Chalcone-based chemical probes as versatile tools for potential target identification and mechanism elucidation of chalcone’s bioactivities

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you.” — 1 Thessalonians 5:16-18 (ESV)
Abstract

Chalcone is an important family of plant-derived compounds. For decades it has attracted great research interest in the field of medicinal chemistry, partly due to its rich natural source, easy synthetic availability, and diverse biological activities. The well-documented biological activities of chalcones include anti-cancer, anti-inflammatory, anti-diabetic, cancer chemopreventive, anti-oxidant, and anti-microbial activities. Numerous studies have attempted to elucidate the mechanisms of action and target interactions responsible for these biological activities. Many putative molecular targets have been reported, which are reviewed in this thesis (Chapter 1). However, lack of evidence of direct target interaction in cells and their biological relevance presents an unmet challenge for chalcone’s mechanistic investigation and optimization. Given the diversity of various putative molecular targets, it is also debated whether chalcone is a privileged or promiscuous template for biological interaction. Therefore, further understanding and improvement of chalcone’s biological activities as well as its translational development are largely dependent on a better understanding of chalcone’s interaction with its direct cellular targets.

To help address this, in this thesis, various chemical probe approaches have been explored to advance the current understanding of chalcone’s target interactions. Specifically, we designed a panel of chalcone-based fluorescent probes and photoaffinity probes (Chapter 2) with varied cytotoxic potencies to study the direct cellular targets responsible for chalcone’s cytotoxicity, which was unambiguously revealed as β-tubulin.
(Chapters 3-5). Besides, we developed a combinatorial synthetic method for the efficient construction of a focused library of chalcone-based photoaffinity probes, and studied their cellular target interaction profiles (Chapter 6), where several novel potential targets were revealed. Overall, our results showcase the usefulness of chemical probe approaches, particularly photoaffinity probes, in the target investigation of chalcone-based compound. These results strongly suggest that chalcone has the potential to be developed as a privileged template for biological interactions, and encourage future research endeavors to further study and optimize chalcone’s various biological activities.
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CHAPTER 1

Introduction – Potential Diverse Molecular Targets of Chalcones

1.1 Chalcones and their diverse biological activities

Chalcone, or (E)-1,3-diphenyl-2-propene-1-one, is an important chemotype that has attracted great research interest for decades due to the abundant natural chalcone-based compounds, the easy synthesis and derivatization, and most importantly, the diverse biological activities of various chalcone-based compounds. The structure scaffold of chalcone contains two phenyl rings connected via a propenone bridge, and the commonly used nomenclature of the two rings and the numbering system is shown in Figure 1A, which is used throughout this thesis. Many natural chalcones isolated from biologically active plants demonstrated a wide variety of bioactivities, including anti-cancer, anti-inflammatory, anti-diabetic, cancer chemopreventive, anti-oxidant, and anti-microbial activities (Figure 1.1.a).\(^{(1-3)}\) In many cases, a single compound can exhibit several bioactivities. For example, xanthohumol revealed anti-cancer, cancer chemopreventive, anti-oxidant, and anti-inflammatory activities; isoliquiritigenin showed anti-inflammatory, anxiolytic, and anti-pigmentation activities; while isobavachalcone demonstrated chemopreventive, anti-cancer, anti-bacterial and anti-fungal activities.\(^{(1)}\) Several chalcone-based compounds have been marketed or clinically tested for various health conditions (e.g. metochalcone – choleric/diuretic; sofalcone – anti-ulcer/mucoprotective; and hesperidin methylchalcone – vascular protective), exemplifying the clinical potential of chalcones (Figure 1.1.b).\(^{(2)}\)
Figure 1.1. (a) Structure scaffold of chalcone and examples of bioactivities; and (b) examples of chalcones that have been marketed or clinically tested.

The wide bioactivity spectrum of chalcones, on the other hand, also presents a challenge for their clinical development as they may have a promiscuous target profile. Therefore, understanding the mechanisms and direct molecular targets is particularly important for the future development of clinically useful chalcone compounds. There have been a great number of reports attempting to reveal potential mechanisms responsible for chalcone’s various bioactivities. Some of such mechanistic knowledge has been extensively reviewed, including anti-cancer,$^{4-9}$ anti-inflammatory,$^{9-11}$ and anti-diabetic activities.$^{12}$ A diverse panel of potential direct molecular targets have also been reported, with varied levels of experimental evidence, which was recently reviewed by our group.$^{13}$ Such knowledge is briefly summarized herein to illustrate the current limited understanding of chalcone’s potential direct molecular targets.
1.2 Potential direct cellular targets of chalcones in literature

1.2.1 Microtubules

Microtubules are the key component of mitotic spindles during cell proliferation. It is formed by heterodimers of α- and β-tubulin through a highly dynamic polymerization-depolymerization process. Anti-microtubule agents, which interact with microtubules and disturb such a dynamic process, typically cause cell cycle arrest. Anti-microtubule agents therefore are potential anticancer agents, among which are paclitaxel, vinblastine, and epothilone-based ixabepilone that have been used clinically to treat a variety of malignancies.\(^{14, 15}\)

A number of chalcones have been demonstrated to directly interact with microtubules, which may be responsible for their cytotoxicity. The earliest example is synthetic cytotoxic chalcone MDL-27048\(^{16, 17}\), which was found to rapidly and reversibly bind to purified tubulin, inhibit tubulin polymerization into microtubules, and disrupt microtubule structure and arrest mitosis in vitro. Binding kinetics was studied taking advantage of changes in spectroscopic properties (absorption and fluorescence) upon tubulin binding, which revealed MDL-27048 as a single-site ligand of tubulin with a binding affinity of \(2.8 \times 10^6 \text{ M}^{-1}\). Classical colchicine binding site ligand podophyllotoxin and MTC competed with MDL-27048 for tubulin binding, suggesting that colchicine binding site was involved in the binding interaction. In combination with other colchicine
binding-site anti-microtubule agents, a 5D-QSAR study\(^{18}\) was performed and concluded that the methyl substitution on the \(\alpha\)-alkene position induced a significant change in the preferred conformation from s-cis to s-trans that favored tubulin binding, which potentially explained the higher potency observed with \(\alpha\)-methyl chalcones. Similarly, \(\alpha\)-methyl chalcone compound 1\(^{(19)}\) was designed and exhibited anti-microtubule activity biochemically and potent cytotoxicity in vitro through disrupting microtubule structure and inducing cell cycle arrest. The binding interaction of MDL-27048 with tubulin was modeled by molecular docking\(^{(20)}\) and revealed an adjacent pocket unoccupied, based on which compound 2 was designed and exhibited micromolar potency of tubulin polymerization inhibition and cancer cell growth. A natural cytotoxic chalcone compound pedicin\(^{(21)}\) was isolated from Fissistigma lanuginosum, which inhibited tubulin polymerization biochemically with an IC\(_{50}\) of 300 \(\mu\)M. Synthetic chalcone 3\(^{(22)}\) from a panel of chalcones developed as potential anti-microtubule agent showed micromolar potency of tubulin inhibition and cytotoxicity against a panel of cancer cell lines. Two closely related cytotoxic chalcones containing an indole moiety, namely JAI51\(^{(23)}\) and IPP51\(^{(24)}\), were synthesized and both compounds inhibited tubulin polymerization biochemically and demonstrated \textit{in vivo} anticancer activity in xenograft mice models. In addition, competition between IPP51 and colchicine was observed, further suggesting the involvement of colchicine-binding site in the binding interaction. A series of dihalogenated chalcones and related dienones\(^{(25)}\) were synthesized and interestingly, among several microtubule inhibitors, compound 4 proved to be a microtubule stabilizer which fell in the
same category as paclitaxel. Similarly, chalcones 5-10\(^{(26-31)}\) were developed as anti-microtubule agents and showed cytotoxicity against tumor cell lines via cell cycle arrest. In addition, compound 6 inhibited tumor cell migration\(^{(27)}\), another microtubule-related activity, and compound 8-9 demonstrated in vivo antitumor activity in xenograft models\(^{(28,30)}\). Other anticancer pharmacophores have also been fused with the chalcone scaffold and yielded several novel anti-microtubule agents. A panel of chalcones fused with a pyran ring were designed to mimic cytotoxic natural product millepachine\(^{(32-34)}\), among which compound 11 showed the highest cytotoxicity towards several cancer cells. Compound 12\(^{(35)}\) was designed by incorporating a resveratrol moiety into chalcone scaffold, and similarly, compound 13\(^{(36)}\) and 14\(^{(37)}\) were designed by incorporating either an amidobenzothiazole or a phenstatin moiety into chalcone core. All of these compounds were shown to be anti-microtubule agents that exhibited cytotoxicity against various cancer cell lines. Many of these compounds have been demonstrated to induce cell cycle arrest, which is the typical outcome of microtubule disruption by anti-microtubule agents.
1.2.2 Kinases

Numerous literature reports have shown the potential of chalcone-based compounds to regulate kinase activities. Such regulations, however, do not necessarily indicate direct interactions with kinases, as they could also result from altered kinase levels or activation status. Nevertheless, several enzymes have been shown to directly bind to and/or be inhibited by chalcones in chemical assays.

1.2.2.1 IKKs

IκB kinases (IKKs) are key regulators of the NF-κB signaling pathway which plays an important role in cell response to various stimuli such as TNF, IL-1, UV radiation, stress,
and pathogenic assaults. The activated IKKs phosphorylate IkB which leads to nuclear translocation of NF-κB to activate downstream transcription of target genes. Inhibiting IKKs is therefore considered a promising approach for intervening NF-κB related diseases, especially cancer and inflammatory diseases.\(^{38, 39}\) Three anti-cancer and anti-inflammatory natural chalcones, namely butein\(^{40}\), licochalcone A\(^{41}\), and xanthohumol\(^{42}\), were found to directly inhibited IKKβ activity both biochemically and in cells, and subsequently reduced the downstream products of NF-κB activation, resulting in elevated apoptosis induced by TNF and other chemotherapeutic agents. In addition, cysteine 179 of IKKβ was found to be crucial to this inhibition, suggesting that a covalent Michael addition-type interaction of the α,β-unsaturated ketone moiety of chalcones with IKKβ at this residue might be involved (Figure 1.3.b). Synthetically, a series of adamantyl chalcones\(^{43-46}\) were developed as cytotoxic agents, many of which, such as compound 15, were found to inhibit IKKα and IKKβ both biochemically and in cells, and the inhibitory activity correlated with their in vitro cytotoxicity.

1.2.2.2 Aurora kinases

Aurora kinases are key regulators of mitosis whose aberrant expression is found in various types of cancer. Aurora A phosphorylates Polo like kinase 1 (PLK1) which then phosphorylates Cdc25C and Wee1 and subsequently activates cyclin B-CDK1 complexes to promote mitotic entry, whereas Aurora B is critical for microtubule-kinetochore attachments, the establishment of the spindle assembly checkpoint and cytokinesis.\(^{47}\) A
natural chalcone xanthoangelol isolated from Psoralea corilifolia\(^{(48)}\) was found to inhibit both Aurora A and B kinases with micromolar potency and induced apoptotic cell death in cancer cell lines. Synthetically, a chromenyl chalcone 16\(^{(49)}\) which demonstrated cytotoxicity against cancer cell lines and inhibited colony formation, was found to inhibit both Aurora A and B kinases with no effect on other kinases. Similarly, a series of chalcones were developed as cytotoxic compounds against ovarian cancer cells, where the most potent compound 17\(^{(50)}\) was found to be an Aurora A inhibitor.

1.2.2.3 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs), such as VEGFR, EGFR, FGFR, and many others, are cell signaling effectors that regulate normal development and homeostasis, whose aberrant activities are responsible for many pathologies, especially cancers. Many small molecule inhibitors of RTKs are clinical targeted cancer therapies.\(^{(51, 52)}\) Natural chalcone xanthoangelol\(^{(48)}\) inhibited not only the Aurora kinases as mentioned above but also EGFR activity in enzymatic assays, which may contribute to its cytotoxicity. Similarly, natural cytotoxic chalcones butein\(^{(53, 54)}\) and isoliquiritigenin\(^{(55)}\) also demonstrated inhibitory activity of EGFR in an ATP-competitive manner, suggesting involvement of the ATP binding site in their interactions. Isoliquiritigenin inhibited not only wild-type EGFR, but also a mutant EGFR prevalent in non-small-cell lung cancer\(^{(55)}\) as well as VEGFR2\(^{(56)}\). Correspondingly, xenograft study revealed its \textit{in vivo} anticancer efficacy against cancer cells expressing mutant EGFR as well as inhibition of endothelial
cell growth and angiogenesis induced by VEGF. The simple unsubstituted chalcone \(^{(57)}\) was also found to inhibit VEGFR in biochemical assays, and suppress growth and angiogenesis in a xenograft model. Interestingly, many other RTKs besides VEGFR were also found to be inhibited by unsubstituted chalcone, such as EphB2, FGFR3, MSPR, and others. \(^{(57)}\)

1.2.2.4 Other kinases

Natural cytotoxic chalcone isobavachalcone \(^{(58)}\) was found to be an inhibitor both biochemically and in cells of the Akt kinase, a key effector in the PI3K/Akt signaling pathway regulating cell proliferation and growth and hence a promising anticancer target. Natural anti-inflammatory chalcone licochalcone A \(^{(59)}\) was found to suppress UV-induced COX-2 expression, an important pro-inflammatory enzyme, through direct binding and inhibition of kinases PI3K, MEK1, and B-Raf. The direct binding study was performed with bead-immobilized compound and revealed that ATP competed for binding of licochalcone A to PI3K and B-Raf, but not MEK1, suggesting different binding sites/modes with different kinases. The mechanisms by which natural chalcone broussochalcone A \(^{(60)}\) attenuates respiratory burst in stimulated neutrophils was studied, where protein kinase C (PKC) activity was found to be directly inhibited by broussochalcone A, and the interaction was shown to be at the catalytic domains instead of the regulatory domains.
Figure 1.3. (a) Structures of chalcone-based inhibitors of various kinases, and (b) proposed mechanism of IKKβ inhibition by chalcones through Michael addition-based covalent modification.

1.2.3 Oxidoreductases

1.2.3.1 Cyclooxygenases and 5-lipoxygenase

Cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) are key enzymes in the arachidonic acid (AA) metabolic pathway, whose end products are inflammatory mediators such as prostaglandins (PGs), leukotrienes (LTs), and thromboxanes (TXs) that are highly involved in the pathology of various inflammatory diseases and cancers. Therefore, COX and 5-LOX have long been considered promising drug targets for these diseases.\textsuperscript{61, 62}
chalcone scaffold has been widely explored as a pharmacophore for inhibition of COX and 5-LOX. A series of 3,4-dihydroxy chalcones\(^{(63)}\) were evaluated for \textit{in vivo} anti-inflammation efficacy using AA-induced mouse ear edema model, and the most potent compound 18 was found to be a potent 5-LOX inhibitor with nanomolar potency and moderate COX inhibitor with micromolar potency. Evaluation of several natural compounds for their inhibitory activity against AA-induced platelet aggregation revealed broussochalcone A\(^{(64)}\) as the most potent compound, which correlated with its COX inhibitory activity. As an expansion of chalcone’s anti-inflammatory scope by inhibiting COX, synthetic chalcone 19\(^{(65)}\) which was a COX inhibitor potently inhibited AA- or adrenaline-induced platelet aggregation, compound 48/80-induced mast cell degranulation, and peptide fMLP-induced neutrophil activation. Compound 20\(^{(66)}\) from a panel of 2-chloroquinolinyl chalcones was found to inhibit both 5-LOX and COX with \textit{in vivo} inhibitory activity of PGE\(_2\) and LTB\(_4\) production by stimulated leukocytes. Natural chalcone xanthohumol\(^{(67)}\) was found to inhibit both isotypes of COX with higher potency against COX-1 than COX-2, however, no further biological relevance was reported. A series of phenylsulphonyl urenyl chalcones\(^{(68)}\) were developed as dual inhibitor of COX-2 and 5-LOX, and the most potent inhibitor 21 demonstrated potent inhibition of PGE\(_2\) and LTB\(_4\) production in cell-based experiments. A para-methylsulphonyl group was introduced to a set of chalcones\(^{(69)}\) as it frequently appears in the structure of COX-2 inhibitors, and the most potent inhibitor 22 showed a more than 100-fold selectivity towards COX-2 than COX-1 without further biological study. Compound 23\(^{(70)}\) from a set of 2'-
hydroxychalcones showed inhibitory activity on LPS-induced cell-based PGE2 production, and molecular docking results suggested potential COX-2 inhibition without further biochemical confirmation. By incorporation of an N-arylpyrazole moiety\(^{(71)}\), the major pharmacophore in celecoxib, into a bis-chalcone scaffold, several selective COX-2 inhibitors were obtained with *in vivo* edema inhibition activity, where compound 24 was the most potent and selective inhibitor with activity profiles comparable to that of celecoxib. Synthetic chalcone 25\(^{(72)}\) inhibited both isotypes of COX as well as 5-LOX in enzymatic assays. Compound 26\(^{(73)}\) among several other heterocyclic chalcones exhibiting cell-based inhibitory activity against AA- and collagen-induced platelet aggregation and TXB2 formation also inhibited COX-1 in enzymatic assays. Compound 27\(^{(74)}\) among a series of naphthyl chalcone derivatives was found to be a non-selective COX inhibitor with micromolar potency. Finally, compound 28\(^{(75)}\) among a series of indole-chalcones was found to be a COX inhibitor with *in vivo* anti-inflammation activity.
1.2.3.2 Tyrosinase

Tyrosinase is the primary enzyme catalyzing the multi-step oxidation of tyrosine in the production of melanin, the skin pigment. Increased tyrosinase activity can cause various types of hyperpigmentation disorders, hence an optimal drug target for such conditions.\(^{76}\) Natural chalcone isoliquiritigenin\(^{77}\) was demonstrated to be a tyrosinase inhibitor both biochemically and in melanocytes. Further studies \(^{78, 79}\) identified butein and a few other chalcones as tyrosinase inhibitors with comparable potency. Interestingly, butein itself was found to be a substrate of tyrosinase and the \(o\)-quinone product was detected with LC-MS. Compound \(29\(^{80}\)\) among a set of 2’,4’,6’-trihydroxychalcones was found to be a tyrosinase inhibitor in a substrate-competitive manner, indicating the potential binding site of chalcone-based tyrosinase inhibitors. Natural chalcone \(30\(^{81}\)\) was found to competitively inhibit tyrosinase activity biochemically and suppress melanin biosynthesis in cells. Several synthetic sulfonylamino chalcones\(^{82}\) were tested for their anti-pigmentary effect,
where compound 31 was demonstrated as the most potent inhibitor of melanin formation in cell-based experiment, although it wasn’t the most potent in tyrosinase enzymatic assay. Part of its cell-based activity may be attributed to reduced protein level of tyrosinase as revealed by Western blotting analysis. Moreover, in vivo activity of compound 31 was demonstrated by the inhibition of UV-induced skin pigmentation in brown guinea pigs.

**Figure 1.5.** Structures of chalcone-based tyrosinase inhibitors.

1.2.3.3 Sex hormone converting enzymes

The sex hormones as important signaling messengers play major roles in the functions of reproductive organs, and their dysregulation is highly associated with diseases such as breast cancer and prostate cancer.\(^{(83, 84)}\) Enzymes involved in the biosynthesis of sex hormones are thus potential drug targets for intervention of such diseases. Chalcones have shown the potential to inhibit some of these enzymes, such as aromatase which is involved in estrogen synthesis, and 5α-reductase and 17β-hydroxysteroid dehydrogenase (17β-HSD) for androgen synthesis. A series of natural and synthetic chalcones\(^{(85)}\) were evaluated for aromatase and 17β-HSD inhibitory activity in biochemical assays, where naringenin chalcone was found to be the most potent aromatase inhibitor, and 4-hydroxychalcone the most potent 17-HSD inhibitor, without further biological evaluation.
Compound 32\(^{(86)}\) among other natural products isolated from *Broussonetia papyrifera* was identified as an aromatase inhibitor with sub-micromolar potency. Five chalcones\(^{(87)}\) were screened for aromatase inhibitory activity in MCF-7aro breast cancer cells and biochemically, where the most potent compound butein also suppressed testosterone-induced MCF-7aro cell growth, which was synergistically enhanced by co-treatment with estrogen receptor antagonist ICI-182780. Similarly, natural chalcone xanthohumol\(^{(88)}\) was identified as an aromatase inhibitor in a cell-based assay, whose anti-proliferative effect was reverted by addition of estradiol. Natural chalcone 33\(^{(89)}\) and β-hydroxyl chalcone 34\(^{(90)}\) were found to be a 5α-reductase inhibitor in biochemical assays, but further biological study is unavailable.

![Figure 1.6. Structures of chalcone-based inhibitors of sex hormone converting enzymes.](image)

1.2.3.4 Aldose reductase

Aldose reductase (ALR2) catalyzes the conversion of glucose to sorbitol, the first step in polyol pathway of glucose metabolism. Increased flux of glucose through polyol pathway under conditions such as diabetes initiates a complex cascade leading to oxidative
stress and inflammation, which renders ALR2 a promising drug target for intervention of inflammatory conditions, particularly the chronic complications associated with diabetes.\textsuperscript{(91, 92)} Natural chalcones isoliquiritin and isoliquiritigenin\textsuperscript{(93, 94)} were found to potently inhibited ALR2 activity biochemically. Furthermore, \textit{in vivo} study showed that isoliquiritigenin significantly inhibited sorbitol accumulation in red blood cells, sciatic nerves, and lenses in diabetic rats. SAR study of isoliquiritigenin\textsuperscript{(95)} revealed the requirement of the three hydroxyl groups for potency. Natural chalcones kuraridin\textsuperscript{(96)} and butein\textsuperscript{(97)} also exhibited ALR2 inhibitory activity, and butein also exhibited inhibitory activity against sorbitol accumulation \textit{in vivo}. Synthetic analogs of isoliquiritigenin 35\textsuperscript{(98)} and 36\textsuperscript{(99)} are equally potent ALR2 inhibitors as isoliquiritigenin, but no further biological investigation was reported. By introducing an additional hydroxyl group, a nanomolar inhibitor 37\textsuperscript{(100)} was obtained, which was 100-fold more potent than isoliquiritigenin.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Structures of chalcone-based ALR2 inhibitors.}
\end{figure}

1.2.3.5 Thioredoxin reductase
Thioredoxin system, consisting of thioredoxin reductase (TrxR), its substrate thioredoxin (Trx), and cofactor NADPH, is one of the major biological antioxidant systems which play crucial roles in maintaining cellular redox homeostasis and regulate various cellular processes. Overexpression of TrxR has been observed in many tumors, rendering it a potential target for chemotherapy.\(^{(101,102)}\) A panel of Michael acceptor-type pharmacophores were developed for TrxR inhibitory activity\(^{(103)}\), among which chalcones (such as 38) and closely related 1,5-diphenyl-pent-1-en-3-ones (DPPen) proved to be potential inhibitors of cellular TrxR activity. The good correlation between TrxR inhibitory potency and cytotoxicity within their library indicated involvement of TrxR inhibition in the cytotoxic mechanisms of these pharmacophores. Interestingly, the covalent adduct of the most potent DPPen analog with TrxR at selenocysteine residue U498 was observed with MS analysis, giving insights into potential interaction modes of chalcones with TrxR. Compound 39\(^{(104)}\) was identified from 44 synthetic chalcones as a TrxR inhibitor selective over other oxidoreductases such as glutathione reductase and glutathione peroxidase, and U498 residue was involved in the interaction. In cells, 39 significantly decreased cellular thiol level and increased ROS level as a result of TrxR inhibition. The cytotoxicity of 39 was attenuated by antioxidant N-acetylcysteine, and enhanced by GSH synthesis inhibitor BSO, further supporting the important role of TrxR inhibition in the cytotoxic mechanism of 39. However, this could also be possibly due to non-specific Michael addition with thiols, which deserves further investigation.
1.2.3.6 Monoamine oxidase

Monoamine oxidases (MAOs) are important metabolic enzymes catalyzing the oxidative degradation of monoamine neurotransmitters. Elevated activities of MAOs can lead to reduced cognitive ability and ROS accumulation, and is therefore associated with depression and neurodegenerative diseases, rendering MAOs important drug targets for such conditions.\(^{(105)}\) Early study with natural products from *Glycyrrhiza uralensis* roots\(^{(106)}\) identified several chalcones, such as compound 40, as competitive inhibitors of mitochondrial MAO of rat liver. Compound 41\(^{(107)}\) was identified from screening sixteen synthetic chalcones against MAO-A and MAO-B as a selective MAO-B inhibitor, four times more potent than clinically used MAO-B inhibitor selegiline, with 10,000-fold selectivity over MAO-A. The inhibition was irreversible as removing compound from enzyme did not result in recovery of enzymatic activity, suggesting potential covalent modification. A panel of furano-chalcones\(^{(108)}\) were also developed as selective MAO-B inhibitor in a competitive manner. Interestingly, inhibition of the most potent compound 42 was reversible, contrary to the previous study. However, no further biological study was performed to investigate the potential contribution of MAO inhibition to chalcone’s bioactivities.
1.2.4 Hydrolases

1.2.4.1 Proteases

a. Cathepsins

Cathepsins are cysteine proteases mainly involved in the bulk protein degradation within lysosomes. Recent findings have shed light on their involvement in many pathological processes, including cancer, arthritis, and atherosclerosis. High cathepsins activity is associated with cancer progression, invasion, and metathesis, thus making them potential drug targets for intervention. A panel of synthetic chalcones were developed as cytotoxic compounds and cathepsin inhibitors, and the most cytotoxic compound 43 was found to be the most potent inhibitor of cathepsin B and L with micromolar potency. Similarly, compound 44 was identified as a cytotoxic compound with cathepsin K inhibitory activity. SAR was studied with 27 synthetic flavonoids for cathepsin B and H inhibitory activity, where 2’-hydroxychalcones showed better potency than flavones and flavanones bearing the same substituents, and 4-nitro substitution was found to possess the best potency, rendering compound 45 as the most potent inhibitor within...
their library. The inhibition kinetics was also studied, revealing a competitive mode of inhibition which suggested potential binding at the catalytic site.

b. β-Secretase

β-Secretase (BACE1) catalyzes the cleavage of β-amyloid precursor protein to produce neurotoxic peptide β-amyloid, whose aggregation is an important pathological hallmark for Alzheimer’s disease (AD). Therefore, development of BACE1 inhibitors has become an attractive anti-AD strategy.\(^ {113} \) Natural products from cognition enhancing herb *Glycyrrhiza uralensis* was screened for BACE1 inhibitory activity\(^ {114} \) where isoliquiritigenin was identified as a moderate BACE1 inhibitor. In an attempt to improve the potency, a 22-membered library was constructed through structural derivatization, among which compound 46 was the most potent inhibitor with sub-micromolar potency. Similar potency was also achieved with sulfonamide chalcone 47\(^ {115} \), and its inhibitory kinetics revealed a reversible, mixed-type mode of action. No further biological investigation was performed.

