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Characterization and Target Identification of AK301: A Novel Mitotic Arrest Agent

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Characterization and Target Identification of AK301: A Novel Mitotic Arrest Agent

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Honors Thesis

B.S. Molecular and Cell Biology
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Chapter 1: Efficient activation of apoptotic signaling during mitotic arrest with A301

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Abstract

Mitotic inhibitors are widely utilized chemotherapeutic agents that take advantage of mitotic defects in cancer cells. We have identified a novel class of piperazine-based mitotic inhibitors, of which AK301 is the most potent derivative identified to date (EC₅₀ < 200 nM). Colon cancer cells arrested in mitosis with AK301 readily exited mitosis and underwent subsequent p53-dependent apoptosis following compound withdrawal. This apoptotic response was significantly higher for AK301 than for other mitotic inhibitors tested (colchicine, vincristine, and BI 2536). AK301-treated cells exhibited a robust mitosis-associated DNA damage response, including ATM activation, gH2AX phosphorylation and p53 stabilization. The association between mitotic signaling and the DNA damage response was supported by the finding that Aurora B inhibition reduced the level of gH2AX staining. Confocal imaging of AK301-treated cells revealed multiple g-tubulin microtubule organizing centers attached to microtubules, but limited centrosome migration, raising the possibility that aberrant microtubule pulling may
underlie DNA breakage. AK301 selectively targeted APC-mutant colonocytes and promoted TNF-induced apoptosis in p53-mutant colon cancer cells. Our findings indicate that AK301 induces a mitotic arrest state with a highly active DNA damage response. The propensity of AK301 to elicit a DNA damage response, along with the readily reversible nature of the arrested state make AK301 a potent promoter of a mitosis-to-apoptosis transition that can target cancer cells with mitotic defects.

**Introduction**

Mitosis is an intricate process in actively dividing cells, orchestrating a myriad of kinases and signaling pathways. Ascribing to this complexity, mitosis is a particularly sensitive phase of the cell cycle (1). A number of mitotic checkpoints ensure the fidelity of chromosome segregation and cytokinesis; failure of mitotic checkpoints often results in chromosomal alterations, culminating in mitotic catastrophe or cancer-promoting chromosomal instability (2, 3). Cancer cells often lack important cell cycle checkpoints and may execute mitosis with improper spindle assembly (2). Therefore, mitotic inhibitors are among the most widely utilized chemotherapeutic agents in the treatment of a number of malignancies (1). Despite their widespread use, the activity of present mitotic inhibitors is limited by their low activity and associated toxicity. The response of the cancer cells to mitotic inhibitors can be distinctly different with varying magnitudes of effect–some cells remain arrested in mitotic phase, while others exit division and undergo apoptosis (4, 5). How microtubule disrupting agents result in apoptosis and what cellular factors influence the transition of mitotic arrest to apoptosis is not completely understood. Previous studies indicate that activation of the spindle assembly
checkpoint (SAC) during mitosis is necessary for an efficient induction of apoptosis, often through the activation of the tumor suppressor protein p53 (6, 7). It is important to elucidate the signaling pathways that associate aberrant mitosis and apoptosis, and how these pathways are affected by mitotic defects in cancer cells. This information may reveal opportunities that could be exploited for the development of novel therapeutics that target aberrant mitotic regulation in cancer cells.

While screening for molecules that might be able to accentuate colon cancer cell sensitivity to the inflammatory microenvironment of a cancer, we identified a family of small molecule inhibitors that dramatically enhanced colon cancer cell death in the presence of TNF and related death ligands (8, 9). The most potent of these compounds, AK301 had activity in the nanomolar range (EC$_{50} < 200$ nM) (8). Notably, AK301 was found to arrest colon cancer cells in a mitotic state that was acutely sensitive to TNF. Further investigation of AK301 showed a robust activation of apoptosis in a p53-normal colon cancer cell line (HCT116 cells), simply by removing AK301 from the medium and releasing the cells from mitotic arrest. Apoptosis induced by this mitotic arrest-and-release protocol was significantly greater than that induced by other mitotic inhibitors tested. Here we characterize the arrest state induced by AK301 to determine the basis of its relationship with apoptosis. We report that colon cancer cells treated with AK301 arrest at a mitotic state with high levels of ATM activation and p53 stabilization. The stabilization of p53 during mitosis culminates in an apoptotic response in cells following AK301 withdrawal, which releases cells from mitosis given the readily reversible arrest state induced by AK301. We propose that AK301 and its derivatives will be beneficial for probing how apoptotic signaling via the ATM-p53 pathway can be activated during
mitosis. A better understanding of this pathway may ultimately be exploitable for developing novel therapies aimed at treating cancers with mitotic defects.

**Materials and Methods**

**Cell Culture**

HT29 and HCT116 colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). HT29 and HCT116 cell lines were cultured in McCoy’s 5A medium, with 10% fetal bovine serum, non-essential amino acids and antibiotic/antimycotic (Life Technologies, Guilford, CT). Immortalized primary colon cell lines Young Adult Mouse Colonocytes (YAMC) and Immorto-Mouse Colonic Epithelial Cells (IMCE) were a gift from Dr. R Whitehead (Vanderbilt University, Nashville, TN)(10, 11). YAMC and IMCE cells were cultured in RPMI medium containing 5% fetal bovine serum, non-essential amino acids, antibiotic/antimycotic, insulin-transferrin-selenium (Life Technologies), and 5 units of murine gamma interferons. The cells were grown at 33°C. AK301 was synthesized from compounds obtained from the ChemBridge DIVERSet™ library (San Diego, CA). Colchicine, Vincristine, and BI 2536 were obtained from Sigma Aldrich (St. Louis, MO), Acros Organics (Pittsburgh, PA), and SelleckChem Chemicals (Houston, TX), respectively. Drug treatments were performed approximately 24 h after passage for 16 h, unless otherwise indicated. TNF was obtained from Pierce Protein Research Products (Rockford, IL).
**Immunofluorescence microscopy**

Cells cultured on coverslips were fixed with 4% paraformaldehyde at room temperature or 100% ice cold methanol at 4°C and then permeabilized with 0.5% Triton X-100 in PBS. Cells were blocked in 5% serum (in PBS) and then incubated with primary antibody (in 5% serum) on shaker for 1 h at room temperature against phospho-histone H3 Ser 28 (sc-12927, Santa Cruz Biotechnology, Santa Cruz, CA), β-tubulin (E7 monoclonal antibody, Developmental Studies Hybridoma Bank, Iowa), gH2AX (sc-101696, Santa Cruz Biotechnology), g-tubulin (GTU-88, Abcam, Cambridge, Massachusetts), Aurora B (ab2254, Abcam). Aurora A (630938, BD Transduction Laboratories, San Jose, CA). Appropriate secondary antibodies (Molecular Probes, Life Technologies or Jackson ImmunoResearch, West Grove, PA) were used for 45 min incubation. Nuclei were visualized using DAPI (5 µg/ml in PBS; DI306, Life Technologies). Coverslips were mounted on slides using ProLong Gold Antifade Reagent (Life Technologies). Images were acquired using Nikon A1R Confocal Microscope (version 2.11, Nikon Instruments Inc.) and NIS-Elements Advanced Research Software (version 4.13.01, build 916, Nikon Instruments Inc.). Quantification of immunostaining was performed using ImageJ image analysis software (http://rsb.info.nih.gov/ij) as previously described [27]. Following background subtraction and image stacking, both DAPI and immunofluorescence images were merged. Image brightness and contrast were modified with Adobe Photoshop software CC 2014 (Adobe Systems).
**Flow cytometry and cell cycle analysis**

Cells were stained for γH2AX using the protocol described above for immunofluorescence staining. Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5% donkey serum. Cells were then incubated with γH2AX antibody (sc-101696, Santa Cruz Biotechnology) followed by incubation with Alexa Fluor® 488 secondary antibody (Life Technologies). Cells were then harvested using trypsin-EDTA for 15 min at 37°C and washed once with PBS. Propidium iodide (30 µg/ml) was added to the cells prior to filtration through 35 µm cell strainer tubes. Cells were promptly analyzed by flow cytometry.

For cell cycle analyses, cells were analyzed for DNA content by ethanol fixation and staining with propidium iodide as previously described (8). Cells were harvested using trypsin-EDTA, centrifuged at 1000 X g for 10 min and resuspended in 500 µl of cold saline GM. Cells were washed once with 1X PBS and then fixed for at least 2 hrs at -20°C in 3X volumes of cold 100% ethanol while vortexing. Cells were then pelleted and washed once with PBS containing 5 mM EDTA. Pelleted cells were stained with 30 µg/ml propidium iodide (Molecular Probes, Life Technologies Corp.) and 0.3 mg/ml RNase A (Sigma-Aldrich, St. Louis, MO) in 500 µl PBS solution for 40 min in the dark at RT. The stained cells were filtered through 35 µm cell strainer tubes (BD Biosciences, San Jose, CA). All flow cytometric analyses were performed on FACSCalibur (BD Biosciences) using Cell Quest software (BD Biosciences). The data were analyzed using FlowJo (v10, TreeStar Inc., Ashland, OR).
**Caspase-3 assay**

Caspase-3 activity was determined as previously described (9). Cells were collected, centrifuged at full speed, and washed once with PBS. Pelleted cells were lysed by two rounds of freeze-thaw in lysis buffer containing 10 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 0.01% Triton X-100 and centrifuged at 10,000 X g for 5 min. The assays were performed on 96 well plate by mixing 50 µl of lysis supernatant with 50 µl of 2X reaction mix (10 mM PIPES pH 7.4, 2 mM EDTA, 0.1% CHAPS, 10 mM DTT) containing 200 nM of the fluorogenic substrate Acetyl-Asp-Glu-Val-Asp-7-Amino-4-methylcoumarin (DEVD-AMC; Enzo Life Sciences). The fluorescence was quantified at the start of the reaction and after 30 min. Protein concentrations were determined using CBQCA Protein Quantitation Kit (Life Technologies). Caspase activity was determined by dividing the change in fluorescence by total protein content of the reaction mixture.

**Western blot**

RIPA buffer was used for total protein extraction. 20 µg of protein was denatured under reducing conditions and separated on 10% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose by voltage gradient transfer. The resulting blots were blocked with 5% (w/v) non-fat dry milk in PBS + 0.1% (v/v) Tween-20. Specific proteins were detected with appropriate antibodies using SignalFire™ Elite ECL Reagent (Cell Signaling Technology). Immunoblotting antibodies were p53 (OP03, Calbiochem, Massachusetts), p-p53 (9284, Cell Signaling Technology, Massachusetts), ATM (2873, Cell Signaling Technology), and p-ATM Ser1981 (13050, Cell Signaling Technology), p21 (C-19, Santa Cruz Biotechnology), Bax (P-19, Santa Cruz
Biotechnology), Bak (G-23, Santa Cruz Biotechnology), Mdm2 (OP115, Calbiochem), β-actin (I-19, Santa Cruz Biotechnology).

**Statistical analyses**

One-way analysis of variance (ANOVA) was used when comparing two groups with Tukey’s post hoc test. For more than two groups, two-way ANOVA was used with Bonferroni correction for multiple comparisons. Significance was calculated at an alpha of 0.05.

