Design and Structure-Activity Relationship of Small Molecule C-terminal Binding Protein (CtBP) Inhibitors and Investigation of the Scope of Palladium Multi-Walled Carbon Nanotubes (Pd-MWCNT) Catalyst in C–H Activation Reactions

Sudha Korwar
Virginia Commonwealth University

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Design and Structure-Activity Relationship of Small Molecule C-terminal Binding Protein (CtBP) Inhibitors and Investigation of the Scope of Palladium Multi-Walled Carbon Nanotubes (Pd-MWCNT) Catalyst in C–H Activation Reactions

A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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Master of Science, Virginia Commonwealth University, August 2012

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May 2016
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<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>ARF</td>
<td>Alternate Reading Frame</td>
</tr>
<tr>
<td>Bik</td>
<td>BCL2-interacting killer</td>
</tr>
<tr>
<td>BRCA1</td>
<td>BReast CAnce gene 1</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB Binding Protein</td>
</tr>
<tr>
<td>CtBP</td>
<td>C-terminal Binding Protein</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial Mesenchymal Transition</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone DeACetylase</td>
</tr>
<tr>
<td>HIPP</td>
<td>2-(hydroxyimino)-3-phenyl propanoic acid</td>
</tr>
<tr>
<td>hTcf4</td>
<td>human T-cell transcription factor-4</td>
</tr>
<tr>
<td>LCoR</td>
<td>Ligand-dependent CoRepressor</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate DeHydrogenase</td>
</tr>
<tr>
<td>LSD</td>
<td>Lysine-Specific histone Demethylase</td>
</tr>
<tr>
<td>MTOB</td>
<td>4-(methylthio)-2-oxobutanoic acid</td>
</tr>
<tr>
<td>PcG</td>
<td>Polycomb Group</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small Ubiquitin-related Modifier</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
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<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>AgOAc</td>
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</tr>
<tr>
<td>BDE</td>
<td>bond dissociation energy</td>
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<tr>
<td>BF4</td>
<td>tetrafluoroborate</td>
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<td>CNT</td>
<td>carbon nanotube</td>
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<tr>
<td>DCE</td>
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<td>DMA</td>
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<tr>
<td>Mes</td>
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<td>MWCNT</td>
<td>multi-walled carbon Nanotube</td>
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</tr>
<tr>
<td>Pd</td>
<td>palladium</td>
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<tr>
<td>Pd(OAc)₂</td>
<td>palladium acetate</td>
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<td>Phl(OAc)₂</td>
<td>diacetoxyiodobenzene</td>
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<tr>
<td>PhlCl₂</td>
<td>dichloroiodo benzene</td>
</tr>
<tr>
<td>SWCNT</td>
<td>single-walled carbon nanotube</td>
</tr>
<tr>
<td>TBPB</td>
<td>tert-butyl peroxybenzoate</td>
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Abstract

DESIGN AND STRUCTURE-ACTIVITY RELATIONSHIP OF C-TERMINAL BINDING PROTEIN (CTBP) INHIBITORS AND INVESTIGATION OF THE SCOPE OF PALLADIUM MULTI-WALLED CARBON NANOTUBES (Pd-MWCNT) CATALYST IN C–H ACTIVATION REACTIONS

By Sudha Korwar, Ph.D.

A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2016

Major Director: Keith C. Ellis, Assistant Professor, Department of Medicinal Chemistry

C-terminal binding proteins (CtBPs) are transcriptional co-repressors involved in developmental processes, and also implicated in a number of breast, ovarian, colon cancers, and resistance against cancer chemotherapy. CtBP is a validated novel potential anti-cancer target. In this project we sought to develop potent and selective small-molecule inhibitors of CtBP. Using a combination of classical medicinal chemistry and modern computational approaches, we designed a potent inhibitor HIPP (hydroxyimino-3-phenylpropanoic acid) that showed an IC₅₀ of 0.24 µM against recombinant CtBP. Further elucidation of the structure-activity relationship (SAR) of HIPP led to the design of more potent inhibitors 3-Cl HIPP (CtBP IC₅₀ = 0.17 µM) and 4-Cl HIPP (CtBP IC₅₀ = 0.18 µM). These compounds also showed inhibition in HCT-116 colon cancer cells with GI₅₀ values ~ 1-4 mM. The compounds showed no off-
target toxicity against a closely related protein. This is a starting point for the
development of CtBP inhibitors as anti-cancer therapeutics.

The second part of this dissertation focuses on C–H activation chemistry. C–H
activation is the most atom-economical method of introducing complexity into a
molecule, even at late stages of drug/product development. We have used solid-
supported palladium nanoparticle catalyst (Pd-MWCNT) to investigate the scope of
C–H activation reactions it can catalyse. Pd-MWCNT was found to efficiently catalyse
N-chelation directed C–H activation reactions – halogenations, oxygenations and
arylations. The turn-over numbers for these reactions were significantly higher than
that of the reported homogenous catalyst. The added advantages of reuse/recyclability
of catalyst, low contamination of metal in the final product make this catalyst very
attractive on an industrial scale. This work serves as a foundation for the further
development of Pd-MWCNT catalyst in late-stage synthesis of drugs and/or
diversification of products.
Design and Structure-Activity Relationship Study of Small Molecule C-terminal Binding Protein (CtBP) Inhibitors

1. Introduction

1.1. Gene Regulation

Gene expression is regulated by a combination of transcriptional activation and transcriptional repression. DNA sequence-specific transcriptional repressors recruit certain co-repressors to specific regions of DNA and inhibit gene expression of a single gene or multiple genes by targeting the transcriptional machinery or altering the chromatin structure. For example, several co-repressors recruit histone deacetylases (HDACs) to remove acetyl group from the N-terminal tails of histones, resulting in chromatin condensation, thereby preventing transcription factors access to DNA. Examples of HDAC-dependent corepressors include Sin3, pRb, Groucho, Mi-2/NuRD. Proper gene regulation through transcriptional regulation allows cells to respond to stimuli, whereas improper gene regulation results in disease states. Molecular control of the transcriptional machinery complex is a highly challenging area, the investigation of which would help in the advancement of cancer therapy. This dissertation focuses on CtBP (C-terminal Binding Protein) – transcriptional corepressors, which have been implicated in development and oncogenesis. Targeting CtBP has been shown to relieve the repression of apoptotic genes, tumor suppression genes and genes involved in cell adhesion. This dissertation focuses on the development of small molecule inhibitors that target CtBP and show potential as novel anti-cancer therapeutics.
1.2. CtBP

The CtBP family of proteins function as transcriptional co-repressors and are highly conserved among vertebrates and invertebrates. CtBP plays an important role during early development, and has been implicated in various cancers. It modulates the activity of oncogenes and tumor suppressor genes. CtBP represses transcription by both chromatin modification-dependent and independent pathways, depending on the promoter. CtBP proteins also act as a link between gene expression and metabolism, as their transcriptional regulation function is dependent on the intracellular NAD⁺/NADH ratio.