![Image of structures](image)

**Figure 1.9.** Structures of chalcone-based inhibitors of proteases.
1.2.4.2 Esterase

a. Histone deacetylases

Histone deacetylases (HDACs) are epigenetic enzymes catalyzing the deacetylation of histone, which in turn regulates the transcription of various genes. Aberrant expression/function of different isoforms of HDACs is involved in the pathology of many diseases, especially cancer, neurological diseases, and immune disorders.\(^{116,117}\) HDAC inhibitors and especially isoform specific inhibitors are therefore potential drug candidates for these diseases. Twenty-one natural chalcones were screened for HDAC inhibitory activity\(^ {118}\), where butein was identified as a moderate HDAC inhibitor. Computational strategies were applied to design selective HDAC2 inhibitors\(^ {119}\) based on structures and mechanisms of different isoforms of HDACs, where \(\beta\)-hydroxymethyl chalcone 48 was identified as a selective HDAC2 inhibitor over HDAC1 and HDAC3. A panel of flavonoids was screened for inhibition of the deacetylase activity of SIRT1\(^ {120}\), a class III HDAC whose substrates include tumor suppressor protein p53, thus a potential anticancer target. Chalcone 49 was identified to inhibit SIRT1 and as a consequence increase the acetylated p53 level in cells and inhibit cell proliferation.

Figure 1.10. Structures of chalcone-based HDAC inhibitors.
b. Cholinesterases

Cholinesterases, including AChE and BChE, are esterases catalyzing the hydrolytic breakdown of neurotransmitter acetylcholine, and are hence involved in various neurological disorders, especially Alzheimer’s disease. Inhibitors of cholinesterases have been widely used in clinic for symptomatic treatment of AD.\textsuperscript{121,122} The study performed by Kang JE \textit{et al}\textsuperscript{115} as mentioned above with sulfonamide chalcones identified compound 47 not only as a potent BACE1 inhibitor, but also a moderate AChE/BChE inhibitor, further indicating its potential anti-AD effect. Synthetic chalcone 50\textsuperscript{123} was identified as an AChE/BChE inhibitor with micromolar potency. Starting from 4-hydroxychalcone and guided by computational docking results, compound 51\textsuperscript{124} was designed by installing a tertiary amine substituent to form a cation-π interaction with Trp84 residue. It was >100-fold more potent in AChE inhibition than the parent compound and also inhibited BChE with lower potency. Kinetic study revealed it as a mixed-type inhibitor of both enzymes. Further study\textsuperscript{125} suggested that introduction of a fluorine atom resulted in enhanced potency, where the most potent compound 52 was 20-fold more potent than 51. However, no further biological study was reported for any of these compounds.

\textbf{Figure 1.11.} Structures of chalcone-based AChE/BChE inhibitors.
c. Protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPs) catalyze the hydrolytic dephosphorylation of phosphotyrosine residues of various protein substrates. Chalcones have shown potential interaction with at least two members of this enzyme family, namely PTP1B and CDC25B. PTP1B dephosphorylates and thereby inactivates insulin receptor kinase, and as a result elevated PTP1B activity is highly associated with decreased insulin sensitivity leading to diabetes and obesity.\(^{(126)}\) CDC25B, on the other hand, is a mitotic regulator by dephosphorylating cyclin dependent kinases (CDKs) whose activity is essential for the G2/M transition in human cells, and thus a potential anticancer target.\(^{(127)}\) Natural chalcones broussochalcone A\(^{(128)}\) and licochalcone A\(^{(129)}\) were identified among a panel of natural products as moderate PTP1B inhibitors. Semisynthetic licochalcone A derivative 53\(^{(129)}\) exhibited 2-fold improved potency over parent compound licochalcone A. Natural chalcone 54\(^{(130)}\) isolated from Angelica keiskei was found to inhibit PTP1B with submicromolar potency in a competitive manner against the model substrate pNPP. 

Synthetically, a set of 2’-hydroxy-4’-isoprenyloxychalcones\(^{(131)}\) was screened for CDC25B inhibitory activity, where compound 55 was identified as the most potent analog. Compound 56\(^{(132)}\) was identified from a series of 2’,4’,6’-trihydroxychalcones as a submicromolar inhibitor of both PTP1B and CDC25B in a competitive manner against pNPP. Similarly, compound 57\(^{(133)}\) was identified from several 2’,4’-dihydroxychalcones as an inhibitor of both CDC25B and PTP1B. This compound also exhibited micromolar
inhibitory activity against cancer cell proliferation as well as in vivo tumor growth inhibition, which might be associated with CDC25B inhibition.

**Figure 1.12.** Structures of chalcone-based PTP inhibitors.

d. Secreted phospholipase A2

Secreted phospholipases A2 (sPLA2) are extracellular enzymes catalyzing the hydrolytic release of fatty acids, including inflammatory intermediate arachidonic acid, from the 2-position of phosphoglycerides. Given its important role in inflammation as the enzyme catalyzing the first step of the arachidonic pathway, sPLA2 has long been considered a promising target for treatment of many inflammation-related diseases.\(^{134}\)

Two studies mentioned above\(^ {72, 74}\) identified not only synthetic chalcones as COX inhibitors, but also moderate sPLA2 inhibitors such as compounds 58 and 59. In addition, chalcone 60 was identified\(^ {135}\) as a sPLA2 inhibitor with cell-based anti-inflammatory activity through inhibiting the production of LTB\(_4\), TXB\(_2\), and elastase by stimulated neutrophils.
Figure 1.13. Structures of chalcone-based sPLA2 inhibitors.

1.2.4.3 α-Glucosidase

α-Glucosidase is an intestinal enzyme to release α-glucose from dietary polysaccharide for glucose absorption, and hence a valid anti-diabetic target on which commercial drugs such as acarbose and miglitol act.\(^{136}\) Chalcones have been shown to possess potential inhibitory activity against α-glucosidase. Natural chalcones kuraridin\(^{137}\) and xanthohumol\(^{138}\) were identified as moderate inhibitors of α-glucosidase, both in a non-competitive manner. Interestingly, interaction with xanthohumol resulted in quenched intrinsic fluorescence, reduced hydrophobicity, and changes in CD spectrum of α-glucosidase, all of which suggested a conformational change which may explain the non-competitive inhibition. Synthetically, an aforementioned study\(^{123}\) not only identified AChE/BChE inhibitory chalcones but also moderate inhibitors of α-glucosidase, among which chalcone 61 showed the best potency. Compound 62\(^{139}\) was identified from a series of chalcone triazoles as a moderate α-glucosidase inhibitors. Introduction of a sulfonamide group was found to significantly increase chalcone’s inhibitory activity against α-glucosidase\(^{140}\), and compound 47 which showed BACE1 inhibitory activity as mentioned above was also the most potent chalcone-based α-glucosidase so far with sub-micromolar potency.
1.2.5 Microbial enzymes

Quite a number of chalcone-based compounds have shown antimicrobial activity against a spectrum of pathogens including bacteria, fungi, parasites, and viruses.\(^1\) Various potential direct molecular targets have been suggested to account for such activities. Several studies\(^{141-143}\) discovered chalcone-based inhibitors of the bacterial enzyme MtbPtpA and MtbPtpB, which are protein tyrosine phosphatases secreted by *Mycobacterium tuberculosis* into host cells that attenuate host immune defenses by interfering with host cell signaling pathways. Compound 63 was identified as the most potent MtbPtpA inhibitor, whereas compound 64 was the most potent inhibitor of MtbPtpB. However, no data for their anti-tuberculosis activity is available. Another bacterial tyrosine phosphatase critical for Yersinia pathogenesis, YopH, was found to be inhibited by several synthetic chalcones such as 65\(^{144}\). A screening was performed with an NCI antiviral compound library for HIV-1 integrase inhibitory activity\(^{145}\) where
chalcone 66 was identified as the most potent inhibitor which also exhibited *in vitro* anti-HIV activity. Compound 67\(^{(146)}\) was designed by incorporating a 3-keto salicylic acid moiety targeting HIV-1 integrase inhibition, and it showed higher potency than 66 in both enzymatic inhibition and *in vitro* anti-HIV assay. Compound 68\(^{(147)}\) was discovered as a potent inhibitor of Pfmrk, a cyclin-dependent kinase critical for the cell cycle of *Plasmodium falciparum* and thus an attractive target for malaria. However, the weak correlation between antimalarial activity and Pfmrk inhibition suggests the potential involvement of other mechanisms. A chemical probe approach was applied to study the antibacterial target of the natural product 4-hydroxyderricin\(^{(148)}\), where probe 69 with similar antibacterial activity as 4-hydroxyderricin covalently pulled out seryl-tRNA synthase (STS) from the lysate of *S. aureus*, which was further confirmed by enzymatic inhibition assay, overall providing compelling evidence that STS is the direct target responsible for the antibacterial activity of 69. With a similar approach, simple unsubstituted chalcone\(^{(149)}\) was found to be a covalent inhibitor of Sortase A (SrtA), a bacterial transpeptidase involved in host cell attachment. The covalent labeling of SrtA by chalcone was revealed by mass spectrometry, and confirmed with biochemical enzymatic inhibition assay as well as biofilm formation inhibition assay with *Staphylococcus aureus*, overall convincingly suggesting SrtA being the direct target. Compound 70\(^{(150)}\) was designed by introducing a quinoline moiety, a well-known pharmacophore for bacterial DNA gyrase inhibition, onto chalcone scaffold, and it inhibited DNA gyrase and exerted antibacterial activity against several strains of bacteria.
1.2.6 Receptors

1.2.6.1 Retinoic acid receptors

Retinoic acid receptors (RARs) are nuclear receptors that function as ligand-dependent transcription factors and thereby play important roles in various physiological as well as pathological processes including cancer and inflammation. Although much cell-based evidence exists in literature for chalcone’s potential effect on RAR activity, these results typically come from classical luciferase-based assays which could be due to a combination of effects on protein level, phosphorylation state, and direct binding interaction. Nevertheless, evidence does exist that chalcones may have the potential to directly interact with RARs. A panel of carboxyl-containing chalcones and flavonoids were synthesized, among which CH55 and CH80 were identified as potential RAR agonists that exhibited retinoidal effect by inducing differentiation in HL60 cells. These compounds were later confirmed to directly bind to all three isoforms of RARs nonspecifically with
sub-nanomolar affinity.\(^{(153, 154)}\) Importantly, co-crystal was obtained\(^{(154)}\) of CH80 bound to the ligand binding domain of RARγ, and the crystal structure clearly showed direct interaction of CH80 with RARs in the ligand binding pocket.

### 1.2.6.2 Estrogen receptor

Estrogen receptors (ERs) are sex hormone receptors acting as transcription factors upon interaction with ligands such as 17β-estradiol. Aberrant ER activity is associated with various pathologies including cancer, menopausal syndrome, inflammatory diseases, and others.\(^{(155)}\) Natural chalcone isoliquiritigenin\(^{(156)}\) was tested for estrogenic activity and found to promote growth of ER positive breast cancer cells T-47D at a low concentration, whereas at a high concentration ER-independent cytotoxicity was observed. Direct binding was studied based on competition with radio labeled ligand 17β-estradiol and revealed low micromolar affinity for isoliquiritigenin, which is in agreement with another study\(^{(157)}\) where it induced ER-responsive mRNA Tff1 level. Similarly, candidachalcone\(^{(158)}\) isolated from Tephrosia candida was identified as a ER ligand with mid-micromolar affinity, although no efficacy data was given.

### 1.2.6.3 Other receptors

Isoliquiritigenin has also been identified as a positive allosteric modulator of GABA\(_A\) receptor\(^{(159)}\), a H\(_2\) histamine receptor antagonist\(^{(160)}\), and a positive allosteric modulator of α7 nicotinic acetylcholine receptor (α7nAChR)\(^{(161)}\), which potentially
explaining its neurological and gastric protective effects. Further synthetic derivatization of isoliquiritigenin yielded compound 71 as a more potent α7nAChR modulator which demonstrated \textit{in vitro} neuroprotective effect and \textit{in vivo} analgesic and cognitive enhancing effect. Compound 72\textsuperscript{(162)} was identified from a set of coumarin-chalcones as a potential ligand of adenosine receptors A\textsubscript{1}, A\textsubscript{2A}, and A\textsubscript{3} with micromolar potency and slight selectivity for A\textsubscript{3} receptor, although no efficacy data was available.

![Structures of chalcone compounds as ligands of various receptors.](image)

**Figure 1.16.** Structures of chalcone compounds as ligands of various receptors.

1.2.7 Others

1.2.7.1 ABC transporters

ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp) and breast cancer resistant protein (BCRP), are a group of efflux pumps capable of transporting drugs out of the cells, whose overexpression are found to be critical in conferring multi-drug resistance (MDR) in many cancer cell lines.\textsuperscript{(163)} Compound 73\textsuperscript{(164)} along with other synthetic chalcones was discovered as a BCRP inhibitor which increased cellular
accumulation of mitoxantrone and sensitized resistant cells to mitoxantrone. None of these chalcones inhibited the ATPase activity of BCRP, suggesting a different interaction mode from canonical ABC inhibitors. Similarly, chalcone 74\textsuperscript{(165)} was found to inhibit BCRP and, being non-cytotoxic itself, was able to sensitize resistant cells to mitoxantrone to a level comparable to the sensitive cells. Symmetric bis-chalcones\textsuperscript{(166)} were also tested as potential BCRP inhibitors with the same method, where activity from zero to complete inhibition was observed with 5 µM of chalcones suggesting a potential sharp SAR, and the most potent compound 75 at sub-micromolar concentration completely sensitized resistant cells to mitoxantrone. Mechanistically, 75 alone slightly stimulated basal ATPase activity of BCRP, but completely inhibited the substrate-induced ATPase activity increase. Canonical BCRP inhibitors through ATPase inhibition significantly synergized with 75, suggesting a non-classical binding interaction of chalcones with BCRP, which is consistent with the aforementioned study. A panel of structurally diverse chalcones were evaluated for inhibitory effect against either P-gp or BCRP using resistant cells specifically expressing either form\textsuperscript{(167)}. Selective inhibitors were found where compound 76 potently inhibited P-gp but not BCRP and 77 selectively inhibited BCRP over P-gp. A series of bifendate-chalcone hybrid\textsuperscript{(168, 169)} were developed as potential P-gp and BCRP dual inhibitors, and the most potent compound 78 potently inhibited P-gp and BCRP mediated efflux and sensitized resistant cells to adriamycin and mitoxantrone without showing cytotoxicity by itself or inhibiting the ATPase activity of P-gp. The potential sharp SAR was studied\textsuperscript{(170)} with a 3D-QSAR model, which gave some structural insights into such interaction.
1.2.7.2 Topoisomerases

Topoisomerases (TOPOs), including TOPO-I and TOPO-II, are enzymes regulating the winding and unwinding of DNA, a critical process for DNA transcription and replication. Therefore, TOPO inhibitors have been widely used as anticancer and antibacterial agents.\textsuperscript{(171)} Several chalcones based compounds have shown TOPO inhibitory activity. Natural compounds from Angelica keiskei was tested for cytotoxicity against various cancer cells\textsuperscript{(172)}, and the most potent compound 4-hydroxyderricin were found to inhibit TOPO-II more potently than etoposide, a clinically used anticancer drug acting as a TOPO-II inhibitor. Synthetically, compound 79\textsuperscript{(173)} was found to be a selective TOPO-II inhibitor over TOPO-I, which also demonstrated potent cytotoxicity against a panel of cancer cell lines. In addition, an aforementioned study\textsuperscript{(110)} not only identified cytotoxic chalcone 43 as a cathepsin inhibitor, but also a nonselective TOPO-I/II inhibitor with comparable potency with clinical drugs camptothecin and etoposide.
1.2.7.3 MD-2

MD-2 is a small secreted glycoprotein that binds to both Toll-like receptor 4 (TLR4) and LPS, and thereby mediates the LPS-induced TLR4 activation which triggers signal transductions related to inflammatory responses.\(^{174}\) Chalcone compound JSH was identified by high-throughput screening for inhibitors of LPS-induced inflammation, and was mechanistically elucidated\(^{175}\) as a MD-2 ligand via biochemical binding assays. Such binding interaction antagonized LPS binding to MD-2 and led to inhibition of LPS-induced NF-κB activation in cells. Similarly, anti-inflammatory mechanisms of chalcone L6H21 was investigated\(^{176}\), which revealed that it bound to MD-2 and antagonized the binding of LPS in biochemical assays. In cells, L6H21 inhibited LPS-induced MAPK phosphorylation, NF-κB activation, and cytokine expression. In vivo, L6H21 enhanced survival and improved lung injury in LPS-induce septic mice. Moreover, Arg90 and Tyr102 were found to be crucial residues as mutation of either of them abolished the binding of L6H21 to MD-2, giving insight into its potential binding site.

1.2.7.4 MDM2
As one of the mechanisms by which cancer cells evade apoptosis, overexpression of the oncoprotein MDM2 inhibits tumor suppressor protein p53 by binding to its transactivation domain and thus prevents p53-induced apoptosis of defective cells. Disruption of the interaction between MDM2 and p53 have thus become an interesting approach to combat cancer. The potential of chalcones to disrupt the MDM2/p53 interaction was studied with ELISA and NMR methods\(^{(177)}\), both of which identified compound 80 as the most potent inhibitor by binding to MDM2. Resolution of individual residue signals with 2D NMR gave insights into the binding site, which was characterized as the tryptophan pocket defined by S92, V93, L54, G58, Y60, V93, and F91. However, no further biological study was reported to confirm this activity in cells.

1.2.7.5 Cholesteryl ester transfer protein

Cholesteryl ester transfer protein (CETP) is a transporter protein shuttling triglycerides and cholesteryl esters among lipoproteins VLDL, LDL, and HDL; it therefore plays important regulatory roles in the lipid metabolism. Modulation of CETP activity is considered a promising strategy to reduce cardiovascular risks.\(^{(178)}\) Screening for CETP inhibitory activity from various plant extractions\(^{(179)}\) led to the discovery of xanthohumol as a moderate CETP inhibitor in a non-competitive manner. Further SAR study revealed the requirement of the prenyl substituent for potency and identified desmethylxanthohumol as a slightly more potent CETP inhibitor.
1.3 Discussions

The diverse bioactivities and target profiles of chalcones is envisioned to be a double-edged sword. On one hand, it gives great opportunities to explore this simple pharmacophore for various target interactions, making it a privileged structure in drug design by definition. On the other hand, it presents the challenge of promiscuity, or poor selectivity, in their biological interactions. Other than in a few limited cases where multiple target interactions are synergistic and therapeutically advantageous, such COX-2/5-LOX dual inhibitors for anti-inflammation and AChE/BACE1 dual inhibitors for anti-Alzheimer’s disease, these multiple-target phenomena would potentially lead to off-target interactions and hence unwanted side effects and compromised potency, which may explain at least partly why there has been very limited clinical success with chalcones in spite of its wide-spread research interest. Therefore, it is of great importance to understand the direct molecular targets responsible for these bioactivities, and ideally their interaction modes, so their target profile can be fine-tuned to achieve better selectivity and
potency. As reviewed above, there have been a wide variety of proteins identified as the potential direct targets of chalcones, with many chalcones implicated in multiple target interactions. (Table 1.1) Such multi-target profile not only suggests potential target promiscuity of the chalcone template, but also complicates the determination of contribution from each individual target to the overall bioactivity. For example, many targets reviewed here are potentially responsible for chalcone’s cytotoxicity, such as microtubules, Aurora kinases, TrxR, HDACs, TOPOs, and others, but knowledge of their relative contributions for a given chalcone compound is largely lacking. Those targets showing sharp SAR, such as microtubules and BCRP, are thus of greater interest than those with flat SAR such as 5-LOX and cathepsins, since sharp SAR typically indicates specific binding modes which may be fine-tuned to achieve optimal interaction and better selectivity.
Table 1.1. Examples of multi-targeting chalcones and potential relevance to their diverse bioactivities.

<table>
<thead>
<tr>
<th>compound</th>
<th>potential direct target</th>
<th>potential bioactivity relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>isoliquiritigenin</td>
<td>EGFR, tyrosinase, ALR2, BACE1, ER, GABA&lt;sub&gt;A&lt;/sub&gt; receptor, H&lt;sub&gt;2&lt;/sub&gt; receptor, α&lt;sub&gt;7&lt;/sub&gt;-nAChR</td>
<td>anticancer, anti-diabetic, anti-inflammation</td>
</tr>
<tr>
<td></td>
<td>IKKβ, EGFR, tyrosinase, aromatase, ALR2, HDAC, CETP</td>
<td>anticancer, anti-inflammation</td>
</tr>
<tr>
<td>xanthohumol</td>
<td>IKKβ, COX, aromatase, α-glucosidase, CETP</td>
<td>anticancer, anti-inflammation, anti-diabetic</td>
</tr>
<tr>
<td>licochalcone A</td>
<td>PI3K, B-Raf, MEK1, PTP1B</td>
<td>anti-inflammation, anti-diabetic</td>
</tr>
<tr>
<td>broussochalcone A</td>
<td>PKC, COX, PTP1B</td>
<td>anti-inflammation, anti-diabetic</td>
</tr>
<tr>
<td>kuraridin</td>
<td>ALR2, α-glucosidase</td>
<td>anti-diabetic, anti-inflammation</td>
</tr>
<tr>
<td>xanthoangelol</td>
<td>Aurora kinases, EGFR</td>
<td>anticancer</td>
</tr>
</tbody>
</table>

Special caution is needed when interpreting different levels of evidence for direct target interactions. With in vivo or cell-based in vitro studies, an observed effect for a target could be due to altered protein level, posttranslational modification, direct interaction with
compound, or their combinations. For this reason, such evidence alone, without some direct biochemical confirmation or ruling out other possibilities, is premature to suggest a direct target interaction. On the other hand, although biochemical assays, including X-ray co-crystal structures, are evidence for direct target interaction, there isn’t a necessary connection to its biological activities without some cell-based in vitro or in vivo experiments, as the compound may not get to its site of action or preferentially interact with other targets. The evidence is most convincing, therefore, when the biochemical studies matches the in vitro and in vivo activities, which is the case with some of the studies reviewed here, such as some of the anti-microtubule, RTK inhibitory, and ALR2 inhibitory chalcones. On the opposite, the data for some other targets are less convincing and deserve further investigations, such as MAO and AChE/BChE.

Another significant limitation of our current knowledge of chalcone’s molecular targets is the lack of evidence of direct interaction in cells. Most of the cell-based evidence reviewed above is downstream effects of a speculated target interaction, which may be regulated by many other targets; while most of the direct evidence is biochemical assays in cell-free conditions. Many factors could lead to potential discrepancy between cell-free and in-cell interactions, such as different subcellular localizations of compound and protein, preferential binding to other proteins, and interactions with other cellular components. The only compelling evidence is from the two studies with chemical probe approach that directly pulled protein targets out of the cells\(^{148, 149}\), which may be applied to study chalcone’s interaction with other potential targets in cells.
In conclusion, we have attempted to summarize the current knowledge in literature of potential direct molecular targets of chalcone-based compounds. The wide variety of their potential targets explains at least partly the diverse bioactivities of chalcones, which presents both opportunities and challenges in developing chalcone-based therapeutic agents. A better understanding of the interaction modes and biological relevance of these targets would greatly facilitate further development of chalcone-based therapeutic agents.
CHAPTER 2

Design and Synthesis of Chalcone-based Chemical Probes

This work in this chapter was performed in collaboration with Dr. Xingxin Yu and Dr. Chunlin Zhuang. Specifically, compounds FC31-FC34, FC53, and FC54 were originally synthesized by Dr. Chunlin Zhuang, and compounds AC1-AC7 were originally synthesized by Dr. Xingxin Yu.
2.1 Introduction

As discussed in Chapter 1, chalcone is an interesting scaffold for medicinal chemistry. However, the lack of target validation in the cellular environment and binding site characterization poses a significant challenge for rational optimization of chalcone based compounds as potential clinically useful agents with high potency and selectivity. Therefore, we envisioned to employ chemical probe approaches for the target study of chalcone based compounds in the whole-cell environment, which may advance our current understanding of such a privileged yet promiscuous pharmacophore and potentially direct its further development.

Various fluorescence-based probe approaches, such as fluorescence microscopy, fluorescence-based binding assay, fluorescence polarization, either alone or in combination with other approaches, have been developed to study small molecule-target interactions.\textsuperscript{(182-185)} These approaches take advantage of the high sensitivity of fluorescence generated by fluorophores to the target binding event, resulting in detectable fluorescence changes.\textsuperscript{(185)} However, these approaches typically require the introduction of a bulky fluorophore which may have a significant impact on their target interaction. It has been reported that chalcones with appropriate substitutions show desirable fluorescence properties and that such chalcones can be explored as fluorescent probes for various purposes.\textsuperscript{(186-189)} For instance, Lee et al\textsuperscript{(186)} developed a fluorescent chalcone library bearing 4-dialkylamino and 4′-amido substitutions, from which one compound was found
to selectively stain mouse embryonic stem cells through interaction with surface glycogen. Ono et al\(^{(187)}\) and Cui et al\(^{(188)}\) discovered 4-dialkylamino and indole-chalcone based compounds respectively that can fluorescently stain Aβ amyloid in mouse brains. Tomasch et al\(^{(189)}\) developed chalcones with tetralone moiety and extended double bonds as potential fluorescent probes which are ligands of human histamine H3 receptor. These examples demonstrated the potential and feasibility of exploring the intrinsic fluorescence property of chalcones as a versatile tool for biological investigations, which does not need to introduce bulky fluorophores that may interfere with its cellular target(s). Although these examples didn’t address the mechanistic questions of chalcone’s bioactivities, it would be interesting to explore the potential of such fluorescence-based approaches to study the mechanisms and target interaction of chalcone’s bioactivities. In order to develop biologically useful fluorescent probes, it is important to understand the fluorescence properties of a fluorophore and the influencing factors, because a major challenge in fluorescence-based applications is the high sensitivity of fluorescence generated by the fluorophore to structural and environmental factors.\(^{(185)}\) Despite a few studies related to the fluorescence properties of selected chalcone compounds,\(^{(190-193)}\) there is very limited knowledge about chalcone’s structural effect on fluorescence and environmental factors affecting it. Therefore, we constructed a focused library of potentially fluorescent chalcones for the study of their fluorescence properties and their potential as probes for target investigation. The design and syntheses of potentially fluorescent chalcone compounds are discussed in this chapter.
Another successful chemical probe approach is photoaffinity labeling (PAL). This approach takes advantage of UV-sensitive functional groups, which upon UV irradiation generate highly reactive intermediate species that subsequently form covalent linkages with adjacent molecules. In the application of small molecular probes for target identification, a small molecular probe possessing a PAL functional group, once bound to its biological target, would covalently modify the target molecule upon UV activation, enabling the enrichment and identification of the target through further biochemical manipulations. The identity of the probe-modified target can be revealed through further biochemical techniques such as biotin-streptavidin based purification and mass spectrometry. Aryl azide, diazirine, and benzophenone represent the three most widely used PAL functional groups, which covalently label their targets through nitrene, carbinol, or ketone radical intermediates. (Figure 2.1) These approaches and successful examples of their applications have been extensively reviewed in literature. To the best of our knowledge, however, no chalcone based photoaffinity probes have been reported for their cellular target identification. We therefore explored this approach for chalcone’s target investigation as well. The design and syntheses of seven chalcone-based photoaffinity probes are also discussed in this chapter.
2.2 Results and discussion

2.2.1 Design and syntheses of fluorescent chalcones (FC) as potential fluorescent probes

By analyzing the structures of the reported fluorescent chalcones in literature\(^\text{186-193}\), it appeared that a key feature for fluorescence is a dialkylamino substituent at the 4-position on the phenyl ring to which the alkene is attached (B ring), while the phenyl ring to which the carbonyl is attached (A ring) seemed to tolerate various substituents. Therefore, we designed our chalcone library by fixing a dialkylamino group on the 4-position of the B ring and varying other substituents. Most analogs (series 1, Table 2.1)
were designed by varying the substituents on the A ring or the alkyl substituents on the 4-dialkylamino group. Some closely related library members, such as constrained backbone analogs (series 2, Table 2.2), extended chalcones (series 3, Table 2.3), bis-chalcones (series 4, Table 4), chalcones with various aromatic systems (series 5, Table 2.5), and two chalcones without 4-dimethyl (series 6, Table 2.6) have also been designed for comparison to study the potential effects of these structural features on fluorescence properties and bioactivity. It is to be mentioned that the design of this library is meant to be diverse but not exhaustive. Since fluorescence characterization and biological evaluation, as detailed below, are performed simultaneously with the ongoing development of the library, the final library construction is partly guided by the feedback of these results, which is why certain categories contain much fewer analogs than others as can possibly be designed and synthesized.
Table 2.1. The structures of chalcone series 1 by varying A ring substituents and 4-dialkylamino group on B ring.
Table 2.2. The structures of chalcone series 2 by constraining the flexibility of the chalcone backbone.