**Results**

**AK301-arrested cells show increased caspase-3 activity**

We were interested in determining how AK301 compared to other mitotic arrest agents with regard to its ability to activate apoptotic signaling. Therefore, we tested a collection of antimitotic agents, including microtubule inhibitors (colchicine and vincristine) and a PLK1 inhibitor (BI2536)(12). Previous work in our lab showed that these compounds could all induce maximal G2/M arrest at concentrations of 250 nM and higher (9, 13). As shown in Figure 1A, flow cytometric analysis of HCT116 treated with either 250 nM or 500 nM of these agents induced a G2/M arrest in over 80% of the cells (P < 0.0001). To examine the relationship between induced mitotic arrest and apoptotic signaling, we tested these agents for their ability to induce capase-3 activation using a DEVD-AMC fluorogenic substrate at 500 nM. As shown in Figure 1B, of the four mitosis-arresting agents, AK301 induced the highest levels of caspase-3 activity (P <
0.0001). Caspase-3 activity suggested a higher apoptotic potential of AK301 relative to the other arrest agents.

**AK301 withdrawal enhances apoptotic response in HCT116 cells**

Since cells may not undergo apoptosis while arrested in mitosis, we withdrew AK301 from arrested HCT116 cells in culture and monitored their progression through the cell cycle. Flow cytometric analysis showed that ~85% of cells are in G1 prior to treatment, with very little sub-G1 cells (Figure 1C, left-most panel). After treatment with AK301 overnight, ~80% of cells were arrested in G2/M as previously reported (8). However, significant increase in sub-G1 cells was observed following AK301 withdrawal. Accumulation of sub-G1 cells appeared as early as 3 hours and progressed through 24 hours (Figure 1C, bottom panels). Cells maintained in AK301 showed persistent arrest with low levels of apoptosis during the 24 hour period (Figure 1C, top panels). We compared the apoptotic effect of AK301 to that of colchicine. HCT116 cells were treated with AK301 or colchicine to induce arrest and were then analyzed post compound withdrawal. As shown in Figure 2, cells subjected to the AK301-arrest and release protocol showed a larger apoptotic sub-G1 population than cells released from a colchicine treatment. Colchicine-treated cells remained arrested in G2/M up to 24 hours post treatment. In addition, some AK301-treated cells underwent cell division (increased proportion of G1 cells from 3 to 24 hours), indicating that mitotic arrest by AK301 is more reversible than arrest induced by colchicine. Figure 3A compares the apoptotic-inducing ability of AK301 to the mitotic inhibitors colchicine, vincristine, and
BI2536 in the arrest-and-withdrawal procedure. AK301 was found to be significantly more potent than these other agents.

**The role of p53 in AK301-induced apoptosis**

To determine whether AK301-induced apoptosis was p53-dependent, we compared the effect of AK301 on caspase-3 activation in p53-normal and mutant cells. As shown in Figure 3B, AK301 caused an increase in caspase-3 activation in p53-normal HCT116 cells, but not in p53-null HCT116 cells. Likewise, p53-normal HCT116 cells underwent a higher level of apoptosis than their p53-mutant counterparts following AK301 withdrawal (Figure 3C). Finally, western blot analysis showed that p53 was stabilized by AK301 treatment (Figure 3D). To assess the mechanism of p53 activation, the level of p53 phosphorylation at the ATM/ATR target residue serine-15 was determined (15-17). We found that AK301 treatment increased phosphorylation at this site (Figure 3D). In addition, ATM was phosphorylated in the presence of AK301 at its autophosphorylation site, serine-1981 (18). Together these data indicate that AK301 activates p53 through a DNA damage response mechanism. To determine if there was an activation of p53-target genes involved in apoptosis and growth arrest, a collection of potential target genes were assayed by western blotting (Figure 3E). These data show that Bax, p21 and Mdm2 (but not Bak) were activate by AK301.

**AK301-induced DNA breakage during mitotic arrest**

To further examine the ability of AK301 to induce a DNA damage response, we measured the level of gH2AX phosphorylation (19, 20). Figure 4A shows a flow
cytometry analysis of gH2AX and PI staining of HCT116 cells treated with AK301, colchicine, vincristine or BI 2536. As previously reported, gH2AX staining is higher in G2/M cells than G1 cells (21, 22). However, gH2AX staining was significantly higher in the AK301 treated cells (32% of AK301-treated cells showed increased gH2AX staining compared to ≈1% with other mitotic inhibitors)(Figure 4B). Analysis of p53-normal and p53-null cells showed a similar level of gH2AX staining both before and after AK301 treatment. This is consistent with DNA damage occurring prior to p53 activation, and not as a result of p53 (Figures 5A and 5B)(23).

To assess the relationship between mitotic arrest and the DNA damage response, we determined the effect of the Aurora B inhibitor AZD1152HQPA on gH2AX levels (24, 25). This inhibitor was chosen since it can reduce histone H3 phosphorylation in mitotically arrested cells and promote mitotic chromatin decondensation. As shown in Figure 5C, treatment of cells with AZD1152HQPA decreased histone H3 phosphorylation and gH2AX staining with a similar dose-dependency, consistent with increased gH2AX being linked with the AK301-induced mitotic arrest state. Potential mechanisms that may link mitotic arrest and the DNA damage response are discussed below.

To further confirm the relationship between gH2AX and mitotic arrest, and to define the features of the AK301-induced mitotic arrest state associated with activation of a DNA damage response, AK301 arrested cells were analyzed by immunofluorescent staining and confocal microscopy. Figure 6A shows an immunofluorescent analysis of gH2AX and g-tubulin in control and AK301 arrested cells. AK301-arrested cells displaying the highest level of gH2AX staining showed g-tubulin clustered amongst the
condensed mitotic chromosomes. A representative cell showing this feature is indicated by a white arrow in the right-most panel of Figure 6A. A second g-tubulin foci is also observed in the AK301 arrested cells (arrowhead in Figure 6A). This second foci colocalizes with other centrosome-associated proteins. gH2AX-positive mitotic cells with these features also appear in the control culture, albeit at a much lower frequency (Figure 6A, second panel, white arrowhead). These findings indicate that AK301 arrests cells in a mitotic state that features an active DNA damage response and that cells in this condition occasionally arise in untreated cultures. These data indicate that cells arrested in a mitotic state with the highest level of gH2AX staining have two distinct g-tubulin foci: centrosome-associated and centrosome-independent foci.

Figure 6B shows Aurora B and microtubule staining in AK301 arrested cells. The arrested cells were found to express elevated levels of chromatin-associated Aurora B. Although the microtubule network in AK301 arrested cells is largely disrupted, short microtubules can be observed in close proximity to the Aurora B foci. The close proximity of the proteins suggests an interaction between the Aurora B/kinetochore complex and microtubules (26). This finding is consistent with microtubule attachments to mitotic chromosomes in AK301-treated cells. The elevated level of Aurora B expression in AK301-treated cells is consistent with reports showing that this kinase can contribute to ATM activation during mitosis (22).

Finally, a TUNEL stain was performed to see if the gH2AX staining was associated with detectable strand breakage. As shown in Figure 7A, TUNEL-positive cells are found in the AK301-treated cultures.
AK301 sensitivity of p53 mutant colon cancer cells

To examine the generality of the effect of AK301 on colon cancer cells, we tested its effects on the HT29 colon cancer cell line. gH2AX and phospho-histone H3 staining of HT29 cells showed an overlap after AK301 treatment consistent with a DNA damage response in these cells during mitosis (Figure 7B). However, since HT29 cells are p53 mutant, they did not undergo apoptosis following the AK301 treatment-and-release protocol. AK301 did, however, increase the sensitivity of HT29 cells to TNF-induced apoptosis. As shown in Figure 7C, neither TNF nor AK301 alone promoted the formation of sub-diploid apoptotic bodies, whereas a 24 hour co-treatment did (Figure 7C)(8). Since apoptosis induced by TNF and AK301 is relatively slow (requiring 16-24 hours), and our findings above indicate that cells must exit an AK301 arrest before they undergo apoptosis, we tested whether cells induced to exit mitotic arrest might have a higher TNF sensitivity. HT29 cells have a strong mitotic checkpoint and remain in mitotic arrest even after AK301 withdrawal. Therefore, we utilized the MPS1 inhibitor SP600125 to release them from arrest. MPS1 is an integral component of the SAC and its inhibition has been shown to release mitotically arrested U2OS cells (27). As shown in Figure 8, AK301-arrested HT29 cells treated with SP600125 readily exit mitotic arrest (27, 28). Cells released from mitotic arrest were more sensitive to subsequent TNF treatment than cells that remained arrested (Figure 8). Consistent with our previous results showing a requirement for mitotic release before apoptosis, colchicine arrested cells were neither released from arrest by SP600125 nor were they sensitive to TNF.
AK301 sensitivity of APC mutant colonocytes

The APC protein is involved in microtubule elongation and mitotic spindle assembly. AK301 targets these processes. Therefore, we tested the sensitivity of APC-normal and APC-mutant mouse colonocyte cell lines to AK301 (YAMCs and IMCEs, respectively)\(^{(10, 11)}\). Release from AK301 arrest by compound withdrawal resulted in a significantly higher level of apoptosis in APC-mutant IMCE cells compared to APC-normal YAMCs (Figure 9A). Interestingly, titration of AK301 on these two cell lines showed that apoptosis occurred at compound concentrations lower than those required to induce optimal mitotic arrest. In addition, cell death could occur without compound removal (Figure 9B). Moreover, this sensitivity was more pronounced in the APC-mutant cell line. Since apoptosis at the lower AK301 concentrations may have resulted from a disruption in mitotic progression, we analyzed the structural features of APC-normal and mutant cells to AK301. We tested a number of antibodies and found that staining for total Aurora A, which associates with the centrosome and mitotic spindle, showed a higher degree of disruption in APC-mutant cells. In untreated cells, Aurora A interacted with the centrosome and the mitotic spindle, regardless of APC status (Figure 9C top panels)\(^{(29)}\). Following AK301 treatment, Aurora A interaction was discreetly localized to centrosomes in APC-normal cells. However, in APC mutant cells Aurora A was dispersed into multiple, disorganized foci (Figure 9C, bottom panels). These data suggest that a more severe mitotic disruption in APC-mutant cells underlies their increased sensitivity to apoptosis.
Discussion

We previously reported the identification of a family of small molecules that induce mitotic arrest in colon cancer cells and increase their sensitivity to TNF and other death ligands (8, 9). In this study, we show that the most potent of these compounds, AK301, is also effective at inducing a mitosis-to-apoptosis transition in the absence of a death ligand in p53-normal colon cancer cells. In this instance, apoptosis can be induced by treating cells with AK301 to induce arrest, and then withdrawing the compound to release cells from arrest and into apoptosis. AK301 appears to function by arresting cells in a mitotic state in which a DNA damage response is activated and p53 is stabilized. Compound withdrawal then allows progression to apoptosis, which likely entails the activation of p53-target genes following the decondensation of mitotic chromatin (14). Although cells arrested in mitosis by other agents have been reported to activate ATM and components of the DNA damage response pathway, AK301 arrests cells in a state in which this response is especially robust (21). In addition, mitotic arrest by AK301 is readily reversible, which facilitates the transition to apoptosis following AK301 withdrawal. The apoptotic signaling pathway activated by AK301 may be exploitable for cancer treatment, particularly for cancer cells with defects in the mitotic apparatus and mitotic checkpoints.

