A Dissertation

entitled

Synthesis and Evaluation of Inducers of Methuotic Cell Death and Preliminary Identification of Their Cellular Targets in Glioblastoma Cells

by

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The overall goal in cancer therapeutics is to do induce cell death in the tumor cells. While many classical anticancer drugs have different targets, they generally induce cell death by stimulating signaling pathways that ultimately activate apoptosis. Unfortunately, many genetic alterations that help drive the progression of a tumor cell also lead to apoptosis resistance. Thus, cancers are often treated with drugs to which they are inherently resistant. In order to circumvent this problem, there is a strong interest in identifying new forms of non-apoptotic cell death, with the goal of developing therapies that can activate these forms of cell death. Our lab has recently identified a form of non-apoptotic cell death called methuosis, characterized by hyperstimulated macropinocytosis and disrupted trafficking of these vesicles to the lysosome. These vesicles overwhelm the cytoplasm, disrupt cellular metabolism, and compromise membrane integrity, resulting in cell death.

Methuosis was originally characterized in glioblastoma cell culture by overexpression of activated Ras or Rac GTPases. However, our lab recently identified a class of small molecules that induce methuosis in cancer cells, providing a potential way
to induce this form of cell death therapeutically. This dissertation describes studies aimed to further understand the molecules that induce methuosis. First, the structure activity relationships of compounds that induce methuosis were evaluated, resulting in the identification of our lead compound, MOMIPP. Next, we sought to identify the cellular targets of MOMIPP that lead to methuosis induction. Accordingly, various chemical tools were synthesized and used to identify MOMIPP target proteins. Ultimately, two proteins that specifically bind MOMIPP were identified as candidates for methuosis-triggering activity. These await further characterization to confirm their relationship to methuosis and cell death.
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Chapter 1

Current Challenges in Glioblastoma Therapy

1.1 Genetic Alterations and Apoptosis in Glioblastoma

A diagnosis of glioblastoma, the most common primary brain tumor in the United States,\textsuperscript{1} remains a dismal prognosis. The current standard therapy, which consists of surgical debulking followed by radiation and adjuvant temozolomide chemotherapy, provides a median survival of only 15 months.\textsuperscript{2} The lack of sustained effect with the current therapy is due to the mechanism of action of these modalities: temozolomide, a small molecule alkylating agent, and ionizing radiation both induce DNA damage – ultimately leading to activation of apoptosis (programmed cell death).\textsuperscript{3,4} However, the apoptotic pathway is often dysfunctional in glioblastoma (GBM) cells due to mutations in various tumor suppressor genes and oncogenes that regulate apoptosis. Tumor suppressor genes such as PTEN, p53, Rb, NF1, are often mutated in glioblastoma; oncogenes such as PDGFR, EGFR, ERBB2, are often overexpressed, amplified, or mutated in a constitutively activated form.\textsuperscript{5,6} Among the pleiotropic functions of these genes pertaining to cancer progression, they all regulate apoptosis. Thus, mutations that help drive a GBM also renders it resistant to apoptotic cell death. As GBM treatments like
temozolomide ultimately induce apoptosis, these tumors are being treated with drugs to which they may rapidly acquire resistance.

The correlation between DNA-damaging chemotherapy and apoptosis was first shown in 1989 when Kaufmann observed that excessive chromatin fragmentation and protein degradation could be induced by etoposide, methotrexate, cisplatin, cytarabine, and colcemid – all of which are drugs used as chemotherapeutics for cancer, each with a different target.⁷ These effects could not be explained simply based on the drug-target interaction; some related downstream effectors were clearly being activated. It is now well understood that the observed DNA and protein degradation is due to the activation of apoptosis, and that most cytotoxic chemotherapy activates the apoptotic machinery.⁸,⁹ Thus, while all of the drugs previously mentioned have different targets, their mechanisms converge in that they all activate apoptosis.

Cell damage-initiated apoptosis is stimulated by a specific signaling pathway. Cellular checkpoint mechanisms have evolved to monitor cellular damage, and once encountered, a signaling cascade can be initiated which eventually stimulates the apoptotic machinery. Figure 1-1 illustrates the apoptotic machinery, the signaling cascade that regulates it, and its relationship with DNA-damaging chemotherapy. Drugs that directly damage DNA, for example cisplatin (“Pt” in Fig. 1-1), stimulate proteins such as ATM, which activate CHK2 and p53. Separately, microtubule-disrupting drugs (“Tx” in Fig. 1-1) can also lead to activation of p53; p53, in turn, leads to mitochondrial membrane permeabilization (through BAX and BAD). Mitochondrial components like cytochrome c and SMAC (second mitochondrial activator of caspases) are released, leading to activation of the caspase proteases. Caspases, the effector enzymes of
apoptosis, are ultimately responsible for the effects of apoptosis. Caspase activation results in the characteristic apoptotic events: cell blebbing, degradation of nuclear membrane proteins and nuclear DNA-repairing enzymes. DNA fragmentation, one of the hallmarks of apoptosis, is carried out by the Caspase Activated DNase (CAD) endonuclease. CAD, in turn, is activated by Caspase 3.

**Figure 1-1** Drug induced apoptosis pathways. Chemotherapy such as DNA disrupting agents (Pt) or microtubule-interfering drugs (Tx) activate various pro-apoptotic proteins (blue), which are regulated by anti-apoptotic proteins (brown). (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, reference 10, copyright 2003)

In terms of drug-stimulated apoptosis, the process can be considered in three parts: first, a drug-induced cellular insult; second, a signaling pathway connecting this cellular insult to apoptosis activation; and third, irreversible activation of apoptosis (i.e.
caspase activation). Common genetic alterations in glioblastoma mostly disrupt the second phase of this system, i.e. the signaling cascade between a drug’s target and caspase activation. Recently, two independent comprehensive genomic analyses of glioblastoma tumor samples have revealed specific genes or loci that are affected in the majority of GBMs. Specifically, components of three distinct pathways are nearly always affected: the RTK/PI3K/AKT pathway, the p53 pathway, and the RB pathway. Indeed, as each pathway regulates apoptosis (among other processes), it is no surprise that GBMs rapidly acquire resistance to apoptosis and apoptosis-inducing therapy. Figure 1-2 summarizes tumor suppressor genes that are mutated or deleted, and the oncogenes that are amplified or mutated to more activated forms in GBM. Interestingly, both independent analyses indicated that alterations within one pathway tended to be mutually exclusive toward other members of that pathway; i.e., most tumor samples had only one alteration in each pathway (e.g. mutation, amplification, or deletion). Thus, any one of a number of potential alterations within a pathway seem to be functionally equivalent.
Figure 1-2. Common genetic alterations in GBM. Three pathways are nearly always altered in GBM: (a), RTK/RAS/PI3K pathway; (b), p53 pathway; (c), RB pathway. Red indicates activating mutations in oncogenes, blue indicates tumor suppressor genes which are inactivated; darker shades correspond to a higher occurrence. (Reprinted by permission from Macmillan Publishers Ltd: Nature, reference 11, Copyright 2008)

Importantly, each pathway is directly related to apoptosis – multiple converging pathways are deregulated, with each pathway regulating different segments of the apoptosis pathway. For example, Akt inhibits caspase-activated DNAse, and also directly stabilizes MDM2 (which inhibits p53);\textsuperscript{12,13} p53 regulates the transcription of various pro-apoptosis factors as well as directly regulating Bcl-2 proteins.\textsuperscript{14} Finally, RB transcriptionally regulates expression of survivin (an apoptosis inhibiting protein), and it can also directly block MDM2-dependent p53 degradation, among other roles.\textsuperscript{15} Thus,
each of the core pathways that are commonly disrupted in glioblastoma cells exerts regulatory control over various components of apoptosis signaling.

To overcome a cancer cell’s tendency to develop resistance to chemotherapy-induced apoptosis, various research groups are designing therapeutics that directly promote apoptosis. While classical chemotherapeutics trigger apoptosis by activating factors upstream in the apoptotic pathway (e.g. p53, death receptor, ATM, etc.), current efforts are attempting to target the Bcl-2 protein family, a group of structurally related proteins with opposing functions – propapoptotic or antiapoptotic, existing in a tightly regulated state to balance each other’s functions. Under proper signaling, they integrate signals from multiple pathways (including the three discussed above which are frequently mutated in glioblastoma) and can lead to mitochondrial outer membrane permeabilization, resulting in eventual caspase activation.\textsuperscript{16,17} However, if the anti-apoptotic members predominate, progression to apoptosis is limited. As shown in Figure 1-1, the anti-apoptotic Bcl-2 members (e.g. Bcl-2, Bcl-XL, MCL-1) serve to sequester and inhibit the pro-apoptotic members (e.g. BIM, BID, BAX, BAD). Thus, inhibiting the Bcl-2–BAX interaction could relieve the inhibition on BAX, and allow apoptosis to be carried out. Similarly, other investigators are targeting the MDM2–p53 interaction. As MDM2 sequesters and inhibits p53, disrupting this interaction could free p53 and allow it to function as both an inducer of apoptosis and as a cell-cycle regulator.\textsuperscript{18} Of course, for the MDM2 approach to work, the tumor must be wild-type for p53. These approaches are currently being evaluated in clinical trials and are producing promising results.\textsuperscript{16}

While apoptosis has generally been considered the primary form of non-necrotic programmed cell death (and therefore received the most research attention), in recent
years other forms of cell death have been identified which are not dependent on apoptosis. From a therapeutic perspective, a new cell death mechanism provides new cellular targets which can be harnessed for killing tumor cells. In theory, if a cell is resistant to conventional apoptosis-inducing therapy, it may still be sensitive to therapy that induces an unrelated cell death mechanism. With the ultimate goal of killing cancer cells, some research groups are already developing therapeutics to target these pathways.  

A growing list of non-apoptotic cell death mechanisms has been recently identified, including autophagy, necroptosis, ferroptosis, oncosis, mitotic catastrophe, paraptosis, lysosome-membrane permeabilization, entosis, and methuosis. While most of these death pathways are still being characterized, some of them are already being therapeutically targeted and evaluated in preclinical models. Below, I will highlight some of the ongoing work in small-molecule drug development toward stimulating these forms of cell death in tumor cells.

Autophagy, or autophagic cell death, is a putative non-apoptotic cell death pathway that has been most extensively studied. Autophagy (Fig. 1-3) is a process by which cytosolic components and organelles are engulfed into a membranous vesicle, thus forming a double-membraned autophagosome. This autophagosome eventually fuses with lysosomes, forming a degradative autophagolysosome (or autolysosome, Fig 1-3). When this process occurs along with cell death, it has been referred to as autophagic cell death, as the autophagolysosome is thought to degrade cellular components until death is unavoidable. A crucial regulator of autophagy, mTOR, is a known protein target of the small molecule rapamycin (structure shown in Figure 1-4); thus, inhibition of mTOR
(and activation of autophagy) is being studied against cancer cells. However, mTOR regulates multiple downstream pathways, and its anticancer effects when inhibited may not be attributed solely to autophagy.

**Figure 1-3.** Autophagosome formation and maturation. (Reproduced with permission from reference 20, Copyright Massachusetts Medical Society)

In addition to rapamycin, multiple reports also attribute temozolomide’s activity against cancer cell growth to stimulating autophagy. However, this remains unclear. Some have shown that autophagy in cells treated with temozolomide provides additional ATP and is thus protective against the temozolomide treatment;30 while others have shown that autophagy is crucial to temozolomide’s cytotoxic effects.31 Logically, autophagy is considered a tumor-suppressive process, as it protects cells from damage during times of stress, thus preventing potential tumor-initiating chromosomal alterations. Indeed, deficiency of Beclin1 or ATG5 (required for autophagosome formation, thus their deficiency blocks autophagy) leads to chromosomal instability and promotes DNA damage, which is a hallmark of cancer and can lead to tumor initiation and promotion.32 However, in an established cancer, deficiency of autophagy could inhibit tumor progression rather than promote it. Along these lines, recent reports show that autophagy protects tumor cells from the effects of traditional chemotherapeutics, and that inhibition of autophagy acts synergistically with chemotherapy to limit the cancer cell growth.33-35
Thus, though there is a considerable body of literature arguing that induction of autophagic cell death can be harnessed to kill tumor cells, the current consensus is that autophagy is generally a cytoprotective process, both for healthy and cancerous cells.  

Figure 1-4. Structures of compounds that induce various forms of cell death (described in text). (a) rapamycin, (b) temozolomide, (c) shikonin, (d) sanguilutine, (e) 3-nitrofluoranthene, (f) necrostatin, (g) Kahalalide F, (h) siramesine.

Other cell death mechanisms less represented in the literature have also been targeted to induce cancer cell death. Necroptosis is a cell death process with similar
characteristics to necrosis, yet can be induced by specific ligands (Tumor Necrosis Factor, TNF; Fas Ligand, FasL) which transmit signals through specific kinases (RIP1 and RIP3), resulting in cell death that is morphologically similar to necrosis. Downstream events between RIP1/RIP3 activation and ultimate cell death have not been well characterized. Necroptosis is best understood as a cell’s alternative to apoptosis for carrying out programmed cell death in response to stress signals, as the two processes are tightly interregulated (Figure 1-5). Normally, when caspase-8 is activated, in addition to activating apoptosis, it blocks the RIP1/RIP3 complex – thus blocking necroptosis. However, when caspase-8’s activity is attenuated due to formation of a heterodimer with FLIP (indicative of a healthy, stress-free scenario), it is unable to activate apoptosis – yet it still maintains residual activity, enough to cleave RIP1/RIP3, and block necroptosis. Finally, if caspase-8 completely lacks function or is inhibited (by mutation, splice variant FLIP, inhibition, or pharmacologically), or if RIP3 is activated, necroptosis is stimulated. Ultimately, cells seem to have evolved a process to principally undergo apoptosis in times of stress. However, if the apoptotic caspase-8 is defective, necroptosis may serve as a backup approach, and this could be harnessed therapeutically as another way to induce cell death in cancer cells.

**Figure 1-5.** Interplay between caspase-8, apoptosis, and RIP1-mediated necroptosis. (a) Caspase-8 homodimerization leads to its full activation, stimulating apoptosis; actived caspase-8 also inhibits RIP1, blocking necroptosis. (b) In some cells, a FLIP–caspase-8
heterodimer inhibits caspase-8 from activating apoptosis, but the complex can still block RIP1 – thus, blocking necroptosis. (c) Various methods of complete caspase-8 inactivation release RIP1 inhibition, activating necroptosis. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, reference 19, copyright 2012)

Some cytotoxic compounds currently in preclinical studies are thought to work by activation of necroptosis (structures shown in Figure 1-4). Shikoni is a natural product that is active against cell lines that are resistant to numerous apoptosis-inducing therapies. Another natural product, sanguilutine, induces necroptosis in melanoma cells. Finally, 3-nitrofluoranthene has been shown to induce necroptosis in liver carcinoma cells. While the exact mechanism of action of these compounds is not yet understood, their activities are all blocked by necrostatin, an inhibitor of RIP1 kinase, which blocks necroptosis. Thus, they were not designed or selected to specifically target necroptosis, but have serendipitously been identified to act via the necroptotic pathway. With a more thorough understanding of the necroptosis signaling cascade, new compounds may be designed to specifically target necroptosis mediators to better stimulate the process.

Lastly, others are developing inducers of lysosomal membrane permeabilization (LMP), a cell death mechanism by which lysosomal membrane integrity is compromised, leaking its lysosomal proteases (cathepsins) into the cytoplasm to degrade vital cell proteins and lead to cell death. For example, siramesine has been reported as an inducer of cell death by lysosomal membrane permeabilization, and its effects were not limited by attenuating apoptosis, via overexpression of Bcl-2 or caspase inhibition. Another inducer of LMP, Kahalalide F, has shown potent activity against multiple cancer cell lines and is already entering clinical trials for some cancers.
Clearly, there are a number of research programs focused on targeting various forms of non-apoptotic cell death to treat aggressive, apoptosis-resistant cancers. In recent years, our lab has emerged as a member of this field by identifying a new form of non-apoptotic cell death, methuosis, as well as small molecules that can induce methuotic cell death. The remainder of this dissertation will describe our studies of methuosis.

1.2 Methuosis: A Novel Form of Non-Apoptotic Cell Death

Recent work in our laboratory has elucidated a previously unidentified form of non-apoptotic cell death. Efforts were originally focused on the paradoxical roles of Ras proteins in inducing cell death, versus its classical oncogenic functions. The Ras GTPases have long been appreciated as inducers of cell growth and proliferation, wherein their activity correlates with tumor progression. This activity has been largely ascribed to its activation of both the Raf→MEK→ERK and PI3K/Akt pathways, leading to increased transcription of pro-growth and anti-apoptotic genes. Indeed, the ras gene family is mutated to a constitutively activated form in numerous cancers, such as colon, pancreatic, bladder, and leukemia.

However, there is scattered evidence that Ras can also lead to cell death in certain instances. Our lab became particularly interested in reports of activated Ras inducing cell death in glioblastoma and gastric carcinoma cells by a non-apoptotic mechanism that was originally described as autophagic cell death. Based on the observation that glioblastoma and gastric carcinoma rarely exhibited mutated ras, a hypothesis emerged that Ras proteins may have novel antiproliferative roles in these cell types. Indeed,
expression of oncogenically mutated ras led to cell death in glioma and gastric cancer cell lines.49 Interestingly, this was considered a non-apoptotic form of cell death, as it did not exhibit hallmarks of apoptosis and was not blocked by caspase inhibition or Bcl-2 overexpression. These cells also displayed a large degree of cytoplasmic vacuole formation, and these were considered to be autophagic vesicles which contributed to the cell death; thus, it was considered an induced form of autophagic cell death.

Our lab began further studying the signaling pathways between Ras and the activation of non-apoptotic cell death in glioblastoma cells. Expression of activated Ras(G12V) induced numerous cytoplasmic vacuoles and led to reduced cell viability (Fig. 1-6). It became apparent that non-classical Ras signaling pathways were involved, as reducing activation of the Raf→MEK→ERK and PI3K/Akt pathways did not attenuate the vacuole formation or cell death.50 Subsequent studies clarified that Ras-induced cell death was mediated through the Rac1 GTPase and relied on Rac1’s indirect inactivation of the protein Arf6.28,51 In addition, the characteristic vacuoles were shown to be derived from macropinosomes, rather than autophagosomes. Thus, the cells were undergoing a form of hyperstimulated macropinocytosis (cell drinking) that led to dysregulated metabolism and compromised membrane integrity. This form of cell death was termed “methuosis” (from the Greek word methuo, to drink to intoxication).28
Figure 1-6. U251 glioblastoma cells expressing activated H-Ras. Left, wild-type U251 cells. Right, U251 cells expressing activated H-Ras(G121V) with extensive cytoplasmic vacuoles characteristic of methuosis. (Reprinted with permission, from reference 28)

Importantly, methuosis was shown to be unique from apoptosis. Cells undergoing methuosis were not rescued by caspase inhibition, nor did they exhibit hallmarks of apoptosis, such as condensed chromatin or DNA fragmentation. Moreover, inhibition of autophagy (via knockdown of beclin-1) did not block the vacuoles or improve cell viability, further arguing against the autophagic origin of the vacuoles. Thus, stimulating cell death by methuosis leads to a form of non-apoptotic cell death, which would be an attractive therapeutic approach for cancers that are highly resistant to apoptosis, like glioblastoma. However, in order to translate this phenotype into a therapeutic option, a druglike small molecule that could induce methuosis is necessary, as overexpressing activated forms of Ras or Rac are clearly not viable therapeutic options.

With the goal of identifying compounds that induce methuosis, our lab was drawn to a report on lysosome-dependent membrane resealing. The authors carried out a phenotypic screen in search of small molecules that block lysosomal exocytosis to the cell membrane. They identified a class of triazine-substituted compounds, termed vacuolins (e.g. Vacuolin-1 in Figure 1-7), which lead to accumulation of numerous lysosome-derived vacuoles due to inhibition of lysosome-to-membrane trafficking.
Importantly, the vacuolins did not induce apoptosis or reduce cell viability, so methuosis clearly wasn’t involved. However, in the manuscript’s supporting information, three additional compounds that were structurally unique from the vacuolins were also identified simply as “vacuole generating compounds” (Figure 1-7, compounds a–c).

![Figure 1-7. Vacuole inducing compounds. Vacuolin-1 induces vacuoles by blocking lysosomal resealing, without affecting cell viability. Compounds a–c, reported as vacuole generating compounds, were evaluated for methuosis inducing activity. While a and b were inactive, compound c induced methuosis. Analog d (MIPP) also induces methuosis.](image)

Of these compounds, when tested in our glioblastoma cells at low micromolar concentrations, compound c induced a seemingly identical phenotype to the Ras- and Rac-induced methuosis previously described (Figure 1-7).53 A search of the Chembridge compound library provided a structural analogue that was slightly more efficacious at inducing methuosis, and was used in further studies (Figure 1-7 compound d). This compound was termed “MIPP” based on its chemical name, 3-(2-Methyl-1-H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one.

At a concentration of 10 µM, MIPP induced overwhelming cytoplasmic vacuolization, morphologically very similar to the Ras- and Rac-induced vacuoles. Next,
the origin of the MIPP-induced vacuoles was evaluated. Live cell imaging with a fluid-phase tracer, Lucifer yellow, confirmed that the vacuoles were derived from macropinosomes and contained extracellular derived fluid. Other compartments that might be associated with the vacuoles were subsequently ruled out (Figure 1-8). There was no overlap between the vacuoles and compartments labeled by either Magic Red (a cathepsin substrate) or Lysotracker Red, which label lysosomes based on their enzymatic activity or acidic pH, respectively. Moreover, the vacuoles were not labeled by markers for the endoplasmic reticulum (ER-tracker) or the mitochondria (MitoTracker Red).53
Figure 1-8. Origins of MIPP-induced vacuoles. MIPP-treated U251 cells were cotreated with the indicated tracer or organelle marker. (Reprinted with permission from reference 53)

The vacuoles were further characterized based on their surface markers that are associated with specific stages of endosomal trafficking. As shown by fluorescence microscopy in Figure 1-9, the vacuoles contained the late endosomal marker, Rab7-GFP, and displayed strong labeling by an antibody against LAMP1. However, the vacuoles were not labeled by the autophagosome marker, LC3II, or markers of other stages of the
endosomal trafficking cycle, such as early endosome (EEA1) or recycling endosome (Rab11). MIPP thus induces a rapid formation of macropinosome-derived vacuoles that are related to late-endosomes, but their progression further along the trafficking cycle is seemingly halted.
Figure 1-9. Surface markers on methuosis vacuoles. Vacuoles were labeled with GFP-Rab7 and analyzed by immunofluorescence for the indicated proteins. (Reprinted with permission from reference 53)
As mentioned above, Ras-induced methuosis is known to function through a Rac1-mediated reduction in the activation of another GTPase, Arf6. In MIPP treated cells, however, there was no significant change in the activation of Rac1 or Arf6. Thus, it seems that while MIPP induces a similar phenotype to activated Ras and Rac, it is interfering with a target downstream of Ras and Rac. This is supported by the observation that EHT1864, a Rac inhibitor, failed to rescue cells from MIPP treatment, which is in contrast to EHT1864’s inhibition of Ras- and Rac-induced methuosis.

Two more GTPases that are known to specifically mediate endosomal trafficking were next evaluated, Rab 5 and Rab7. MIPP induced a significant decline in active Rab5, while simultaneously inducing an increase in active Rab7. In further evaluating Rab5 and Rab7, expression of constitutively activated Rab5 does not block the effects of MIPP. Similarly, expression of a dominant-negative Rab7 did not block MIPP’s effects. Thus, it seems the changes in the Rab5 and Rab7 pools are effects of MIPP treatment, rather than direct targets of the compound.

Finally, the response of glioblastoma cells to MIPP in the context of cell viability and apoptosis was evaluated (Figure 1-10). As shown in the colony forming assay, cells that had been treated with 10 µM MIPP for 2 days displayed markedly reduced viability (Figure 1-10 A). In agreement with Ras-induced methuosis, the cells’ response to MIPP was not dependent on activation of apoptosis and typical hallmarks of apoptosis were not observed (Figure 1-10 B–D). By electron microscopy, the vacuoles were empty, devoid of any cellular contents or organelles. Moreover, the vacuoles were surrounded by a single, not double membrane. These observations are in contrast with the characteristics of autophagic vacuoles. Moreover, the MIPP-treated cells did not stain positive for
TUNEL, ruling out nucleosomal DNA fragmentation that is characteristic of apoptosis. Finally, when caspase enzymes were effectively inhibited (as evidenced by the lack of PARP cleavage in Figure 1-10 D), cell viability in response to MIPP was essentially unaffected (Figure 1-10 D). Overall, these results indicate that MIPP reduces U251 cell viability by a mechanism independent from apoptosis.

Figure 1-10. MIPP-induced methuosis and non-apoptotic cell death. (A) U251 cells were treated with 10 μM MIPP or DMSO (control) for 2 days and assayed by colony formation. Mean ± SD of triplicate dishes, with representative dishes shown. * p < 0.0001. (B) U251 cells were examined by electron microscopy after two days of treatment with 10 μM MIPP; “att” represents an attached cell, “det” represents a cell detached from the dish. Arrows point to regions of plasma membrane discontinuity indicative of cell rupture. Nuclei (N) do not show changes in chromatin distribution typical of apoptosis. Scale bar equals 10 microns. (C) U251 cells treated with 10 μM MIPP for 2 days are negative for TUNEL stain. (D) Inhibition of caspase activity with zVAD does not prevent MIPP-induced loss of cell viability. Western blot confirms zVAD activity by the lack of PARP cleavage. Values are means ± SD of quadruplicate samples. (Reprinted with permission from reference 53)
The next parts of this dissertation describe the further study of small-molecule induced methuosis. Chapter 2 describes the synthesis of indole-based chalcone analogs of MIPP to evaluate the structure-activity relationships of methuosis-inducing molecules. Chapter 3 describes efforts to develop essential chemical tools to identify the cellular target(s) of compounds that induce methuosis, involving a combination of synthetic chemistry and biochemical approaches. Finally, chapter 4 outlines subsequent experiments that will continue this work, and ultimately validate the target identification efforts, as well as directions to further improve the feasibility of MIPP-type compounds toward use as cancer therapeutics.
Chapter 2

Synthesis and Evaluation of Indole-Based Chalcones as Inducers of Methuosis, a Novel Type of Non-Apoptotic Cell Death.*

2.1 Introduction

Despite recent advances in developing therapeutic agents that target regulatory pathways unique to certain types of cancer cells, the mainstays for post-surgical adjuvant therapy of many tumors remain radiation and DNA alkylating agents. An example is the highly malignant brain tumor, glioblastoma multiforme (GBM), where the current standard of care involves surgery when possible, followed by adjuvant therapy with radiation and oral temozolomide (TMZ). A limitation of the latter approaches is that they work by damaging DNA, triggering the intrinsic apoptotic pathway. Since GBM cells typically harbor mutations in tumor suppressor genes (e.g., PTEN, p53, pRB), they are relatively insensitive to apoptotic stimuli. Moreover, glioblastoma cells develop

resistance to alkylating agents by increasing their capacity to repair DNA lesions. We believe that it may be possible to develop new approaches to treat such drug resistant cancers through the induction of alternative non-apoptotic forms of cell death, which do not depend on DNA damage as a trigger. Toward this end, we have defined a unique form of cell death termed ‘methuosis’. The hallmark of methuosis is the displacement of much of the cellular cytoplasmic space by vacuoles derived from macropinosomes. The latter are formed when membrane ruffles (lamellipodia) enclose pockets of extracellular fluid and are internalized. In methuosis, impairment of the recycling and lysosome-directed trafficking of macropinocytotic vesicles locks them in an intermediate stage where they fuse to form progressively larger vacuoles. This eventually causes a decrease in metabolic activity and rupture of the cell membrane. Death is considered to be non-apoptotic, as it is not accompanied by nuclear chromatin condensation, cell blebbing, or nucleosomal DNA fragmentation. Methuosis is also caspase-independent, as it cannot be prevented by broad-spectrum caspase inhibitors such as zVAD-fmk.

Methuosis was initially characterized in GBM cells, where this form of cell death was triggered by ectopic expression of activated Ras and Rac GTPases. However, the potential for exploiting this non-conventional cell death pathway to kill cancer cells that are refractory to apoptosis depends on the identification of molecules with druglike properties that can induce methuosis. We recently described a prototype chalcone-related compound that can induce cell death with the hallmarks of methuosis in both TMZ-resistant and non-resistant GBM cells, as well as other cancer cell lines derived from breast, colon, and pancreas. Herein we report synthesis and structure-activity relationship (SAR) studies of a directed library of related compounds leading to: 1) the
identification of key structural features required for methuosis-inducing activity; 2) the identification of a derivative with improved biological activity; and 3) insight toward the design of chemical tools that can be used as probes to identify the molecular target of methuosis-inducing compounds.

2.2 Results

2.2.1. Structure-Activity Relationships (SARs)

![Structures of compounds](image)

**Figure 2-1.** Structures of compounds 1 and 2, initially found to be active inducers of methuosis as compared with commercially available analogues 3-8, which failed to induce the hallmarks of methuosis in cultured U251 GBM cells.

As we began to seek druglike small molecules that could trigger methuosis, we noted a report from Kirchhausen and colleagues\(^5\) in which they described a molecule termed vacuolin-1, along with several other triazine-based compounds, that were capable of inhibiting Ca\(^{2+}\)-dependent lysosomal exocytosis. Although these compounds induced marked cellular vacuolization, they did not cause cell death. However, in the Supporting Information included with their report, a structurally distinct vacuole-inducing compound captured our attention because of its resemblance to a class of molecules termed
chalcones. The structure of this compound is depicted in Figure 2-1 (compound 1). Chalcones consist of a 1,3-diphenyl-2-propen-1-one framework that serves as a precursor for flavonoid natural products. However, the term chalcone has been applied more broadly to describe a number of synthetic derivatives built on this framework. Several chalcones have been found to have significant anticancer activity but have not been reported to induce vacuolization of cells. This prompted us to ask whether compound 1 might represent a novel type of chalcone that can kill cancer cells by inducing methuosis. We found that compound 1 caused extensive vacuolization of glioblastoma cells within a few hours and substantial loss of cell viability within 48 h. Chemical database searches yielded additional compounds with >75% similarity to 1. Among these, compound 2 (Figure 2-1) was selected for further study. It induced vacuoles that were larger and more numerous than those produced by compound 1. Compound 2 was assigned the acronym MIPP, that is, 3-(2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one. Detailed evaluation of the biological effects of MIPP indicated that the form of cell death induced by this compound matched the profile of methuosis. Furthermore, MIPP was effective in reducing the growth and viability of GBM cells that were highly resistant to TMZ.

Because concentrations of MIPP ≥ 10 µM were required to effectively induce methuosis, we began to assemble a directed library of MIPP-related compounds and conducted initial SAR comparisons with the goal of identifying analogues with improved potency. We began by comparing the methuosis-inducing activity of MIPP with several closely related compounds that were commercially available (compounds 3 – 8 in Figure 2-1). The latter did not trigger cellular vacuolization when added to U251 GBM cells at a
concentration of 10 µM. Examination of compounds 1–8 (Figure 2-1) suggested that the specific relationship between an indole and a pyridinyl ring was likely to be playing a significant role in determining activity. Specifically, comparison of compound 1 (active) with compound 7 (inactive) demonstrated the importance of the pyridinyl ring, because replacement of the latter with a para-methoxy phenyl ring rendered the compound inactive. Therefore, an initial set of analogues was synthesized to investigate the influence of the position of the pyridine nitrogen on biological activity.

**Figure 2- 2. Synthesis of analogs of MIPP (compound 2).**

Analogues based on the α,β-unsaturated ketone core can be prepared by Claisen–Schmidt condensations between indole-3-carboxaldehydes and aryl ketones.\(^{63} \)

Condensation of acetophenone or various acetyl-pyridines with indole-3-carboxaldehyde yielded compounds 9-12 (Figure 2-2A). The activities of these compounds were compared to MIPP at a concentration of 10 µM using three criteria: (1) morphological...
vacuolization of live cells assessed by phase contrast microscopy at 24 and 48 h; (2) cell viability at a 48 h end point assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, with fresh compound added after the first 24 h; and (3) colony-forming assays (2 week end point) performed on cells exposed to the compounds for 48 h. The results of this analysis (Table 2.1) indicated that a para-nitrogen orientation of the pyridine ring is a key feature required for activity. The ortho and meta analogues 10 and 11, as well as the acetophenone analogue 9, were all relatively ineffective at inducing methuosis as compared to MIPP. In contrast, removal of the 2-methyl from the indole ring of MIPP (compound 12) reduced but did not eliminate activity.

We next explored the consequences of functionalizing the 5-position of the indole ring using commercially available 5-methoxy and 5-benzyloxy indole-3-carboxaldehydes to prepare compounds 13 and 14, respectively. The 5-methoxy compound 13, in turn, was demethylated with BBr3 to afford the 5-OH compound 15 (Figure 2-2B). As shown in Table 2.1, the activities of compounds 13 and 14 were similar to MIPP when compared by colony-forming assay and cell morphology, although they were not as cytotoxic in the short-term growth/viability assay. In contrast, the 5-OH derivative, compound 15, exhibited greatly reduced biological activity in all of the assays. To confirm that the location of the pyridine nitrogen was still crucial for activity of the 5-methoxy-substituted compounds, analogues 16 and 17 were generated. Loss of activity confirmed the necessity of the para-nitrogen (Table 2.1).
Table 2.1. Summary of SAR Studies Done on MIPP (Compound 2) and Related Compounds Generated in Figures 2-2 and 2-3. *Results are expressed as percent of controls that received vehicle alone (DMSO). Values are the mean ± SD of quadruplicate (MTT) or triplicate (colony formation) determinations (colony formation assay was not done on compound 58).

<table>
<thead>
<tr>
<th>cmpd #</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Ar</th>
<th>Viability (MTT*)</th>
<th>Colony Formation*</th>
<th>Vacuoles?</th>
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<tr>
<td>2 (MIPP)</td>
<td>H</td>
<td>CH₃</td>
<td>H</td>
<td></td>
<td>24 ± 3</td>
<td>8 ± 4</td>
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<tr>
<td>9</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td></td>
<td>109 ± 7</td>
<td>76 ± 7</td>
<td>no</td>
</tr>
<tr>
<td>10</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td></td>
<td>96 ± 9</td>
<td>53 ± 10</td>
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<td>H</td>
<td>H</td>
<td></td>
<td>72 ± 6</td>
<td>71 ± 10</td>
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<td>H</td>
<td>H</td>
<td>H</td>
<td></td>
<td>58 ± 7</td>
<td>22 ± 2</td>
<td>yes</td>
</tr>
<tr>
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<td>H</td>
<td></td>
<td>58 ± 7</td>
<td>2 ± 1</td>
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<td>H</td>
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<td>56 ± 4</td>
<td>8 ± 2</td>
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<td>H</td>
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<td>107 ± 22</td>
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<td>H</td>
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<td>43 ± 17</td>
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<tr>
<td>58</td>
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<td>CH₃</td>
<td>H</td>
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<td>107 ± 8</td>
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</table>

Because comparisons of compounds 12–15 versus MIPP suggested that modifications at the 5- and 2-positions of the indole ring both affect activity, we
generated a 2-methyl-5-methoxy analogue starting from commercially available 2-methyl-5-methoxy-indole. Key intermediate 18 was synthesized by a Vilsmeier-Haack formylation of 2-methyl-5-methoxyindole, followed by coupling with 4-acetyl-pyridine to yield 19 (Figure 2-2C). The inhibitory activity of this compound exceeded that of MIPP in both the MTT viability assay and the colony formation assay (Table 2.1).

Alternatively, methylation of the indole-nitrogen of compound 13 with NaH/CH$_3$I in dimethylformamide (DMF) (Figure 2-2D) to yield 20 produced a compound that induced some cytoplasmic vacuolization but had only modest effects on cell viability (Table 2.1). Thus, after comparing compounds 13, 19, and 20, it became clear that the optimal activity is achieved when the 1- and 2-positions of the indole are occupied by H and methyl, respectively. The 5-OH compound 21, made by BBr$_3$ demethylation of 19 (Figure 2-2B), showed a marked reduction in activity as compared to the 5-methoxy compound 19 (Table 2.1), consistent with the detrimental effect of the 5-OH previously observed in compound 15.