Table 2.3. The structures of chalcone series 3 by extending the conjugation system with an additional double bond.
Table 2.4. The structures of chalcone series 4, namely bis-chalcones.

<table>
<thead>
<tr>
<th>Compound</th>
<th>position of substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC45</td>
<td>meta–</td>
</tr>
<tr>
<td>FC46</td>
<td>para–</td>
</tr>
</tbody>
</table>

Table 2.5. The structures of chalcone series 5 with various aromatic systems.
Table 2.6. The structures of chalcone series 6 by varying A ring substituents and 4-dialkylamino group on B ring.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
<th>R₅</th>
<th>R₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC55</td>
<td>H</td>
<td>NMe₂</td>
<td>H</td>
<td>OMe</td>
<td>OMe</td>
<td>OMe</td>
</tr>
<tr>
<td>FC56</td>
<td>OMe</td>
<td>OMe</td>
<td>OMe</td>
<td>H</td>
<td>OMe</td>
<td>H</td>
</tr>
</tbody>
</table>

As shown in Tables 2.1-2.6, the synthesis of all chalcones was accomplished through a conventional base catalyzed aldol condensation reaction between the corresponding acetophenone-type and benzaldehyde-type building blocks with sodium hydroxide as the catalyst and ethanol as the solvent. Overall, we have obtained 56-chalcones with structural diversity. A simple observation of the DMSO stock solutions of representative compounds under the lab UV lamp revealed interesting fluorescence phenomena, where they exhibited different fluorescence colors and brightness, despite their similar yellow color under visible light. (Figure 2.2) Detailed fluorescence characterizations are described in Chapter 4.
2.2.2 Design and synthesis of azido chalcone (AC) library as potential photoaffinity probes

Chalcone-based PAL probes were constructed by introducing both an aryl azide and an alkyne moiety onto the chalcone scaffold. Aryl azide was selected as the PAL functional group for its facile synthesis as well as reliable photo-reactivity. The alkyne moiety serves as a common tagging handle through which a biotin functional group can be attached via a copper-catalyzed alkyne-azide cycloaddition reaction (click reaction). These functional groups were separately introduced onto the building blocks, acetophenones/propiophenone and benzaldehydes, through established methods (see Chapter 2.4 for details and references). A final aldol condensation of the two building blocks
blocks gave the fully functionalized chalcone PAL probes. (Scheme 2.1) Seven PAL probes were synthesized, shown in Table 2.7. It is to be mentioned that the design and synthesis is partly directed by our SAR observations on the structural influence of chalcones on their cytotoxicity, with obtaining structural similar positive (cytotoxic) and negative (non-cytotoxic) probes as the goal. Success was achieved with the first round of synthesis of positive and negative probes, and hence a limited numbers of the analogs were synthesized.

**Scheme 2.1.** Synthetic route of chalcone PAL probes containing aryl azide and alkyne functionalities.
Table 2.7. The structures of chalcone series 7 as potential photoaffinity probes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \mathbf{R_1} )</th>
<th>( \mathbf{R_2} )</th>
<th>( \mathbf{R_3} )</th>
<th>( \mathbf{R_4} )</th>
<th>( \mathbf{R_5} )</th>
<th>( \mathbf{R_6} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>H</td>
<td>H</td>
<td>-OCH(_2)C≡CH</td>
<td>H</td>
<td>H</td>
<td>-Me</td>
</tr>
<tr>
<td>AC2</td>
<td>H</td>
<td>-OMe</td>
<td>-OCH(_2)C≡CH</td>
<td>-OMe</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>AC3</td>
<td>H</td>
<td>-OMe</td>
<td>-OCH(_2)C≡CH</td>
<td>-OMe</td>
<td>H</td>
<td>-Me</td>
</tr>
<tr>
<td>AC4</td>
<td>-OMe</td>
<td>H</td>
<td>-OMe</td>
<td>H</td>
<td>-OCH(_2)C≡CH</td>
<td>H</td>
</tr>
<tr>
<td>AC5</td>
<td>-OCH(_2)C≡CH</td>
<td>H</td>
<td>-OCH(_2)C≡CH</td>
<td>H</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>AC6</td>
<td>-OMe</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>-OCH(_2)C≡CH</td>
<td>H</td>
</tr>
<tr>
<td>AC7</td>
<td>H</td>
<td>-OMe</td>
<td>-OMe</td>
<td>-OCH(_2)C≡CH</td>
<td>H</td>
<td>-Me</td>
</tr>
</tbody>
</table>

2.3 Conclusion

In conclusion, we have constructed an FC library of fifty-six potential fluorescent probes, and an AC library of seven potential photoaffinity probes. These compounds were further studied as potential chemical probes for chalcone’s target investigation as detailed in the following chapters.

2.4 Experimental procedures

2.4.1 General procedures for the synthesis of substituted acetophenones
The propargyl substituted acetophenones were obtained through an alkylation reaction of the corresponding hydroxylated acetophenones.\(^{(19)}\) Potassium carbonate (3 eq) was added to the hydroxy acetophenone (1 eq) dissolved in acetonitrile, followed by the addition of propargyl bromide (1.2 eq). The reaction mixture was stirred at room temperature for overnight and diluted with ethyl acetate. The organic layer was washed with 1N HCl and dried over magnesium sulfate. Solvent was evaporated in vacuo and the residue was purified by column chromatography on silica gel to give the desired compounds.

2.4.2 General procedures for the synthesis of substituted propiophenones

The propargyl substituted propiophenones were prepared from the corresponding hydroxylated benzaldehydes via multiple steps including phenol protection, Grignard’s reaction, oxidation, deprotection, and alkylation.\(^{(19)}\)

In the first step, imidazole (2 eq) was added to a mixture of the benzaldehyde (1 eq) and t-butyldimethylchlorosilane (1 eq) in dry dimethylformamide at room temperature and stirred for 3 hours. Upon reaction completion, reaction mixture was quenched with water and extracted with ethyl acetate (2 x50 mL). Organic layers were combined, washed with saturated brine solution and dried over magnesium sulfate. The solvent was evaporated in vacuo and the residue was purified by flash column chromatography on silica gel to give silyl-protected benzaldehyde intermediate.
In the second step, Grignard reagent ethylmagnesium bromide (1.2 eq) was added drop-wise to silyl-protected benzaldehyde (1 eq) in dry tetrahydrofuran under nitrogen atmosphere at -78°C and stirred for 30 min. Then the reaction mixture was brought up to room temperature and stirred for another 2 hours. Reaction mixture was quenched with saturated aqueous ammonium chloride solution upon completion as judged by TLC and extracted with ethyl acetate. The combined organic layers were washed with saturated brine solution and dried over magnesium sulfate. Solvent was evaporated in vacuo and the residue was purified by column chromatography on silica gel to give the secondary alcohol intermediate.

In the third step, pyridinium chlorochromate (1.2 eq) was added to the secondary alcohol (1 eq) in dry dichloromethane at room temperature and stirred for 3 hours. Upon reaction completion by TLC, the reaction mixture was filtered and evaporated in vacuo and the residue was purified by column chromatography on silica gel to give the silyl-protected propiophenone intermediate.

In the fourth step, tetra-n-butylammonium fluoride (1.2 eq) was added to the silyl-protected propiophenone (1 eq) at room temperature and stirred for 1 hour. Upon reaction completion by TLC, the reaction mixture was evaporated in vacuo to give the hydroxy propiophenone intermediate.

In the fifth step, the exactly same procedure as in 2.4.1 was applied for the propargylation of the hydroxyl group of the hydroxy propiophenone intermediate.
2.4.3 General procedures for the synthesis of substituted benzaldehydes

The 3-azidobenzoaldehyde was converted from 3-formylphenylboronic acid following an established copper(II)-catalyzed method. To the aryl boronic acid (1.0 mmol, 1.0 eq) in methanol (5 mL) were added sodium azide (1.5 mmol, 1.5 eq) and Copper(II) acetate (0.2 mmol, 0.2 eq). The solution was vigorously stirred in an 8-mL round-bottom vial at 60 °C under air. Upon completion of reaction by TLC, the mixture was concentrated in vacuo to remove methanol, and the residue was re-suspended in 50 mL ethyl acetate and washed with water (20 mL × 3) through extraction. The combined organic portions were dried over magnesium sulfate, concentrated, and purified by chromatography to afford the title compound.

2.4.4 General procedures for the synthesis of chalcone compounds

For fluorescent probes (FC1 to FC56), all acetophenone-type and benzaldehyde-type starting materials were directly purchased and used without further purification. To a stirred solution of the corresponding acetophenone or other aryl ketones (3.33 mmol, 1.0 eq) and benzaldehyde or other aryl carbaldehydes (3.67 mmol, 1.1 eq) dissolved in ethanol (10 ml, 0.33 M) was added sodium hydroxide (10 mmol, 3.0 eq). This mixture was then stirred under room temperature (or at 50°C for slow reactions) for 6-24 hours, during which time a yellow precipitate formed in most cases. After the precipitate stopped increasing in
amount, crude product was collected by filtration. Recrystallization from pure methanol yielded pure products as colored crystalline solids. For products that did not precipitate from the reaction mixture, extraction and column chromatography was performed to obtain pure product. Purities of all compounds were over 95% by HPLC.

For photoaffinity probes (AC1 to AC7), a different procedure was applied based on condition optimization. Acetophenone or propiophenone (0.2 mmol, 1.0 eq) and 3-azidobenzaldehyde (0.4 mmol, 2.0 eq) were dissolved in ethanol (2 mL) in a glass vial, to which potassium hydroxide (0.6 mmol, 3.0 eq) was added. The reaction mixture was stirred at room temperature for 5 days when no further progress could be observed via TLC. The mixture was diluted with water and extracted with ethyl acetate, followed by chromatography purification with ethyl acetate and hexanes as the eluent to yield the final pure compounds.

(E)-1-(3,5-dimethoxyphenyl)-3-(4-(dimethylamino)phenyl)prop-2-en-1-one (FC8)

Yield: 75%. $^1$H NMR (400MHz, CDCl$_3$): $\delta$ 7.79 (1H, d, $J$ = 15.6 Hz), 7.54 (2H, d, $J$ = 8.8 Hz), 7.27 (1H, d, $J$ = 15.6 Hz), 7.14 (2H, d, $J$ = 2.2 Hz), 6.68 (2H, d, $J$ = 8.8 Hz), 6.64 (1H, t, $J$ = 2.2 Hz), 3.86 (6H, s), 3.03 (6H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 190.16, 160.73, 152.02, 145.92, 141.12, 130.39, 122.54, 116.82, 111.76, 106.11, 104.40, 55.55, 40.06. MS (ESI, positive) calculated m/z for [M+H]$^+$: 312.2; found: 312.1.

(E)-3-(4-(dimethylamino)phenyl)-1-(o-tolyl)prop-2-en-1-one (FC14)
Yield: 70%. \(^1\)H NMR (400MHz, CDCl\(_3\)): δ 7.44 (2H, d, \(J = 9.0 \) Hz), 7.43 (1H, d, \(J = 6.8 \) Hz), 7.37 (1H, d, \(J = 16.0 \) Hz), 7.35 (1H, t, \(J = 7.2 \) Hz), 7.26 (1H, d, \(J = 7.2 \) Hz), 7.25 (1H, t, \(J = 6.8 \) Hz), 6.91 (1H, d, \(J = 16.0 \) Hz), 6.67 (2H, d, \(J = 9.0 \) Hz), 3.03 (6H, s), 2.42 (3H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 197.39, 152.22, 147.43, 140.22, 136.44, 131.12, 130.48, 129.86, 127.88, 125.45, 122.35, 122.17, 111.94, 40.23, 20.14. MS (ESI, positive) calculated m/z for [M+H]\(^+\): 266.2; found: 266.2.

\( (E)\)-3-(4-(dimethylamino)phenyl)-1-(3,5-dimethylphenyl)prop-2-en-1-one (FC16)

Yield: 74%. \(^1\)H NMR (400MHz, CDCl\(_3\)): δ 7.78 (1H, d, \(J = 15.6 \) Hz), 7.61 (2H, s), 7.56 (2H, d, \(J = 9.0 \) Hz), 7.32 (1H, d, \(J = 15.6 \) Hz), 7.19 (1H, s), 6.69 (2H, d, \(J = 9.0 \) Hz), 3.04 (6H, s), 2.40 (6H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 190.99, 151.93, 145.41, 139.14, 137.99, 133.78, 130.31, 126.05, 122.71, 117.25, 111.77, 40.07, 21.26. MS (ESI, positive) calculated m/z for [M+H]\(^+\): 280.2; found: 280.1.

\( (E)\)-3-(4-(dimethylamino)phenyl)-1-(4-(trifluoromethyl)phenyl)prop-2-en-1-one (FC17)

Yield: 81%. \(^1\)H NMR (400MHz, CDCl\(_3\)): δ 8.07 (2H, d, \(J = 8.2 \) Hz), 7.80 (2H, d, \(J = 15.6 \) Hz), 7.74 (2H, d, \(J = 8.2 \) Hz), 7.55 (2H, d, \(J = 8.8 \) Hz), 7.27 (1H, d, \(J = 8.2 \) Hz), 6.69 (2H, d, \(J = 8.8 \) Hz), 3.05 (6H, s), 2.40 (6H, s). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)): δ 189.71, 152.42, 147.24, 142.19, 133.44 (q, \(J = 32.3 \) Hz), 130.82, 128.66, 125.56 (q, \(J = 3.8 \) Hz), 58
123.93 (q, J = 271.0 Hz) 122.32, 116.33, 111.91, 40.18. MS (ESI, positive) calculated m/z for [M+H]^+: 319.1; found: 319.1.

(E)-3-(4-(dimethylamino)phenyl)-1-(4-ethoxy-2,5-difluorophenyl)prop-2-en-1-one (FC18)

Yield: 68%. ¹H NMR (400MHz, CDCl₃): δ 7.77 (1H, dd, J = 15.6, 2.0 Hz), 7.64 (1H, dd, J = 11.6, 6.8 Hz), 7.52 (2H, d, J = 8.8 Hz), 7.24 (1H, dd, J = 15.6, 2.4 Hz), 6.69 (1H, dd, J = 12.0, 6.8 Hz), 6.67 (2H, d, J = 8.8 Hz), 4.14 (2H, q, J = 6.8 Hz), 3.03 (6H, s), 1.49 (3H, t, J = 6.8 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 186.03 (d, J = 4.3 Hz), 158.12 (dd, J = 249.0, 1.5 Hz), 152.21, 151.17 (dd, J = 12.6, 11 Hz), 148.74 (dd, J = 242.3, 2.2 Hz), 145.78, 130.69, 122.62, 119.97 (d, J = 9.0 Hz), 119.27 (dd, J = 15.2, 4.7 Hz), 117.25 (dd, J = 21.2, 4.9 Hz), 111.89, 102.25 (dd, J = 30.0, 1.3 Hz), 65.43 40.19, 14.57. MS (ESI, positive) calculated m/z for [M+H]^+: 332.1; found: 332.1.

(E)-3-(4-((2-hydroxyethyl)(methyl)amino)phenyl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (FC31)

Yield: 59%. ¹H NMR (500MHz, CDCl₃): δ 7.76 (1H, d, J = 15.5 Hz), 7.52 (2H, d, J = 9.0 Hz), 7.26 (1H, d, J = 15.5 Hz), 7.25 (2H, s), 6.74 (2H, d, J = 9.0 Hz), 3.93 (6H, s), 3.92 (3H, s), 3.84 (2H, t, J = 5.5 Hz), 3.56 (2H, t, J = 5.5 Hz), 3.05 (3H, s), 2.08 (1H, bs). ¹³C NMR (125 MHz, CDCl₃): δ 189.59, 153.14, 151.56, 145.77, 142.05, 134.45, 130.63,
123.05, 116.77, 112.12, 105.99, 61.07, 60.24, 56.47, 54.66, 39.06. MS (ESI, positive) calculated m/z for [M+H]^+: 372.2; found: 372.2.

*(E)-3-(4-(pyrrolidin-1-yl)phenyl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (FC32)*

Yield: 72%. ^1^H NMR (500MHz, CDCl3): δ 7.80 (1H, d, J = 15.5 Hz), 7.54 (2H, d, J = 8.5 Hz), 7.26 (1H, d, J = 15.5 Hz), 7.26 (2H, s), 6.55 (2H, d, J = 8.5 Hz), 3.94 (6H, s), 3.92 (3H, s), 3.39-3.30 (4H, m), 2.07-1.98 (4H, m). ^13^C NMR (125 MHz, CDCl3): δ 189.45, 153.12, 149.73, 146.23, 141.90, 134.70, 130.73, 122.13, 115.91, 111.86, 105.91, 61.05, 56.45, 47.70, 25.54. MS (ESI, positive) calculated m/z for [M+H]^+: 368.2; found: 368.2.

*(2E,4E)-5-(4-(dimethylamino)phenyl)-1-(2-methoxyphenyl)penta-2,4-dien-1-one (FC38)*

Yield: 75%. ^1^H NMR (400MHz, CDCl3): δ 7.55 (1H, dd, J = 7.6, 2.0 Hz), 7.46-7.34 (4H, m), 7.01 (1H, td, J = 7.4, 1.0 Hz), 6.97 (1H, d, J = 7.6 Hz), 6.88 (1H, d, J = 15.2 Hz), 6.80 (1H, dd, J = 15.2, 11.2 Hz), 6.78 (1H, d, J = 15.2 Hz), 6.67 (2H, d, J = 8.4 Hz), 3.89 (3H, s), 3.00 (6H, s). ^13^C NMR (125 MHz, CDCl3): δ 193.43, 157.85, 151.09, 145.64, 142.50, 132.26, 130.12, 129.90, 128.90, 128.02, 124.41, 122.74, 120.66, 112.12, 111.64, 55.80, 40.30. MS (ESI, positive) calculated m/z for [M+H]^+: 308.2; found: 308.1.

*(2E,4E)-5-(4-(dimethylamino)phenyl)-1-(3-methoxyphenyl)penta-2,4-dien-1-one (FC39)*
Yield: 75%. ¹H NMR (400MHz, CDCl₃): δ 7.63 (1H, dd, J = 14.8, 10.8 Hz), 7.55 (1H, d, J = 7.6), 7.52 (1H, d, J = 2.4 Hz), 7.40 (2H, d, J = 8.8 Hz), 7.10 (1H, dd, J = 8.0, 1.2 Hz), 7.00-6.93 (2H, m), 6.85 (1H, dd, J = 14.8, 10.8 Hz), 6.68 (2H, d, J = 8.8 Hz), 3.87 (3H, s), 3.01 (6H, s). ¹³C NMR (100 MHz, CDCl₃): δ 190.33, 159.91, 151.23, 146.54, 143.28, 140.27, 129.52, 129.04, 124.30, 122.78, 122.56, 120.94, 118.99, 112.70, 112.13, 55.56, 40.31. MS (ESI, positive) calculated m/z for [M+H]⁺: 308.2; found: 308.2.

(2E,4E)-5-(4-(dimethylamino)phenyl)-1-(3,5-dimethylphenyl)penta-2,4-dien-1-one
(FC41)

Yield: 71%. ¹H NMR (400MHz, CDCl₃): δ 7.61 (1H, dd, J = 15.2, 11.0 Hz), 7.58 (2H, s), 7.40 (2H, d, J = 8.8 Hz), 7.18 (1H, s), 6.98 (1H, d, J = 15.2 Hz), 6.95 (1H, d, J = 15.2 Hz), 6.85 (1H, dd, J = 15.2, 11.0 Hz), 6.68 (2H, d, J = 8.8 Hz), 3.01 (6H, s), 2.39 (6H, s). ¹³C NMR (100 MHz, CDCl₃): δ 190.99, 159.91, 146.08, 142.91, 138.95, 138.17, 134.05, 128.96, 126.20, 124.39, 123.16, 122.66, 112.15, 40.31, 21.41. MS (ESI, positive) calculated m/z for [M+H]⁺: 306.2; found: 306.2.

(2E,4E)-5-(4-(dimethylamino)phenyl)-1-(2-methylphenyl)penta-2,4-dien-1-one
(FC42)

Yield: 71%. ¹H NMR (400MHz, CDCl₃): δ 7.45-7.30 (4H, m), 7.27-7.17 (3H, m), 7.40 (2H, d, J = 8.8 Hz), 6.85 (1H, d, J = 15.2 Hz), 6.79 (1H, dd, J = 15.2, 9.6 Hz), 6.67 (2H, d, J = 8.4 Hz), 6.55 (1H, d, J = 15.2 Hz), 3.01 (6H, s), 2.42 (3H, s). ¹³C NMR (100
MHz, CDCl$_3$): $\delta$ 197.21, 151.21, 147.92, 143.07, 139.88, 136.53, 131.16, 129.98, 129.06, 127.88, 127.70, 125.45, 124.18, 122.33, 112.15, 40.33, 20.17. MS (ESI, positive) calculated m/z for [M+H]$^+$: 292.2; found: 292.1.

(2E,4E)-5-(4-(dimethylamino)phenyl)-1-(2-nitrophenyl)pent-2,4-dien-1-one (FC43)

Yield: 79%. $^1$H NMR (500MHz, CDCl$_3$): $\delta$ 8.12 (1H, dd, $J = 8.0$, 1.0 Hz), 7.72 (1H, td, $J = 7.5$, 1.0 Hz), 7.60 (1H, ddd, $J = 8.0$, 7.5, 1.5 Hz), 7.47 (1H, dd, $J = 7.5$, 1.0 Hz), 7.34 (2H, d, $J = 8.8$ Hz), 7.03 (1H, dd, $J = 15.0$, 10.0 Hz), 6.80 (1H, d, $J = 15.0$ Hz), 6.74 (1H, dd, $J = 15.5$, 10.0 Hz), 6.64 (2H, d, $J = 8.8$ Hz), 6.45 (1H, d, $J = 15.5$ Hz), 3.00 (6H, s). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 192.88, 151.40, 148.35, 146.95, 144.08, 136.91, 133.87, 130.27, 129.27, 129.01, 126.52, 124.53, 123.81, 121.75, 112.05, 40.25. MS (ESI, positive) calculated m/z for [M+H]$^+$: 323.1; found: 323.1.

(2E,2’E)-1,1’-(1,3-phenylene)bis(3-(4-(dimethylamino)phenyl)prop-2-en-1-one) (FC45)

Yield: 71%. $^1$H NMR (400MHz, CDCl$_3$): $\delta$ 8.61 (1H, t, $J = 1.6$ Hz), 8.18 (2H, dd, $J = 7.6$, 1.6 Hz), 7.84 (2H, d, $J = 15.6$ Hz), 7.61 (1H, t, $J = 7.6$ Hz), 7.58 (4H, d, $J = 8.8$ Hz), 7.39 (2H, d, $J = 15.6$ Hz), 6.70 (4H, d, $J = 8.8$ Hz), 3.01 (6H, s), 2.42 (3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 190.10, 152.31, 146.62, 139.40, 131.88, 130.78, 128.85, 128.07, 122.63, 116.62, 111.95, 40.26. MS (ESI, positive) calculated m/z for [M+H]$^+$: 425.2; found: 425.2.
(E)-3-(3-azidophenyl)-2-methyl-1-(4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (AC1)

Yield: 20%. $^1$H NMR (400 MHz, CDCl$_3$): 2.22 (s, 3H), 2.56-2.57 (m, 1H), 4.76 (d, $J = 2.4$ Hz, 2H), 6.99-7.07 (m, 4H), 7.18 (d, $J = 7.8$ Hz, 1H), 7.36-7.40 (m, 1H), 7.80 (d, $J = 8.6$ Hz, 2H), 7.94-8.03 (m, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 14.9, 55.8, 76.1, 77.8, 114.4, 118.7, 119.8, 125.9, 129.8, 130.6, 131.1, 131.8, 138.0, 138.6, 140.3, 160.7, 197.8. MS (ESI, positive) calculated m/z for [M+H]$^+$: 318.1; found: 318.1.

(E)-3-(3-azidophenyl)-1-(3,5-dimethoxy-4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (AC2)

Yield: 61%. $^1$H NMR (400 MHz, CDCl$_3$): 2.45-2.46 (m, 1H), 3.94 (s, 6H), 4.83 (d, $J = 2.4$ Hz, 2H), 6.92-6.94 (m, 2H), 7.03 (s, 1H), 7.25-7.26 (m, 1H), 7.40-7.47 (m, 3H), 7.74 (dd, $J = 15.6$, 3.1 Hz, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 56.4, 75.3, 77.0, 106.0, 118.5, 120.7, 122.7, 125.0, 130.3, 133.8, 136.6, 140.2, 142.9, 143.5, 153.5, 189.0. MS (ESI, positive) calculated m/z for [M+H]$^+$: 364.1; found: 364.1.