1.2.1 Discovery

CtBP is a 48 kDa cellular phosphoprotein (441 amino acids) that was first identified in 1993 by its binding to the C-terminal region (PLDLS motif) of E1A human adenovirus oncoprotein,² ³ during a search for proteins involved in negative modulation of oncogenic transformation. This initial isoform was named CtBP1. CtBP2 (445 amino acids, 48 kDa, 83% sequence homology to CtBP1) was identified by analysing expressed sequence tag (EST) data bank sequences.⁴

1.2.2. Isoforms and genes

Invertebrates possess a single CtBP gene. They have different isoforms of CtBP as a result of differential RNA processing. Vertebrates possess CtBP1 (Ctbp1 gene 4p16 chromosome) and CtBP2 (Ctbp2 gene 21q21.3 chromosome).⁴ Both vertebrate genes code for different CtBP isoforms – CtBP-L (long) and CtBP-S (short). CtBP1-S lacks the first 13 amino acids of the CtBP1-L N-terminal domain. CtBP1-S is also known as CtBP3/BAR50, is N-terminally truncated CtBP1, which has acyltransferase activity in Golgi.⁵
CtBP2-S does not possess the first 25 amino acids of the CtBP2-L N-terminal domain. In the retina, a form of CtBP2 called RIBEYE protein (120 kDa) is expressed. It has an N-terminal domain fused to CtBP2 (amino acids 21-445).

1.2.3. Localization

CtBP isoforms are present in nucleus or cytoplasm based on the post-translational modifications and the presence/absence of nuclear localization signal (NLS). The NLS sequence is KRQR and corresponds to the residues 10-13 of hCtBP2. CtBP2-L can heterodimerize with CtBP1 and translocate CtBP1 to the nucleus. CtBP1 isoforms do not have NLS sequence, and are concentrated in the nucleus, significant quantities are present in the cytosol. Neuronal nitric acid synthase (nNOS) binds to CtBP1 and translocates it to the cytoplasm from the nucleus. CtBP2-S lacks the NLS sequence, and hence is localised in the cytoplasm. Localization is not solely dependent on NLS, as CtBP2-L dimer interface mutants localise in the cytoplasm. RIBEYE is localized in the cytoplasm and is mainly present in the ribbon synapses. The localization of CtBP depends on the post-translational modifications, and is not consistent throughout the literature. CtBP1-L and CtBP-1S have been observed both in cytoplasm and nucleus. Phosphorylation of S158 by Pak1 kinase translocates CtBP1 to the cytoplasm, and inhibits the corepressor activity of CtBP1. SUMOylation of K428 by SUMO-1 (blocked by nNOS) localises it in the nucleus and is required for corepression of E-cad by CtBP1. Dimerization, which is dependent on NADH binding, also affects nuclear localization. Inhibition of CtBP dimerization prevents nuclear localization.
1.2.4. Oligomerization

CtBP forms dimers through the dehydrogenase domain similar to D-isomer specific NAD-dependent 2-hydroxy acid dehydrogenase (D2-HDH) family members. Oligomerization has been observed in bacterial D2-HDH enzymes – D-Lactate Dehydrogenases\(^\text{16}\).\(^\text{17}\) Hydroxyisocaproate Dehydrogenase,\(^\text{18}\) Formate Dehydrogenase,\(^\text{19}\) D-Glycerate Dehydrogenase,\(^\text{20}\) and in human D2-HDH enzymes – Glyoxylate Reductase/Hydroxypyruvate Reductase\(^\text{21}\) and D-3 Phosphoglycerate Dehydrogenase.

CtBP is a redox sensor as its activity is dependent on the metabolic status of the cells – NADH/NAD\(^+\) ratio. It binds to NADH with a 100-fold more affinity than NAD\(^+\).\(^\text{22}\) Once NADH binds to CtBP, the protein undergoes dimerization,\(^\text{23}\) which is essential for the activity of CtBP.\(^\text{10, 24-26}\) When NADH levels increase – conditions of hypoxia and high extracellular glucose levels – NADH binds to CtBP, and the activity of CtBP increases. Each monomer of CtBP contains a single PXDLS motif to which other proteins bind. The presence of a single monomer results in competition between different factors binding to CtBP, which disrupts transcriptional regulation.\(^\text{10}\) Dimerization of CtBP increases the number of PXDLS sites, thereby providing a scaffold for other transcriptional factors to bind. CtBP1 dimerization is required for its interaction with E1A protein. NADH mediated dimerization enhances repression activity of CtBP. CtBP1 mutants that cannot dimerize fail to effect transcriptional repression. NADH has been found to be essential for dimerization and hence nuclear localization, but not for binding of other factors. Binding of other factors depends on the PXDLS motif.
1.2.5. CtBP Domain Arrangement

CtBP is highly homologous to D-isomer specific NAD-dependent 2-hydroxy acid dehydrogenases (D2-HDH) – catalytic Histidine residue, Arg, Glu, and an NAD-binding region (Figure 1-1). It has 3 domains – C-terminal domain, dehydrogenase domain and N-terminal domain. The dehydrogenase domain has two domains – substrate binding domain and coenzyme binding domain. Two features of CtBP that are involved in recruiting cofactors/proteins are:

a. Hydrophobic cleft formed by N-terminal region in CtBP1-L (AA 27-121) that recruits PLDLS motif containing factors (DNA binding proteins).

b. Surface groove on NADH-binding domain that recruits RRT motif containing factors

C-terminal unstructured region has sites for SUMOylation and PDZ (structural domain 80-90 AA) binding.

Figure 1-1. Structural Features of CtBP

CtBP1-L has the entire Ctbp sequence, CtBP1-S has a truncation (blue region) in the N-terminal region. In CtBP2-S, this truncation (25 AA) causes loss of NLS sequence, thus localising it to the cytoplasm. D2-HDH domain has a substrate binding domain (yellow, has PXDLS-binding motif), and coenzyme NADH binding domain (green, has RRT-binding motif and catalytic triad REH), which are connected by hinges (Figure 1-1).
1.3. Cytosolic and Nuclear functions of CtBP

1.3.1. Nuclear functions

CtBP forms large DNA-bound chromatin remodelling complexes and is involved in transcriptional regulation (Figure 1-2). CtBP is recruited to DNA by several DNA binding proteins through PXDLS-binding motif (substrate binding domain of N-terminal region) of CtBP. DNA-binding proteins such as ZEB 1/2 (zinc finger protein) function as bridges between CtBP PXDLS-binding motif and the promoters (for example E-cadherin).\textsuperscript{10, 27-31} CtBP coenzyme binding domain has RRT binding motif (RRTGXPPXL) that binds to cofactors that are involved in repression.\textsuperscript{32} Znf217 binds to CtBP through both PXDLS motif and RRT motif,\textsuperscript{15, 33} thus it binds to CtBP dimers (RRT-binding motif of one monomer, and PXDLS-binding motif of the second monomer).