One of the limitations for using MIPP under physiological conditions is its sparing solubility in aqueous solutions. Therefore, we explored the effects of modifying the 5-position of the indole with a group that would add polarity at physiological pH. Because our previous SAR studies showed that there was some flexibility at the 5-position of the indole ring (compare compounds 13 and 14), we designed an analogue of 14 that added charge and also included a methyl at the indole’s 2-position. We envisaged that the OH analogue 21 could be alkylated with methyl-4-(bromomethyl)benzoate, which could then be hydrolyzed to its acid to provide a highly water-soluble analogue at pH 7.4. However, we were somewhat surprised that no appreciable amount of product
could be produced by direct alkylation of 21. Multiple bases were screened with varying $pK_a$ values, from $K_2CO_3$ and $Cs_2CO_3$, as well as triethylamine, tetramethylguanidine, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and NaH. In all reactions, alkylation at the pyridine nitrogen was observed. Stronger bases such as TMG and NaH, even using 1 equiv, produced appreciable indole-N-alkylation as well as pyridine and indole-OH alkylation. The yield of singly alkylated product at the 5-indole position was negligible. Thus, a new route was designed by functionalizing the indole before introduction of the pyridine moiety (Figure 2-3). Commercially available 2-methyl-5-methoxy-indole was demethylated with BBr$_3$ to provide 22. Alkylation with 4-methyl-(bromomethyl)-benzoate under phase-transfer conditions afforded mono-O-alkylated product 23. After formylation with POCl$_3$/DMF, intermediates 24 and 25 were independently prepared. Workup in mild base (NaHCO$_3$) produced the ester 24, while workup in 5 N NaOH gave acid 25. Condensations with acetyl-pyridine provided the corresponding targets 26 and 27. However, testing in glioblastoma cultures revealed that neither 26 nor 27 induced methuosis (Table 2.1).

**Figure 2-3.** Analogues with 5’ modifications of the indole ring generated by functionalizing the indole prior to introduction of the pyridine moiety.

### 2.2.2. Biological Activity of Compound 19 (MOMIPP) vs. Compound 2 (MIPP)

The foregoing studies identified compound 19 as the most potent for inducing methuosis. Hereafter, we refer to this compound by the acronym “MOMIPP” for 3-(5-
methoxy-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one. Superior activity of MOMIPP versus compound 2 (MIPP) was confirmed in studies with U251 glioblastoma cells, using MTT viability assays, cell growth assays, morphological assessment, and colony-forming assays to compare MOMIPP and MIPP. Figure 2-4 shows the dose-response curves for the effects of the drugs on cell viability. Each compound was added at the indicated concentration for 2 days, with medium and compounds replenished after the first day.

**Figure 2-4.** Comparison of the effects of MOMIPP (compound 19) an MIPP (compound 2) on growth and viability of U251 GBM cells. (A) One day after plating the cells in 96-well dishes, MOMIPP (●) or MIPP (■) was added at the indicated concentrations. Controls consisted of cells in parallel wells treated with an equivalent volume of vehicle (DMSO). Medium containing fresh compound was added after 24 h, and MTT assays were performed after a total 48 h of treatment. Each point represents the mean ± SD from four separate wells, with the results expressed as percent of the mean of the parallel control wells. (B) U251 cell sseeded in parallel 35 mm dishes were treated with MOMIPP (●), MIPP (■), or an equivalent volume of DMSO (▲), and cells were harvested for counting on each of three consecutive days. Each point is a mean ± SD from three separate cultures (Assay performed by Jean Overmeyer, PhD).

The relative IC$_{50}$ for MOMIPP was 1.9 µM versus 4.8 µM for MIPP. To obtain a measure of the comparative duration of activity for each compound, their effects on cell growth and survival were assessed by counting the number of cells in parallel cultures treated for three consecutive days with 2.5, 5, or 10 µM compound (Figure 2-4B). Unlike the viability studies, in these experiments, the compounds were added at the beginning of the study and were not replenished for the duration. Under these conditions, MOMIPP was
clearly more effective than MIPP in reducing cell growth and viability. The reduction of cell number in the cultures treated with MOMIPP coincided with massive early
**Figure 2- 5.** Comparison of the abilities of MOMIPP and MIPP to induce the morphological hallmarks of methuosis. One day after plating, U251 GBM cells were treated with MOMIPP or MIPP at final concentrations of 2.5 (A) or 10 μM (B). Controls received an equivalent volume of vehicle (DMSO). Cells were observed by phase contrast microscopy on three sequential days after addition of the compounds, without changing the medium or replenishing the compounds. Methuosis is characterized by extensive accumulation of phase-lucent cytoplasmic vacuoles, with eventual cell rounding and detachment from the substratum as viability is compromised (Assay performed by Jean Overmeyer, PhD).

Vacuolization of the cells and loss of nonviable cells from the substratum (Figure 2-5A,B). In contrast, the cells treated with MIPP initially underwent vacuolization on days 1 and 2 but tended to recover, especially at the 2.5 μM concentration (Figure 2-5A). These studies demonstrate that a single application of MOMIPP has a much more sustained effect than MIPP on cell morphology and cell viability. The difference between MOMIPP and MIPP was underscored when colony-forming assays were used to evaluate proliferative capacity and long-term cell viability (Figure 2-6). MOMIPP was clearly more effective than MIPP in reducing colony formation when cells were treated for 2 days (Figure 2-6A). If treatment was shortened to just 4h, MOMIPP was still more effective than MIPP, but higher concentrations of both compounds were required to reduce colony formation (Figure 2-6B).
Figure 2-6. Comparison of the abilities of MOMIPP and MIPP to inhibit survival of U251 GBM cells in colony-forming assays. (A) Cells were plated for colony-forming assays as described in the Experimental Section. One day after plating the cells, MOMIPP (●) or MIPP (■) was added to the medium at the indicated concentrations, and cells were maintained in the presence of the compounds for 48 h. Thereafter, the compounds were removed, and colonies >50 cells were counted after 2 weeks. Each point represents the mean ± SD from three separate dishes, with the results expressed as percent of the mean of the parallel control dishes containing DMSO. (B) The effects MOMIPP (●) and MIPP (■) on colony formation were compared as in panel A, except that cells were exposed to the compounds for only 4 h instead of 48 h (Assay performed by Ashley Young)

To determine if the methuosis-inducing compounds might be effective for targeting TMZ-resistant glioblastoma, we used a previously generated TMZ-resistant U251 cell line that was essentially unaffected by concentrations of TMZ as high as 100 µM²³ (Figure 2-7A). As shown in Figure 2-7B, both MIPP and MOMIPP were able to induce methuosis and reduce cell viability in the TMZ-resistant cells, but the efficacy of
MOMIPP was clearly superior to MIPP. The ability of MOMIPP to kill drug-resistant tumor cells by meethuosis extends beyond glioblastoma. For example, we observed similar MOMIPP-induced vacuolization and loss of colony-forming ability in both parental and doxorubicin-resistant MCF7 breast cancer cells (Figure 2-7C,D). To determine if the concentration of MOMIPP that is maximally toxic in cancer cells might also be cytotoxic to normal cells, we treated human mammary epithelial cells (HMEC) with 10 µM MOMIPP. As shown in Figure 2-7E, HMECs underwent extensive cytoplasmic vacuolization within 24 h after addition of MOMIPP. By 48 h, cell viability was reduced approximately 40% as compared with a 70% reduction seen in MCF7 breast carcinoma cells treated for the same period at a similar cell density (Figure 2-7F). To further assess the effects of MOMIPP on normal cells, we applied the compound to human skin fibroblasts. As in all of the other cell lines tested, 10 µM MOMIPP induced obvious vacuolization in the fibroblasts by 24 h (Figure 2-7G). Similar to the HMECs, normal fibroblasts plated at subconfluent density to maintain exponential growth during exposure to MOMIPP exhibited a 40% decline in viability by 48 h. However, when the fibroblasts were plated at a high initial density, so that they would be in stationary phase at the time that MOMIPP was added, cell viability was essentially unaffected (Figure 2-7H), even though the cells underwent vacuolization (not shown). These observations suggest that although MOMIPP can be cytotoxic to normal cells, the latter are somewhat less sensitive to the compound than the cancer cell lines, U251 and MCF7. A possible explanation for this is suggested by the differential effects of MOMIPP on subconfluent versus confluent fibroblasts, which imply that the disruptions of endosomal trafficking
typified by vacuolization may be relatively well tolerated in cells that are not dividing as compared with cells that are actively proliferating.
Figure 2-7. MOMIPP effectively inhibits the viability of drug-resistant GBM and breast cancer cells. (A) TMZ-resistant U251 glioblastoma cells (U251-TR) were derived as described previously. The graph shows that in contrast to the parental U251 cells, the viability of U251-TR cells is not reduced by treatment with TMZ. (B) The U251-TR cells were treated with the indicated concentrations of MIPP or MOMIPP for 48 h and then subjected to colony-forming assays as described in the Experimental Section. Each point is based on colony counts from three dishes (mean ± SD), with the results expressed as percent of the mean from parallel control dishes treated with an equivalent volume of vehicle alone (DMSO). (C) Parental and doxorubicin-resistant (DoxR) MCF-7 breast cancer cells were treated with 10 µM MOMIPP for 24 h and then examined by phase-contrast microscopy. (D) Long-term viability of parental or DoxR MCF-7 cells was assessed by colony-forming assay after a 48 h of treatment with the indicated concentrations of MOMIPP. The results are the mean ± SD of determinations performed on three parallel dishes. (E) Normal HMECs were treated with 10 µM MOMIPP or an equivalent volume of DMSO (control) and examined by phase-contrast microscopy after 24 h. (F) MCF-7 cells or HMECs were plated in 96-well plates. After 48 h, while the cells were still subconfluent, fresh medium containing 10 µM MOMIPP or an equal volume of vehicle (DMSO) was added. MTT viability assays were performed at a 48 h end point. Values are the means (±SD) from four separate wells. (G) Normal human skin fibroblasts were treated with 10 µM MOMIPP or an equivalent volume of DMSO (control) and examined by phase-contrast microscopy after 24 h. (H) Normal human fibroblasts were seeded in 96-well plates at subconfluent density (1000 cells/well) or confluent density (10000 cells/well). After 24 h, fresh medium containing 10 µM MOMIPP or an equal volume of vehicle (DMSO) was added. MTT viability assays were performed at a 48 h end point. Values are the means (±SD) from four separate wells (Assay performed by Ashley Young (A,B) and Jean Overmeyer, PhD (C–H)).

2.3 Discussion

Methuosis is a newly discovered form of nonapoptotic cell death that is triggered by alterations of macropinocytotic vesicular trafficking, resulting in massive cellular vacuolization and loss of cellular metabolic integrity. We recently described MIPP as a chalcone-like small molecule that is capable of inducing methuosis in GBM and other cancer cell lines. Here, we present SAR studies that have led to the identification of a 5-methoxy analogue termed MOMIPP, which demonstrates improved potency and stability in cell culture systems.
In the course of generating a directed library of compounds for SAR studies, we carried out Claisen-Schmidt condensation reactions between various indole-3-carboxaldehydes and aromatic ketones catalyzed by piperidine, which efficiently provided indole-chalcones. As piperidine acts as a catalyst, we initially carried out the aldol condensations in the presence of catalytic amounts of piperidine. However, we generally observed higher yields when piperidine was used in excess. In almost all instances, the products precipitated from solution and were thus easily purified from excess catalyst (or starting material) by simple rinsing.

Our SAR studies provided several useful insights into the molecular features required for the methuosis-inducing activity of this class of compounds. First, regarding the indole ring, methylation of the nitrogen (compound 20) or removal of the 2-methyl group (compound 13) reduced but did not eliminate activity. At the 5-position, different modifications had opposite consequences. The activity of the 5-methoxy (19, MOMIPP) was substantially improved, and the 5-benzyl (compound 14) was similar to unsubstituted MIPP. In contrast, modification of the 5-position with OH (compound 21), p-methyl ester benzyloxy (compound 26), and p-COOH benzyloxy (compound 27) all caused a major loss of activity. Together, these findings demonstrate that there may be some flexibility at these positions on the indole ring for future attempts to further improve potency, create more water-soluble derivatives, or tether the compound to an affinity matrix. Regarding the second aryl system of the chalcone framework, we learned that the para-nitrogen orientation of the pyridine ring is critical for activity. Analogues with a 2-pyridine (compound 10), 3-pyridine (compounds 11 and 16), pyrazine (compound 17), or phenyl (compound 9) were inactive.
In considering the potential mechanisms through which MOMIPP induces cell death by methuosis, one possibility is that it could be acting as an electrophile (i.e., Michael acceptor). Compounds possessing electrophilic moieties that render them potential substrates to cellular nucleophiles are not often used in drug design because they can arbitrarily modify many biomolecules. This can lead to off-target effects, including the formation of immunoreactive hapents. The SAR studies summarized in Table 2.1 show that while many of the compounds in our series possess the α,β-unsaturated ketone scaffold and could act as putative Michael acceptors, only MOMIPP and a few other compounds were effective inducers of methuosis at micromolar concentrations. Thus, it seems unlikely that MOMIPP is inducing cell death via general electrophilic modification of proteins. It remains possible that MOMIPP may be functioning as a target-specific Michael acceptor, with certain features of the molecule mediating association with a specific protein, where proximity of the α,β-unsaturated ketone to nucleophilic residues (e.g., cysteine) may then promote covalent modification. Examples of such protein-specific modifications have been reported for compounds that bind to the receptor protein ErbB2, the TRPA1 ion channel, the nuclear protein KSRP/FUBP2, and sortase enzymes in Gram-positive bacteria. These types of observations have spurred a general resurgence of interest in drugs that work by covalent modification of their specific targets.

Compounds broadly classified as chalcones have been shown to exhibit anticancer activity through multiple mechanisms, including disruption of p53 interactions with MDM2, inhibition of p-glycoprotein, and disruption of microtubule polymerization. Many of the latter compounds were designed as analogues of
colchicine and combretastatins, natural products known to bind β-tubulin. A number of publications have since established that chalcones and related molecules can act as antimitotic agents, and substantial progress has been made in understanding their SAR.\textsuperscript{78,79} While our active methuosis-inducing compounds (e.g., MOMIPP) can be classified as chalcones, their specific features are quite distinct from most of the antimitotic chalcones previously described. The stringent structural specificity for induction of methuosis, with dependence on the specific substitution patterns of both the indolyl and the pyridinyl moieties, appears to differentiate MOMIPP from chalcones previously reported as antimitotic agents. We have not observed mitotic arrest prior to loss of viability in cells treated with these compounds (unpublished observation). Conversely, massive endosomal vacuolization akin to what we have observed with MOMIPP has not been reported with the antimitotic chalcones. Of all the cytotoxic chalcones described in the literature, the one most related to MOMIPP contains an unsubstituted indole ring linked to a 3,4,5-trimethoxy phenyl moiety.\textsuperscript{62} However, we found that replacement of the 4-pyridine of MOMIPP with a 3,4,5-trimethoxy phenyl group yielded a compound that did not induce appreciable cellular vacuolization or death when applied to U251 cells at 10 µM for 48 h (not shown). This is consistent with the lack of tolerance for modifications to the 4-pyridinyl moiety and suggests that the cytotoxicity previously reported for the trimethoxysphenyl indolyl chalcone\textsuperscript{62} probably was not due to methuosis.

In general, cell death induced by antimitotic chalcones is thought to occur by classical apoptosis, not methuosis. A possible exception was noted in a recent report where a chalcone-derivative termed “C2” may have induced death in glioblastoma cells.
by a nonapoptotic mechanism involving accumulation of autophagic vacuoles.\textsuperscript{80} However, as we have previously reported, the vacuoles induced during methuosis arise from macropinosomes and endosomes, which are distinct from autophagosomes.\textsuperscript{28,53} It would be premature to rule out the possibility that MOMIPP might bind tubulin in a manner similar to colchicine and related chalcones, but so far, the preponderance of evidence suggests that the compounds described in this study act by a different mechanism to trigger abnormal macropinocytosis, swelling of endosomal compartments, and nonapoptotic cell death.\textsuperscript{53} Ultimately, clarification of this mechanism will depend on identification of the specific molecular target(s) of MOMIPP and related compounds.

The key observation that MOMIPP effectively induced methuosis in TMZ-resistant GBM cells, as well as doxorubicin-resistant breast cancer cells, raises the possibility that further development of this compound could lead to useful therapeutic agents for treating cancers that are resistant to drugs that commonly work by inducing apoptosis. Ultimately, deployment of MOMIPP or related compounds as anticancer agents will need to address some challenges. Preliminary studies indicate that MOMIPP’s ability to induce vacuolization is not restricted to cancer cells (Figure 2-7E-H). However, the studies with normal HMECs and skin fibroblasts suggest that the consequences of vacuolization for cell viability are more severe for rapidly dividing cancer cells than normal cells, particularly when the normal cells enter stationary phase at high cell density (Figure 2-7H). This raises a possibility that a therapeutic window might be identified for selective effects on cancer cells. A second challenge relates to the poor aqueous solubility of MOMIPP and its active analogues. However, similar solubility issues have been encountered with other hydrophobic anticancer drugs (e.g., taxol, camptothecin) and have
been circumvented through use of appropriate excipients\textsuperscript{81} or novel nanoparticle\textsuperscript{82,83} or liposome\textsuperscript{84,85} based delivery strategies. The latter strategies may offer the additional benefit of allowing selective targeting of non-specific agents by surface modifications of the delivery vehicle with tumor-specific peptides or antibodies.\textsuperscript{82,86,87} Thus, given the observed cytotoxic effects of MOMIPP on drug-resistant cancer cells and the range of available options for its delivery in vivo, we believe that MOMIPP can serve as a valuable prototype for further preclinical testing.

2.4 Methods

For synthetic chemistry experimental details and NMR spectra, see the Appendix.

**Library Compounds.** Compounds 1-8 used for initial screening of methuosis-inducing activity (Fig. 1) were obtained from Hit2Lead.com, a division of Chembridge Corp. The identification numbers of the compounds were: 1, 5224450; 2, 5224466; 3, 5312531; 4, 7995005; 5, 7916760; 6, 6161388; 7, 5267766; 8, 6155359. All compounds are certified by the vendor to be at least 90\% pure with NMR confirmation of structure.

**Cell Culture.** U251 human glioblastoma cells were purchased from the DCT Tumor Repository (National Cancer Institute, Frederick, MD). MCF-7 mammary carcinoma cells were obtained from The American Type Culture Collection, Rockville, MD. Temozolomide-resistant U251 cells (U251-TR) were derived in our laboratory as described previously.\textsuperscript{53} MCF-7 cells selected for resistance to doxorubicin were provided
by Amadeo Parissenti, Northeastern Ontario Regional Cancer Centre. Normal human skin fibroblasts were derived from a skin biopsy as described previously. Unless stated otherwise, cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) (JR Scientific, Woodland, CA) at 37°C in an atmosphere of 5% CO₂/95% air. Normal pre-stasis human mammary epithelial cells (specimen 184) were provided by Martha Stampfer, Lawrence Berkeley Lab, Berkeley, CA. The HMECs were maintained in M87A medium supplemented with cholera toxin and oxytocin, essentially as described.

**Cell Proliferation and Morphology.** To generate cell growth curves, U251 cells were plated in 35 mm dishes (100,000 cells/dish, Fig. 2-4 B, 2-5) and allowed to attach for 24 h. Thereafter, cells were treated with the indicated compounds dissolved in DMSO or with vehicle alone. At daily intervals, three parallel cultures were harvested from each group by trypsinization and aliquots of cell suspension were counted in a Coulter Z1 particle counter. Phase-contrast images of live cells were obtained using an Olympus IX70 inverted microscope equipped with a digital camera and SPOT imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Cell Viability.** Cells were seeded in 96-well plates, with four replicate wells for each culture condition. After addition of the indicated concentrations of compounds, cell viability was determined at a 48 h end-point using a 3-(4,5-dimethylthiazol-2-yl)-2,5-
diphenyl tetrazolium bromide (MTT)-based assay as described. Absorbance at 570 nm was quantified on a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA).

**Colony Formation.** Cells were plated in 100 mm dishes at 2,500 (U251 and U251-TR) or 1,500 (MCF-7 and MCF-7 DoxR) cells per dish. Beginning on the day after plating, the cells were exposed to the indicated compounds for the periods of time noted in the figure legends. The medium was then replaced (without compounds) and cells were incubated for 10 days, with fresh medium added every 2 d. Colonies were visualized by washing with phosphate-buffered normal saline, fixing for 10 min with ice-cold 100% methanol, and staining with 1% (w/v) crystal violet (Acros Organics, Fisher Scientific, Pittsburgh, PA) in 35% methanol. After 2-3 washes with water, colonies containing at least 50 cells were counted using a dissecting microscope or a Protocol 2 colony counter (Synbiosis, Frederick MD).

**ABBREVIATIONS USED**

DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene; DMF, dimethylformamide; DMSO, dimethylsulfoxide; GBM, glioblastoma multiforme; HMEC, human mammary epithelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RT, room temperature; TMZ, temozolomide.
ACKNOWLEDGEMENT

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Chapter 3

Identifying the Cellular Target of Methuosis-Inducing Small Molecules

Since the advent of the genomic era, medicinal chemistry efforts have largely relied on target-guided hypotheses for the design of new drugs. As new technologies emerged such as RNAi, molecular cloning, X-ray crystallography, molecular modeling, etc., designing a drug based on its intended target became a priority in many drug discovery programs. This approach, target-guided drug discovery, is in contrast to an alternative approach, i.e. phenotypic-guided drug discovery. Phenotypic screening generally involves testing a somewhat arbitrary collection of small molecules in some biological or biochemical system, and assaying for a specific readout: reduced activation of a specific pathway, disruption of bacterial cell wall synthesis, reduced cancer cell growth, etc. This can be done with an unbiased approach; i.e. with no knowledge or hypothesis of the potential targets that require activation/inactivation to induce the desired biologic response.
Despite the power afforded by genomic approaches toward target-guided drug discovery, the phenotypic approach may actually be a more productive means of developing new and effective medicines. Indeed, a recently analysis of the 259 new molecular entities (NMEs) and biologics that were FDA approved between 1998 and 2008 showed that the majority of first-in-class small molecule drugs were discovered through phenotypic screening. Specifically, of the 50 small molecule NMEs approved, 28 were discovered through phenotypic screening while 17 were discovered by target-based methods. The discrepancy is especially notable considering these drugs were approved during a period when target-guided approaches were the primary focus of most pharmaceutical companies.

With each compound that garners interest after a positive phenotypic screen, the subsequent challenge is to determine its cellular target(s). This is crucial in order to understand the mechanism of action, to predict toxicity, etc. Accordingly, there has been a corresponding increase in target-identification efforts, along with improvements in existing methodologies to label and identify protein targets of small molecules.

Through phenotypic screening, our lab recently identified specific indole-based chalcones that induce methuosis in glioblastoma cells (as described in Chapter 2). Thus, we were subsequently interested in the cellular target of the MIPP compounds (specifically MOMIPP) for a variety of reasons. First, identification of MOMIPP’s target should provide a more thorough understanding of the mechanism of MOMIPP-induced methuosis. Current studies by our lab have characterized the compound’s biologic effects on vesicle trafficking and cell death (section 1.2); however, we do not know the cellular target that interacts with the compound to initially stimulate methuosis. Moreover, an
understanding of the target could explain the different sensitivity of glioblastoma cells and non-transformed cells to MOMIPP, and perhaps predict other tumors that will be more or less sensitive, as well as predicting the toxicity to normal cells (on-target toxicity). Along these lines, the target could serve as a biomarker, such that its expression in a tumor biopsy might predict that tumor’s sensitivity to methuosis-stimulating treatment. Finally, structural information about the target could lead to the design of better methuosis-inducing compounds using molecular modeling and target-guided SAR. Toward that goal, the sections below describe the synthesis of various chemical tools and their use in biochemical experiments toward identifying target proteins of MOMIPP in glioblastoma cells.

3.1 Current Approaches in Small Molecule Target Identification

The most straightforward and proven method for target isolation and enrichment is analogous to affinity chromatography, commonly referred to as a ‘pulldown’ approach. A small-molecule of interest is covalently appended to a solid support, typically a small resin, which is then incubated with cellular extract. The unbound components are rinsed away while the target proteins remain bound; a final elution step releases the target proteins, which are typically identified by mass spectrometry-based peptide sequencing. Many successful examples of target identification have been carried out with covalently resin-bound drug, followed by competitive elution of target proteins using free ligand.93-97

In a related approach, resin-bound streptavidin coupled to a biotin-drug conjugate is used to isolate target proteins.98-101 In this approach, the drug is synthetically coupled to
biotin. Next, the biotin-drug conjugate is incubated with the streptavidin resin to ‘load’ the column. As the streptavidin-biotin interaction is one of the strongest known non-covalent interactions, with a dissociation constant of $10^{-14}$, column loading is essentially irreversible and allows for a known amount of drug to be loaded (in contrast to standard activated resins as described above). While biotinylating a compound often renders it impermeable to the cell membrane, in some cases the biotin-drug conjugate may retain activity in a cell-based system, and thus SAR evaluation and cell-based target binding studies may be possible.

While there are many examples of success, small-molecule affinity chromatography still presents significant disadvantages. Specifically, appending a small drug-like molecule introduces two main concerns. First, the molecule needs a chemical moiety that can react with the solid support. This is typically in the form of an amine, alcohol, or acid, such that it can react with a resin’s reactive group, such as an epoxide, n-hydroxysuccinimide, or amine. In the case of a biotin-drug conjugate, the molecule similarly needs a moiety to covalently bind biotin. Second, appending the compound (or biotin conjugate) on a solid support may have drastic effects on its SAR. Thus, prior SAR knowledge is crucial in order to predict which portion of the molecule can be linked to the solid support (or biotin) without affecting the compound’s ability to bind its target. Likewise, the presence of the solid support even when distanced from the active drug via an extended linker may interfere with the drug’s ability to bind its target.

To overcome some of these drawbacks, a more recent approach, target labeling by in-situ click-chemistry, has proven successful by multiple groups. In this case, click chemistry refers to the coupling reaction between two unique reaction partners (typically
azide and acetylene) with the goal of labeling a target-drug interaction that occurs among a complex mixture of biomolecules. The target-bound drug is often reacted, or “clicked” with an azide-functionalized biotin derivative, to allow its identification and/or isolation from the lysate mixture. This is analogous to the first approach of using resin-bound drug, but in this case, the drug-resin bond (via biotin-streptavidin) is made after the drug binds its target, using click chemistry. This allows use of a less-modified drug analog, thus one that is more likely to maintain the original compound’s target binding characteristics. The acetylene-azide “click” coupling reaction is bioorthogonal; that is, largely unaffected by other biomolecules. As acetylene and azide are functionalities that are not present in biological molecules, and as they are relatively inert outside of their affinity to couple each other (with requisite Cu\(^{1}\) catalysis), there should be no background labeling, i.e. no reaction with the drug’s acetylene and the biotin’s azide other than their own coupling.

![Figure 3-1](image.png)

**Figure 3-1** Target Identification by In-situ Click-Chemistry. An active compound (green triangle) is modified with a terminal acetylene moiety. Once the compound binds its target protein (yellow), the biotin-azide conjugate (purple) is added (along with a copper catalyst), leading to an azide-acetylene cycloaddition reaction, forming the triazole linker. The complex is purified with streptavidin-coated solid resin to enrich target proteins.
Finally, proteins are digested and sequenced by LC/MS. (Reprinted with permission from reference 109)

First, the drug compound is minimally modified with a terminal acetylene functionality. After applying the drug to the biological sample (e.g. cell lysate), azide-functionalized biotin is introduced. The azide-acetylene coupling reaction occurs, thus linking the target protein-drug-biotin. Finally, the complex is isolated using streptavidin beads. This small chemical modification with an acetylene introduces much less structural variation compared to appending the drug molecule to a biotin moiety or a resin linker, as described previously. Thus, this approach improves upon the limitations of the pulldown approach as SAR should be much less affected by the additional acetylene.

Unfortunately, the nature of the drug-target interaction is crucial for the success of these approaches. Indeed, all of the click-chemistry approaches mentioned above, and some of the pulldown approaches mentioned, used small molecules which bound their targets covalently or with extremely high affinity. If not covalent, the interaction needs to be strong enough and with a long enough residence time to endure multiple washing steps during the enrichment stages, as well as the click reaction steps used in that specific approach. When this is the major limitation, photoaffinity labeling is the desired approach.

Photoaffinity labeling refers to the use a photoreactive functional group that, upon irradiation, is excited to a highly reactive intermediate, which then covalently binds molecules in its immediate vicinity, i.e. its protein target in this case.\textsuperscript{110,111} The small molecule of interest must first be functionalized with a photoreactive group. Importantly, the molecule must retain biological activity after introduction of the additional photoreactive group (similar to the acetylene addition above). This can be optimized
through the standard SAR evaluation for a given system. When the photoreactive derivatized drug is additionally labeled by incorporating a radioactive label, the molecule can be used as a tracer to irreversibly tag its binding protein.\textsuperscript{112,113} This approach has been used to identify ligand-protein interactions of all scales, from identifying the specific region or residue that a ligand binds\textsuperscript{114-116} to identifying a ligand’s unknown protein target or target complex.\textsuperscript{113,117-119}

To briefly highlight some of those examples, photoaffinity labeling was recently used to identify $\gamma$-secretase activating protein (GSAP), a previously uncharacterized protein, as a target of imatinib.\textsuperscript{117} Imatinib had previously garnered interest in neurodegeneration applications as it had been shown to reduce amyloid-β levels without inhibiting Notch cleavage.\textsuperscript{120} Using a photoreactive, radiolabeled derivative of imatinib, a

**Figure 3-2.** Target Identification by Photoaffinity Labeling. (A) Normally, a drug (red triangle) binds its target protein (blue) reversibly, among other cellular proteins (yellow, purple, green). (B) In the photoaffinity labeling method, the drug is modified with a photoreactive group (orange) and a radioactive label (cyan). (C) Upon UV irradiation, the photoreactive drug irreversibly binds its nearby protein(s). (D) Proteins are separated by SDS-PAGE. Labeled bands are visualized by phosphorimaging or autoradiography/fluorography and excised from the gel. (E) Excised proteins are digested and analyzed by mass spectrometry, (F) ultimately providing sequence information.
16 kD protein named GSAP was identified. GSAP was further shown to coordinate with γ-secretase and increase its affinity for the amyloid-precursor protein, but not Notch, thus validating it as an attractive target for Alzheimer’s treatment.

Others have used photoaffinity labeling to challenge the current dogma regarding NAADP and calcium signaling. While nicotinic acid adenine dinucleotide phosphate (NAADP) had been thought to mediate calcium signaling by directly stimulating the two-pore channel (TPC), recent work with radiolabeled, photoreactive NAADP derivatives failed to label the TPC, yet did selectively label a small number of other proteins.\textsuperscript{118,121} One or some of these putative targets are thought to function in complex with, but separate from the TPC, ultimately leading to its activation. Clearly, photoaffinity labeling is a powerful technique to precisely identify specific binding partners.

In all instances, after labeling and/or enrichment of protein targets, proteins are identified by mass-spectrometry peptide sequencing.\textsuperscript{122} In addition to the techniques discussed above, other less common approaches have also been demonstrated. While their effectiveness is less proven based on their representation in the literature, these interesting methods may eventually become more prevalent, and will be briefly discussed here.

The first of these approaches evolved from protein folding and stability studies, from which it is well-known that certain proteins display increased resistance to proteolysis when bound to their ligands, such as in DNA-transcription factor binding\textsuperscript{123} or carbohydrate-binding proteins when bound to their appropriate carbohydrate.\textsuperscript{124} Recently, these concepts have expanded toward target identification by considering that a drug, like a natural ligand, can also render its target more thermodynamically stable in the
folded state, thus more resistant to proteolysis. At least three labs have recently reported similar approaches toward target identification based on these principles, and each has coined their own phrase: DARTS (drug affinity responsive target stability),\textsuperscript{125} energetics-based target discovery by pulsed proteolysis,\textsuperscript{126} and SPROX (stability of proteins from rates of oxidation).\textsuperscript{127} All follow the same general approach and have one major advantage over other methods of target identification: no structural modification of the ligand is necessary. There is no need to link to a resin, to introduce a tag or radiolabel, or a group to induce covalent bond formation; the active molecule itself is used without any modifications. Moreover, the detection sensitivity is tunable by adjusting ligand concentration (or by adjusting the denaturant concentration, as the method reported by Liu et al. additionally introduces urea to selectively induce more denaturation in unbound versus ligand-bound proteins), as increasing ligand concentration will increase the chance of identifying differential proteolysis, though this will of course increase nonspecific interactions as well. However, at this point, this approach has been evaluated on proof-of-principle experiments using compounds with known targets, and there are limited examples of using it to identify truly unknown targets.\textsuperscript{128}
Figure 3- 3. Drug Affinity Responsive Target Stability (DARTS). A drug is added to cell lysate, and the mixture is degraded with proteases. Compared to a parallel sample with no drug, the target protein will be protected from degradation, and the differential degradation can be assessed by parallel SDS-PAGE analysis. (Reprinted with permission from reference 125)

The three-hybrid assay$^{129,130}$ represents another powerful method, which is an extension of the original two-hybrid assay.$^{131}$ Like the two hybrid assay, a transcriptional activator is severed in two parts, its DNA binding domain (DBD) and its transcriptional activation domain (TAD). In the three hybrid approach, the DBD is fused to dihydrofolate reductase (DHFR). The small molecule of interest is synthetically linked to methotrexate, a DHFR ligand. Finally, a fusion protein, between the TAD and a library of candidate target cDNA, is expressed. As the DBD-DHFR binds the methotrexate-drug conjugate, when drug-target binding occurs, DBD and TAD are brought in proximity, activating transcription of a reporter gene. The putative target protein is ultimately
identified by sequencing. This method has recently identified the target protein for acecortave acetate, a drug used for glaucoma therapy, previously without an understood mechanism of action. While extremely powerful, this method too has its limitations. First, the target proteins are not in their native state – they are expressed as fusion proteins in yeast or bacteria, and in some cases have other added constructs as well (e.g. nuclear localization signal). Thus, they may not interact with ligand in a predictable or physiologic manner. Further, as is seen with most target identification strategies, the ligand of interest must be chemically modified; here, it is linked to methotrexate. This is similar to linking a compound to a solid support for chromatographic approaches, which may reduce a compound’s target affinity.
Figure 3-4. Yeast Three Hybrid Technique. The drug or ligand of interest ("L") is chemically linked to Methotrexate ("Mt"x). A fusion protein between DHFR (target of Mt) and a DNA-binding-domain (DBD) is expressed. Finally, multiple fusion proteins between a transcription-activating domain (TAD) and a cDNA library of potential target genes is expressed in separate clones. If a ligand’s target is expressed, the target–ligand interaction results in the TAD relocating to the gene’s transcription start site, initiating transcription of a reporter gene. Reprinted with permission from reference [130]

Given the advantages and limitations to each, we chose chromatographic and photoaffinity approaches to identify candidate targets of MOMIPP.