(E)-3-(3-azidophenyl)-1-(3,5-dimethoxy-4-(prop-2-yn-1-yloxy)phenyl)-2-methylprop-2-en-1-one (AC3)

Yield: 14%. $^1$H NMR (400 MHz, CDCl$_3$): 2.25 (s, 3H), 2.45-2.46 (m, 1H), 3.89 (s, 6H), 4.81 (d, $J = 2.4$ Hz, 2H), 7.02-7.04 (m, 4H), 7.11 (s, 1H), 7.19 (d, $J = 7.8$ Hz, 1H), 7.36-7.40 (m, 1H), 7.74 (dd, $J = 15.6$, 3.1 Hz, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 14.9, 55.8, 76.1, 77.8, 114.4, 118.7, 119.8, 125.9, 129.8, 130.6, 131.1, 131.8, 138.0, 138.6, 140.3, 160.7, 197.8. MS (ESI, positive) calculated m/z for [M+H]$^+$: 364.1; found: 364.1.
7.38-7.42 (m, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 14.8, 56.3, 75.2, 78.9, 107.0, 118.9, 119.8, 123.1, 126.0, 133.6, 137.4, 139.6, 140.2, 140.4, 142.9, 153.1, 198.1. MS (ESI, positive) calculated m/z for [M+H]$^+$: 378.1; found: 378.1.

*(E)*-3-(3-azidophenyl)-1-(2,4-dimethoxy-6-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (AC4)

Yield: 54%. $^1$H NMR (400 MHz, CDCl$_3$): 2.47-2.48 (m, 1H), 3.76 (s, 3H), 3.84 (s, 3H), 4.66 (d, $J = 2.4$ Hz, 2H), 6.19 (s, 1H), 6.30 (s, 1H), 6.94 (d, $J = 16.0$ Hz, 1H), 6.99-7.01 (m, 1H), 7.13 (s, 1H), 7.31 (d, $J = 16.0$ Hz, 1H), 7.25-7.35 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 55.4, 55.9, 56.4, 75.9, 78.2, 91.8, 92.2, 112.2, 118.3, 120.4, 124.8, 129.9, 130.1, 136.8, 140.6, 142.6, 156.7, 158.9, 162.3, 193.2. MS (ESI, positive) calculated m/z for [M+H]$^+$: 364.1; found: 364.1.

*(E)*-3-(3-azidophenyl)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (AC5)

Yield: 62%. $^1$H NMR (400 MHz, CDCl$_3$): 2.57-2.58 (m, 1H), 2.60-2.61 (m, 1H), 4.75 (d, $J = 2.4$ Hz, 2H), 4.78 (d, $J = 2.4$ Hz, 2H), 6.67-6.71 (m, 2H), 7.02-7.04 (m, 1H), 7.35-7.37 (m, 2H), 7.54-7.64 (m, 2H), 7.79 (d, $J = 8.6$ Hz, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 56.0, 56.5, 76.2, 76.5, 77.6, 77.7, 100.8, 107.2, 118.1, 120.2, 123.1, 125.2, 128.1, 130.1, 132.9, 137.2, 140.6, 140.8, 158.1, 161.8, 189.6. MS (ESI, positive) calculated m/z for [M+H]$^+$: 358.1; found: 358.1.
(E)-3-(3-azidophenyl)-1-(2-methoxy-6-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (AC6)

Yield: 59%. $^1$H NMR (400 MHz, CDCl$_3$): 2.45-2.46 (m, 1H), 3.78 (s, 3H), 4.68 (d, $J = 2.4$ Hz, 2H), 6.66 (d, $J = 8.6$ Hz, 1H), 6.75 (d, $J = 8.6$ Hz, 1H), 6.94 (d, $J = 16.0$ Hz, 1H), 7.01-7.03 (m, 1H), 7.14 (s, 1H), 7.25-7.37 (m, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 55.9, 56.3, 75.7, 78.3, 104.9, 105.7, 118.5, 119.0, 120.6, 124.8, 129.5, 130.1, 130.8, 136.6, 140.7, 143.7, 155.3, 157.7, 194.2. MS (ESI, positive) calculated m/z for [M+H]$^+$: 334.1; found: 334.1.

(E)-3-(3-azidophenyl)-1-(3,4-dimethoxy-5-(prop-2-yn-1-yloxy)phenyl)-2-methylprop-2-en-1-one (AC7)

Yield: 12%. $^1$H NMR (400 MHz, CDCl$_3$): 2.24 (s, 3H), 2.40-2.42 (m, 1H), 3.91 (s, 3H), 3.94 (s, 3H), 4.78 (d, $J = 2.4$ Hz, 2H), 7.00-7.05 (m, 2H), 7.11-7.20 (m, 4H), 7.38-7.41 (m, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 14.8, 56.2, 57.0, 61.1, 76.0, 78.2, 107.7, 110.1, 118.8, 119.8, 126.0, 129.8, 132.7, 137.5, 137.6, 139.6, 140.3, 142.5, 150.3, 153.3, 197.7. MS (ESI, positive) calculated m/z for [M+H]$^+$: 378.1; found: 378.1.
CHAPTER 3

Cytotoxicity Evaluation, Cell Cycle Arrest, and Microtubule Disruption

The work in this chapter was performed in collaboration with Dr. Xingxin Yu and Dr. Junxuan Lu. Specifically, initial screening of cytotoxicity of compounds AC1-AC7 was performed by Dr. Xingxin Yu. Figure 3.3.c was performed by Dr. Junxuan Lu.
3.1 Introduction

Among chalcone’s various biological activities, cytotoxicity as a potential anti-cancer indicator has been of particular interest. Numerous studies have reported chalcones with various substituents, both natural and synthetic, as potential cytotoxic agents. Many hit compounds, including natural chalcones from cancer chemopreventive plants such as isoliquiritigenin, xanthohumol, and licochalcone-A, exhibited cytotoxic activity against a panel of cancer cell lines.\(^{(7, 198-202)}\) The cytotoxicity of chalcones has also shown sharp structure activity relationships, with IC\(_{50}\) values ranging from single-digit nanomolar to high micromolar.\(^{(18, 203-205)}\) A number of putative direct cellular targets potentially responsible for chalcone’s cytotoxicity against cancer cell lines, including microtubules, kinases, thioredoxin reductase, cathepsins, HDACs, CDC25B, topoisomerases, MDM2, and sex hormone converting enzymes, have been reported and summarized in Chapter 1, but the evidence of such direct interactions with these putative targets in cells or their contribution to chalcone’s cytotoxicity in cells has not been demonstrated. Therefore, we selected cytotoxicity as the bioactivity of our interest for target study.

We performed cytotoxicity evaluation as new analogs were synthesized, and results were analyzed in a timely manner to make SAR observations which in turn aided design of new analogs. As a preliminary mechanistic investigation, we analyzed chalcone’s effect on cell cycle arrest, because many of the putative targets listed above, such as microtubules, kinases, and HDACs, participate in cell cycle regulation. Upon confirmation of cell cycle
arrest effect by selected cytotoxic chalcones, we proceeded to investigate their effect on cellular microtubule network structure, as microtubules play an important role in cell cycle regulation and have been suggested as the potential cytotoxic target of chalcones. These results will be discussed in this chapter.

3.2 Results and discussion

3.2.1 Cytotoxicity and structure-activity relationship

All fifty-six FC compounds and seven AC compounds were screened for growth inhibition of A549 lung cancer cells as an indication of their cytotoxicity. (Table 3.1 and Figure 3.1) Their cytotoxicity IC₅₀ values (concentration at which cell growth was inhibited by 50%) covered a wide range, from sub-micromolar to high micromolar. Interestingly, cytotoxic potency was found to be highly sensitive to subtle structural differences within this set of chalcones, demonstrating a sharp structure-activity relationship (SAR). (Figure 3.1.b) First, substituents on the para-position of A ring (4’-position) decreases the potency, regardless of the nature of the substituent; compounds with any 4’-substitution all showed lower potency than their unsubstituted counterparts (FC4, FC15, FC17, FC20-FC23, FC27-FC29 compared to FC1; FC5 compared to FC2; FC7 compared to FC3, and FC9 compared to FC8). This suggests that the binding pocket for A ring has limited space to accommodate
substituents. Second, any substituent, other than a hydroxyl, on the ortho-position of A ring (2’-position) is favored for potency, as FC2, FC14, FC24, FC25 all exhibited lower IC_{50} values compared to the unsubstituted counterpart FC1. However, if both ortho positions (2’-position and 6’-position) were substituted, the potency was lost (FC6, FC10, and FC12). Third, a methoxy substitution at the meta-position of A ring (3’-position) improved potency (FC3 compared to FC1; FC7 compared to FC4), and substitution of both meta-positions (3’-position and 5’-position) with methoxy or methyl was further preferred (FC8, FC9, and FC16). Fourth, as for the B ring, the dimethylamino group on the para-position of B ring (4-position) is not essential for potency, as compounds with other dialkylamino groups (FC30-FC34) or a methoxy group (FC56) at the same position, or an azido group (AC3) at the meta-position (3-position) also gave comparable potency. Fifth, a methyl substitution at the α–position of the alkene portion (FC33 compared to FC1, AC3 compared to AC2) considerably increased potency, which is consistent with the observation in literature. Finally, compounds with a modified scaffold, such as the extended chalcones (FC38-FC44), the bis-chalcones (FC45, FC46), the ring-constrained chalcones (FC36, FC37), the heterocyclic chalcones (FC49, FC50), or the indole-chalcones (FC51-FC54), all showed reduced potency compared to their chalcone counterparts. Altogether, the cytotoxicity SAR of our chalcones revealed favorable biological diversity, which provides an optimal window for positive/negative probe selection for mechanistic study.
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<th>IC50 (μM)</th>
<th>Cmpd.</th>
<th>IC50 (μM)</th>
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<th>Cmpd.</th>
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<td>FC1</td>
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<td>FC17</td>
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<td>FC33</td>
<td>19.0 ± 1.58</td>
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<td>FC2</td>
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<td>FC19</td>
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<td>FC47</td>
<td>2.42 ± 0.04</td>
<td>AC7</td>
<td>2.75 ± 0.06</td>
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<tr>
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<td>FC32</td>
<td>&gt; 60</td>
<td>FC48</td>
<td>24.1 ± 0.13</td>
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Table 3.1. Cytotoxicity IC50 values of all chalcone probe compounds.
3.2.2 Synergism between non-cytotoxic and cytotoxic compounds

Since chalcones potentially have been shown to interact with various cellular proteins, we speculated that the potency of cytotoxic chalcones might be compromised due to off-target interactions that are not related to cytotoxicity. We thereby hypothesize that co-treatment of cells with a cytotoxic chalcone and a structurally similar but non-cytotoxic chalcone, which may share some common off-target interactions but not interaction with
the responsible cytotoxic target, would show a synergistic effect, because the competition for off-target interactions would make more cytotoxic chalcone available for interaction with the cytotoxic target. As a proof of concept, we tested the cytotoxicity of FC8 or AC3 against A549 cells in the presence of non-cytotoxic chalcone FC4.

As shown in Figure 3.2, FC4 at 20 μM alone did not cause any cell growth inhibition, but it significantly enhanced the cytotoxicity of FC8 and AC3. The IC\textsubscript{50} values were reduced by ~79% for FC8 and ~28% for AC3. The relatively smaller difference observed for AC3 might indicate either a less optimal off-target overlapping with FC4 or a relatively cleaner target interaction profile than FC8, both of which would result in a weaker effect of off-target competition by non-cytotoxic FC4.

![Synergistic effect between non-cytotoxic FC4 and cytotoxic FC8 or AC3.](image)

**Figure 3.2.** Synergistic effect between non-cytotoxic FC4 and cytotoxic FC8 or AC3.
3.2.3 Cytotoxic compound FC8 arrests cell cycle at the M phase in a time- and dose-dependent manner

Since cell cycle arrest has been reported to be involved in chalcone’s cytotoxicity\(^{(30)}\), we first characterized the effect of cytotoxic chalcone FC8 on cell cycle distribution. Consistent with literature, FC8 was found to arrest A549 cells in the G2/M phases in a dose- and time-dependent manner (Figure 3.3.a and 3.3.b). The dose to reach maximal effect (1 µM) is slightly higher than its IC\(_{50}\) (0.79 µM). Treatment of FC8 at this concentration for 6 hours resulted in an increase in G2/M population from 25.2% to 67.5%. Interestingly, such an effect was readily reversible, as cells treated with FC8 at 1 µM for 6 hours (third bar in Figure 3.3.b) re-distributed to the normal cell cycle population upon FC8 removal for 3 hours (last bar in Figure 3.3.b), suggesting a non-covalent interaction of FC8 with its responsible cellular target for cytotoxicity. Finally, by separating G2 and M phases, FC8’s time-dependent cell cycle arrest effect was found to be specifically in the M phase (Figure 3.3.c).
Figure 3.3. Cell cycle arrest and reversibility of FC8. (a) Dose-response and (b) time-dependence of FC8-induced reversible G2/M cell cycle arrest; and (c) FC8 arrests cells specifically at M phase.

3.2.4 Cytotoxic potency does not correlate with Michael addition reactivity

As discussed in Chapter 3.2.3, the results suggested a likely non-covalent interaction between chalcones and their cytotoxic target. To further support this, we tested and
compared the Michael addition reactivity of non-potent compound FC1 ($IC_{50} = 23.2 \mu M$) and potent compound FC35 ($IC_{50} = 0.43 \mu M$) with a model thiol compound, namely, N-acetylcysteamine (NAC). (Figure 3.4.a) The thiol reactant NAC was in large excess (10 times concentration of that of the FC compounds), so that the initial reaction would follow a pseudo-first order kinetics. The reaction was performed under physiological pH (pD 7.1) and the reaction progress was monitored by $^1$H-NMR. (Figure 3.4.b) The results showed that the pseudo-first order reaction rate of the Michael addition with FC35 is 200-times slower than that with FC1. (Figure 3.4.c) This was expected since their only structural difference is the $\alpha$-methyl substitution of FC35 which would disfavor the Michael addition reaction through steric hindrance and disruption of the conjugation. The fact that compound FC35 which is a much weaker Michael-acceptor than FC1 showed 50-fold higher cytotoxic potency strongly argues against the potential involvement of Michael addition reaction in the target interaction of cytotoxic chalcones, and further supports a non-covalent mode of binding interaction.
Figure 3.4. Michael reactivity of FC1 and FC35 with N-acetylcysteamine. (a) Reaction scheme, (b) real-time monitoring of reaction progress, with peaks characteristic of the products and starting materials highlighted, and (c) initial rate plots based on integration of the characteristic peaks highlighted in (b).
3.2.5 Cytotoxic potency parallels the ability to cause cell cycle arrest among different compounds

We then tested several chalcone compounds with varied cytotoxicity, including both potential fluorescent probes (FCs) and photoaffinity probes (ACs), for cell cycle arrest effect (Figure 3.5). Five FCs were tested at 1 µM, and their abilities to cause cell cycle arrest nicely paralleled their different cytotoxic potencies. Compared to the control G2/M population (27.6%), the most potent compound FC2 (IC$_{50}$ = 0.25 µM) resulted in an increase of G2/M population to 67.7%, and the slightly less potent compounds FC8 (IC$_{50}$ = 0.79 µM) and FC16 (IC$_{50}$ = 1.25 µM) increased it to 64.1% and 49.6%, respectively. On the contrary, the non-potent compounds FC1 (IC$_{50}$ = 23.2 µM) and FC4 (IC$_{50}$ > 60 µM) did not cause any significant difference in cell cycle population compared to the control. Three ACs were tested at 0.5 µM (for the IC$_{50}$ of potent AC3 is about half of that of FC8) as well as at concentrations of the same fold-relationship to their own IC$_{50}$ values (1.3×IC$_{50}$). Similar to FCs, potent compound AC3 (IC$_{50}$ = 0.38 µM) increased G2/M population to 70.4%, whereas the non-potent compounds AC1 (IC$_{50}$ > 60 µM) and AC2 (IC$_{50}$ = 28.6 µM) did not cause any significant cell cycle arrest even at high concentrations corresponding to 1.3×IC$_{50}$. This suggests that the cytotoxicity of the non-potent chalcones at high concentrations is caused by other mechanisms irrelevant to cell cycle arrest, which further supports chalcone’s multi-target interaction potential, and provides a potential
explanation for the various putative targets reported for chalcone’s cytotoxicity. Overall these results suggest a good correlation between cytotoxic potency and the ability to cause G2/M phase cell cycle arrest within our chalcone library, indicating a potential mechanistic link.

![Figure 3.5](image_url)

**Figure 3.5.** Cell cycle arrest of various chalcone probes with different cytotoxicity.

3.2.6 **Cytotoxic potency parallels the ability to disrupt the microtubule network structure in cells**

We next investigated the effect of these four FC compounds on microtubule structure in A549 cells since chalcone-based compounds have been suggested to disrupt cellular microtubule structure that further leads to cell cycle arrest, a common mechanism
for anti-tubulin agents.\textsuperscript{(30)} A549 cells were treated with FC1, FC4, FC8 and FC16, at 5 µM and the cellular microtubule structure was visualized via anti-tubulin immunostaining as previously described.\textsuperscript{(30)} (Figure 3.6) As expected, non-cytotoxic FC1 and FC4 had no obvious effect on the microtubule structure in comparison to the control cells. A significant decrease of the microtubule structure was observed in cells treated with the cytotoxic FC8 and FC16, indicating an impaired tubulin-microtubule dynamic stability by promoting microtubule depolymerization. The extent of decrease was higher in FC8-treated cells than in FC16-treated cells, consistent with the higher cytotoxic potency of FC8 than FC16. These results together with the cell cycle arrest results overall strongly support that M-phase cell cycle arrest caused by cellular microtubule depolymerization is likely involved in the mechanism of chalcone’s cytotoxicity.
Figure 3.6. The effect of different chalcone compounds on cellular microtubule structures in A549 cells via immunofluorescence microscopy.

3.2.7 NCI-60 screening of AC3 against various cancer cells and mechanistic implication by CellMiner and COMPARE studies

Finally, potential PAL probe AC3 was evaluated against a panel of human cancer cell lines through the NCI-60 screening service (Figure 3.7). AC3 had an average IC$_{50}$ of 0.26 µM among the cell lines screened, which is comparable to its IC$_{50}$ value in A549 cells determined in our studies (0.38 µM). AC3 also showed a wide range of potencies among the screened cancer cell lines (IC$_{50}$ ranging from 26.9 nM to 28.8 µM). NCI screening data
are frequently analyzed by COMPARE and CellMiner for pattern comparison with known compounds for potential mechanistic implications.\(^{(207, 208)}\) Therefore, we applied these tools to compare AC3 with other compounds that have been evaluated through the same NCI service for their cytotoxic potency fingerprint similarity. Ten anti-mitotic agents showed a correlation of \(> 0.5\) with AC3, suggesting a similar mechanism of cytotoxicity. (Table 3.2) Except for S-trityl-L-cysteine and estramustine, the other eight compounds all have been shown to exert their anti-mitotic activity through direct interactions with tubulin,\(^{(30, 209-212)}\) suggesting that cytotoxic chalcones might act as anti-mitotic agents by directly interacting with tubulin.

<table>
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<th>Anti-mitotic agents</th>
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<tr>
<td>nakiterpiosin</td>
<td>0.641(^c)</td>
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<td>desacetylcolchicine d-tartrate</td>
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<td>S-trityl-L-cysteine</td>
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<td>N-benzoyl deacetylcolchicine</td>
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**Table 3.2.** The top anti-mitotic agents that have high correlation with AC3 via CellMiner/COMPARE analysis of their cytotoxic potency among the NCI-60 human cancer cell lines.

\(^a\). Correlation was calculated using both CellMiner and COMPARE services, with the higher value reported; \(^b\). correlation by COMPARE; and \(^c\). correlation by CellMiner.
3.3 Conclusion

In summary, we have characterized the cytotoxic potencies of all potential chalcone-based probes, and performed initial mechanistic investigations. The wide range of cytotoxic potencies and the sharp structure-activity relationship observed thereof indicated
a well-defined target interaction mode and that chalcone may be a privileged template instead of a promiscuous one for developing potent cytotoxic agents. Primary mechanistic investigations suggested a good positive correlation among chalcone’s cytotoxic potencies, their M phase cell cycle arrest effect and their cellular microtubule depolymerization effect. These results together with the NCI-60 pattern comparison results strongly suggested tubulin as the potential target for chalcone’s cytotoxicity, and provided guidance for the probe studies discussed in Chapters 4 and 5 to investigate the direct target interaction in cells.

3.4 Experimental procedures

3.4.1 Cell culture

A549 cells were purchased from ATCC and grown in DMEM media supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine at 37°C with 5% CO₂. Cells grown for less than two weeks were harvested for storage in FBS containing 10% DMSO at -80°C. All experiments were performed with cells grown for less than five weeks after they were recovered from storage.

3.4.2 In vitro cytotoxicity assay
The in vitro cytotoxicity of chalcones was assayed by determining the GI50 (the concentration of chalcones to reduce the cell growth by 50%). In brief, the cells were plated in a 96-well plate. After attachment, cells were treated with chalcones at different concentrations in media containing 1% DMSO (cells treated with 1% DMSO media as control). After 48 h of treatment, the relative cell viability in each well was determined using CellTiter-Blue cell viability assay kit. The IC$_{50}$ of each agent was determined by fitting the relative viability of the cells to the drug concentrations by using a sigmoidal dose-response model of varied slope in Prism GraphPad. The IC$_{50}$ reported herein is the average of at least three replicates, with standard error, in most cases, of no more than 20% of the average IC$_{50}$.

3.4.3 Cell cycle analysis by flow cytometry

Cell cycle population was analyzed from DNA content detected with flow cytometry as previously described.$^{(21,2)}$ Briefly, A549 cells were cultured as described above. When cells reach about 70% confluency in a petri dish, the culture media was replaced by media containing corresponding treatment (with 1% DMSO). After specified time period, cells were trypsinized and centrifuged and cell palate was collected by pouring out the supernatant. The cell pellet was washed once with PBS, re-pelleted, and re-suspended in 1ml PBS. This suspension was added into 9ml of 70% ethanol and stored at
4°C overnight for fixation. Cell pellets were collected by centrifugation, washed twice with PBS, then resuspended in 1 mL PI staining solution (50 µg/mL PI, 200 µg/mL RNase A, and 0.1% Triton-X 100 in PBS) and incubated for 30 min at room temperature in the dark. DNA content and cell cycle population was then analyzed with a BD FACSCalibur flow cytometry system.

3.4.4 Michael addition reactivity test

Phosphate buffered saline (PBS, deuterated, pD 7.1) was prepared by adding a 0.1 M NaH₂PO₄ solution to a 0.1 M Na₂HPO₄ solution, both dissolved in D₂O, until the pD reached 7.1 by a pH meter. The stock solution of N-acetylsysteamine (NAC, 2.5 M) was prepared by dissolving 133 µL of NAC with 367 µL DMSO-d₆ as the NMR solvent. Calculated amount of the FC starting material (3.1 mg for FC1 and 3.3 mg for FC35, so that the final reaction concentration is 25 mM) was dissolved with 425 µL DMSO-d₆ in an Eppendorf tube. Each reaction was started by adding to the tube containing the FC starting material 25 µL of PBS and 50 µL of NAC stock prepared above. Then the 500 µL total reaction was immediately transferred to a NMR tube for reaction monitoring.

