Biological Characterization of an Improved Pyrrole-Based Colchicine Site Agent Identified Through Structure-based Design

Cristina C. Rohena, Nakul Telang, Chenxiao Da, April L. Risinger, James A. Sikorski, Glen E. Kellogg, John T. Gupton, Susan L. Mooberry

Department of Pharmacology and Cancer Therapy & Research Center, University of Texas Health Science Center at San Antonio, San Antonio, Texas (C.C.R., A.L.R., S.L.M.);
Department of Chemistry University of Richmond, Richmond Virginia (N.T., J.T.G.);
Department of Medicinal Chemistry and Institute of Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, Virginia (C.D., G.E.K.); and Medicinal Chemistry & Drug Discovery, Chesterfield, Missouri (J.A.S.).
**Running Title:** Modeling identifies an improved colchicine site agent

**Corresponding Author:** Susan L. Mooberry, Department of Pharmacology, Mail Code 7764, 7703 Floyd Curl Drive, University of Texas Health Science Center at San Antonio, San Antonio, TX, 78229-3900.

Telephone: 210-567-4788; fax: 210-567-4300; email: Mooberry@uthscsa.edu.

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**ABBREVIATIONS:** CA-4, combretastatin A-4; DAPI, 4',6-diamidino-2-phenylindole; EC$_{50}$, the concentration that causes 50% loss of cellular microtubules; HINT, Hydropathic INTeractions (scoring function); IC$_{50}$, the concentration that causes 50% inhibition of proliferation; MTA, microtubule targeting agent; SRB, sulforhodamine B; Pgp, P-glycoprotein.
ABSTRACT
A refined model of the colchicine site on tubulin was used to design an improved analog of the pyrrole parent compound JG-03-14. The optimized compound, NT-7-16, was evaluated in biological assays that confirm that it has potent activities as a new colchicine site microtubule depolymerizer. NT-7-16 exhibits antiproliferative and cytotoxic activities against multiple cancer cell lines with IC₅₀ values of 10-16 nM and it is able to overcome drug resistance mediated by the expression of P-glycoprotein and the βIII isotype of tubulin. NT-7-16 initiated the concentration-dependent loss of cellular microtubules and caused the formation of abnormal mitotic spindles leading to mitotic accumulation. The direct interaction of NT-7-16 with purified tubulin was confirmed and it was more potent than combretastatin A-4 in these assays. Binding studies verified that NT-7-16 binds to tubulin within the colchicine site. The antitumor effects of NT-7-16 were evaluated in an MDA-MB-435 xenograft model and it had excellent activity at concentrations that were not toxic. A second compound, NT-9-21, that contains dichloro moieties in place of the 3,5-dibromo substituents of NT-7-16, had a poorer fit within the colchicine site as predicted by modeling and the HINT score. Biological evaluations showed that NT-9-21 has 10-fold lower potency than NT-7-16, confirming the modeling predictions. These studies highlight the value of the refined colchicine site model and identify a new pyrrole-based colchicine site agent with potent in vitro activities and promising in vivo antitumor actions.
Introduction

Microtubules are dynamic structures that play critical roles in intracellular transport, protein trafficking and cell division. The ability to disrupt these processes has proven useful for anticancer therapy and microtubule disrupting drugs continue to be a mainstay in the treatment of a wide variety of adult and pediatric cancers (Dumontet and Jordan, 2010; Jordan and Wilson, 2004). Chemically diverse MTAs were initially derived from a variety of natural products, including paclitaxel and vinblastine, but advances in synthetic chemistry have led to the development of new microtubule targeting drugs via semi or total-synthesis, including, docetaxel, cabazitaxel, vinorelbine and eribulin. New MTAs with improved clinical efficacy and different spectrums of activity, including the ability to overcome drug resistance mechanisms, continue to advance into clinical use.

To date, five distinct binding sites for MTAs on tubulin/microtubules have been identified: two for microtubule stabilizers and three for microtubule depolymerizers. The microtubule stabilizer sites are the taxoid site on β-tubulin in the interior of the microtubule (Nogales et al., 1995; Xiao et al., 2006) and the laulimalide/peloruside site that is also located on β-tubulin, but on the exterior of the microtubule (Bennett et al., 2010; Huzil et al., 2008). Drug occupancy within these sites stimulates tubulin polymer formation, leading to a higher density of cellular microtubules. In contrast, microtubule depolymerizers inhibit tubulin polymerization and cause a loss of cellular microtubules. Three microtubule destabilizer binding sites have been identified; the vinca domain (Hamel, 2002), the maytansine site (Prota et al., 2014) and the colchicine site (Hamel, 2003). Vinblastine binds within a deep pocket formed between 2 adjacent α-β tubulin heterodimers and occupancy within this site disrupts both the longitudinal and lateral interactions between tubulin heterodimers (Gigant et al., 2005). Multiple clinically useful MTAs bind within the vinca domain. The maytansine site is close to, but non-overlapping
with the vinca domain and binding prevents the formation of longitudinal associations of microtubule protofilaments (Prota et al., 2014). Maytansine analogs have recently found utility as antibody-drug conjugates (Verma et al., 2012). The colchicine site consists of a deep pocket in β-tubulin at the α-β tubulin interface (Dorleans et al., 2009; Ravelli et al., 2004). Colchicine prevents microtubule elongation and destabilizes protofilament interactions leading to microtubule depolymerization. While colchicine was the first MTA to be identified, it was found to be too toxic for the treatment of cancer, yet did find clinical utility in the treatment of gout and Mediterranean familial fever. Some less toxic colchicine site compounds that have been evaluated clinically include 2-methoxyestradiol, the combretastatins CA-4P and CA-1P, ABT-751, and NPI-2358 and newer generation colchicine site agents continue to advance to clinical trials with the hope that improved anticancer drugs can be identified (Lu et al., 2012). One of the most important attributes of colchicine site agents is their ability to circumvent multiple modes of resistance to clinically approved MTAs, including expression of the P-glycoprotein (Pgp) drug efflux pump or the βIII isotype of tubulin. The discovery and development of novel compounds that bind in a distinct manner within the colchicine binding site is important to more fully exploit the clinical potential of this site in hopes of identifying a therapeutic lead compound with excellent efficacy and low toxicity.