3.2 Results

3.2.1 Attempts at MOMIPP Target Enrichment Using In-Situ Click-Chemistry
Toward identifying the target of methuosis-inducing compounds, we first
designed MOMIPP analogs for in-situ click-chemistry. As described above, the ligand is
derivatized with a terminal acetylene moiety, and the enrichment tag (biotin) is
derivatized with a terminal azide moiety. While the opposite orientation can also be
successful (i.e., ligand-azide, biotin-acetylene), comparisons between the two approaches
have shown that azide-derivatized ligands lead to more non-specific background labeling
compared to acetylene-derivatized ligands.\textsuperscript{104,106,133}

As the MOMIPP scaffold contains an $\alpha,\beta$-unsaturated ketone (enone), we
hypothesized it may function as a covalent modifier of its target protein, serving as a
Michael acceptor at its $\beta$-carbon. However, we know that MOMIPP-induced methuosis is
not a general result of covalent binding via the enone moiety, as our strict SAR results
prove that multiple enone-containing MOMIPP analogs do not induce methuosis, yet still
contain the electrophilic enone scaffold. Therefore, assuming MOMIPP’s enone moiety is
crucial to interact with its target and stimulate methuosis, other components of the
compound must also interact with the target to situate the molecule in the necessary
conformation to induce the observed phenotype. Indeed, the rational design of covalent
drugs harnesses a similar idea: compounds that contain electrophilic moities can be
designed to target nucleophilic protein residues (e.g. sulfhydryls) only after the SAR is
optimized to situate the reactive moiety adjacent to a nucleophilic residue, but these
moieties often are not reactive enough to bind general, abundant proteins (which would
effectively eliminate the active compound, reducing activity).\textsuperscript{70}

Based on SAR results (as described in chapter 2), we sought a region of the
molecule to incorporate a terminal acetylene. Functionalizing the indole ring was the
natural approach, as the exquisite necessity of the para-pyridine moiety suggested that it should not be altered in any manner. The most chemically accessible positions on MOMIPP to incorporate an acetylene are at the indole-nitrogen, as well as the 2- and 5-indole positions. Alkylating the indole nitrogen was previously shown to reduce activity, and incorporating new groups at carbon 2 would require a longer synthetic approach, so we began with the 5-position.

Figure 3-5 illustrates the synthetic approach to compound 30, termed POMIPP (propargyl-oxy-MIPP). 2-Methyl-5-methoxyindole was demethylated using BBr₃ to the hydroxy indole, which was then alkylated with propargyl bromide. Formylation and aldol condensation provided POMIPP (30). For later use as negative controls, potential inactive POMIPP analogs were similarly made by modifying the pyridine ring as shown in Figure 3-5.

**Figure 3-5.** Synthesis of Probes for Click-Chemistry Based Target Identification. (22): BBr₃, CH₂Cl₂, –78 °C, 93%. (28): Propargyl bromide, Cs₂CO₃, acetone, 40 °C, 73%. (29): POCl₃, DMF, 0 °C, 79 %. (30–33): piperidine, MeOH, 64 °C and (30): 4-acetylpyridine, 65%, (31): acetonaphone, 21%, (32): acetylpyrazine, 51%, (33) 3-acetylpyridine, 36%. (34): 1. SOCl₂, toluene 2. NaN₃, H₂O, 57%. (35): biotin, EDC, DMAP, DMF, 76%.
The biotinylated enrichment tag, biotin-azide 35, was synthesized from 6-aminohexanol, which was activated with thionyl chloride followed by substitution with azide to compound 34. Next, 6-azidohexylamine was coupled with biotin in the presence of EDC and DMAP, yielding biotin-azide conjugate 35.

Before attempting actual target identification experiments, the analogs must prove to maintain methuosis-inducing activity. Viability of U251 cells treated with POMIPP and analogs confirms that POMIPP does affect cell viability at similar concentrations to MOMIPP (Figure 3-6). Of the potential inactive analogs, the pyrazine-substituted compound (compound 32) proved best (i.e. affected viability the least). At low concentrations (< 10 µM), the difference in activity between POMIPP (30) and POMIPP-pyrazine (32) is most pronounced. Thus, as POMIPP-pyrazine is presumed to have a much weaker binding affinity to the methuosis-inducing target protein(s), it may serve as a negative control to differentiate true targets from those that bind the chalcone scaffold nonspecifically.
Figure 3-6. MTT Viability Assay for POMIPP and Derivatives in U251 Cells. Each point represents the mean ± SD of results from quadruplicate wells (Assay performed by Jean Overmeyer, PhD).

The effects of POMIPP and the inactive pyrazine analog are further shown morphologically, as POMIPP induces excessive formation of macropinocytotic vacuoles, similar to MOMIPP (Figure 3-7). POMIPP-pyrazine does not induce vacuoles, further supporting its inability to induce methuosis and confirming it as an inactive POMIPP analog.

Figure 3-7. Morphology of Cells Treated with POMIPP. One day after plating 100,000 per 35mm dish, cells were treated with 10 μM of POMIPP (left), MOMIPP (middle), or POMIPP-pyrazine (right), and surveyed for morphology by phase contrast microscopy.
after 48 hours. Notice the lack of signs of methuosis for POMIPP-pyrazine, compared to POMIPP and MOMIPP.

With the acetylene compounds confirmed for methuosis activity, the POMIPP click experiment was performed with cell lysate from U251 glioblastoma cells. Cell lysate was first incubated with POMIPP or POMIPP-pyrazine. Next, the click-chemistry reaction cocktail (CuSO$_4$, TCEP as a reducing agent, TBTA as copper-stabilizing agent$^{104}$) was added along with azide-functionalized biotin (compound 35). Avidin-decorated agarose resin was then added, followed by thorough washings to remove unbound protein. Finally, specifically bound proteins were eluted from the beads by boiling in denaturing buffer and the entire sample was resolved by SDS-PAGE.

The eluted proteins were visualized by both silver staining and Western blotting with streptavidin-conjugated horseradish peroxidase (HRP). Silver staining should reveal any proteins that were eluted from the avidin resin, while streptavidin-HRP blotting will reveal any biotinylated proteins that were eluted: it will identify proteins that are irreversibly bound to biotin (through the POMIPP-biotin conjugate), as well as any endogenously biotinylated proteins.
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Figure 3- 8 A.
Figure 3-8 B. Attempted Target Enrichment by In-Situ Click-Chemistry with POMIPP. U251 Cell lysate was incubated with POMIPP. Next, the click-chemistry cocktail (CuSO₄, TCEP, TBTA) was added along with biotin-azide (compound 35). Parallel samples were treated with the indicated modifications: excess MOMIPP (lane 2), inactive analog meta-MOMIPP (lane 3), no drug (lane 4), no biotin-azide (lane 5), no CuSO₄ (lane 6). Samples were resolved by SDS-PAGE, and silver stained (A) or blotted with Streptavidin-HRP and visualized by ECL (B). (MM = MOMIPP, mM = meta-MOMIPP)
Ultimately, the POMIPP in-situ click-chemistry approach did not isolate specific binding proteins. While a number of proteins were labeled in the streptavidin-HRP blot (Figure 3-8 B), the control samples show that these are not specific. While lane 1 is the experimental sample, in the subsequent two lanes (2, 3) excess MOMIPP or an inactive analog (meta-MOMIPP, compound 58, Table 2.1) was added, respectively, to selectively attenuate POMIPP-target interactions. The following three lanes are additional controls to identify proteins biotinylated in the absence of POMIPP (lane 4), proteins labeled in the absence of biotin-azide (lane 5), and proteins labeled in the absence of exogenous copper (which the acetylene-azide couple reaction requires, lane 6).104,108 The most prominent band just below 80 kD is most likely the endogenously-biotinylated prioprionyl CoA (72 kD), which appears in all samples. The other bands can also be eliminated by the various control samples. For example, the ~ 65 kD band is also present in all samples. The remaining proteins, which initially looked promising, were ruled out as the POMIPP-free sample shows a similar protein profile, i.e. the proteins are biotinylated in a POMIPP-independent mechanism (clearly, the azide-acetylene coupling is not truly bioorthogonal).

3.2.2 Attempts at MOMIPP Target Enrichment Using Biotinylated Methuosis-Inducing Derivatives

A related approach to the click-chemistry based biotinylation is the use of biotinylated drug probes for direct pulldown type experiments, as described earlier in this chapter. The synthetic approach toward POMIPP and analogs (Figure 3-5 above) was continued as it allowed for the subsequent synthesis of biotinylated MOMIPP conjugates.
that could be used for pulldown assays. Accordingly, POMIPP was coupled with azido-biotin to make MIPP-biotin conjugate 36 (Figure 3-9).

![Synthesis of MIPP-Biotin Conjugate.](image)

**Figure 3-9.** Synthesis of MIPP-Biotin Conjugate. (36): CuSO₄, sodium ascorbate, DMF, 60 °C, 41%.

We first tested if MIPP-biotin could induce methuosis in glioblastoma cells. In concentrations up to 40 µM, cells showed no signs of vacuole formation and no obvious reduction in growth (not shown). However, we cannot assume that the compound no longer binds its original target, as biotin often renders compounds impermeable to the cell membrane. Although MIPP-biotin does not induce methuosis in whole cells, it may still bind its target in cell-free lysate conditions.

Next, various pulldown experiments were carried out. In general, biotinylated-MIPP derivatives were incubated with cell lysate, and streptavidin-decorated beads were used to enrich the labeled proteins. In attempt to maximize streptavidin bead efficiency and reduce enrichment of endogenously biotinylated proteins, streptavidin beads were initially loaded with MIPP-biotin conjugate, and excess compound was washed away (as opposed to treating lysate with MIPP-biotin conjugates, followed by straptavidin...
addition). Next, cell lysate (at 1 mg/mL) was incubated with the MIPP-decorated beads. Various competition experiments were done wherein excess non-biotinylated, free MOMIPP was preincubated with the lysate, with the goal of saturating its target protein. Parallel samples were similarly pretreated with inactive derivatives of MOMIPP, with the goal to not compete away the physiologic target, while still competing away proteins which bind the chalcone scaffold nonspecifically.

Some of the experimental considerations will be discussed before evaluating the results. First, after incubating lysate with the MIPP-decorated beads, washing steps are necessary to remove nonspecifically bound proteins. The stringency of washing steps can have significant impact on the proteins that are ultimately eluted in the final steps. Moreover, the elution method is also important. Similar to affinity chromatography, target proteins can be eluted by incubating the lysate–MIPP–resin mixture with excess free MOMIPP. This should compete with the interaction between target proteins and MIPP-biotin, and the target protein will elute, free of biotin label. However, there are certain drawbacks to this. First, MOMIPP’s solubility is limited at the high concentrations of free compound that are necessary for this approach. Also, this method may not elute proteins with covalent interactions to MIPP. Thus, if the MIPP-loaded beads have covalently bound target proteins, eluting with free MOMIPP may not be successful.

The alternate elution approach is to boil the resin/conjugate/lysate mixture in denaturing SDS-PAGE sample buffer. The buffer’s detergent, in combination with boiling, will eliminate all of the reversible binding interactions present, including the streptavidin-biotin bond. Thus, enriched proteins will be eluted from the resin, and can
then be separated by gel electrophoresis and viewed by general protein staining.

However, if a protein was covalently bound to the MIPP-biotin conjugate, and this protein-MIPP bond survived the boiling step, an additional visualizing approach is possible – blotting with streptavidin-HRP (horseradish peroxidase), followed by ECL (enhanced chemiluminescence) detection. Importantly, this detection method will only work if proteins are covalently biotinylated, i.e. if they are covalently bound to MIPP-biotin (or if they are endogenously biotinylated). Proteins that reversibly interact with the MIPP-biotin conjugate will presumably be separated from the small-molecule conjugate during gel electrophoresis, and thus will not be labeled in the streptavidin-HRP blot.

The final parameter to discuss is selectivity: discriminating between proteins that bind MIPP specifically, i.e. those that do not bind inactive analogs of MIPP, versus proteins that bind MIPP in a nonspecific manner, through general interactions with the hydrophobic chalcone scaffold, its enone, etc. Two approaches were used to address this. First, preincubating the target proteins with free MOMIPP, or its inactive analogs, should differentially saturate specific targets, and accordingly alter the pulldown profile. A separate approach is to conduct pulldown experiments directly with inactive MOMIPP derivatives – i.e., loading inactive-MIPP-biotin conjugates onto streptavidin resin and performing the pulldown. Comparison with the active pulldown may show a differential binding profile.
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**Figure 3- 10 A.**
Figure 3-10 B. Affinity pulldown with MIPP-Biotin in U251 Cell Lysate. Streptavidin-resin was loaded with MIPP-biotin and incubated with U251 cell lysate. The resin was washed with 0.1% Triton-X-100, and eluted in three steps: twice by incubating with an excess of free MOMIPP (Fig 3-10 B) and finally by boiling the resin in denaturing sample buffer (Fig 3-10 A, right). Parallel samples contained excess MOMIPP (lanes 2,3,8,9) or meta-MOMIPP (lanes 4,5,10,11) or naked streptavidin beads (lanes 6, 12). The flowthrough gel (A, left) represents 2% of the total (MM = MOMIPP, mM = meta-MOMIPP).

A representative pulldown experiment is shown in Figure 3-10. Lysate was preincubated with DMSO, free MOMIPP, or free meta-MOMIPP, a MOMIPP analog that does not induce methuosis (compound 58, Table 2.1). Separately, streptavidin-resin was loaded with MIPP-biotin. Finally, lysate was added to the MIPP-decorated resin,
incubated overnight, and washed multiple times. Bound proteins were eluted in three steps: twice by incubating with free MOMIPP, and a final step by boiling in denaturing sample buffer (the bands between ~55–65 are attributed to an artifact of silver staining with buffer containing 2-mercaptoethanol\textsuperscript{134,135}).

In the second MOMIPP eluate, an extremely faint band is present near the 45 kD marker (Figure 3-10 B, lanes 7-12, arrow). This same band is well represented in the final elution (Figure 3-10 A, lanes 7-12), along with a number of other proteins. Unfortunately, the binding profile is essentially identical for all 6 samples – including the naked streptavidin resin sample (lane 6, 12 Figure 3-10, A & B) which had no MIPP-biotin loaded, and thus is clearly not a MIPP-binding protein. The one unique band that is observed was isolated from the naked streptavidin resin (lane 6, 12 Figure 3-10, A & B), and is presumably the endogenously-biotinylated propionyl–CoA carboxylase (72 kD).
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Figure 3- 11 A.
Figure 3-11 B. Affinity Pulldown with MIPP-Biotin in U251 Cell Lysate. Streptavidin-resin was loaded with MIPP-biotin and incubated with U251 cell lysate, washed with 1% Triton-X-100, and eluted in three steps: twice by incubating with an excess of free MOMIPP (Fig 3-11 B) and finally by boiling the resin in denaturing sample buffer (Fig 3-11 A, right). Parallel samples contained excess MOMIPP (lanes 2,3,8,9) or meta-MOMIPP (lanes 4,5,10,11) or naked streptavidin beads (lanes 6, 12). The flowthrough gel (A, left) represents 2% of the total (MM = MOMIPP, mM = meta-MOMIPP).

To reduce the enrichment of non-specific proteins, the experiment was repeated with harsher washing steps (higher detergent concentration), followed by the same three step elution protocol (Figure 3-11). In this case, much fewer proteins eluted in the final step. Two proteins were seen in all samples, however there was no selectivity as the pulldown was not reduced by incubating with excess free MOMIPP. Moreover, by
comparison to the unbound proteins (i.e. the supernatant or original flow-through, lanes 1-6, Fig 3-11 A), these two bands seem to be high abundance proteins, and thus might be explained simply by non-specific interactions.

The lack of success of the biotinylated approach may be related to the chemical modifications of the biotin-linker group. The MIPP-biotin conjugate (36) used above has a 6-carbon linker between the two compounds. Others have reported that the hydrophobicity and/or length of the linker region can have significant impact on both non-specific protein pulldown and on specific target binding.\textsuperscript{136,137}

With this in mind, biotin-MIPP conjugates with longer and more hydrophilic linkers (polyethylene glycol, PEG) were synthesized. Tetraethylene glycol was used as the starting material, which would serve as the hydrophobic linker between biotin and MIPP. The terminal alcohols were activated with mesyl chloride, followed by substitution with sodium azide. The diazido compound 37 was selectively reduced to the monoazide 38 using a biphasic system which extracts the singly-reduced azido-amine into an acidic aqueous layer, preventing its further reduction by triphenylphosphine.\textsuperscript{138}
This was coupled with n-hydroxysuccinimide-activated biotin

![Chemical structure diagram]

Figure 3-12. Synthesis of Hydrophilic MIPP-PEG-Biotin Conjugates. (37): 1, MsCl, Et$_3$N, THF, 0 °C; 2, NaN$_3$, NaHCO$_3$, H$_2$O, 77 °C, 85%. (38): [0.65] H$_3$PO$_4$, H$_2$O, PPh$_3$, ether, 73%. (39): EDC, DMF, 88%. (40): 39, Et$_3$N, DMF, 48%. (41): 30, sodium ascorbate, TBTA, CuSO$_4$, DMF, 72%. (42): 33, sodium ascorbate, CuSO$_4$, DMF, 34%. (43): 31, sodium ascorbate, CuSO$_4$, TBTA, DMF, 51%.

(39), providing biotin-PEG-azide conjugate 40 that could undergo a copper-catalyzed azide-acetylene coupling with POMIPP, similar to what was done previously for compound 36. In addition, inactive analogs were also made using this linker – from meta-POMIPP (33) and from phenyl-POMIPP (31). These three POMIPP derivatives were coupled with the biotin-PEG-azide, providing biotin-PEG-MIPP conjugates (compounds 41, 42, and 43).
These conjugates were loaded onto streptavidin-resin, as before. Resins were made with each derivative: the active (para-pyridine orientation), and two inactive analogs (meta-pyridine and phenyl). These resins were incubated with glioblastoma cell lysate overnight. To repeat the competition approach, lysate for two additional samples was separately preincubated with MOMIPP and meta-MOMIPP, and this lysate was then incubated with the active MIPP-resin. Resins were washed with increasing detergent concentrations (including both the low and high detergent concentrations described previously), followed by final elution in boiling denaturing sample buffer.

As before, no protein bands appear to be specifically pulled down by the MIPP-loaded resin (Figure 3-13, 2-mercaptoethanol impurity is still seen in all silver stained gels, as described earlier). The one clear protein isolated in the main experimental sample (Figure 3-13, lane 7) is actually present in all samples, including the eluate from naked streptavidin beads (Figure 3-13, lane 10). In that sample, additional bands are visible, which probably correspond to other endogenously biotinylated proteins (acetyl-CoA carboxylase, 240 kD; pyruvate-CoA carboxylase, 130 kD, propionyl-CoA carboxylase, 72 kD).

Around the time these experiments were being carried out, colleagues in the lab found evidence that MOMIPP probably does not function as an irreversible binder and the binding may be quite transient, i.e. exhibiting short drug-target residence time (not shown). This characteristic renders the above approaches unlikely to succeed. Thus, we turned to photoaffinity labeling.
Figure 3-13. Affinity Pulldown with MIPP-PEG-Biotin in U251 Cell Lysate. Streptavidin-resin was loaded with MIPP-PEG-biotin (lane 1, 5, 9, and 7, 11, 12), Phenyl-MIPP-PEG-Biotin (lane 2, 8), meta-MIPP-PEG-Biotin (lane 3, 9), or unloaded (naked resin, lane 4 and 10) and incubated with U251 cell lysate, washed with 1% Triton-X-100, and eluted in three step: twice by incubating with a excess of free MOMIPP (bottom gels) and finally by boiling the resin in denaturing sample buffer (top gel, right). Parallel samples contained excess MOMIPP (lane 5, 11) or meta-MOMIPP (lane 6, 12). The flowthrough gel, lanes 1–6, represents 2% of the total (MM = MOMIPP, mM = meta-MOMIPP).

3.2.3 Preliminary MOMIPP Target Identification Using Photoaffinity Probes

As MOMIPP may not bind covalently, we sought to develop photoaffinity analogs that could irreversibly label their target protein(s). Two of the most common photoreactive groups used in photoaffinity labeling are aromatic azides and
When both groups are irradiated in the UV range, they are excited to intermediates which can covalently label protein residues. Figure 3-14 illustrates the mechanism of insertion to various protein moieties.

Figure 3-14. Mechanism for Photoaffinity Ligand Excitation and Insertion. (Top): Aromatic azides undergo UV-induced loss of nitrogen, forming a short-lived nitrene which can insert into various protein functional groups. Often, the nitrene rearranges within its neighboring aromatic ring, forming a highly electrophilic seven-membered ketenimine, which reacts with nucleophilic residues. (Bottom): Benzophenones are reversibly excited to a diradical, which can insert into various protein moieties, especially C-H bonds.

Importantly, both functional groups are relatively inert before irradiation, and thus will not label random cellular components (cf. a classical affinity reagent). Moreover, the groups are relatively inert to visible light, and thus meticulous care is not needed to
handle them in ambient light. Finally, both of these groups can be incorporated into the MIPP scaffold.

### 3.2.3.1 Synthesis and Evaluation of Methuosis-Inducing Activity of Photoaffinity Probes

Initially, we designed a pseudobenzophenone analogue of MIPP as a potential photoaffinity probe. On the basis of the structure of compound 14 (Table 2.1), we postulated that a 5-benzoyl analogue might retain activity and function as a photoaffinity probe. The benzophenone analogue (46, 5-benzoyl-MIPP, Figure 3-15) was prepared in two steps, starting with acylation of the Vilsmeier-Haack intermediate of 2-methylindole. With unmethylated indole, this system has been shown to direct acylation of the Vilsmeier-Haack intermediate to the 5- and 6-positions.\(^{142}\) In our hands, acylation of the Vilsmeier-Haack (iminium) intermediate of 2-methylindole gave a 1:3 mixture of 5- and 6-benzoyl-2-methylindole-3-carboxaldehyde, respectively (44 and 45). This regioselectivity was characterized by 1D nuclear Overhauser effect (nOe) as described in the experimental section. In the final step, the target compounds were made by coupling the aldehydes with 4-acetyl-pyridine in the presence of piperidine. Subsequent morphology and viability studies revealed that unlike the benzyloxy derivative (14), neither of the benzophenone analogues (46 and 47) induced methuosis when tested in U251 glioblastoma cells (not shown).
Aromatic azides represent another photoreactive moiety commonly used in photoaffinity labeling.\textsuperscript{110,117,119,143} Thus, we next explored the possibility that an azide could be added to either the 5- or the 6-position of the indole ring without loss of methuosis-inducing activity, as the azide represents less of a structural change from MOMIPP than the benzoyl group of compounds 46/47. The synthesis of compound 52 (5-azido-MIPP) was based on the directed nitration of 2-alkylindoles toward the 5-position,\textsuperscript{144} with further conversion to an azide by diazotization (Figure 3-15). 2-Methylindole was selectively nitrated in concentrated sulfuric acid to yield compound 48 (see the experimental section in the Appendix for validation of regiospecificity), which
was then hydrogenated to aromatic amine 49. Under mild diazotization conditions, the aminooindole was converted to the aromatic azide 50. The azide was formylated to obtain 51 and then condensed with 4-acetylpyridine, providing 52, 5-azido-MIPP. Separately, the same reaction scheme was performed on 2-methyl-5-methoxyindole, wherein nitration led predominately to the 6-nitrated product 53. The same sequence of reaction steps described above was then completed to obtain 57, 6-azido-MOMIPP.

Evaluation of the biological activities of 52 (5-azido-MIPP) and 57 (6-azido-MOMIPP) indicated that both compounds retained methuosis-inducing activity as judged by both cellular vacuolization (Figure 3-16A) and inhibition of colony formation (Figure 3-16B,C) in U251 cells. Although not as potent as MOMIPP, 5-azido-MIPP was clearly superior to the 6-azido compound in terms of its biological activity, and it was selected as the photoaffinity derivative of MOMIPP moving forward.
Figure 3-16. Activity of Azido-MIPP Derivatives in U251 Cells. (A) MOMIPP, 5-Azido-MIPP (52), and 6-Azido-MOMIPP (57) were added to cells at concentrations of 2.5 or 10 μM, and the cells were observed by phase-contrast microscopy after 48 h. (B &C) U251 cells were treated with varying concentrations of the indicated compound for 48 h, and long-term cell viability was assessed by colony-forming assays. Each represents the counts from three dishes (mean ± SD), with the results expressed as percent of the mean from parallel control dishes treated with an equivalent volume of vehicle alone (DMSO) (Assay performed by Jean Overmeyer, PhD (A) and Ashley Young (B,C).

3.2.3.2 Evaluation of 5-Azido-MIPP’s Photoaffinity Properties

After establishing that 5-azido-MIPP induces methousis with acceptable potency, its photoaffinity characteristics were evaluated. First, the UV/visible absorbance spectrum of 5-azido-MIPP was measured. Subsequently, the solution was irradiated at 254 nm (5, 15-watt bulbs, 10 cm distance) for various time intervals. After each irradiation period, the absorbance spectrum was taken, and then the same sample was re-irradiated. Cumulative irradiation times for each spectrum taken were 0, 0.5, 2, 5, 15, and
30 minutes. The absorbance spectra (Figure 3-17, left) show that 5-azido-MIPP has an absorbance shoulder at 275 nm that is gradually eliminated upon irradiation (asterisk in Figure 3-17). This experiment was repeated using a borosilicate glass filter between the sample and UV bulbs (Figure 3-17, right); borosilicate glass acts as a filter to block wavelengths below ~ 280 nm. As expected, when using the filter, longer irradiation periods are necessary to alter the absorbance spectrum, at which point the 275 nm absorbance begins to weaken; without the filter, the same peak is reduced after much shorter periods of irradiation. Thus, 5-azido-MIPP undergoes irradiation-dependent modifications in the < 300 nm range, as has been reported for other aromatic azides. This will presumably lead to formation of a reactive species that can irreversibly tag nearby proteins.
Figure 3-17 Absorbance Spectra of Irradiated 5-Azido-MIPP Over Time. (Left) A solution of 5-Azido-MIPP (150 μM in MeOH) was irradiated at 254 nm for the indicated times and its absorbance spectrum was measured. (Right) Same experiment with a borosilicate glass filter between the light source and sample to block < 300 nm. (Spectra abscissae are 200–700 nm).
In addition to photoreactivity, a putative MOMIPP photoaffinity analog requires a label to allow identification of tagged proteins. We decided to substitute a carbon atom in the backbone of 5-azido-MIPP with radioactive $^{14}$C. In the synthetic scheme of 5-azido-MIPP, the aldehyde carbon inserted in the penultimate step is derived from dimethylformamide (DMF). $^{14}$C-labeled DMF (labeled at its carbonyl carbon) is commercially available, providing a simple solution to inserting nearly 100% labeled radioactive material (Figure 3-18). Previously, this formylation step was done in neat DMF; here, that excess use of radiolabeled DMF would be cost prohibitive, and diluting $^{14}$C-DMF with regular DMF would lead to reduced radioactive incorporation. Thus, the reaction was modified to conditions where DMF was used in stochiometric amounts. This step (and the subsequent aldol condensation) was done by a commercial radiolabeling vendor (Tjaden Biosciences, Burlington, IA), providing 5-azido-MIPP with a delivered specific activity of 55 mCi/mmol (nearly total incorporation; theoretical maximum specific activity for $^{14}$C is 62 mCi/mmol). An alternative synthesis to tritium-labeled 5-Azido-MIPP was designed based on a related approach, but this was not done experimentally (Figure 3-18).

Figure 3-18. Synthesis of Radiolabeled 5-Azido-MIPP. The top route shows synthesis of $^{14}$C radiolabeled 5-Azido-MIPP via a near identical route as presented above (Fig 3-15)
but using dilute DMF in the formylation step. Alternatively, at bottom is a proposed synthesis toward a $^3$H-labeled version, via reduction of a methyl ester with a 100% $^3$H-labeled reducing agent, followed by oxidation to the aldehyde; the same reduction/oxidation approach could be done on the original aldehyde, but would only lead to 50% final $^3$H labeling.

Finally, we confirmed that $^{14}$C-azido-MIPP irreversibly labels protein in a UV-dependent mechanism; that is, it serves as a photoaffinity reagent, but not a simple affinity reagent (i.e. an affinity reagent irrespective of UV excitation). Concentrated solutions of bovine serum albumin in phosphate buffered saline (BSA, 100 µM, 7 mg/mL) were incubated with 15 µM $^{14}$C-azido-MIPP. High concentrations of both protein and drug were chosen intentionally to ensure interaction between compound and protein. Samples were irradiated for various lengths of time, from 0.5 to 30 minutes. Finally, they were resolved by SDS-PAGE. While only one protein was used (and thus no true protein separation was needed), the gel electrophoresis was done to remove any reversibly bound radiolabeled compound. Further, evaluation of the gel’s proteins could crudely indicate the degree of protein degradation due to irradiation. Gels were analyzed for radioactivity by phosphorimaging.

Indeed, $^{14}$C-azido-MIPP does irreversibly label protein in a UV-dependent mechanism (Figure 3-19). SDS-PAGE gels were first Coomassie stained to illustrate equal protein loading among samples and to crudely assess protein degradation. Degradation of the BSA signal does not seem to be a serious concern, though at extended irradiation times (15 and 30 minutes) there is a noticeable increase in proteins above and below the main band (perhaps due to UV induced oligomerization and degradation). Next, gels were dried and exposed to a storage phosphor screen, revealing a UV-dependent incorporation of radioactive signal on BSA. Maximum signal incorporation
occurs after 15 minutes of irradiation, but after only 2 minutes, 80% of maximum labeling is achieved. After 30 minutes of irradiation, the signal intensity of the 66 kD BSA band decreases to 74% of maximum. BSA molecules probably undergo UV-dependent oligomerization and degradation after extended irradiation; the increased labeling above/below the 66 kD BSA band is indicative of this. In the results presented hereafter, 2 minute irradiation periods were used to achieve adequate labeling while minimizing degradation.

Figure 3-19. Photoaffinity Labeling of BSA with $^{14}$C-N$_3$-MIPP. Separate samples of concentrated solution of bovine serum albumin (BSA, 100 μM) were incubated with $^{14}$C-N$_3$-MIPP (15 μM) and irradiated for the indicated time. Samples were resolved by SDS-PAGE, gels were Coomassie stained (Left), dried and analyzed by phosphorimaging (Right). Label incorporation at the 66 kD band (BSA = 66 kD) was quantified by densitometry (Bottom).

3.2.3.3 Evaluation of the Activity of 5-Azido-MIPP Irradiation Products
Once irradiated, 5-azido-MIPP loses $N_2$ and undergoes rearrangement to a nitrene, which can insert into C-H bonds (Figure 3-20). Before that can occur, however, the nitrene-containing compound typically rearranges to a seven membered ketenimine – a highly electrophilic intermediate – and is susceptible to attack by any cellular nucleophile. In the case that the compound is situated in its target-binding pocket, a nearby residue such as a cysteine, serine, or lysine can react with the ketenimine, forming a covalent bond with the ligand. However, if this excited photoaffinity ligand is not situated in its target, it can react with any other nucleophile – water, glutathione, carbohydrates and other metabolites, amino acids, etc. This effectively “quenches” the ligand such that it can no longer serve to label target proteins.

**Figure 3-20.** Proposed Azido-MIPP Excitation, Protein-Insertion or Quenching Scheme. Azido-MIPP is excited to a nitrene, which can covalently label proteins, but typically rearranges to an electrophilic ketenimine. The ketenimine intermediate is either quenched (e.g. by $H_2O$) or labels proteins to which it’s bound.

There exists a chance that this quenched derivative still maintains the ability to induce methuosis by interacting with the original methuosis-inducing target. This would ultimately reduce the effectiveness of our photoaffinity labeling experiment, as the
quenched ligand would compete with free $^{14}\text{C}-\text{N}_3\text{-MIPP}$ for target binding, which would attenuate the labeling efficiency.

To evaluate this possibility, we essentially carried out a cell-based SAR assay on the quenched products of irradiated 5-azido-MIPP. First, solutions of 5-azido-MIPP in cell-free culture medium were irradiated. Next, MOMIPP was added, and the mixture was added to glioblastoma cells. After four hours, the cells were crudely assessed morphologically for early signs of methuosis (i.e. by vacuole formation). When concentrations of irradiated 5-azido-MIPP from 2.5 µM to 40 µM were irradiated and mixed with 10 µM MOMIPP, the intensity of vacuole formation was only slightly attenuated as 5-azido-MIPP concentrations increased (see Fig 3-21 for representative images, complete image set is in the Appendix). Moreover, irradiated 5-azido-MIPP added directly to cells did not induce any signs of methuosis (Figure 3-21 A). It appears that the quenched products of irradiated 5-azido-MIPP do not induce methuosis, and thus presumably do not compete with 5-azido-MIPP for binding at the relevant target – which is promising for ultimate photoaffinity labeling experiments.
Figure 3-21. Effects of 5-Azido-MIPP Photolysis Products on U251 Cells. A solution of 5-Azido-MIPP in cell culture medium (supplemented with 10% FBS) was irradiated for 2 mins at 254 nm, and immediately transferred to a 35 mm dish containing U251 cells (in the exponential growth phase) at the indicated concentrations, followed by addition of fresh MOMIPP as indicated: (A): 10 μM irradiated 5-Azido-MIPP only (B) 2.5 μM irradiated 5-Azido-MIPP + 10 μM fresh, non-irradiated MOMIPP, (C) 40 μM irradiated 5-Azido-MIPP + 10 μM fresh, non-irradiated MOMIPP. Phase contrast microscopy images taken 4 hours after treatment.
3.2.3.4 Photoaffinity Labeling of Glioblastoma Cell Lysate

Photoaffinity labeling experiments done in U251 glioblastoma cell lysate initially identified 5 cytosolic and 5 membrane-associated proteins that were labeled with 5-Azido-MIPP. Subsequent control experiments narrowed this pool to one potential target in each fraction. Cells were lysed in detergent-free, hypotonic buffer and crudely fractionated by differential centrifugation into a nuclear fraction, a mitochondrial and membranous fraction (pelleted at 100,000 g, P100), and a soluble fraction (supernatant at 100,000 g, S100). Lysates were incubated with $^{14}$C-$\text{N}_3$-MIPP, irradiated, resolved by SDS-PAGE and evaluated for radioactivity by phosphorimaging and fluorography.

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<td>band 1, 50 kD</td>
<td>band 2, 45 kD</td>
<td>band 3, 42 kD</td>
</tr>
<tr>
<td></td>
<td>band 4, 36 kD</td>
<td>band 5, 31 kD</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-22. Photoaffinity Labeling of U251 Cytosolic Cell Lysate. After fractionation, the soluble lysate (S100) was first incubated with the indicated cold compounds (or an
equivalent amount of DMSO) for 1 h. Next, $^{14}$C-N$_3$-MIPP was added to each sample and incubated for 1 h, followed by irradiation for 2 mins. Samples were concentrated and resolved by SDS-PAGE. Dried gels were exposed to storage phosphor screens for ~ 100 h, and analyzed on a phosphorimager. Note: these samples were boiled immediately before SDS-PAGE.

In the soluble cell lysate, initial experiments showed strong labeling of five protein bands, all between 31–50 kD (Figure 3-22). To differentiate specifically labeled proteins from abundant, and/or hydrophobic proteins which are labeled with N$_3$-MIPP nonspecifically, lysate was pretreated with 50-fold excess cold MOMIPP (lane 2). In theory, this should saturate any specific binding sites, blocking the ability of radiolabeled-azido-MIPP from labeling its target. However, other proteins which interact with N$_3$-MIPP due to their hydrophobicity and/or high concentration, or in any arbitrary manner, are often less attenuated by presaturation.\textsuperscript{119,121} In comparing lanes 1 and 2 (Figure 3-22), all five bands are candidate targets based on this criterion, as their signal intensities are all attenuated by MOMIPP pretreatment. Band quantification by densitometry (Figure 3-23) illustrates the overall signal intensity of the five bands of interest (Fig 3-23, left), as well as a ratio of signal in lane 1 to lane 2, i.e. of $^{14}$C-N$_3$-MIPP-treated samples to $^{14}$C-N$_3$-MIPP + excess-MOMIPP-treated samples (Fig 3-23, right). While bands 1, 3, and 5 clearly have the most intense signal, all labeled bands show a roughly 2–4 fold difference in signal in the presence of MOMIPP, identifying each band as a potentially specific MOMIPP target. The largest reduction in labeling was seen in band 5, the 31 kD band, as its signal was nearly 4 times as intense compared to pretreatment with unlabeled MOMIPP, implying that this band may exhibit the most specific azido-MIPP binding.
Densitometry For Figure 3-22. Bands are numbered corresponding to those in Figure 3-22. Band 1 = 50 kD, 2 = 45 kD, 3 = 42 kD, 4 = 36 kD, 5 = 31 kD. (Left) Each band’s signal density is shown relative to the strongest overall signal from that experiment. (Right): Ratio of signal density for lane 1 / lane 2 (illustrating the fold-change in signal with excess cold compound), or lane 1 / lane 3 (illustrating the fold-change in non-irradiation dependent labeling vs. irradiation-dependent labeling). Note: these samples were boiled immediately before SDS-PAGE.