3.4.5 Immunomicroscopy imaging of microtubule network structure in cells
A549 cells were seeded in 96-well confocal plates at 6000/well. After attachment, cells were treated with media containing 5 μM chalcone compounds with 1% DMSO for 6 hours. Media was removed and cells were washed with PBS twice and treated with 4% PFA fixative at 37°C for 12 min. After three washes with PBS, cells were permeabilized with 0.05% Triton X-100/PBS for 5 min at 37°C, and then blocked with 5% donkey serum/PBS at 37°C for 8 min, followed by incubation with 1:100 sheep anti-tubulin antibody (Cat# ATN02, Cytoskeleton) at 37°C for 15 min. After three PBS washes, cells were then treated with 1:100 APC-conjugated donkey-anti-sheep antibody (Cat# 713-136-147, Jackson ImmunoResearch) at 37°C for 8 min. After two more PBS washes, fluorescence images were taken with a Nikon TE2000 inverted confocal microscope.
CHAPTER 4

Characterization of Fluorescent Properties of Fluorescent Probes and
Their Application in Cytotoxic Mechanistic Studies
4.1 Introduction

The potential of a fluorescence-based probe is to a great extent determined by the fluorescent properties of the fluorophore.\textsuperscript{(214)} The absorption and emission wavelengths determine the dynamic range of detection, while the sensitivity is largely dictated by the fluorescence brightness (B), which is the product of the extinction coefficient (ε) at the excitation wavelength and the quantum yield (Φ).\textsuperscript{(215)} Moreover, it is also known that fluorescence is highly sensitive to its physicochemical environment. Many biologically relevant factors, such as solvent polarity, pH, and potential interactions with biological components and additives may have significant effects on the fluorescence behavior of a fluorophore. We have shown the intrinsic fluorescence potential of chalcones in Chapter 2.2.1. In order to explore the potential of these chalcone compounds to be used as useful biological probes, we further characterized the fluorescence properties of all the fifty-six chalcones synthesized in Chapter 2.2.1, and the effects of the environmental factors mentioned above on their fluorescence. These results are discussed in this chapter to obtain a better understanding of chalcone’s fluorescence properties. Finally, potential fluorescent probes were selected to explore chalcone’s target interaction with tubulin for their cytotoxicity via fluorescence-based binding assay and cell-based confocal fluorescence microscopy imaging. These results gave novel evidence for such a long-proposed target interaction, which will be discussed in this chapter.
4.2 Results and discussion

4.2.1 Intrinsic fluorescent properties characterized in DMSO

We characterized the absorption and emission maximum wavelengths, extinction coefficient ($\varepsilon$), quantum yield ($\Phi$), and fluorescence brightness ($B$) of all the fifty-six FC compounds in DMSO. Overall, most compounds showed similar absorption properties, whereas large variation was observed for their fluorescence emission (Table 4.1, and Figure 4.1). Their maximal absorption wavelengths are typically between 390 nm and 460 nm, with extinction coefficients of 28,000 to 38,000. The few outliers of these ranges were compounds with significant structural variations, such as lack of the 4-dialkylamino group on B ring (FC55, FC56), limited confirmation flexibility by steric hindrance or fused ring (FC35, FC37), indole-chalcones (FC51-FC53), and bis-chalcones (FC45, FC46). Their emission spectra, on the other hand, is much more sensitive to structural differences, with the maximal emission ranging between 450 and 620 nm and the quantum yield between 0 and 0.40. Eighteen of these chalcone compounds showed a fluorescent brightness ($B > 6,000 \text{ M}^{-1}\text{cm}^{-1}$, e.g. FC7 – 13,200 M$^{-1}$cm$^{-1}$), comparable to fluorophores that have been widely used in biological detection/imaging (e.g. Cy3.18 – 6,000 M$^{-1}$cm$^{-1}$, Atto740 – 12,000 M$^{-1}$cm$^{-1}$)$^{(214)}$. These results overall suggest that a number of the synthesized chalcones have adequately bright intrinsic fluorescence as potential fluorescent probes.
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<th>Emi $\lambda_m$ (nm)</th>
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Table 4.1. The optical parameters of 56 FC compounds characterized in DMSO.
Figure 4.1. The optical properties of 56 chalcone compounds. (a) The absorption and (b) emission spectra of representative chalcones (FC3, FC4, FC7, FC9, FC11, FC16, FC39, FC41, FC48, FC49). (c) The extinction coefficient (ε) and the quantum yield (Φ), and (d) the brightness (compounds with brightness > 6,000 M⁻¹cm⁻¹ highlighted in red).
4.2.2 Structure-activity relationship regarding fluorescence properties

Some structural effects on chalcone’s fluorescence properties were observed (Figure 4.2). First, an electron-donating dialkylamino group at the 4-position on the B ring is essential for fluorescence, because compounds lacking this feature (FC55, FC56) were non-fluorescent. Second, the planar conformation of chalcone’s core structure, encompassing both rings and the propenone bridge, is important for optimal fluorescence, because compounds with steric hindrance that disfavors such a planar conformation (FC6, FC10, FC12, and FC35–C37) all showed significant decreases in quantum yield. Third, electron donating/withdrawing properties of the substituents on the A ring greatly influence the quantum yield. Weak electron-donating substituents were preferred (FC4, FC7, and FC11, relative to FC1), while either electron withdrawing (FC17, FC25–FC27, relative to FC1) or strong electron donating substituents (FC28 relative to FC1) significantly decreased the quantum yield. Fourth, an internal hydrogen bonding interaction with chalcone’s ketone moiety (FC13 relative to FC1) decreased the quantum yield. Lastly, an extended conjugation system (FC38–FC44) resulted in a red shift of the maximum emission wavelength and a decrease in quantum yield.
4.2.3 Environmental factors affecting fluorescence

Since the fluorescence of many fluorophores are highly sensitive to their environment\(^{(185)}\), the influence of biologically relevant factors, such as solvent, pH, and common biological components, should be characterized in developing biologically useful fluorophores (Figure 4.3). Compound C9 was chosen as the model compound to characterize such effects. We first evaluated the solvent effect and observed an overall positive correlation between solvent polarity and fluorescence quantum yield, with the exception of ethanol and water (Figure 4.3.a). Compound C9 showed no fluorescence in ethanol or water despite their high polarity. This is potentially due to the hydrogen bond formation between the solvent and the basic nitrogen atom of the dialkylamino group on chalcone’s B ring.\(^{(216)}\) With such an interaction, the nitrogen lone pair electrons cannot delocalize into the conjugation system, which is essential for chalcone’s fluorescence.\(^{(186)}\)

Given the importance of water in all biological systems, we characterized the effect of the
amount of water on C9’s fluorescence in DMSO. A sharp decrease in fluorescence was observed with increasing percentage of water; and near-complete quenching occurred with > 50% water (Figure 4.3.b). Nevertheless, such an effect could be partially reversed with low concentrations of additives, such as BSA (Figure 4.3.c), Triton-X100 and Tween-20 (not shown). The quantum yields of C9 under these conditions were 0.12 with 0.2% BSA, 0.12 with 0.1% Triton-X100, and 0.11 with 0.1% Tween-20. Such a fluorescence recovery is potentially due to the formation of local hydrophobic environments by the additives. The hydrophobic environment may sequester C9, making the dialkylamino nitrogen less available for hydrogen-bonding interaction with water. We next evaluated the effect of pH on C9’s fluorescence in aqueous solutions with 0.1% Triton-X100 as the additive. The fluorescence intensity remained decently constant within the biological pH range (pH 5-9) but decreased significantly when pH was lower than 3 (Figure 4.3.d). This may be due to the protonation of the dialkylamino nitrogen under low pH conditions. Lastly, since chalcones are well-known electrophiles to react with biological thiols via Michael addition (217), we tested the effect of such a reaction on C9’s fluorescence. Using N-acetylcysteamine as the model thiol as previously described (218), we monitored the extent of Michael addition reaction by 1H-NMR as the ratio of C9 to its thiol adduct, with the corresponding fluorescence change measured. The same extent of Michael addition and fluorescence reduction was observed (Figure 4.3.e), indicating that the thiol adduct was essentially non-fluorescent. This suggests that chalcone-based fluorescent probes would lose their
fluorescence if the mechanism of action involves covalent modification of the cellular
target via Michael addition.
Figure 4.3. Environmental factors that influence C9’s fluorescence properties. (a) The quantum yield of C9 in different solvents. (b) The effect of different percentage of water on C9’s fluorescence in DMSO. (c) Fluorescence recovery of C9 by BSA in water. (d) The effect of pH on C9’s fluorescence. (e) The effect of thiol adduct formation on C9’s fluorescence.
4.2.4 **Fluorescence-based tubulin binding assay**

Since tubulin is a potential target responsible for chalcone’s cytotoxicity as discussed above, we studied the potential interaction between chalcones and tubulin. Based on efficient fluorescence quenching by water and the possibility of fluorescence recovery upon protein binding, we first performed fluorescence-based binding assays with purified tubulin protein with three potent (FC8, FC16, FC35), one medium potent (FC1), and one inactive (FC4) compound. (Figure 4.4) Fluorescence increase and plateauing was observed with increasing concentration of compounds, indicating a typical one-site binding-saturation event. Interestingly, the potent α-methylated compound FC35, which doesn’t fluoresce in DMSO due to steric hindrance that disfavors the planar conformation, also exhibited fluorescence emission in the presence of tubulin, suggesting a conformational change that favors fluorescence upon tubulin binding. This assay also offers a convenient way to characterize their binding affinity to tubulin, which was determined as the Kd values from the curve. The inactive compound FC4 has a Kd value more than 20 times higher than the potent compounds, potentially explaining its poor cytotoxicity. However, the Kd values do not perfectly parallel their cytotoxic potencies, as compound FC1 with a moderate potency showed a higher affinity to tubulin than the potent FC16. This might be explained by either the difference between protein-based assay condition and cellular condition which contains other proteins that may sequester compounds through off-target binding, or a lack
of necessary causal relationship between tubulin binding and conformational disruption leading to microtubule destabilization. Since BSA was found to recover chalcone’s fluorescence from water quenching, the same fluorescence-based binding assay was carried out with BSA and the Kd values for were 4 μM for FC8 and 9 μM for FC4, on the same order of magnitude as tubulin binding affinity, again arguing for possible off-target binding with other proteins that are hard to characterize in detail with fluorescent probe approaches, posing a potential limitation of fluorescent probes for mechanistic studies.

Figure 4.4. Fluorescence-base tubulin binding assay of selected chalcones.

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4.2.5 Confocal-immunomicroscopy imaging with tubulin

We then sought evidence of potential direct interaction of cytotoxic chalcone FC8 with tubulin in cells via confocal microscopy imaging taking advantage of FC8’s intrinsic fluorescence. Pre-fixed and permeabilized A549 cells were stained with fluorescently...
labeled β-tubulin antibody and then treated with FC8. Fluorescence images were taken with a green channel for FC8 and a red channel for tubulin. Under the experimental conditions, FC8 unexpectedly showed wide-spread fluorescence in the cytosol with significant bright spots not corresponding to the tubulin network (Figure 4.5). These bright spots overall do not have well defined structures, suggesting potential precipitation of FC8. Nevertheless, a closer inspection revealed weak but discernible co-localization of FC8 with tubulin at the mitotic spindles, where tubulin was highly concentrated (Figure 4.5). Further attempts to reduce the bright spots and/or increase specific signal by varying FC8 concentration did not result in better images (data not shown). Given the high sensitivity of chalcone’s fluorescence to environmental factors, the non-optimal pattern may not accurately reflect the extent and specificity of biological interactions, as the high background noise may be due to compound precipitation or interaction with unknown environmental factors that favors fluorescence, while the low specific signal may be due to undesirable binding conformation that disfavors fluorescence. Nevertheless, the weak co-localization signal in cells did provide supporting evidence of direct cellular interaction of cytotoxic chalcones with tubulin.
**Figure 4.5.** Co-localization of FC8 with tubulin at mitotic spindles in A549 cells via confocal microscopy. Green channel: FC8 fluorescence; red channel: anti-tubulin fluorescence. White arrows indicate the mitotic spindle structures where weak but discernible co-localization of FC8 and tubulin.

### 4.3 Conclusion

In summary, we have investigated the influence of structural and environmental factors on chalcone’s intrinsic fluorescence, and explored their potential as chemical probes for the mechanistic investigation of chalcone’s cytotoxicity. Important structural effects were observed with chalcone’s fluorescence properties. The 4-dialkylamino group on the B ring and the overall planar conformation are essential for optimal fluorescence, while the electronic properties of the substituents on the A ring affect the quantum yield. With respect to environmental factors, water quenches chalcone’s fluorescence, which can be partially reversed by the addition of a minimal amount of biologically friendly additives.
Based on this observation, the fluorescence in detected cells may derive from chalcone’s binding to its cellular target(s) while the free form would have minimal fluorescence, which can be of great benefit to target identification. Finally, chalcone’s fluorescence would disappear upon covalent modification by thiols via Michael addition. This property is interesting as well for target/mechanistic investigation. For instance, we have observed the co-localization of FC8 with tubulin via fluorescence, which suggests that such an interaction is not mediated via covalent modification, consistent with the reversibility of cell cycle arrest effect (3.2.3). Taking advantage of their fluorescence properties, the potential interaction between chalcones and tubulin was studied for their cytotoxicity mechanistic investigation via fluorescence-based binding assay and cell-based confocal fluorescence microscopy. These results, though less than optimal, overall provided novel evidence of such direct target interaction, especially the direct physical interaction of cytotoxic chalcones with tubulin in cells revealed by confocal fluorescence microscopy, that deserves further investigation.

4.4 Experimental procedures

4.4.1 Fluorescence characterization
All optical properties were measured with a Varian Cary Eclipse fluorescence spectrophotometer. Compounds were made into 10-50 mM DMSO stock solutions, which were diluted to 3 μM with DMSO in a 1 cm quartz cuvette for the measurement of the maximum absorption wavelength. The same sample was then excited at the maximum absorption wavelength for the measurement of the maximum emission wavelength. The extinction coefficient was obtained by a linear regression of the absorption measured at 10, 20, and 30 μM in DMSO. The quantum yield was determined following an established procedure using fluorescein (Φ = 0.79) as the standard. Briefly, a linear regression of the total fluorescence emission against absorption at different concentrations was performed, with the absorption not exceeding 0.12. For solvent effect, the DMSO stock solution of FC9 (50 mM) was diluted to 3 μM in different solvents for quantum yield characterization. For pH effect, phosphate solutions at pH 1.0-9.0 containing 0.1% Triton X-100 were prepared by mixing 0.1 M monosodium phosphate with 0.1 M disodium phosphate or 1N HCl if needed to the specific pH, followed by addition of the detergent. The DMSO stock solution of FC9 (50 mM) was diluted to 3 μM in these solutions and the fluorescence was measured.

4.4.2 Fluorescence quenching by N-acetylcysteamine

FC9 (4.3 mg) was dissolved in 425 μL acetonitrile-d₃. To it, 25 μL phosphate buffer in deuterium oxide at pD 9.2 was added. The reaction started by the addition of N-
acetylcysteamine (NAC) stock solution in acetonitrile-d$_3$ (50 μL, 2.5 M). The final concentrations of FC9 and NAC were 25 and 250 mM, respectively. The formation of the thiol adduct was monitored by $^1$H-NMR. The doublets at 6.63-6.71 ppm, the characteristic signal of the adduct, and the doublets at 6.71-6.79 ppm, the characteristic signal of unreacted FC9, were integrated; and their ratio was calculated. One microliter of the mixture was diluted 10,000 folds with DMSO for fluorescence measurement at the beginning of the reaction and when it reached equilibrium.

### 4.4.3 Fluorescence–based binding assay

Tubulin was dissolved in general tubulin buffer at a concentration of 1 μM, and compounds were made into DMSO stock solution at varying concentrations between 0 and 2 mM. Binding was initiated by adding 5 μL of compound stock into 995 μL of tubulin solution so that the final compound concentrations were 0, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5 and 10 μM, with 0.5% DMSO. Fluorescence spectra were recorded after 10 minutes of incubation when the fluorescence becomes stable. The Kd of each compound was determined by fitting the total fluorescence to the drug concentrations by using a one-site binding model in Prism GraphPad.

### 4.4.4 Co-localization of FC8 and tubulin by confocal microscopy

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A549 cells were cultured the same way as detailed in 3.4.1. Cells were seeded in 96-well confocal plates at 6000/well. After attachment, cells were treated with media containing 5 μM chalcone compounds with 1% DMSO for 6 hours. Media was removed and cells were washed with PBS twice and treated with 4% PFA fixative at 37°C for 12 min. After three washes with PBS, cells were permeabilized with 0.05% Triton X-100/PBS for 5 min at 37°C, and then blocked with 5% donkey serum/PBS at 37°C for 8 min, followed by incubation with 1:100 sheep anti-tubulin antibody (Cat# ATN02, Cytoskeleton) at 37°C for 15 min. After three PBS washes, cells were then treated with 1:100 APC-conjugated donkey-anti-sheep antibody (Cat# 713-136-147, Jackson ImmunoResearch) at 37°C for 8 min. After two more PBS washes, cells were exposed to FC8 at 0.25 μM for 3 hours at 37°C. The medium was replaced with PBS and confocal images were taken immediately with a Nikon TE2000 inverted confocal microscope.
CHAPTER 5

Photoaffinity Labeling with Photoaffinity Probes for Cytotoxicity

Target Identification

The work in this chapter was performed in collaboration with Dr. Xingxin Yu. Specifically, initial labeling screening in Chapter 5.2.1 was performed together with Dr. Xingxin Yu.
5.1 Introduction

Among the various approaches to identify the direct cellular targets of bioactive compounds, a photoaffinity labeling (PAL) approach is of particular importance because of its ability to capture the direct drug-target interactions through the formation of a covalent bond upon UV exposure (Figure 5.1.a). Facile target identification is usually achieved by linking the on-target PAL probe to a fluorescent or biotin functional group, through synthetic methods such as the click reaction, for target visualization or enrichment. This approach, if applied to the intact cell system, can to a great extent unbiasedly reveal the direct cellular target profile of the drug candidate under physiologically relevant biological conditions.\(^{(183, 194-196)}\)

To the best of our knowledge, no chalcone-based PAL probes have been explored for the identification of the direct cellular targets of chalcones. In this chapter, we employed this approach to search for the cellular targets responsible for chalcone’s cytotoxicity. Among the seven azido chalcones (AC1-AC7) synthesized in Chapter 2.2.2, three compounds were selected as PAL probes (AC1, AC2, and AC3, Figure 5.1.b) for cytotoxicity mechanistic study, owing to their similar structures yet distinct cytotoxic potencies. Cell-based PAL experiments revealed clear and distinct target protein labeling patterns among these three probes, suggesting that these chalcones have a high level of discrimination in the cellular proteome. Interestingly one 52-kD protein was selectively labeled by AC3, the cytotoxic PAL probe, but not by the non-cytotoxic ones. Competition
with cytotoxic and non-cytotoxic chalcones lacking the PAL functional groups further supported this protein as the target responsible for chalcone’s cytotoxicity. Subsequent streptavidin-biotin-based target enrichment and mass spectrometry analysis unambiguously identified the target protein as β-tubulin. Our MS analysis also revealed the potential binding site for chalcones on β-tubulin. These data are valuable for the understanding and future optimization of chalcone’s cytotoxicity. The success also demonstrates the feasibility of PAL as a general approach to explore the cellular targets responsible for chalcone’s diverse bioactivities.

**Figure 5.1.** Design of chalcone-based photoaffinity probes for cytotoxicity target investigation. (a) The scheme of target protein labeling, enrichment, and MS identification
with chalcone-based photoaffinity labeling (PAL) probes. (b) Structures of the chalcone-based PAL probes and their cytotoxicity IC\textsubscript{50} values against A549 cells.

5.2 Results and discussion

5.2.1 Dose-dependent labeling of photoaffinity probe AC3 as compared to negative controls AC1 and AC2 reveals potential target protein

We first performed intact cell-based photoaffinity labeling experiments with our PAL probes AC1-AC3 with PAGE analysis visualized via the biotin tag. Briefly, A549 cells were pre-treated with the PAL probes, followed by the covalent bond formation between the PAL probes and their cellular targets upon UV activation of the azido functional group. Cells were lysed and the lysates were subjected to the click reaction via the propargyl functional group on the PAL probe with an azido-functionalized biotin (Figure 5.8 in Chapter 5.2.8, biotin-PEG-N\textsubscript{3}) so that the PAL probe-labeled targets were selectively tagged with a biotin moiety. Such modified lysates were then resolved by SDS-PAGE, followed by streptavidin-based Western Blotting (WB) to visualize the biotinylated proteins. The results (Figure 5.2.a) showed that these probes had distinct labeling patterns. For the non-cytotoxic probes, AC2 barely labeled any proteins while AC1 labeled predominantly one protein at ~ 55 kD and slightly another protein at ~ 36 kD. The cytotoxic probe AC3, on the other hand, dose-dependently labeled mostly three proteins (~ 36 kD, ~
52 kD, and ~ 55 kD). Among these three proteins, AC3 preferentially labeled the one at 52 kD (red arrow in Figure 5.2.a). The different labeling patterns among the three PAL probes in the cellular proteome suggest that they have distinct and highly selective cellular target profiles instead of a promiscuous non-specific protein interaction. In addition, the absence of any protein labeling by AC3 without UV activation (the last lane in Figure 5.2.a) indicated a non-covalent nature of the interactions between AC3 and its cellular targets, despite its α,β-unsaturated ketone functional group as a potential Michael acceptor.\textsuperscript{(149, 217)}

The distinct labeling profiles of structurally very similar probes support that the chalcone core structure in these probes is likely a privileged structure. More interestingly, the protein at 52 kD was only labeled by the cytotoxic probe and not by either of the two non-cytotoxic probes. These data suggest that this 52-kD protein may be the direct target responsible for chalcone’s cytotoxicity.
Figure 5.2. Intact cell-based photoaffinity labeling of the cellular target proteins via the cytotoxic and non-cytotoxic photoaffinity probes. (a) A dose-response photoaffinity labeling of the cellular proteins with the cytotoxic probe AC3 in comparison to the non-cytotoxic probes AC1 and AC2 and the necessity of UV activation for the covalent labeling; (b) Differential competition of AC3 labeled cellular proteins with the cytotoxic chalcone FC8, and the non-cytotoxic chalcone FC4. Red arrows indicate the 52 kD protein in the enriched samples.

5.2.2 Labeling competition between probe AC3 and cytotoxic/non-cytotoxic compounds lacking labeling ability confirms target protein

To further explore this, a set of competitive labeling experiments were performed (Figure 5.2.b). In brief, A549 cells were co-treated with AC3 and either a cytotoxic
chalcone FC8 (IC$_{50}$ = 0.79 µM) or a non-cytotoxic chalcone FC4 (IC$_{50}$ > 60 µM), both of which are structurally similar to AC3 but do not possess the PAL group (for structures of FC4 and FC8 see Table 2.1), followed by the standard photoaffinity labeling procedures as described above. The results (Figure 5.2.b, red arrow) showed that the cytotoxic FC8 significantly reduced the extent of labeling of the 52-kD protein by AC3, with no significant competing effects on the other two proteins. The non-cytotoxic FC4, on the other hand, reduced the extent of labeling of the proteins at 55 kD and 36 kD by AC3, with no competing effect on the 52-kD protein. These data provide additional evidence further supporting that the 52-kD protein is chalcone’s direct cellular target responsible for their cytotoxicity. Given that both α- and β-tubulin have a monomeric molecular weight of about 50 kD, these labeling results further pointed to tubulin as the potential direct target of chalcone’s cytotoxicity.

5.2.3 Interaction between probe AC3 and known anti-microtubule agents revealed by photoaffinity labeling

The hypothesis of tubulin as chalcone’s cytotoxic target was further investigated by PAL for potential interaction between AC3 and known anti-microtubule agents. Three anti-microtubule agents, namely paclitaxel, vinblastine, and colchicine, were selected as they are known to each bind at a different site on tubulin.$^{(14, 15)}$ As shown in Figure 5.3, while co-treatment of vinblastine did not significantly affect the labeling of the 52-kD
protein by AC3, both paclitaxel and colchicine significantly reduced the labeling extent with a positive dose response. All samples showed similar total β-tubulin level, suggesting the difference of labeling extent was not due to regulation of tubulin expression or degradation. These results not only further supported the hypothesis that cytotoxic chalcones directly interact with tubulin in cells, but also provided information of potential binding site. The vinca-binding site is not likely involved in chalcone’s binding to tubulin, since no labeling competition was observed with vinblastine. The fact that both paclitaxel and colchicine reduced the labeling extent may be explained by the hypothesis that one of them directly competes with AC3 for tubulin binding at the same site while the other inhibits AC3 binding allosterically by inducing conformational changes. This hypothesis is supported by previous literature report where a phenotypical antagonism between paclitaxel and colchicine was observed. (221)
Figure 5.3. Interaction between AC3 and anti-microtubule agents taxol, vinblastine, and colchicine by photoaffinity labeling. The three concentrations of each anti-microtubule agent were determined as 10×, 40×, and 100× of their individual IC\textsubscript{50} values obtained with the same cell line. Red arrows indicate samples where significant inhibition of AC3 labeling of the 52-kD protein was observed. Red arrows indicate the 52 kD protein in the enriched samples.

5.2.4 Enrichment of target protein through subcellular fractionation

Since the non-cytotoxic probe AC2 had minimal protein labeling in the intact cell-based target profiling, while AC1 showed an otherwise similar labeling pattern with AC3 except for the 52-kD putative target protein, AC1 was selected as the control probe to AC3 for later studies. In order to isolate the target proteins for mass spectrometry (MS)-based
analysis, we first attempted target enrichment based on a standard subcellular fractionation, hoping that the target proteins would be distributed in specific subcellular fractions. A549 cells, upon PAL experiments, were lysed into four fractions using a commercial kit – the cytoplasmic fraction (CF), the membrane fraction (MF), the nuclear fraction (NF) and the pellet fraction (PF). These four fractions were subjected to click reaction with an azido-functionalized biotin and then analyzed via streptavidin-based WB (Figure 5.4). It is clear that the majority of the PAL probe-labeled proteins were in the cytoplasmic fraction. The membrane fraction contained only a small amount of these proteins, which might be partly caused by cross-over among adjacent fractions. The non-specific signals (~ 75 kD and ~ 150 kD respectively, likely due to endogenous biotin-modified proteins) were not detectable in the cytoplasmic fraction. Through this standard cellular fractionation, we were able to enrich the proteins selectively labeled by the PAL probes and significantly reduce the endogenous background.
Figure 5.4. Subcellular fractionation of AC3 and AC1 treated cells. CF: cytoplasmic fraction; MF: membrane fraction; NF: nuclear fraction; PF: pellet fraction; Mix: combined fractions based on their relative abundance. The same amount of proteins was loaded onto each lane. Red arrow indicates the 52-kD target protein, enriched in CF. Red arrow indicates the 52 kD protein in the enriched samples.

5.2.5 Enrichment of labeled target protein through affinity purification with streptavidin-coated beads

To further enrich the PAL probe-labeled target proteins from unlabeled protein in the cytoplasmic fraction, streptavidin-biotin interaction-based affinity purification was performed. After the click reaction-based selective biotin modification of labeled proteins, the lysate was incubated with streptavidin-coated beads to separate the PAL probe-labeled proteins from the rest of the proteome. As expected, the supernatants after probe-labeled
target enrichment showed no biotin signal (Figure 5.5.a) while their Coomassie blue staining patterns were indistinguishable from the non-enriched control lysates (Figure 5.5.b), indicating successful removal of majority of proteins and capture of probe-labeled proteins. The enriched samples, upon release from the beads under denaturing conditions, showed similar patterns in biotin signal as the non-enriched control lysates (Figure 5.5.a) while their Coomassie blue staining showed minimal signal other than the two proteins at ~55 kD and ~52 kD (Figure 5.5.b), indicating successful release of probe-labeled proteins from streptavidin-coated beads. These two proteins are not the most abundant endogenous proteins in the non-enriched control lysates, further supporting chalcone’s specific interactions with certain cellular proteins instead of promiscuous interactions. These results demonstrated that the PAL-probe labeled proteins were efficiently and selectively enriched from the cytoplasmic fraction with minimal contamination of unlabeled proteins.
Figure 5.5. Enrichment of PAL probe labeled proteins using streptavidin-coated beads.

(a) Biotin-streptavidin-based Western blot, and (b) Coomassie blue staining of cytoplasmic lysates before and after enrichment with streptavidin-coated beads. Red arrows indicate the 52 kD protein in the enriched samples.

5.2.6 In-gel digestion followed by mass spectrometry analysis identifies the target protein as β-tubulin

The 52-kD protein band in the AC3 treated enriched sample lane was collected from the Coomassie blue stained gel (Figure 5.5.b). The gel regions of the same molecular weight from AC1 treated and DMSO samples were also collected as controls. These gel bands were subjected to in-gel trypsin digestion followed by MS analysis of the digested peptides for protein target identification. Five rounds of experiments were performed; the first three rounds were biological repeats while the last two rounds had slight procedural
modifications as detailed later. The MS-based peptide identification results from each round were searched against a UniProt human protein database using Proteome Discoverer (PD). The combined MS results from all five rounds revealed several isoforms of β-tubulin along with one α-tubulin isoform as the only target candidates for AC3 (Figure 5.6.a) that meet the following two criteria: a) having a PD score of greater than 10 in every round, and b) not keratin. The PD scores and #PSM (number of peptide-spectrum matches), two commonly used parameters for protein identification\(^{222, 223}\), of these protein hits in AC3 treated sample were also compared to the DMSO control sample and the AC1 treated sample in each round to eliminate false positives due to potential nonspecific interactions. All tubulin isoforms showed significantly higher PD scores and #PSM in AC3 treated sample than those in AC1 or DMSO control samples in all five rounds (Table 5.1), confirming that they were preferentially labeled by the cytotoxic AC3. The one α-tubulin isoform showed much lower PD score and # PSM than the top β-tubulin isoforms, suggesting that β-tubulin was the main protein labeled by C95, with a much weaker interaction with α-tubulin potentially through interacting at a site near the dimer interface on β-tubulin such as the colchicine-binding site\(^{224}\).
Figure 5.6. Identification of PAL probe labeled proteins. (a) Combined report of protein hits identified in five rounds of MS experiment of AC3 treated sample, and (b) overall coverage of tubulin beta chain (TUBB) from all five rounds combined. Hit filters applied for (a) are “description doesn’t contain ‘keratin’” and “score is > 10”.
### Table 5.1
Proteins identified as the top candidates from the PAL probe-based intact-cell proteome labeling experiment.

*a* Protein IDs, names, and gene symbols are based on UniProtKB database.