Numerous chromatin modifying proteins have been observed in CtBP transcriptional complexes. It has been seen that class I HDACs – HDAC 1/2 and class II HDAC proteins interact with CtBP.\textsuperscript{15, 34, 35} Other proteins such as HMT (Histone Methyl Transferase), LSD-1 (Lysine Demethylase), G9A, GLP bind to the CtBP complex.\textsuperscript{15, 34} Histone-modifying proteins bind to CtBP either through other proteins or bind directly. HDAC1/2, CoREST (corepressor of REST) bind directly through non-PXDLS interactions.\textsuperscript{15, 34} CoREST recruits both HDAC1/2 and LSD-1 to CtBP.\textsuperscript{36, 37} However LCoR corepressor interacts through PXDLS motif of CtBP to recruit HDAC1/2.\textsuperscript{38} Some examples of chromatin modifying complexes include: Znf217 binding to CoREST that recruits HDAC1/2 and LSD-1 to CtBP;\textsuperscript{33} Wiz protein that binds to G9A/GLP and CtBP directly.\textsuperscript{39} Thus, depending on the context, various histone modifying proteins are recruited through different DNA-binding proteins in different CtBP multi-subunit regulatory complexes to regulate transcription.\textsuperscript{10}
1.3.2. Cytosolic functions

CtBP1 and CtBP2 play an important role in Golgi fission, vesicle formation and synapse signalling. CtBP1-S has functions in the Golgi – tubule constriction and fissioning using acyl-coenzyme A (acyl-CoA) molecules. CtBP1-S also has acyl transferase activity towards lysophosphatidic acid (LPA) and alters the membrane properties in Golgi. RIBEYE also has acyl transferase activity and is involved in vesicle formation at ribbon synapses.

1.4. CtBP as a Transcriptional Corepressor

The transcriptional repression role of CtBP was first suggested in a tethering transcriptional assay involving E1A protein. Interaction of CtBP with the C-terminal region of E1A inhibited the activity of conserved region 1 (CR1) of E1A. In-depth studies on Drosophila CtBP (dCtBP) showed that dCtBP functions as a transcriptional corepressor during development of embryo. dCtBP has been found to interact with Knirps, Snail (short range repressors) and Hairy (long range repressors). CtBPs have
been shown to be recruited through PLDLS motif by several DNA-binding transcriptional repressors.\textsuperscript{27, 31} CtBPs exert their transcriptional regulation by interacting with DNA-binding repressors that have PLDLS motifs and chromatin modifying proteins (HDACs-1 that do not contain PXDLS motif). CtBPs form a hydrophobic cleft that interacts with both PXDLS and non-PXDLS containing factors. In vertebrates, mouse CtBP2 (mCtBP2) has been found to interact with mouse basic Kruppel-like factor (BKLF). This was dependent on PXDLS motif in the repressor domain. mCtBP1 has been found to interact with Net – Ets family transcriptional repressor.\textsuperscript{47} Several CtBP-interacting proteins have been identified through a two hybrid screening study.\textsuperscript{48}

1.4.1. Mechanism of Transcriptional Repression by CtBP

The exact mechanism by which CtBP mediates transcriptional repression is not yet clear. It can occur in a HDAC-dependent or –independent manner. hCtBP1 has been reported to associate with HDAC1 in cotransfection experiments,\textsuperscript{49} with endogenous HDAC2 and Sin3.\textsuperscript{50} Repression of certain promoters by CtBP has been reported to be affected by trichostatin A (TSA, HDAC inhibitor), such as c-fos promoter repression by Net; whereas repression of SV40 promoter by pRb and p130 is not affected by TSA.\textsuperscript{51, 52} pRb and p130 are involved in HDAC-independent repression by recruiting PcG complex through CtIP and CtBP. Human polycomb protein hPC2 has been shown to interact with hCtBP1 and hCtBP2.\textsuperscript{53} CtIP is an adaptor protein that interacts with both pRb, p130 proteins (Rb binding motif, LXCXE) and with CtBP (PXDLS motif), thus linking both families of proteins. Thus, mammalian CtBPs repress transcription either through HDACs or through PcGs (Figure 1-3\textsuperscript{27}).
In *Drosophila*, the involvement of deacetylases in dCtBP-mediated repression is uncertain. The activity of dCtBP is not significantly affected in Rpd3 (HDAC mammalian homolog) mutant embryos. The mutant embryos might be expressing other deacetylases.

In contrast to the repressor functions, dCtBP has also been shown to possess context-dependent weak transcriptional activational functions. This activation has been observed in human HEK293 cells expressing E1A proteins. In the context of Hairy, dCtBP might be antagonizing the activity of Gro. The regions of dCtBP that are required for activation (His residue) and repressor functions are adjacent to each other.

The corepressor complex of CtBP causes deacetylation and methylation of histone H3-K9, demethylation of histone H3-K4.
1.5. Regulation of CtBP Activity

CtBP is a phosphoprotein, its phosphorylation is cell-cycle dependent. It possess consensus phosphorylation sites of DNA-PK (TQ or SQ) and has been found to interact with DNA-PK.\textsuperscript{48, 56} Phosphorylation changes the localization of CtBP. The activity of CtBP is also modulated by cellular energy levels/homeostasis as it binds to NAD. CtBP binding to E1A C-terminal region is regulated by the nuclear acetylases p300/CBP and P/CAF that acetylate the Lys residue (PLDLSCK) flanking CtBP binding motif of E1A,\textsuperscript{57} resulting in decrease of CtBP binding.

1.6. Role of CtBP

CtBP plays an important role in development and oncogenesis

1.6.1. Role in Development

In \textit{Drosophila}, dCtBP is involved in transcriptional regulation and plays an important role during early embryo development and also during later developmental processes.\textsuperscript{58, 59} The CtBP gene is involved in regulatory functions in the wing,\textsuperscript{60} eye,\textsuperscript{61} sensory organs.\textsuperscript{63} Decrease in the levels of dCtBP leads to severe segmentation defects, disruptions in anterior-posterior patterning.\textsuperscript{46} This is a result of loss of repression of genes – eve, runt and hairy by short range repressors Knirps, Snail and Kruppel. A transcriptional repressor Tramtrack69 (Ttk69) binds to dCtBP and acts as a neural inhibitor during early eye development. This interaction determines the number of photoreceptor cells produced.\textsuperscript{64}

In \textit{Xenopus}, the transcription factors xTcf-3, xFOG, xPc have been shown to interact with xCtBP. xCtBP is involved in the development and is localized in the head, tail bud
and in central nervous system. xFOG represses RBC formation partially by interacting with xCtBP.