Of these five labeled protein bands of interest, two were also labeled in the no-irradiation control (50 and 42 kD bands, lane 3, Figure 3-22). Two more bands were also labeled in the no-irradiation control, to a much lower extent (45 and 36 kD bands). Figure 3-23 (right) also illustrates the proportion of signal from lane 1 to lane 3. Only band 5 shows virtually no UV-independent labeling, illustrated by a 735-fold signal increase for lane 1/lane 3. Initially, bands 1–4 seem to be labeled in an irradiation-independent mechanism – i.e., they are proteins that covalently bind $^{14}$C-N$_3$-MIPP, regardless of photoexcitation. However, this labeling was eventually attributed to the sample preparation. Immediately before resolving samples by SDS-PAGE, samples were boiled: a routine exercise to ensure protein denaturation and complete solubilization by the SDS detergent. However, in this case, boiling led to labeling by free $^{14}$C-N$_3$-MIPP with
random, relatively abundant and/or hydrophobic proteins, as all $^{14}$C-N$_3$-MIPP was in its native form before the boiling step for the sample in lane 3 (since this sample was never irradiated). Thus, heating the lysate with $^{14}$C-N$_3$-MIPP at 100 °C for ~10 minutes could induce labeling by at least two mechanisms: exciting the aryl-azide, leading to similar excitation as if the compound was irradiated; separately, the increased temperature may simply promote covalent modifications onto electrophilic sites of azido-MIPP, e.g. its enone scaffold.

When this experiment was repeated while omitting the boiling step, essentially no labeling was observed in the no-irradiation sample (Figure 3-24). Thus, of the five original bands of interest, the 50 kD and 42 kD bands (first and third bands) are presumably hydrophobic and/or high abundance proteins that maintain interactions with $^{14}$C-N$_3$-MIPP, even after addition of ionic detergents, as opposed to specific targets. However, they are not proteins that inherently bind N$_3$-MIPP covalently. These proteins were induced to irreversibly bind $^{14}$C-N$_3$-MIPP due to heating at 100 °C, as they are not labeled by $^{14}$C-N$_3$-MIPP without the heating step (Figure 3-22, lane 3 vs Figure 3-24, lane 3). To a lesser degree, the 45 kD and 36 kD bands (second and fourth bands) show a similar pattern. There is no appreciable signal for the 31 kD band in the no-irradiation control, regardless of boiling – implying that its binding is eliminated by the detergent and is not rescued during boiling, in contrast to the other four bands.
Figure 3-24. Photoaffinity Labeling of U251 Cytosolic Cell Lysate. After fractionation, the soluble lysate (S100) was first incubated with the indicated cold compounds (or an equivalent amount of DMSO) for 1 h. Next, $^{14}$C-$N_3$-MIPP was added to each sample and incubated for 1 h, followed by irradiation for 2 mins. Samples were concentrated and resolved by SDS-PAGE. Dried gels were exposed to storage phosphor screens for ~ 100 h, and analyzed on a phosphorimager. Note: samples were not boiled, in contrast to Figure 3-22.

Again, the 31 kD band 5 shows the most intense labeling (Fig 3-25, left).

Furthermore, the repeated experiment again shows a 2–3.6 fold difference in signal when cold MOMIPP is added for all proteins (Figure 3-24, lane 2 vs. lane 1; Figure 3-25, right). Band 5 (31 kD) again shows the strongest attenuation by free MOMIPP, with a 3.6-fold change in signal (Figure 3-24, lane 2 vs. 1, band 5; Fig 3-25, right), as well as the most UV-dependent labeling compared with its no irradiation control (Fig 3-25, right).
Figure 3-25. Densitometry For Figure 3-24. Bands are numbered corresponding to those in Figure 3-24. Band 1 = 50 kD, 2 = 45 kD, 3 = 42 kD, 4 = 36 kD, 5 = 31 kD. (Left) Each band’s signal density is shown relative to the strongest overall signal from that experiment. (Right): Ratio of signal density for lane 1 / lane 2 (illustrating the fold-change in signal with excess cold compound), or lane 1 / lane 3 (illustrating the fold-change in non-irradiation dependent labeling vs. irradiation-dependent labeling).

All labeling described above was done with 5 µM $^{14}$C-5-azido-MIPP. Next, lower concentrations of $^{14}$C-N$_3$-MIPP were evaluated to further determine which bands were specific to azido-MIPP. Concentrations as low as 100 nM (Figure 3-26, lane 1) continue to produce detectable signal for the protein band at 31 kD, while labeling of the four other bands was drastically reduced. The dose-labeling densitometry curve (Figure 3-27) shows that saturation of the 31 kD band occurs around 1 µM in this system. In contrast, with respect to bands 1–4, the degree of labeling increased linearly with concentration, a characteristic of nonspecific incorporation,$^{146}$ further supporting that bands 1–4 are nonspecifically labeled.
Figure 3-26. Photoaffinity Labeling of U251 Cytosolic Cell Lysate. After fractionation, the soluble lysate (S100) was incubated with the indicated concentrations of $^{14}\text{C}-\text{N}_3\text{-MIPP}$ for 1 h, followed by irradiation for 2 mins. Samples were concentrated and resolved by SDS-PAGE. Dried gels were exposed to storage phosphor screens for ~100 h, and analyzed on a phosphorimager.

Figure 3-27. Dose-Labeling Densitometry For Figure 3-26. Bands are numbered corresponding to those in Figure 3-26. Band 1 = 50 kD, 2 = 45 kD, 3 = 42 kD, 4 = 36 kD,
$5 = 31 \text{ kD}$. The absolute density at each point is shown relative to the signal with highest intensity.

The photoaffinity experiment was repeated in cytosolic U251 cell extracts with lower probe concentrations, wherein the preincubation with MOMIPP was at 50 µM, and the main incubation was with 500 nM $^{14}$C-N3-MIPP (Figure 3-28). In this case, selective labeling of the 31 kD band is illustrated by the 6.5-fold increase in signal compared to the MOMIPP-pretreated sample (Figure 3-28, lane 2 vs. 1; Figure 3-29, right). In contrast, bands 1–5 only showed 1.8–2.7-fold difference in signal compared to MOMIPP pretreatment. Moreover, the 31 kD band has markedly higher probe incorporation compared to the other bands (Fig 3-29, left).
Figure 3-28. Photoaffinity Labeling of U251 Cytosolic Cell Lysate. After fractionation, the soluble lysate (S100) was first incubated with the indicated cold compounds (or an equivalent amount of DMSO) for 1 h. Next, $^{14}\text{C}$-N$_2$-MIPP was added to each sample and incubated for 1 h, followed by irradiation for 2 mins. Samples were concentrated and resolved by SDS-PAGE. Dried gels were exposed to storage phosphor screens for ~ 72 h, and analyzed on a phosphorimager.

In attempt to further identify the 31 kD band as highly-specific to MOMIPP, an additional sample was done by pretreating with excess meta-MOMIPP (compound 58, Table 2.1), a compound that does not induce any signs of methuosis, yet is structurally very similar to MOMIPP (Fig 3-28, lane 3). While the 31 kD band showed a 6-fold increase in signal compared to MOMIPP preincubation, the same band showed only a 4-fold increase in signal compared to meta-MOMIPP preincubation (Figure 3-29, right).
Though not drastic, there is moderate selectivity of the 31 kD protein for MOMIPP vs. *meta*-MOMIPP. However, other factors may render *meta*-MOMIPP inactive at inducing methuosis, and thus it may still bind the MOMIPP target, yet in an unproductive manner.

**Figure 3-29.** Densitometry For Figure 3-28. Bands are numbered corresponding to those in Figure 3-28. Band 1 = 50 kD, 2 = 45 kD, 3 = 42 kD, 4 = 36 kD, 5 = 31 kD. (Left) Each band’s signal density is shown relative to the strongest overall signal from that experiment. (Right): Ratio of signal density for lane 1 / lane 2 (illustrating the fold-change in signal with excess cold MOMIPP), lane 1 / lane 3 (illustrating the fold-change in signal with excess cold *meta*-MOMIPP), or lane 1 / lane 4 (illustrating the fold-change in non-irradiation dependent labeling vs. irradiation-dependent labeling).

One limitation of the MOMIPP-target binding interaction may be that it is extremely short-lived; i.e., it displays a short residence time. Preliminary evidence implies that MOMIPP’s time spent target-bound is short lived (as evidenced by its rapid efflux from cultured cells, not shown). Regardless of the theoretical target saturation by preincubation with excess cold MOMIPP, if the drug-target residence time is brief, the competition will be less dramatic. In an attempt to improve the target saturation to further illustrate specific binding, lysate was pretreated with a large excess of non-radiolabeled 5-azido-MIPP and irradiated (Figure 3-30). Next, the same sample was treated with 500
nM of $^{14}\text{C-N}_3\text{-MIPP}$, and irradiated a second time. As before, a parallel sample was pretreated with excess inactive analog, this time using cold *meta*-azido-MIPP, (also inactive at inducing methuosis). Results are shown in Figure 3-30 and 3-31. The main experimental sample (Figure 3-30, lane 1) was preincubated with DMSO and similarly irradiated, to control for any UV-dependent protein degradation that might attenuate ligand-target interactions.

**Figure 3-30.** Photoaffinity Labeling of U251 Cytosolic Cell Lysate. After fractionation, the soluble lysate (S100) was first incubated with the indicated cold compounds (or an equivalent amount of DMSO) for 1 h, followed by irradiation for 2 mins. Next, $^{14}\text{C-N}_3\text{-MIPP}$ was added to each sample and incubated for 1 h, followed by irradiation for 2 mins. Samples were concentrated and resolved by SDS-PAGE. Dried gels were exposed to storage phosphor screens for ~72 h, and analyzed on a phosphorimager.
**Figure 3-31.** Densitometry For Figure 3-30. Bands are numbered corresponding to those in Figure 3-30. Band 1 = 50 kD, 2 = 45 kD, 3 = 42 kD, 4 = 36 kD, 5 = 31 kD. (Left) Each band’s signal density is shown relative to the strongest overall signal from that experiment. (Right): Ratio of signal density for lane 1 / lane 2 (illustrating the fold-change in signal with cold N$_3$-MIPP prelabeling), lane 1 / lane 3 (illustrating the fold-change in signal with cold meta-N$_3$-MIPP prelabeling), or lane 1 / lane 4 (illustrating the fold-change in non-irradiation dependent labeling vs. irradiation-dependent labeling).

As shown in the labeled gel and the corresponding graphs (Figure 3-30 & 3-31), the 31 kD band showed a 9-fold change in labeling with prelabeled with cold azido-MIPP. Bands 1-4 (though bands 2 and 4 were barely labeled at all) were minimally affected by prelabeling with cold azido-MIPP, with 1–1.7 fold differences: not indicative of specific binding. Prelabeling with *meta*-azido-MIPP did attenuate binding, though to a lesser degree than azido-MIPP (6-fold difference, Fig 3-30, lane 3 vs. 2; Fig 3-31, right).

The *meta* analog seems to still compete with azido-MIPP for target binding, though to a slightly lesser degree than its active analog, as in the previous experiment. Thus, the inactivity for the *meta* analog is likely not entirely attributed to a lack of target interaction; rather, its subtle structural modifications may eliminate a crucial interaction.
necessary to stimulate methuosis. Another possibility is that the meta-pyridine may have a different metabolic fate than the para compound.

In addition to the interesting 31 kD band in the soluble S100 cellular fraction, experiments with the P100 fraction (sedimented at 100,000 × g, which contains mitochondrial, lysosomal, and membranous proteins) demonstrated selective labeling of an 18 kD protein (Figure 3-32). While the signal is not as intense as the S100 bands, the most intensely labeled band is at 18 kD. Moreover, this labeling was largely eliminated when the sample was pre-incubated with 50-fold excess MOMIPP (5-fold difference in labeling intensity, Figure 3-32, lane 2 vs. 1; Figure 3-32, right). However, the labeling is also blocked when excess meta-MOMIPP is added (4-fold difference, lane 3 vs. lane 1), thus the specificity for MOMIPP over meta-MOMIPP is not observed, as before. At least four other bands are labeled at low levels (Figure 3-32, lane 1), and these bands show essentially no reduction in signal after excess cold drug pre-treatment (Figure 3-32, lanes 2 and 3 vs. 1; Figure 3-33, right), indicating probable non-specific interaction with a relatively abundant protein.
Figure 3-32. Photoaffinity Labeling of U251 P100 Cell Lysate. After fractionation, the P100 lysate fraction was first incubated with the indicated cold compounds (or an equivalent amount of DMSO) for 1 h. Next, $^{14}$C-N$_3$-MIPP was added to each sample and incubated for 1 h, followed by irradiation for 2 min. Samples were concentrated and resolved by SDS-PAGE. Dried gels were exposed to storage phosphor screens for ~100 h, and analyzed on a phosphorimager.
Figure 3-33. Densitometry For Figure 3-32. Bands are numbered corresponding to those in Figure 3-32. Band 1 = 40 kD, 2 = 37 kD, 3 = 30 kD, 4 = 26 kD, 5 = 18 kD. (Left) Each band’s signal density is shown relative to the strongest signal from that experiment. (Right): Ratio of signal density for lane 1 / lane 2 (illustrating the fold-change in signal with excess cold MOMIPP), lane 1 / lane 3 (illustrating the fold-change in signal with excess cold meta-MOMIPP), or lane 1 / lane 4 (illustrating the fold-change in non-irradiation dependent labeling vs. irradiation-dependent labeling).

Finally, the P0.5 (nuclei, sedimented at 500 × g) fraction did not display any selective labeling. Only minimal labeling, barely above background levels, was seen with 5 µM 14C-5-azido-MIPP (Figure 3-34). As MOMIPP initially stimulates methuosis via an overwhelming flux of vacuole formation within minutes, the target is most likely outside the nucleus; therefore the lack of specific labeling of nuclear proteins is not surprising.
Figure 3-34. Photoaffinity Labeling of U251 Nuclear Cell Lysate. After fractionation, the P0.5 lysate fraction was first incubated with the indicated cold compounds (or an equivalent amount of DMSO) for 1 h. Next, \(^{14}\text{C}\text{-N}_2\text{-MIPP}\) was added to each sample and incubated for 1 h, followed by irradiation for 2 min. Samples were concentrated and resolved by SDS-PAGE. Dried gels were exposed to storage phosphor screens for \(\sim 100\) h, and analyzed on a phosphorimager.

3.3 Discussion

3.3.1 Potential Targets
Two targets were identified as specific targets of MOMIPP in U251 glioblastoma cells: a 31 kD soluble protein and an 18 kD protein that pelleted with the P100 fraction. These two proteins were shown to specifically interact with MOMIPP, in that their labeling by $^{14}$C-N$_3$-MIPP was largely reduced by competition with unlabeled MOMIPP or N$_3$-MIPP. While specific, this does not give any indication to the pharmacologic relevance of this interaction, i.e. its role in inducing methuosis.

However, assuming one or both of the identified protein bands are relevant targets, we can speculate which protein targets may be stimulating methuosis. Previous studies confirmed that methuosis-inducing compounds interfere with the endosomal trafficking cycle.\textsuperscript{53} A simplified cartoon of the macropinosome/endosome/lysosome maturation pathway is shown in Figure 3-35.\textsuperscript{147} The cycle is initiated as extracellular material is internalized by ongoing endocytosis, wherein both macropinosomes and clathrin-coated endosomes may merge with the sorting or early endosome (EE) – the majority of which are quickly recycled back to the plasma membrane. However, some of the EEs mature toward multivesicular endosomes and late endosomes (LE). LEs undergo homotypic fusion, growing in size. Eventually, LEs undergo heterotypic fusion with lysosomes, and the endosomal cargo is degraded by lysosomal enzymes. Continuous trafficking also occurs between the trans-golgi network (TGN) and the endosomal compartment.
**Figure 3- 35.** Macropinosome – Endosome – Lysosome Maturation Pathway. Macropinosomes emerging during large-scale endocytosis fuse into early endosomes. Typically, most early endosomes are recycled, however, they can mature toward a late endosome (LE), acquiring specific signaling and effector components at each stage. LEs ultimately fuse with lysosomes, and their cargo is degraded. Methuosis involves some combination of excessive macropinocytosis and failed endosome–lysosome fusion, and pseudo-late endosomes overwhelm the cytoplasm, the characteristic vacuoles seen in cells undergoing methuosis. MT = microtubules. (Reprinted with permission from reference 147)

As illustrated by previous work (see chapter 1), in methuosis, vacuoles are labeled by some markers characteristic of LEs, such as Rab7 and LAMP1. However, the macropinosome-derived vacuoles do not proceed to fuse with lysosomes, and thus appear stuck in the LE stage. Moreover, there is evidence that the early maturation into the LE is also abnormal, as effectors of early endosome formation were significantly reduced, even at early timepoints. Thus, it seemed that MIPP led to abnormal maturation of early endocytic vesicles into late endosomal-like vacuoles, which lacked the necessary
effectors to fuse with lysosomes. However, direct LE—lysosomal fusion could also be inhibited by MOMIPP, instead of a maturation defect. With this in mind, we can further speculate about the target of MOMIPP (below).

As the endosomal/lysosomal cycle is better understood, the protein machinery involved at each step is becoming characterized. After surveying the recent literature, a list of roughly 100 proteins that function along the macropinosomal – endosomal – lysosomal maturation cycle was made, along with their corresponding protein mass (available in the Appendix). From this list, proteins that have at least one isoform with a mass of 18 ± 2 kD or 31 ± 2 were selected (Table 3.1). A total of 21 candidates were identified; 7 that correspond to the 18 kD labeled band and 14 that correspond to the 31 kD labeled band. Of the 21 selected proteins, most are associated with one of a few core protein complexes, as listed in Table 3.1.

Seven of the selected proteins are members of the Endosomal Sorting Complexes Required for Transport (ESCRT) family, of which three main complexes exist: ESCRT-I, II, and III. These multi-protein complexes are responsible for the membrane budding and scission that occurs as an EE matures into a LE (also known as a multivesicular body, MVB, due to EEs budding into multiple intraluminal vesicles). Interestingly, in addition to their membrane-cleaving functions in endosome maturation (i.e., in forming MVBs/LEs), ESCRT-I and III are also involved in a similar membrane-cleaving step that occurs during cytokinesis. Thus, inhibition of one of these components by MOMIPP may directly reconcile its effects on vacuole formation with its antiproliferative effects.
Table 3.1 Potential MOMIPP Targets. Proteins reported to play key roles in the maturation of a macropinosome through endosome–lysosome fusion with a mass of 18 ± 2 kD or 31 ± 2 kD are listed.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Size (kD)</th>
<th>Comments</th>
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<tbody>
<tr>
<td>VPS25 (EAP20)</td>
<td>20</td>
<td>ESCRT-II component</td>
</tr>
<tr>
<td>CHMP5</td>
<td>20</td>
<td>ESCRT-III component, associated with cytokinesis</td>
</tr>
<tr>
<td>VPS24 (CHMP3)</td>
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<td>ESCRT-III component, associated with cytokinesis</td>
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<td>Arf1, Arf6</td>
<td>20</td>
<td>EE maturation</td>
</tr>
<tr>
<td>Vps8</td>
<td>17</td>
<td>CORVET component, EE maturation</td>
</tr>
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<td>SNAP23</td>
<td>18</td>
<td>SNARE complex, assoc. w/ fusion, assoc. with glucose transport</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>17</td>
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</tr>
<tr>
<td>MVBL2A, MVBB</td>
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<td>ESCRT-I component</td>
</tr>
<tr>
<td>VPS37A-D (37B)</td>
<td>31</td>
<td>ESCRT-I component</td>
</tr>
<tr>
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<tr>
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<td>GAP for Rab35 (Rab35: also assoc. w/ cytokinesis)</td>
</tr>
<tr>
<td>LAPTM5</td>
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<td>aids TGN to lysosome trafficking</td>
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</table>

Numerous other selected candidates are SNAP proteins or members of SNARE complexes. SNAP proteins are required to form tethers between endosomes or endosomes and lysosomes; formation of such tethers is the first step in homotypic endosomal and heterotypic endosome-lysosome fusion (see Figure 3-35). After tether formation, SNARE (i.e. SNAP receptor) complexes are assembled, immediately preceding fusion. Based on the process of methuosis, MOMIPP clearly does not interfere with homotypic fusion between macropinosomes or endosomes, but it may interfere with heterotypic fusion between endosomes and lysosomes. Interestingly, two of the identified proteins in Table 3.1 are specifically associated with heterotypic endosomal–lysosomal fusion, a step which indeed may be inhibited in methuosis: VAMP7 and calmodulin. Calmodulin associated calcium release is thought to be necessary for heterotypic fusion, but not for homotypic fusion, as shown in Figure 3-36. Inhibition of any of these proteins may be occurring by MOMIPP.
Figure 3-36. Proteins Complexes in Homotypic Endosomal Fusion and Heterotypic Endosomal–Lysosomal Fusion. Fusion is thought to involve three steps: (A) tether formation, requiring Rab7, NSF, and SNAP proteins, (B) SNARE protein complex assembly follows tethering. Some SNARE components are ubiquitous to homotypic and heterotypic fusion, some are thought to be specific to one type of fusion (C) calmodulin-dependent Ca$^{2+}$ release is necessary specifically for heterotypic fusion. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, reference 149, copyright 2007)

Finally, MOMIPP may also serve as an agonist, or an allosteric stabilizer to stimulate a protein’s activity. In this instance, any of the identified proteins may be targeted by MOMIPP, leading to premature and abnormal vesicle maturation, resulting in a defective pseudo-late endosome that cannot progress to lysosomal fusion. This would agree with previous work. Of course, MOMIPP may not interfere with any of these
proteins directly, but rather it may function to disrupt proteins that regulate the function of vesicle maturation and fusion proteins.

Of course, we will proceed with identifying the azido-MIPP labeled proteins before evaluating these 21 candidate proteins that may be targeted by methuosis-inducing compounds. Accordingly, labeled proteins will be sequenced by mass spectrometry-based peptide mass fingerprinting (see chapter 4 for more on how this may be approached). Following identification of candidate protein sequences, we must determine if they are physiologically relevant to the methuosis phenotype, perhaps matching one of the proteins discussed in Table 3.1.

Eventually, candidate targets will then be evaluated biologically. For example, the gene of interest will be knocked down using siRNA. If MOMIPP is inhibiting an enzyme or protein interaction, the knockdown should induce a phenotype similar to MOMIPP treatment. Alternatively, overexpression of the protein would render these cells less sensitive to MOMIPP. Though less likely, MOMIPP may activate or stabilize its target; in this case, target knockdown should lead to MOMIPP resistance. Successful target validations of this sort are reported in a number of target identification studies.68,101,117,137

3.3.2 Limit of Detection

To evaluate the advantages and limitations of the target identification approaches used, a discussion on each method’s theoretical limit of detection is necessary. Beginning with photoaffinity labeling with radioactive ligands, the limit of detection equals the ability to visualize a radiolabeled protein band; using our phosphorimager, the limit of detection for a $^{14}$C-labeled band is 2 dpm/mm$^2$ in 1 hour (per the manufacturer’s
literature, using the instrument’s largest pixel settings). As the gels described herein contained bands of about 1 cm × 1 mm, a band would need 20 dpm total for detection in 1 hour. While I typically expose dried gels to phosphor screens for approximately 100 hours, I also used a finer pixel setting for better resolution (5× smaller); thus, the original estimate of 20 dpm is an appropriate starting point for visualizing a usable signal. Finally, assume the protein of interest has a mass of 25 kD.

A 20 dpm signal correlates to 0.16 pmol $^{14}$C-labeled compound, given our compound’s specific activity of 55 mCi/mmol. If 0.16 pmol $^{14}$C-N$_3$-MIPP stochiometrically labeled its protein target, i.e. labeling 0.16 pmol protein, this would correlate to 4.0 ng of protein (of a 25 kD protein). In experiments described here, samples (S100 or P100) typically contained roughly 600 µg of protein. In this hypothetical case, 0.16 pmol of our protein (4.0 ng) would represent 0.00067% of the proteome – which is on the low end of the spectrum of protein concentrations.

Recent proteomic analyses have shown that protein copies-per-cell in human cancer cells range over many orders of magnitude, from 20,000,000 down to 5,000 copies per cell.$^{152,153}$ Our estimated requirement of 0.16 pmol target protein equates to roughly 100 billion total copies of the individual protein molecule. In the typical photoaffinity experiment described here, each sample contained fractionated lysate from roughly 8 million cells (and the entire sample was used in one lane per gel). Thus, the number of proteins needed to reach our predicted limit of detection, i.e. 100 billion copies, divided by the number of cells used per experiment, 8 million, would require our target protein to exist at a concentration of at least 10,000 copies per cell. As this is near the lowest
predicted levels for protein copies per cell,\textsuperscript{152,153} our approach should be able to identify low abundance proteins.

However, these approximations are all dependent on the labeling efficiency. Based on the above analysis, proteins would need to be 100\% labeled by our radioactive probe – which is not likely. In our initial experiments, 5 \mu M 14C-N\textsubscript{3}-MIPP was used; this correlates to roughly 500 pmol of 14C-N\textsubscript{3}-MIPP in every 100 \mu L sample (P100 samples were done in \sim 100 \mu L volumes, S100 in \sim 300 \mu L). Even if the target of interest is a low abundance protein, we are saturating the system with more than enough compound. It is difficult to predict the actual efficiency with which our probe will label its target. For each order of magnitude loss of labeling efficiency (\textit{e.g.} only 10\% of target sites are labeled), a corresponding increase of protein copies would need to exist to reach our limit of detection (\textit{i.e.} 100,000 copies per cell, to achieve 10,000 14C-labeled copies per cell).

One cause for reduced labeling efficiency is tight binding of photolysis products (irradiated 14C-N\textsubscript{3}-MIPP that is not inserted into a protein, but instead “quenched” by water or other molecules) at the MOMIPP binding site. However, we have shown preliminary evidence that the photolysis products do not display tight binding at the MOMIPP target site (Figure 3-21). Another cause would be destruction of the receptor during irradiation, which is possible; however, our results show similar labeling after preirradiating the sample (Figures 3-30 vs. 3-28). Ultimately, our molecule’s properties and our experimental approach should be able to detect low-abundance target proteins, and higher abundance proteins have an even greater likelihood of identification.

Finally, the amount of labeled protein must be amenable to characterization by mass spectrometry in order to be useful. These pmol protein levels are well above the
single-digit fmol levels (i.e. ~ 100 pg protein) that can be used for peptide mass fingerprinting to identify an unknown protein.\textsuperscript{154}

This discussion focused on identifying low abundance proteins, and using an excess of radiolabel. Of course, that can lead to excessive nonspecific labeling. However, this can be discriminated from specific labeling using competition and dose-labeling analyses as explained in the results section.

In contrast to the photoaffinity approach, wherein the limit of detection relies on the limit of radioactivity detection, the pulldown approach depends on silver staining limit of detection, roughly 1 ng, which, for a 25 kD protein, is only 0.04 pmol. In the typical experiment described in this chapter, resin was loaded with roughly 1 nmol of MIPP-biotin conjugate. Clearly, only a small fraction of compound needs to bind its target, and remain bound through the washing steps. However, the nature of the biotin-MIPP–target interaction is not known, and it very well may not interact with its specific target due to the structural modifications. Therefore, the photoaffinity labeling approach is much more reliable for identifying MOMIPP-interacting proteins.

The in-situ click-chemistry approach most likely failed due to the reversibility of the POMIPP-target interaction. Any POMIPP-target interactions were probably lost during the click-chemistry reaction steps, and/or the pulldown processing steps (resin washing, similar to the direct pulldown approach). An alternative approach to optimize the click-chemistry approach is presented in the subsequent chapter.
3.4 Methods

Synthetic chemistry experimental details and NMR spectra are provided in the Appendix.

Cell Culture. U251 human glioblastoma cells were purchased from the DCT Tumor Repository (National Cancer Institute, Frederick, MD). Unless stated otherwise, cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) (JR Scientific, Woodland, CA) at 37°C in an atmosphere of 5% CO₂/95% air. Phase-contrast images of live cells were obtained using an Olympus IX70 microscope equipped with a digital camera and SPOT imaging software (Diagnostic Instruments, Inc.)

In-situ click-chemistry. U251 glioblastoma cells were harvested by scraping and lysed by homogenization in PBS with 1% Triton-X-100 with protease inhibitor cocktail (PBS-T-PIC) (Roche, cOmplete mini EDTA free protease inhibitors). After pelleting nuclei by centrifugation (1,000 × g for 10 mins at 4 °C), cell lysate was diluted to 1 mg/mL with PBS-T-PIC. To each reaction sample of 1 mL lysate, POMIPP with or without competitor drug was added from 100x stock solutions in DMSO, and incubated for 1 h at RT. Next, the click-chemistry reagents TCEP, TBTA, CuSO₄, and biotin-azide were added (final concentrations: 1 mM TCEP from fresh 100 mM stock in H₂O, 100 μM TBTA from 10 mM stock in 1:4 DMSO:tBuOH, 1 mM CuSO₄ from 100 mM fresh stock in H₂O, 100 μM biotin-azide from 20 mM stock in DMSO) and incubated 1 h at RT. After removing 20 μL aliquots (input sample for gel), samples were moved to 15 mL tubes and 5 volumes of cold acetone was added. Samples were incubated at –20 ºC for 30
min to extract excess biotin-azide. Precipitated proteins were pelleted by centrifugation (4600 × g for 10 min at 4 °C) and the acetone was aspirated. This was repeated two more times to ensure removal of excess biotin-azide. Precipitated proteins were dissolved with 1% SDS in PBS (100 µL, with brief sonication), followed by addition of 900 µL PBS-T (PBS with 1% Triton-X-100), for a final 0.1% SDS (20 µL sample saved for gel to evaluate resolubilization efficiency). Entire 1 mL samples were added to 100 uL Neutravidin agarose resin (Thermo, prewashed with 0.1% SDS-PBS) and rotated end-over-end at 4 °C for 16 h. Resin was centrifuged (4000 × g for 3 min at 4 °C) and the supernatant was discarded (20 µL sample saved for gel to evaluate efficiency of affinity purification). Resin was washed three times by adding 0.5% SDS-PBS, vortexing, and collected by centrifugation. Resin was suspended in sample buffer (100 µL) and boiled for 5 min to elute bound proteins. Resin was sedimented by centrifugation (4000 × g for min) and supernatant was directly used for SDS-PAGE.

**Streptavidin-bead pulldowns.** U251 glioblastoma cells were harvested by scraping and lysed by homogenization in PBS with 1% Triton-X-100 with protease inhibitor cocktail (PBS-T-PIC) (Roche, cOmplete mini EDTA free protease inhibitors). After pelleting nuclei by centrifugation (1,000 × g for 10 mins at 4 °C), cell lysate was diluted to 1 mg/mL with PBS-PIC (final Triton-X-100 ~ 0.2%). Lysate was pre-incubated with free competitor compound (1:100 dilution from DMSO stock) or an equivalent amount of blank DMSO for 3 h at 4 °C. Separately, M-280 magnetic streptavidin Dynabeads (Invitrogen) were washed twice (PBS with 0.1% Triton-X-100) and loaded with biotinylated-drug conjugate. Typically, 100 µL of bead suspension was used per sample
and was incubated with 7.5 nmols of biotinylated-compound (5 µL of 1.5 mM DMSO stock) in 1 mL of PBS with 0.1% Triton-X-100 (per the manufacturer’s instructions, 100 µL resin can bind 1 nmol biotin), rotated end-over-end for 4 h at RT, and washed three times with PBS-0.1%-Triton-X-100 to remove excess biotinylated-compound. After incubation with free drug or DMSO, lysate was added to the resin and rotated end-over-end at 4 °C overnight. Resin was collected and washed three times with PBS-Triton-X-100 (0.1% or 1%, as described in the text). In some instances, resin was competitively eluted by adding 100 µL MOMIPP (50 µM in PBS with 1% Triton-X-100) and vortexing/rotating; to this was added 5× sample buffer for SDS-PAGE. Resin was finally suspended in 1× sample buffer and boiled for 5 min. Supernatant was collected and resolved by SDS-PAGE.

**Western blot analysis.** SDS-PAGE gels were transferred onto PVDF membrane at 0.1 Amp overnight at 4 °C in transfer buffer (10 mM NaHCO₃, 3 mM Na₂CO₃, pH 9.5). Membranes were blocked in PBS-TM (PBS with 0.2% Tween20, 5% dry milk) for 1 h at RT. Blocking buffer was then removed, and PBS-TM with 1:1000 Streptavidin-HRP (Invitrogen) and 1:1000 anti-biotin-HRP-antibody (Cell Signaling) was added for 1-2 h. The membrane was washed with fresh PBS-TM three times, at 15, 5, and 5 minutes each. Blots were incubated in ECL solution for 1 min, then exposed to film and/or gel imaging system (AlphaInnotech) (ECL: done by mixing 10 mL solution A and 10 mL solution B, immediately adding to blot for 1 min; soln A: 9.9 mL 0.1 M Tris-HCl, pH8.5, 100 µL 250 mM luminol, 44 µL 90 mM p-coumaric acid; soln B: 10 mL 0.1 M Tris-HCl, pH 8.5, 6 µL 30% H₂O₂).
Colony Formation. Cells were plated in 100 mm dishes at 2,500 (U251 and U251-TR) or 1,500 (MCF-7 and MCF-7 DoxR) cells per dish. Beginning on the day after plating, the cells were exposed to the indicated compounds for the periods of time noted in the figure legends. The medium was then replaced (without compounds) and cells were incubated for 10 days, with fresh medium added every 2 d. Colonies were visualized by washing with phosphate-buffered normal saline, fixing for 10 min with ice-cold 100% methanol, and staining with 1% (w/v) crystal violet (Acros Organics, Fisher Scientific, Pittsburgh, PA) in 35% methanol. After 2-3 washes with water, colonies containing at least 50 cells were counted using a dissecting microscope or a Protocol 2 colony counter (Synbiosis, Frederick MD).

Phosphorimaging. Protein gels were dried and covered with plastic wrap, and exposed to storage phosphor screen for the indicated times. Phosphorimaging was done using a Typhoon 7000 instrument, and data was analyzed using ImageQuantTL software.

Fluorography. After Coomassie staining, wet gels were immersed in Amplify (Amersham) for 30 minutes, and then dried and exposed directly to BioMax MR autoradiograph film (Kodak) with an intensifying screen and placed at –80 °C for the indicated times.

Photoaffinity labeling of cell lysate.
Cells at no more than 70% confluency were harvested by scraping into HBSS and washed with cold PBS (2×). Cells were suspended in 5 pellet cell volumes (PCV) of hypotonic lysis buffer (10 mM sodium phosphate, pH 7.4, 1.5 mM MgCl₂) on ice for 15 min to swell. Cells were broken by passing through a 27 gauge needle 30–35 times until lysis was complete (by trypan blue). 1/3 volume of sucrose buffer (1 M sucrose in lysis buffer) was added such that the final sucrose was 0.25 M. Lysate was fractioned by differential centrifugation. Lysate was centrifuged (500 × g, 10 min, 4 °C) providing P0.5, which was resuspended in 2.5 PCVs (based on the original PCV) of 0.25 M sucrose in hypotonic lysis buffer. The supernatant was centrifuged (100,000 × g, 1 h, 4 °C) providing P100, which was resuspended in 1 PCV (based on the original PCV) of 0.25 M sucrose in hypotonic lysis buffer, and supernatant, S100. Typical protein concentrations for P100 and S100 were 5.9 mg/mL and 1.9 mg/mL, respectively. Samples were pre-treated with appropriate cold competitor compounds or DMSO, mixed and incubated for 1 h, followed by addition of radioactive material for another 1 h. Mixtures were then irradiated in a Stratalinker 2400 (5 254 nm 15-watt bulbs) for 2 min. A 24-well plate was covered tightly with parafilm; using a gloved finger, the film was indented over individual wells, and sample was pipetted onto the well (≤ 350 µL per well) and irradiated. Samples were treated with 5× sample buffer and the entire sample was resolved by SDS-PAGE (however, if sample volume was > 125 µL, they were concentrated to an appropriate volume before adding sample buffer, using centrifugal filter units (Amicon Ultra-4 Ultracel 10K). Samples were not boiled unless otherwise mentioned. Presented experiments contained roughly 1.8 mg/sample for P100, and 800 µg/sample for S100.
Gels were stained using Coomassie or colloidal blue, and analyzed by fluorography and/or phosphorimaging.
Chapter 4

Summary and Future Work.