Compounds with a wide range of structural diversity can interact within the colchicine site (Lu et al., 2012). X-ray crystallography has defined multiple binding modes within the site and these differences in binding mode might underlie some of the differences in activities among colchicine site agents (Dorleans et al., 2009). Analogous to the vinca domain, the different binding poses prompted the suggestion that the colchicine site be referred to as the colchicine domain, taking into account these various orientations (Dorleans et al., 2009). A continuing
challenge in the rational development of new colchicine site agents is the low resolution (~3.6 Å) of the existing crystal structures of this site and a further challenge in designing new colchicine site agents with improved binding properties is the inherent flexibility of the colchicine binding pocket as predicted by molecular simulations (Chakraborti et al., 2012; Dorleans et al., 2009; Ravelli et al., 2004) and the fact that over 56 chemical scaffolds can interact within this pocket. Over the past 5 years, our goal has been to iteratively refine the model of the colchicine site to allow more informed design of selective, potent compounds that might overcome the limitations of earlier generation colchicine site agents (Da et al., 2013a; Da et al., 2012; Da et al., 2013b; Tripathi et al., 2008). Combining an ensemble docking approach (applying 5 crystal structures of αβ-tubulin) with the biological activities of 59 compounds allowed us to identify optimal substituents for favorable hydrophobic interactions and hydrogen bonding opportunities. Consequently, a near atomic-resolution molecular model of the colchicine binding site was revealed that possesses three major binding pockets (Da et al., 2013a). This information allowed us to design an optimized analog of the previously described pyrrole-based colchicine site agent JG-03-14 (Mooberry et al., 2007). The new analog, NT-7-16, is a potent microtubule depolymerizing agent that overcomes multiple drug resistance mechanisms and has excellent antitumor effects with no evident toxicities. These results validate our docking model for the rational design of improved pyrrole-based colchicine site agents.
Materials and Methods

Materials. Paclitaxel and combretastatin A-4 (CA-4) were purchased from Sigma-Aldrich (St. Louis, MO). All compounds were solubilized in DMSO (Sigma-Aldrich).

Modeling. Molecular modeling procedures are generally as reported previously (Da et al., 2013a). Briefly, X-ray crystal structure models of αβ-tubulin (pdbids: 1SA0, 1SA1, 3HKC, 3HKD and 3HKE) (Dorleans et al., 2009; Ravelli et al., 2004) were obtained from the RCSB protein data bank. We used Sybyl 8.1 (Tripos, LP, St. Louis, MO, USA) to prepare protein and small molecule models for docking, and the Tripos forcefield with Gasteiger-Hückel charges for model optimization. Ligands were docked with GOLD 5.1 (Jones et al., 1995) by generating 100 conformations for each compound that were initially analyzed by GoldScore and further by rescoring with HINT (Kellogg and Abraham, 2000; Sarkar and Kellogg, 2010). HINT is a scoring algorithm developed to enumerate and evaluate hydrophobic as well as polar interactions (e.g., Coulombic, hydrogen bonding) that is based on the experimental measurements of small molecule log Poctranol/water. Log P is the free energy for solute transfer between the two solvents. In previous studies, HINT scores have been shown to correlate with ΔΔG such that ~500 HINT score units = 1 kcal mol⁻¹ (Burnett et al., 2001; Cozzini et al., 2004). The “active” conformation was selected from the models at all five colchicine site structures as the conformation with both a high HINT score and high similarity to the conformation of the complexed ligand in these structures. It should be noted that the crystallographic models for the bound ligands are only approximate because of the low resolution of the protein structures.

Chemical Synthesis of NT-7-16 and NT-9-21. Detailed methods for the synthesis and structure determination of these new chemical entities are provided in Supplemental Data. All purified
reaction products gave TLC results, flash chromatograms, and proton and carbon NMR spectra consistent with a single, homogenous substance with purity exceeding 95%.

**Cell Lines.** The A-10, HeLa and SK-OV-3 cell lines were purchased directly from the ATCC (Manassas, VA). MDA-MB-435 cells were obtained from the Lombardi Cancer Center of Georgetown University and validated by ATCC. The HeLa wild-type βIII (WT βIII) and SK-OV-3/MDR-1-6/6 cell lines were described previously (Risinger et al., 2008). MDA-MB-435 cells were maintained in Improved Minimum Essential Medium (Richter’s Modification) (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum, 25 µg/mL gentamicin in a humidified 37°C incubator with 5% CO₂. The WT βIII cell line was grown and maintained in DMEM (Gibco, Life Technologies) with 10% fetal bovine serum, 50 µg/mL gentamicin in a humidified 37°C incubator with 5% CO₂. The other cell lines were maintained in Basal Medium Eagle (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 50 µg/mL gentamicin in a humidified 37°C incubator with 5% CO₂. Cell stocks were stored in liquid nitrogen and all experiments performed within six months of retrieval.