In mammals, mCtBP2 is expressed during embryogenesis, and mCtBP1 is expressed throughout. CtBP1 is expressed in spinal chord, and CtBP2 is expressed in spinal chord, limb buds, and root ganglia. Mutation in CtBP2 causes embryonic lethality. CtBP1-null mice are smaller in size than the wt mice, and have about 23% mortality rate after birth within 20 days.

In humans, both hCtBP1 and hCtBP2 are ubiquitously expressed in most human tissues. TGIF is a transcriptional repressor of TGF-β activated genes. It binds to CtBP protein. Mutations in TGIF leads to holoprosencephaly – brain malformations, due to the loss of interaction with hCtBP1. The wt huntingtin protein mediates transcriptional repression by interacting with CtBP. In Huntington’s disease (HD), the protein contains polyglutamine expansion, which leads to reduced interaction with CtBP, possibly leading to HD.

1.6.2. Role of CtBP in Oncogenesis

CtBP has been shown to bind to more than 1,800 promoters throughout the mammalian genome. CtBP has been linked to the development of cancer in the context of EMT (epithelial-to-mesenchymal) transition. EMT transition is an essential process during development, in which epithelial cells lose cell adhesion property and polarity, and acquire migratory, invasive properties. Though EMT is essential for wound healing and during development, it increases the resistance to apoptosis and metastasis of malignant tumors.
CtBP has been shown to repress the transcription of pro-apoptotic factors,\textsuperscript{73} tumor suppressors,\textsuperscript{74} cell adhesion molecules – E-cadherin,\textsuperscript{73} cell-cell junction proteins and cytoskeletal proteins. Transcriptional repression of E-cadherin causes EMT. CtBP has been shown to play a role in the progression of melanoma,\textsuperscript{75} pituitary tumors,\textsuperscript{76} prostate cancer,\textsuperscript{77} colon cancer\textsuperscript{78-83} and breast cancer.\textsuperscript{70, 84-87}

Levels of CtBP are regulated by tumor suppressors, disruption of which leads to cancers. In colorectal tumors, mutations in the tumor suppressor APC (adenomatous polyposis coli) have been observed.\textsuperscript{88} APC degrades CtBP1.\textsuperscript{78} The levels of APC and CtBP1 are inversely correlated during cancer initiation.\textsuperscript{78, 79}

ARF is another tumor suppressor which targets CtBP for proteosomal degradation. Binding of ARF to CtBP relieves the repression of a pro-apoptotic factor Bik.\textsuperscript{81} Bik inhibits the function of anti-apoptotic factors, and sensitizes cells to apoptosis.\textsuperscript{89} Samples of colon cancer tissue showed high levels of CtBP and low levels of ARF, whereas adjacent healthy tissues showed low levels of CtBP and normal levels of ARF.\textsuperscript{83}

In colon cancer cells, increase in NADH levels due to hypoxia increased cell migration.\textsuperscript{24, 82} ARF antagonizes the function of CtBP, leading to Bik-mediated apoptosis. Hence, loss of APC and ARF increases CtBP levels and activity leading to tumorigenesis.

The role of CtBP in breast cancer has been highly studied. Depletion of CtBP stops cell growth, whereas elevated CtBP levels lead to cell survival and metastasis. Elevated CtBP1 levels have been observed in invasive ductal carcinoma tissues.\textsuperscript{85} Breast cancer cells show loss of E-cadherin, and low levels of the DNA repair protein BRCA1 breast cancer gene. Knockdown of CtBP restores the levels of E-cadherin and
Decrease in the NADH/NAD⁺ ratio removes HDAC1 for the promoter, leading to increase in BRCA1 levels. This might be the result of failure of CtBP dimerization under low NADH levels, and failure to form a repressor complex. This is the opposite effect of the “Warburg effect” in which high glycolysis increases NADH levels leading to increased CtBP activity in cancers. CtBP knockdown reduces cell proliferation and sensitizes breast cancer cells to Cisplatin. Thus, breast cancer involves downregulation of E-cadherin and BRCA1 by CtBP. CtBP-mediated repression might be resulting in BRCA1 loss even in the absence of BRCA1 mutation.

CtBP has been shown to be involved in oncogenesis through multiple mechanisms/pathways explained below.

1.6.2.1. E1A Model – Tumor suppressor role

CtBP negatively regulates oncogenesis by interacting with E1A protein. E1A mutants that lack the CtBP binding motif (PLDLS) in the C-terminal region cooperate with activated Ras oncogene, and cause high-frequency in vitro transformation of primary rat kidney cells. The resulting cells (E1A mutants) are very tumorigenic in mice models, and the tumors are metastatic. There are three possible pathways by which CtBP negatively regulates oncogenesis through E1A interaction (Figure 1-4):

a). Interaction of CtBP with the PLDLS motif in the C-terminal region might antagonise the acetyl transferase activities of p300/CBP and P/CAF. This would result in the inhibition of cell proliferation activity of the N-terminal region. It has also been shown that pRb is acetylated by the E1A complex, which increases interaction between pRb and Mdm2, resulting in the inactivation of pRb.
b). CtBP interaction with E1A represses certain cellular genes. Acetylation of Lys residues or deletion of the CtBP-interacting region (PLDLS motif) relieves repression.
c). wt E1A competitively interacts with CtBP and relieves the repression in endogenous repression complexes resulting in the activation of certain genes that modulates oncogenesis,$^{49,95,96}$ whereas mutant E1A fails to relieve the repression.

\[\text{Figure 1-4. Negative regulation of oncogenesis by CtBP through E1A (ref 27)}\]

1.6.2.2. Wnt Signaling Model – Tumor suppressor role

In mammals, CtBP might be playing a role in the Wnt signalling pathway during development and oncogenesis. In the absence of Wnt signalling pathway, APC tumor
suppressor protein degrades β-catenin; and hTcf-4 functions as a constitutive repressor of Wnt target genes. hTcf-4 has two binding motifs for CtBP binding. When Wnt signalling is activated, β-catenin levels increase. β-catenin binds to hTcf-4, recruits the p300/CBP coactivator, which activates Wnt target genes cMyc, cyclin D1 resulting in oncogenesis. Mutations in APC and β-catenin have been shown to cause tumorigenesis. Oncogenic mutations increase β-catenin levels. Mutations that affect interaction of CtBP with hTcf-4 might contribute to colorectal carcinogenesis.\textsuperscript{97,98} CtBP might be contributing to the transcriptional repression activity of hTcf-4, and antagonizing the β-catenin/CBP coactivator complex activity.