The goal of the studies presented in this dissertation was to better understand small-molecule induced methuosis. As presented in the second chapter, we have identified structural requirements of indole-based chalcones necessary for inducing methuosis, leading to our current lead compound, MOMIPP. MOMIPP is active at inducing non-apoptotic cell death in glioblastoma cells, with a GI50 of about 2 µM. Moreover, MOMIPP induces methuotic cell death in glioblastoma cells that are resistant to the current first-line chemotherapeutic, temozolomide. Thus, mechanisms that cells evolve to overcome the TMZ-induced DNA damage are not sufficient to protect from methuosis. Moving forward, this can be evaluated in animal models as an approach to limit or reverse tumor growth. However, its utility will most likely be displayed in combination with other drugs.

Specifically, combining MOMIPP with therapies that induce other forms of cell death, such as those that induce apoptosis (TMZ, radiation, etc.), should provide the best chance of treating a tumor while limiting its development of resistance. Moreover, as
therapeutics become available that induce even more forms of cell death (necroptosis, lysosomal membrane permeabilization, etc., as discussed in chapter 1), these should be included as a cocktail approach.

The SAR requirements that were discovered for MOMIPP were crucial toward the design of putative photoaffinity analogs. After identifying a photoreactive MOMIPP derivative which had similar methuosis-inducing activity (i.e. 5-azido-MIPP), two specific protein targets were identified. Soon, those will be sequenced by peptide mass fingerprinting with mass spectrometry (MS). However, at least two problems may arise that render that step difficult. First, the protein of interest may co-migrate with other protein(s) by gel electrophoresis. Thus, when the labeled protein of interest is excised from the gel and analyzed by MS, other proteins may contaminate the sample. Discriminating between the contaminants and the actual target would be impossible at this stage.

In order to determine which protein in this mixture is the true target protein, we can take advantage of two isotopic labeling techniques. The first method is depicted in Figure 4-1 and is somewhat related to traditional SILAC (Stable Isotope Labeling of Amino acids in Cell culture) applications. In our case, cells are labeled with an isotopically labeled amino acid, e.g. $^{13}$C-labeled leucine. After sufficiently passaging cells in medium supplemented with this amino acid, the proteins will be essentially 100% labeled, i.e. “heavy.” Next, a photoaffinity labeling experiment is done on lysate from the “heavy” cells using N$_3$-MIPP. Cell lysate from a parallel dish of unlabeled “light” cells will also be used, and the two samples are then mixed 1:1 and resolved by SDS-PAGE. The band of interest is excised from the gel, along with any co-migrating proteins.
inadvertently included (the band of interest is identified from previous photoaffinity experiments using the radioactive analog). Finally, when analyzed by MS, the expected mixture of proteins will be visualized as doublets on MS, with a m/z separation equal to the number of $^{13}$C leucines in each peptide. However, one peptide should be represented by a larger doublet with an m/z difference of the excited azido-MIPP molecule plus the number of $^{13}$C leucines in that peptide, which will be drastically different than any other doublet.

**Figure 4-1.** SILAC approach to discriminate probe-labeled proteins from non-labeled, contaminating proteins. Traditionally photoaffinity labeling is done using isotopically labeled cells. Next, non-isotopically labeled, non-photolabeled cells are mixed 1:1, and
cell lysate is evaluated by mass spectrometry. Protein which has been labeled by the photoaffinity ligand will display a large doublet, while non-photolabeled cells will display doublets with much smaller m/z differences.

An alternate approach is to isotopically label the photoprobe. N₃-MIPP can be made substituting deuterium for multiple hydrogen atoms.¹⁵⁶ Next, a photoaffinity experiment is done with a 1:1 mixture of heavy:light N₃-MIPP. Once the protein is resolved, excised (again, along with co-migrating proteins), and analyzed by MS – one peptide will be a doublet, separated by an m/z difference equal to the number of deuterium atoms used. All other non-labeled, co-migrating bands will be singlets on MS.

The second potential problem relates to limit of detection. As explained in the Chapter 3 discussion, our approach most likely accounts for low abundance proteins. However, in the case that we cannot identify or isolate enough of a putative target protein, we can combine our photoaffinity-induced covalent bond formation with the in-situ click-chemistry based enrichment approach. This would effectively address the failed click-chemistry approach described in chapter 3. Here, a MOMIPP analog that contains a click-chemistry handle (like POMIPP) but also contains a photoreactive group (like azido-MIPP) is needed. A potential synthesis is shown in Figure 4-2, based on an updated
Figure 4-2. Proposed synthesis for a bifunctional MOMIPP photoprobe. The synthesis shown is a proposed route to provide a photolabile MOMIPP analog with a click-chemistry acetylene handle.

Madelung indole for the synthesis of 2-alkylindoles. The other steps are analogous to the original 5-azido-MIPP synthesis already accomplished. Recent SAR studies have determined that substitution of ethyl for methyl at MOMIPP’s 2 position does not reduce activity, thus there is good reason to believe that the 2-acetylene derivative would also be active at inducing methuosis. Thus, cell lysate will be labeled with 2-acetylene-5-azido-MIPP in a typical photoaffinity approach. Next, the click-chemistry reaction with biotin-azide will be done, without the concern of losing the MIPP–protein interaction (which most likely was why the approach presented in Chapter 3 failed). This biotin–MIPP–protein complex is finally enriched using a streptavidin resin, potentially providing much more target protein that can be isolated from the previous photoaffinity labeling.
approach. This will also address the first potential issue, identifying a band within a gel that contains all other cellular proteins. Here, the isolated protein should be enriched from contaminating proteins, providing a purer protein sample for mass spectrometry.

Once identified, knowledge of MOMIPP’s target will improve our understanding of methuosis as well as provide many future directions for studying methuosis. First, it should help clarify our understanding of the methuosis process. As described in chapter 1, MIPP-induced methuosis disrupts the macropinosome-endosome-lysosomal maturation process. However, it isn’t known how MOMIPP is acting; is it inhibiting the function of a crucial endosomal maturation effector, thus rendering the vesicles incapable of fusing with lysosomes? Is it simply hyperstimulating macropinocytosis to levels that overwhelm the lysosomes’ ability to fuse with and degrade them? In addition to the vesicle trafficking effects, is MOMIPP affecting other factors, such as those necessary for cytokinesis, metabolism, etc.? Thoroughly identifying MOMIPP’s target(s) is crucial to answer these questions. In addition to providing a more thorough understanding of the mechanism of action regarding MOMIPP’s potential as a drug, its disruption of a specific vesicle maturation step may render it an extremely useful tool to study the biology of vesicle trafficking.

As the overarching goal of the work done by our laboratory is to develop a new treatment strategy for drug-resistant cancers, we hope that the further characterization of drug-induced methuosis will ultimately provide a new therapeutic option for patients with intractable tumors. Along those lines, the protein target of MOMIPP may one day be used as a biomarker to stratify patient tumors based on its expression level. This will predict
which patients will benefit from methuosis-inducing therapy. Continued work evaluating the methuosis process and therapeutic potential may help realize that goal.
The key accomplishments of the work described in this dissertation are:

- Identification of MOMIPP, potent inducer of methuosis in glioblastoma cells, including temozolomide-resistant glioblastoma cells
- Evaluation of structure-activity relationships for indole-based chalcones and their methuosis-inducing activity. Importantly, compounds inactive at inducing methuosis were identified that have structures very similar to MOMIPP, displaying the specific structural requirements for methuosis induction
- Synthesis of biotinylated MOMIPP derivatives for target identification studies
- Synthesis of photoreactive MOMIPP derivatives, including 5-Azido-MIPP, and synthesis of radiolabeled 5-Azido-MIPP to be used as a photoaffinity labeling derivative of MOMIPP
- Preliminary identification of two proteins that specifically bind 5-Azido-MIPP. A 31 kD soluble protein and an 18 kD membrane protein were identified to specifically bind MOMIPP as their labeling with 5-Azido-MIPP was strongly reduced in the presence of non-labeled MOMIPP.
• The biotinylated and photoreactive MOMIPP derivatives that were developed will continue to serve as useful tools in future target identification studies, to be carried out by other members of the lab
References


35 Hammerova, J., Uldrijan, S., Taborska, E., Vaculova, A. H. & Slaninova, I. 
Necroptosis modulated by autophagy is a predominant form of melanoma cell 
death induced by sanguilutine. Biological chemistry 393, 647-658, 

Rev Mol Cell Biol 9, 1004-1010, doi:nrm2529 [pii] 
10.1038/nrm2529 (2008).

37 Kornienko, A., Mathieu, V., Rastogi, S. K., Lefranc, F. & Kiss, R. Therapeutic 
Agents Triggering Nonapoptotic Cancer Cell Death. J Med Chem, 

38 Xuan, Y. & Hu, X. Naturally-occurring shikonin analogues--a class of necroptotic 
inducers that circumvent cancer drug resistance. Cancer letters 274, 233-242, 

39 Asare, N. et al. 3-Nitrofluoranthene (3-NF) but not 3-aminofluoranthene (3-AF) 
elicits apoptosis as well as programmed necrosis in Hepa1c1c7 cells. Toxicology 

40 Degterev, A. et al. Identification of RIP1 kinase as a specific cellular target of 


Note that although no published analytical data were found, the following compounds have CAS numbers: compound 2, 373618-91-8; compound 10, 1237055-51-4.


Appendix A

Experimental Details, Chapter 2

EXPERIMENTAL SECTION

Chemistry. General methods. Reagents and starting materials were obtained from commercial suppliers without further purification. Thin layer chromatography (TLC) was done on 250 µm fluorescent silica gel 1B-F plates and visualized with UV light. Flash column chromatography was performed using silica gel 230-400 µm mesh size. Melting points (MP) are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on either a 600, 400 or 200 MHz instrument. Peak locations were referenced using the residual solvent peak (7.26 and 77.16 for CDCl$_3$ $^1$H and $^{13}$C, respectively, and 2.50 and 39.51 for DMSO $^1$H and $^{13}$C). Proton coupling constants ($J$ values) and signals are expressed in hertz using the following designations: s (singlet), d (doublet), br s (broad singlet), m (multiplet), t (triplet), dd (doublet of doublets) and qd (quintet of doublets). NMR spectra are available in the supplementary information. Elemental analyses were done by Atlantic Microlabs (Norcross, GA) with results regarded as acceptable when
within ± 0.4% of theoretical values. The composite of analytical methods suggests that all
tested compounds were at least 95% pure.

2-Methylindole-3-carboxaldehyde (2a). To a dried 300 mL two-neck round
bottom flask under argon at 0 °C, N,N-dimethylformamide (3 mL) was added, followed
by POCl\textsubscript{3} (1.05 mL, 11.3 mmol). After stirring for ten minutes, 2-methylindole (1.23 g,
9.37 mmol) dissolved in DMF (6 mL) was added dropwise via an addition funnel under
argon. After two hours, 1 N NaOH (70 mL) was added slowly, upon which a white
precipitate formed. The solid was filtered and dried under vacuum, yielding 1.32 g (89%)
of white solid. \textsuperscript{1}H NMR (600 MHz, \textit{d}_6-DMSO): δ 11.998 (s, 1H, N-H), 10.050 (s, 1H,
CHO), 8.044-8.032 (d, \textit{J} = 7.2, 1H, indole-4H), 7.388-7.375 (d, \textit{J} = 7.8, 1H, indole-7H),
7.183-7.135 (m, 2H, indole-5,6H), 2.679 (s, 3H, methyl). \textsuperscript{13}C NMR (150 MHz, \textit{d}_6-
DMSO): δ 184.3, 148.6, 135.4, 125.6, 122.7, 121.9, 120.0, 113.7, 111.4, 11.5. Melting
Point: 195 – 200 °C (published\textsuperscript{158}: 197-200 °C). TLC (ethyl acetate:hexanes 4:1) \textit{R_f} =
0.39. Elemental analysis calculated for C\textsubscript{10}H\textsubscript{9}NO: C, 75.45; H, 5.70; N, 8.80; found: C,
75.23; H, 5.70; N, 8.93.

\textit{trans}-3-(2-Methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (2). To a
dried 500 mL round bottom flask under argon, 2-methylindole-3-carboxaldehyde (400
mg, 2.51 mmol) was dissolved in anhydrous MeOH (10 mL). 4-Acetyl-pyridine (305 µL,
2.76 mmol, 1.1 equiv.) and piperidine (82 µL, 0.83 mmol) were added and the reaction
was stirred under reflux. A red-orange precipitate gradually formed, and after twelve
hours this solid was isolated by filtration, rinsed with chilled methanol and dried under
vacuum, producing 458 mg (69%) of yellow solid. NMR: \textsuperscript{1}H NMR (600 MHz, \textit{d}_6-
DMSO): δ 12.017 (s, 1H, N-H), 8.816-8.806 (dd, \textit{J}_1 = 4.5, \textit{J}_2 = 2.1, 2H, pyr-2,6H), 8.121-
8.095 (d, J = 15.6, 1H, C=CH), 8.077-8.063 (m, 1H, indole-4H), 7.975-7.965 (dd, J₁ = 4.5, J₂ = 1.5, 2H, pyr-3,5H), 7.489-7.464 (d, J = 15.6, 1H, C=CH), 7.415-7.400 (m, 1H, indole-7H), 7.220-7.200 (m, 2H, indole-5,6H), 2.596 (s, 3H, methyl).

13C NMR (150 MHz, d6-DMSO): δ 188.0, 150.7, 145.6, 144.9, 139.5, 136.3, 125.8, 122.4, 121.50, 121.46, 120.4, 113.1, 111.7, 109.4, 11.9. Melting Point: 268 – 272 °C. TLC: (ethyl acetate:hexanes 4:1) Rf = 0.18. Elemental analysis calculated for C₁₇H₁₄N₂O: C, 77.84; H, 5.38; N, 10.68; found: C, 77.96; H, 5.28; N, 10.59.

trans-3-(1H-Indol-3-yl)-1-phenyl-2-propen-1-one (9). In a dried, 25 mL round bottom flask under argon, indole-3-carboxaldehyde (300 mg, 2.07 mmol) was dissolved in anhydrous methanol (8 mL). Acetophenone (240 µL, 2.07 mmol) and piperidine (100 µL, 1.00 mmol) were added. The reaction was stirred under reflux for 18 hours. 10% Acetic acid was added (10 mL), precipitating 248 mg of a crude yellow solid. This was recrystallized in 100% EtOH, filtered, and dried under vacuum, yielding a pure, yellow solid (198 mg, 39%). 1H NMR (600 MHz, d6-DMSO): δ 8.133-8.114 (m, 3H, phenyl-2,6H, indole-2H), 8.095-8.081 (m, 1H, indole-4H), 8.075-8.049 (d, J = 15.6, 1H, C=CH), 7.668-7.642 (d, J = 15.6, 1H, C=CH), 7.655-7.629 (m, 1H, phenyl-4H), 7.581-7.556 (t, J = 1.5, 2H, phenyl-3,5H), 7.504-7.490 (m, 1H, indole-7H), 7.254-7.231 (m, 2H, indole-5,6H). 13C NMR (150 MHz, d6-DMSO): δ 188.8, 139.1, 138.5, 137.5, 133.4, 132.4, 128.7, 128.2, 125.1, 122.8, 121.2, 120.4, 115.3, 112.8, 112.5. Melting point: 167 – 170 °C (published: 166-167 °C). TLC (2:1 ethyl acetate:hexanes) Rf = 0.48. Elemental analysis calculated for C₁₇H₁₃NO: C, 82.57; H, 5.30; N, 5.66; found: C, 82.19; H, 5.35; N, 5.63.
trans-3-(1H-Indol-3-yl)-1-(2-pyridinyl)-2-propan-1-one (10).\textsuperscript{159} Indole-3-carboxaldehyde (200 mg, 1.38 mmol) was added to a dried 100 mL round bottom flask under argon and anhydrous methanol (8 mL) was added. 2-Acetyl-pyridine (232 µL, 2.07 mmol) and piperidine (69 µL, 0.7 mmol) were added and the reaction was stirred under reflux for 24 hours, after which still no precipitate had formed (AcOH did not lead to precipitation). The crude reaction mixture was concentrated and directly applied to a silica column for chromatography (ethyl acetate:hexanes 1:1). The product was partially separated from the aldehyde starting material (some product coeluted with aldehyde and these fractions were discarded), and 80 mg (23%) of purified product was isolated. NMR: 

1H NMR (600 MHz, d6-DMSO): δ 11.989 (s, 1H, N-H), 8.831-8.819 (m, pyr-H2), 8.220-8.194 (d, J = 15.6, 1H, C=CH), 8.154-8.105 (m, 3H, C=CH, indole-H2, pyr-H3), 8.052-8.027 (m, 1H, pyr-H4), 7.976-7.962 (m, 1H, indole-H4), 7.679-7.656 (m, 1H, pyr-H5), 7.522-7.508 (m, 1H, indole-H7), 7.285-7.262 (m, 2H, indole-H5,6). 13C NMR (150 MHz, d6-DMSO): δ 188.2, 154.3, 149.1, 139.3, 137.7, 137.6, 134.2, 127.1, 125.1, 122.3, 122.2, 121.4, 120.1, 114.3, 113.1, 112.7. Melting Point: 141 – 145 °C. TLC: (ethyl acetate:hexanes 4:1) R\textsubscript{f} = 0.40. Elemental analysis calculated for C\textsubscript{16}H\textsubscript{12}N\textsubscript{2}O \cdot 0.1 C\textsubscript{4}H\textsubscript{5}O\textsubscript{2}: C, 76.62; H, 5.02; N, 10.90; found: C, 76.41; H, 5.03; N, 10.80.

trans-3-(1H-Indol-3-yl)-1-(3-pyridinyl)-2-propan-1-one (II).\textsuperscript{160} Indole-3-carboxaldehyde (100 mg, 0.69 mmol) was added to a dried 100 mL round bottom flask and dissolved in anhydrous methanol (5 mL). 3-Acetyl-pyridine (113 µL, 1.03 mmol, 1.5 equiv.) and piperidine (69 µL, 0.7 mmol) were added and the reaction stirred under reflux. After twelve hours, the reaction was cooled to room temperature, upon which a precipitate formed. The solid was filtered, yielding 54 mg. However, there was
significant aldehyde present on a NMR. This mixture was dry-loaded onto silica and purified by column chromatography (methylene chloride:methanol 9:1), producing 33 mg pure, yellow solid (19%). NMR: $^1$H NMR (600 MHz, d6-DMSO): $\delta$ 12.004 (s, 1H, N-H), 9.292-9.289 (d, $J = 1.8$, 1H, pyr-H2), 8.808-8.797 (dd, $J_1 = 4.8$, $J_2 = 1.8$, 1H, pyr-H6), 8.467-8.447 (dt, $J_1 = 7.8$, $J_2 = 2.1$, 1H, pyr-H4), 8.174 (s, 1H, indole-H2), 8.154-8.140 (dd, $J_1 = 6.6$, $J_2 = 1.8$, 1H, indole-H4), 8.120-8.095 (d, $J = 15.0$, 1H, C=CH), 7.665-7.639 (d, $J = 15.6$, 1H, C=CH), 7.607-7.585 (m, 1H, pyr-H5), 7.506-7.492 (dd, $J_1 = 6.9$, $J_2 = 1.5$, 1H, indole-H7), 7.268-7.223 (qd, $J_1 = 6.6$, $J_2 = 1.5$, 1H, indole-H5,6). $^{13}$C NMR (150 MHz, d6-DMSO): $\delta$ 187.9, 152.7, 149.3, 139.9, 137.6, 135.7, 134.1, 133.7, 125.1, 122.9, 121.3, 120.7, 115.0, 112.9, 112.5. Melting Point: 192 – 194 °C (published: 191 °C). TLC (methylene chloride:methanol 9:1) $R_f = 0.26$. Elemental analysis calculated for C$_{16}$H$_{12}$N$_2$O: C, 77.40; H, 4.87; N, 11.28; found: C, 77.00; H, 4.80; N, 11.12.

*trans*-3-(1H-Indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (12). In a dried, 25 mL round bottom flask under argon, indole-3-carboxaldehyde (300 mg, 2.07 mmol) was dissolved in anhydrous methanol (8 mL). 4-Acetyl-pyridine (229 µL, 2.07 mmol) and piperidine (100 µL, 1.00 mmol) were added. The reaction was stirred under reflux; gradually, yellow product precipitated. After 14 hours, the reaction was cooled to room temperature, filtered and washed with chilled methanol and hexanes. Drying under vacuum for two hours yielded a pure, yellow solid (343 mg, 67%). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 8.826-8.816 (dd, $J_1 = 4.2$, $J_2 = 1.8$, 2H, pyr-H2,6), 8.186-8.182 (d, $J = 2.4$, 1H, indole-H2), 8.128-8.117 (m, 1H, indole-H4), 8.121-8.095 (d, $J = 15.6$, 1H, C=CH), 7.978-7.968 (dd, $J_1 = 4.2$, $J_2 = 1.8$, 2H, pyr-H3,5), 7.592-7.566 (d, $J = 15.6$, 1H, C=CH), 7.512-7.498 (m, 1H, indole-H7), 7.267-7.243 (m, 2H, indole-H5,6). $^{13}$C NMR (150 MHz, d6-DMSO): $\delta$ 187.9, 152.7, 149.3, 139.9, 137.6, 135.7, 134.1, 133.7, 125.1, 122.9, 121.3, 120.7, 115.0, 112.9, 112.5. Melting Point: 192 – 194 °C (published: 191 °C). TLC (methylene chloride:methanol 9:1) $R_f = 0.26$. Elemental analysis calculated for C$_{16}$H$_{12}$N$_2$O: C, 77.40; H, 4.87; N, 11.28; found: C, 77.00; H, 4.80; N, 11.12.
MHz, $d_6$-DMSO): $\delta$ 188.4, 150.7, 144.7, 140.9, 137.6, 134.6, 125.0, 123.0, 121.5, 121.4, 120.6, 114.6, 112.9, 112.6. Melting point: 266 – 268 °C (published\textsuperscript{160}: 257-258 °C).

TLC (in 4:1 ethyl acetate:hexanes) $R_f = 0.23$. Elemental analysis calculated for $C_{16}H_{12}N_2O$: C, 77.40; H, 4.87; N, 11.28; found: C, 77.35; H, 4.84; N, 11.23.

trans-3-(5-Methoxy-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (13). In a dried, 25 mL round bottom flask under argon, 5-methoxyindole-3-carboxaldehyde (100 mg, 0.57 mmol) was dissolved in anhydrous methanol (3 mL). 4-Acetyl-pyridine (63 µL, 0.57 mmol) and piperidine (30 µL, 0.3 mmol) were added. The reaction was stirred under reflux, during which a crude yellow solid precipitated. After 15 hours, 10% acetic acid (10 mL) was added to further the precipitation. The solid was filtered and dried under vacuum, yielding a pure, yellow solid (121 mg, 76%). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 8.821-8.811 (dd, $J_1 = 4.5, J_2 = 1.5$, 2H, pyr-H2,6), 8.163-8.159 (d, $J = 2.4$, 1H, indole-H2), 8.117-8.091 (d, $J = 15.6$, 1H, C=CH), 7.953-7.943 (dd, $J_1 = 4.5, J_2 = 1.5$, 2H, pyr-H3,5), 7.532-7.506 (d, $J = 15.6$, 1H, C=CH), 7.489-7.485 (d, $J = 2.4$, 1H, indole-H4), 7.405-7.391 (d, $J = 8.4$, 1H, indole-H7), 6.902-6.883 (dd, $J_1 = 9.0, J_2 = 2.4$, 1H, indole-H6), 3.866 (s, 3H, methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): $\delta$ 188.4, 155.2, 150.7, 144.9, 140.9, 134.2, 132.4, 126.0, 121.5, 114.3, 113.3, 112.7, 112.4, 102.4, 55.6. Melting point: 235 – 237 °C. TLC (in 4:1 ethyl acetate:hexanes) $R_f = 0.20$. Elemental analysis calculated for $C_{17}H_{14}N_2O_2$: C, 73.37; H, 5.07; N, 10.07; found: C, 73.55; H, 5.00; N, 10.04.

trans-3-(5-Phenylmethoxy-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (14). In a dried, 25 mL round bottom flask under argon, 5-benzylxyindole-3-carboxaldehyde (100 mg, 0.40 mmol) was dissolved in anhydrous methanol (3 mL). 4-Acetyl-pyridine
(75 µL, .68 mmol) and piperidine (20 µL, 0.2 mmol) were added. The reaction was stirred under reflux, during which a crude yellow solid precipitated. The solid was filtered, rinsed with cold methanol and dried under vacuum. This crude product (107 mg) was purified from residual aldehyde by column chromatography in ethyl acetate:hexanes (1:1 → 3:1 gradient), yielding pure, yellow solid (70 mg, 49%). \(^1\)H NMR (600 MHz, \(d_6\)-DMSO): δ 8.837-8.827 (dd, \(J_1 = 4.5, J_2 = 1.5, 2\)H, pyr-2,6H), 8.148-8.143 (d, \(J = 3.0, 1\)H, indole-2H), 8.095-8.069 (d, \(J = 15.6, 1\)H, C=CH), 7.932-7.922 (dd, \(J_1 = 4.5, J_2 = 1.5, 2\)H, pyr-3,5H), 7.556-7.553 (d, \(J = 1.8, 1\)H, indole-4H), 7.523-7.511 (d, \(J = 7.2, 2\)H, phenyl-2,6H), 7.460-7.434 (d, \(J = 15.6, 1\)H, C=CH), 7.411-7.370 (m, 3H, phenyl-3,5H, indole-7H), 7.330-7.306 (t, \(J = 7.2, 1\)H, phenyl-4H), 6.979-6.961 (dd, \(J_1 = 8.4, J_2 = 2.4, 1\)H, indole-6H), 5.252 (s, 2H, methylene). \(^{13}\)C NMR (150 MHz, \(d_6\)-DMSO): 188.4, 154.1, 150.7, 144.9, 140.9, 137.7, 134.6, 132.5, 128.4, 127.7, 127.6, 125.8, 121.5, 114.2, 113.3, 113.1, 112.7, 104.1, 69.8. Melting point: 218 – 221 °C. TLC (in 4:1 ethyl acetate:hexanes) \(R_f = 0.26\). Elemental analysis calculated (for C\(_{23}\)H\(_{19}\)N\(_2\)O\(_2\) • 0.2 C\(_6\)H\(_{14}\) • 0.05 H\(_2\)O): C, 78.02; H, 5.93; N, 7.52; found: C, 77.69; H 5.62; N 7.24.

**trans-3-(5-Hydroxy-1H-Indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (15).** To a dried two-neck 250 mL round bottom flask under argon at – 40 °C, 13 (352 mg, 0.99 mmol) was partially dissolved in CH\(_2\)Cl\(_2\) (30 mL). BBr\(_3\) (10 mL, 1.0 M in CH\(_2\)Cl\(_2\), 10 mmol) was added dropwise via an addition funnel under argon. After four hours the reaction was poured onto ice and treated with 5 N NaOH until pH ≈ 12. The aqueous solution was isolated and treated with 5 N HCl until pH ≈ 7, forming a brown precipitate which was extracted with ethyl acetate (3 x 40 mL). Extracts were combined, dried with Na\(_2\)SO\(_4\), filtered, concentrated and dried under vacuum to yield 158 mg of an orange
solid (61%). NMR: $^1$H NMR (600 MHz, d$_6$-DMSO): $\delta$ 11.840 (s, 1H, N-H), 9.109 (s, 1H, O-H), 8.826-8.816 (dd, $J_1 = 4.5, J_2 = 1.5$, 2H, pyr-2,6H), 8.063-8.058 (d, $J = 3.0$, 1H, indole-2H), 8.048-8.022 (d, $J = 15.6$, 1H, C=CH), 7.902-7.892 (dd, $J_1 = 4.5, J_2 = 1.5$, 2H, pyr-3,5H), 7.349-7.346 (d, $J = 1.8$, 1H, indole-4H), 7.307-7.293 (d, $J = 8.4$, 1H, indole-H7), 6.762-6.744 (dd, $J_1 = 8.4, J_2 = 2.4$, 1H, indole-H6). $^{13}$C NMR (150 MHz, d$_6$-DMSO): $\delta$ 188.2, 152.9, 150.6, 145.0, 141.4, 134.8, 131.7, 126.0, 121.3, 113.6, 113.1, 112.7, 112.3, 104.9. Melting Point: 262 – 268 °C. TLC (in ethyl acetate:hexane 4:1) $R_f$ = 0.35. Elemental analysis calculated for C$_{16}$H$_{12}$N$_2$O$_2$ • 0.1 H$_2$O: C, 72.22; H, 4.62; N, 10.53; found: C, 72.21; H, 4.42; N, 10.26.

**trans-3-(5-Methoxy-1H-indol-3-yl)-1-(3-pyridinyl)-2-propen-1-one (16).** In a dried, 50 mL round bottom flask under argon, 5-methoxyindole-3-carboxaldehyde (100 mg, 0.57 mmol) was dissolved in anhydrous methanol (4 mL). 3-Acetyl-pyridine 63 µL, 0.57 mmol) and piperidine (30 µL, 0.30 mmol) were added. The reaction was stirred under reflux, during which a crude orange solid precipitated. After twenty hours, the solid was isolated by vacuum filtration, rinsed with chilled methanol and dried under vacuum, yielding a pure, orange solid (98 mg, 60%). $^1$H NMR (600 MHz, d$_{6}$-DMSO): $\delta$ 11.891 (s, 1H, N-H), 9.276-9.273 (d, $J = 1.8$, 1H, pyr-2H), 8.798-8.790 (m, 1H, pyr-6H), 8.440-8.421 (m, 1H, pyr-4H), 8.155-8.150 (d, $J = 3.0$, 1H, indole-2H), 8.113-8.088 (d, $J = 15.0$, 1H, C=CH), 7.607-7.582 (d, $J = 15.0$, 1H, C=CH), 7.598-7.584 (m, 1H, pyr-5H), 7.500-7.497 (d, $J = 1.8$, 1H, indole-4H), 7.397-7.383 (d, $J = 8.4$, 1H, indole-7H), 6.892-6.874 (dd, $J_1 = 8.4, J_2 = 2.4$, 1H, indole-6H), 3.867 (s, 3H, methyl). $^{13}$C NMR (150 MHz, d$_6$-DMSO): $\delta$ 187.9, 155.1, 152.6, 149.3, 139.9, 135.7, 133.8, 133.7, 131.3, 126.1, 123.9, 114.7, 113.2, 112.7, 112.4, 102.6, 55.6. Melting point: 169 – 173 °C. TLC (in 4:1 ethyl
acetate:hexanes) \( R_f = 0.17 \). Elemental analysis calculated for \( \text{C}_{17}\text{H}_{14}\text{N}_2\text{O}_2 \): C, 73.37; H, 5.07; N, 10.07; found: C, 73.09; H, 5.10; N, 10.01.

\( \text{trans-3-} \left(5\text{-Methoxy-1H-indol-3-yl}\right)\text{-1-(pyrazine)-2-propen-1-one (17).} \)

In a dried, 25 mL round bottom flask under argon, 5-methoxyindole-3-carboxaldehyde (75 mg, 0.43 mmol) was dissolved in anhydrous methanol (4 mL). Acetyl-pyrazine (52 mg, .43 mmol) and piperidine (23 µL, 0.23 mmol) were added. The reaction was stirred under reflux, during which a crude, yellow solid precipitated. After three hours, the solid was isolated by vacuum filtration, rinsed with chilled methanol and dried under vacuum. This crude product was recrystallized from EtOH (8 mL) to remove residual aldehyde, yielding a pure, yellow solid (28 mg, 24%). \( ^1\text{H NMR (600 MHz, } d_6\text{-DMSO): } \delta \ 11.960 \text{ (s, 1H, N-H), 9.244 (s, 1H, pyr-2H), 8.905-8.873 (m, 2H, pyr-4,5H), 8.204-8.178 (d, } J = 15.6, \text{ 1H, } \text{C=CH), 8.155 (s, 1H, indole-2H), 7.987-7.961 (d, } J = 15.6, \text{ 1H, C=CH), 7.427-7.412 (m, 2H, indole-4,7H), 6.934-6.915 \text{ (dd, } J_1 = 9.0, J_2 = 2.4, \text{ 1H, indole-6H), 3.853 (s, 3H, methyl).} \)

\( ^{13}\text{C NMR (150 MHz, } d_6\text{-DMSO): } \delta \ 187.3, 155.1, 148.8, 147.6, 144.0, 143.7, 140.2, 134.6, 132.5, 126.0, 113.4, 113.2, 112.8, 111.9, 102.9, 55.6. \) Melting point: 176 – 180 °C. TLC (in 4:1 ethyl acetate:hexanes) \( R_f = 0.30 \). Elemental analysis calculated \( \text{C}_{16}\text{H}_{13}\text{N}_3\text{O}_2 \): C, 68.81; H, 4.69; N, 15.05; found: 68.76; H, 4.64; N, 14.99.

\( 5\text{-Methoxy-2-methyl-1H-indole-3-carboxaldehyde (18).} \)

To a dried two-neck 25 mL round bottom flask at 0 °C, POCl\(_3\) (1.00 mL, 10.8 mmol) was added to \( N,N\)-dimethylformamide (2.5 mL). After ten minutes of stirring, 2-methyl-5-methoxyindole (1.45 mg, 9.00 mmol) dissolved in DMF (5 mL) was added dropwise. After 45 minutes, 1N NaOH (50 mL) was slowly added, forming a white precipitate. The solid was isolated by filtration, washed with cold H\(_2\)O and dried under vacuum, yielding 1.52 g of white
solid (90 %). $^1$H NMR (600 MHz, $d_6$-DMSO): δ 11.875 (s, 1H, N-H), 10.009 (s, 1H, CHO), 7.570-7.566 (d, $J = 2.4$, 1H, indole-4H), 7.282-7.268 (d, $J = 8.4$, 1H, indole-7H), 6.797-6.778 (dd, $J_1 = 9.0$, $J_2 = 2.4$, 1H, indole-6H), 3.764 (s, 3H, o-methyl), 2.646 (s, 3H, c-methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): δ 184.1, 155.5, 148.6, 130.0, 126.4, 113.7, 112.1, 111.8, 102.3, 55.2, 11.5. Melting Point: 188 – 192 °C (published $^{161}$: 191-194 °C).

TLC (ethyl acetate:hexane 4:1) $R_f = 0.37$. Elemental analysis calculated for C$_{11}$H$_{11}$NO$_2$: C, 69.83; H, 5.86; N, 7.40; found: C, 69.65; H, 5.95; N, 7.25.

$trans$-3-(5-Methoxy-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one (19).

