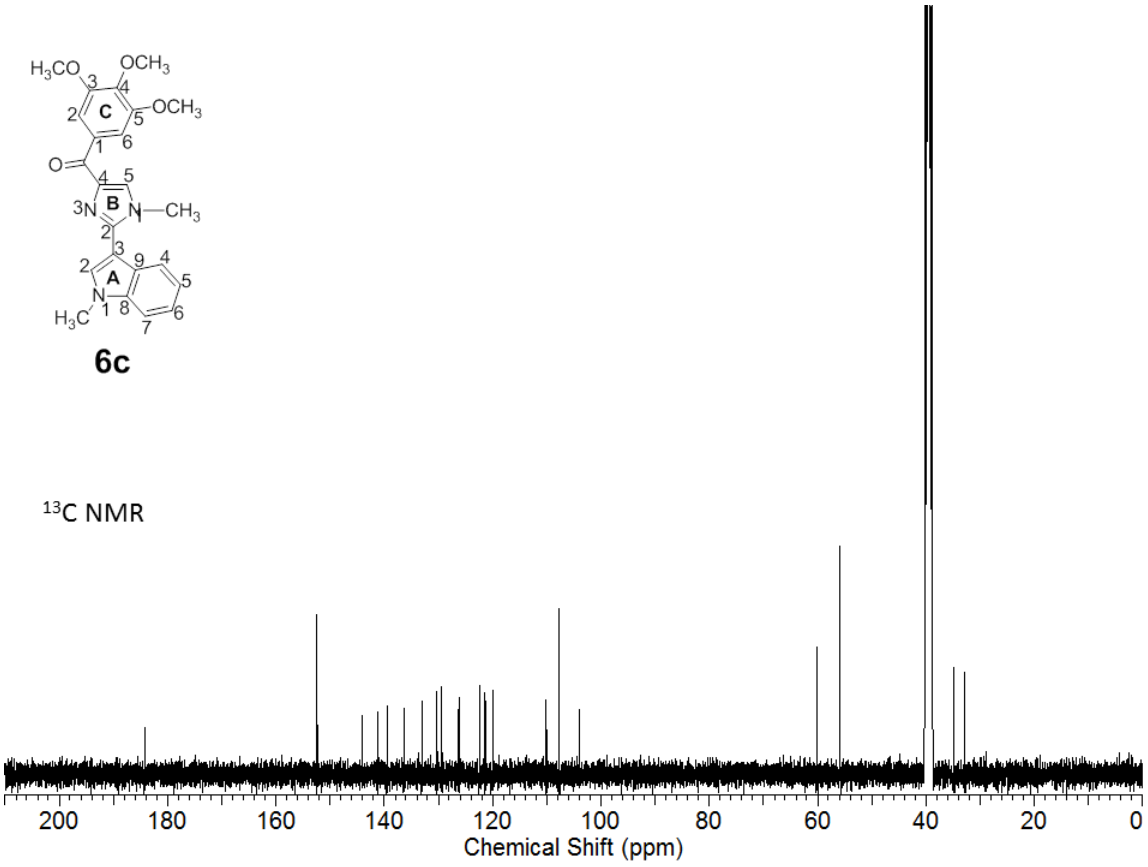
S10: ^{1}H-^{13}C HMBC from N1-methyl of **6c**