To better understand the relationship between mitotic arrest and the DNA damage response, we analyzed the arrest state generated by AK301. AK301-arrested cells exhibiting high levels of gH2AX displayed condensed chromatin adjacent to a central cluster of g-tubulin. However, this g-tubulin was not centrosome-associated. Instead, the centrosomes (and their associated g-tubulin) remained at the cell periphery
with minimal migration to the mitotic poles. Cells in this state are common in AK301 cultures but also appear in untreated cultures, albeit at much lower frequency, indicating that cells encounter this type of arrest during a normal cell division. How the DNA is broken under these conditions is not clear. One possibility is that g-tubulin-seeds the formation of aberrant spindles that pull the chromosomes to induce breakage. Consistent with this possibility, we find microtubules in close association with the Aurora B passenger protein in AK301-arrested cells. Aurora B’s proximity to microtubules indicates that microtubule attachment to the kinetochore is being established (26). The relationship between microtubule attachment and gH2AX staining is also supported by the finding that colchicine-treated cells have completely disassembled microtubules and lower levels of gH2AX. Although microtubule pulling is one possible mechanism for ATM activation, Aurora B can also directly activate ATM in the absence of DNA breakage (22). Since AK301 arrested cells show elevated levels of Aurora B, it is possible that direct activation of ATM by chronic Aurora B activity may be occurring, thereby accentuating the DNA damage response. Our finding that the Aurora B inhibitor AZD1152-HQPA can reduce gH2AX levels in AK301-treated cells is consistent with this possibility. Understanding how the DNA damage response is optimally activated during mitotic arrest could provide insight into how this event might be best targeted to cancer cells with mitotic defects.

AK301 was originally identified by virtue of its ability to arrest cells in a mitotic state that is highly sensitive to the apoptotic actions of TNF (8, 9). The basis of this sensitivity was determined to be an increased coupling between TNFR1 and capase-8 activation (8). Based on our findings here, and reports by other groups, the increased
caspase-8 activation by TNF in the presence of AK301 may be mediated in part by ATM activation; siRNA knock down of ATM has been reported to reduce TNF-induced caspase-8 activation in HeLa cells (30). The synergistic action of the TNF and AK301 may also result in part from the ability of TNF to promote cells to exit mitotic arrest and enter apoptosis. Caspase activation by death ligands has been reported to promote degradation of the spindle checkpoint proteins, which releases cells from mitotic arrest and allows them to enter apoptosis (31). We find that cells arrested by AK301 must first exit mitosis before they can enter apoptosis and TNF may facilitate this exit from mitotic arrest.

Our findings here suggest that the impact of AK301 on cells is complex and depends on a range of genetic and tissue-specific factors. For instance, cells with a normal p53 gene but a weak spindle assembly checkpoint may be more directly sensitive to AK301. In addition, our analysis indicates that cells carrying an APC mutation are more sensitive to AK301, compared to p53-mutant cells with a robust SAC that may require a death ligand to undergo apoptosis. APC mutations frequently occur at an early stage of colon cancer development and are well documented to increase Wnt signaling by increasing b-catenin stability (32-34). However, the APC protein is also known to facilitate spindle assembly during mitosis by stabilizing microtubule plus (growth) ends (35-39). C-terminal truncating mutations of APC, most commonly found in colon cancers, act in a dominant negative fashion to disrupt spindle assembly (40, 41). Since AK301 also affects microtubules, we tested the sensitivity of APC-normal and mutant colonocytes to AK301 and found that the mutant cells were more sensitive. The basis of this higher sensitivity is not entirely clear, but may result from a more severe
disruption of mitotic events resulting from the dual targeting of the mitotic spindle. Consistent with this possibility, we observed a more severe disruption of centrosome regulation and localization (using total Aurora A staining) in APC-mutant cells treated with AK301. How this disruption is translated into increased apoptosis in these and other APC-mutant cells is presently under study.

Although microtubule and mitosis targeting chemotherapies are not typically used to treat colon cancer, our findings here, and reports by other groups, suggest that these therapies may be advantageous in some cases (42-44). Colon cancers with microsatellite instability are usually p53-normal and have a defective CHFR mitotic checkpoint. Therefore, these cancers are interesting targets for AK301 (and similarly acting agents), particularly if they also have an APC mutation. Although colon cancers with this combination of defects represent only a subset of all colon cancers, the specific targeting of this type of cancer may provide an avenue for patient stratification. Moreover, microsatellite unstable colon cancers are particularly interesting since they respond poorly to present 5-fluorouracil-based therapies (45, 46). Understanding how AK301 disrupts cellular components to induce a reversible mitotic arrest state that includes elevated levels of ATM signaling and p53 stabilization could provide valuable information into how cancers with the appropriate vulnerability might best be targeted.
Figures and Figure legends

**Figure 1.**

A) G2/M arrest in HCT116 colon cancer cells. HCT116 cells were treated with the indicated concentrations of AK301, colchicine or vincristine (microtubule inhibitors), or BI2536 (a PLK1 inhibitor) for 16 hours. Cells were then fixed and stained with propidium iodide (PI), and analyzed by flow cytometry. All four drugs induced high levels of G2/M arrest at both concentrations (P < 0.0001) with no significant differences between the compounds.

B) HCT116 cells were treated with 500 nM of each of the indicated compounds for 16 h. Cell lysates were prepared and tested for caspase-3 activity using DEVD-AMC fluorogenic assay. AK301 (**P < 0.0001) and BI2536 (*P <
0.05) induced significantly higher levels of caspase-3 activation relative to control cells. 

**C** Apoptosis in AK301 treated cells released from arrest. The left-most panel shows the cell cycle distribution of HCT116 cells under normal growth conditions. In the remaining panels, HCT116 cells were treated with 500 nM of AK301 for 16 hours. AK301 was then removed and cells were allowed to grow in fresh medium for 3, 6, 12, and 24 hours (bottom panel) or returned to AK301-containing medium (top panel). Cells were harvested at the indicated times following the medium change. Cells maintained in AK301 show a relatively stable G2/M arrest, whereas those switched to new medium showed increasing levels of sub-G1 apoptotic cells.
Figure 2. AK301 withdrawal induces more apoptosis than colchicine withdrawal. HCT116 cells were treated with 500nM of AK301 or colchicine for 16 hours, as indicated. Cells were then switched to fresh growth medium for the indicated lengths of time. Flow cytometric analysis of DNA content showed that both AK301 and colchicine arrested HCT116 cells in G2/M phase of the cell cycle. However, upon drug withdrawal, cells arrested with AK301 showed the formation of more sub-diploid cells than those released from colchicine arrest.
Figure 3. A) Apoptosis of cells exiting mitotic arrest. HCT116 cells treated with 500 nM of each of the indicated compounds for 16 hours. Cells where then either switched to drug-free medium for 8 hours, or treated with fresh drug-containing media. Flow cytometric analysis of the DNA content showed that AK301 treated cells showed a significantly higher levels of apoptosis after release than cells treated with the other compounds (*P < 0.0001). B) Wild type and p53-null HCT116 cells were treated with 500 nM of AK301 for 16 hours. Cell lysates were prepared and tested for caspase-3 activity using DEVD-AMC fluorogenic assay. The p53-normal HCT116 cells showed more caspase-3 activation than the null cells (*P < 0.001). C) Wild type and p53-null HCT116 cells were treated and released with AK301 as described in 3A. Cells were
then processed for flow cytometric analysis. Apoptosis was significantly higher in p53-normal HCT116 cells (*P<0.001). D) ATM activation and p53 stabilization following AK301 treatment. HCT116 cells were treated with 500 nM AK301 for 16 hours, followed by transfer into fresh medium for 0, 4, or 6 hours. Protein was then extracted for analysis. Immunoblot analysis shows phosphorylation of ATM at Ser1981 and phosphorylation and stabilization of p53 (p-p53 Ser15) in treated and released cells. Increased MDM2 expression was observed after AK301 treatment and release, which is consistent with a release from arrest being required for gene activation by p53. β-actin was used as a loading control.
Figure 4. A) γH2AX levels in response to treatment with mitotic arrest agents. HCT116 cells were treated for 16 hours with AK301, colchicine, vincristine, or BI2536 at 500 nM. Treated cells were analyzed for γH2AX immunofluorescent staining (Y-axis) and DNA content/PI staining (X-axis) by flow cytometry. B) Quantification of γH2AX staining in mitotically arrested cells. Using the gates indicated in 4A, the percentage of cells entering quadrant 2 (Q2) was calculated and compared for the arrest agents shown. AK301-treated HCT116 cells showed a significantly greater proportion of cells with γH2AX activation (*P < 0.0001).
Figure 5. A) γH2AX levels in p53-normal and p53-null HCT116 cells treated with AK301. Cells were treated with 500 nM AK301 for 16 hours. Cells were then analyzed for gH2AX immunofluorescent staining (Y-axis) and DNA content/PI staining (X-axis). B) Quantification of γH2AX staining in p53-normal and mutant cells mitotically arrested with AK301. Using the gates indicated in 5A, the percentage of cells entering quadrant 2 (Q2) was calculated and compared. AK301-treated HCT116 cells showed a significantly greater proportion of cells with γH2AX activation (*P < 0.0001) but no significant differences between the wild type and null cells. C) Aurora B inhibitor-induced reduction
in γH2AX. HCT116 cells were arrested with AK301 and then treated with the indicated concentrations of the Aurora B inhibitor AZD1152-HQPA for 1 hour. Immunofluorescent images of γH2AX and phospho-histone H3 staining were then captured and quantified. The Aurora B inhibitor induced a reduction in both phospho-histone H3 Ser 28 (a direct Aurora B target) and γH2AX (*P<0.01).
**Figure 6.** HCT116 cells were examined by immunofluorescence confocal microscopy. Cells were treated with 500 nM AK301 for 16 hours, and then processed for γH2AX and γ-tubulin staining (**A**) or Aurora B and b-tubulin staining (**B**). The color key and 20 nm bars are shown. The arrow and the arrowheads indicate structures referred to in the text. **C**) TUNEL staining shows DNA breakage in AK301-treated cells. HCT116 cells were treated with 500 nM AK301 for 16 hours, and then processed for TUNEL staining. Images of representative field is shown with a 20 mm bar. End-labeled DNA is shown in red and DAPI-stained DNA is blue.
Figure 7. A) γH2AX levels in HT29 colon cancer cells following AK301 treatment. HT29 cells were treated with 500 nM AK301 for 16 hours. Treated cells were then analyzed for γH2AX (red) and phospho-histone H3 Ser28 (green) by immunofluorescent staining and confocal microscopy. DAPI-stained DNA is in blue. Two representative images from both control and AK301 treated cultures are shown. A 20 mm bar is shown in the left panel. B) Cell cycle analysis of HT29 cells treated with AK301 in the presence or absence of TNF. HT29 cells were treated with AK301 (500 nM) and TNF (50 ng/ml) as indicated for 24 hours. Cells were then fixed and stained with PI for cell cycle analysis by flow cytometry.
Figure 8. Enhanced TNF sensitivity of AK301-treated HT29 cells after release from mitotic arrest. HT29 cells were arrested in mitosis with AK301 or colchicine. Arresting agents were removed and cells were released from arrest by treatment with the MPS1 inhibitor SP600125 for 2 hours as indicated. Cells were then treated with TNF (as indicated) for 4 additional hours. Under these conditions, AK301-treated cells released from mitotic arrest are the most sensitive to TNF-induced apoptosis as determined by sub-diploid formation.
Figure 9. Influence of APC mutation on AK301 sensitivity. A) Mouse colonocyte cell lines that are APC-normal (YAMC) or APC heterozygous with a Min mutation (IMCE cells) were treated with 500 nM AK301 for 16 hours. Cells were then released from arrest by medium replacement and analyzed by flow cytometry at the indicated time points. APC-mutant IMCE cells underwent apoptosis more readily than wild type cells following release from arrest (*P>0.0001). B) Titration of AK301 on APC-normal and heterozygous mutant colonocytes. YAMC and IMCE cells were treated with the indicated concentrations of AK301 for 16 hours and then assessed for sub-diploid
formation by flow cytometry and for mitotic arrest by the phospho-histone H3 staining. Significantly higher levels of apoptosis were observed for IMCE cells at AK301 concentrations from 75-125 nM. C) Comparison of Aurora A localization in YAMC and IMCE cells treated with 100 nM AK301. Cells were immunostained for Aurora A (green) with nuclei counterstained with DAPI (blue). Untreated YAMCs and IMCE cells show a normal bipolar localization of Aurora A to the centrosome and spindle in mitotic cells. AK301 treatment of YAMCs restricted Aurora A association with the centrosome, whereas treatment of IMCE cells induced the formation of multiple diffuse Aurora A foci.
Chapter 2: Structural Activity Relationship Study, Molecular Probe Synthesis and Target Identification of AK301