**Fluorescence Microscopy.** A-10 and HeLa cells were plated on glass coverslips and allowed to attach for 24 h. Cells were treated with vehicle (DMSO), CA-4, NT-7-16 or NT-9-21 at specified concentrations for 18 h. Microtubules were visualized with a β-tubulin antibody (Clone Tub2.1, Sigma-Aldrich), the DNA stained with DAPI (Sigma-Aldrich), and images acquired with a Nikon Eclipse Ti80 microscope with the Nikon Advanced Research Imaging Software. To calculate the EC₅₀ for microtubule depolymerization, the percent microtubule loss in A-10 cells was estimated microscopically over a range of concentrations as previously described (Gangjee et al., 2010; Lee et al., 2010). All experiments were performed a minimum of three times.
**Inhibition of Cellular Proliferation.** The sulforhodamine B (SRB) assay was used to measure the antiproliferative and cytotoxic effects of the compounds as previously described. Briefly, cells were plated in 96-well plates and allowed to attach for 24 h. Cells were then treated with a range of drug concentrations for 48 h, fixed and protein stained with SRB dye. IC$_{50}$ values were calculated from the linear portions of the log-dose response curves of three independent experiments, each conducted in triplicate. Values are expressed as mean ± SD.

**Cell Cycle Analysis.** HeLa cells were treated for 18 h with NT-7-16, NT-9-21, vehicle (DMSO), or paclitaxel and the drug’s effects on cell cycle progression was evaluated by flow cytometry as previously described (Gangjee et al., 2010; Lee et al., 2010). Cells were harvested and stained with Krishan’s reagent and analyzed with a BD Biosciences BD™ LSRII flow cytometer.

**Tubulin Polymerization.** The effects of the compounds on purified porcine brain tubulin polymerization (Cytoskeleton, Denver, CO) were monitored at 340 nm with a SpectraMax plate reader. The assay mixture contained 2 mg/ml tubulin in GPEM buffer (80mM PIPES pH 6.8, 1 mM MgCl$_2$, and 1 mM EGTA) containing 1 mM GTP and 10% glycerol and DMSO as vehicle (0.5 % v/v) or specified drug in 100 µl reactions at 37°C.

**Electron Microscopy.** Aliquots from tubulin polymerization experiments as described above were collected after 60 min reaction time and they were fixed by mixing with equal volumes of 4% gluteraldehyde solution (Electron Microscopy Sciences, Hatfield, PA). Reaction mixtures were mounted on 200 mesh Cu grids, washed with a 10% cytochrome C solution (Sigma-Aldrich) and negatively stained with 8% uranyl acetate. Microtubules were visualized using a JEOL100CX transmission electron microscope with a range of 2,000-100,000 X magnification.
Colchicine Displacement. The ability of NT-7-16 to displace colchicine from tubulin was evaluated using a fluorescent colchicine displacement assay (Bhattacharyyya and Wolff, 1974). Reaction mixtures containing 2 μM tubulin with or without 2 μM colchicine were incubated for 2 h at 37°C with vehicle (DMSO), 20 μM CA-4, 100 μM vinblastine or a range of concentrations (2 μM-10 μM) of NT-7-16. The fluorescence of the samples was analyzed using a Horiba Fluoromax-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) using an excitation wavelength of 380 nm and an emission wavelength of 438 nm. The fluorescence values were normalized by subtracting the buffer alone and setting the fluorescence of tubulin and colchicine as 100%.

In Vivo Studies. Six-week old athymic nude (Foxn1
\(^{\text{nu}}\)/Foxn1
\(^{\text{nu}}\)) female mice were obtained from Harlan (Indianapolis, Indiana) and bilaterally injected with MDA-MB-435 tumor fragments on each flank. When tumors reached approximately 200 mm\(^3\) mice were placed into 3 groups of five mice each (10 tumors) that were assigned to give the same average tumor size per group and randomly assigned a treatment condition. Mice were injected i.p. with either 20 mg/kg paclitaxel 2x weekly for 2 weeks or 75 mg/kg NT-7-16 daily for 14 days. Tumor volume was measured as width x length x height in mm\(^3\) and weight gain/loss was monitored. NT-7-16 was prepared in a 2:1:7 solution (Tween 80:DMSO:PBS) in a total volume of 0.2 mL per injection. Paclitaxel was prepared in 1:1:18 (Cremophor:EtOH:PBS) in a total volume of 0.2 ml per injection. The studies were conducted in accordance with the NIH guidelines as described in the Guide for the Care and Use of Laboratory Animals and were approved by the UTHSCSA Institutional Animal Care and Use Committee. The animals were housed in an AALAC approved facility where food and water were provided ad libitum.
Statistical Studies. For all antiproliferative studies an average of at least three independent experimental values were used to generate the IC$_{50}$ values and a standard deviation was calculated to illustrate the variability of the data. For in vivo studies, statistical analysis of final tumor volumes was performed using a one way ANOVA with a Tukey posthoc test. Statistical analysis of animal weight loss on day 14 was performed using a one way ANOVA with a Dunnet’s posthoc test to compare each drug treated condition to the control. An unpaired t-test was used to compare the wet weights of control and NT-7-16 treated tumors at the completion of the trial.
Results

The initial lead pyrrole compound JG-03-14 (Fig. 1A), a tetra-substituted brominated pyrrole, has microtubule depolymerizing activities and antitumor effects (Mooberry et al., 2007). The chemical structure and positioning of JG-03-14 within the colchicine site is shown in Fig. 1B. Studies were initiated to identify the structure activity relationships within this class of pyrrole compounds that lead to high potencies for both inhibition of cancer cell proliferation and microtubule depolymerizing effects. As part of this optimization process, the HINT scores of the pyrroles were calculated because HINT scores have been shown in a large and diverse number of computational experiments to correlate with the free energy of binding, and are useful in comparative studies of drug binding (Spyrakis et al., 2007). Our goal was to design analogs of the parent compound with higher HINT scores to optimize the hydrogen binding within the Cys241β and Ala354β containing sub-pocket of the colchicine site.