To a dried 250 mL two-neck round bottom flask under argon, 2-methyl-5-methoxy-1H-indole-3-carboxaldehyde (1.51 g, 7.98 mmol) was dissolved in anhydrous MeOH (30 mL). 4-Acetyl-pyridine (1.32 mL, 11.97 mmol, 1.5 equiv.) and piperidine (0.788 mL, 7.98 mmol) were added and the reaction was stirred under reflux. An orange solid gradually precipitated, and this was isolated by filtration, rinsed with chilled MeOH and dried under vacuum, yielding 2.08 g of orange solid (89%). $^1$H NMR (600 MHz, $d_6$-DMSO): δ 11.909 (s, 1H, N-H), 8.812-8.802 (dd, $J_1 = 4.2$, $J_2 = 1.8$, 2H, pyr-2,6H), 8.097-8.072 (d, $J = 15.0$, 1H, C=CH), 7.947-7.937(dd, $J_1 = 4.2$, $J_2 = 1.8$, 2H, pyr-3,5H), 7.434-7.430 (d, $J = 2.4$, 1H, indole-4H), 7.374-7.349 (d, $J = 15.0$, 1H, C=CH), 7.315-7.301 (d, $J = 8.4$, 1H, indole-7H), 6.851-6.833 (dd, $J_1 = 8.4$, $J_2 = 2.4$, 1H, indole-6H), 3.860 (s, 3H, o-methyl), 2.572 (s, 3H, c-methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): δ 188.1, 155.2, 150.6, 145.8, 145.1, 136.6, 131.0, 126.6, 121.5, 112.8, 112.3, 110.9, 109.3, 103.5, 55.6, 12.2. Melting Point: 252 – 256 °C. TLC (ethyl acetate:hexane 4:1) $R_f = 0.16$. Elemental analysis calculated for C$_{18}$H$_{16}$N$_2$O$_2$: C, 73.95; H, 5.52; N, 9.58; found: C, 73.76; H, 5.46; N, 9.47.
trans-3-(5-Methoxy-1-methyl-Indol-3-yl)-1-(4-pyridinyl)- 2-propen-1-one (20).

*N,N*-dimethylformamide (3 mL) was added to a dried 100 mL two-neck round bottom flask under argon containing NaH (21 mg, 0.52 mmol, 60% in mineral oil, 1.2 equiv., unwashed). After stirring for five minutes, the starting material (120 mg, 0.43 mmol) dissolved in DMF (1 mL) was added slowly and stirred for five minutes until a homogenous red-solution was formed. Methyl iodide (40 µL, 0.65 mmol, 1.5 equiv.) was added slowly. After two hours, sat. NH₄Cl (20 mL) and H₂O (20 mL) were added; this was extracted with ethyl acetate (3 x 30 mL), dried with Na₂SO₄, filtered and concentrated. The crude product was purified by column chromatography (methylen chloride:methanol 95:5), providing 103 mg (82%) of solid. NMR: \(^1\)H NMR (400 MHz, \(d_6\)-DMSO): \(\delta\) 8.821-8.806 (dd, \(J_1 = 4.4, J_2 = 1.6, 2\)H, pyr-2,6H), 8.143 (s, 1H, indole-2H), 8.081-8.042 (d, \(J = 15.6, 1\)H, C=CH), 7.950-7.935 (dd, \(J_1 = 4.4, J_2 = 1.6, 2\)H, pyr-3,5H), 7.517-7.473 (m, 3H, C=CH, indole-4,7H), 6.971-6.942 (dd, \(J_1 = 9.2, J_2 = 2.4, 1\)H, indole-6H), 3.875 (s, 3H, o-methyl), 3.838 (s, 3H, n-methyl). \(^{13}\)C NMR (100 MHz, \(d_6\)-DMSO): \(\delta\) 188.2, 155.5, 150.6, 144.9, 140.2, 137.3, 133.1, 126.5, 121.5, 114.2, 112.3, 111.9, 111.5, 102.9, 55.7, 33.4. Melting Point: 178 – 182 °C. TLC (in methylene chloride:methanol 95:5) \(R_f = 0.35\). Elemental analysis calculated for C\(_{18}\)H\(_{16}\)N\(_2\)O\(_2\) • 0.1 H\(_2\)O: C, 73.50; H, 5.55; N, 9.52; found: C, 73.34; H, 5.77; N, 9.25.

trans-3-(5-Hydroxy-1H-Indol-3-yl)-1-(4-pyridinyl)- 2-propen-1-one (21). In a dried 250 mL two-neck round bottom flask under argon, 19 (500 mg, 1.71 mmol) was partially dissolved in anhydrous CH\(_2\)Cl\(_2\) (35 mL) and placed at – 78 °C. BBr\(_3\) (17 mL, 1.0 M in CH\(_2\)Cl\(_2\), 17 mmol) was added dropwise via an addition funnel under argon. After addition, the reaction was warmed to room temperature and stirring continued for one
hour. Ice-water (50 mL) was added, followed by 1N NaOH until the pH = 12 (~ 60 mL). The CH₂Cl₂ was further extracted with 1N NaOH (3 x 20 mL). The basic extracts were neutralized with 8N HCl to a neutral pH (~ 8 mL), upon which the product precipitated. The precipitate was filtered and dried under vacuum, providing 454 mg (95%) of a yellow solid. NMR: ¹H NMR (600 MHz, d₆-DMSO): δ 11.833 (s, 1H, N-H), 9.072 (s, 1H, O-H), 8.818 (s, 2H, pyr-2,6H), 8.064-8.039 (d, J = 15.0, 1H, C=CH), 7.897 (s, 2H, pyr-3,5H), 7.342 (s, 1H, indole-4H), 7.282-7.257 (d, J = 15.0, 1H, C=CH), 7.209-7.195 (d, J = 8.4, 1H, indole-7H), 6.687-6.674 (d, J = 7.8, 1H, indole-6H), 2.542 (s, 3H, methyl). ¹³C NMR (150 MHz, d₆-DMSO): δ 187.7, 153.0, 150.7, 145.9, 145.4, 140.0, 130.2, 126.8, 121.3, 112.2, 112.0, 111.6, 109.1, 105.2, 12.0. Melting Point: 296 – 299 °C. TLC (in ethyl acetate:hexane 4:1) Rᵣ = 0.19. Elemental analysis calculated for C₁₇H₁₄N₂O₂ • 0.1 CH₂Cl₂: C, 71.55; H, 4.99; N, 9.79; found: C, 71.89; H, 5.02; N, 9.67.

2-Methyl-1H-indol-5-ol (22) A dried 250 mL two-neck round bottom flask was charged with 2-methyl-5-methoxyindole (1.00 g, 6.20 mmol), purged with argon, and CH₂Cl₂ (30 mL) was added and stirred vigorously until the indole was dissolved. After placing the reaction at -78 °C, BBr₃ (37.2 mL, 1.0 M in CH₂Cl₂, 37.2 mmol, 6 equiv.) was added dropwise via an addition funnel under argon. After addition, the reaction was allowed to slowly warm to room temperature. Thirty minutes after removing the cooling bath, the reaction was poured into ice-water (~ 50 mL) and sat. NaHCO₃ (50 mL), resulting in a neutral pH. This was extracted with CH₂Cl₂ (3 x 50 mL); the aqueous phase retained a yellow color and was acidified to pH ~3 (with 5N HCl) and extracted with ethyl acetate (2 x 50 mL). The combined organic extracts were washed with brine, dried with Na₂SO₄, filtered and concentrated to a brown oil. After drying under vacuum for 6
hours, 845 mg of a pure brown solid was isolated (93%). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 10.538 (s, 1H, N-H), 8.476 (s, 1H, O-H), 7.028-7.014 (d, $J = 8.4$, 1H, indole-7H), 6.701-6.698 (d, $J = 1.8$, 1H, indole-4H), 6.487-6.468 (dd, $J_1 = 9.0$, $J_2 = 2.4$, 1H, indole-6H), 5.906 (s, 1H, indole-3H), 2.305 (s, 3H, methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): $\delta$ 150.4, 135.8, 130.6, 129.4, 110.6, 109.8, 103.3, 98.5, 13.5. Melting point: 131 – 134 °C (published$^{162}$: 134 °C). TLC (in 1:1 ethyl acetate:hexanes) $R_f$ = 0.28. Elemental analysis calculated for C$_9$H$_8$NO: C, 73.45; H, 6.16; N, 9.52; found: C, 73.09; H, 6.29; N, 9.28.

5-(4-Methylbenzoate)methoxy-2-methyl-1H-indole (23). In a 250 mL round bottom flask, 2-methyl-1H-indole-5-ol (670 mg, 4.55 mmol) was partially dissolved in CH$_2$Cl$_2$ (50 mL). Tetra-n-butylammonium bromide (808 mg, 2.5 mmol) was added, followed by NaOH (50 mL of a 5 N solution, 250 mmol), and methyl 4-(bromomethyl)benzoate (1.15 g, 5.01 mmol, 1.1 equiv.). After 8 hours, the organic layer was removed, and the aqueous phase was extracted with CH$_2$Cl$_2$ (1 x 30 mL). The combined extracts were washed with brine, dried with Na$_2$SO$_4$, filtered and concentrated to an oil. Purification by column chromatography (1:3 ethyl acetate:hexane) provided 839 mg of pure product (63%) [followed by the 3,5-doubly alkylated product (115 mg, 5%)]. $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 10.755 (s, 1H, N-H), 7.980-7.966 (d, $J = 8.4$, 2H, phenyl-3,5H), 7.602-7.588 (d, $J = 8.4$, 2H, phenyl-2,6H), 7.150-7.136 (d, $J = 8.4$, 1H, indole-7H), 6.982-6.978 (d, $J = 2.4$, 1H, indole-4H) 6.717-6.699 (dd, $J_1 = 8.4$, $J_2 = 2.4$, 1H, indole-6H), 6.005 (s, 1H, indole-3H), 5.157 (s, 2H, methylene), 3.849 (s, 3H, o-methyl), 2.331 (s, 3H, c-methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): $\delta$ 166.1, 151.9, 143.6, 136.3, 131.4, 129.3, 129.0, 128.7, 127.4, 111.0, 110.1, 102.8, 99.0, 69.0, 52.1, 13.4.
Melting point: 151 – 154 °C. TLC (in 1:1 ethyl acetate:hexanes) \( R_f = 0.49 \) (doubly alkylated product \( R_f = 0.42 \)). Elemental analysis calculated for \( \text{C}_{19}\text{H}_{17}\text{NO}_3 \): C, 73.20; H, 5.80; N, 4.74; found: C, 73.02; H, 5.82; N, 4.55.

5-(4-Methylbenzoate)methoxy-2-methyl-1H-indole-3-carboxaldehyde (24). To a dried two-neck 250 mL round bottom flask under argon, POCl\(_3\) (325 µL, 3.5 mmol) was added to \( N,N \)-dimethylformamide (8 mL) at 0 °C. After stirring for five minutes, 23 (320 mg, 1.08 mmol) dissolved in DMF (4 mL) was added dropwise. The yellow solution was slowly warmed to room temperature and after one hour sat. NaHCO\(_3\) was added (50 mL), producing a white precipitate, followed by 1N NaOH (20 mL) to complete precipitation (direct workup with NaOH resulted in roughly 1:1 mixture of ester product to ester-hydrolyzed analog). The solid was filtered, rinsed with cold H\(_2\)O and dried under vacuum, yielding 315 mg of white solid (90%). \(^1\)H NMR (600 MHz, \( d_6 \)-DMSO): \( \delta \) 11.902 (s, 1H, N-H), 10.000 (s, 1H, CHO), 7.991-7.977 (d, \( J = 8.4 \), 2H, phenyl-3,5H), 7.664-7.660 (d, \( J = 2.4 \), 1H, indole-4H), 7.633-7.619 (d, \( J = 8.4 \), 2H, phenyl-2,6H), 7.304-7.290 (d, \( J = 8.4 \), 1H, indole-7H), 6.906-6.887 (dd, \( J_1 = 9.0 \), \( J_2 = 2.4 \), 1H, indole-6H), 5.211 (s, 2H, methylene), 3.854 (s, 3H, o-methyl), 2.646 (s, 3H, c-methyl). \(^{13}\)C NMR (150 MHz, \( d_6 \)-DMSO): \( \delta \) 184.0, 166.1, 154.3, 148.7, 143.2, 130.4, 129.3, 128.8, 127.4, 126.4, 113.6, 112.3, 112.2, 104.0, 69.0, 52.1, 11.5. Melting point: 224 – 227 °C. TLC: (in ethyl acetate:hexanes 4:1) \( R_f = 0.35 \). Elemental analysis calculated for \( \text{C}_{19}\text{H}_{17}\text{NO}_4 \): C, 70.58; H, 5.30; N, 4.33; found: C, 70.45; H, 5.41; N, 4.51.

5-(4-Benzoylate)methoxy-2-methyl-1H-indole-3-carboxaldehyde (25). To a dried two-neck 250 mL round bottom flask under argon, POCl\(_3\) (565 µL, 6.1 mmol) was added to \( N,N \)-dimethylformamide (12 mL) at 0 °C. After stirring for five minutes, 23 (600 mg,
2.03 mmol) dissolved in DMF (9 mL) was added dropwise. The yellow solution was slowly warmed to room temperature. After one hour, the reaction was cooled to 0 °C and 5N NaOH (90 mL) was added. After stirring for 30 minutes, 5N HCl (95 mL) was added to precipitate the product, which was filtered and dried overnight under vacuum, yielding 626 mg (99%) of white solid. $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 11.944 (s, 1H, N-H), 10.001 (s, 1H, CHO), 7.961-7.947 (d, $J = 8.4$, 2H, phenyl-3,5H), 7.664-7.660 (d, $J = 2.4$, 1H, indole-4H), 7.590-7.576 (d, $J = 8.4$, 2H, phenyl-2,6H), 7.304-7.290 (d, $J = 8.4$, 1H, indole-7H), 6.902-6.884 (dd, $J_1 = 8.4$, $J_2 = 2.4$, 1H, indole-6H), 5.194 (s, 2H, methylene), 2.647 (s, 3H, methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): $\delta$ 184.0, 167.3, 154.4, 148.8, 142.5, 130.4, 129.4, 127.3, 127.2, 126.4, 113.6, 112.3, 112.2, 104.0, 69.1, 11.5. Melting point: 267 – 270 °C. TLC: (in ethyl acetate:hexanes 4:1) $R_f = 0.25$.

$trans$-3-[5-((4-Methylbenzoate)methoxy)-1H-Indol-3-yl]-1-(4-pyridinyl)-2-propen-1-one (26). In a dried 100 mL two-neck round bottom flask under argon, 24 (50 mg, 0.15 mmol) was partially dissolved in anhydrous methanol (2 mL). 4-Acetyl-pyridine (26 µL, 0.23 mmol) and piperidine (7 µL, 0.075 mmol) were added and the reaction was refluxed. A yellow precipitate gradually formed, and after 24 hours the reaction was brought to room temperature and the solid was isolated by filtration, rinsed with cold methanol and dried under vacuum, yielding 36 mg. However, NMR showed 1:0.15 product:aldehyde. This mixture was dry loaded onto silica and purified by column chromatography (ethyl acetate:hexanes 2:1), providing 22 mg pure product (34 %). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 11.937 (s, 1H, N-H), 8.829-8.819 (d, $J = 6.0$, 2H, pyr-2,6H), 8.069-8.044 (d, $J = 15.0$, 1H, C=CH), 7.971-7.957 (d, $J = 8.4$, 2H, phenyl-3,5H), 7.905-7.896 (d, $J = 5.4$, 2H, pyr-3,5H), 7.662-7.649 (d, $J = 7.8$, 2H, phenyl-2,6H), 7.479-
7.475 (d, J = 2.4, 1H, indole-4H), 7.326-7.312 (d, J = 8.4, 1H, indole-7H), 7.284-7.259 (d, J = 15.0, 1H, C=CH), 6.938-6.920 (dd, J₁ = 8.7, J₂ = 2.1, 1H, indole-6H), 5.358 (s, 2H, methylene), 3.838 (s, 3H, o-methyl), 2.561 (s, 3H, c-methyl). 

13C NMR (150 MHz, d₆-DMSO): δ 188.1, 166.0, 153.9, 150.6, 146.0, 145.1, 143.5, 139.5, 131.3, 129.3, 128.8, 127.4, 126.4, 121.4, 112.8, 112.4, 111.9, 109.3, 104.8, 69.2, 52.2, 12.1.

Melting Point: 236 – 239 °C. TLC (ethyl acetate:hexanes 4:1) Rf = 0.26. Elemental analysis calculated for C₂₆H₂₂N₂O₄ • 0.25 C₄H₈O₂: C, 72.31; H, 5.39; N, 6.25; found: C, 72.62; H, 5.64; N, 5.95.

trans-3-[(4-Carboxyphenyl)-methoxy]-1H-Indol-3-yl]-1-(4-pyridinyl)-2-propen-1-one (27). In a dried 100 mL round bottom flask under argon, 25 (200 mg, 0.65 mmol) was partially dissolved in anhydrous methanol (15 mL). 4-Acetyl-pyridine (107 µL, 0.97 mmol, 1.5 equiv.) and piperidine (256 µL, 0.65 mmol) were added and the reaction was refluxed. After 24 hours, the solid precipitate was isolated by filtration, yielding 75 mg of product contaminated with aldehyde. Due to both solubility and poor filtration, a large amount of product remained in the filtrate; thus, the filtrate was dry loaded onto silica and purified by column chromatography (methylene chloride:methanol 9:1), yielding 85 mg of purified acid product (21%). 

1H NMR (600 MHz, d₆-DMSO): δ 11.947 (s, 1H, N-H), 8.831-8.821 (dd, J₁ = 4.2, J₂ = 1.8, 2H, pyr-2,6H), 8.073-8.047 (d, J = 15.6, 1H, C=CH), 7.954-7.941 (d, J = 7.8, 2H, phenyl-3,5H), 7.904-7.894 (dd, J₁ = 4.2, J₂ = 1.8, 2H, pyr-3,5H), 7.630-7.616 (d, J = 8.4, 2H, phenyl-2,6H), 7.479-7.476 (d, J = 1.8, 1H, indole-4H), 7.326-7.312 (d, J = 8.4, 1H, indole-7H), 7.289-7.264 (d, J = 15.0, 1H, C=CH), 6.939-6.920 (dd, J₁ = 9.0, J₂ = 2.4, 1H, indole-6H), 5.349 (s, 2H, methylene), 2.562 (s, 3H, methyl). 

13C NMR (150 MHz, d₆-DMSO): δ 188.1, 167.3,
154.0, 150.6, 146.0, 145.1, 142.8, 139.6, 131.2, 130.3, 129.5, 127.2, 126.4, 121.4, 112.8, 112.4, 111.9, 109.3, 104.8, 69.3, 12.1. Melting point: 269 – 273 °C. TLC (methylene chloride:methanol 9:1) Rf = 0.41. Elemental analysis calculated for C25H20N2O4 • 0.25 CH2Cl2 • 0.25 CH3OH: C, 69.34; H, 4.91; N, 6.34; found: 69.28; H, 5.22; N, 6.72.

**Library Compounds.** Compounds 1-8 used for initial screening of methusosis-inducing activity (Fig. 1) were obtained from Hit2Lead.com, a division of Chembridge Corp. The identification numbers of the compounds were: 1, 5224450; 2, 5224466; 3, 5312531; 4, 7995005; 5, 7916760; 6, 6161388; 7, 5267766; 8, 6155359. All compounds are certified by the vendor to be at least 90% pure with NMR confirmation of structure.

**Cell Culture.** U251 human glioblastoma cells were purchased from the DCT Tumor Repository (National Cancer Institute, Frederick, MD). MCF-7 mammary carcinoma cells were obtained from The American Type Culture Collection, Rockville, MD. Temozolomide-resistant U251 cells (U251-TR) were derived in our laboratory as described previously. MCF-7 cells selected for resistance to doxorubicin were provided by Amadeo Parissenti, Northeastern Ontario Regional Cancer Centre. Normal human skin fibroblasts were derived from a skin biopsy as described previously. Unless stated otherwise, cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) (JR Scientific, Woodland, CA) at 37°C in an atmosphere of 5% CO2/95% air. Normal pre-stasis human mammary epithelial cells (specimen 184) were provided by Martha Stampfer, Lawrence Berkeley Lab, Berkeley,
CA. The HMECs were maintained in M87A medium supplemented with cholera toxin and oxytocin, essentially as described.

**Cell Proliferation and Morphology.** To generate cell growth curves, U251 cells were plated in 35 mm dishes (100,000 cells/dish, Fig. 4B) and allowed to attach for 24 h. Thereafter, cells were treated with the indicated compounds dissolved in DMSO or with vehicle alone. At daily intervals, three parallel cultures were harvested from each group by trypsinization and aliquots of cell suspension were counted in a Coulter Z1 particle counter. Phase-contrast images of live cells were obtained using an Olympus IX70 inverted microscope equipped with a digital camera and SPOT imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI).

**Cell Viability.** Cells were seeded in 96-well plates, with four replicate wells for each culture condition. After addition of the indicated concentrations of compounds, cell viability was determined at a 48 h end-point using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based assay as described. Absorbance at 570 nm was quantified on a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA).

**Colony Formation.** Cells were plated in 100 mm dishes at 2,500 (U251 and U251-TR) or 1,500 (MCF-7 and MCF-7 DoxR) cells per dish. Beginning on the day after plating, the cells were exposed to the indicated compounds for the periods of time noted in the
figure legends. The medium was then replaced (without compounds) and cells were incubated for 10 days, with fresh medium added every 2 d. Colonies were visualized by washing with phosphate-buffered normal saline, fixing for 10 min with ice-cold 100% methanol, and staining with 1% (w/v) crystal violet (Acros Organics, Fisher Scientific, Pittsburgh, PA) in 35% methanol. After 2-3 washes with water, colonies containing at least 50 cells were counted using a dissecting microscope or a Protocol 2 colony counter (Synbiosis, Frederick MD).

**ABBREVIATIONS USED**

DBU, 1,8-Diazabicyclo[5.4.0]undec-7-ene; DMF, dimethylformamide; DMSO, dimethylsulfoxide; GBM, glioblastoma multiforme; HMEC, human mammary epithelial cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; RT, room temperature; TMZ, temozolomide.

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Appendix B

Experimental Details, Chapter 3

**Chemistry.** *General methods.* Reagents and starting materials were obtained from commercial suppliers without further purification. Thin layer chromatography (TLC) was done on 250 µm fluorescent silica gel 1B-F plates and visualized with UV light. Flash column chromatography was performed using silica gel 230-400 µm mesh size. Melting points (MP) are uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on either a 600, 400 or 200 MHz instrument. Peak locations were referenced using the residual solvent peak (7.26 and 77.16 for CDCl$_3$ $^1$H and $^{13}$C, respectively, and 2.50 and 39.51 for DMSO $^1$H and $^{13}$C). Proton coupling constants ($J$ values) and signals are expressed in hertz using the following designations: s (singlet), d (doublet), br s (broad singlet), m (multiplet), t (triplet), dd (doublet of doublets) and qd (quintet of doublets). NMR spectra are available in the supplementary information. Elemental analyses were done by Atlantic Microlabs (Norcross, GA) with results regarded as acceptable when within ± 0.4% of theoretical values. The composite of analytical methods suggests that all
tested compounds were at least 95% pure. High resolution mass spectrometry (HRMS) was done by University of Michigan, mass spectrometry technical services.

22, 2-Methyl-1H-indol-5-ol. A dried 250 mL two-neck round bottom flask was charged with 2-methyl-5-methoxyindole (1.00 g, 6.20 mmol), purged with argon, and CH₂Cl₂ (30 mL) was added and stirred vigorously until the indole was dissolved. After placing the reaction at -78 °C, BBr₃ (37.2 mL, 1.0 M in CH₂Cl₂, 37.2 mmol, 6 equiv.) was added dropwise via an addition funnel under argon. After addition, the reaction was allowed to slowly warm to room temperature. Thirty minutes after removing the cooling bath, the reaction was poured into ice-water (~ 50 mL) and sat. NaHCO₃ (50 mL), to a neutral pH. This was extracted with CH₂Cl₂ (3 x 50 mL); the aqueous phase retained a yellow color and was acidified to pH ~3 (with 5N HCl) and extracted with ethyl acetate (2 x 50 mL). The combined organic extracts were washed with brine, dried with Na₂SO₄, filtered and concentrated to a brown oil. After drying under vacuum for 6 hours, 845 mg of a pure brown solid was isolated (93%, 147.17 MW). ¹H NMR (600 MHz, d₆-DMSO): δ 10.538 (s, 1H, N-H), 8.476 (s, 1H, O-H), 7.028-7.014 (d, J = 8.4, 1H, indole-7H), 6.701-6.698 (d, J = 1.8, 1H, indole-4H), 6.487-6.468 (dd, J₁ = 9.0, J₂ = 2.4, 1H, indole-6H), 5.906 (s, 1H, indole-3H), 2.305 (s, 3H, methyl). ¹³C NMR (150 MHz, d₆-DMSO): δ 150.4, 135.8, 130.6, 129.4, 110.6, 109.8, 103.3, 98.5, 13.5. Melting point: 131 – 134 °C (published: 134 °C). TLC (in 1:1 ethyl acetate:hexanes) Rₜ = 0.28. Elemental analysis calculated for C₉H₇NO: C, 73.45; H, 6.16; N, 9.52; found: C, 73.09; H, 6.29; N, 9.28.
28, 5-(2-propyn-1-yloxy)-2-methyl-1H-indole. In a dried two-neck 250 mL round bottom flask under argon, acetone (40 mL) was added to 2-methyl-5-hydroxy-indole (681 mg, 4.63 mmol, 1 equiv.), propargyl bromide (1.24 mL, 13.9 mmol, 3 equiv.) and cesium carbonate (4.53 g, 13.9 mmol, 3 equiv.) and stirred at 40 °C. After 12 hours, the reaction was concentrated and partitioned into CH$_2$Cl$_2$ (75 mL) and H$_2$O (75 mL). The organic phase was washed with 0.1 N NaOH (2 x 50 mL) and brine (1 x 50 mL), dried (Na$_2$SO$_4$), filtered and concentrated to a brown oil. The residue was purified by column chromatography (2 cm column, packed to 6”) with ethyl acetate and hexanes (1:4). The product was isolated, which yielded a light brown solid after drying under vacuum (629 mg, 73%). (A slower-eluting 1,5-dialkylated side product –160 mg, 15% – was also recovered.) $^1$H NMR (400 MHz, d$_6$-DMSO): δ 10.765 (s, 1H, N-H), 7.152-7.130 (d, J = 8.8, 1H, indole-\&-H), 6.971-6.966 (d, J = 2.0, 1H, indole-4-H), 6.662-6.634 (dd, J$_1$ = 8.8, J$_2$ = 2.0, 1H, indole-6-H), 4.708-4.702 (d, J = 2.4, 2H, O–CH$_2$–), 3.504-3.493 (t, J = 2.2, 1H, C≡CH), 2.341 (s, 3H, –CH$_3$). Melting Point: 90 – 95 °C. TLC (ethyl acetate:hexane 1:1) R$_f$ = 0.63

29, 5-(2-propyn-1-yloxy)-2-methyl-1H-indole-3-carboxaldehyde. To a dried two-neck 100 mL round bottom flask under argon at 0 °C, POCl$_3$ (450 µL, 4.86 mmol, 1.5 equiv.) was added to N,N-dimethylformamide (2.0 mL). After ten minutes of stirring, 2-methyl-5-(2-propyn-1-yloxy)-indole (600 mg, 3.24 mmol, 1.0 equiv.) dissolved in DMF (3 mL) was added dropwise. The reaction slowly precipitates to a thin slurry; after 1 hr the mixture was poured into H$_2$O (75 mL) and stirred, dissolving the reaction mixture. 1N NaOH (25 mL) was added and a white precipitate gradually emerged. After 20 minutes
the product was isolated by vacuum filtration and rinsed with water and dried under vacuum, yielding a light brown solid (544 mg, 79%). $^1$H NMR (400 MHz, $d_6$-DMSO): $\delta$ 11.908 (s, 1H, N-H), 10.006 (s, 1H, CHO), 7.647-7.641 (d, $J = 2.4$, 1H, indole-4-H), 7.305-7.283 (d, $J = 8.8$, 1H, indole-7-H), 6.849-6.822 (dd, $J_1 = 8.4$, $J_2 = 2.4$, 1H, indole-6-H), 4.776-4.771 (d, $J = 2.0$, 2H, O–CH$_2$–), 3.544-3.533 (t, $J = 2.2$, 1H, C=CH), 2.651 (s, 3H, –CH$_3$). Melting Point: 200 – 205 °C. TLC (ethyl acetate:hexane 3:1) $R_f$ = 0.38.

30, Trans-3-(5-(2-propyn-1-yloxy)-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propenal. To a dried 100 mL two-neck round bottom flask under argon, 2-methyl-5-propargyloxy-1H-indole-3-carboxaldehyde (90 mg, 0.42 mmol) was partially dissolved in anhydrous MeOH (5 mL); 4-acetylpyridine (139 µL, 1.27 mmol, 3 equiv.) and piperidine (249 µL, 2.52 mmol, 6 equiv.) were added and the reaction was refluxed. The solution gradually became colored yellow and a yellow product eventually began to precipitate. After 18 hours, the precipitate was isolated by vacuum filtration and rinsed with cold MeOH and dried under vacuum (5 hrs), yielding a yellow solid product (87 mg, 65%). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 11.926 (s, 1H, N-H), 8.820-8.809 (dd, $J_1 = 4.8$, $J_2 = 1.8$, 2H, 3,5-pyr-H), 8.097-8.072 (d, $J = 15.0$, 1H, C=CH), 7.945-7.935 (dd, $J_1 = 4.2$, $J_2 = 1.5$, 2H, 2,6-pyr-H), 7.573-7.569 (d, $J = 2.4$, 1H, indole-4-H), 7.419-7.393 (d, $J = 15.6$, 1H, C=CH), 7.330-7.316 (d, $J = 8.4$, 1H, indole-7-H), 6.885-6.866 (dd, $J_1 = 9.0$, $J_2 = 2.4$, 1H, indole-6-H), 4.913-4.909 (d, $J = 2.4$, 2H, O–CH$_2$–), 3.610-3.602 (t, $J = 2.4$, 1H, C=CH), 2.577 (s, 3H, –CH$_3$) $^{13}$C NMR (100 MHz, $d_6$-DMSO): $\delta$ 187.9, 153.1, 150.1,
146.0, 145.0, 139.4, 131.4, 126.3, 121.4, 112.8, 112.3, 111.9, 109.4, 104.9, 79.9, 78.1, 56.2, 12.1. Melting Point: 233–234 °C. TLC (ethyl acetate:hexane 4:1) Rf = 0.27

Elemental analysis calculated for C_{20}H_{16}N_{2}O_{2}: C, 75.93; H, 5.10; N, 8.86; found: C, 75.98; H, 4.98; N, 8.86.

31, Trans-3-(5-(2-propyn-1-yloxy)-2-methyl-1H-indol-3-yl)-1-phenyl-2-propen-1-one. To a dried 100 mL two-neck round bottom flask under argon, 2-methyl-5-propargyloxy-1H-indole-3-carboxaldehyde (50 mg, 0.23 mmol) was partially dissolved in anhydrous MeOH (5 mL); acetophenone (109 µL, 0.94 mmol, 4 equiv.) and piperidine (93 µL, 0.94 mmol) were added and the reaction was refluxed. The solution gradually became colored yellow. After 72 hours, both starting materials and a yellow product peak were identified by TLC (1:1 ethyl acetate:hexanes, indole-aldehyde Rf = 0.13, acetophenone = 0.62, yellow product = 0.23). The reaction mixture was concentrated and applied directly to a silica chromatography column (packed to 6”, 2 cm diameter) and eluted with a gradient of 40–60 % ethyl acetate in hexanes. Fractions (10 mL tubes) 4-8: acetophenone, 18-37: yellow product, 39-47: indole-aldehyde. Concentration and drying of the yellow product yielded 31 mg of product with impurities by NMR. The product was crystallized in 5 mL hot ethanol; this cooled overnight at RT, then three days at 4 °C. The yellow crystals were then filtered, rinsed with cold ethanol, and dried under high vacuum overnight, yielding 15 mg of a yellow solid (21%). 1H NMR (400 MHz, d6-DMSO): δ 11.818 (s, 1H, N-H), 8.103-8.085 (d, J = 7.2, 2H, phenyl-2,6-H), 8.058-8.020 (d, J = 15.2, 1H, C=CH), 7.649-7.548 (m, 4H, indole-4-H, phenyl-3,4,5-H), 7.506-7.468 (d, J = 15.2, 1H, C=CH), 7.323-7.301 (d, J = 8.8, 1H, indole-7H), 6.869-6.841 (dd, J...
8.8, J₂ = 2.4, 1H, indole-6H), 4.910-4.900 (d, J = 2.4, 2H, O−CH₂−), 3.629-3.619 (t, J = 2.0, 1H, C≡CH), 2.571 (s, 3H, −CH₃).

\[ ^{13}C \text{ NMR (100 MHz, } d_6\text{-DMSO)}: \delta 193.9, 158.3, 150.1, 144.2, 143.3, 137.7, 136.7, 134.1, 133.4, 131.7, 119.0, 117.6, 117.2, 114.6, 109.9, 85.3, 83.5, 61.5, 17.5. \]

\[ \text{Melting Point: } 188–190 \degree C. \text{ TLC (ethyl acetate:hexane 1:1) } R_f = 0.23. \text{ Elemental analysis calculated for } C_{21}H_{17}NO_2: C, 79.98; H, 5.43; N, 4.44; \text{ found: C, 79.90; H, 5.46; N, 4.48.} \]

32, **Trans-3-(5-(2-propyn-1-yloxy)-2-methyl-1H-indol-3-yl)-1-(pyrazine)-2-propen-1-one.** To a dried 100 mL two-neck round bottom flask under argon, 2-methyl-5-propargyloxy-1H-indole-3-carboxaldehyde (50 mg, 0.23 mmol) was partially dissolved in anhydrous MeOH (5 mL); acetylpyrazine (114 mg, 0.94 mmol, 4 equiv.) and piperidine (93 µL, 0.94 mmol) were added and the reaction was refluxed. The solution gradually became colored yellow and a yellow precipitate appeared. After 16 hours, both starting materials and a yellow product peak were identified by TLC (4:1 ethyl acetate:hexanes, indole-aldehyde R₇ = 0.42, pyrazine = 0.53, yellow product = 0.34). The mixture was filtered and dried under vacuum 3 hrs, yielding 37 mg of a yellow solid (51%).

\[ ^1H \text{ NMR (400 MHz, } d_6\text{-DMSO): } \delta 11.963 (s, 1H, N-H), 9.242 (s,1H, pyr-H2), 8.901-8.891 (m, 2H, pyr-H4,5), 8.179-8.140 (d, J = 15.6, 1H, C=CH), 7.959-7.920 (d, J = 15.6, 1H, C=CH), 7.481-7.475 (d, J = 2.4, 1H, indole-4H), 7.356-7.334 (d, J = 8.8, 1H, indole-7H), 6.932-6.905 (dd, J₁ = 8.6, J₂ = 2.2, 1H, indole-6H), 4.854-4.849 (d, J = 2.0, 2H, O−CH₂−), 3.601 (s, 1H, C=CH), 2.598 (s, 3H, −CH₃). \]

\[ ^{13}C \text{ NMR (100 MHz, } d_6\text{-DMSO): } \delta 187.1, 153.1, 148.9, 147.5, 146.2, 144.1, 143.7, 138.7, 131.6, 126.6, 112.4, 112.1, 111.4, 109.6, 105.3, 79.7, 78.2, 56.4, 12.2. \text{ Melting Point: } 186–188 \degree C. \text{ TLC (ethyl acetate:hexane 4:1) } R_f = 0.23. \]
$R_f = 0.34$. Elemental analysis calculated for $C_{19}H_{15}N_3O_2$: C, 71.91; H, 4.76; N, 13.24; found: C, 71.77; H, 4.91; N, 13.09.