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Abstract

The Giardina Laboratory has identified [(4-(3-chlorophenyl)piperazin-1-yl)2-ethoxyphenyl)methanone], named AK301, as a novel mitotic inhibitor [9]. Previously our lab has shown that colon cancer cells treated with AK301 arrest at the G2/M phase of the cell cycle with characteristic multipolar spindle (8). The arrest state of AK301 has been found to be readily reversible upon compound withdrawal, with cells efficiently entering apoptosis after release from mitotic arrest (47). This release effect has been shown to be unique to AK301 when compared to conventional spindle poisons like colchicine and vincristine, which arrest cells in mitosis irreversibly and induce lower levels of apoptosis (47). Therefore, we hypothesized that AK301 may be modulating cellular proteins other than tubulin. To test this hypothesis we developed several molecular probes. A structural activity relationship (SAR) study was performed to
increase AK301’s potency in hope of offsetting the loss in activity that normally accompanies probe development. We used the most potent compound, MJB6, as the scaffold for a biotinylated derivative. Affinity chromatography followed by mass spectrometry identified clathrin heavy chain (CHC) as a potential target. A western blot of elution fractions and an endocytosis assay provided additional evidence for clathrin being the target of the AK301/MJB6 family of compounds. AK301 compounds were also found to disrupt clathrin localization in vitro. Our data in this study suggests that AK301 induces mitotic arrest by inhibiting the actions of clathrin, which would make it the third known clathrin inhibitor identified. MJB6 (IC$_{50}$ = 75nM) is markedly more potent than the other known inhibitors like Pitstop 2 (IC$_{50}$ = 12µM) (48,49). Overall, our findings suggest that interfering with the mitotic functions of clathrin arrests cells in a mitotic state that is prone to undergo apoptosis. Continued work on this compound could illuminate a novel apoptotic signaling pathway that may be ultimately be targeted for cancer therapy.

Introduction

As biochemical assaying techniques have become more sophisticated, the paradigm in drug discovery has shifted. In the formative years of small molecule inhibitor research, scientists took a target-based approach to therapeutic discovery. This method, now called reverse chemical genetics, relies upon identifying proteins, usually enzymes, known to be associated with disease states (50, 51). Compounds are then screened for activity against purified extracts of the target protein. This is typically done by monitoring the progress of a reaction catalyzed by the enzyme. Early chemotherapeutics were identified in this way. Methotrexate, initially called
amethopterin, was discovered as a treatment for solid tumors and acute lymphoblastic leukemia by Dr. Roy Hertz and Dr. Sidney Farber respectively (52, 53). Both men tested the compound in patients afflicted by cancer because of reverse chemical genetic thinking. Methotrexate was known to be an inhibitor of the dihydrofolate reductase (DHFR) enzyme (54). DHFR’s main substrate is folic acid. Also called vitamin B9, folic acid is necessary for proper DNA synthesis (55). Hertz and Farber hypothesized that since cancer is caused by unregulated growth, inhibiting DHFR would disrupt DNA synthesis, preventing growth of cancer cells (52, 53). Their hypothesis was correct, as methotrexate remains a first line chemotherapeutic for non-metastatic solid cancers. Hertz and Farber were assisted in their discovery by previous study that showed methotrexate could inhibit DHFR (56). However, in most target-based discovery efforts, inhibitors for the proteins are not known. Instead a library of compounds is screened for activity against purified protein. Active compounds are then tested in cells and/or model organisms (50, 51). A major problem with this type of screening is that activity against a purified protein does not always correlate to activity in an in vitro or in vivo system due to pharmacokinetic issues like solubility and absorption.

One way around this problem is to test small molecules on living cells and assay for a desired phenotypic response like mitotic arrest or cell death. This method of drug discovery, known as phenotype-based discovery or forward chemical genetics, maintains the cellular environment of target proteins (57, 58). Thus, it has a higher predictive power for compounds that will be effective in vivo (59). Phenotype-based identification has been made possible through advances in assay technologies like flow cytometry and cell imaging. These techniques allow scientist to better analyze
phenotypic changes in cells (60). Phenotypic screens have an advantage over target-based approaches because growing cells in culture is generally easier and less expensive than purifying proteins (61). However, forward chemical genetics has its own set of issues. The main problem with this process is protein target identification (62). While there may be proteins known to be involved in the phenotypic effect observed, it is impossible to know definitively what protein is being modulated based on screening data alone (63, 64).

Due to the relative ease of designing phenotypic screens and identifying lead compounds there has been much interest in developing techniques to identify protein targets. This work lies at the crossroads of chemistry and biology, a field now known as chemical biology. Over the years chemical biologists have devised several systems to identify drug targets. A classic example, which was employed in this work, is biotin-avidin affinity chromatography (65). This method takes advantage of the extremely strong interaction between biotin, also known as vitamin B7, and avidin, a protein found in egg white (66). Biotin can be conjugated to small molecules, typically through amide linkages (65, 67). The biotinylated compound is then mixed with a cell lysate. Target proteins bind to the small molecule and the entire biotin-small molecule-target protein complex is fished out using an avidin column that binds the biotin (65, 67). Proteins are eluted by either competing them off the column with unbiotinylated compound or changing the chemical environment, such as dropping the pH to 2. SDS-PAGE is then used to analyze elution fractions. Proteins can be excised from the gel and identified using mass spectrometry (67). Additional biochemical analysis is then performed to validate the targets.
In recent years other techniques have been developed. Photochemistry and cycloadditions are two hot areas of target identification development (68-70). Photo-reactive probes can be used to covalently bind small molecules to their target. This ensures that all proteins the small molecule binds to, both specific and non-specific, are analyzed (68-70). To identify target proteins a reporter moiety, like a terminal alkyne, can be attached to the photo reactive compound. Azides conjugated to fluorophores can be reacted with terminal alkynes in a copper catalyzed Huisgen cycloaddition, commonly called “click chemistry” (70, 71). In a typical experiment, the photo reactive compound containing a terminal alkyne is mixed with a cell lysate. The lysate is exposed to UV light that radicalizes the photo-reactive element. This radical irreversible binds to bound proteins. The proteins can then be separated by 1D or 2D gel electrophoresis. An azide conjugated to a fluorophore will then be washed over the blot along with the components necessary for the click reaction. The resulting fluorescent protein bands can be excised and sent to mass spectrometry as in biotin affinity chromatography (70).

Affinity chromatography performed in this work using MJB6-linked to biotin provided evidence for the binding of MJB6 to clathrin. Most widely known for its role in endocytosis, clathrin is a protein that has recently been considered as a target for cancer therapeutics due to its role in mitosis (49, 72). Whether clathrin is a good therapeutic target or not remains to be determined. But it is clear that the MJB6 compound has illuminated the role of clathrin in apoptosis regulation and could serve to provide new insight into the link between mitotic aberrations and cell death signaling.
During endocytosis clathrin serves to stabilize membrane curvature in budding endocytic pits (73). Clathrin triskelia assemble into polyhedral cages via their clathrin heavy chain repeat domains. The cage surrounds nascent invaginations and may promote further deformation of the budding membrane (74). Once the pit has matured dynamin, a mechanochemical enzyme, is recruited and is responsible for both the scission of the vesicle and resealing of the vesicle membrane after detachment from the cellular membrane (75, 76). Upon vesicle scission from the plasma membrane, the clathrin coat is disassembled by the ATPase heat shock cognate 70 (HSC70) and a cofactor, GAK or auxilin (77, 78). Since the budding vesicle is attached to the cellular membrane, the clathrin coat is unable to enclose the entire vesicle (79, 80). The opening in the cage structure that results following vesicle membrane scission serves as a docking site for auxilin (81, 82). The cofactor binds to the terminal domains and ankles of the exposed triskelia where it then recruits up to three HSC70 complexes to disassemble the clathrin cage (83-85). Clathrin uncoating is necessary as it allows for the nascent vesicle to fuse with target organelles or endosomes. Once the cage is disassembled triskelia are recycled and recruited to new sites of invagination.

Clathrin has many cellular roles beyond endocytosis. These include endosomal sorting via adaptor proteins, secretion of cargo from the trans-Golgi network, and stabilization of the Golgi apparatus (86, 87). However, its most interesting function from a cancer biology perspective is its role in mitotic spindle stabilization, since spindle assembly is necessary for proper cell division (88). Improper attachment of microtubules to chromosome kinetochores or uneven tension on MTOCs results in mitotic arrest. During mitosis clathrin localizes along spindle microtubules (88). Together with TACC3,
clathrin stabilizes the spindles that grow from kinetochores, called “K-fibers,” allowing them to be captured by the microtubules that emanate from the centrosomes (89). TACC3 is a protein commonly upregulated in cancers. This protein is essential for proper spindle formation through poorly understood interactions with microtubules, clathrin, and the tubulin polymerase, ch-TOG (89). Aurora Kinase A (AKA) activates TACC3. Only after phosphorylation on S558 can TACC3 complex with ch-TOG and clathrin to stabilize K fibers (90). Recent studies have found that clathrin and TACC3 have two different sites of interaction. Clathrin’s ankle and β-propeller domain are able to interact with TACC3’s CID and TACC domain after phosphorylation of TACC3 by AKA (89). The third player in the complex, ch-TOG is a microtubule-associated protein responsible for tubulin polymerization, stabilization, and depolymerization at different stages of the cell cycle. It is responsible for carrying clathrin to the mitotic spindle. The protein’s interaction with clathrin is necessary for clathrin to bind TACC3 (91). Both TACC3 and clathrin have been found to be essential for recruitment of the other to the spindle. TACC3 will only localize to the spindle if a clathrin-ch-TOG complex is formed. ch-TOG alone is not sufficient for TACC3 localization or spindle formation. Likewise, the ch-TOG-clathrin complex is not sufficient for spindle formation (92, 93). Any disruption to these three proteins results in aberrant spindle formation and mitotic arrest (89-93). Thus, much interest has been drawn toward these proteins as potential therapeutic targets for cancer. MJB6 is therefore a useful compound for studying how this disruption can be accomplished.
Materials and Methods

General Experimental

All reactions, unless specified, were conducted under an atmosphere of Argon in glassware that had been flame dried. Methylene chloride (CH$_2$Cl$_2$) was used from Baker Cycle-Tainers, anhydrous toluene, triethylamine and dimethylformamide (DMF) were purchased from Sigma-Aldrich. N-Boc-piperazine, 2-ethoxy benzoic acid, 2-propoxy benzoic acid, and 1,4-benzodioxan-5-carboxylic acid were purchased from AK Scientific. 2-ethoxybenzoyl chloride and 1-bromo-3(trifluoromethoxy)benzene were bought from Alfa Aesar. 1-bromo-3,5-dichlorobenzenes was bought from Acros Organics. 2-ethoxy-4-nitro benzoic acid was purchased from Sigma-Aldrich. Boc-5-aminovaleric acid was purchased from Chem-Impex Int’L INC. NHS-Biotin was purchased from APExBIO. BDP FL NHS ester was bought from Lumiprobe Life Science Solutions. Where appropriate, control of temperature was achieved with a Neslab Cryocool CC-100 II immersion cooler, ice-bath or a heated oil bath. Flash chromatography was performed on Silica Gel, 40 microns, 32-63 flash silica and/or -NH$_2$ capped spherical silica gel. High Performance Liquid Chromatography was performed on Phenomenex C18 silica gel column, 5 microns 250 x 4.60 mm, and monitored using the Shimadzu SIL-20AC equipped with a UV detector. Thin layer chromatography was performed on silica gel (Silica Gel 60 F254) glass plates and the compounds were visualized by UV and/or potassium permanganate stain.
Synthetic Procedures

Procedure A

Synthesis of 1-Piperazinecarboxylic acid, 4-(2-ethoxybenzoyl)-1,1-dimethylethyl ester

To a cold solution of N-Boc-piperazine (5g, 26.8 mmol) in triethylamine (40.3 mmol) was added 2-ethoxybenzoyl chloride (5.45 g; 4.6mL; 29.5 mmol) followed by a catalytic amount of DMAP and stirred for 16h at 4°C. Reaction was concentrated and purified by flash chromatography on silica gel (Hexanes/EtOAc 80:20) to yield desired product (7.84 g, 89% yield) as a white solid.