The lack of an optimal pose of JG-03-14 within the colchicine site was predicted based on its high EC50/IC50 ratio. This ratio of the concentration that causes 50% depolymerization of cellular microtubules (EC50) divided by the concentration that causes 50% inhibition of cancer cell proliferation (IC50) has proven useful to compare on-target microtubule-dependent effects with off-target cytotoxic actions. The low EC50/IC50 ratio of 2.3 seen with the colchicine site binding agent CA-4 (Table 1) is indicative of a close correlation between the potencies of cytotoxicity and microtubule depolymerization, suggesting that interruption of microtubule-dependent activities is largely responsible for its cytotoxic effects. In comparison, higher ratios suggest that off-target effects, independent of microtubule depolymerization, are major contributors to a compound’s cytotoxic activities. The high EC50/IC50 ratio of 13 for JG-03-14
suggests that JG-03-14 has off-target cytotoxic effects in addition to its microtubule depolymerizing activity, which was confirmed (Gupton et al., 2000).

Our goal was to design improved analogs of JG-03-14, based on a large data set of diverse colchicine site interacting compounds that was used to refine the model of the colchicine site (Da et al., 2013a). Based on molecular simulations and acknowledgement of the importance of hydrophobic interactions within the colchicine site, a 2,3,4 trimethoxyphenyl analog, designated NT-7-16, was identified with an optimal fit (Fig. 1 A,B). The additional methoxy group at the 2-position of the phenyl ring in NT-7-16 provides opportunities for enhanced hydrophobic interactions with Ala354β that are not present in the parent compound (Fig. 1B).

NT-7-16 was synthesized and its biological activities evaluated. A second compound, designated NT-9-21 (Fig. 1A), which is identical to NT-7-16 but with dichloro substituents replacing the dibromo groups at C3 and C5, has a lower HINT score (Table 1) and it was also synthesized and evaluated as an additional test for the modeling predictions. The biological evaluations showed that NT-7-16 is more potent than the parent compound with an IC50 for inhibition of proliferation of 10.4 nM in the MDA-MB-435 cell line, a 3.4-fold improvement over JG-03-14 (Table 1). Additionally, as would be expected for a better fit with in the colchicine site, NT-7-16 has an EC50 for loss of cellular microtubules of 37 nM, a 13-fold improvement over the parent compound. The EC50/IC50 ratio of NT-7-16 of 3.6 indicates a close association between the concentration that inhibits cellular proliferation and that causes loss of cellular microtubules, consistent with on-target effects. The potencies and EC50/IC50 ratio of NT-7-16 are comparable to those obtained with CA-4, a compound that sits in a distinct pocket of the colchicine site and is the active product of the clinically evaluated prodrug, CA-4P.

These results show that the HINT score and modeling predictions were accurate in predicting
optimal interactions of pyrrole compounds within the colchicine binding pocket yielding a compound with high biological potencies and a low EC₅₀/IC₅₀ ratio. The biological evaluations are consistent with NT-7-16 having an improved fit within the colchicine site consistent with the modeling predictions.

The second pyrrole analog NT-9-21 (Fig 1A), has a HINT score of 772, which suggests an improved fit as compared to JG-03-14, but not as optimal as NT-7-16 (Table 1). Consistent with a predicted poorer fit within the colchicine site, NT-9-21 had a 27-fold higher EC₅₀ value than NT-7-16 and 11-fold decrease in potency for inhibition of proliferation in the MDA-MB-453 cell line (Table 1). The EC₅₀/IC₅₀ ratio of 8.6 for this compound, suggests additional cytotoxic mechanisms outside of its ability to interact with the colchicine site. These results confirm the value of the docking site model we have developed for the binding of pyrroles within the colchicine domain and its quantification with HINT scores can help predict optimal interactions within the refined model and facilitate the design of new pyrrole compounds with much improved activities.

One advantage of many compounds that bind within the colchicine domain is the ability to overcome drug resistance mediated by the expression of the βIII isotype of tubulin. One difference between βIII compared to other isotypes is the substitution of Cys241 with a serine in the A-pocket of the colchicine site, which can limit drug binding (Joe et al., 2008). The ability of NT-7-16 to overcome drug resistance mediated by expression of the βIII isotype of tubulin was evaluated using an isogenic cell line pair of parental and βIII-overexpressing HeLa cells (WT βIII). βIII tubulin-mediated drug resistance to paclitaxel was observed in the WT βIII cells as indicted by a relative resistance (Rr) value of 8.6, which is obtained by dividing the IC₅₀ of the βIII-expressing cell line by the IC₅₀ of the parental HeLa cell line. In contrast, in this cell line
pair, CA-4 has an Rr value of 1.0, showing that CA-4 is able to overcome drug resistance mediated by βIII tubulin (Table 2). NT-7-16 and NT-9-21, with Rr values of 1.2 and 1.1 respectively, can also circumvent drug resistance mediated by βIII tubulin (Table 2).

A second isogenic cell line pair was used to evaluate the ability of the new compounds to circumvent drug resistance mediated by the drug efflux pump Pgp. The SK-OV-3-MDR-1/M-6-6 line is a Pgp expressing clone derived from the parental SK-OV-3 cell line. This Pgp expressing subline is resistant to the effects of paclitaxel as evidenced by an Rr value of 220 (Table 2), but retains sensitivity to CA-4, with a Rr value of 0.61 (Table 2). Both NT-7-16 and NT-9-21 have the ability to circumvent drug resistance mediated by Pgp as indicated by the low Rr values of 0.70 or 0.67 for each compound, respectively (Table 2). These results demonstrate that cells expressing Pgp remain sensitive to NT-7-16 and NT-9-21, consistent with the effects of CA-4. Thus, in contrast to many clinically useful anticancer drugs, including paclitaxel, the new pyrrole-based colchicine site agents are able to overcome drug resistance mediated by βIII tubulin or Pgp expression.