1.6.2.3. Evi-1 oncogene – Oncogenesis role

Evi-1 is a nuclear protein that functions as a sequence-specific transcriptional repressor. It inhibits TGF-β signalling by causing repression of Smad-induced transcription of TGF-β responsive genes,\textsuperscript{99} resulting in cell proliferation. Evi-1 is a cellular oncogene that has been implicated in myeloid leukemogenesis in humans and mice. It is highly expressed in human myeloid leukemias and in chronic ML. Evi-1 repressor domain contains two CtBP binding motifs. Mutants in these motifs do not bind well with CtBP and fail to cause transcriptional repression.\textsuperscript{100,101} CtBP might be playing an important role in Evi-1 mediated leukemogenesis.

1.6.2.4. Ras signalling

CtBP modulates the activity of Net belonging to the family of Ets transcriptional repressors.\textsuperscript{47} Net is regulated by Ras. Net interacts with serum response element (SRE) and represses c-fos promoter. Ras signalling reverses this repression. Net recruits CtBP and mediates repressor activity in the absence of Ras.
1.7. Dehydrogenase Activity of CtBP

CtBP is similar to the D2-HDH family of proteins. D2-HDH enzymes function by transferring a hydride anion to substrate by simultaneous NADH oxidation, and in that process convert the ketone carbonyl group of α-keto acid to hydroxyl group (Scheme 1-1, Figure 1-5).\textsuperscript{18, 102, 103} Keto-acid portion of the substrate is stabilized by arginine by electrostatic interactions, glutamate makes hydrogen bonding interaction with histidine residue, histidine proton polarizes carbonyl oxygen. NADH transfers hydride anion to the carbon of substrate carbonyl group, carbonyl oxygen abstracts proton from histidine giving the D-hydroxyacid product.

![Figure 1-5. Mechanism of hydride transfer](image)

The dehydrogenase activity of CtBP was first discovered by its ability to convert pyruvate to lactate by oxidation of NADH.\textsuperscript{23, 104} Search for potential substrates resulted in the discovery of MTOB (4-methylthio-2-oxobutyric acid), as the putative endogenous substrate for CtBP. MTOB is an intermediate in the methionine salvage
pathway – following reduction, transamination of MTHB gives methionine.\textsuperscript{105} MTOB was found to be 80-fold better substrate for CtBP1 than pyruvate.\textsuperscript{106} When the sulfur in MTOB was replaced by a methylene group (2-oxohexanoic acid), the enzymatic activity reduced by 8-fold. Thus, sulfur is essential for selectivity.\textsuperscript{106}

\begin{equation}
\begin{align*}
\text{Me-S-} & \text{O} \text{H} \\
\text{substrate MTOB} & \text{CtBP} \\
\text{NADH} & \text{NAD}\textsuperscript{+} \\
\text{Me-S-} & \text{O} \text{H} \\
\text{MTHB}
\end{align*}
\end{equation}

\textbf{Scheme 1-1. Reduction of MTOB by CtBP}

The transcriptional regulation by CtBP does not require the dehydrogenase activity of CtBP, which suggests that NADH binding is conserved for the purpose of transcriptional regulation, and not for catalytic turnover.\textsuperscript{73}

Though MTOB is a better substrate for CtBP than pyruvate, it is still a poor substrate compared to the other D2-HDH family of proteins. For example, \textit{E. coli} DGDH catalyses substrate 2600 times more efficiently than MTOB catalysis by CtBP.\textsuperscript{106-108}

1.8. Targeting CtBP to Treat Cancer

Reports show that CtBP can be inhibited by high levels (millimolar) of putative substrate MTOB (shows bi-phasic kinetics), and that MTOB has anti-tumor effects in breast and colon cancer cells.\textsuperscript{83} Although MTOB was shown to have an apoptosis-inducing effect earlier, its targets were not known at that time.\textsuperscript{109} Straza et al reported that MTOB at concentrations of around 4 mM displays cytotoxicity in HCT-116 cells.

MTOB acts by displacing CtBP from the Bik promoter, thereby relieving Bik-mediated repression, finally resulting in apoptosis. MTOB has been shown to be effective in a mouse xenograft model using p53\textsuperscript{−/−} HCT-116 cells, but it showed no effect in normal healthy mice, suggesting that it has no off-target toxicity issues. MTOB (10 mM) has
been shown to have anti-cancer effect in MCF-7 and MDA-MD231 cells through inhibition of CtBP; it inhibited the repression of several genes associated with EMT and genome stability.\textsuperscript{75} These preliminary data provide insights into targeting CTBP to treat cancers. Other than the small molecule inhibitor MTOB, there are two other CtBP inhibitors reported – peptide inhibitor, and a small molecule inhibitor NSC95397.

1.8.1. Peptide inhibitor of CtBP

Birts et al. reported a CtBP dimerization cyclic peptide inhibitor – cyclo-SGWTVVRMY by high-throughput screening that assesses SICLOPPS (split-intein circular ligation of peptides and proteins)\textsuperscript{110}, \textsuperscript{111} cyclic peptide libraries (cyclic heptamer/octamer/nonamer) of about 64 million members (genetically encoded).\textsuperscript{112} This study proved that CtBP dimerization links cellular metabolism with mitotic fidelity. The assay used a bacterial reverse two-hybrid system (RTHS) to analyse the link between bacterial (\textit{E. coli}) survival on a selective media and disruption of CtBP1 homodimer (NADH-dependent) fused to bacteriophage 434 repressor. The repressor 434 (bacteriophage DNA binding protein) is reconstituted when CtBP1 fusion protein homodimerizes, leading to repressor binding to operator sites in \textit{E. coli} chromosome, this prevents transcription of three downstream reporter genes (HIS3, Kan and LacZ), resulting in cell death in selective media. If the target proteins do not interact or when the protein-protein interaction is inhibited by an inhibitor, the reporter genes are expressed and cells survive in selective media (Figure 1-6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1-6.png}
\caption{Bacteria reverse two hybrid system (ref 112)}
\end{figure}

The peptides contained these residues in common – serine (nucleophile for intein processing), glycine (prevents racemization during synthesis), tryptophan (chromophore for HPLC purification). Transformation was carried out on CtBP1 RTHS with SICLOPPS plasmids, split-inteins were expressed that underwent processing to give cyclic peptides. Only plasmids that produce cyclic peptides disrupting CtBP1 homodimerization allow for cell survival on selective media. After picking bacterial colonies, plasmids were isolated and re-screened for non-specific inhibitors of the RTHS (by using a different RTHS system). The SICLOPPS plasmids which gave rise to three most potent peptides were sequenced for identity (Table 1-1).