Procedure B

Synthesis of 1-(2-Ethoxybenzoyl)piperazine

To a mixture of 1-Piperazinecarboxylic acid, 4-(2-ethoxybenzoyl)-1,1-dimethylethyl ester (3 g, 8.97 mmol) in methanol (359 mmol) was added acetyl chloride (2.5mL 35.9 mmol) and stirred for 1 hr on ice. Reaction was concentrated and dissolved in water to which 1 M NaOH was added until pH 12 was reached. Dichloromethane was used to extract from the aqueous layer three times. Organic layer was concentrated to yield desired product (1.96 g, 93% yield) as a white solid.

Procedure C

Synthesis of 1-(3,5-dichlorophenyl)-4-(2-ethoxybenzoyl)piperazine (MJB6)

To solid 1-(2-Ethoxybenzoyl)piperazine (100 mg, 0.427 mmol), 1-bromo-3,5-dichlorobenzene (482 mg, 2.13 mmol), palladium acetate (2.87 mg, 12.8 µmol), BINAP (15.9 mg, 25.6 µmol) and sodium tert-butoxide (41.0 mg, 0.427 mmol) was added
toluene (0.33 M) and the reaction was stirred for 21 hr at 65°C. Reaction worked up with sodium bicarbonate and extracted with dichloromethane (2 X 10 mL). Organic layer washed twice with brine (15 mL), dried with sodium sulfate, and filtered through celite. Filtrate was concentrated and purified by gradient flash chromatography (Hexane/EtOAC 95:5 followed by 80:20) to yield MJB6 (112 mg, 30% yield) as a pale yellow solid.

Procedure D

Synthesis of 1-(2-ethoxybenzoyl)-4-[3-(trifluoromethoxy)phenyl]piperazine (MJB7)

To solid 1-(2-Ethoxybenzoyl)piperazine (100 mg, 0.427 mmol), palladium acetate (2.87 mg, 12.8 µmol), BINAP (15.9 mg, 25.6 µmol) and sodium tert-butoxide (41.0 mg, 0.427 mmol) was added 1-bromo-3(trifluoromethoxy)benzene (514 mg, 217 µL, 2.13 mmol) and toluene (0.33 M). The reaction was stirred for 2.5 hr at 95°C. Reaction washed with sodium bicarbonate (3 X 10 mL) and then filtered through celite. Filtrate was concentrated and purified by gradient flash chromatography (Hexane/EtOAC 95:5 followed by 80:20 and 75:25) to yield MJB7 (70 mg, 42% yield) as a pale yellow solid.

Procedure E

Synthesis of tert-butyl 4-(3,5-dichlorophenyl)piperazine-1-carboxylate

To a solution of BINAP (386 mg, 620 µmol) and sodium tert-butoxide (923 mg, 10.3 mmol) in toluene (0.33 M) was added N-Boc-piperazine (1.92 g, 10.3 mmol), 1-bromo-3,5-dichlorobenze (3.5 g, 15.5 mmol) and palladium acetate (70 mg, 310 µmol). The reaction was degrassed with Argon and then stirred at 65°C for 18 hr. Reaction
concentrated and purified by gradient flash chromatography (Hexane/EtOAC 95:5 followed by 90:10 and 80:20) to yield tert-butyl 4-(3,5-dichlorophenyl)piperazine-1-carboxylate (2.4 g, 70% yield) as a white solid.

Procedure F

Synthesis of 1-(3,5-dichlorophenyl)piperazine hydrochloride

To a mixture of tert-butyl 4-(3,5-dichlorophenyl)piperazine-1-carboxylate (2.4 g, 6.33 mmol), in methanol (8.11 g; 10.3 mL, 50.6 mmol) and THF (0.4 M) was added acetyl chloride (4.00 g; 3.61 mL, 35.9 mmol) and stirred for 30 min at room temperature. Ether added to reaction, white solid precipitated. Reaction filtered to yield 1-(3,5-dichlorophenyl)piperazine hydrochloride (1.18 g, 81% yield) as a white solid.

Procedure G

Synthesis of 1-(3,5-dichlorophenyl)-4-(2-propoxybenzoyl)piperazine (MJB11)

To solid 1-(3,5-dichlorophenyl)piperazine hydrochloride (100 mg, 432.7 µmol), 2-propoxy benzoic acid (117 mg, 649 µmol), DCC (133 mg, 649 µmol), DMAP (7.93 mg, 6.49 µmol) was added triethylamine (52.5 mg; 72.4 µL, 519 µmol) and reaction-grade dichloromethane (43 mM). The reaction was stirred for 3 hr at room temperature. Reaction concentrated and purified by gradient flash chromatography (Hexane/EtOAC 95:5 followed by 80:20) to yield MJB11 (44.4 mg; 26% yield) as a pale yellow solid.
Procedure H

1-(3,5-dichlorophenyl)-4-(2,3-dihydro-1,4-benzodioxine-5-carbonyl)piperazine (MJB13)

To solid 1-(3,5-dichlorophenyl)piperazine hydrochloride (100 mg, 432.7 µmol), 1,4-benzodioxan-5-carboxylic acid (78.0 mg, 649 µmol), DCC (133 mg, 649 µmol), DMAP (7.93 mg, 6.49 µmol) was added triethylamine (52.5 mg; 72.4 µL, 519 µmol) and reaction-grade dichloromethane (43 mM). The reaction was stirred for 1 hr at room temperature. Reaction washed with 10 mL sodium bicarbonate and extracted with EtoAc (2 X 15 mL). Combined organic extracts dried with sodium sulfate and filtered through celite. Filtrate was concentrated and purified by gradient flash chromatography (Hexane/EtOAC 95:5 followed by 80:20) to yield MJB13 (11.0 mg; 6.47% yield) as a pale yellow solid.

Procedure I

Synthesis of 1-(3,5-dichlorophenyl)-4-(2-ethoxy-4-nitrobenzoyl)piperazine

To solid 1-(3,5-dichlorophenyl)piperazine hydrochloride (1.00 g, 4.33 mmol), 2-ethoxy-4-nitro benzoic acid (1.10 g, 519 mmol), DCC (1.07 g, 519 mmol), DMAP (63.0 mg, 0.433 mmol) was added triethylamine (1.32 g; 1.82 mL, 12.9 mmol) and reaction-grade dichloromethane (0.1 M). The reaction was stirred for 18 hrs at room temperature. Reaction washed with 75 mL sodium bicarbonate and extracted with DCM (2 X 50 mL). Combined organic extracts dried with sodium sulfate and filtered through celite. Filtrate was concentrated and purified by flash chromatography (Hexane/EtOAC 85:15) to yield
1-(3,5-dichlorophenyl)-4-(2-ethoxy-4-nitrobenzoyl)piperazine (1.37 g; 72% yield) as a yellow solid.

Procedure J

Synthesis of 4-[4-(3,5-dichlorophenyl)piperazine-1-carbonyl]-3-ethoxyaniline (MJB27)

To a solution of Nickel (II) chloride hexahydrate (280 mg, 1.18 mmol) in methanol (0.1 M) was added sodium borohydride (134 mg, 3.54 mmol) in three portions to yield a black mixture. Reaction was degassed with Argon and stirred for 30 minutes at room temperature. Vessel then cooled to 4°C with ice and 1-(3,5-dichlorophenyl)-4-(2-ethoxy-4-nitrobenzoyl)piperazine (500 mg, 1.18 mmol) dissolved in methonal/THF. An additional 12 equivalents of sodium borohydride (267 mg, 7.08 mmol) was added and the reaction was stirred on ice for 10 minutes. Reaction filtered through celite. Filtrate washed with sodium bicarb (15 mL) and extracted from with dichloromethane (3 X 10 mL). Organic washes combined, dried with sodium sulfate, filtered through cotton and concentrated to yield MJB27 (446 mg, 96% yield) as a pale yellow solid.

Procedure K

Synthesis of N-[4-[4-(3,5-dichlorophenyl)piperazine-1-carbonyl]-3-ethoxyphenyl]-5-{2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl}pentanamide (MJB28)

To solid MJB28 (20 mg, 50.7 µmol) and NHS-Biotin (20.8 mg, 60.9 µmol) was added DMF and reaction stirred for 6 days at 50°C. Reaction concentrated and purified by flash chromatography (dichloromethane/methanol 95:5) to yield MJB28 (2.84 mg, 9.0% yield) as a pale yellow solid.
Procedure L

Synthesis of $N\{4\{4\{3,5\text{-dichlorophenyl}\}piperazine-1\text{-carbonyl}\}\{3\text{-ethoxyphenyl}\}\{3\text{-methylbutanamide} (MJB30)$. 

To a solution of the hydrochloride salt of MJB27 (75 mg, 190 µmol) in dichloromethane (0.1 M) and triethylamine (39 mg; 53.5 µL, 380 µmol) was added isovaleryl chloride (230 mg; 230 µL, 1.9 mmol) dropwise and solution was stirred at 35°C for 3 hr. Reaction washed with sodium bicarbonate (15 mL) and extracted from with ethyl acetate (2 X 10 mL). Organic washes combined, dried with sodium sulfate, filtered through cotton and concentrated. Concentrate was purified by flash chromatography (Hexane/EtOAc 80:20) to yield MJB30 (73 mg, 80% yield) as an off-white solid.

Procedure M

Synthesis of $N\{4\{4\{3,5\text{-dichlorophenyl}\}piperazine-1\text{-carbonyl}\}\{3\text{-ethoxyphenyl}\}\{7\text{-heptynamide} (MJB31)$

To a solution of DCC (43 mg, 210 µmol) and DMAP (2.3 mg, 19 µmol) in reaction grade dichloromethane (0.1 M) was added the hydrochloride salt of MJB27 (75 mg, 190 µmol), triethylamine (39 mg; 54 µL, 380 µmol), and 6-heptynoic acid (48 mg; 48 µL, 380 µmol). Reaction stirred for 12 hrs at room temperature. TLC showed 50% completion so 3 more equivalences of 6-heptynoic acid (72 mg; 72 µL, 570 µmol) and DCC (129 mg, 630 µmol) was added. Solution stirred for 3 hr at 40°C. Reaction filtered through celite, concentrated, purified by gradient flash chromatography (Hexane/EtOAc 70:30 followed by 60:40). NMR showed contaminant believed to be the urea intermediate of DCC and 6-heptynoic acid. To remove urea, product was dissolved in diethyl ether and placed at
80°C causing the urea to crash out of solution. Ether was filtered through cotton and concentrated to yield MJB31 (41 mg, 44% yield) as a white solid.