The effects of NT-7-16 and NT-9-21 on interphase and mitotic microtubules were evaluated in A-10 and HeLa cells respectively. Normal microtubule arrays were present in vehicle-treated cells with the microtubules extending from the microtubule organizing center in the center of the cell toward the periphery (Fig. 2). The positive control, CA-4, caused a loss of microtubules with an EC₅₀ for cellular microtubule depolymerization of 10 nM, where loss of microtubules was observed at the cell periphery but were retained in the vicinity of the microtubule organizing center (Fig. 2). Consistent with the effects of CA-4, NT-7-16 and NT-9-21 each caused loss of interphase microtubules. NT-7-16 caused extensive, concentration-
dependent microtubule loss between 25 – 50 nM (Fig. 2, Supplemental Fig. 1). NT-9-21 caused similar microtubule loss at concentrations in the low micromolar range (Fig. 2).

The loss of interphase microtubules initiated by either NT-7-16 or NT-9-21 was accompanied by a notable increase of the number of cells in mitosis, consistent with interruption of the formation of functional mitotic spindles. The effects of the compounds on mitotic spindle structures were evaluated in HeLa cells. Cells treated with vehicle had normal, bipolar mitotic spindles with the chromosomes aligned at the metaphase plate (Fig. 3A). In contrast, in cells treated with 15 nM NT-7-16, the appearance of lagging chromosomes was evident and was more pronounced at 25 nM (Fig. 3A, right panels). NT-9-21 caused similar effects but at much higher concentrations (data not shown). Consistent with these mitotic spindle defects, an accumulation of cells in G2/M was also observed (Fig. 3B). The cell cycle profile of vehicle- treated cells indicates that the majority of cells were in the G1 phase of the cell cycle (Fig. 3B). The microtubule stabilizer, paclitaxel, caused the majority of the HeLa cells to accumulate in G2/M, consistent with mitotic arrest (Fig. 3B). NT-7-16 and NT-9-21 also caused G2/M accumulation with NT-7-16 being much more potent, causing pronounced G2/M accumulation at 25 nM, while 500 nM NT-9-21 was required to cause the same change in cell cycle distribution (Fig. 3B).

Due to its superior potency and better predicted fit within the colchicine site, we focused further studies on NT-7-16. The ability of NT-7-16 to interact directly with porcine brain tubulin was evaluated turbidimetrically. Robust polymerization was observed in vehicle-treated samples while the positive control, CA-4, inhibited this polymerization (Fig. 4A). Consistent with our cellular studies showing depolymerization of interphase microtubules (Fig. 2), NT-7-16 inhibited the polymerization of purified tubulin in a concentration-dependent manner (Fig. 4A). Interestingly, NT-7-16 was more potent than CA-4 in this assay as 5 µM NT-7-16 was more...
effective than 10 µM CA-4 for inhibiting microtubule polymerization. Once this direct interaction with tubulin was confirmed, the effects of NT-7-16 and CA-4 on tubulin structures were evaluated by electron microscopy. Consistent with the increased turbidity observed in Figure 4A, microtubule polymers were observed in samples treated with vehicle while a 10 µM concentration of CA-4 reduced the number and length of these microtubule polymers (Fig. 4B). Similarly, very few microtubules were observed in the presence of 2.5 µM NT-7-16 and with a 5 µM concentration essentially no microtubules were observed by electron microscopy (Fig. 4B). The finding that NT-7-16 was more potent than CA-4 for inhibiting tubulin polymerization is striking, suggesting that NT-7-16 does have optimal interactions with tubulin, while in the cellular assays CA-4 was more potent.

Colchicine displacement assays were performed to evaluate whether NT-7-16 is able to prevent colchicine binding consistent with occupancy of the colchicine site. When bound to tubulin, colchicine is constrained, which produces fluorescence that was defined as 100% colchicine binding. The effects of NT-7-16, CA-4, and vinblastine on colchicine binding were evaluated (Fig. 4C). At 20 µM, a concentration stoichiometric to tubulin, the colchicine-site binding agent CA-4 decreased colchicine fluorescence by 72%, consistent with occupancy at the colchicine site. In contrast, the negative control, vinblastine, which binds to the distinct vinca domain on tubulin, had little effect even at the super-stoichiometric concentration of 100 µM (Fig. 4C). Consistent with occupancy of the colchicine site, NT-7-16 caused a dose-dependent decrease in the fluorescence of tubulin-bound colchicine with a 10 µM concentration of NT-7-16 inhibiting this fluorescence by 78% (Fig. 4C).