Among the β-tubulin isoforms, a high sequence coverage (77.03%) was achieved for the top hit TUBB (Figure 5.6.b). The theoretical peptides from TUBB upon trypsin digestion were compared to the detected peptides and all medium-sized theoretical peptides (5-35 amino acids) were unambiguously detected with high-confidence MS² spectra in AC3 sample (Table 5.2). These results strongly suggested tubulin, particularly β-tubulin, is the 52-kD protein that selectively interacted with the cytotoxic PAL probe AC3 in intact A549 cells.
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Table 5.2. Theoretical (blue and black) and detected (blue only) peptides from trypsin digestion of tubulin beta chain (TUBB). A lower case “c” denotes a carbamidomethylated cysteine, and a lower case “m” denotes an oxidated methionine.
5.2.7 Attempts to directly identify C95-modified peptides on β-tubulin

We first attempted to directly search for AC3-modified peptides from the trypsin-digested peptide samples (Rounds 1 and 2). The input for the MS² data search was based on the data of our model reaction of the UV-activated reaction of the probe (Chapter 5.2.8, Figure 5.7) with the theoretical modification of the azido-functionalized biotin (Figure 5.8, biotin-PEG-N₃). We also tried to enrich AC3-modified peptides from the trypsin-digested samples via streptavidin beads followed by MS² analysis (Round 3). All these attempts, however, resulted in no hits. We reasoned that the lack of success may be caused by the large hydrophobic probe-biotin functional group, which might significantly change the property of the modified peptide(s) and prevent their recovery from the PAGE or the C18 column (C18 column was used for desalting before MS analysis). The probe-biotin modification may also compromise the ionization of the modified peptide or MS² fragmentation. We therefore modified the experimental protocol to remove the desalting step and acquired the MS² data with CID (collision-induced dissociation) fragmentation mode (Round 4). This attempt again did not lead to any potential hits.

We then employed an UV-cleavable biotin azide (Figure 5.8), wishing to optimize the property of the C95-modified peptide(s) (Round 5). The labeled cytoplasmic lysates were subjected to click reaction with the UV-cleavable biotin azide, followed by enrichment, in-solution digestion, beads capture and UV cleavage of the labeled peptides. Again the experimental conditions were optimized using our model compound and MS
search was based on the MS fragmentation of the model compound (Chapter 5.2.8, Figure 5.9). Disappointingly, this attempt also did not identify any modified peptides with an unambiguous MS² spectrum.

5.2.8 Model reaction of UV-activated labeling, and model reaction of click reaction and photocleavage with UV-cleavable biotin azide

The binding sites on the target protein(s) for the PAL probes could be theoretically characterized via identifying PAL probe-labeled peptides and even the specific amino acids by data mining the MS² data.\(^{194, 225, 226}\) In order to confirm the exact structure of the probe modification and the exact molecular weight increase for MS searching, we performed model reactions. We first modeled the UV-activated labeling reaction. Literature evidence suggests that the UV-activated labeling reaction of azides, through formation of a key nitrene intermediate by eliminating a dinitrogen, results in an apparent insertion of the nitrene into the target molecule.\(^{183, 225, 226}\) This was confirmed with our model reaction. The results (Figure 5.7) clearly suggest that nitrene-insertion is the major reaction pathway as expected with a high-confidence MS² spectrum, without any significant undesired side-reaction to the chalcone scaffold.
In order to confirm the expected conversions during click reaction and final UV-cleavage from the beads, we performed model reaction as shown in Figure 5.9. The model compound with a propargyl group but no azido group was designed and synthesized in the same fashion as described in Chapter 2.4.4. Click reaction was performed for biotin tagging of the model compound, and the product purified by HPLC and confirmed with LC-MS$^2$ (not shown) was subjected to affinity purification with streptavidin-coated beads and photocleavage. The final mixture was analyzed by LC-MS$^2$, and the expected product was confirmed with a high-confidence MS$^2$ spectrum. These results altogether suggest that the click reaction-based biotin tagging, biotin-streptavidin-based affinity enrichment, and UV-cleavage reaction would work as expected in the real experiments with labeled proteins, despite the unsuccessful results with the real experiments (Chapter 5.2.6).
Figure 5.8. Structures of the two azido-functionalized biotins applied in this study.
Figure 5.9. Model reaction of capture and release with UV-cleavable biotin-azide analyzed by LC-MS².
5.2.9 Indirect binding-site characterization

We then resorted to using MS-based peptide quantitation as an indirect approach to search for probe-modified peptides. Our rationale was that if a trypsin-digested peptide from β-tubulin was modified by AC3, which has not been detected in our previous attempts, the relative abundance of this natural peptide detected then should decrease. The data analysis was performed following the reported label-free MS quantitation method. Briefly, the extracted ion chromatograms (EIC) of peptides with high-confidence MS spectra were obtained from the full MS chromatograms. The peak areas of the same peptide among different samples in the same experiment were integrated and compared to determine the relative abundance of such a peptide in the samples. Trypsin peptides resulting from autolysis were analyzed as quality controls for quantitation since they should have equal abundance in all the samples within the same experiment. As shown in Table 5.3, four unique peptides derived from trypsin consistently showed similar abundances in AC3, AC1 and DMSO treated samples, establishing the feasibility and reliability of this quantification method. Since TUBB was the top hit among tubulin isoforms, peptides of TUBB detected in Rounds 1 – 3 were analyzed. Rounds 1 – 3 were used for analysis because they were performed under the same conditions. The ratios of the abundance of the TUBB peptides in AC3 treated samples to that in the DMSO control samples were calculated (Table 5.4). Among the 14 peptides detected, the ratio of peptide N337-K350
(NSSYFVEWIPNNVK) was the only one that was out of the 99% confidence interval in all three rounds of experiments. The ratio was also the lowest among the 14 peptides in each round. The unusually low ratio of this peptide in AC3 to control samples suggested that AC3 likely modified this peptide, leading to a decrease in the amount of its natural form. A close inspection of the X-ray structure of a colchicine-tubulin heterodimer complex referred to in the text revealed that the terminal lysine residue of this peptide resides in the colchicine-binding site, suggesting that AC3’s binding site on tubulin may overlap with the colchicine-binding site (embedded figure in Table 5.4). This potential binding site is consistent with several other literature reports. For instance, docking studies suggested that cytotoxic chalcones fit well in the colchicine-binding site, and colchicine was shown to compete with cytotoxic chalcones for tubulin binding. It is also consistent with our previous labeling competition results in Chapter 5.2.3, and the fact that AC3 labeled α-tubulin as well, which indicated that AC3 may bind around the interface of the α-tubulin and β-tubulin heterodimer as discussed in Chapter 5.2.6.
Table 5.3. Quality control for MS-based quantitative analysis\(^a\) based on trypsin autolysis peptides. Integrated peak area in ctrl sample was normalized as 1.0 for the same peptide in the same round.

\(^a\) Parameters for quantitation: mass tolerance 10 ppm; Gaussian peak smoothing at 5 points.
Table 5.4. MS-based quantitative analysis\(^a\) of the natural peptides from β-tubulin (\textit{TUBB}) in Rounds 1 – 3. The embedded figure on the right (produced form PDB #1SA0) shows the structural relationship among α-tubulin (yellow), β-tubulin (green), the potential labeled peptide (red), and the colchicine (blue) binding site on β-tubulin.

\(^a\) Parameter for quantitation: mass tolerance 10 ppm; Gaussian peak smoothing at 5 points; only peaks integrated > 50000 in all samples in all three rounds were analyzed. \(^b\) Averages were given as the mean values of all of the analyzed peptides with 99% confidence interval.
5.2.10 Target validation through anti-β-tubulin Western blotting and tubulin polymerization assay

Anti-β-tubulin Western blotting was performed to further validate β-tubulin as the specifically labeled protein by AC3. The result (Figure 5.10.a) confirmed the presence of β-tubulin in the enriched AC3 treated cell samples which was absent in AC1 treated samples, which unambiguously validated the success of target identification described above. To further establish the mechanistic link between AC3’s direct interaction with tubulin and its cytotoxicity, we evaluated the effect of AC3 on tubulin polymerization following the standard microtubule formation assay.\(^{(30)}\) AC3 (3 µM) significantly inhibited microtubule formation, whereas AC1 and AC2 at the same concentration had much weaker inhibitory effects (Figure 5.10.b). This was highly consistent with the earlier observation that cytotoxic chalcones induce microtubule depolymerization in cells, and further established that AC3 upon direct interaction with tubulin in cells causes microtubule depolymerization which leads to M-phase cell cycle arrest and hence its cytotoxicity (Chapter 3.2.5).
Figure 5.10. Target validation by anti-β-tubulin WB and tubulin polymerization assay. (a) Anti-β-tubulin WB of non-enriched samples (8 µg) and streptavidin-enriched samples (from 40 µg of non-enriched sample). (b) Effect of compounds (3 µM) on tubulin polymerization by monitoring absorbance at 340 nm due to light scattering of polymerized microtubules.

5.3 Conclusion

We have developed three chalcone-based PAL probes to characterize chalcone’s direct interaction with their cellular targets, particularly the target(s) responsible for their cytotoxicity. Integrating photoaffinity labeling approach and other techniques, the results herein have provided compelling evidence that cytotoxic chalcones directly interact with β-tubulin in cells, leading to the disruption of cellular microtubule dynamics and consequently cell cycle arrest at G2/M phase. Although tubulin and a list of other proteins have been previously suggested to be involved in chalcone’s cytotoxicity, this work
demonstrates the direct chalcone-tubulin interaction in intact cells and the critical role of such an interaction in chalcone’s cytotoxicity. With indirect MS evidence about labeled peptide with the help of molecular docking, the colchicine-site on β-tubulin was identified as the putative binding site of chalcones. Detailed knowledge with respect to the binding site on tubulin deserves further investigation, which will provide structural information to explain the sharp SAR and guide further optimization of chalcone-based anti-microtubule agents. The PAL methodology employed herein can also be explored to search for the cellular targets responsible for chalcone’s other biological activities. Lastly the high target selectivity of the three chalcones in intact cells and their sharp SAR in cytotoxicity suggest that chalcone can be developed as a privileged structure for medicinal chemistry and chemical biology.

5.4 Experimental procedures

5.4.1 Photoaffinity labeling

A549 cells were cultured the same way as detailed in Chapter 3.4.1. Cells were seeded in 6-well plates (2×10^5 cells/well in 2 mL medium) or 10 mm petri dishes (2×10^6 cells/plate in 10 mL medium) and incubated for 20-24 h for attachment. The old medium was removed and the new medium, containing the corresponding probe at the specified
concentrations, was added. After 1.5 h incubation, plates or dishes were exposed to UV light via a UV illuminator (312 nm) for 8 min to activate the azide for probe-target covalent labeling. Cells were then collected via standard trypsin treatment, pelleted and lysed with the EDTA-free RIPA buffer. The protein concentration of the lysates were determined via the standard BCA assay. The lysates were diluted to a final concentration of 1 µg/µL with PBS and stored at -80°C till use.

5.4.2 Click reaction with biotin-N3

To the lysate sample (30 µL) was added 3 µL of each of these reagents in the following order: azido-functionalized biotin (0.35 mM in DMSO), tris(2-carboxyethyl)phosphine (14 mM in H₂O), tris-(benzyltriazolylmethyl)amine (2.8 mM in 3:1 t-butanol:DMSO), and copper(II) sulfate (16.8 mM in H₂O), to reach a final concentration of 25 µM, 1 mM, 200 µM, and 1.2 mM, respectively. For the click reaction using subcellular fraction lysates, which contained EDTA (1 mM), concentration of copper(II) sulfate was increased to 2 mM to achieve optimal reaction efficiency. The reaction mixture was incubated at room temperature for 1 h in the dark. For larger scale sample preparation, up to 500 µL of lysate was used and all reagents were proportionally adjusted.

5.4.3 Western blotting analysis
Protein samples were resolved with the Novex NuPAGE gel electrophoresis system following the manufacturer’s protocol using commercial 4 – 12% SDS-PAGE gels (Invitrogen #NP0322). The proteins in the gel was transferred onto a cellulose membrane and blocked with 1% BSA in PBST (0.05% Tween-20/PBS) for 1h. The membrane was washed twice with PBST, then incubated with streptavidin-HPR conjugate (1:5000, BioLegend #405210) in PBST for 1h. For anti-β-tubulin experiments, membrane was blocked with 5% BSA in TBST (0.1% Tween-20/TBS) for 1 h and then incubated with HRP-conjugated β-tubulin antibody (1:1000, Cell Signaling Technology #5346) in TBST containing 5% BSA for overnight at 4°C. After three washes with PBST, the membrane was incubated in SuperSignal West Pico chemiluminescent substrate mix (Thermo Scientific #34080) for 8 min with gentle shaking. The autoradiology film (GeneMate #F-9023-5X7) was then exposed to the membrane for a suitable period (typically within 2 min) and developed in an automatic film developer (Konica Minolta SRX-101A).

5.4.4 Subcellular fractionation

A549 cells, cultured in 10 mm petri dishes, were treated by the PAL probes and exposed to UV the same way as described above. Cells were then collected via trypsin treatment and fractionated by using a cellular fractionation kit (Thermo Scientific #78840) following the manufacturer’s protocol. Briefly, cell pellet (≈ 10^7 cells) was sequentially
extracted with cytoplasmic extraction buffer (400 µL, 10 min at 4°C, centrifuged at 500×g for 5 min), membrane extraction buffer (200 µL, 10 min at 4°C, centrifuged at 3,000×g for 5 min), nuclear extraction buffer (75 µL, 30 min at 4°C, centrifuged at 5,000×g for 5 min), RNase-containing nuclear extraction buffer (50 µL, 5 min at 37°C, centrifuged at 17,000×g for 5 min), and pellet extraction buffer (50 µL, 10 min at room temperature, centrifuged at 17,000×g for 5 min). Protein concentrations in each fraction were determined following the BCA method and then diluted to 1 µg/µL accordingly with PBS. Protein lysates were stored at -80°C till use.

5.4.5 Affinity purification with streptavidin-coated beads

Streptavidin T1® beads slurry (67 µL, Invitrogen #65601) was washed thoroughly with PBS in a microcentrifuge tube. The beads were then incubated with the cytoplasmic fraction lysate (200 µL) with gentle rotation via a rotary shaker (4°C, overnight). The beads were washed with PBST (400 µL × 2), 1M NaCl in 0.1% Tween-20/PBS (400 µL × 2), and 0.5% SDS/PBS (100 µL). The beads were then mixed with 0.5% SDS/PBS (14 µL) and heated (95°C, 5 min) to release the proteins from the beads. The protein sample was then subjected to SDS-PAGE analysis. Typically 5% of such protein sample was used for WB analysis while the remaining sample was used for Coomassie blue staining, in-gel digestion and MS analysis.
5.4.6 Staining and in-gel digestion

The SDS-PAGE gel was stained with Imperial® Coomassie staining (Thermo Scientific #24615) to visualize the target proteins. The gel region corresponding to protein MW of around 52 kD was cut out and diced into 1 mm cubes. To the diced gel samples was added ammonium bicarbonate (25 mM, 100 µL) and dithiothreitol (300 mM, 10 µL). The mixture was incubated for 15 min at 50°C. Then saturated iodoacetamide (10 µL) was added, and incubated for 15 min at room temperature. The supernatant was removed and the gel pieces were washed with a mixture of 1:1 (v/v) acetonitrile:25 mM ammonium bicarbonate (100 µL × 2) and acetonitrile (100 µL). The residual solvent was removed via speed-vac and the gel pieces were re-suspended in ammonium bicarbonate (25 mM, 75 µL) and trypsin (0.1 µg/µL, 25 µL) was added to the gel pieces and incubated at 37°C for overnight. The supernatant was collected. The gel pieces were washed with 0.1% formic acid in 60% acetonitrile/25 mM ammonium bicarbonate (100 µL × 2). The washes were combined with the supernatants and dried via speed-vac. The dried samples were reconstituted in 0.1% formic acid (15 µL) and then desalted with ZipTip C18 column (Millipore #ZTC18S096) following manufacturer’s instruction.

5.4.7 Mass spectrometry (MS) analysis
The final desalted samples were analyzed by LC-MS$^2$ using a 75 µm × 11 cm, 15 µm orifice, Luna C18 (Phenomenex), 5 µm, 80 Å LC column, and a Thermo Scientific Orbitrap Velos (Rounds 1-4) or Fusion (Round 5) MS system. The MS$^1$ spectra were collected with an orbitrap detector (at a resolution of 30,000), and the MS$^2$ spectra were collected with either HCD (for rounds 1-3, 5; at a normalized collision energy of 40.0) or CID (for round 4, at a normalized collision energy of 40.0) MS$^2$ fragmentation with an orbitrap detector (at a resolution of 7,500). The eluent was acetonitrile/H$_2$O (0.1% formic acid) with a gradient from 2% to 35% within 1 hour at a flow rate of 300 µL/min. The raw peptide data thus collected was searched by Proteome Discoverer with Sequest algorithm against UniProt human proteome database to identify protein hits. Default protein grouping was performed, and results were filtered with the following two criteria: a) “description” field doesn’t contain “keratin”, and b) “score” is greater than 10 by default.

5.4.8 Model reaction of UV-activated labeling, and model reaction of click reaction and photocleavage with UV-cleavable biotin azide

The model reaction of UV-activated labeling (Figure 5.8) was performed as follows: AC3 stock solution was diluted in water at 5 µM, and 100 µM of the diluted aqueous solution was placed in a well on a 96-well plate. The plate was then exposed to 312 nm UV light the same way as the cell-based labeling experiments for 8 minutes. This sample was then subjected to LC-MS$^2$ analysis (Agilent 1100 LC/MSD). The
fragmentation of major peaks was manually assigned based on the structure of the starting material and the expected product.

The model compound in Figure 5.10 was synthesized in a similar fashion as the other chalcones. The biotinylated model compound was prepared by a click reaction with the UV-cleavable biotin-N₃ in a similar fashion as the real experiment and the product was collected by preparative HPLC. The product was confirmed by LC-MS² and made into ~60 µM methanol stock solution, which was then diluted to ~3 µM with PBS for enrichment with streptavidin-coated beads (100 µL sample with 50 µL beads). The enrichment supernatant was confirmed by HPCL to not have any remaining biotinylated model compound. The beads were then exposed to a hand-held UV lamp (365 nm) for 30 minutes in 30 µL 60% acetonitrile/water and the final supernatant was analyzed by LC-MS² (Agilent 1100 LC/MSD) to confirm the expected product.

5.4.9 MS-based peptide quantitation

Xcalibur Qual browser was used to view and analyze the raw data from the MS experiments. Exact m/z values were calculated for peptides identified with Proteome Discoverer, and used to extract exact mass ion chromatograms from the full MS data. Parameters for peak extraction were as follows: 10 ppm for mass tolerance and Gaussian peak smoothing at 5 points. Integrated peak areas below 50000 were marked as < 50000
based on the detection limit. The peaks thus obtained were confirmed by manually inspecting the retention time, the charge state, and the isotopic pattern.
CHAPTER 6

Other Potential Targets Revealed by Combinatorial Probes

The work in this chapter was performed in collaboration with Jiewei Jiang and Alex Strom, who helped with the synthesis of the probes.
6.1 Introduction

As detailed in the preceding chapter, we have successfully applied photoaffinity labeling approach to identify chalcone’s cytotoxic target, where β-tubulin was unambiguously identified as the direct cellular target. Encouraged by these successful results, we believe that further exploration of chalcone-based photoaffinity probes presents a promising approach to unravel chalcone’s direct cellular target interactions that are potentially responsible for their various bioactivities. Combinatorial chemistry, as an efficient way to synthesize large numbers of compounds, has been widely applied in library synthesis for various applications,\((229-231)\) including the syntheses of chemical probes.\((232, 233)\) In a notable example of such an application, Sieber et al.\(^{234}\) constructed a peptide library containing PAL moiety and studied their interaction with different types of metalloproteases. The simple and reliable synthesis of chalcones via the aldol condensation reaction of substituted acetophenones and benzaldehydes has made it possible to construct chalcone compound libraries through combinatorial chemistry.\((186, 235-237)\) In this chapter, by combining the concepts of combinatorial chemistry and PAL approach, we constructed a focused library of thirty-six novel chalcone-based photoaffinity probes, and investigated their cellular protein interactions by PAL approach for the identification of potential cellular targets of chalcone-based compounds.
6.2 Results and discussion

6.2.1 Design and synthesis of combinatorial probe library

The chalcone-based PAL probe library was constructed by a similar strategy as described in Chapter 2.2.2, where an aryl azide for photoaffinity labeling and an alkyne moiety biotin tagging were separately introduced onto different positions of the building blocks, acetophenones (A) and benzaldehydes (B), through established methods. (Scheme 6.1) A methoxy substituent was also incorporated onto some of these building blocks at various positions to introduce some structural diversity to the library. A final cross-coupling of A and B via an aldol condensation reaction gave the fully functionalized chalcone PAL probes (C). (Scheme 6.1)

The protocol of the aldol condensation was adapted for efficient combinatorial chemistry. Briefly, ethanolic stock solutions of building blocks A (0.25 M) and B (1 M) were prepared. Each reaction was carried out by mixing 400 µL of A stock (0.1 mmol), 150 µL of B stock (0.15 mmol), and 16.7 µL of aqueous KOH solution (15M, 0.25 mmol) in a 1.5 mL Eppendorf tube. The tube was mixed on a rotary tube mixer in dark for overnight. In most cases, the product precipitated out from the reaction mixture. Product thus formed was collected by centrifugation and washed with ice-cold ethanol four times for purification. The product was dried in a speed-vac for four hours to remove residual ethanol. This protocol, in most cases, gave good to excellent yields with > 90% purity by
NMR without any further purification. The ease of operation and eco-friendliness added to the advantages of this modified method, making it especially suitable for combinatorial synthesis. For the few compounds that did not precipitate, normal phase chromatography was performed to obtain the pure product.

Overall, we have obtained six building block A’s and six building block B’s, (Table 6.1). A library of thirty-six PAL probe C’s was constructed by the combinatorial cross-coupling of A and B. (Table 6.2)

Scheme 6.1. General synthetic route of chalcone probes containing alkyne and azide functionalities.
Table 6.1. Structures of building block acetophenones (A’s) and benzaldehydes (B’s).

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Table 6.2. Final chalcone probes from building blocks A’s and B’s, with yield showing in parentheses.

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<td>A4</td>
<td>C41 (62%)</td>
<td>C42 (74%)</td>
<td>C43 (78%)</td>
<td>C44 (92%)</td>
<td>C45 (81%)</td>
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<tr>
<td>A5</td>
<td>C51 (84%)</td>
<td>C52 (87%)</td>
<td>C53 (78%)</td>
<td>C54 (90%)</td>
<td>C55 (84%)</td>
<td>C56 (85%)</td>
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<td>A6</td>
<td>C61 (40%)</td>
<td>C62 (75%)</td>
<td>C63 (87%)</td>
<td>C64 (68%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C65 (80%)</td>
<td>C66 (85%)</td>
</tr>
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</table>

<sup>a</sup>. Product was isolated by column chromatography.

6.2.2 Whole cell-based screening of chalcone PAL probes to profile potential targets

We then screened the probe library for cell-based photoaffinity labeling in A549 lung carcinoma cells following our established method described in Chapter 5. Briefly, A549 cells treated with the probe compounds were exposed to UV (312 nm) to initiate the
PAL reaction of the probes with potential target proteins. The cells were then lysed, and the probe-modified proteins in the lysates were tagged with a biotin moiety through a click reaction with an azido-functionalized biotin. Such samples were analyzed with streptavidin-HRP-based Western blotting to visualize the biotin-containing protein bands (Figure 6.1). Overall, the labeling patterns of different probes shared some level of similarity, with a few proteins being widely labeled by many probes, such as the bands at ~ 55 kD, ~ 50 kD, and ~ 38 kD (red arrows for C23 and C66 in Figure 6.1). However, significant differences were observed as well. First, some compounds (C21-C26 and C31-C36 with the propargyloxy group at the 3'- or 4'-position respectively) have lighter overall labeling than others, which may indicate less non-specific interactions with proteins. Second, the labeling preference is different among the probes. For example, C23 almost exclusively labeled the ~ 55 kD band, whereas C66 preferentially labeled the ~38 kD band. Third, one unique protein at ~ 58 kD was specifically labeled by C33 and not by any other probe (yellow arrow for C33 in Figure 6.1). Such a degree of diversity, though not perfect, is decently satisfactory considering the close similarity of their chemical structures. Such a high sensitivity of protein interaction profiles to subtle structural differences strongly suggest that chalcone can be a privileged template for medicinal chemistry research, and encourages the exploration of more structurally diverse chalcone PAL probes. Based on their labeling patterns, we selected probe C23 (for ~ 55 kD band), C33 (for ~ 58 kD band), and C66 (for ~50 kD and ~ 38 kD bands) to proceed further for target enrichment and MS-based target identification.
Figure 6.1. Labeling results of all thirty-six chalcone probes at 5 µM. Red arrows indicate the 55-kD, 50-kD, and 38-kD bands labeled by multiple probes, while the yellow arrow indicates the 58-kD band specifically labeled by C33. All of these highlighted bands were selected for further enrichment and identification.

6.2.3 Identification of selected protein hits through target enrichment and mass spectrometry

Through subcellular fractionation according to our established protocol (Chapter 5.2.1), we found all signals of interest mainly in the cytoplasmic lysates (Figure 6.2.a). To further enrich the labeled proteins from the total cytoplasmic lysates, samples were subjected to click reaction for biotin tagging, and the proteins tagged with biotin were then enriched by affinity purification with streptavidin-coated beads. The non-enriched and enriched samples were analyzed with streptavidin-HRP-based WB (Figure 6.2.a) to confirm the success of enrichment. As expected, all signals of interest in the initial labeling
screening were retained in the enriched cytoplasmic lysate samples. The enriched samples were then analyzed with SDS-PAGE with Coomassie blue staining (Figure 6.2b). Bands of interest were cut out from the stained gel for in-gel trypsin digestion and MS-based identification following established protocol (Chapter 5.2.5).

**Figure 6.2.** Streptavidin-biotin-based enrichment of labeled proteins by selected probes. (a) Anti-biotin WB of non-enriched and enriched cytoplasmic lysates labeled with probes at 5 µM, and (b) Coomassie blue staining of the same enriched samples. Red arrows indicate the signals of interest for target identification.