Table 1-1. Peptide inhibitors identified by SICLOPPS

<table>
<thead>
<tr>
<th>Rank</th>
<th>Name</th>
<th>Target</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CP61</td>
<td>CtBP1/CtBP2</td>
<td>SGW TVVRMY</td>
</tr>
<tr>
<td>2</td>
<td>CP68</td>
<td>CtBP1/CtBP2</td>
<td>SGW PLSTWY</td>
</tr>
<tr>
<td>3</td>
<td>CP65</td>
<td>CtBP1/CtBP2</td>
<td>SGW RLIRLY</td>
</tr>
</tbody>
</table>

CP61 (Figure 1-7) has been shown to disrupt CtBP dimerization both in vitro and in cells. It binds to CtBP1 (3 \( \mu \text{M} \) affinity), inhibits CtBP homo- and heterodimerization with an in vitro IC\(_{50}\) of 19 ± 4 \( \mu \text{M} \), but it requires fusion to a cell penetrating molecule to enter the cell. CP61 does not bind to the NADH-binding pocket of CtBP, so probably it is an allosteric inhibitor. It did not show any inhibition of LDH, thus it is very selective for CtBP. CP61 was used to establish that dimerization of CtBP regulates mitotic fidelity in cancer cells. In breast cancer cells with high rate
of glycolysis, this compound reduced the mitotic fidelity, proliferation and colony formation; but had no effect in cells with lower rate of glycolysis. This provides evidence that the glycolytic state of cells is linked to mitotic cell cycle checkpoint control through NADH regulation/detection by CtBPs. This inhibitor of dimerization is important to study the roles of NADH-unbound CtBP.

1.8.2. NSC95397 – A Small-molecule inhibitor of CtBP

Blevins et al. reported a NSC95397, small molecule inhibitor of CtBP. They used a high-throughput screening assay (AlphaScreen) to screen LOPAC library (Sigma-Aldrich, 1280 bioactive compounds), and found NSC95397 (Figure 1-8) to be a good inhibitor of CtBP with an IC$_{50}$ of 2.9 µM (inhibits CtBP-E1A interaction). NSC95397 was found to be a weaker substrate of CtBP1 compared to MTOB; and was not found to inhibit LDH. MTOB failed to inhibit CtBP-E1A interaction, in contrast NSC95397 inhibits CtBP-E1A interaction. This rules out the suggestion that NSC95397 mode of action could be by it acting as a CtBP1 substrate. NSC95397 reversed the repression of E-cadherin promoter (in H1299 small cell lung carcinoma cells) by CtBP. It is a known inhibitor of cdc25 phosphatase activity and spliceosomal activity. Thus, in order for it to be a useful CtBP inhibitor, potency and specificity has to be improved.

The possible mechanism of action of this compound is that it might be locking CtBP1 in a conformation which prevents it from binding to transcriptional factors, or it could be binding to the surface groove on CtBP at the conserved binding motif PXDLS, thereby directly inhibiting interaction of CtBP with transcriptional factors.
1.9. Scope of this dissertation

This first project in my dissertation focuses on design and development of small molecule inhibitors that target CtBP for use as anti-cancer therapeutics. We have used a blend of classic traditional medicinal chemistry as well as modern computational techniques to approach the lead compounds. We succeeded in obtaining two compounds that are slightly more active in inhibiting CtBP and growth of cancer cells compared to the reported lead compound HIPP. These two compounds were found to be stable oximes, and did not have any off-target toxicity issues as determined by their lack of activity against LDH, a closely related enzyme of CtBP.
2. Structure-Guided Design of CtBP Inhibitors

2.1. MTOB as an Inhibitor of CtBP

MTOB (2-1), a putative substrate of CtBP shows bi-phasic kinetics – it acts as a substrate at lower concentrations, but acts as an inhibitor at higher concentrations. It has been shown to interfere with the oncogenic activity of CtBP in cells and mice.\textsuperscript{114} MTOB displaced CtBP from the Bik promoter and thereby induced apoptosis in HCT-116 colon cancer cells.\textsuperscript{83} In a mouse xenograft model, MTOB treated mice showed prolonged survival and less tumor burden compared to non-treated mice. MTOB shifted phenotypic indicators such as E-cadherin from mesenchymal to epithelial phenotype.\textsuperscript{70}

MTOB has provided a direction to develop small molecule inhibitors of CtBP (Figure 2-1). Hilbert et al have reported the crystal structures of human CtBP1 (28-253) and CtBP2 (33-364) complexed with NAD(H) and ligand MTOB at 2.38 Å and 2.86 Å resolution respectively.\textsuperscript{114}

Figure 2-1. Crystal structure of CtBP1 in complex with MTOB

MTOB binds in the active site cleft between the coenzyme binding domain (125-319) and the substrate binding domain (28-120, 327-353). MTOB does not cause any tertiary or quarternary changes in the protein conformation upon binding. So, the mechanism of CtBP inhibition by MTOB could be through the substrate turnover → NAD⁺ generation and release → dimer dissociation → transcriptional regulation. Or, high MTOB concentrations (via substrate inhibition) → inhibit NADH to NAD⁺ conversion → inhibit monomer-dimer cycling → transcriptional regulation.

Using the CtBP1(28-353)/MTOB/NAD⁺ crystal structure (Figure 2-2) as a starting point, we wished to design, synthesize, and evaluate small molecules that would inhibit the dehydrogenase activity of CtBP.

The interactions between MTOB and CtBP1 in the binding site show sulphur-pi interactions between ‘S’ of MTOB and tryptophan 318 of CtBP (4 Å distance between the two). This Trp has been shown to function as a dimerization switch in CtBP.¹¹⁵

![Figure 2-2. Interactions of MTOB with CtBP active site](image)
Hydrogen-bonding interactions are observed between the carbonyl group of MTOB and Arg266, His315 of CtBP; between carboxylic acid portion of MTOB and Arg97, Arg266 of CtBP. The carbonyl is properly oriented towards His315 for hydride transfer. The backbone amides in substrate-binding domain also provide two hydrogen bonding interactions with MTOB.

There is a hydrophilic cavity between MTOB binding site and NAD⁺. This cavity has four water molecules – W1, W2, W3 and W4 which link MTOB with the phosphate of NAD⁺ through hydrogen bonding (Figure 2-3). The conformation of A123 (hinge residue) is responsible for the presence of the cavity that holds water molecules. This cavity is absent in other D2-HDH enzymes because of large side chains which fill this volume and stabilize the substrates.