The promising potent antiproliferative effects of NT-7-16 against multiple cancer cell lines prompted its evaluations in in vivo models of cancer to evaluate its potency, efficacy and
toxicity. The antitumor effects of NT-7-16 were evaluated in a MDA-MB-435 human xenograft model in athymic nude female mice. Initial dose tolerance tests were conducted to identify an optimal dose and schedule. A maximal tolerated dose was not identified in these trials and 75 mg/kg was found to be the highest dose possible based on the limited aqueous solubility of NT-7-16. The in vivo trial was initiated when the MDA-MB-435 cells had formed tumors with an average size of 200 mm³. A 75 mg/kg dose of NT-7-16 was administered i.p. daily over a period of 14 days for a total dose of 1,050 mg/kg. The positive control, paclitaxel, was dosed twice a week i.p. at 20 mg/kg for a total dose of 80 mg/kg. The results of this trial show that NT-7-16 had antitumor effects that were significantly different from control tumors (p = 0.0018) whereas paclitaxel had no significant effect compared to control or NT-7-16 treated tumors at these doses and schedules (Fig. 5A,B). When the individual tumor volumes were graphed at the conclusion of the trial, it was interesting that the tumors in the NT-7-16 treatment group were much smaller with less variability in tumor size as compared to either the paclitaxel or control groups (Fig. 6B). At day 14, the average tumor burden for NT-7-16 treated mice was 521 mm³ (range 197-858 mm³), whereas the average tumor volume for the control and paclitaxel groups were 1,182 (range 564-1,871 mm³), and 833 mm³ (range 237-1,694 mm³), respectively. Additionally when the wet weights of the tumors were evaluated at the conclusion of the trial, the animals treated with NT-7-16 had an average tumor weight of 381 mg whereas the control animals had an average tumor weight of 899 mg, further demonstrating that NT-7-16 significantly inhibits tumor growth (p = 0.0007) (Supplemental Fig. 2). These tumor measurements highlight the excellent antitumor effects of NT-7-16. Daily dosing of NT-7-16 at 75 mg/kg did not lead to any evidence of overt toxicity and no notable change in the weight of the animals was measured as compared to the controls (Fig. 6C). In comparison, significant cumulative weight loss compared to control
mice \( p = 0.0014 \) was observed in the paclitaxel treated group at day 14 with no significant inhibition of tumor size.

In conclusion, our data show that NT-7-16 is a potent microtubule depolymerizer that binds to the colchicine site and possesses significant antitumor effects with no evidence of toxicity. The modeling predictions and the activities of NT-7-16 demonstrate the value of our refined model for the design of improved agents that bind within the pyrrole sub-pocket of the colchicine site.
Discussion

This study presents the biological validation of the rational synthesis of pyrrole-containing colchicine site agents based on our refined colchicine binding site model, docking and 3D QSAR methodologies. With this refined binding model, we designed molecules that, as predicted, bind more optimally within the colchicine domain and have superior biological potencies and specificity as compared to earlier generation pyrrole-based colchicine site agents. In particular, the 2,3,4 trimethoxyphenyl analog, NT-7-16, showed excellent, low nanomolar potency in a variety of sensitive and drug resistant cancer cell lines, was more potent than CA-4 in its ability to inhibit the polymerization purified tubulin, and had excellent antitumor activity in a MDA-MB-435 xenograft model without evidence of toxicities.

Our previous modeling and biological studies showed that an ethyl ester is optimal at the C2 substitution of the pyrrole, providing favorable alkyl length and hydropathic properties into the site’s C2 pocket, named based on the 2nd ring of colchicine (Da et al., 2012). Shortening or lengthening the C2 ethyl ester group or adding more polar C2 amide functionality diminished optimal interactions and decreased potency and selectivity. We previously evaluated pyrrole C4 phenyl substituents and their interaction within the A pocket of the colchicine site. These modeling simulations and biological evaluation of 18 compounds with various phenyl substitutions at C4 identified the importance of hydrogen bonding between the compound and Cys241β, which is in an otherwise hydrophobic pocket (Da et al., 2013b). The refined model shows that the precise placement of hydrogen bond acceptors and hydrophobes in this pocket is critical: the 3-methoxyphenyl substituent at C4 has a 5-fold higher EC₅₀, and the 4-methoxyphenyl substituent has a 14-fold higher EC₅₀ than the 3,4-dimethoxyphenyl substituent found in JG-03-14 (Da et al., 2013a).
Furthermore, the potency provided by the 2,3,4-trimethoxyphenyl group at C4 in NT-7-16 stands in sharp contrast to the dramatically lower activity reported for the corresponding 3,4,5-trimethoxy-phenyl analog (Da et al., 2013a). Although maximizing the engagement of pocket hydrophobic residues was seemingly desirable, placing a 3,4,5-trimethoxyphenyl at C4 yielded a compound with a 145-fold higher EC₅₀ compared to JG-03-14, presumably due to clashes of the ring’s 5-position methoxy with the pocket wall. Interestingly, one of the key differences between the βIII isotype of tubulin and other isotypes is the replacement of the A-pocket Cys241β with a serine, which modeling indicates provides a key hydrogen-bonding interaction with the most active pyrrole compounds, particularly those with a low EC₅₀/IC₅₀ ratio. The –OH group of serine would be expected to be a stronger hydrogen bond donor to the 2-methoxy group than the –SH of cysteine, yielding tighter interactions between compounds with appropriately-placed acceptors and this residue, such as NT-7-16. Overall, such compounds would be expected to interact more strongly with the βIII isotype of tubulin, which is implicated in multidrug resistance to other classes of microtubule targeted agents. This is indeed what is seen with these compounds in that they show no loss in potency or efficacy in HeLa cells overexpressing the βIII isotype.

Our molecular model also suggested opportunities for further optimization of the pyrroles. First, while optimizing substituents for the C2 position of the pyrrole, we observed that alkyl groups longer than butyl led to a substantive decrease in IC₅₀ values, which we rationalized as a second binding mode where the C2 group was splayed in the pocket entrance as it became too large for the pocket. In the higher-activity binding mode, the pocket entrance is occupied by the pyrrole C5 substituents, Br (NT-7-16) and Cl (NT-9-21), neither of which appear to take full advantage of the properties of residues in the entrance of the pocket. Second, the C3 position of
pyrrole has been explored only cursorily. The 3,5-dibromo substituted NT-7-16 outperformed the 3,5-dichloro substituted NT-9-21 in terms of both efficacy and potency. Bromine is a) larger than Cl, b) more hydrophobic than Cl, and c) a C-Br bond is longer than a C-Cl bond, therefore our models predict that NT-7-16 has stronger hydrophobic interactions within the pocket than NT-9-21. This is further corroborated by our biological data. The model indicates that the site, which is really more of a corner, provides a relatively small volume and is notably hydrophobic. One lesson learned from our earlier optimization of the C2 pocket (Da et al., 2012) was that very minor structural differences that compromise tight hydrophobic associations can have large effects on EC50: the ethyl to methyl ester substitution resulted in a 17-fold increase in EC50. In that context, the differences between Br and Cl at C3 are not surprising; the Br of NT-7-16 similarly should make tighter contacts with the pocket than the Cl of NT-9-21. Nonetheless, the 27-fold difference in EC50 between the NT-9-21 dichloro and the NT-7-16 dibromo compounds suggests that some future optimization of that position may yield unanticipated results.