Procedure N

*Synthesis of N-{4-[4-(3,5-dichlorophenyl)piperazine-1-carbonyl]-3-ethoxyphenyl}benzamide (MJB32)*

To a solution of DCC (40 mg, 190 µmol) and DMAP (2.3 mg, 19 µmol) in reaction grade dichloromethane (0.1 M) was added the hydrochloride salt of MJB27 (75 mg, 190 µmol), triethylamine (39 mg; 54 µL, 380 µmol), and benzoic acid (46 mg; 380 µmol). Reaction stirred for 18 hrs at 40°C. TLC showed 50% completion so 6 more equivalences of benzoic acid (138, 1.14 mmol) and 1 more equivalence of DCC (40 mg, 190 µmol) was added. Solution stirred for 3 hr at 40°C. Reaction filtered through celite, concentrated, purified by gradient flash chromatography (Hexane/EtOAc 70:30 followed by 60:40). NMR showed contaminant believed to be the urea intermediate of DCC and benzoic acid. To remove urea, product was dissolved in diethyl ether and placed at 80°C causing the urea to crash out of solution. Ether was filtered through cotton and concentrated to yield MJB32 (45 mg, 48% yield) as a white solid.

Procedure O

*Synthesis of tert-butyl N-{4-[4-{4-(3,5-dichlorophenyl)piperazine-1-carbonyl]-3-ethoxyphenyl}carbamoyl}butyl]carbamate*

To a solution of DCC (160 mg, 760 µmol), DMAP (6.20 mg, 50.7 µmol), and Boc-5-aminovaleric acid (330 mg, 1.52 mmol) in reaction grade dichloromethane (0.1 M) was
added MJB27 (200 mg, 507 µmol). Reaction was stirred for 16 hr at 50°C. TLC showed 50% progression so 1 more equivalent of both Boc-5-aminovaleric acid (110 mg, 507 µmol) and DCC (107 mg, 507 µmol) was added and the reaction was stirred at 60°C for 2 hrs. Reaction washed with ammonium chloride (10 mL), then washed with brine (10 mL), dried with sodium sulfate, filtered through cotton and concentrated to yield methane tert-butyl N-[4-((4-(3,5-dichlorophenyl)piperazine-1-carbonyl)-3-ethoxyphenyl)carbamoyl]butyl]carbamate (360 mg, 96% yield) as a colorless oil.

Procedure P

Synthesis of 5-amino-N-{4-[4-(3,5-dichlorophenyl)piperazine-1-carbonyl]-3-ethoxyphenyl}pentanamide (MJB39)

To a solution of N-[4-((4-(3,5-dichlorophenyl)piperazine-1-carbonyl)-3-ethoxyphenyl)carbamoyl]butyl]carbamate (360 mg, 606 µmol) and dichloromethane (0.6 M) was added trifluoroacetic acid (2.23 g; 1.50 mL, 19.7 mmoles) and the reaction was stirred at room temperature for 6 hr. Reaction washed with sodium bicarbonate (3 mL), washed with brine (3 mL), basified with 1 M NaOH, and then extracted from with dichloromethane (2 x 10 mL). Organic washes combined, dried with sodium sulfate, filtered through cotton, concentrated and then purified by gradient flash chromatography (dichloromethane/methanol 98:2, 95:5, 90:10 and then dichloromethane/methanol/triethylamine 90:9:1) to yield MJB39 (240 mg, 80% yield) as a pale yellow solid.
Procedure Q

Synthesis of 2,2-difluoro-10,12-dimethyl-4-{2-[[4-[[4-(piperazine-1-carbonyl)phenyl]carbamoyl]butyl]carbamoyl]ethyl]-1\textsuperscript{5},3-diaza-2-boratricyclo[7.3.0.0\textsuperscript{3,7}]dodeca-1(12),4,6,8,10-pentaen-1-ylium-2-uide (MJB67)

To solid MJB39 (12.7 mg, 25.6 µmol) and solid BDP FL NHS ester (5 mg, 12.8 µmol) was added DMF (0.06 M) and the reaction was stirred at 37°C for 3 days. The resulting red solution was azeotroped with toluene (3 X 3 mL) and placed under high vacuum for 2 days to remove DMF. Resulting red solid was brought up in 65% acetonitrile/H\textsubscript{2}O and purified by high performance liquid chromatography (acetonitrile/H\textsubscript{2}O 60:40, R\textsubscript{T}: 27.5 minutes) to yield MJB67 (8.36 mg, 85% yield) as a red solid.

Cell Culture

HCT116 colon cancer cells were obtained from the American Type Culture Collection (Manassas, VA). HT29 cell lines were cultured in McCoy’s 5A medium and MEM medium, respectively, with 10% fetal bovine serum, nonessential amino acids and antibiotic/antimycotic (Life Technologies, Guilford, CT). Drug treatments were performed approximately 24 h after passage for 18 h, unless otherwise indicated.

Flow Cytometry

For cell cycle analyses, cells were analyzed for DNA content by ethanol fixation and staining with propidium iodide as previously described (8). Cells were harvested using trypsin-EDTA, centrifuged at 1000 X g for 10 min and resuspended in 500 µl of cold saline GM. Cells were washed once with 1X PBS and then fixed for at least 2 hrs at -
20°C in 3X volumes of cold 100% ethanol while vortexing. Cells were then pelleted and washed once with PBS containing 5 mM EDTA. Pelleted cells were stained with 30 µg/ml propidium iodide (Molecular Probes, Life Technologies Corp.) and 0.3 mg/ml RNase A (Sigma-Aldrich, St. Louis, MO) in 500 µl PBS solution for 40 min in the dark at RT. The stained cells were filtered through 35 µm cell strainer tubes (BD Biosciences, San Jose, CA). All flow cytometric analyses were performed on FACSCalibur (BD Biosciences) using Cell Quest software (BD Biosciences). The data were analyzed using FlowJo (v10, TreeStar Inc., Ashland, OR).

**Affinity Chromatography**

RIPA buffer was used to prepare whole cell extracts from 8 T75 flasks of HCT116 cells. Pierce™ Avidin Agarose (Thermo Fischer) was washed once with 10 times its packed volume of 1X PBS to dilute azide in the packing solution. Two chromatography columns were run in tandem, one containing MJB28, the biontynlated derivative, and one containing D-(+)-biotin (Sigma Aldrich) as a control. Equimolar amounts of MJB28 or D-(+)-biotin and Pierce™ Avidin Agarose were mixed for 1 hour at room temperature. Columns were washed twice with 10 column volumes of 1X PBS to remove excess MJB28 or biotin. Both columns were then incubated with equal amounts of cell extract at 4°C overnight under mild agitation. Columns were then packed into gravity chromatography columns and flow through was collected. To remove unbound proteins, each column was washed with 10 column volumes of Wash Buffer (PBS, 0.1% protease inhibitor cocktail, 1mM DTT). The first and last washes were collected to confirm efficacy of the wash step using SDS-PAGE. After the wash, bound proteins were eluted.
from the column using Elution Buffer (PBS, 1X protease inhibitor cocktail, 1mM DTT, 1µM). Between 2 and 5 column volumes of Elution Buffer was passed through the column and collected. To remove proteins that remained adherent to the column, columns were suspended in elution buffer supplemented with 1% SDS and then heated to 90°C for 10 minutes. The heated solution was placed back into the chromatography column and collected. To prepare for SDS PAGE, elution fractions were concentrated using Pierce™ 3K MWOC PES Protein Concentrators (Thermo Fischer). One percent SDS was added to each fraction to limit proteins from adhering to the concentrators, which otherwise would stop solution from passing through the membrane of the concentrators. Concentrated fractions from each column, cell extract, flow through and washes from both columns were then analyzed using SDS PAGE. Polyacrylamide ranged from 8-10% (Bio-Rad Laboratories, Hercules, CA). Percentage was lowered to make excision of clathrin easier. Proteins from fraction were stained with SYPRO Ruby Red Gel stain (Bio-Rad). Bands present in the MJB28 column, but not the biotin column, were excised from the gel and sent to the Yale/Keck MS & Proteomics Resource facility for MS analysis.

**Western Blotting**

Elution fractions from affinity chromatography experiments were analyzed by western blot to confirm presence of clathrin. 20 µl of elution fraction was denatured under reducing conditions and separated on 8% polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose by voltage gradient transfer. The resulting blots were
blocked with 5% (w/v) non-fat dry milk in PBS + 0.1% (v/v) Tween-20. Clathrin Heavy Chain antibody (P1663; Cell Signaling Technology) was detected using the appropriate infrared secondary antibody (LiCor, Lincoln, Nebraska). Blots were analyzed using the LiCor Odyssey® CLx.

**Endocytosis Assay**

HCT116 cells plated onto glass cover slips were serum starved overnight in McCoy’s media plus 0.5% BSA. Cells were then treated with DMSO or varying concentrations of MJB6 dissolved in DMSO for 30 minutes at 37°C. MJB6 treatment was limited to 30 minutes to limit the mitotic arrest action of the compound, which may have skewed data since endocytosis does not occur during mitosis. Media was then removed and fresh media containing 0.5% Transferrin (Tfn) Alexa Fluor® 488 Conjugate (Thermo Fischer) was added. Cells were then placed at 37°C for 30 minutes. After exposure to transferrin, media was removed, cells were washed once with PBS, and then fixed with 4% PFA for 10 minutes. Cells were washed twice with PBS after fixation and mounted with ProLong Gold Antifade Reagent (Life Technologies). Images were acquired using Nikon A1R Confocal Microscope (version 2.11, Nikon Instruments Inc.) and NIS-Elements Advanced Research Software (version 4.13.01, build 916, Nikon Instruments Inc.). Following background subtraction and image stacking, both DAPI and fluorescent Tfn channels were merged. Images were processed using Cells were analyzed using the particle analysis plugin, standard with ImageJ image analysis software (http://rsb.info.nih.gov/ij). Image brightness and contrast was modified with Adobe.
Photoshop software CS6 (Adobe Systems).

**In vitro click chemistry staining**

Cells plated into a glass bottomed 8-well chamber plate (Lab-Tek) were fixed with 4% PFA for 15 minutes at room temperature and then permeabilized with 0.5% Triton-X100 for 20 minutes at room temperature. During the permeabilization step, a click reaction using MJB31, the alkyne derivative, and reagents from the Click-iT® Plus Alexa Fluor® Picolyl Azide Toolkit (Life Technologies) was conducted in a microcentrifuge tube. The reaction was allowed to proceed for 15 minutes. Cells were then incubated with the clicked MJB31 for 2.5 hrs at room temperature in the dark. The clicked MJB31 was removed, 200µL of PBS was added, and images of stained cells were immediately captured using the Nikon A1R Confocal Microscope (version 2.11, Nikon Instruments Inc.) and NIS-Elements Advanced Research Software (version 4.13.01, build 916, Nikon Instruments Inc.). Image brightness and contrast was modified with Adobe Photoshop software CS6 (Adobe Systems).