The database search of MS results of the trypsin digests of these bands was performed with Proteome Discoverer (PD) against a human protein database for hit identification, and the top five hits for each band are summarized in Table 6.3. For the ~58 kD band, pyruvate kinase PKM (*PKM*) was identified as the predominant protein species, as its PD score and #PSM (number of peptide-spectrum match), two commonly used parameters for protein identification, were both significantly greater than any
other hits by more than 7 folds. These parameters were also significantly higher in the treated sample than the control sample by ~ 4 folds. Similar observation was made for the ~ 55 kD band, where retinal dehydrogenase 1 (ALDH1A1) was unambiguously identified as the predominant protein species for this band. For the ~ 50 kD band, however, the predominance of a single protein was not clearly observed. Instead, several proteins, including two isoforms of elongation factors 1-alpha (EEF1A1 and EEF1A2), two isoforms of tubulin beta (TUBB and TUBB4), and alpha-enolase (ENO1), were identified with less than 2-fold difference in PD score and #PSM. These parameters were higher in the treated sample than the control by ~ 2-3 folds for all these protein hits, indicating specific labeling by the probe compound and potential co-contribution to the overall labeling signal. Similar observation was made for the ~ 38 kD band, where glyceraldehyde-3-phosphate dehydrogenase (GAPDH), two aldo-keto reductase family 1 members (AKR1C2 and AKR1B10), and aldose reductase (AKR1B1) were identified with similar PD scores and #PSMs, all higher in the treated sample than the control. These results suggest that some probes have higher target specificity than others.
### 58 kD band

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<td>retinal dehydrogenase 1</td>
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### 38 kD band

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<tr>
<td>P04406</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>GAPDH</td>
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<td>21</td>
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Table 6.3. MS identification of target hits of selected protein bands. The following filters were applied for result display: 1) “description” field does not contain “keratin”, and 2) MW (molecular weight) is within ±5 kD from the expected values based on PAGE observations. Top five hits of each protein band are shown.

* Protein IDs, names, and gene symbols are based on UniProtKB database.
6.2.4 Target validation by Western immunoblotting with specific antibodies

In order to confirm that these proteins were indeed labeled by the probe compounds and enriched through biotin-streptavidin interaction, we performed Western immunoblotting for the top-ranking protein hits with the highest scores of each band (Figure 6.3). Among the enriched lysate samples of C33, C23, C66, and control, the PKM signal only showed in the C33 treated sample. This was in agreement with the initial labeling screening results (Chapter 6.2.2) and suggested that PKM was specifically labeled by C33, but not by other probes with close structural similarity such as C23. Similarly, the ~ 50 kD hit EEF1A (including both EEF1A1 and EEF1A2 isoforms) showed more labeling by C66 as compared to C33 and C23. For the ~ 55 kD band ALDH1A1, although it was selected as a hit for C23 because of its overall clean labeling background in C23, it actually showed slightly stronger signal in C66 and C33. This is not surprising since the ~ 55 kD band showed up in the initial labeling results of the other two compounds in good intensity as well (Figure 6.1). Finally, we were not able to confirm the specific labeling and enrichment of the ~ 38 kD hit GAPDH, as no signal showed up in the enriched samples. This could be due to a low labeling efficiency or the possible blockage of antibody recognition epitope by the labeling. Since none of these proteins were detectable in the control sample, these results confirmed the selective and direct interactions of PKM with C33, EEF1A mostly with C66, and ALDH1A1 with C23, C33 and C66 in A549 cells. The differential targeting
profiles among C23, C33 and C66 provide evidence suggesting that chalcone can be chemically modified to tailor its cellular target profile.

![Figure 6.3. Validation of top target hits by Western immunoblotting.](image)

**6.3 Conclusion**

In this chapter, we have constructed a combinatorial library of thirty-six chalcone-based PAL probes by combining six alkyne-containing acetophenones and six azido-substituted benzaldehydes via a simple and environmentally friendly method. In order to explore the sensitivity of protein target profile to subtle structural changes, limited structural diversity was introduced into the library by varying the positions of the azido and the propargyloxy groups, and the introduction of a methoxy substituent at different positions. To our satisfaction, such minor structural variations indeed resulted in a decent degree of difference in target profiles among library members, further supporting chalcone
as a privileged template instead of a promiscuous one. Especially, pyruvate kinase was selectively labeled by compound C33 among all the thirty-six probes. In addition, retinal dehydrogenase 1 and elongation factor 1-alpha were also confirmed as being labeled by selected chalcone probes, although with less selectivity among probes. These results indicate that they may be the potential molecular targets of chalcones, which deserves further investigation. It would be interesting to study the functional outcomes of such interactions, which may potentially account for their various biological activities. These results also strongly encourage further expansion of the probe library to incorporate more structural diversity, so that more potential targets of chalcones may be revealed.

6.4 Experimental procedures

6.4.1 General procedures for the synthesis of substituted acetophenone (A) and benzaldehyde (B) intermediates

General procedures described in Chapters 2.4.1 and 2.4.3 were followed to synthesize the intermediates. The only exception is with 2-azidobenzaldehyde, where the copper-catalyzed method did not give a clean conversion. An alternative route for 2-azidobenzaldehyde was adopted.$^{(238)}$ Starting material 2-nitrobenzaldehyde (1.0 mmol, 1 eq) was dissolved in hexamethylphosphoramide (5 mL, 0.2 M) at room temperature and
sodium azide (2.0 mmol, 2 eq) was added with stirring. The reaction was allowed to proceed at room temperature with stirring for 24 hours and the resulting mixture was diluted with diethyl ether (50 mL) and washed with brine (50 mL × 2). The mixture was concentrated and purified by column chromatography to give the pure product. Characterizations of novel compounds are listed below.

**1-(4-methoxy-2-(prop-2-yn-1-yloxy)phenyl)ethan-1-one (A4)**

$^1$H NMR (500 MHz, CDCl$_3$): δ 7.88-7.81 (1H, m), 6.62-6.54 (2H, m), 4.79 (2H, d, $J = 2.5$ Hz), 3.86 (3H, s), 2.60 (3H, s), 2.56 (1H, t, $J = 2.5$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 197.57, 164.26, 158.85, 132.79, 121.75, 106.12, 99.74, 77.74, 76.25, 56.22, 56.59, 31.97. MS (ESI, positive) calculated m/z for C$_{12}$H$_{12}$O$_3$ ([M+H]$^+$): 205.1; found: 205.1.

**1-(5-methoxy-2-(prop-2-yn-1-yloxy)phenyl)ethan-1-one (A5)**

$^1$H NMR (500 MHz, CDCl$_3$): δ 7.29 (1H, dd, $J = 2.0$ Hz, 1.0 Hz), 7.06-6.99 (2H, m), 4.75 (2H, d, $J = 2.5$ Hz), 3.80 (3H, s), 2.65 (3H, s), 2.53 (1H, t, $J = 2.5$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 199.36, 154.35, 151.32, 129.71, 120.26, 115.42, 113.92, 78.30, 76.14, 57.22, 55.93, 32.07. MS (ESI, positive) calculated m/z for C$_{12}$H$_{12}$O$_3$ ([M+H]$^+$): 205.1; found: 205.1.

**1-(2-methoxy-6-(prop-2-yn-1-yloxy)phenyl)ethan-1-one (A6)**
1H NMR (500 MHz, CDCl₃): δ 7.26 (1H, t, J = 8.5 Hz), 6.68 (1H, d, J = 8.5 Hz), 7.60 (1H, d, J = 8.5 Hz), 4.69 (2H, d, J = 2.5 Hz), 3.80 (3H, s), 2.49 (1H, t, J = 2.5 Hz), 2.48 (3H, s). 13C NMR (125 MHz, CDCl₃): δ 202.38, 156.88, 154.70, 130.58, 121.53, 105.73, 104.98, 78.35, 75.92, 56.60, 55.99, 32.48. MS (ESI, positive) calculated m/z for C₁₂H₁₂O₃ ([M+H]+): 205.1; found: 205.1.

3-azido-2-methoxybenzaldehyde (B4)

1H NMR (500 MHz, CDCl₃): δ 10.37 (1H, s), 7.61 (1H, dd, J = 8.0 Hz, 1.5 Hz), 7.33 (1H, dd, J = 8.0 Hz, 1.5 Hz), 7.22 (1H, t, J = 8.0 Hz), 3.99 (3H, s). 13C NMR (125 MHz, CDCl₃): δ 189.26, 154.92, 134.15, 130.60, 126.14, 125.06, 124.89, 63.92. MS (ESI, positive) calculated m/z for C₈H₇N₃O₂ ([M+H]+): 178.1; found: 178.1.

3-azido-4-methoxybenzaldehyde (B5)

1H NMR (500 MHz, CDCl₃): δ 9.86 (1H, s), 7.65 (1H, dd, J = 8.5 Hz, 2.0 Hz), 7.56 (1H, d, J = 2.0 Hz), 7.01 (1H, d, J = 8.5 Hz), 3.98 (3H, s). 13C NMR (125 MHz, CDCl₃): δ 190.22, 156.83, 130.43, 129.86, 129.15, 120.55, 111.68, 56.43. MS (ESI, positive) calculated m/z for C₈H₇N₃O₂ ([M+H]+): 178.1; found: 178.1.

5-azido-2-methoxybenzaldehyde (B6)

1H NMR (500 MHz, CDCl₃): δ 9.86 (1H, s), 7.64 (1H, dd, J = 8.5 Hz, 2.0 Hz), 7.55 (1H, d, J = 2.0 Hz), 7.01 (1H, d, J = 8.5 Hz), 3.97 (3H, s). 13C NMR (125 MHz, CDCl₃): δ
190.22, 156.83, 130.42, 129.86, 129.15, 120.54, 111.68, 56.43. MS (ESI, positive) calculated m/z for C₈H₇N₃O₂ ([M+H]⁺): 178.1; found: 178.1.

6.4.2 General procedures for the synthesis of chalcone (C) final products

Stock solutions of building blocks A (0.25 M) and B (1 M) were prepared by dissolving appropriate amount of compound in ethanol, while concentrated KOH stock solution (15 M) was prepared in H₂O. Each reaction was started by adding into a 1.5 mL Eppendorf tube 400 µL of A stock (0.1 mmol), 150 µL of B stock (0.15 mmol), and 16.7 µL of KOH stock (0.25 mmol). The tube was mixed on a rotary tube mixer in dark for overnight. In most cases, the product precipitated out from the reaction mixture. Product thus formed was collected as a pellet by centrifugation and washed with ice-cold ethanol four times for purification. After decanting the final wash, the product pellet was dried in a speed-vac for four hours to remove residual ethanol. This protocol, in most cases, gave good to excellent yields with > 90% purity by NMR without any further purification. For the few compounds that did not precipitate, column purification was performed to obtain the pure product. All thirty-six chalcones are novel compounds, and their characterizations are listed below.

(E)-3-(2-azidophenyl)-1-(2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C11)
Yield: 66%. $^1$H NMR (500 MHz, CDCl$_3$): δ 7.85 (1H, d, $J = 16.0$ Hz), 7.68 (1H, d, $J = 7.5$ Hz), 7.66 (1H, dd, $J = 7.5$ Hz, 1.5 Hz), 7.49 (1H, td, $J = 7.5$ Hz, 1.5 Hz), 7.44 (1H, d, $J = 16.0$ Hz), 7.41 (1H, td, $J = 7.5$ Hz, 1.5 Hz), 7.20 (1H, d, $J = 8.0$ Hz), 7.16 (1H, t, $J = 7.5$ Hz), 7.12 (1H, d, $J = 8.5$ Hz), 7.11 (1H, t, $J = 7.5$ Hz), 4.79 (2H, d, $J = 2.5$ Hz), 2.53 (1H, t, $J = 2.5$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 192.67, 156.16, 139.69, 137.70, 132.90, 131.34, 130.81, 130.10, 128.66, 128.52, 126.99, 125.00, 122.02, 118.99, 113.48, 78.24, 76.15, 56.60. MS (ESI, positive) calculated m/z for C$_{18}$H$_{13}$N$_3$O$_2$ ([M+H]$^+$): 304.1; found: 304.1.

**(E)-3-(3-azidophenyl)-1-(2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C12)**

Yield: 95%. $^1$H NMR (500 MHz, CDCl$_3$): δ 7.68 (1H, dd, $J = 8.0$ Hz, 2.0 Hz), 7.58 (1H, d, $J = 16.0$ Hz), 7.50 (1H, ddd, $J = 8.5$ Hz, 7.5 Hz, 1.5 Hz), 7.44 (1H, d, $J = 16.0$ Hz), 7.41-7.34 (2H, m), 7.26 (1H, d, $J = 1.5$ Hz), 7.14-7.08 (2H, m), 7.05 (1H, dt, $J = 7.0$ Hz, 2.0 Hz), 4.80 (2H, d, $J = 2.5$ Hz), 2.56 (1H, t, $J = 2.5$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 192.23, 156.22, 141.91, 140.86, 137.12, 133.14, 130.86, 130.33, 129.82, 128.25, 125.34, 122.05, 120.69, 118.42, 113.38, 78.13, 76.38, 56.52. MS (ESI, positive) calculated m/z for C$_{18}$H$_{13}$N$_3$O$_2$ ([M+H]$^+$): 304.1; found: 304.1.

**(E)-3-(4-azidophenyl)-1-(2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C13)**

Yield: 59%. $^1$H NMR (500 MHz, CDCl$_3$): δ 7.66 (1H, dd, $J = 7.5$ Hz, 1.5 Hz), 7.60 (2H, d, $J = 8.5$ Hz), 7.59 (1H, d, $J = 16.0$ Hz), 7.49 (1H, td, $J = 8.0$ Hz, 1.5 Hz), 7.37 (1H,
d, \( J = 16.0 \text{ Hz} \)), 7.11 (1H, d, \( J = 8.0 \text{ Hz} \)), 7.11 (1H, t, \( J = 7.5 \text{ Hz} \)), 7.04 (2H, d, \( J = 8.5 \text{ Hz} \)), 4.80 (2H, d, \( J = 2.0 \text{ Hz} \)), 2.53 (1H, t, \( J = 2.0 \text{ Hz} \)). \(^{13}\text{C NMR} \) (125 MHz, CDCl\(_3\)): \( \delta \) 192.44, 156.12, 142.27, 142.02, 132.91, 132.15, 130.77, 130.14, 130.12, 126.74, 122.05, 119.61, 113.48, 78.24, 76.23, 56.59. MS (ESI, positive) calculated m/z for \( \text{C}_{18}\text{H}_{13}\text{N}_{2}\text{O}_{2} \) ([M+H]\(^+\)): 304.1; found: 304.1.

\( (E)-3-(3\text{-azido-2-methoxyphenyl})-1-(2-(\text{prop-2-yn-1-yloxy})phenyl)\text{prop-2-en-1-one} \)  
(C14)

Yield: 72\%. \(^{1}\text{H NMR} \) (500 MHz, CDCl\(_3\)): \( \delta \) 7.86 (1H, d, \( J = 16.0 \text{ Hz} \)), 7.66 (1H, dd, \( J = 7.5 \text{ Hz} \), 1.5 Hz), 7.50 (1H, ddd, \( J = 8.5 \text{ Hz} \), 7.5 Hz, 1.5 Hz), 7.47 (1H, d, \( J = 16.0 \text{ Hz} \), 7.45 (1H, dd, \( J = 7.5 \text{ Hz} \), 2.0 Hz), 7.16-7.07 (4H, m), 4.79 (2H, d, \( J = 2.5 \text{ Hz} \)), 3.85 (3H, s), 2.52 (1H, t, \( J = 2.5 \text{ Hz} \)). \(^{13}\text{C NMR} \) (125 MHz, CDCl\(_3\)): \( \delta \) 192.63, 156.21, 151.60, 137.06, 134.00, 133.01, 130.80, 130.49, 130.06, 129.23, 124.94, 124.53, 122.05, 121.97, 113.48, 78.19, 76.30, 62.65, 56.65. MS (ESI, positive) calculated m/z for \( \text{C}_{19}\text{H}_{15}\text{N}_{3}\text{O}_{3} \) ([M+H]\(^+\)): 334.1; found: 334.1.

\( (E)-3-(3\text{-azido-4-methoxyphenyl})-1-(2-(\text{prop-2-yn-1-yloxy})phenyl)\text{prop-2-en-1-one} \)  
(C15)

Yield: 88\%. \(^{1}\text{H NMR} \) (500 MHz, CDCl\(_3\)): \( \delta \) 7.65 (1H, dd, \( J = 8.0 \text{ Hz} \), 2.0 Hz), 7.54 (1H, d, \( J = 16.0 \text{ Hz} \)), 7.50 (1H, td, \( J = 7.5 \text{ Hz} \), 1.5 Hz), 7.33 (1H, dd, \( J = 8.5 \text{ Hz} \), 2.0 Hz), 7.31 (1H, d, \( J = 15.5 \text{ Hz} \)), 7.28 (1H, d, \( J = 1.5 \text{ Hz} \)), 7.11 (1H, d, \( J = 8.0 \text{ Hz} \)), 7.10 (1H, t, \( J
= 7.0 Hz), 6.90 (1H, d, J = 8.5 Hz), 4.80 (2H, d, J = 2.5 Hz), 3.92 (3H, s), 2.55 (1H, t, J = 2.5 Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 192.41, 156.07, 153.77, 142.28, 132.84, 130.74, 130.17, 129.09, 128.80, 127.16, 126.11, 122.03, 119.58, 113.43, 112.12, 78.24, 76.30, 56.53, 56.24. MS (ESI, positive) calculated m/z for C$_{19}$H$_{15}$N$_3$O$_3$ ([M+H]$^+$): 334.1; found: 334.1.

(E)-3-(5-azido-2-methoxyphenyl)-1-(2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C16)

Yield: 84%. $^1$H NMR (500 MHz, CDCl$_3$): δ 7.65 (1H, dd, J = 8.0 Hz, 2.0 Hz), 7.54 (1H, d, J = 16.0 Hz), 7.50 (1H, td, J = 7.5 Hz, 1.5 Hz), 7.33 (1H, dd, J = 8.5 Hz, 2.0 Hz), 7.31 (1H, d, J = 16.0 Hz), 7.28 (1H, d, J = 2.0 Hz), 7.11 (1H, d, J = 8.0 Hz), 7.10 (1H, t, J = 7.0 Hz), 6.90 (1H, d, J = 8.5 Hz), 4.80 (2H, d, J = 2.5 Hz), 3.92 (3H, s), 2.55 (1H, t, J = 2.5 Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 192.41, 156.07, 153.77, 142.27, 132.84, 130.74, 130.17, 129.09, 128.80, 127.16, 126.10, 122.02, 119.58, 113.43, 112.11, 78.24, 76.30, 56.53, 56.24. MS (ESI, positive) calculated m/z for C$_{19}$H$_{15}$N$_3$O$_3$ ([M+H]$^+$): 334.1; found: 334.1.

(E)-3-(2-azidophenyl)-1-(3-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C21)

Yield: 14%. $^1$H NMR (500 MHz, CDCl$_3$): δ 8.01 (1H, d, J = 16.0 Hz), 7.69 (1H, d, J = 8.0 Hz), 7.64 (1H, d, J = 8.0 Hz), 7.62 (1H, d, J = 2.0 Hz), 7.54 (1H, d, J = 16.0 Hz), 7.44 (1H, t, J = 8.0 Hz), 7.26-7.16 (3H, m), 4.78 (2H, d, J = 2.5 Hz), 2.55 (1H, t, J = 2.5
\( ^{13}\text{C} \text{ NMR (125 MHz, CDCl}_3 \): \delta 190.31, 157.97, 139.86, 139.71, 139.53, 131.64, 129.85, 128.10, 126.69, 125.10, 124.00, 122.11, 120.14, 119.10, 114.39, 78.26, 76.08, 56.17. \text{ MS (ESI, positive) calculated m/z for C}_{18}\text{H}_{13}\text{N}_3\text{O}_2 ([\text{M+H}]^+): 304.1; \text{ found: 304.1}. \)

\( (E)-3-(3\text{-azidophenyl})-1-(3\text{-prop-2-yn-1-yloxy)phenyl} prop-2\text{-en-1-one (C22)} \)

\text{ Yield: 24\%. } ^{1}\text{H NMR (500 MHz, CDCl}_3 \): \delta 7.76 (1H, d, \( J = 15.5 \text{ Hz} \)), 7.65 (1H, d, \( J = 7.5 \text{ Hz} \)), 7.62 (1H, t, \( J = 2.0 \text{ Hz} \)), 7.49 (1H, d, \( J = 15.5 \text{ Hz} \)), 7.45 (1H, t, \( J = 8.0 \text{ Hz} \)), 7.43-7.38 (2H, m), 7.27 (1H, s, br), 7.22 (1H, dd, \( J = 7.5 \text{ Hz, 2.5 Hz} \)), 7.09 (1H, dt, \( J = 6.0 \text{ Hz, 2.5 Hz} \)), 4.78 (2H, d, \( J = 2.5 \text{ Hz} \)), 2.56 (1H, t, \( J = 2.5 \text{ Hz} \)). \text{ ^{13}\text{C NMR (125 MHz, CDCl}_3 \): \delta 189.91, 158.00, 143.81, 141.06, 139.52, 136.79, 130.49, 129.93, 125.25, 123.28, 122.05, 121.04, 120.31, 118.56, 114.35, 78.22, 76.13, 56.18. MS (ESI, positive) calculated m/z for C}_{18}\text{H}_{13}\text{N}_3\text{O}_2 ([\text{M+H}]^+): 304.1; \text{ found: 304.1}. \)

\( (E)-3-(4\text{-azidophenyl})-1-(3\text{-prop-2-yn-1-yloxy)phenyl} prop-2\text{-en-1-one (C23)} \)

\text{ Yield: 23\%. } ^{1}\text{H NMR (500 MHz, CDCl}_3 \): \delta 7.77 (1H, d, \( J = 15.5 \text{ Hz} \)), 7.65 (1H, d, \( J = 7.5 \text{ Hz} \)), 7.64 (2H, d, \( J = 8.0 \text{ Hz} \)), 7.62 (1H, d, \( J = 2.0 \text{ Hz} \)), 7.45 (1H, d, \( J = 16.0 \text{ Hz} \)), 7.44 (1H, t, \( J = 8.0 \text{ Hz} \)), 7.21 (1H, dd, \( J = 8.0 \text{ Hz, 2.0 Hz} \)), 7.08 (2H, d, \( J = 8.5 \text{ Hz} \)), 4.78 (2H, d, \( J = 2.5 \text{ Hz} \)), 2.55 (1H, t, \( J = 2.5 \text{ Hz} \)). \text{ ^{13}\text{C NMR (125 MHz, CDCl}_3 \): \delta 189.96, 157.97, 143.95, 142.41, 139.77, 131.82, 130.20, 129.86, 121.96, 121.63, 120.08, 119.73, 114.36, 78.25, 76.09, 56.17. MS (ESI, positive) calculated m/z for C}_{18}\text{H}_{13}\text{N}_3\text{O}_2 ([\text{M+H}]^+): 304.1; \text{ found: 304.1}. \)
(E)-3-(3-azido-2-methoxyphenyl)-1-(3-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one

(C24)

Yield: 22%. ¹H NMR (500 MHz, CDCl₃): δ 8.02 (1H, d, J = 16.0 Hz), 7.65 (1H, d, J = 8.0 Hz), 7.63 (1H, d, J = 2.0 Hz), 7.59 (1H, d, J = 16.0 Hz), 7.45 (1H, d, J = 7.5 Hz), 7.44 (1H, t, J = 7.5 Hz), 7.22 (1H, dd, J = 8.0 Hz, 2.0 Hz), 7.16 (1H, t, J = 7.5 Hz), 7.13 (1H, dd, J = 8.0 Hz, 2.0 Hz), 4.78 (2H, d, J = 2.5 Hz), 3.88 (3H, s), 2.56 (1H, t, J = 2.5 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 190.14, 157.99, 151.65, 139.62, 138.97, 134.17, 130.21, 129.88, 125.08, 124.66, 124.44, 122.28, 122.06, 120.24, 114.39, 78.23, 76.13, 62.55, 56.17. MS (ESI, positive) calculated m/z for C₁₉H₁₅N₃O₃ ([M+H]⁺): 334.1; found: 334.1.

(E)-3-(3-azido-4-methoxyphenyl)-1-(3-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one

(C25)

Yield: 88%. ¹H NMR (500 MHz, CDCl₃): δ 7.72 (1H, d, J = 16.0 Hz), 7.64 (1H, d, J = 8.0 Hz), 7.61 (1H, d, J = 2.5 Hz), 7.44 (1H, t, J = 8.0 Hz), 7.37 (1H, d, J = 15.5 Hz), 7.36 (1H, dd, J = 7.5 Hz, 2.0 Hz), 7.30 (1H, d, J = 2.0 Hz), 7.21 (1H, dd, J = 8.0 Hz, 2.0 Hz), 6.92 (1H, d, J = 8.5 Hz), 4.78 (2H, d, J = 2.0 Hz), 3.94 (3H, s), 2.55 (1H, t, J = 2.0 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 189.94, 157.96, 154.09, 143.91, 139.83, 129.85, 129.23, 128.48, 127.22, 121.96, 120.95, 120.06, 119.68, 114.30, 112.18, 78.28, 76.09, 56.27, 56.16. MS (ESI, positive) calculated m/z for C₁₀H₁₅N₃O₃ ([M+H]⁺): 334.1; found: 334.1.
(E)-3-(5-azido-2-methoxyphenyl)-1-(3-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C26)

Yield: 92%. 1H NMR (500 MHz, CDCl3): δ 7.72 (1H, d, J = 15.5 Hz), 7.64 (1H, d, J = 7.5 Hz), 7.61 (1H, d, J = 2.0 Hz), 7.44 (1H, t, J = 8.0 Hz), 7.37 (1H, d, J = 15.5 Hz), 7.36 (1H, dd, J = 7.0 Hz, 1.5 Hz), 7.30 (1H, d, J = 2.0 Hz), 7.21 (1H, dd, J = 8.0 Hz, 2.0 Hz), 6.92 (1H, d, J = 8.5 Hz), 4.78 (2H, d, J = 2.5 Hz), 3.94 (3H, s), 2.55 (1H, t, J = 2.5 Hz). 13C NMR (125 MHz, CDCl3): δ 189.94, 157.95, 154.09, 143.91, 139.83, 129.85, 129.23, 128.48, 127.22, 121.96, 120.94, 120.05, 119.67, 114.29, 112.18, 78.28, 76.09, 56.26, 56.16. MS (ESI, positive) calculated m/z for C19H15N3O3 ([M+H]+): 334.1; found: 334.1.

(E)-3-(2-azidophenyl)-1-(4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C31)

Yield: 45%. 1H NMR (500 MHz, CDCl3): δ 8.04 (2H, d, J = 8.5 Hz), 7.99 (1H, d, J = 15.5 Hz), 7.68 (1H, d, J = 8.0 Hz), 7.57 (1H, d, J = 15.5 Hz), 7.43 (1H, td, J = 8.0 Hz, 1.0 Hz), 7.23 (1H, d, J = 8.0 Hz), 7.18 (1H, t, J = 7.5 Hz), 7.07 (2H, d, J = 9.0 Hz), 4.78 (2H, d, J = 2.0 Hz), 2.57 (1H, t, J = 2.0 Hz). 13C NMR (125 MHz, CDCl3): δ 189.03, 161.37, 139.73, 138.74, 131.94, 131.42, 130.97, 128.76, 126.87, 125.06, 123.90, 119.07, 114.87, 77.93, 76.31, 56.03. MS (ESI, positive) calculated m/z for C18H13N3O2 ([M+H]+): 304.1; found: 304.1.
(E)-3-(3-azidophenyl)-1-(4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C32)

Yield: 53%. $^1$H NMR (500 MHz, CDCl$_3$): δ 8.05 (2H, d, $J = 9.0$ Hz), 7.75 (1H, d, $J = 15.5$ Hz), 7.53 (1H, d, $J = 16.0$ Hz), 7.44-7.36 (2H, m), 7.27 (1H, s), 7.08 (2H, d, $J = 9.0$ Hz), 7.08 (1H, d, $J = 9.0$ Hz), 4.79 (2H, d, $J = 2.0$ Hz), 2.57 (1H, t, $J = 2.0$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 188.54, 161.51, 142.99, 141.00, 136.97, 131.76, 130.94, 130.44, 125.21, 123.08, 120.82, 118.46, 114.94, 77.88, 76.36, 56.05. MS (ESI, positive) calculated m/z for C$_{18}$H$_{13}$N$_3$O$_2$ ([M+H]$^+$): 304.1; found: 304.1.