The colchicine domain is well known for the wide range of chemical structures and poses that can occupy this largely unstructured site. The orientations of colchicine, ABT-751 and podophyllotoxin were used to describe the “main site” by Dorléans and colleagues (Dorleans et al., 2009). However, even within this “main site” there are major differences in binding poses and interactions with tubulin. ABT-751 overlaps extensively with colchicine, but is buried further into β-tubulin, which facilitates interactions with Tyr202β but eliminates the interaction with the α subunit. Dorléans et al. additionally describe a deeper pocket buried in β-tubulin that contains Cys241β, which is the site predicted to bind our optimized pyrroles. In the current study, we focused on identifying compounds with optimal binding into this sub-pocket, but have
not evaluated how the refined model predicts interactions with compounds that would occupy the other sites and subpockets within the colchicine domain.

The major drawbacks of the pyrrole-based compounds are their limited aqueous solubility and low in vivo potency. The parent compound JG-03-14 had poor aqueous solubility, which necessitated use of DMSO as the solvent for in vivo studies. The aqueous solubility of NT-7-16 has been improved from JG-03-14 and allowed the use of a 70% aqueous vehicle, but is still not optimal. Even though excellent antitumor effects were observed with daily administration of 75 mg/kg, an MTD was never reached due to limited solubility. Multiple colchicine site agents with excellent aqueous solubility, in vivo potency and metabolic stability have been identified (Gangjee et al., 2013; Lu et al., 2014; Zhang et al., 2014) so it is reasonable that newer pyrrole analogs with improved aqueous solubility can be generated. Efforts will also be made to increase the in vivo potency. The metabolic liabilities of the trimethoxyphenyl moieties were identified in other colchicine site binding agents (Li et al., 2011) and medicinal chemistry optimization has improved these liabilities while still retaining potent cytotoxic activities (Lu et al., 2014). Identification of analogs with better aqueous solubility and in vivo potency will be a future priority.

In spite of these limitations, an advantage of NT-7-16 is its low toxicity. In the in vivo trial, NT-7-16 had excellent antitumor effects with no weight loss or other side effects, suggesting that it is less toxic than CA-4, which has an MTD of 500-1,000 mg/kg in a variety of xenograft models (Chaplin and Hill, 2002; Dark et al., 1997; Grosios et al., 1999; Horsman et al., 1998; Nabha et al., 2001). We hypothesize that the optimal fit of NT-7-16 deep within the β-tubulin pocket that allows interactions with both Cys241β and Ala354β might reduce toxicity. This is consistent with clinical trials results of T138067, which also is buried deep in β-tubulin
and binds covalently with Cys241β and had no dose limiting toxicities or neurotoxicity (Berlin et al., 2008; Kirby et al., 2005). The low toxicity of NT-7-16 is also reminiscent of 2-methoxyestradiol, a drug that was advanced into the clinic due to its anti-angiogenic activities. A Clinical trial of 2-methoxyestradiol showed that it was safe and a maximal tolerated dose was not achieved (James et al., 2007); unfortunately it did not have the efficacy necessary to advance to Phase III trials.

A number of other colchicine site agents that rapidly disrupt tumor vasculature have entered clinical evaluation. The failure of these vascular disrupting agent drugs to advance clinically, due in part to toxicity, brings up a question of whether different poses and interactions within the colchicine site might have different effects on tumor vasculature. Recent data suggests that vascular normalization might be a more optimal therapeutic strategy than vascular disruption (Rivera and Bergers, 2015) and thus colchicine site agents without vascular disrupting action might have advantages in clinical trials. When we identify a clinical lead candidate it will be valuable to conduct functional MRI studies to evaluate tumor perfusion.

In summary, these studies demonstrate that modeling of pyrrole compounds within the refined colchicine site can identify optimized colchicine site agents with excellent in vitro and in vivo activities. Further optimization of their medicinal chemical properties will now be required to translate this improved fit within the site into greater potency and efficacy both in vitro and in vivo. We can also not rule out potential metabolic and pharmacokinetic liabilities that will need to be optimized as this class of pyrrole compounds progresses through further lead optimization and preclinical studies.
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Authorship Contributions

 Participated in research design: Rohena, Risinger, Mooberry, Gupton, Telang, Sikorski

 Conducted experiments: Rohena, Risinger, Da, Telang

 Contributed new reagents or analytic tools: Telang

 Performed data analysis: Rohena, Risinger, Mooberry, Da, Kellogg, Gupton

 Wrote or contributed to the writing of the manuscript: Rohena, Risinger, Kellogg, Gupton, Sikorski, Mooberry, Telang
References


Footnotes:

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Legends for Figures

**Fig. 1.** Chemical structures of JG-03-14, NT-7-16 and NT-9-21 (A). The modeling of JG-03-14 (purple) and NT-7-16 (green) within the colchicine site of tubulin. The methoxy group at the 2-position of the phenyl ring in NT-7-16 has opportunities for enhanced hydrophobic interactions with Ala354β that the parent JG-03-14 does not have.