33, **Trans-3-(5-(2-propyn-1-yloxy)-2-methyl-1H-indol-3-yl)-1-(3-pyridinyl)-2-propen-1-one.** To a dried 100 mL two-neck round bottom flask under argon, 2-methyl-5-propargyloxy-1H-indole-3-carboxaldehyde (50 mg, 0.23 mmol) was partially dissolved in anhydrous MeOH (5 mL); 3-acetylpyridine (103 µL, 0.94 mmol, 4 equiv.) and piperidine (93 µL, 0.94 mmol) were added and the reaction was refluxed. The solution gradually became colored yellow and a yellow precipitate appeared. After 48 hours, both starting materials and a yellow product peak were identified by TLC (4:1 ethyl acetate:hexanes, indole-aldehyde $R_f = 0.39$, pyridine = 0.26, yellow product = 0.33). The mixture was filtered and dried under vacuum overnight, yielding 26 mg of a yellow solid (36%). $^1$H NMR (400 MHz, $d_6$-DMSO): $\delta$ 11.892 (s, 1H, N-H), 9.266-9.261 (d, $J = 2.0$, 1H, pyr-H2), 8.798-8.783 (dd, $J_1 = 4.4$, $J_2 = 1.6$, 1H, pyr-4H), 8.439-8.419 (d, $J = 8.0$, 1H, pyr-6H), 8.099-8.061 (d, $J = 15.2$, 1H, C=CH), 7.610-7.578 (m, 2H, indole-4H, pyr-5H), 7.486-7.448 (d, $J = 15.2$, 1H, C=CH), 7.326-7.304 (d, $J = 8.8$, 1H, indole-7H), 6.877-6.850 (dd, $J_1 = 8.8$, $J_2 = 2.0$, 1H, indole-6H), 4.924-4.918 (d, $J = 2.4$, 2H, O–CH₂–), 3.593-3.582 (t, $J = 2.0$, 1H, C=CH), 2.579 (s, 3H, –CH₃). $^{13}$C NMR (100 MHz, $d_6$-DMSO): $\delta$ 188.2, 153.7, 153.2, 149.9, 146.1, 139.2, 136.2, 134.6, 132.0, 127.0, 124.5, 114.0, 112.9, 112.6, 109.9, 105.6, 80.6, 78.7, 56.9, 12.8. Melting Point: 222–225 °C.

TLC (ethyl acetate:hexane 4:1) $R_f = 0.33$. Elemental analysis calculated for $C_{20}H_{16}N_2O_2$:
C, 75.93; H, 5.10; N, 8.86; found: C, 75.44; H, 5.21; N, 8.74. Calculated for C_{20}H_{16}N_{2}O_{2}•0.1\text{H}_{2}\text{O}: C, 75.50; H, 5.13; N, 8.81; found: C, 75.44; H, 5.21; N, 8.74.

34, 6-Azido-1-hexanamine. In a dried 100 ml round bottom flask under argon, 6-aminohexanol (1.00 g, 8.53 mmol) was dissolved in toluene (10 mL), with mild heating, and placed in an ice-bath. Thionyl chloride (3 equiv., 1.86 mL, 25.6 mmol) was added, and the reaction was stirred at 80 °C. After two hours, the reaction was concentrated under vacuum, forming a brown residue. The flask was again placed in an ice-bath, and H_{2}O (10 mL) and sodium azide (3 equiv., 1.66 g, 25.6 mmol) were added. After two hours, the reaction was diluted with H_{2}O (50 mL), and 5 N NaOH was added (5 mL) raising the pH to 12. The product was extracted with methlene chloride (3x60 mL), washed with brine (150 mL), dried (Na_{2}SO_{4}), filtered, concentrated and dried under vacuum, yielding 696 mg of a brown oil (57% over two steps).

35, N-(6-azidohexyl)-hexahydro-2-oxo-1H-thieno[3,4-d]imidazole-4-pentanamide. In a dry 100 mL round bottom flask under argon, biotin (100 mg, 0.41 mmol) was dissolved in anhydrous DMF (5 mL). EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 157 mg, 0.82 mmol) and DMAP (dimethylaminopyridine, 100 mg, 0.82 mmol) were added, and the mixture was stirred for 5 minutes. Next, 6-azidohexylamine (58 mg, 0.41 mmol) was added and the reaction was stirred overnight. After 16 hours, TLC (10% MeOH/DCM) shows product formation at R_{f}=0.20 (by I_{2} chamber stain). The starting material was consumed as verified by a negative ninhydrin stain. The reaction was poured into H_{2}O (100 mL) and extracted with ethyl acetate (3 x 50 mL), washed with
brine, dried with Na₂SO₄, filtered and concentrated to a gelatinous colorless solid. This mixture was purified by silica column chromatography (6”, 2cm diameter, 5% MeOH/DCM → 10%). The product eluted in fractions 34-43 (10 mLfractions, visible by I₂ chamber stain). The purified product was concentrated to 114 mg of a white amorphous solid (76%). ¹H NMR (600 MHz, d₆-DMSO): δ 7.735 (s, 1H, N-H), 6.421 (s, 1H, biotin N-H), 6.356 (s, 1H, biotin N-H), 4.310-4.288 (dd, J₁ = 7.8, J₂ = 5.4, 1H, biotin-N–CH–CH₂–S), 4.129-4.111 (m, 1H, biotin-N–C₃H–C(H)C), 3.319-3.296 (t, J = 6.9, 2H, CH₂–N₃), 3.094-3.082 (m, 1H, biotin-N–CH–C(H)C), 3.025-2.992 (q, J = 6.6, 2H, –NH–CH₂–), 2.829-2.800 (dd, J₁ = 12.6, J₂ = 4.8, 1H, biotin-N–CH–CH(endo)H–S), 2.582-2.561 (d, J = 12.6, 1H, biotin-N–CH–CH(endo)H–S), 2.049-2.025 (t, J = 7.2, 2H, N–C(O)–CH₂), 1.639-1.258 (m, 14H, methylenes). ¹³C NMR (150 MHz, d₆-DMSO): δ 172.3, 163.2, 61.5, 59.6, 55.9, 51.0, 38.7, 35.7, 29.5, 28.69, 28.66, 28.5, 26.4, 26.3, 25.8.

Melting Point: 154–157 °C. TLC (10% MeOH/CH₂Cl₂) Rf = 0.20 by I₂ chamber stain.
Elemental analysis calculated for C₁₆H₂₉N₆O₂S: C, 52.15; H, 7.66; N, 22.81; found: C, 51.25; H, 7.26; N, 22.09. Calc for C₁₆H₂₉N₆O₂S·0.1CH₂Cl₂: C, 51.29; H, 7.54; N, 22.29.

36, Biotin-MIPP. N-[6-[4-Trans-3-(5-oxyethyl-2-methyl-1H-indol-3-yl)-1-phenyl-2-propen-1-one]-1H-1,2,3-triazol-1-yl]hexyl]hexahydro-2-oxo- H-Thieno[3,4-d]imidazole-4-pentanamide. In a dry 50 mL round bottom flask, biotin-azide (30 mg, 81 µmol), POMIPP (23 mg, 74 µmol), CuSO₄ (1.2 mg, 8 µmol), and sodium ascorbate (3.2 mg, 16 µmol) were dissolved in DMF (2 mL). The reaction was stirred for 24 hours at 60 °C. At this point, TLC (15% MeOH/DCM) showed one yellow product spot (Rf = 0.41; POMIPP = 0.80 and was no longer present). The reaction was poured into H₂O (50 mL)
and extracted with ethyl acetate (3x50 mL). Saturated NaHCO$_3$ (10 mL) was added to the water (pH 5 $\rightarrow$ 8) and extracted again (1x50 mL). The extracts were washed with brine, dried with NaSO$_4$. The mixture was purified by column chromatography (7", 1.5 cm diameter column, 5 $\rightarrow$ 15% MeOH/DCM). The product, eluted in fractions 22-33 (10 mL fractions), was combined and dried under vacuum for three hours, yielding an orange solid (23 mg, 41%). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 11.921 (s, 1H, indole N-H), 8.813-8.805 (dd, $J_1 = 4.5$, $J_2 = 1.5$, 2H, pyr-3,5H), 8.251 (s, 1H, triazole-5H), 8.106-8.081 (d, $J = 15.0$, 1H, C=CH), 8.044-8.034 (dd, $J_1 = 4.5$, $J_2 = 1.5$, 2H, pyr-2,6H), 7.724-7.706 (m, 1H, amide N-H), 7.689-7.685 (d, $J = 2.4$, 1H, indole-4H), 7.464-7.439 (d, $J = 15.0$, 1H, C=CH), 7.308-7.293 (d, $J = 9.0$, 1H, indole-7H), 6.886-6.868 (dd, $J_1 = 8.4$, $J_2 = 2.4$, 1H, indole-6H), 6.416 (s, 1H, biotin N-H), 6.351 (s, 1H, biotin N-H), 4.366-4.342 (t, $J = 7.2$, 2H, triazole-N-CH$_2$), 4.287-4.275 (m, 1H, biotin-N–CH–CH$_2$–S), 4.115-4.107 (m, 1H, biotin-N–CH–C(H)C), 3.091-3.052 (m, 1H, biotin-N–CH–C(=C)=CH$_2$–S), 2.995-2.964 (q, $J = 6.3$, 2H, –NH–CH$_2$–), 2.811-2.782 (dd, $J_1 = 12.6$, $J_2 = 4.8$, 1H, biotin-N–CH–CH$_2$(exo)H–S), 2.570 (s, 3H, indole-CH$_3$), 2.570-2.550 (d, $J = 12.0$, 1H, biotin-N–CH–CH$_2$(endo)H–S), 2.041-2.016 (t, $J = 7.5$, 2H, CH$_2$–C(O)N–) 1.806-1.783 (t, $J = 6.9$, 2H, triazole-N-CH$_2$–CH$_2$–), 1.631-1.205 (m, 10H, methylenes), 0.885-0.847 (m, 2H, methylenes). TLC (15% MeOH/CH$_2$Cl$_2$) $R_f = 0.41$. Elemental analysis calculated for C$_{36}$H$_{244}$N$_8$O$_4$: C, 63.14; H, 6.48; N, 16.36; found: C, 58.46; H, 6.67; N, 12.52.

37, 1,11-Diazido-3,6,9-trioxaundecane.$^{138}$ In an oven-dried 250 mL round bottom flask under argon, tetraethylene glycol (1x, 6.67 mL, 38.6 mmol) was added to anhydrous THF (30 mL) and the reaction was placed over an ice bath. Methanesulfonyl chloride (2.2x,
6.57 mL, 84.9 mmol) was added through an addition funnel, followed by triethylamine (2.2x, 11.9 mL, 84.9 mmol, dissolved in 7 mL THF) was added dropwise, during which a white precipitate formed. The stirred reaction was then allowed to reach room temperature and stirred for 30 mins. The THF was removed under reduced pressure, and H₂O (25 mL) was added, dissolving the white residue. The stirred flask (now with a reflux condenser) was again placed over an ice bath, and sodium bicarbonate (1.8 g) was added, followed by sodium azide (2.1x, 5.27 g, 81.1 mmol). The solution was stirred at 77 °C. After 24 hrs, the reaction was cooled to room temperature and extracted with ether (4 x 30 mL), washed with brine, dried (Na₂SO₄), filtered, and dried under vacuum for 5 hrs, yielding 8.04 g of an amber oil (85%). ¹H NMR (600 MHz, CDCl₃): δ 3.675 (m, 12H), 3.391 (t, J = 5.1, 4H). ¹³C NMR (150 MHz, CDCl₃): δ 70.72, 70.71, 70.07, 50.67. TLC: 1:1 Ethyl acetate:Hexanes Rᶠ = 0.61 (KMnO₄ stain).

38, 1-Amino-11-azido-3,6,9-trioxaundecane.¹³⁸ In a 200 mL round bottom flask, the diazide-PEG (1.15x, 5.00 g, 20.5 mmol) was dissolved in dilute phosphoric acid (1.5x, 50 mL of [0.65] H₃PO₄, 2.22 mL 85% H₃PO₄ + 47.78 mL H₂O) and stirred vigorously. Triphenylphosphine (1x, 4.64 g, 17.7 mmol) dissolved in diethyl ether (40 mL) was added dropwise via an addition funnel, and a white precipitate slowly formed. After 24 hrs, the aqueous layer was removed, and the white precipitate (presumed PPh₃O) was isolated from the ether by filtration. The acid was extracted with ether (3 x 15 mL) to remove any residual diazide. Potassium hydroxide (4.75 g, 87 mmol) was added to the acid solution (pH = 7), and placed at 4 °C overnight to precipitate residual PPh₃O, which was removed by filtration. Potassium hydroxide (10.7 g) was added to the filtrate (pH =
13), forming some a white precipitate. The basic solution was extracted with CH$_2$Cl$_2$ (16x 10 mL), dried (Na$_2$SO$_4$), filtered, concentrated and dried under vacuum for 3 hrs, yielding 3.73 g of an amber oil. By TLC, some PPh$_3$O still remained, which was confirmed by NMR to be in a 0.25:1 ratio with the product, giving a 73% yield of intended product (purity 75% by mass, 80% by molar ratio). The PPh$_3$O can be easily removed in the subsequent step, therefore the crude product was used in the subsequent step without purification. $^1$H NMR (600 MHz, CDCl$_3$): $\delta$ 3.65 (m, 10H), 3.49 (t, $J = 5.4$, 2H), 3.38 (q, $J = 5.4$, 2H), 2.85 (t, $J = 5.1$, 2H), 2.28 (bs, 2H); (with impurities: $\delta$ 7.64, 7.51, 7.44 (PPh$_3$O impurity)). TLC: 10% NH$_4$OH in EtOH: $R_f$ = 0.60 (ninhydrin stain) and 0.86 (PPh$_3$O impurity, by UV); 10% MeOH in CH$_2$Cl$_2$: $R_f$ = 0.03 (ninhydrin) and 0.55 (PPh$_3$O impurity, by UV); 1:1 ethyl acetate:hexanes: $R_f$ = 0.23 (PPh$_3$O impurity).

39, N-hydroxysuccinimido-Biotin.$^{163}$ In an oven-dried 250 mL round bottom flask under argon, biotin (1x, 3.00 g, 12.3 mmol), n-hydroxysuccinimide (1.6x, 2.26 g, 19.7 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 1.3x, 3.05 g, 16.0 mmol) were dissolved in dimethylformamide (30 mL) and stirred. After 24 hrs, the solution was poured into ice-water (400 mL) and stirred (20 minutes), forming a white precipitate. The residue was filtered using a Buchner funnel under vacuum, and dried under vacuum overnight, yielding 3.68 g white solid product (88 %). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 6.4318 (s, 1H, N-H), 6.3712 (s, 1H, N-H), 4.3139 (m, 1H, N–CH–CH$_2$–S), 4.1549 (m, 1H, N–CH–C(H)C), 3.1134 (m, 1H, N–CH–C(H)C), 2.8394 (dd, $J_1 = 12.5$, $J_2 = 5.2$, 1H, N–CH–CH(exo)H–S), 2.8171 (s, 4H, NHS-methylene), 2.6782 (t, $J = 7.0$, 2H, O–C(O)–CH$_2$), 2.5887 (d, $J = 12.4$, 1H, N–CH–CH(endo)H–S), 1.6832–1.5100 (m, 6H,
methylenes). $^{13}$C NMR (100 MHz, $d_6$-DMSO): $\delta$ 170.7, 169.4, 163.2, 61.5, 59.6, 55.7, 40.4, 30.5, 28.3, 28.0, 25.9, 24.8. TLC in 10% MeOH in DCM: $R_f = 0.30$ (PMA or I$_2$ stain). MP 209 – 211 °C.

40, **Biotin-PEG-azide.**$^{164}$ In an oven-dried 100 mL round bottom flask under argon, azido-PEG-amine (1.1x, 1.31 g of oil with 75% mass purity with PPh$_3$O contaminant, 4.50 mmol) was added to anhydrous DMF (10 mL), followed by triethylamine (1.2x, 669 µL, 4.80 mmol). NHS-biotin (1x, 1.37 g, 4.00 mmol), partially dissolved in anhydrous DMF (10 mL), was added, forming a cleanly dissolved solution. The reaction was concentrated under vacuum after 24 hrs and dry loaded onto silica gel, and purified by column chromatography (2 $\rightarrow$ 10% methanol in CH$_2$Cl$_2$, 2.5 cm column, 7” deep), providing a pure white solid (845 mg, 48%). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 7.81 (t, $J$ = 5.6, 1H, C(О)NH–PEG), 6.41 (s, 1H, biotin-NH), 6.35 (s, 1H, biotin-NH), 4.30 (m, 1H, N–CH–CH$_2$–S), 4.12 (m, 1H, N–CH–C(H)C), 3.60 (t, $J$ = 5.0, 2H, PEG-CH$_2$-N$_3$), 3.53 (m, 8H, PEG), 3.39 (t, $J$ = 5.6, 4H, PEG-O-CH$_2$-CH$_2$-N), 3.09 (m, 1H, N–CH–C(H)C), 2.81 (dd, $J_1$ = 12.4, $J_2$ = 5.2, 1H, N–CH–CH(exo)H–S), 2.57 (d, $J$ = 12.4, 1H, N–CH–CH(endo)H–S), 1.62-1.26 (m, 6H, biotin methylenes). $^{13}$C NMR (150 MHz, $d_6$-DMSO): $\delta$ 170.1, 162.7, 69.79, 69.76, 69.68, 69.55, 69.25, 69.16, 61.0, 59.2, 55.4, 50.0, 40.1, 38.4, 35.1, 28.2, 28.0, 25.24. TLC: 10% MeOH in CH$_2$Cl$_2$: $R_f = 0.18$ (I$_2$). MP: 110 – 112 °C.

41, **Biotin-PEG-MIPP.** In a scintillation vial, biotin-PEG-azide (1.0x, 50.5 mg, 0.114 mmol), para-POMIPP (1.2x, 30 mg, 0.0948 mmol), sodium ascorbate (0.45x, 8.5 mg),
CuSO$_4$.5H$_2$O (0.15x, 3.5 mg), and Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 0.15x, 8.0 mg) were stirred in DMF (3 mL). After 24 hours, the reaction mixture was concentrated under vacuum, dissolved in methanol and dry loaded onto silica gel. The product was purified by column chromatography (5 → 15% MeOH/CH$_2$Cl$_2$, 2 cm column, 6” deep) and dried under vacuum, providing an orange solid (58 mg, 72%).

TLC: 15% MeOH in CH$_2$Cl$_2$: $R_f = 0.34$ (para-POMIPP starting material = 0.77, TBTA = 0.41). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 11.90 (s, 1H, indole-N-H), 8.81 (d, $J = 4.6$, 2H, pyr-2,6H), 8.22 (s, 1H, triazole-H), 8.09 (d, $J = 15.2$, 1H, C=CH), 8.03 (d, $J = 6.1$, 2H, pyr-3,5H), 7.77 (t, $J = 5.6$, 1H, C(O)NH–PEG), 7.70 (d, $J = 2.3$, 1H, indole-4H), 7.45 (d, $J = 15.2$, 1H, C=CH), 7.30 (d, $J = 8.7$, 1H, indole-7H), 6.88 (dd, $J_1 = 8.7$, $J_2 = 2.4$, 1H, indole-6H), 6.38 (s, 1H, biotin-NH), 6.33 (s, 1H, biotin-NH), 5.28 (s, 2H, triazole-CH$_2$-O-), 4.55 (t, $J = 5.2$, 2H, triazole-CH$_2$-PEG), 4.28 (m, 1H, N–CH–CH$_2$–S), 4.10 (m, 2H, N–CH–C(H)C), 3.82 (t, $J = 5.3$, 2H, triazole-CH$_2$-CH$_2$-PEG), 3.50 (m, 2H, PEG), 3.44 (m, 6H, PEG), 3.35 (t, $J = 6.0$, 2H, PEG-CH$_2$-CH$_2$-NH-biotin), 3.14 (m, 2H, PEG-CH$_2$-CH$_2$-NH-biotin), 3.06 (m, 1H, N–CH–C(H)C), 2.80 (dd, $J_1 = 12.4$, $J_2 = 5.1$, 1H, N–CH–CH(exo)H–S), 2.57 (s, 3H, indole-CH$_3$), 2.56 (d, $J = 10$, 1H, N–CH–CH(endo)H–S), 2.03 (t, $J = 7.4$, 2H, NH-C(O)-CH$_2$-), 1.62–1.23 (m, 6H, biotin methylenes). $^{13}$C NMR: 187.6, 172.1, 162.7, 153.9, 152.4, 149.2, 145.3, 143.2, 138.6, 135.6, 133.9, 131.1, 126.4, 124.9, 123.8, 113.2, 112.2, 111.8, 109.3, 104.5, 69.64, 69.56, 69.51, 69.48, 69.11, 68.66, 61.8, 61.0, 59.2, 49.4, 40.1, 38.4, 35.1, 28.1, 28.0, 25.2, 12.0. HRMS [M+Na]$^+$ calc 783.3259, found 783.3250.
**42, Biotin-PEG-meta-MIPP.** In a scintillation vial, biotin-PEG-azide (56 mg, 0.126 mmol), meta-POMIPP (40 mg, 0.126 mmol), sodium ascorbate (0.2x, 5.0 mg), and CuSO₄ (0.1x, 2.0 mg) were stirred in DMF (3 mL). After two weeks, an appreciable amount of product had formed by TLC. The reaction mixture was concentrated under vacuum, dissolved in methanol and dry loaded onto silica gel. The product was purified by column chromatography (5 → 15% MeOH/CH₂Cl₂, 2 cm column, 6” deep) and dried under vacuum, providing a yellow solid (33 mg, 34%). 

**¹H NMR:** (600 MHz, d₆-DMSO): δ 11.91 (s, 1H, indole-NH), 9.29 (d, J = 1.9, 1H, pyr-2H), 8.77 (dd, J₁ = 4.7, J₂ = 1.5, 1H, pyr-6H), 8.53 (dt, J₁ = 7.9, J₂ = 1.9, 1H, pyr-4H), 8.21 (s, 1H, triazole-H), 8.09 (d, J = 15.2, 1H, C=CH), 7.76 (t, J = 5.5, 1H, C(O)NH–PEG), 7.69 (d, J = 2.3, 1H, indole-4H), 7.58 (m, 1H, pyr-5H), 7.50 (d, J = 15.2, 1H, C=CH), 7.29 (d, J = 8.7, 1H, indole-7H), 6.88 (dd, J₁ = 8.7, J₂ = 2.3, 1H, indole-6H), 6.38 (s, 1H, biotin-NH), 6.33 (s, 1H, biotin-NH), 5.28 (s, 2H, triazole-CH₂-O-), 4.54 (t, J = 5.1, 2H, triazole-CH₂-PEG), 4.28 (m, 1H, N–CH–CH₂–S), 4.10 (m, 1H, N–CH–C(H)C), 3.82 (t, J = 5.2, 2H, triazole-CH₂-PEG), 3.50 (m, 2H, PEG), 3.44 (m, 6H, PEG), 3.35 (m, 2H, PEG-CH₂-CH₂-NH-biotin), 3.15 (m, 2H, PEG-CH₂-CH₂-NH-biotin), 3.06 (m, 1H, N–CH–C(H)C), 2.80 (dd, J₁ = 12.4, J₂ = 5.1, 1H, N–CH–CH(exo)H–S), 2.58 (s, 3H, indole-CH₃), 2.56 (d, J = 11.3, 1H, N–CH–CH(endo)H–S), 2.03 (t, J = 7.4, 2H, NH-C(O)-CH₂-), 1.60-1.27 (m, 6H, biotin methylenes). 

**¹³C NMR:** 187.6, 172.1, 162.7, 153.9, 152.4, 149.2, 145.3, 143.2, 138.6, 135.6, 133.9, 131.1, 126.4, 124.9, 123.8, 113.2, 112.2, 111.8, 109.3, 104.5, 69.64, 69.56, 69.51, 69.48, 69.11, 68.66, 61.8, 61.0, 59.2, 55.4, 49.4, 40.1, 38.4, 35.1, 28.1, 28.0, 25.2, 12.0. 

**TLC:** 15% MeOH in CH₂Cl₂: R_f = 0.39, (meta-POMIPP starting material = 0.70).

**HRMS [M+Na]^+** calc 783.3259, found 783.3240.
**43, Biotin-PEG-phenyl-MIPP.** In a scintillation vial, biotin-PEG-azide (1x, 42 mg, 0.095 mmol), phenyl-POMIPP (1x, 30 mg, 0.095 mmol), sodium ascorbate (0.45x, 8.5 mg, 0.043 mmol), CuSO$_4$·5H$_2$O (0.15x, 3.6 mg, 0.0143 mmol), and Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 0.15x, 7.6 mg, 0.0143 mmol) were stirred in DMF (3 mL). After 24 hours, the reaction mixture was concentrated under vacuum, dissolved in methanol and dry loaded onto silica gel. The product was purified by column chromatography (2.5 → 15% MeOH/CH$_2$Cl$_2$, 2 cm column, 6” deep) and dried under vacuum, providing a yellow solid (37 mg, 51%). 

$^1$H NMR (600 MHz, $d_6$-DMSO): δ

11.76 (s, 1H, indole-NH), 8.19 (s, 2H, triazole-H), 8.16 (m, 2H, phenyl-2,6H), 8.04 (d, $J$ = 15.3, 1H, C=CH), 7.77 (t, $J$ = 5.6, 1H, C(O)NH–PEG), 7.66 (d, $J$ = 2.3, 1H, indole-4H), 7.62 (m, 1H, phenyl-4H), 7.57 (m, 2H, phenyl-3,5H), 7.52 (d, $J$ = 15.3, 1H, C=CH), 7.30 (d, $J$ = 8.6, 1H, indole-7H), 6.88 (dd, $J_1$ = 8.7, $J_2$ = 2.4, 1H, indole-6H), 6.39 (s, 1H, biotin-NH), 6.34 (s, 1H, biotin-NH), 5.28 (s, 2H, triazole-CH$_2$-O-), 4.54 (t, $J$ = 5.1, 2H, triazole-CH$_2$-CH$_2$-PEG), 4.29 (m, 1H, N–CH–CH–S), 3.82 (t, $J$ = 5.3, 2H, triazole-CH$_2$-CH$_2$-PEG), 3.50 (m, 2H, PEG), 3.44 (m, 6H, PEG), 3.36 (t, $J$ = 6.0, 2H, PEG-CH$_2$-CH$_2$-NH-biotin), 3.16 (m, 2H, PEG-CH$_2$-CH$_2$-NH-biotin), 3.07 (m, 1H, N–CH–C(H)C), 2.80 (dd, $J_1$ = 12.4, $J_2$ = 5.2, 1H, N–CH–CH(exo)H–S), 2.56 (s, 3H, indole-CH$_3$), 2.55 (d, $J$ = 12.2, 1H, N–CH–CH(endo)H–S), 2.04 (t, $J$ = 7.4, 2H, NH-C(O)-CH$_2$), 1.63-1.29 (m, 6H, biotin methylenes). 

$^{13}$C NMR: 188.6, 172.1, 162.6, 153.8, 144.5, 143.23, 138.7, 137.8, 132.1, 131.1, 128.6, 128.1, 126.4, 124.8, 113.6, 112.1, 111.7, 109.2, 104.3, 69.64, 69.55, 69.51, 69.48, 69.11, 68.7, 61.8, 61.0, 59.2, 55.4, 49.4, 40.1, 38.4, 35.1, 28.1, 28.0, 25.2, 12.0. TLC: 15% MeOH in CH$_2$Cl$_2$: $R_f$ = 0.38 (phenyl-
POMIPP starting material = 0.94, TBTA = 0.24, biotin-azide = 0.51). HRMS [M+Na]$^+$
calc 782.3306, found 782.3297

44, 45, 2-Methyl-5-benzoyl-indole-3-carboxaldehyde and 2-Methyl-6-benzoyl-indole-3-
carboxaldehyde. In a dried two-neck 250 mL round bottom flask under argon, N,N-
dimethylformamide (339 µL, 4.4 mmol) was added to 1,2-dichloroethane (8 mL). The
reaction was cooled to 0 °C and oxalyl chloride (377 µL, 4.4 mmol) dissolved in 1,2-
DCE (dichloroethane, 8 mL) was slowly added, forming a white heterogeneous mixture.
The mixture was allowed to warm to room temperature while stirring. After fifteen
minutes, the reaction was cooled to 0 °C and 2-methylindole (577 mg, 4.0 mmol)
dissolved in 1,2-DCE (8 mL) was slowly added, forming a dark red solution. After one
hour, AlCl$_3$ (1.96 g, 14.7 mmol) was added and stirred vigorously. Benzoyl chloride (510
µL, 4.4 mmol) dissolved in 1,2-DCE (4 mL) was slowly added and the reaction was
warmed to room temperature and stirred overnight. After 24 hours, cold H$_2$O (50 mL)
was added, followed by 5N NaOH (10 mL), and the mixture was stirred. After one hour,
5N HCl (18 mL) was added and this was extracted with methylene chloride (3 x 50 mL).
The combined 1,2-DCE and methylene chloride extracts were combined, dried with
Na$_2$SO$_4$, filtered and concentrated. The crude product mixture was purified by column
chromatography (ethyl acetate: hexanes 1:1 $\Rightarrow$ 4:1), yielding 5-benzoyl product 28 (142
mg, 13%) and 6-benzoyl product 29 (429 mg, 41%) (1:3 regioselectivity for 5 vs. 6
benzyolation).
44. 2-Methyl-5-benzoyl-indole-3-carboxaldehyde: $^1$H NMR (600 MHz, $d_6$-DMSO): δ 12.371 (s, 1H, N-H), 10.073 (s, 1H, CHO), 8.462 (s, 1H, indole-4H), 7.723-7.711 (m, 2H, phenyl-2,6H), 7.673-7.656 (m, 2H, indole-6H, phenyl-4H), 7.587-7.561 (t, $J = 7.8$, 2H, phenyl-3,5H), 7.553-7.539 (d, $J = 8.4$, 1H, indole-7H), 2.728 (s, 3H, methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): δ 196.1, 184.8, 150.8, 138.3, 138.0, 132.1, 130.9, 129.5, 128.5, 124.9, 124.7, 123.4, 114.4, 111.6, 11.6. 1-D nOe: irradiation of peak at δ 12.371 (N-H): nOe signal enhancement seen at δ 7.549 (C-H) and 2.728 (CH$_3$). The H peak at 7.549 has ortho coupling ($J = 8.4$), proving the benzoyl group inserted at the 5 position, not 6. Separately, irradiation of peak at δ 7.540 (C-H with ortho coupling): nOe signal enhancement seen at δ 12.370 (N-H), again proving the proximity of N-H to an ortho-coupled C$_7$-H; signal enhancement also seen at δ 7.66 for C$_6$-H (the benzoyl 2H triplet peak at δ 7.574 was also irradiated by proximity, thus signal enhancement was seen for the other benzoyl C-H peaks at δ 7.72 and δ 7.67). Melting point: 227 – 230 °C. TLC (ethyl acetate:hexanes 4:1) $R_f$ = 0.32. Elemental analysis calculated for C$_{17}$H$_{13}$NO$_2$ • 0.2 C$_4$H$_8$O$_2$ (trace ethyl acetate): C, 76.11; H, 5.24; N, 4.99; found: C, 75.74; H, 4.94; N, 5.06.

45. 2-Methyl-6-benzoyl-indole-3-carboxaldehyde: $^1$H NMR (600 MHz, $d_6$-DMSO): δ 12.323 (s, 1H, N-H), 10.116 (s, 1H, CHO), 8.173-8.159 (d, $J = 8.4$, 1H, indole-4H), 7.776-7.774 (d, $J = 1.2$, 1H, indole-7H), 7.741-7.729 (m, 2H, phenyl-2,6H), 7.683-7.659 (t, $J = 7.2$, 1H, phenyl-4H), 7.627-7.611 (dd, $J_1 = 7.8$, $J_2 = 1.8$, 1H, indole-5H), 7.583-7.558 (t, $J = 7.5$, 2H, phenyl-3,5H), 2.744 (s, 3H, methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): δ 196.3, 185.4, 152.2, 138.7, 135.3, 132.8, 131.8, 130.1, 129.8, 129.1, 124.4,
1-D nOe: irradiation of peak at δ 12.323 (N-H): nOe signal enhancement seen at δ 7.775 (C-H with meta coupling, thus proving benzoyl addition to position 6 of indole) and δ 2.744 (CH₃). Separately, irradiation of the peak at δ 7.775 (C-H with meta coupling: J = 1.2) led to signal enhancement at δ 12.323 (N-H peak), thus proving proximity of meta-coupled C-H to N-H (the benzoyl 2,6 2H peak at 7.73 was also irradiated by proximity, leading to signal enhancement of benzoyl 3,5 2H peak at δ 7.57). Melting point: 192 – 196 °C. TLC (ethyl acetate:hexanes 4:1) Rf = 0.40. Elemental analysis calculated for C₁₇H₁₃NO₂: C, 77.55; H, 4.98; N, 5.32; found: C, 77.28; H, 4.97; N, 5.15

46, trans-3-(5-Benzoyl-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one. In a dried 100 mL round bottom flask under argon, 2-Methyl-5-benzoyl-indole-3-carboxaldehyde (50 mg, 0.19 mmol) was dissolved in anhydrous methanol (3 mL). 4-Acetyl-pyridine (21 µL, 0.19 mmol) and piperidine (4 µL, 0.04 mmol) were added and the reaction was refluxed. After twelve hours, 0.3 equivalents of 4-acetyl-pyridine (6.3 µL, 0.06 mmol) was added. After a total of 24 hours reaction time, the reaction was cooled and the precipitate was isolated by filtration and rinsed with cold methanol, providing 37 mg of yellow solid. A ¹H NMR showed a 1:3.5 ratio of product to aldehyde. The filtrate was concentrated and added to the product/aldehyde mixture, and 0.8 equivalents of both 4-acetyl-pyridine (17 µL, 0.15 mmol) and piperidine (15 µL, 0.15 mmol) were added to the reaction. This was refluxed for another 24 hours, after which the reaction was cooled, filtered and rinsed with cold methanol, providing 9 mg (13%) pure, yellow product. ¹H NMR (600 MHz, d₆-DMSO): δ 12.397 (s, 1H, N-H), 8.815-8.805 (m, 2H, pyr-2.6H),
8.321 (s, 1H, indole-4H), 8.081-8.056 (d, J = 15.0, 1H, C=CH), 7.820-7.808 (d, J = 7.2, 2H, phenyl-2,6H), 7.761-7.728 (m, 4H, phenyl-4H, indole-6H, pyr-3,5H), 7.644-7.619 (t, J = 7.5, 2H, phenyl-3,5H), 7.587-7.573 (d, J = 8.4, 1H, indole-7H), 7.347-7.321 (d, J = 15.6, 1H, C=CH), 2.639 (s, 3H, methyl).

13C NMR (150 MHz, d6-DMSO): δ 195.7, 187.9, 150.6, 146.9, 144.7, 138.8, 138.5, 138.4, 131.9, 130.1, 129.4, 128.4, 125.2, 123.9, 123.6, 121.1, 114.6, 111.9, 110.1, 12.1. Melting point: 258–262 °C. TLC (ethyl acetate:hexanes 4:1) Rf = 0.24. Elemental analysis calculated for C24H18N2O2 • 0.65 H2O: C, 76.24; H, 5.14; N, 7.41; C, 75.96; H, 4.75; N, 7.16.

47. trans-3-(6-Benzoyl-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one. In a dried 100 mL round bottom flask under argon, 2-Methyl-6-benzoyl-indole-3-carboxaldehyde (50 mg, 0.19 mmol) was dissolved in anhydrous methanol (3 mL). 4-Acetyl-pyridine (21 µL, 0.19 mmol) and piperidine (19 µL, 0.19 mmol) were added and the reaction was refluxed. After 24 hours, the reaction was cooled, filtered and rinsed with cold hexanes and dried under vacuum, yielding 52 mg (74 %) of pure yellow solid. 1H NMR (600 MHz, d6-DMSO): δ 12.350 (s, 1H, N-H), 8.832-8.222 (d, J = 6.0, 2H, pyr-2,6H), 8.255-8.241 (d, J = 8.4, 1H, indole-4H), 8.125-8.099 (d, J = 15.6, 1H, C=CH), 8.008-7.999 (d, J = 5.4, 2H, pyr-3,5H), 7.801 (s, 1H, indole-7H), 7.766-7.754 (d, J = 7.2, 2H, phenyl-2,6H), 7.683-7.642 (m, 2H, phenyl-4H, indole-5H), 7.585-7.563 (m, 3H, C=CH, phenyl-3,5H), 2.659 (s, 3H, methyl). 13C NMR (150 MHz, d6-DMSO): δ 195.5, 188.2, 150.6, 148.5, 144.6, 138.7, 138.2, 135.4, 132.0, 130.6, 129.4, 129.2, 128.4, 123.1, 121.5, 119.9, 114.5, 114.1, 109.6, 12.3. Melting point: 267–270 °C. TLC (ethyl
acetate:hexanes 4:1) $R_f = 0.29$. Elemental analysis calculated for $C_{24}H_{18}N_2O_2 \cdot 0.05 H_2O$: C, 78.48; H, 4.97; N, 7.63; found: C, 78.08; H, 5.01; N, 7.50.