Two features seen in the CtBP cocrystal structure that are not shared by other D2-HDH enzymes are: conserved Trp (contributing to substrate specificity) and hydrophilic cavity (linking MTOB with phosphate of NAD⁺). These features could be used to develop highly selective CtBP inhibitors.
2.2. Design of CtBP Inhibitors Based on MTOB

Based on the interactions observed in the crystal structure of CtBP1(28-353)/MTOB/NAD\(^+\), it was hypothesized that increasing the pi-interactions with CtBP Trp318 might result in a potent inhibitor. Replacing the sulfur of MTOB with a methylene group decreased the enzymatic activity by 8-fold.\(^{106}\) This showed that pi-interaction is important for activity. Thus, the first step was to increase the pi-interactions of MTOB. We replaced the sulfur in MTOB (2-1, IC\(_{50}\) = 300 μM), with a phenyl ring to increase the π-interactions with Trp318 of CtBP (Scheme 2-1) giving rise to phenylpyruvic acid 2-2 (PPA). Hilbert et al arrived at this compound computationally through the Schrodinger Suite Glide program. Phenylpyruvic acid (2-2) was tested in an NADH consumption assay, and was found to inhibit the reduction reaction of MTOB to MTHB by CtBP. Phenylpyruvic acid (2-2) was found to have an IC\(_{50}\) of 116 μM, which is about ~3-fold better inhibitor activity than MTOB (Table 2-1). Hilbert et al reported the crystal structure of PPA complexed with CtBP1 and NAD\(^+\) at 2.1 Å resolution.\(^{116}\) The crystal structure of CtBP1 complexed with NAD\(^+\) and phenylpyruvic acid shows similar interactions as that of MTOB, and does not induce major conformational changes. The phenyl group makes pi-stacking interactions with Trp318. One interesting feature is that PPA assumes two different and proportionally

![Figure 2-4. Substrate conformation (yellow Coulombic)](image-url)
equal conformations in the crystal structure (substrate and non-canonical). The substrate conformation (Figure 2-4) is similar to that of MTOB, where the carbonyl of PPA interacts through H-bonding with His315 and Arg266; and is oriented for hydride transfer from His315. In contrast, in the non-canonical conformation (Figure 2-5), the carboxylic acid group is oriented towards His315 and Arg266; and the carbonyl group is oriented towards Ser100 and away from His315, so is not positioned for hydride transfer. The binding affinities (energies) of both the conformations are not substantially different.

**Figure 2-5. Non-canonical conformation**


The water network is disrupted (different from that of MTOB, Figure 2-6). Because of a different conformation of A123, the cavity is collapsed, as a result of which the waters W2 and W3 are absent. In the substrate conformation, W1 (orange) is present; whereas W1 is absent in the non-canonical conformation. W4 is present similar to the position in the MTOB structure.
2.3. Structure-Activity Relationship (SAR) Study

We further investigated the SAR of phenylpyruvic acid by synthesizing and testing various analogues.

2.3.1. Deconstruction analogues of phenylpyruvic acid

Phenylpyruvic acid was deconstructed to investigate which structural features of the molecule are important for activity. Three analogues were designed (Scheme 2-1): an analogue in which phenyl ring was removed – pyruvate (2-3), an analogue in which ketone was removed – hydrocinnamic acid (2-4), and an analogue in which carboxylic acid group was removed – 1-phenylpropan-2-one (2-5). All of these compounds were less active than phenylpyruvic acid.
2.3.2. Linker length analogues of phenylpyruvic acid

In order to investigate what effect the linker length has on the activity, compounds 2-6 (phenylglyoxylic acid – methylene spacer between phenyl ring and α-ketoacid removed), 2-7 and 2-8 were designed. These compounds were also either synthesized or purchased and tested for their ability to inhibit CtBP. 2-6 was less active than phenylpyruvic acid (we were unable to synthesize 2-7 and 2-8).

Scheme 2-1. Deconstruction Analogues
2.3.3. Non-reducible Ketone Isosteres of phenylpyruvic acid

Though phenylpyruvic acid (2-2) inhibits the dehydrogenase activity of CtBP, it is a substrate for the enzyme. To improve inhibition of CtBP, as well as to further establish the SAR of phenylpyruvic acid, we hypothesized that stopping this chemical reduction of phenylpyruvic acid (2-2) would result in better inhibitors. Thus, we designed compounds based on the phenylpyruvic acid structure by replacing α-ketone with isosteres that cannot be reduced by NADH.

The hypothesis was that, if CtBP is unable to reduce the compound, then the compound would remain bound to CtBP and the turnover of the enzyme would be greatly reduced, resulting in inhibition of the protein. The following analogues were designed and tested (Scheme 2-2): ketone replaced by a sulfur – α-thioketone 2-9, ketone replaced by a methylene – acrylic acid 2-10, ketone replaced by a carboxylic acid – malonic acid 2-11, amide 2-12, ketone replaced by a hydrazine – hydrazone 2-13, ketone replaced by an oxime – hydroxyimine (HIPP) 2-14.
All of these compounds were either synthesized or purchased and tested for their ability to inhibit CtBP. Although most of these compounds were less active than phenylpyruvic acid (Table 2-1), the compound hydroxyimine 2-14 inhibited CtBP with an IC$_{50}$ of 0.24 μM, a ~480-fold improvement over PPA (2-2). Hydroxyimine 2-14 was therefore chosen for further structure-activity relationship studies.
Table 2-1. IC\textsubscript{50} values of deconstruction analogues and PPA ketone isosteres

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (\textmu M)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTOB (2-1)</td>
<td>300</td>
</tr>
<tr>
<td>2-2</td>
<td>116.3</td>
</tr>
<tr>
<td>2-3</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-4</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-5</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-6</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-9</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-10</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-11</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-12</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-13</td>
<td>&gt;300</td>
</tr>
<tr>
<td>2-14</td>
<td>0.24</td>
</tr>
</tbody>
</table>