**Fig. 2.** Effects on interphase microtubules. A-10 cells were treated with vehicle (DMSO), 10 nM CA-4, 25 nM NT-7-16 or 1 μM NT-9-21 for 18 hr. Microtubules were visualized by indirect immunofluorescence using a β-tubulin antibody.

**Fig 3.** Effects on mitotic spindles, chromosomes and cell cycle distribution. The effects of NT-7-16 on mitotic structures were evaluated in HeLa cells by indirect immunofluorescence and compared to vehicle treated controls. (A). Microtubule structures are shown in green and DNA in blue. Lagging chromosomes are indicated by arrows. (B) The effects of NT-7-16 and NT-9-21 on cell cycle distribution in HeLa cells were determined by flow cytometry of propidium iodide stained cells and compared to cells treated with vehicle (DMSO) or paclitaxel.

**Fig. 4.** Effects on tubulin polymerization and colchicine displacement. The ability of NT-7-16 to interact directly with tubulin was evaluated. (A) The polymerization of purified porcine brain tubulin was monitored by absorbance at 340 nm after incubation with vehicle (DMSO), 10 μM CA-4, or NT-7-16 at 1 or 5 μM. (B) The effects of NT-7-16 on microtubule structure were evaluated by electron microscopy. Samples were treated similarly as in (A) and microtubules visualized by electron microscopy. Shown are representative images at 2,000 x magnification of
tubulin in the presence of vehicle, 10 µM CA-4, or NT-7-16 at 2.5 or 5 µM. (C) The effects of NT-7-16 on colchicine binding were evaluated fluorometrically. CA-4 was used as a positive control and vinblastine as a negative control. Data is presented as an average of 3 experiments ± SD.

**Fig. 5.** Antitumor effects of NT-7-16. (A) The effects of NT-7-16 on MDA-MB-435 tumors were evaluated in nude mice. The graph shows the average tumor volume ± SD starting on day 0. After the tumors were established, mice were treated daily by i.p. injection of 75 mg/kg NT-7-16. Paclitaxel was dosed at 20 mg/kg twice weekly. (B) Individual tumor volumes on day 14 are presented; the mean is represented by the horizontal line in each panel ± 95% confidence intervals. Statistical analysis was performed using a one way ANOVA with a Tukey posthoc test to compare each condition to every other condition. A significant difference (p = 0.0018) was only found between the control and NT-7-16 groups. (C) Average percent weight change for each treatment group over the course of the trial ± SD. Statistical analysis was performed using a one way ANOVA with a Dunnet’s posthoc test to compare each drug treated condition to the control. A significant difference (p = 0.0014) was only found between the control and NT-7-16 groups.
Table 1. Biological potencies and HINT Scores. The effects of the compounds on the inhibition of cancer cell proliferation were evaluated using the SRB assay in MDA-MB-435 cells (n = 3 ± SD). The EC$_{50}$ values for microtubule loss in A-10 cells were calculated from concentration response curves for cellular microtubule loss (n = 3 ± SD). The HINT scores of the pyrrole compounds were calculated.
Table 2. The ability of compounds to overcome drug resistance mediated by βIII tubulin or P-glycoprotein overexpression was evaluated using pairs of isogenic cell lines. The IC₅₀ values were determined in each cell line using the SRB assay and are expressed (n = 3 ± SD). The relative resistance (Rr) values were determined by dividing the IC₅₀ of the resistant cell line by the IC₅₀ of the parental cell line. Paclitaxel and CA-4 were used as a positive and negative controls, respectively.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa (nM)</th>
<th>HeLa WTβIII (nM)</th>
<th>Rr</th>
<th>SK-OV-3 (nM)</th>
<th>SK-OV-3-MDR-1/M-6-6 (nM)</th>
<th>Rr</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT-7-16</td>
<td>12.9 ± 0.8</td>
<td>15.7 ± 0.2</td>
<td>1.2</td>
<td>15.2 ± 0.8</td>
<td>10.6 ± 0.3</td>
<td>0.70</td>
</tr>
<tr>
<td>NT-9-21</td>
<td>131 ± 8</td>
<td>140 ± 20</td>
<td>1.1</td>
<td>210 ± 70</td>
<td>140 ± 80</td>
<td>0.67</td>
</tr>
<tr>
<td>CA-4</td>
<td>3.3 ± 0.4</td>
<td>3.3 ± 0.3</td>
<td>1.0</td>
<td>3.3 ± 0.3</td>
<td>2.0 ± 0.9</td>
<td>0.61</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>2.8 ± 0.4</td>
<td>24 ± 4</td>
<td>8.6</td>
<td>5.0 ± 0.6</td>
<td>1,120 ± 60</td>
<td>220</td>
</tr>
</tbody>
</table>
Figure A: Absorbance over time for different treatments: Vehicle, 10 μM CA-4, 1 μM NT-7-16, and 5 μM NT-7-16.

Figure B: Microscopy images showing the effects of different treatments on cell morphology:
- Vehicle
- 10 μM CA-4
- 2.5 μM NT-7-16
- 20 μM CA-4

Figure C: Percent Colchicine Binding for different treatments:
- Colchicine
- 20 μM CA-4
- 100 μM Vinblastine
- 2 μM NT-7-16
- 5 μM NT-7-16
- 10 μM NT-7-16
**A**

Average Tumor volume (mm$^3$)

Days

- Control
- Paclitaxel
- NT-7-16

**B**

Day 14

Individual Tumor Volumes (mm$^3$)

- Control
- Paclitaxel
- NT-7-16

- $p = 0.0018$

**C**

Percent Weight Change

Days

- Control
- Paclitaxel
- NT-7-16

- $p = 0.0018$

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