48, 2-Methyl-5-nitro-1H-indole.\textsuperscript{144} In a 250 mL round bottom flask, 2.62 grams of 2-methyl-indole (20 mmol) was dissolved in 20 mL of $H_2SO_4$ after vigorous stirring. In a separate flask, 1.87 grams of NaNO$_3$ (1.1x, 22 mmol, 84.99 MW) was dissolved in 20 mL of $H_2SO_4$, also after vigorous stirring, and added dropwise via addition funnel to the indole. After addition, the reaction was stirred for another 10 minutes, and then poured into 400 mL of ice-water, precipitating a yellow product. The product was isolated via filtration and washed with cold water. After 12 hours of drying under vacuum, 3.35 grams of yellow product was isolated (95%). $^1H$ NMR (600 MHz, $d_6$-DMSO): $\delta$ 11.703 (s, 1H, N-H), 8.4003-8.400 (d, $J = 1.8$, 1H, indole-4H), 7.916-7.898 (dd, $J_1 = 9.0$, $J_2 = 1.8$, 1H, indole-6H), 7.422-7.407 (d, 1H, $J = 9.0$, 1H, indole-7H), 6.408 (s, 1H, indole-3H), 2.419 (s, 1H, methyl). $^{13}C$ NMR (150 MHz, $d_6$-DMSO): $\delta$ 140.5, 140.0, 139.4, 128.0, 115.9, 115.7, 110.7, 101.6, 13.4. 1-D nOe: irradiation of peak at $\delta$ 7.422-7.407 (C$_7$-H): nOe signal enhancement seen at $\delta$ 11.690 (N-H) and 7.916-7.897 (C$_6$-H). Irradiation of peak at $\delta$ 11.690 (N-H): nOe signal enhancement seen at $\delta$ 7.422-7.407 (C$_7$-H) and 2.419 (C$_2$-CH$_3$) (nOe observed between the C-H proton with ortho coupling and N-H proton, thus proving NO$_2$ inserted at indole C-5 vs. C-6). Melting point: 166 – 169 °C (published\textsuperscript{144}: 176-176.5 °C). TLC (in 1:1 ethyl acetate:hexanes) $R_f = 0.38$. Elemental analysis calculated for $C_9H_8N_2O_2$: C, 61.36; H, 4.58; N, 15.90; found: C, 61.54; H, 4.63; N, 15.89.
49. 2-Methyl-5-amino-1H-indole.\textsuperscript{144} 2-methyl-5-nitro-1H-indole (1.00 grams, 5.68 mmol) was dissolved in 75 mL ethanol. 10% Pd/C was added (200 mg) and the mixture was subjected to hydrogenation (38 psi) using a Parr hydrogenator for 3.5 hours. The mixture was filtered over celite, which was washed with methanol. After concentration and drying under vacuum, 801 mg of a brown powder (97%) was isolated. \textsuperscript{1}H NMR (600 MHz, $d_6$-DMSO): δ 10.370 (s, 1H, N-H), 6.943-6.929 (d, $J = 8.4$, 1H, indole-7H), 6.552-6.549 (d, $J = 1.8$, 1H, indole-4H), 6.373-6.355 (dd, $J_1 = 8.4$, $J_2 = 2.4$, 1H), 5.815 (s, 1H, indole-6H), 4.436 (bs, 2H, NH$_2$), 2.288 (s, 3H, methyl). \textsuperscript{13}C NMR (150 MHz, $d_6$-DMSO): δ 140.8, 135.0, 129.9, 129.6, 110.5, 110.3, 102.9, 98.0, 13.5. Melting point: 147 – 150 °C (published\textsuperscript{144}: 157-159 °C). TLC (in 3:1 ethyl acetate:hexanes) $R_f = 0.30$; (in 1:1 ethyl acetate:hexanes) $R_f = 0.16$. Elemental analysis calculated for C$_9$H$_{10}$N$_2$ • 0.05 C$_2$H$_6$O: C, 73.61; H, 6.99; N, 18.87; found: C, 73.48; H, 6.77; N, 18.81.

50. 2-Methyl-5-azido-1H-indole. In an oven dried, 250 mL round bottom flask flushed with argon, 2-methyl-5-amino-1H-indole (400 mg, 2.74 mmol, 146.19 MW) was dissolved in 90 % AcOH (20 mL). After complete solvation, the reaction was placed at 0 °C and protected from light with foil. NaNO$_2$ (1.1x, 3.01 mmol, 208 mg, 69.00 MW) dissolved in cold H$_2$O (2 mL) was added dropwise and stirred for ten minutes. NaN$_3$ (1.1x, 3.01 mmol, 196 mg, 65.01 MW) dissolved in cold H$_2$O (2 mL) was added dropwise. After one hour the reaction was poured into water (40 mL), which was extracted 3 times with CH$_2$Cl$_2$ (40 mL each). The extracts were washed with sodium bicarbonate and brine (100 mL each), dried with sodium sulfate, filtered and concentrated. Purification by silica flash chromatography (100% CH$_2$Cl$_2$) yielded the
azide as a light brown solid (310 mg, 66%). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 11.025 (s, 1H, N-H), 7.295-7.281 (d, $J = 8.4$, 1H, indole-7H), 7.131-7.127 (d, $J = 2.4$, 1H, indole-4H), 6.737-6.719 (dd, $J_1 = 8.4$, $J_2 = 2.4$, 1H, indole-6H), 6.108-6.106 (d, $J = 1.2$, 1H, indole-3H), 2.369 (s, 1H, methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): $\delta$ 137.5, 133.9, 130.0, 129.5, 111.7, 111.6, 108.5, 99.0, 13.4. Melting point: 57 – 60 °C. TLC (in 1:1 ethyl acetate:hexanes) $R_f$ = 0.56. IR (film): 3409 cm.$^{-1}$, 2111 (aryl-azide). Elemental analysis calculated for C$_9$H$_8$N$_4$: C, 62.78; H, 4.68; N, 32.54; found: C, 62.95; H, 4.68; N, 32.53

51, 2-Methyl-3-carboxaldehyde-5-azido-1H-indole. In an oven dried, 100 mL round bottom flask under argon, POCl$_3$ (1.5x, 3.14 mmol, 291 µL) was added to anhydrous DMF (2.0 mL) at 0 °C. 2-Methyl-5-azido-1H-indole (dissolved in DMF, 2 mL) was added dropwise. The stirred reaction was slowly warmed to RT. 1N NaOH (25 mL) and water (25 mL) was added, forming a precipitate which was filtered and rinsed with cold water. The resulting white solid was dried under vacuum, affording 369 mg (88%). $^1$H NMR (600 MHz, $d_6$-DMSO): $\delta$ 12.078 (s, 1H, N-H), 10.031 (s, 1H, CHO), 7.754-7.751 (d, $J = 1.8$, 1H, indole-4H), 7.423-7.409 (d, $J = 8.4$, 1H, indole-7H), 6.913-6.896 (dd, $J_1 = 8.4$, $J_2 = 1.8$, 1H, indole-6H), 2.676 (s, 3H, methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): $\delta$ 184.3, 149.7, 133.4, 133.0, 126.6, 114.4, 113.4, 112.8, 109.4, 11.5. Melting point: degradation ca. 150 °C. TLC (in 1:1 ethyl acetate:hexanes) $R_f$ = 0.19. Elemental analysis calculated for C$_{10}$H$_8$N$_4$O: C, 59.99; H, 4.03; N, 27.99; found: C, 60.03; H, 4.07; N, 27.96
trans-3-(5-Azido-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one. In a dried, 100 mL round bottom flask under argon and protected from light, 2-Methyl-3-carboxaldehyde-5-azido-1H-indole (80 mg, 0.40 mmol) was partially dissolved in anhydrous methanol (5 mL). 4-Acetyl-pyridine (1.5 equiv., 66 µL, 0.60 mmol) and piperidine (40 µL, 0.40 mmol) were added and the reaction was stirred at 40 °C. After 24 hours, the reaction was cooled to RT and the yellow precipitate was isolated via filtration and rinsed with cold methanol. A 1H NMR of this product showed a ca. 1:3 ratio of product to the indole starting material. This crude product was redissolved in anhydrous methanol (5 mL) along with the concentrated filtrate, and another equivalent of 4-acetyl-pyridine (44 µL, 2.5 equiv. total) and five equivalents of piperidine (200 µL, 6 equiv. total) were added. The stirred reaction was heated at 40 °C. After 12 hours, a yellow precipitate was filtered and rinsed with cold methanol and dried under vacuum, affording 47 mg (39%) of pure product. 1H NMR (600 MHz, d6-DMSO): δ 12.105 (s, 1H, N-H), 8.816-8.806 (dd, J1 = 4.8, J2 = 1.8, 2H, pyr-2,6H), 8.066-8.041 (d, J = 15, 1H, C=CH), 7.956-7.945 (dd, J1 = 4.8, J2 = 1.8, 2H, pyr-3,5H), 7.661-7.657 (d, J = 2.4, 1H, indole-4H), 7.455-7.441 (d, J = 8.4, 1H, indole-7H), 7.401-7.375 (d, J = 15.6, 1H, C=CH), 7.001-6.984 (dd, J1 = 8.4, J2 = 1.8, indole-6H), 2.587 (s, 3H, methyl). 13C NMR (150 MHz, d6-DMSO): δ 188.2, 150.6, 146.5, 144.9, 139.0, 133.9, 132.9, 126.8, 121.5, 113.9, 113.8, 113.0, 110.3, 109.2, 12.2. Melting point: decomposes ca. 190 °C. TLC (in 4:1 ethyl acetate:hexanes) Rf = 0.21. Elemental analysis calculated for C17H13N5O: C, 67.32; H, 4.32; N, 23.09; found: C, 67.37; H, 4.33; N, 23.12
53. 2-Methyl-5-methoxy-6-nitro-1H-indole. In a 100 mL round bottom flask, 2-methyl-5-methoxyindole (370 mg, 2.30 mmol) was dissolved in H₂SO₄ (4 mL) after vigorous stirring, then placed at 0 °C. In a separate flask, 1.87 grams of NaNO₃ (214 mg, 2.52 mmol) was dissolved in H₂SO₄ (4 mL) also after vigorous stirring, chilled to 0 °C, and added dropwise to the indole. After addition, the reaction was stirred for another 10 minutes, and then poured into ice-water (200 mL), precipitating a yellow product, which was extracted with ethyl acetate (3 x 100 mL). Extracts were washed with sat. NaHCO₃ and brine, dried with Na₂SO₄, filtered and concentrated. Purification by column chromatography (ethyl acetate:hexane 2:3 → 3:2) yielded 296 mg of pure product (62%), (followed by 73 mg of 4-nitrated product, 15%; 4:1 regioselectivity). ¹H NMR (600 MHz, CDCl₃): δ 8.350 (s, 1H, N-H), 7.983 (s, 1H, indole-7H), 7.066 (s, 1H, indole-4H), 6.211 (s, 1H, indole-3H), 3.958 (s, 3H, o-methyl), 2.477 (s, 3H, c-methyl). ¹³C NMR (150 MHz, CDCl₃): δ 148.7, 142.8, 134.7, 134.2, 129.1, 109.2, 102.7, 101.3, 57.1, 14.2. Melting point: 134 – 138 °C. TLC (in 1:1 ethyl acetate:hexanes) Rₚ = 0.37 (4-nitro product Rₚ = 0.25). Elemental analysis calculated for C₁₀H₁₀N₂O₃ • 0.1 C₆H₁₄: C, 59.27; H, 5.35; N, 13.04; found: C, 59.33; H, 5.10; N, 12.98

54. 2-Methyl-5-methoxy-6-amino-1H-indole. In a hydrogenation flask, 2-Methyl-5-methoxy-6-nitro-1H-indole (270 mg, 1.31 mmol) was dissolved in 100% EtOH (35 mL) and 10 % Pd/C (40 mg) was added. The mixture was hydrogenated on a Parr hydrogenator at 40 p.s.i. for 45 minutes. The red-pink solution was filtered over celite and rinsed with MeOH, concentrated and dried under vacuum, yielding 230 mg of pure product (99%). ¹H NMR (400 MHz, CDCl₃): δ 7.516 (s, 1H, N-H), 6.907 (s, 1H), 6.607
(s, 1H), 6.052 (s, 1H, indole-3H), 3.873 (s, 3H, o-methyl), 3.733 (bs, 2H, NH2), 2.365 (s, 3H, c-methyl). $^{13}$C NMR (150 MHz, $d_6$-DMSO): δ 142.8, 132.9, 131.5, 131.4, 119.5, 100.5, 98.7, 95.7, 55.6, 13.5

Melting point: 144 – 148 °C. TLC (in 3:1 ethyl acetate:hexanes) $R_f = 0.33$. Elemental analysis calculated for C$_{10}$H$_{10}$N$_2$O$_3$ 0.1 C$_6$H$_{14}$: C, 59.27; H, 5.35; N, 13.04; found: C, 59.33; H, 5.10; N, 12.98.

55. 2-Methyl-5-methoxy-6-azido-1H-indole. In a dried, 100 mL round bottom flask under argon, 2-Methyl-5-methoxy-6-amino-1H-indole (227 mg, 1.29 mmol) was dissolved in 90% AcOH (10 mL) and placed at 0 °C. The flask was covered with foil and the reaction was conducted in low light. NaNO$_2$ (98 mg, 1.43 mmol, 1.1 equiv) dissolved in H$_2$O (1 mL) was added dropwise, and the mixture stirred for 10 minutes. NaN$_3$ (92 mg, 1.43 mmol, 1.1 equiv.) dissolved in H$_2$O (1 mL) was added dropwise. After 45 minutes, the mixture was slowly poured into H$_2$O (25 mL) and sat. K$_2$CO$_3$ (19 mL) to form a neutral pH. This was extracted with ethyl acetate (4 x 50 mL), washed with brine, dried with Na$_2$SO$_4$, filtered and concentrated. The crude product was purified by column chromatography (ethyl acetate:hexanes 1:4), yielding 139 mg of pure oil (53 %). $^1$H NMR (600 MHz, CDCl$_3$): δ 7.782 (s, 1H, N-H), 7.007 (s, 1H), 6.883 (s, 1H), 6.145 (s, 1H, indole-3H), 3.890 (s, 3H, o-methyl), 2.388 (s, 3H, c-methyl). $^{13}$C NMR (150 MHz, CDCl$_3$): δ 147.0, 136.1, 130.7, 126.5, 123.1, 102.4, 102.0, 100.4, 56.5, 13.7. TLC (in 1:1 ethyl acetate:hexanes) $R_f = 0.60$

56. 2-Methyl-3-carboxaldehyde-5-methoxy-6-azido-1H-indole. In an oven dried, 100 mL round bottom flask, under argon and protected from light with foil, POCl$_3$ (84 µL, 0.90
mmol, 1.5 equiv.) was added to anhydrous DMF (1.0 mL) at 0 °C and stirred for 5 minutes. Next, 2-Methyl-5-methoxy-6-azido-1H-indole (dissolved in DMF, 1 mL) was added dropwise. The stirred reaction was slowly warmed to RT. 1N NaOH (10 mL) and water (10 mL) were added, and this was extracted with CH₂Cl₂ (3 x 20 mL). Extracts were washed with brine, dried with Na₂SO₄, filtered, concentrated and dried overnight under vacuum, producing 95 mg pure product (69%). ¹H NMR (600 MHz, d₆-DMSO): δ 11.865 (s, 1H, N-H), 10.004 (s, 1H, CHO), 7.645 (s, 1H, indole-4H), 7.032 (s, 1H, indole-7H), 3.857 (s, 3H, o-methyl), 2.645 (s, 3H, c-methyl). ¹³C NMR (150 MHz, d₆-DMSO): δ 184.8, 149.5, 149.4, 130.3, 124.1, 124.0, 114.4, 104.2, 103.5, 56.9, 12.2. TLC (in 1:1 ethyl acetate:hexanes) Rₜ = 0.12.

57, trans-3-(6-Azido-5-methoxy-2-methyl-1H-indol-3-yl)-1-(4-pyridinyl)-2-propen-1-one. In a dried, 100 mL round bottom flask under argon and protected from light, 2-Methyl-3-carboxaldehyde-5-methoxy-6-azido-1H-indole (70 mg, 0.30 mmol) was partially dissolved in anhydrous methanol (5 mL). 4-Acetyl-pyridine (50 µL, 0.45 mmol, 1.5 equiv.) and piperidine (148 µL, 1.5 mmol, 5 equiv.) were added and the reaction was stirred at 40 °C. After 7 hours, the reaction was cooled to RT and the precipitate was isolated via filtration and rinsed with cold methanol. A ¹H NMR of this product showed a mixture of aldehyde with trace product. This mixture was redissolved in the filtrate and additional 4-acetyl-pyridine (165 µL, 5 equiv.) and piperidine (148 µL, 5 equiv.) were added and the mixture was set to react at 40 °C. After 48 hours, although starting materials were still seen on TLC, the crude reaction mixture was dry loaded onto silica and purified by column chromatography (ethyl acetate:hexane 3:1), eluting first the
aldehyde, followed by the ketone and finally the product as a yellow solid (39 mg, 39%).

$^1$H NMR (600 MHz, $d_6$-DMSO): δ 11.784 (s, 1H, N-H), 8.815-8.805 (dd, $J_1 = 4.2, J_2 = 1.8, 2H, \text{pyr-2,6H}$), 8.073-8.047 (d, $J = 15.6, 1H, C=\text{CH}$), 7.957-7.947 (dd, $J_1 = 4.2, J_2 = 1.8, 2H, \text{pyr-3,5H}$), 7.538 (s, 1H, indole C=CH), 7.410-7.384 (d, $J = 15.6, 1H, \text{indole C}=\text{CH}$), 7.392 (s, 1H, 3H, o-methyl), 2.569 (s, 3H, c-methyl).

$^{13}$C NMR (150 MHz, $d_6$-DMSO): δ 188.2, 150.6, 148.5, 145.6, 145.0, 139.1, 130.6, 123.6, 123.3, 121.5, 113.5, 109.5, 103.8, 103.7, 56.9, 12.3. Melting point: degrades to black substance ca. 160 °C. TLC (in 4:1 ethyl acetate:hexanes) R$_f$ = 0.15. Elemental analysis calculated for C$_{18}$H$_{15}$N$_5$O$_2$ • C$_4$H$_8$O$_2$ (trace ethyl acetate): C, 64.72; H, 4.60; N, 20.74; found: C, 65.11; H, 4.71; N, 20.39.

58, meta-MOMIPP. trans-3-(5-Methoxy-2-methyl-1H-indol-3-yl)-1-(3-pyridinyl)-2-propen-1-one. To an oven dried 50 mL round bottom flask kept under an argon atmosphere, 5-Methoxy-2-methyl-1H-Indole-3-carboxaldehyde (75 mg, 0.40 mmol, 1 equiv.) was added, followed by anhydrous methanol (10 mL), 3-acetylpyridine (174 µL, 1.6 mmol, 4 equiv.) and piperidine (158 µL, 1.6 mmol, 4 equiv.) and refluxed for 16 hours. The yellow solution was cooled to room temperature, but no precipitation occurred. By TLC, both starting materials and product were visible (4:1 ethyl acetate:hexanes: R$_f$ aldehyde: 0.46, pyridine: 0.30, product: 0.20). The solution was concentrated and the residue was applied directly to a silica column (2 cm diameter, packed to 6”) and eluted with 50% → 70% ethyl acetate in hexanes. The yellow product was isolated and dried under vacuum, yielding 20 mg of a yellow solid (17%). $^1$H NMR (600 MHz, $d_6$-DMSO): δ 11.848 (s, 1H, N-H), 9.249-9.246 (d, $J = 1.8, 1H, \text{pyr-2-H}$),
8.790-8.779 (dd, $J_1 = 4.8$, $J_2 = 1.8$, 1H, pyr-6-H), 8.432-8.419 (m, 1H, pyr-4-H), 8.093-8.068 (d, $J = 15.0$, 1H, C=CH), 7.598-7.577 (dd, $J_1 = 7.8$, $J_2 = 4.8$, 1H, pyr-5-H), 7.453-7.449 (d, $J = 2.4$, 1H, indole-4-H), 7.434-7.409 (d, $J = 15.0$, 1H, C=CH), 7.308-7.293 (d, $J = 9.0$, 1H, indole-7-H), 6.841-6.823 (dd, $J_1 = 8.4$, $J_2 = 2.4$, 1H, indole-6-H), 3.861 (s, 3H, –O–CH$_3$), 2.576 (s, 3H, C–CH$_3$). $^{13}$C NMR (150 MHz, $d_6$-DMSO): δ 188.1, 155.6, 153.0, 149.6, 145.7, 139.2, 136.0, 134.4, 131.4, 127.1, 124.3, 113.7, 112.7, 111.4, 109.7, 103.9, 56.0, 12.6. Melting point: 185 – 189 °C. TLC (in 4:1 ethyl acetate:hexanes) $R_F = 0.20$. Elemental analysis calculated for C$_{18}$H$_{16}$N$_2$O$_2$•0.2 C$_6$H$_{14}$ (trace hexanes): C, 74.50; H, 6.12; N, 9.05; found: C, 74.18; H, 6.36; N, 8.83.
### Appendix C

**Full Protein List Associated With Table 3.1**

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<td>Vps39</td>
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<td>Vps39</td>
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<td></td>
<td>Vps41</td>
<td>829 AA</td>
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<tr>
<td>UVRAG</td>
<td></td>
<td>UVRAG</td>
<td>699 AA</td>
<td></td>
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<tr>
<td>MOTILITY</td>
<td>Name (metazoan)</td>
<td>Size(s)</td>
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<td></td>
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<td>Yeast</td>
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<tr>
<td>Cytoplasmic Dynein</td>
<td>DYNC1H1, 2H1</td>
<td>989 AA</td>
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<tr>
<td>Heavy Chain</td>
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<tr>
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<tr>
<td>Light. Int. Chain</td>
<td>DYNC1LI1, 2</td>
<td>523 AA</td>
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<tr>
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<td>DYNLL1, 2</td>
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<tr>
<td>Light Chains</td>
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<td>560+ AA</td>
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<tr>
<td>Heavy Chains</td>
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<td>957+ AA</td>
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Appendix D

Additional images for Figure 3-21.

Effects of 5-Azido-MIPP Photolysis Products on U251 Cells. A solution of 5-Azido-MIPP in cell culture medium (supplemented with 10% FBS) was irradiated for 2 mins at 254 nm, and immediately transferred to a 35 mm dish containing U251 cells (in the exponential growth phase) at the indicated concentrations, followed by addition of fresh MOMIPP as indicated: (A) 10 µM irradiated 5-Azido-MIPP only (B) 10 µM irradiated 5-Azido-MIPP + 2.5 µM fresh, non-irradiated MOMIPP, (C) 10 µM irradiated 5-Azido-MIPP + 5 µM fresh, non-irradiated MOMIPP, (D) 10 µM irradiated 5-Azido-MIPP + 10 µM fresh, non-irradiated MOMIPP, (E) 10 µM irradiated 5-Azido-MIPP + 20 µM fresh, non-irradiated MOMIPP, (F) 10 µM irradiated 5-Azido-MIPP + 40 µM fresh, non-irradiated MOMIPP, (G) 2.5 µM irradiated 5-Azido-MIPP + 10 µM fresh, non-irradiated MOMIPP, (H) 5 µM irradiated 5-Azido-MIPP + 10 µM fresh, non-irradiated MOMIPP, (I) 10 µM irradiated 5-Azido-MIPP + 10 µM fresh, non-irradiated MOMIPP, (J) 20 µM irradiated 5-Azido-MIPP + 10 µM fresh, non-irradiated MOMIPP, (K) 40 µM irradiated 5-Azido-MIPP + 10 µM fresh, non-irradiated MOMIPP, (L) 2.5 µM un-irradiated 5-Azido-MIPP, (M) cell culture medium (with 10% FBS) irradiated and added to cells, (N) untreated cell culture medium (with 10% FBS) on U251 cells. Phase contrast microscopy images taken 4 hours after treatment.
Irradiated N$_3$-MIPP: 10 μM

MOMIPP: 0

10 μM

2.5 μM
Irradiated N$_3$-MIPP: 10 μM
MOMIPP: 5 μM
Irradiated $N_3$-MIPP: 10 μM
MOMIPP: 20 μM

10 μM
40 μM
Irradiated $\text{N}_3$-MIPP: 2.5 μM

MOMIPP:

- 2.5 μM
- 5 μM
- 10 μM
- 10 μM
Irradiated N₃-MIPP: 10 μM 20 μM
MOMIPP: 10 μM 10 μM
Irradiated N₃-MIPP: 40 μM
MOMIPP: 10 μM

2.5 μM (no-irradiation)
medium irradiated  
n/a (no treatment)

218
Appendix E – Spectra

2a

219
STANDARD CARBON PARAMETERS
Pulse Sequence: tippi
Solvent: DMSO
Ambient Temperature
File: 831080
Sample-Comb "geneses"

Pulse 8.8 degrees
Acq. time 0.064 sec
VRMR 8650.9 Hz
OSCARING HR: 595.9781446 Mhz
Data processing:
line broadening 6.4 Hz
FT size 131072
Total time 1 min, 14 sec

STANDARD PROTON PARAMETERS
Pulse Sequence: tippi
Solvent: DMSO
Ambient Temperature
File: 831080
Sample-Comb "geneses"

Pulse 8.8 degrees
Acq. time 0.064 sec
VRMR 8650.9 Hz
OSCARING HR: 595.9781446 Mhz
Data processing:
line broadening 6.4 Hz
FT size 131072
Total time 1 min, 14 sec
223
S11
STANDARD PROTON PARAMETERS
Pulse Sequence: z12p1
Solvent: DMSO
Ambient temperature
File: 100230
Date: 000
Path: "\n
Pulse delay: 1.1953 sec
Pulse 60.0 degrees
Amp. time 0.988 sec
Sweep 0.996 Hz
Sweep time 5 sec

DATA PROCESSING
Line broadening 3.4 Hz
FT time 13472
Total time 3 min, 0 sec

10

S12
STANDARD CARBON PARAMETERS
Pulse Sequence: z12p1
Solvent: DMSO
Ambient temperature
File: 100230
Path: "

Pulse 40.4 degree
Amp. time 1.690 sec
Sweep 0.7765 Hz
Sweep time 5 sec

DATA PROCESSING
Sweep width 3.1 Hz
center at 3.1 Hz
FT time 13472
Total time 3 min, 0 sec
10

11
SIS

STANDARD CARBON PARAMETERS
Pulse Sequence: sipul
Solvent: DMSO
Ambient temperature
Field: 400 MHz
Instrument: INOVA-600 "Nova-west"

Pulse 90.4 degrees
Acq. time: 1.20 sec
Slices: 32
Reps: 128

Observe H, 15.583297 M00
Decouple H, 55.9871636 M00
Power: 32 dB
Quad off

Data Processing
Gain window 0.009 sec
Sampling 9.100 sec
FT T1 16.918 sec
Total Time 28 hr, 1 min, 31 sec

SIS

STANDARD PROTON PARAMETERS
Pulse Sequence: gocsy
Solvent: DMSO
Ambient temperature
Field: 400 MHz
Instrument: INOVA-600 "Nova-west"

Pulse delay: 1.000 sec
Acq. time: 0.032 sec
Voxel: 201.1 Hz
Slices: 1024
Single scan

Observe H, 55.9871455 MHz
Data Processing
FT T1 18.999 sec
F1 Data Processing
Slices 1024 x 1024
Total Time 1 min, 58 sec
23

SSO
STANDARD SPECTRAL PARAMETERS
Pulse Sequence: TIPPI
Solvent: DMSO
Ambient temperature
Date: 14-97
File: 5501150

PW=60.6 deg
Acq. time: 0.98 sec
Decoupler: 397.5 MHz
Recycle delay: 22.0 sec
Power: 32 mW
Continuously on

Diff: 0.0 ppm
Gain: 0.0 ppm
COSY window: 106.6 Hz
Center at 2.0 ppm
FID size: 256K
Total time: 28 hr, 1 min, 21 sec
553

STANDARD CARBON PARAMETERS
Pulse Sequence: zg5u
Solvent: DMSO
Temp: 298.1 K
Field: 400 MHz
IPR: 15-30 Hz
Filter: 0.005-0.30 ppm

Pulse 88.4 degrees
Acq. time 2.146 sec
Wait 3.55 sec
mix repetitions
ORDER: 121, 59.815788 MHz
ECOUPLE: 8, 59.78818 MHz
Power: 22 mW
Gain: 300 K
VOLT: 7.3 modulated
DATA PROCESSING
Gauss window 8.098 sec
cramp 40.160 sec
rt 97.100 sec
Total time 2 hr, 48 min, 3 sec

24

554

STANDARD PROTON PARAMETERS
Pulse Sequence: zg5u
Solvent: DMSO
Temp: 298.1 K
IPR: 15-30 Hz

Delay: 1,300 sec
Acq. time 6.162 sec
Wait 3.55 sec
Filter 15-30 Hz
Mix repetitions
ORDER: 3, 87.11358 MHz
ECOUPLE: 8, 87.11358 MHz
Power: 22 mW
Gain: 300 K
VOLT: 7.3 modulated
DATA PROCESSING
Gauss: 8.098 sec
rt 97.100 sec
Total time 2 hr, 48 min, 3 sec

24

245
246

556
STANDARD PROTON PARAMETERS
Pulse Sequence: 62ps1
Solvent: DMDO
Temp: 25 ± 0.1 °C
Pulse Protocols
ENDO-ER/ "Tough-watt"
Relax. Delay 1.000 sec
Pulse 60.5 Degree
Acq. Time 6.000 sec
# of Repetitions
1
Data Processing
Spin Locking 8.4 Hz
Total time 1 min, 4 sec

25

556
STANDARD CARBON PARAMETERS
Pulse Sequence: 62ps1
Solvent: DMDO
Temp: 25 ± 0.1 °C
Pulse Protocols
ENDO-ER/ "Tough-watt"
Pulse 60.4 degree
Acq. time 1.000 sec
Wait 37.795 sec
Data Repetition
64
DECoupling rf. 108.637962 MHz
Power 50 W
Conjugated by
Gauss window 0.638 sec
Gain 3.750 sec
Total time 20 hr, 1 min, 85 sec

246
STANDARD PROTON PARAMETERS

Pulse Sequence: gpro
Solvent: DMSO
Temp: 303 K

20 MHz, 201.5 Hz
Single scan
20 acquisitions

F2 (ppm)

F1 (ppm)

13C OBSERV

Pulse Sequence: sgp13
Solvent: DMSO
Ambient Temperature: 303 K
20 MHz, 201.5 Hz

20 MHz, 201.5 Hz
20 acquisitions

Li 20 MHz
DSSO 0.96 ppm

140 120 100 80 60 40 20 ppm
Pulse Sequence: shg1
Solvent: CDCl3
Ambient Temperature
File: 24-1-N_150

1H NMR (400 MHz) 

259
biotin-PEG-azide
biotin-PEG-phenyl-MIPP

**Current Data Parameters**
- **MASS**: 5-75
- **XMODE**: 0
- **RELAX**: 1

**P2 - Acquisition Parameters**
- **DS**: 500.00 MHz
- **馔**: 10.58 Hz
- **PIECE**: 400.00 MHz
- **PIRED**: 20.00 Hz
- **ACQ**: 10.00 Hz
- **T1**: 4.00 Hz
- **T2**: 1.50 Hz

**P1 - Processing Parameters**
- **DS**: 500.00 MHz
- **P1**: 20.00 Hz
- **P2**: 50.00 Hz
- **P3**: 100.00 Hz
- **P4**: 200.00 Hz
- **P5**: 500.00 Hz

**Current Data Parameters**
- **MASS**: 0-200
- **XMODE**: 0
- **RELAX**: 1

**P2 - Acquisition Parameters**
- **DS**: 500.00 MHz
- **馔**: 10.58 Hz
- **PIECE**: 400.00 MHz
- **PIRED**: 20.00 Hz
- **ACQ**: 10.00 Hz
- **T1**: 4.00 Hz
- **T2**: 1.50 Hz

**P1 - Processing Parameters**
- **DS**: 500.00 MHz
- **P1**: 20.00 Hz
- **P2**: 50.00 Hz
- **P3**: 100.00 Hz
- **P4**: 200.00 Hz
- **P5**: 500.00 Hz
STANDARD CARBON PARAMETERS

Pulse Sequence: 1p1p1
Solvent: DMSO
Temperature: 298 K
Field: 399.81 MHz
Mode: NMR

Pulse train: 4, 4, 4
Front time: 1.256us
Back time: 1.256us
Reps: 65

Decoupler: 14.9979489 MHz
Power: 12.0 W
Gain: 22 dB

/data processing
Gauss window 0.039

/FT
Total time: 19 min, 1 sec

STANDARD PROTON PARAMETERS

Pulse Sequence: gcosy
Solvent: DMSO
Temperature: 298 K
Field: 399.81 MHz

Pulse train: 2.299us
Front time: 1.256us
Back time: 1.256us
2D width: 2291.1 Hz
Spin rate: 133.3 Hz
Gauss window: 0.039

/FT
Total time: 19 min, 1 sec
STANDARD PROTON PARAMETERS
Pulse Sequence: cyclohexane
Solvent: DMSO
Ambient Temperature
FID: #34306654-000
Delay: 2056.9 sec
Acq. time: 3.020 sec
WIF: 5000.0 Hz
Data Processing
DOSY: 1.0 Hz
Total time 17 min, 6 sec

STANDARD PROTON PARAMETERS
Pulse Sequence: cyclohexane
Solvent: DMSO
Ambient Temperature
FID: #34306654-000
Delay: 2056.9 sec
Acq. time: 3.020 sec
WIF: 5000.0 Hz
Data Processing
DOSY: 1.0 Hz
Total time 17 min, 6 sec
**STANDARD PROTON PARAMETERS**

**Pulse Sequence:** zgad

**Solvent:** DMSO

**Ambient temperature:** 298 K

**Relaxation delay:** 1.000 sec

**Data matrix:** 2048 x 2048

**F1 resolution:** 1000 Hz

**F2 resolution:** 1000 Hz

**Data Processing:**

**F1:** Zero-filling 1x

**F2:** Zero-filling 4x

**Total time:** 4 min 28 sec

---

**STANDARD PROTON PARAMETERS**

**Pulse Sequence:** zgad

**Solvent:** DMSO

**Temp:** 298 K

**Gradient:** 1000 Hz

**Relaxation delay:** 1.000 sec

**Data matrix:** 2048 x 2048

**F1 resolution:** 1000 Hz

**F2 resolution:** 1000 Hz

**Data Processing:**

**F1:** Zero-filling 1x

**F2:** Zero-filling 4x

**Total time:** 4 min 28 sec
Standard Proton Parameters

Pulse Sequence: gndy
Solvent: DMSO
Temperature: 298K
Dilution: "none-what"

Relax. delay: 1.0 sec
Avg. time 0.250 sec
FID Acquisition 1024
2964 ppm for 11 ppm
Single scan
32 increments
Delay before sweep
0.034 sec
0.202 sec
300 KHz
Total time 2.5 min, 10 sec

![Chemical Structure](image)

![2D NMR Spectrum](image)