**Immunofluorescence microscopy**

Cells cultured on coverslips were fixed with 4% paraformaldehyde or 100% ice cold methanol and then permeabilized with 0.5% Triton X-100 in PBS. Cells were blocked in 5% serum (in PBS) and then incubated for 1 h at room temperature on the shaker with the primary antibody (in 5% serum) against β-tubulin (E7 monoclonal antibody,
Developmental Studies Hybridoma Bank), Clathrin (P1663; Cell Signaling Technology), and TACC3 (D9E4; Cell Signaling Technology). Appropriate secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used for 45 min incubation. Nuclei were visualized using DAPI (5 µg/ml in PBS; DJ306, Life Technologies). Coverslips were mounted on slides using ProLong Gold Antifade Reagent (Life Technologies). Images were acquired using Nikon A1R Confocal Microscope and NIS-Elements Advanced Research Software. Following background subtraction and image stacking, both DAPI and immunofluorescence images were merged. Image brightness and contrast was modified with Adobe Photoshop software CS6 (Adobe Systems).

**Statistical analyses**

One-way analysis of variance (ANOVA) was used for comparing more than two groups. Tukey’s post-hoc test was employed to determine the significance of differences between multiple groups, with $P < 0.05$ considered significant. Two-way ANOVA was used for more than two independent variables and Bonferroni correction was used for multiple comparisons ($P < 0.05$).

**Results**

**SAR Study**

We decided to perform further SAR on AK301 with the intention of synthesizing molecular probes to identify possible cellular targets. Our goal was to create more
potent compounds to offset the loss in efficacy that usually accompanies the attachment of biotin or fluorescent molecules. From previous study we knew that the position of the chlorine on the phenyl ring and both the position and length of the ether group on the benzoyl ring were important for maximal activity [REF]. AK301, the most potent compound from the first round of SAR, features a chloro group on the meta-position of the phenyl ring and a methoxy group in the ortho-position of the benzoyl ring. Since we knew the position of substituents on these rings could influence efficacy and it is synthetically straightforward to introduce new functionalities to aromatic rings, we focused on these two areas of the molecule in our second round of SAR. Figure 1 showcases the new molecules resulting from this study and their respective activity in HCT116 colon cancer cells. A cell cycle assay was used to measure the percentage of cells arrested in G2/M. EC\textsubscript{50} numbers were obtained from titration curves plotting the percent of arrested cells (Figure 2).

The phenyl group was the first aromatic ring that we manipulated. Since having one chloro-group in the meta-position decreased EC\textsubscript{50}, we decided to incorporate another chloro in the other available meta-position. We hypothesized that the chloro group was interacting with an important hydrophobic pocket in the target protein. In AK301 the phenyl group is attached to the piperazine linker by a single bond. Free-rotation is allowed around the attachment. Thus, configurations can exist where the chloro group is not in the position necessary to interact with the hypothetical hydrophobic pocket. In adding another chloro-group we added symmetry to the phenyl ring in hopes that there would be double the number of configurations were the chlorine was in the proper position to interact with the pocket. Alternatively, the addition of the
chlorine could have resulted in interaction with another hydrophobic space in the protein. This hypothesis lead to the development of a dichlorobenzene derivative named MJB6. The compound was more active than AK301 in HCT116 colon cancer cells. EC$_{50}$ decreased two fold from ~150nM for AK301 to ~75nM for MJB6.

Our next compound aimed to examine the size of the hydrophobic pocket. To test this, we developed a molecule with a trifluoromethyl ether (-OCF$_3$) replacing the chlorine on AK301. OCF$_3$ encompasses more space than a chloro group, but is similarly hydrophobic. Interestingly, the OCF$_3$ derivative, called MJB7, was significantly less potent than AK301 with an EC$_{50}$ near 800nM. This data implies that the hydrophobic pocket in which the chlorine is interacting with is small. It also suggests that a possible halogen bond may be formed between the small molecule and its target protein.

With the success of MJB6 we decided to fix the dichloro phenyl group and continue SAR with the benzoyl ring. In our first SAR it was determined that the length of the ether contributed to efficacy. An ethoxy group was more effective than a methoxy group. To test the limit of the effective chain length a derivative was synthesized with a propoxy group replacing the methoxy group. This derivative, MJB11 was also more potent than AK301 with a slightly higher, but not significant, EC$_{50}$ than MJB6. It appeared that extending the length of the ether group was not enhancing potency and therefore we did not pursue a 4 or 5 carbon ether functionality.

A second modified benzoyl derivative was synthesized to test the effect of flexibility on the compounds efficacy. We replaced the ether moiety with 1,4 dioxane, a cyclic acetyl. This created rigidity in the molecule, while still maintaining the ability of the ring to make polar contacts with the protein. The derivative, named MJB13, was more
effective than AK301 at inducing mitotic arrest, however had an EC\textsubscript{50} around 100nM. This suggested that flexibility in the ether was beneficial to the molecules mode of action.

After investigating aspects of AK301’s binding pocket we began constructing molecular probes. First we wanted to stitch biotin to MJB6 for use in affinity chromatography. This required a reactive handle. Biotin is equipped with a free carboxylic acid, which can be attached to an amino group (-NH\textsubscript{2}) via a peptide bond. NHS esters are also available, as was used in our synthesis, to speed up the formation of this peptide bond. Therefore, we wanted to introduce an amino group to MJB6. We decided to introduce the functional handle to the benzoyl ring because it appeared that the dichloro phenyl ring was most important for this newfound activity. Due to the restraints of commercially available starting materials, we synthesized an intermediate that contained a nitro group (-NO\textsubscript{2}) meta to the ethoxy group on the benzoyl ring. The nitro group was then reduced to the desired NH\textsubscript{2} functionality using NaBH\textsubscript{4} and Ni\textsuperscript{II}Cl. This aniline derivative, called MJB27, was tested in HCT116 colon cancer and found to have similar mitotic arresting activity to MJB6 and MJB11. While its activity was better than AK301, MJB27 may not be the best choice to pursue as a drug candidate due to possible metabolic reactions that could occur at the amino group.

Once MJB27 was synthesized we began attaching functional groups that could aid in the biochemical identification of target proteins. As mentioned previously, biotin was the first probe we created. Called MJB28, this molecule showed some activity in HCT116 colon cancer cells. This made us hopeful that it would bind to MJB6’s target in a complex cell lysate. Next, we synthesized a derivative containing an alkyne attached
to the benzoyl ring by a long methylene chain. Alkynes are reactive carbon triple bonds that can be used in Husigen’s cycloaddition reaction with an azide, commonly called “click chemistry.” We hoped to use such a probe to fluorescently label the target in both live and fixed cells using an azide conjugated to a fluorophore. The alkyne derivative, called MJB31, had some activity *in vitro*, but was 10 fold weaker than MJB6.

Our next compounds tested the solvent accessibility of the benzoyl group. Both biotin and the alkyne had long, flexible carbon linkers that we presumed, based on their *in vitro* activity, were solvent accessible. In order to see how much room we had to work with, we synthesized MJB30, an isovaleric acid derivative, and MJB32, a benzoic acid derivative. Isovaleric acid incorporates steric hindrance due to a branching point after two methylene groups. Interestingly, this derivative was completely inactive, unable to induce mitotic arrest at concentrations as high as 4uM. Next we tested MJB32. Benzoic acid was used to mimic benzophenone, a photo reactive molecule we hoped to incorporate in order to build a photo affinity/click chemistry probe. Like with MJB30, MJB32 was also inactive at concentrations up to 4uM. These data indicated that a carbon linker of at least three methylene groups was necessary for solvent accessibility.

Based on the finding that a long carbon linker was needed to ensure solvent accessibility MJB39 was synthesized. This compound contains a four-chain carbon linker and a free amine for further attachment of probe elements. With this scaffolds we created two more molecules. MJB68, a long chain biotin derivative, and MJB67, a BODIPY derivative. The long chain biotin compound allowed us to determine if improved solvent accessibility increased the number of identified targets. Preliminary study suggests that clathrin is still the main protein pulled down. The BODIPY derivative
will enable us to verify the target using fluorescence anisotropy experiments. However, while testing the MJB67 we found that it does not induce mitotic arrest even at concentrations of 8uM (Figure 3). However, its binding to discreet cell sites is competed by MJB6, indicating that it may bind to clathrin, but not strongly enough to block association with other cellular proteins involved in mitosis. In the future we would like to use MJB39 to synthesize a photo affinity/click chemistry probe using benzophenone and a terminal alkyne.

**Biotinylated Probe Pulls Down Clathrin**

The biotin derivative synthesized during SAR was used to pull down target protein from whole cell lysates. Briefly, an equimolar amount of avidin agarose and MJB28 or MJB6? were mixed for 1 hour on ice. The agarose was then washed with 10 column volumes of Buffer A (PBS, 1x protease inhibitor cocktail, 1mM DTT) at 4°C to remove excess MJB28 or MJB68. Next, the avidin-biotin-small molecule complex was incubated with cell lysate from HCT116 colon cancer cells overnight at 4°C. The agarose was then packed into a chromatography column and washed with 10 column volumes of Buffer A in the cold. An elution buffer, comprised of buffer A and 1uM MJB6, was used to compete off bound protein at room temperature. Finally, the avidin agarose was suspended in Buffer A, 1% SDS was added, and the slurry was heated at 90°C for 10 minutes to remove any protein that remained bound to the column. Five column volumes of the elution buffer and heated column were collected and analyzed by SDS-PAGE. Gels were stained with SYPRO Ruby Red Gel stain (Figure 4). Bands were excised from the gel and sent to the Yale/Keck MS & Proteomics Resource facility for
MS analysis. Clathrin was identified as a top hit. Western blotting was used to confirm the MS data (Figure 4).

**MJB6 interferes with clathrin-mediated transferrin uptake in HCT116 colon cancer cells**

A classic method for determining clathrin inhibition utilizes a fluorogenic transferrin (Tfn) assay [2,3]. HCT116 cells were treated with DMSO or various concentrations of MJB6 for 1 hour. Cells were not treated with MJB6 overnight as is customary for cell cycle experiments because the mitotic arresting effects of the compound could skew the endocytosis data; mitotic cells do not undergo endocytosis. After compound treatment, cells were exposed to Tfn bound to the Alexa 488 fluorophore for 30 minutes. Cells were then analyzed by confocal microscopy and images were processed using Fiji. MJB6 was able to inhibit the aggregation of Tfn in the cytoplasm both visually and quantitatively (Figure 5). DMSO did not inhibit endocytosis of Tfn. The majority of cells in MJB6 treated images, even at 100nM concentrations, did not have condensed chromosomes indicating that cells were not in mitosis implying that all inhibition of Tfn was due to the actions of MJB6 interacting with clathrin and not the resulting mitotic arrest. These data indicate that MJB6 inhibits clathrin-mediated endocytosis providing more evidence for clathrin as its relevant cellular target.

**In vitro staining with an alkyne-containing derivative mirrors clathrin immunofluorescent staining**
The alkyne derivative, MJB31, was used to stain cells after reaction with an azide conjugated to a fluorescein fluorophore. Briefly, the azide was attached to MJB31 using the molecular probes Click-iT® Plus Alexa Fluor® 488 picoly azide tool kit in a test tube. Cells were fixed with 4% PFA, permeabilized with 0.1% Triton-X100, and then incubated with the clicked probe for about one hour. Excess MJB31 was removed with a quick PBS wash. Cells were immediately analyzed using confocal microscopy to minimize dissociation of the reversible MJB31. Images revealed that MJB31 localized to the perinuclear region of the cell (Figure 6). We compared these images to immunofluorescent staining of clathrin. Clathrin in IF images is also concentrated in the perinuclear region of the cell. These data suggest that MJB31 is binding to clathrin.