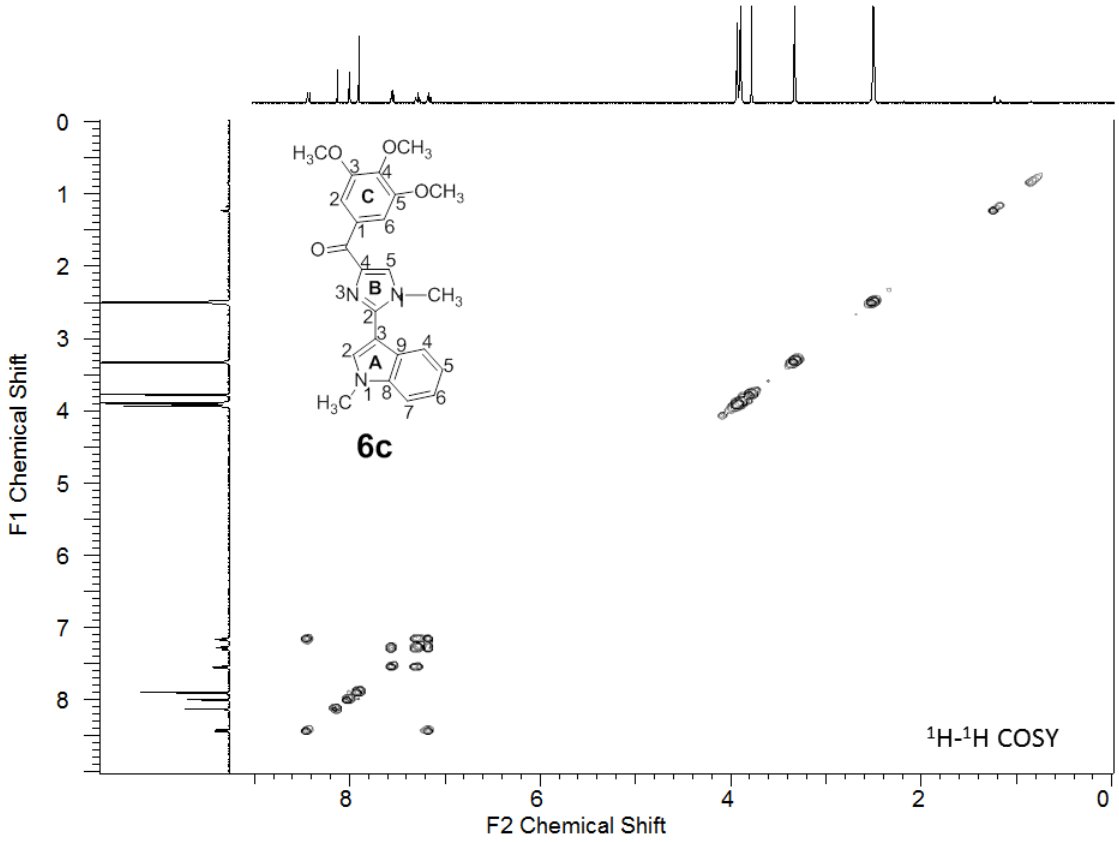
S11: ^{1}H NMR of **6c**



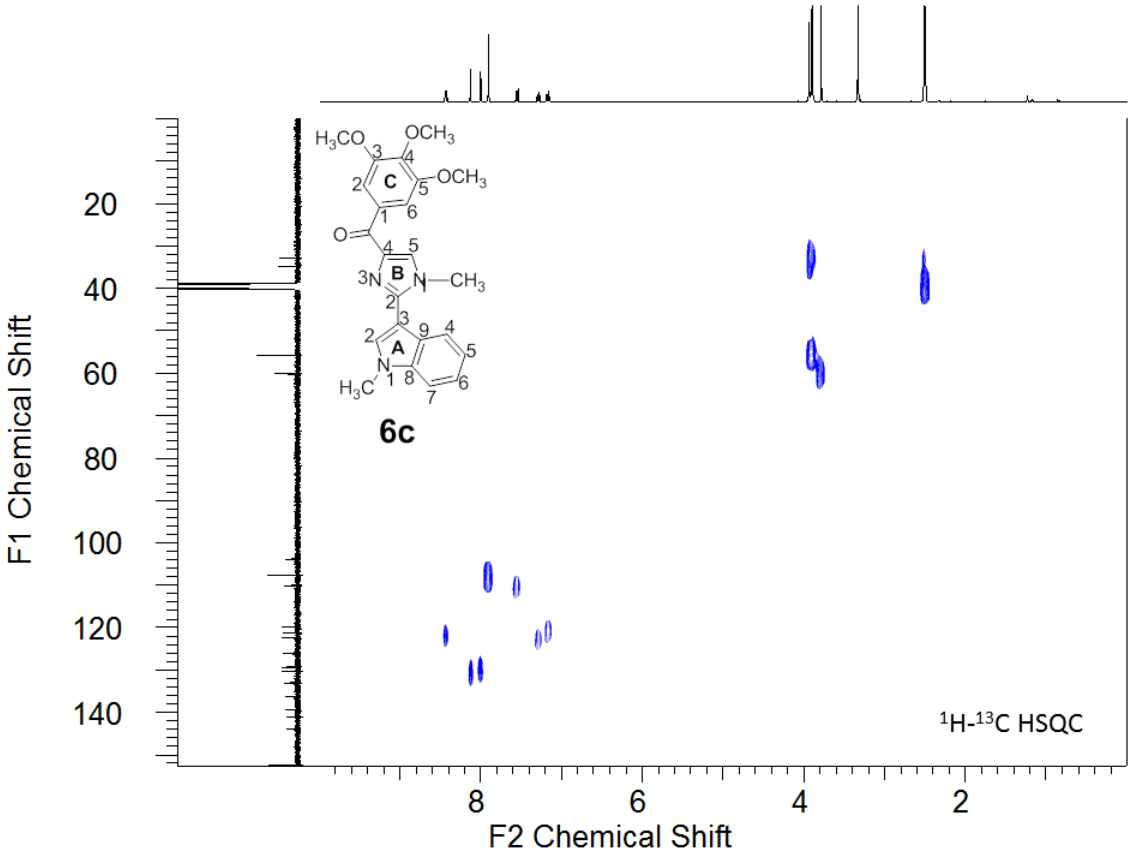
S12: ^{13}C NMR of **6c**



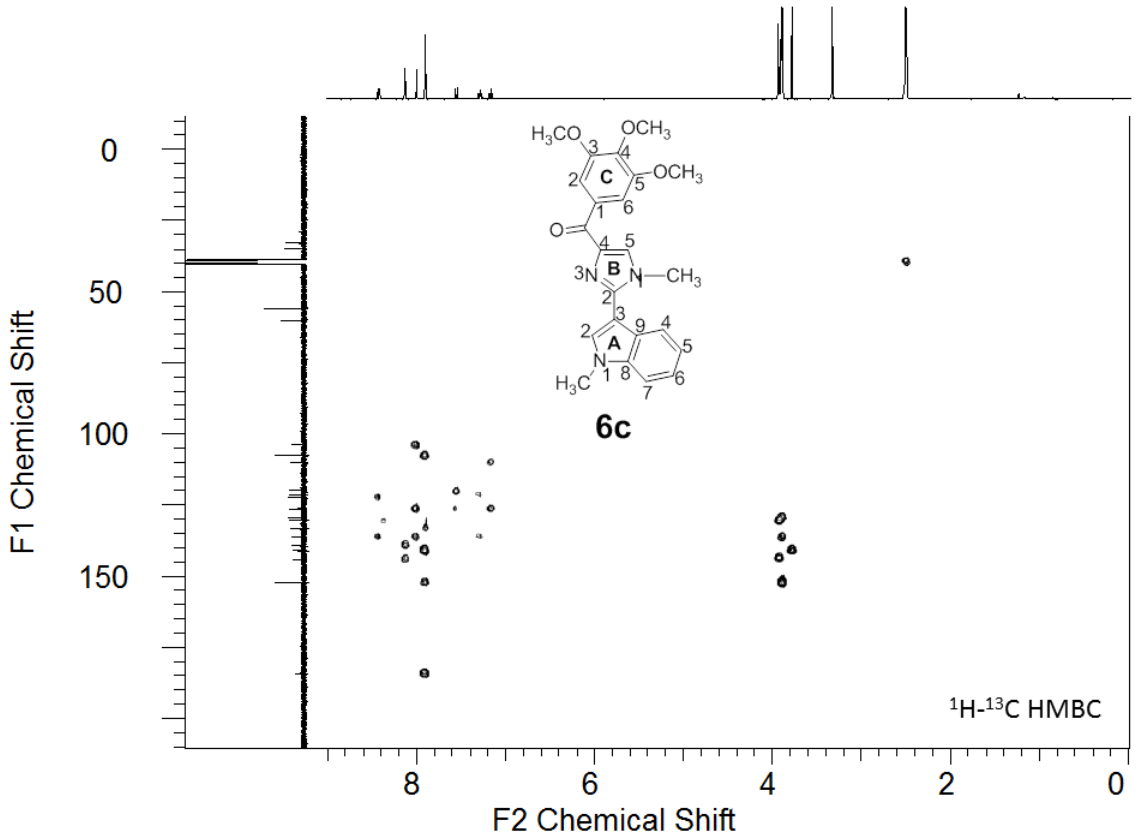
S13: ^{1}H-^{1}H COSY of **6c**



S14:^{1}H-^{13}HSQC of **6c**



S15:^{1}H-^{13}HMBC of **6c**



Cell Culture and Cytotoxicity Assay

Melanoma cell lines (A375 and WM-164) and prostate cancer cell lines (LNCaP, DU 145, and PC-3) were purchased from ATCC (American Type Culture Collection, Manassas, VA). Paclitaxel-resistant PC-3/TxR, and its parental cell lines were gifts from Dr. Evan Keller at the University of Michigan. Melanoma cells were cul-tured in DMEM (Cellgro Mediatech, Inc., Herndon, VA), and prostate cancer cells were cultured in RPMI 1640 (Cellgro Mediatech, Inc.,) supplemented with 10% FBS (Cellgro Medi-atech). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. 1000 to 5000 cells were plated into each well of 96-well plates depending on growth rate and exposed to different concentrations of a test compound for 48 h (fast growing melanoma cells) or 96 h (slow growing prostate cancer cells) in three to five replicates. Cell numbers at the end of the drug treatment were measured by the sulforhodamine B (SRB) assay. Briefly, the cells were fixed with 10% trichloroacetic acid and stained with 0.4% SRB, and the absorbances at 540 nm were measured using