(E)-3-(4-azidophenyl)-1-(4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C33)

Yield: 61%. $^1$H NMR (500 MHz, CDCl$_3$): δ 8.04 (2H, d, $J = 9.0$ Hz), 7.77 (1H, d, $J = 15.5$ Hz), 7.64 (2H, d, $J = 8.5$ Hz), 7.49 (1H, d, $J = 15.5$ Hz), 7.07 (2H, d, $J = 9.0$ Hz), 7.07 (2H, d, $J = 8.5$ Hz), 4.78 (2H, d, $J = 2.0$ Hz), 2.57 (1H, t, $J = 2.0$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 188.64, 161.39, 143.13, 142.17, 132.00, 131.97, 130.85, 130.09, 121.43, 119.69, 114.89, 77.91, 76.32, 56.03. MS (ESI, positive) calculated m/z for C$_{18}$H$_{13}$N$_3$O$_2$ ([M+H]$^+$): 304.1; found: 304.1.

(E)-3-(3-azido-2-methoxyphenyl)-1-(4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C34)

Yield: 74%. $^1$H NMR (500 MHz, CDCl$_3$): δ 8.05 (2H, d, $J = 9.0$ Hz), 8.00 (1H, d, $J = 16.0$ Hz), 7.62 (1H, d, $J = 15.5$ Hz), 7.45 (1H, dd, $J = 7.5$ Hz, 1.5 Hz), 7.16 (1H, t, $J = 7.5$ Hz), 7.12 (1H, dd, $J = 7.5$ Hz, 1.5 Hz), 7.07 (2H, d, $J = 8.5$ Hz), 4.79 (2H, d, $J = 2.5$ Hz), 3.97 (3H, s), 3.84 (3H, s).
Hz), 3.88 (3H, s), 2.57 (1H, t, J = 2.5 Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 188.87, 161.45, 151.58, 138.16, 134.14, 131.87, 130.95, 130.39, 125.04, 124.66, 124.35, 122.08, 114.92, 77.90, 76.34, 62.51, 56.04. MS (ESI, positive) calculated m/z for C$_{19}$H$_{15}$N$_3$O$_3$ ([M+H]$^+$): 334.1; found: 334.1.

(E)-3-(3-azido-4-methoxyphenyl)-1-(4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C35)

Yield: 96%. $^1$H NMR (500 MHz, CDCl$_3$): δ 8.04 (2H, d, J = 9.0 Hz), 7.71 (1H, d, J = 16.0 Hz), 7.41 (1H, d, J = 16.0 Hz), 7.37 (1H, dd, J = 8.5 Hz, 2.0 Hz), 7.30 (1H, d, J = 1.5 Hz), 7.07 (2H, d, J = 8.5 Hz), 6.92 (1H, d, J = 8.5 Hz), 4.78 (2H, d, J = 2.5 Hz), 3.93 (3H, s), 2.56 (1H, t, J = 2.5 Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 188.62, 161.34, 153.92, 143.08, 132.03, 130.83, 129.16, 128.67, 127.15, 120.73, 119.54, 114.88, 112.16, 77.93, 76.31, 56.25, 56.03. MS (ESI, positive) calculated m/z for C$_{19}$H$_{15}$N$_3$O$_3$ ([M+H]$^+$): 334.1; found: 334.1.

(E)-3-(5-azido-2-methoxyphenyl)-1-(4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C36)

Yield: 96%. $^1$H NMR (500 MHz, CDCl$_3$): δ 8.04 (2H, d, J = 8.5 Hz), 7.71 (1H, d, J = 15.5 Hz), 7.41 (1H, d, J = 15.5 Hz), 7.37 (1H, dd, J = 8.5 Hz, 1.5 Hz), 7.30 (1H, d, J = 2.0 Hz), 7.07 (2H, d, J = 9.0 Hz), 6.92 (1H, d, J = 8.5 Hz), 4.78 (2H, d, J = 2.5 Hz), 3.93 (3H, s), 2.56 (1H, t, J = 2.5 Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 188.62, 161.34, 153.92,
143.08, 132.03, 130.83, 129.16, 128.67, 127.15, 120.73, 119.54, 114.87, 112.16, 77.93, 76.31, 56.25, 56.03. MS (ESI, positive) calculated m/z for C_{19}H_{15}N_{3}O_{3} ([M+H]^+): 334.1; found: 334.1.

(E)-3-(2-azidophenyl)-1-(4-methoxy-2-(prop-2-ynyl)oxy)phenyl)prop-2-en-1-one

(C41)

Yield: 62%. \(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 7.90 (1H, d, \(J = 16.0\) Hz), 7.79 (1H, d, \(J = 8.0\) Hz), 7.70 (1H, d, \(J = 7.5\) Hz), 7.58 (1H, d, \(J = 16.0\) Hz), 7.40 (1H, td, \(J = 7.5\) Hz, 1.0 Hz), 7.20 (1H, d, \(J = 8.0\) Hz), 7.15 (1H, t, \(J = 7.5\) Hz), 6.66-6.58 (2H, m), 4.78 (2H, d, \(J = 2.0\) Hz), 3.88 (3H, s), 2.56 (1H, t, \(J = 2.0\) Hz). \(^1^3\)C NMR (125 MHz, CDCl\(_3\)): \(\delta\) 190.28, 164.11, 158.37, 139.59, 136.55, 133.24, 131.05, 128.87, 128.49, 127.33, 124.94, 122.86, 118.97, 106.57, 100.32, 78.04, 76.34, 56.69, 55.76. MS (ESI, positive) calculated m/z for C_{19}H_{15}N_{3}O_{3} ([M+H]^+): 334.1; found: 334.1.

(E)-3-(3-azidophenyl)-1-(4-methoxy-2-(prop-2-ynyl)oxy)phenyl)prop-2-en-1-one

(C42)

Yield: 74%. \(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 7.81 (1H, d, \(J = 8.5\) Hz), 7.63 (1H, d, \(J = 16.0\) Hz), 7.59 (1H, d, \(J = 16.0\) Hz), 7.41-7.34 (2H, m), 7.28 (1H, s, br), 7.09-7.00 (1H, m), 6.63 (1H, dd, \(J = 8.0\) Hz, 2.0 Hz), 6.59 (1H, d, \(J = 2.0\) Hz), 4.79 (2H, d, \(J = 2.0\) Hz), 3.88 (3H, s), 2.61 (1H, t, \(J = 2.0\) Hz). \(^1^3\)C NMR (125 MHz, CDCl\(_3\)): \(\delta\) 189.85, 164.34, 158.51, 140.81, 140.80, 137.49, 133.34, 130.28, 128.47, 125.38, 122.57, 120.40, 118.32,
106.65, 100.17, 77.93, 76.61, 56.67, 55.79. MS (ESI, positive) calculated m/z for C$_{19}$H$_{15}$N$_3$O$_3$ ([M+H]$^+$): 334.1; found: 334.1.

**(E)-3-(4-azidophenyl)-1-(4-methoxy-2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C43)**

Yield: 78%. $^1$H NMR (500 MHz, CDCl$_3$): δ 7.80 (1H, d, $J = 8.5$ Hz), 7.64 (1H, d, $J = 16.0$ Hz), 7.61 (2H, d, $J = 8.5$ Hz), 7.53 (1H, d, $J = 16.0$ Hz), 7.04 (2H, d, $J = 8.5$ Hz), 6.63 (1H, dd, $J = 8.5$ Hz, 2.0 Hz), 6.60 (1H, d, $J = 2.0$ Hz), 4.79 (2H, d, $J = 2.0$ Hz), 3.88 (3H, s), 2.57 (1H, t, $J = 2.0$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 190.03, 164.15, 158.38, 141.69, 141.10, 133.22, 132.51, 130.04, 126.91, 122.82, 119.55, 106.61, 100.29, 78.03, 76.42, 56.70, 55.77. MS (ESI, positive) calculated m/z for C$_{19}$H$_{15}$N$_3$O$_3$ ([M+H]$^+$): 334.1; found: 334.1.

**(E)-3-(3-azido-2-methoxyphenyl)-1-(4-methoxy-2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C44)**

Yield: 92%. $^1$H NMR (500 MHz, CDCl$_3$): δ 7.90 (1H, d, $J = 16.0$ Hz), 7.80 (1H, d, $J = 8.5$ Hz), 7.20 (1H, d, $J = 16.0$ Hz), 7.45 (1H, d, $J = 7.5$ Hz), 7.12 (1H, t, $J = 7.5$ Hz), 7.09 (1H, dd, $J = 8.0$ Hz, 1.5 Hz), 6.67-6.59 (2H, m), 4.78 (2H, d, $J = 2.0$ Hz), 3.88 (3H, s), 3.87 (3H, s), 2.56 (1H, t, $J = 2.0$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 190.18, 164.21, 158.46, 151.56, 135.88, 133.96, 133.28, 130.82, 129.43, 124.87, 124.53, 122.79, 121.69,
106.63, 100.31, 78.00, 76.49, 62.62, 56.73, 55.77. MS (ESI, positive) calculated m/z for C_{20}H_{17}N_{3}O_{4} ([M+H]^{+}): 364.1; found: 364.1.

(E)-3-(3-azido-4-methoxyphenyl)-1-(4-methoxy-2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C45)

Yield: 81%. ¹H NMR (500 MHz, CDCl₃): δ 7.79 (1H, d, J = 8.5 Hz), 7.59 (1H, d, J = 15.5 Hz), 7.46 (1H, d, J = 16.0 Hz), 7.34 (1H, dd, J = 8.5 Hz, 2.0 Hz), 7.31 (1H, d, J = 2.0 Hz), 6.89 (1H, d, J = 8.5 Hz), 6.62 (1H, dd, J = 8.5 Hz, 2.0 Hz), 6.59 (1H, d, J = 2.0 Hz), 4.79 (2H, d, J = 2.0 Hz), 3.92 (3H, s), 3.88 (3H, s), 2.60 (1H, t, J = 2.0 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 190.01, 164.10, 158.33, 153.58, 141.09, 133.19, 129.15, 128.99, 127.16, 126.24, 122.86, 119.42, 112.08, 106.58, 100.24, 78.01, 76.54, 56.67, 56.22, 55.77. MS (ESI, positive) calculated m/z for C_{20}H_{17}N_{3}O_{4} ([M+H]^{+}): 364.1; found: 364.1.

(E)-3-(5-azido-2-methoxyphenyl)-1-(4-methoxy-2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C46)

Yield: 86%. ¹H NMR (500 MHz, CDCl₃): δ 7.79 (1H, d, J = 8.5 Hz), 7.59 (1H, d, J = 15.5 Hz), 7.46 (1H, d, J = 15.5 Hz), 7.34 (1H, dd, J = 8.5 Hz, 2.0 Hz), 7.31 (1H, d, J = 1.5 Hz), 6.89 (1H, d, J = 8.5 Hz), 6.62 (1H, dd, J = 8.5 Hz, 2.0 Hz), 6.59 (1H, d, J = 2.5 Hz), 4.79 (2H, d, J = 2.5 Hz), 3.92 (3H, s), 3.88 (3H, s), 2.60 (1H, t, J = 2.5 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 190.01, 164.10, 158.33, 153.58, 141.09, 133.19, 129.15, 128.99,
127.16, 126.24, 122.86, 119.42, 112.08, 106.58, 100.24, 78.01, 76.54, 56.66, 56.22, 55.77.

MS (ESI, positive) calculated m/z for C_{20}H_{17}N_{3}O_{4} ([M+H]^+): 364.1; found: 364.1.

(\textit{E})-3-(2-azidophenyl)-1-(5-methoxy-2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one

(C51)

Yield: 84%. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}): \textsuperscript{\delta} 7.88 (1H, d, \textit{J} = 16.0 Hz), 7.69 (1H, d, \textit{J} = 7.5 Hz), 7.47 (1H, d, \textit{J} = 16.0 Hz), 7.41 (1H, td, \textit{J} = 8.0 Hz, 1.0 Hz), 7.23-7.18 (2H, m), 7.16 (1H, t, \textit{J} = 7.5 Hz), 7.07 (1H, d, \textit{J} = 9.0 Hz), 7.04 (1H, dd, \textit{J} = 9.0 Hz, 3.0 Hz), 4.73 (2H, d, \textit{J} = 2.0 Hz), 3.82 (3H, s), 2.51 (1H, t, \textit{J} = 2.0 Hz). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}): \textsuperscript{\delta} 192.16, 154.68, 150.50, 139.73, 137.78, 131.38, 130.91, 128.53, 128.43, 126.97, 125.00, 119.32, 118.99, 116.02, 114.54, 78.52, 76.06, 57.74, 55.97. MS (ESI, positive) calculated m/z for C_{19}H_{18}N_{3}O_{3} ([M+H]^+): 334.1; found: 334.1.

(\textit{E})-3-(3-azidophenyl)-1-(5-methoxy-2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one

(C52)

Yield: 87%. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}): \textsuperscript{\delta} 7.61 (1H, d, \textit{J} = 16.0 Hz), 7.49 (1H, d, \textit{J} = 16.0 Hz), 7.42-7.34 (2H, m), 7.27 (1H, s, br), 7.22 (1H, s, br), 7.09-7.01 (3H, m), 4.74 (2H, d, \textit{J} = 2.5 Hz), 3.82 (3H, s), 2.54 (1H, t, \textit{J} = 2.5 Hz). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}): \textsuperscript{\delta} 191.77, 154.70, 150.58, 142.04, 140.90, 137.14, 130.60, 130.34, 128.09, 125.38, 120.73, 119.60, 118.49, 115.83, 114.54, 78.43, 76.29, 57.60, 55.98. MS (ESI, positive) calculated m/z for C_{19}H_{15}N_{3}O_{3} ([M+H]^+): 334.1; found: 334.1.
(E)-3-(4-azidophenyl)-1-(5-methoxy-2-(prop-2-yn-1-yl oxy)phenyl)prop-2-en-1-one (C53)

Yield: 78%. ¹H NMR (500 MHz, CDCl₃): δ 7.61 (1H, d, J = 16.0 Hz), 7.61 (2H, d, J = 8.0 Hz), 7.42 (1H, d, J = 16.0 Hz), 7.21 (1H, d, J = 3.0 Hz), 7.09-7.01 (4H, m), 4.73 (2H, d, J = 2.0 Hz), 3.82 (3H, s), 2.51 (1H, t, J = 2.0 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 191.93, 154.71, 150.45, 142.39, 142.06, 132.14, 130.92, 130.18, 126.53, 119.61, 119.28, 115.99, 114.53, 78.52, 76.13, 57.71, 55.97. MS (ESI, positive) calculated m/z for C₁₉H₁₅N₃O₃ ([M+H]+): 334.1; found: 334.1.

(E)-3-(3-azido-2-methoxyphenyl)-1-(5-methoxy-2-(prop-2-yn-1-yl oxy)phenyl)prop-2-en-1-one (C54)

Yield: 90%. ¹H NMR (500 MHz, CDCl₃): δ 7.88 (1H, d, J = 16.0 Hz), 7.51 (1H, d, J = 16.0 Hz), 7.45 (1H, dd, J = 8.0 Hz, 2.0 Hz), 7.21 (1H, d, J = 2.5 Hz), 7.13 (1H, t, J = 8.0 Hz), 7.10 (1H, dd, J = 8.0 Hz, 2.0 Hz), 7.07 (1H, d, J = 9.0 Hz), 7.04 (1H, dd, J = 9.0 Hz, 2.5 Hz), 4.72 (2H, d, J = 2.0 Hz), 3.86 (3H, s), 3.82 (3H, s), 2.50 (1H, t, J = 2.0 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 192.13, 154.70, 151.63, 150.56, 137.16, 134.01, 130.85, 130.48, 129.01, 124.95, 124.53, 122.01, 119.41, 116.00, 114.57, 78.48, 76.20, 62.68, 57.78, 55.97. MS (ESI, positive) calculated m/z for C₂₀H₁₇N₃O₄ ([M+H]+): 364.1; found: 364.1.
(E)-3-(3-azido-4-methoxyphenyl)-1-(5-methoxy-2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C55)

Yield: 84%. $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.56 (1H, d, $J = 15.5$ Hz), 7.35 (1H, d, $J = 16.0$ Hz), 7.34 (1H, dd, $J = 8.5$ Hz, 2.0 Hz), 7.29 (1H, d, $J = 1.5$ Hz), 7.20 (1H, d, $J = 2.5$ Hz), 7.06 (1H, d, $J = 9.0$ Hz), 7.03 (1H, dd, $J = 9.0$ Hz, 3.0 Hz), 6.90 (1H, d, $J = 8.5$ Hz), 4.73 (2H, d, $J = 2.5$ Hz), 3.92 (3H, s), 3.82 (3H, s), 2.54 (1H, t, $J = 2.5$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 191.92, 154.68, 153.81, 150.39, 142.41, 130.97, 129.10, 128.79, 127.20, 125.89, 119.64, 119.19, 115.91, 114.52, 112.11, 78.53, 76.20, 57.64, 56.24, 55.97. MS (ESI, positive) calculated m/z for C$_{20}$H$_{17}$N$_3$O$_4$ ([M+H]$^+$): 364.1; found: 364.1.

(E)-3-(5-azido-2-methoxyphenyl)-1-(5-methoxy-2-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C56)

Yield: 85%. $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.56 (1H, d, $J = 15.5$ Hz), 7.35 (1H, d, $J = 16.0$ Hz), 7.34 (1H, dd, $J = 8.5$ Hz, 2.0 Hz), 7.29 (1H, d, $J = 1.5$ Hz), 7.20 (1H, d, $J = 2.5$ Hz), 7.06 (1H, d, $J = 8.5$ Hz), 7.03 (1H, dd, $J = 9.0$ Hz, 3.0 Hz), 6.90 (1H, d, $J = 8.5$ Hz), 4.73 (2H, d, $J = 2.5$ Hz), 3.92 (3H, s), 3.82 (3H, s), 2.54 (1H, t, $J = 2.5$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 191.91, 154.68, 153.81, 150.39, 142.41, 130.97, 129.10, 128.79, 127.20, 125.89, 119.64, 119.19, 115.91, 114.52, 112.11, 78.53, 76.20, 57.64, 56.24, 55.97. MS (ESI, positive) calculated m/z for C$_{20}$H$_{17}$N$_3$O$_4$ ([M+H]$^+$): 364.1; found: 364.1.
(E)-3-(2-azidophenyl)-1-(2-methoxy-6-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C61)

Yield: 40%. ¹H NMR (500 MHz, CDCl₃): δ 7.63 (1H, d, J = 8.0 Hz), 7.57 (1H, d, J = 16.5 Hz), 7.40 (1H, td, J = 8.0 Hz, 1.0 Hz), 7.35 (1H, t, J = 7.5 Hz), 7.16 (1H, d, J = 7.5 Hz), 7.15 (1H, t, J = 8.0 Hz), 6.97 (1H, d, J = 16.5 Hz), 6.78 (1H, d, J = 8.5 Hz), 6.67 (1H, d, J = 8.5 Hz), 4.69 (2H, d, J = 2.5 Hz), 3.79 (3H, s), 2.46 (1H, t, J = 2.5 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 194.84, 157.87, 155.63, 139.58, 139.42, 131.50, 130.88, 130.05, 128.44, 126.70, 125.06, 119.35, 118.93, 106.00, 105.17, 78.57, 75.76, 56.67, 56.16. MS (ESI, positive) calculated m/z for C₁₉H₁₅N₃O₃ ([M+H]⁺): 334.1; found: 334.1.

(E)-3-(3-azidophenyl)-1-(2-methoxy-6-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C62)

Yield: 75%. ¹H NMR (500 MHz, CDCl₃): δ 7.36 (1H, t, J = 8.0 Hz), 7.30 (1H, d, J = 8.0 Hz), 7.28 (1H, d, J = 16.0 Hz), 7.15 (1H, s, br), 7.03 (1H, d, J = 8.0 Hz, br), 6.95 (1H, d, J = 16.0 Hz), 6.76 (1H, d, J = 8.5 Hz), 6.70 (1H, d, J = 8.5 Hz), 4.69 (2H, d, J = 2.5 Hz), 3.79 (3H, s), 2.46 (1H, t, J = 2.5 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 194.46, 157.89, 155.55, 143.91, 140.89, 136.83, 130.99, 130.32, 129.75, 125.04, 120.85, 119.23, 118.69, 105.52, 105.14, 78.50, 75.90, 56.52, 56.15. MS (ESI, positive) calculated m/z for C₁₉H₁₅N₃O₃ ([M+H]⁺): 334.1; found: 334.1.
(E)-3-(4-azidophenyl)-1-(2-methoxy-6-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C63)

Yield: 87%. ¹H NMR (500 MHz, CDCl₃): δ 7.51 (2H, d, J = 8.5 Hz), 7.35 (1H, t, J = 8.5 Hz), 7.28 (1H, d, J = 16.5 Hz), 7.02 (2H, d, J = 8.5 Hz), 6.90 (1H, d, J = 16.0 Hz), 6.76 (1H, d, J = 8.5 Hz), 6.66 (1H, d, J = 8.5 Hz), 4.68 (2H, d, J = 2.5 Hz), 3.79 (3H, s), 2.45 (1H, t, J = 2.5 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 194.54, 157.84, 155.53, 144.19, 142.15, 131.83, 130.83, 130.14, 128.28, 119.57, 119.42, 105.95, 105.14, 78.55, 75.83, 56.56, 56.15. MS (ESI, positive) calculated m/z for C₁₉H₁₅N₃O₃ ([M+H]+): 334.1; found: 334.1.

(E)-3-(3-azido-2-methoxyphenyl)-1-(2-methoxy-6-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C64)

Yield: 68%. ¹H NMR (500 MHz, CDCl₃): δ 7.56 (1H, d, J = 16.5 Hz), 7.38 (1H, dd, J = 8.0 Hz, 1.5 Hz), 7.35 (1H, t, J = 8.5 Hz), 7.12 (1H, t, J = 8.0 Hz), 7.07 (1H, dd, J = 8.0 Hz, 1.5 Hz), 6.97 (1H, d, J = 16.0 Hz), 6.77 (1H, d, J = 8.5 Hz), 6.66 (1H, d, J = 8.5 Hz), 4.67 (2H, d, J = 2.5 Hz), 3.78 (3H, s), 3.75 (3H, s), 2.45 (1H, t, J = 2.5 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 195.01, 157.79, 155.59, 151.36, 139.26, 133.89, 130.96, 130.73, 130.25, 125.06, 124.41, 122.17, 119.18, 105.88, 105.08, 78.49, 75.91, 62.70, 56.67, 56.10. MS (ESI, positive) calculated m/z for C₂₀H₁₇N₃O₄ ([M+H]+): 364.1; found: 364.1.
(E)-3-(3-azido-4-methoxyphenyl)-1-(2-methoxy-6-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C65)

Yield: 80%. $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.35 (1H, t, $J = 8.5$ Hz), 7.28 (1H, dd, $J = 8.5$ Hz, 2.0 Hz), 7.23 (1H, d, $J = 16.0$ Hz), 7.16 (1H, d, $J = 2.0$ Hz), 6.88 (1H, d, $J = 8.5$ Hz), 6.84 (1H, d, $J = 16.0$ Hz), 6.76 (1H, d, $J = 8.5$ Hz), 6.66 (1H, d, $J = 8.5$ Hz), 4.68 (2H, d, $J = 2.0$ Hz), 3.91 (3H, s), 3.79 (3H, s), 2.46 (1H, t, $J = 2.0$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 194.48, 157.82, 155.49, 153.74, 144.18, 130.78, 129.05, 128.47, 127.69, 126.70, 119.97, 119.47, 112.11, 105.93, 105.14, 78.58, 75.84, 56.53, 56.24, 56.15. MS (ESI, positive) calculated m/z for C$_{20}$H$_{17}$N$_3$O$_4$ ([M+H]$^+$): 364.1; found: 364.1.

(E)-3-(5-azido-2-methoxyphenyl)-1-(2-methoxy-6-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (C66)

Yield: 85%. $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.35 (1H, t, $J = 8.5$ Hz), 7.28 (1H, dd, $J = 8.5$ Hz, 2.0 Hz), 7.23 (1H, d, $J = 16.0$ Hz), 7.16 (1H, d, $J = 2.0$ Hz), 6.88 (1H, d, $J = 9.0$ Hz), 6.84 (1H, d, $J = 16.0$ Hz), 6.76 (1H, d, $J = 8.5$ Hz), 6.66 (1H, d, $J = 8.5$ Hz), 4.68 (2H, d, $J = 2.5$ Hz), 3.91 (3H, s), 3.79 (3H, s), 2.46 (1H, t, $J = 2.5$ Hz). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 194.48, 157.82, 155.49, 153.74, 144.18, 130.78, 129.05, 128.47, 127.69, 126.70, 119.97, 119.47, 112.11, 105.93, 105.14, 78.58, 75.84, 56.53, 56.24, 56.15. MS (ESI, positive) calculated m/z for C$_{20}$H$_{17}$N$_3$O$_4$ ([M+H]$^+$): 364.1; found: 364.1.

6.4.3 Photoaffinity labeling, click reaction, Western blotting analysis, subcellular
enrichment, affinity purification, in-gel digestion, and mass spectrometry

The procedures for these experiments are detailed in the previous chapter (5.4.1-5.4.7). Conditions for Western immunoblotting for target validation are as follows. The membrane was blocked with 5% milk in TBST for 1 hour and then incubated with the corresponding rabbit-originated primary antibody (1:200, Fisher Scientific #50-608-728 for PKM; 1:8000, Pierce #PIPA532127 for ALDH1A1; 1:200, Cell Signaling #2551S for eEF1A; and 1:1000, Santa Cruz #sc-25778 for GAPDH) for overnight at 4°C. The membrane was washed twice with TBST and then incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:2000, Pierce #PI31466) in TBST containing 5% milk for 1 hour at room temperature. After three washes with TBST, the membrane was incubated in SuperSignal West Pico chemiluminescent substrate mix (Thermo Scientific #34080) for 8 min with gentle shaking. The autoradiology film (GeneMate #F-9023-5X7) was then exposed to the membrane for a suitable period (typically within 2 min) and developed in an automatic film developer (Konica Minolta SRX-101A).
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