**MJB6 disrupts clathrin, but not TACC3 localization to β-tubulin**

Untreated and MJB6 treated cells were immunofluorescently co-stained for clathrin/β-tubulin and TACC3/β-tubulin as described previously. In untreated cells both clathrin and TACC3 localized with β-tubulin near the centrosome. Clathrin stain also appeared around mitotic spindle fibers near kinetochores in agreement with its role as a stabilizer of K-fibers. After treatment with MJB6 clathrin stain was no longer localized with β-tubulin. Rather than concentrating in the interior of the cell near centrosomes, clathrin localized to the periphery of the cell. TACC3 was less affected by MJB6 treatment. In untreated cells a TACC3 foci was present at each centrosome and co-stained heavily with β-tubulin. After MJB6 treatment smaller foci of TACC3 appeared. However, the staining was not as dispersed as clathrin. At least one TACC3 foci was present in arrested cells that co-localized heavily with β-tubulin. Figure 7 shows a side-by-side
comparison of clathrin and TACC3 training. These data demonstrates that MJB6 potentially disrupts the interaction between clathrin and TACC3, releasing clathrin from the centrosome and K-fibers.

Discussion

We have successfully synthesized several novel derivatives of AK301. The most potent compound, MJB6, was used as the scaffold for several different molecular probes. Biochemical experiments employing the probes identified clathrin as a possible target protein for AK301 and its derivatives. MJB6 limited cellular uptake of transferrin and modified cellular clathrin distribution in vitro based on immunofluorescence staining. This evidence suggests that clathrin is the cellular target of this class of small molecules. This is the third small molecule clathrin inhibitor to be identified and shows remarkably more potency than other clathrin disrupting compounds like Pitstop2 (IC$_{50} = 12\mu$M) (49).

The SAR study performed on AK301 in this work revealed important aspects of the small molecule’s binding interactions with clathrin. MJB6, the dichlorobenzene derivative, was the most potent. This indicates that AK301’s binding site contains two hydrophobic pockets in which two different chlorines can interact. Alternatively, the presence of two chlorines may result in an increased number of confirmations from which tight binding results. The latter was the thought behind synthesis of MJB6. We would like to perform X-ray crystallography to determine the correct binding mechanism and identify possible sites of halogen bonding. MJB7 was the first inactive compound synthesized. Its inactivity suggests the point of interaction between the protein and
AK301 is not large enough to incorporate an –OCF₃ group, which spans ~3Å. While MJB11 and MJB13 were more active than AK301, they were not as active as MJB6. These data suggest that the dichlorobenzene ring contains the interactions necessary for a potent arrest response. Lastly, in developing the probes it was found that a linker of at least four –CH₂ groups was necessary for biological activity. These data imply that the site of interaction of the benzoyl ring cannot accommodate sterically hindered functionalities, like the isovaleryl group.

Our SAR studies led to the development of a biotinylated MJB6. The compound was used to perform affinity chromatography. Pull-down experiments of whole cell extracts yielded a protein band that was identified as clathrin heavy chain after mass spectrometry analysis. Clathrin is an interesting target for AK301 compounds because of previous work in our lab. The first generation of AK301 derivatives was identified for their ability to induce apoptosis only in the presence of tumor necrosis factor (TNF) (8). TNF is a potent inflammatory cytokine whose cancer cell killing potential is associated with uptake of its membrane bound receptor after TNF binding. TNF is both pro-tumorigenic and apoptotic. If TNF binds to its receptor and remains on the cell surface, survival signaling through the NF-κB pathway is favored. Endocytosis of the receptor-ligand complex inhibits survival signaling and death signaling predominates (94). Modulation of clathrin could explain the increased sensitivity of colon cancer cells to TNF after treatment with AK301. Disruption of proper receptor uptake may limit trafficking to lysosomes and result in persistent death signaling. Knockdown of clathrin in vitro has revealed that clathrin is not necessary for bud formation, but is necessary for bud maturation (95). Thus, invaginations will form in the membrane. It is possible that
upon TNF binding it is receptor or bud will begin to form and the change in membrane curvature may be enough to inhibit survival signaling allowing caspase activation and death signaling to predominate. In the future we would like to test these hypotheses and uncover the mechanism by which AK301 compounds sensitize colon cancer cells to TNF induced apoptosis.

Several biochemical experiments were performed to provide evidence for clathrin as the target of AK301 compounds. First, an endocytosis assay was performed. As with the other clathrin inhibitors identified, MJB6 limited the uptake and trafficking of fluorogenic transferrin. Next, we used a derivative containing a terminal alkyne, MJB31, to stain the target protein in vivo using an azide conjugated to a fluorophore. MJB31 treated cells showed concentrated fluorescence in the peri-nuclear region of the cell. This staining pattern is consistent with clathrin immunofluorescent staining. In the future we would like to co-stain with a clathrin antibody and measure the overlap in fluorescence. We will also use the BODIPY probe to image clathrin, AK301’s assumed target, in real time. In addition, this compound will be used to conduct fluorescence anisotropy experiments to further provide evidence for clathrin as the target of AK301.

In our final experiments were visualized the effect of MJB6 on clathrin and TACC3 by immunofluorescence staining. Upon treatment with MJB6 clathrin, but not TACC3, is disrupted. In untreated cells clathrin collects in the Golgi apparatus in the cell’s perinuclear region. Upon treatment with MJB6, the staining is more dispersed throughout the cell. TACC3 staining places TACC3 at the MTOC during mitosis in untreated cells. In cells exposed to MJB6 most of the TACC3 remains congregated in one foci despite there being several nucleation sites for b-tubulin. These data provide
evidence that the phenotype observed is from clathrin inhibition and not TACC3 inhibition. In the future we would like to complete further SAR to design molecules that can disrupt clathrin’s interaction with TACC3, but does not limit its endocytic activity.
Figures and Figure Legends

A.

![Chemical structures]

B.

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Figure 1. A) Line drawings of AK301 and its derivatives. ChemDraw 15.0 (PerkinElmer) was used to draw and format images. B) Table comparing the EC\textsubscript{50} and percent of cells arrested in G2-M by AK301 and each of its derivatives. EC\textsubscript{50} concentrations were determined by cell cycle analysis of HCT116 colon cancer cells as previously described [8]. Maximal arrest is the percentages of cells arrested in G2-M when HCT116 cells were treated with compound overnight at a concentration at least five times the derivatives EC\textsubscript{50}. An ideal compound should have a low nano-molar EC\textsubscript{50} and produce the largest maximal arrest. MJB6, the most potent derivative, has both the lowest EC\textsubscript{50} and produces the largest maximal arrest.
Figure 2. A) Dose response curve for MJB6. Plotted points were obtained by cell cycle analysis of HCT116 colon cancer cells. Non-linear regression curves were determined using the analyze function of GraphPad Prism 6. B) Dose response curves of AK301 and the three most potent derivatives. Over the titration concentration range AK301 fails to reach its maximal arrest. In addition, no arrest effect is observed for AK301 between 85 and 65nM, the EC$_{50}$ range for the three derivatives. All three derivatives reach their maximal arrest around 100nM, greater than half the value of AK301’s reported EC$_{50}$ in HCT116 cells. MJB6, MJB11, and MJB27 are clearly more effective at inducing mitotic arrest in HCT116 cells.
Figure 3. Dose response for MJB67 between the concentrations of 0 and 8uM. Data was determined by cell cycle analysis of HCT116 cells treated overnight with MJB67, the BODIPY derivative. Even at 8uM, MJB67 is unable to elicit an arrest response that is more significant than the control. These data indicate that MJB67 is not biologically active. Future competition experiments with MJB6 and/or other derivatives will be conducted to confirm binding to clathrin.
Figure 4. A) Methodology for affinity chromatography conducted with the biotinylated derivative, MJB28. RIPA buffer was used to prepare whole cell extracts from HCT116 colon cancer cells. Biotin or MJB28 was incubated with avidin agarose for 1 hour at room temperature. Cell extract was then mixed with the biotin/MJB28-avidin column overnight at 4°C under slight agitation. Columns were then packed and washed with 10 columns of Wash Buffer. The columns were then suspended in Elution Buffer and heated to 90°C for ~10 minutes to release bound protein. Eluted proteins were then analyzed by SDS-PAGE. B) SDS-PAGE gel produced from an affinity chromatography experiment. SYPRO Ruby Red Gel Stain was used to visualize proteins. Proteins bands present in the MJB28 column, but not the biotin column, were sent for MS analysis at the Keck Biotechnology Resource Laboratory at Yale University. The arrow indicates the band excised that produced clathrin-heavy chain (CHC) as the top hit after MS analysis. C) Western blot confirming presence of clathrin-heavy chain in elution fraction from MJB28 column. Clathrin was also detected in the flow through and was for the column. This indicates that either the column is being saturated or there is not enough time allowed for maximal protein binding. IR antibodies were used to probe proteins after transfer to nitrocellulose paper. Blots analyzing using the Odyssey® CLx.
Figure 5. A) HCT116 colon cancer cells were treated with differing concentrations of MJB6 for 30 minutes at 37°C followed by incubation with Tfn conjugated to Alexa Flour 488 for 30 minutes at 37°C. Tfn was visualized by confocal microscopy. In untreated cells Tfn accumulated at the perinuclear region, consistent with other studies (48). When cells were treated with MJB6 the Tfn signal became dispersed, failing to localize at the perinuclear region. Signal appeared to accumulate around cell membranes. The color key and 20 nm bars are shown. B) Quantification of Tfn fluorescence using ImageJ’s particle analysis plugin compared to the dose response curve for MJB6. Tfn internalization mirrors the dose response curve for MJB6.
Figure 6. MJB31-FITC staining compared to clathrin immunofluorescent staining in HCT116 colon cancer cells. The color key for the immunofluorescent image and 20 nm bars are shown. The MJB31-FITC probe appeared to accumulate at the perinuclear region of the cell. White arrows on left-most panel indicate concentrated areas of MJB31-FITC. The right-most panel shows immunofluorescent staining using a clathrin heavy chain antibody. Clathrin also accumulate at the cell’s perinuclear region (white arrow). The similarities in staining suggest that MJB31-FITC stains clathrin.
Figure 7. Immunofluorescent staining of clathrin (top) and TACC3 (bottom) in untreated and MJB6 treated HCT116 colon cancer cells. Color keys and 20 nm bars are shown. In untreated cells clathrin localizes to the mitotic spindle and K-fibers of dividing cells as well as the cell membrane of interphase cells. TACC3 also localizes to the mitotic spindle, however it is not visible in interphase cells. Upon treatment with MJB6, clathrin staining is delocalized from the mitotic spindle and appears to gather along the membrane (as seen with Tfn after MJB6 treatment). Clathrin’s overlap with β-tubulin also decreased when compound was present. TACC3 was less affected by MJB6. A
The focus of TACC3 was present in treated cells that localized heavily with β-tubulin. Clathrin and its interactions with β-tubulin appear to be more affected by MJB6 than TACC3 suggesting disruption of clathrin, and not TACC3, is the mechanism by which the AK301 family of compounds induces mitotic arrest.
References:


40. Caldwell CM, Kaplan KB. The role of APC in mitosis and in chromosome instability. Advances in experimental medicine and biology. 2009;656:51-64.