a plate reader (DYNEX Technologies, Chantilly, VA). Percentages of cell survival versus drug concentrations were plotted, and the IC_{50} (concentration that inhibited cell growth by 50% of untreated control) values were obtained by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA).

Pgp ATPase assay

Pgp ATPase assay was conducted using the Pgp-Glo Assay System (Promega, Madison, WI) according to the manufacturer's protocol. The luminescence of the sample reflects the ATP level in the sample, which is negatively correlated with the activity of Pgp ATPase and was detected using a Synergy 4 Hybrid Multi-Mode Reader (BioTek Instruments, Winooski, VT). Accordingly, the stimulator of Pgp ATPase will result in significantly lower signals than untreated samples. Na_3VO_4, a selective inhibitor of Pgp without Pgp ATPase activity, and verapamil, a known Pgp substrate with the ATPase activity stimulation, were used as control substrates as provided by Pgp-Glo™ Assay System.

In Vitro Microtubule Polymerization Assay

Bovine brain tubulin (0.4 mg) (Cytoskeleton, Denver, CO) was mixed with 5, 10 μM of the test compounds or colchicine at 5 μM (positive control) and incubated in 110 μL of general tubulin buffer (80 mM PIPES, 2.0 mM $MgCl_2$, 0.5 mMEGTA, and 1 mM GTP) at pH 6.9. The absorbance at 340 nm was monitored every 1 min for 20 min by the SYNERGY 2 microplate reader (Bio-Tek Instruments, Winooski, VT). The spectrophotometer was set at 37 °C for tubulin polymeri-zation.

Molecular Modeling

All molecular modeling studies were performed with Schrodinger Molecular Modeling Suite 2011 (Schrodinger LLC, New York, NY) running on a Dell Linux workstation. We selected tubulin complex with TN16 (PDB code: 3HKD) as our modeling system because of the structure similarity between ABI-III analogs and TN16. ABI-IIIs were built and prepared using the Ligprep module, and they were docked into the TN16 site by the Glide module in the Schrodinger Suite. The best docking complexes were subject to restricted molecular dynamics to release any strains by using the Macromodel module with OPLS-

2005 force field. The ligand and its surrounding residues within 15Å were allowed to move freely while the outer atoms are frozen.

References:

1. H.M. Daviesand X. Dai. Lewis acid-catalyzed tandem Diels-Alder reaction/retro-Claisen rearrangement as an equivalent of the inverse electron demand hetero Diels-Alder reaction. J Org Chem. 70:6680-6684 (2005).
2. J. Chen, Z. Wang, C.M. Li, Y. Lu, P.K. Vaddady, B. Meibohm, J.T. Dalton, D.D. Miller, and W. Li. Discovery of novel 2-aryl-4-benzoyl-imidazoles targeting the colchicines binding site in tubulin as potential anticancer agents. J Med Chem. 53:7414-7427 (2010).