2.3.4. SAR of the Lead Compound HIPP

With the above results, which helped us understand the minimum structural elements needed to inhibit CtBP, we designed a large set of analogues based on the best inhibitor in our series – hydroxyimine 2-14. In the next series, we worked on exploring the structure-activity relationship of the phenyl ring. Our set of analogues included electronically and sterically diverse substituents. All analogues were evaluated computationally by docking them into the site identified in the CtBP-2-14 crystal structure to prioritize synthesis and evaluation. Docking scores were calculated with the HINT scoring function;\textsuperscript{117} this identified thirteen compounds 2-15 through 2-27 that we selected for synthesis and evaluation as inhibitors of CtBP (Figure 2-7).
All these compounds were tested against recombinant CtBP and in HCT-116 cells. Compounds 2-21 and 2-22 were found to be the most potent analogues in this series based on the lead compound 2-14 (Table 2-2).
Table 2-2. IC<sub>50</sub> values of oxime analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Substituent</th>
<th>CtBP IC&lt;sub&gt;50&lt;/sub&gt; (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cellular IC&lt;sub&gt;50&lt;/sub&gt; (mM)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-14</td>
<td>H</td>
<td>0.24 (0.21, 0.27)</td>
<td>4.12 (2.96, 5.73)</td>
</tr>
<tr>
<td>2-15</td>
<td>4-Me</td>
<td>0.32 (0.29, 0.37)</td>
<td>3.28 (2.51, 4.28)</td>
</tr>
<tr>
<td>2-16</td>
<td>3-Me</td>
<td>0.48 (0.43, 0.54)</td>
<td>3.26 (2.71, 3.93)</td>
</tr>
<tr>
<td>2-17</td>
<td>2-Me</td>
<td>8.73 (6.19, 12.29)</td>
<td>0.23 (0.16, 0.35)</td>
</tr>
<tr>
<td>2-18</td>
<td>4-OMe</td>
<td>2.16 (1.19, 3.90)</td>
<td>1.93 (1.65, 2.25)</td>
</tr>
<tr>
<td>2-19</td>
<td>3-OMe</td>
<td>0.88 (0.81, 0.97)</td>
<td>5.60 (3.56, 8.84)</td>
</tr>
<tr>
<td>2-20</td>
<td>2-OMe</td>
<td>&gt; 100</td>
<td>1.24 (1.02, 1.51)</td>
</tr>
<tr>
<td>2-21</td>
<td>4-Cl</td>
<td>0.18 (0.16, 0.20)</td>
<td>1.74 (1.47, 2.06)</td>
</tr>
<tr>
<td>2-22</td>
<td>3-Cl</td>
<td>0.17 (0.15, 0.19)</td>
<td>0.85 (0.76, 0.96)</td>
</tr>
<tr>
<td>2-23</td>
<td>2-Cl</td>
<td>7.65 (5.93, 9.86)</td>
<td>2.37 (1.83, 3.08)</td>
</tr>
<tr>
<td>2-24</td>
<td>4-OH</td>
<td>7.34 (5.26, 10.25)</td>
<td>&gt;10</td>
</tr>
<tr>
<td>2-25</td>
<td>3-OH</td>
<td>0.72 (0.67, 0.78)</td>
<td>&gt;10</td>
</tr>
<tr>
<td>2-26</td>
<td>4-F</td>
<td>0.30 (0.27, 0.33)</td>
<td>3.97 (3.52, 4.49)</td>
</tr>
<tr>
<td>2-27</td>
<td>4-CN</td>
<td>0.90 (0.82, 0.98)</td>
<td>1.10 (0.81, 1.49)</td>
</tr>
<tr>
<td>MTOB (2-1)</td>
<td>---</td>
<td>n.d.</td>
<td>4.0</td>
</tr>
</tbody>
</table>

2.3.5. Second generation analogues

Based on the above results, we synthesized second generation analogues (Figure 2-8). As the compounds 2-21 (4-Cl HIPP) and 2-22 (3-Cl HIPP) had very good activity, the next obvious compound to synthesize was 3, 4-dichloro HIPP (2-28) to see if incorporation of chloro groups at both the positions would give a better inhibitor. We also synthesized 2,4-dichloro HIPP (2-29) to see if there would be any improvement
in the activity. The next compound we synthesized was 4-trifluromethyl HIPP (2-30) to investigate if a more electron withdrawing group in the para position would improve the activity relative to 4-Cl HIPP (2-21).

![Figure 2-8. Second generation analogues](image)

In parallel with this study to explore the structure-activity relationship of hydroxyimine 2-14, our collaborators co-crystallized 2-14 with CtBP1 and NADH at a resolution of 2.3 Å (Figure 2-9). It assumes the non-canonical conformation, i.e. the oxime group is oriented away from His315, so hydride transfer cannot occur. If this compound were to adopt a substrate conformation (i.e. oxime oriented towards His315), then a large conformational change in the protein would be necessary to prevent oxime from clashing with His315. The carboxylic acid group forms H-bonds with His315 and Arg266, Coulombic interactions with Arg97. The oxime forms H-bond with Ser100. It also forms a H-bond with an active site water molecule, which stabilizes it.

![Figure 2-9. CtBP1-NADH-HIPP crystal structure](image)
The phenyl ring forms pi-stacking interactions with Trp318 (Figure 2-10, from PyMol). This van der Waals interaction is 2-3 fold greater than the sulfur-pi interaction in MTOB.

![Figure 2-10. Interactions of HIPP at CtBP active site](image)

The water network is different from that of MTOB. W1 is displaced completely by the hydroxyl group of the oxime. Both W2 and W3 have shifted. HIPP interacts directly with W2. The positions of W2 and W3 clash (1.9 Å apart). There is no change in the position of W4 (Figure 2-11).
HIKP \((K_d = 0.37 \ \mu M)\) has been shown to bind to CtBP1 with 1000-fold more affinity than that of MTOB \((K_d = 1.26 \ mM)\) through Isothermal Titration Calorimetry (ITC) experiment. Though HIPP is expected to show competitive inhibition (no change in \(V_{max}\), but increase in \(K_m\)) due to its binding in the substrate binding site, kinetic experiments indicate non-competitive inhibition (decrease in \(V_{max}\), but no change in \(K_m\)). This could be because NADH binding causes a conformational change in the protein leading to domain closure, which prevents release of NAD\(^+\) after product (MTHB) release, and permits binding of a molecule of inhibitor (HIKP) or substrate (MTOB). This forms an abortive ternary complex resulting in enzyme non-competitive inhibition by HIKP. This model has not been completely investigated yet.
2.4. Syntheses and Biological Assays of CtBP Inhibitors

2.4.1. Syntheses of the compounds

The compounds MTOB (2-1), phenylpyruvic acid (2-2), pyruvic acid (2-3), hydrocinnamic acid (2-4), phenylglyoxylic acid (2-6), 2-benzylacrylic acid (2-10), benzylmalonic acid (2-11) and anilino(oxo)acetic acid (2-12) were purchased from commercial sources.

The compound 2-5 was synthesized by reacting phenylacetic acid (3-1) with dimethylhydroxylamine hydrochloride under EDC coupling conditions to give the Weinreb amide 3-2. This underwent Grignard reaction with methylmagnesium bromide under anhydrous conditions to furnish the product 2-5 (Scheme 2-3).

The compound 2-9 was synthesized by the reaction of benzaldehyde (3-3) and rhodanine (3-4) to give the condensed intermediate benzalrhodanine (3-5), which underwent hydrolysis under basic conditions to furnish the thioketone product 2-9 (Scheme 2-4).
The hydrazone 2-13 was synthesized by reacting phenylpyruvic acid (3-6) with hydrazine under basic conditions. The hydroxyimine compound 2-14 was synthesized by reacting phenylpyruvic acid (3-6) with hydroxylamine hydrochloride under basic conditions (Scheme 2-5).

![Scheme 2-5. Synthesis of compound 2-14]

To synthesize the hydroxyimine analogues, we chose hydantoin chemistry as a general route that would offer the flexibility needed to introduce a variety of substituents on the phenyl ring (Scheme 2-6). Condensation of substituted benzaldehydes (3-7a through 3-17a) with hydantoin under basic conditions afforded the 5-benzylideneimidazolidine-2,4-diones (3-7b through 3-17b), which were then hydrolyzed to afford a variety of substituted phenylpyruvic acid analogues (3-7c through 3-17c). These phenylpyruvic acid analogues were then condensed with hydroxylamine to form the hydroxyimine analogues (2-15 through 2-27). We found this synthetic route to be highly tolerant of substitutions on the starting benzaldehyde and were able to synthesize eleven of the thirteen analogues using this methodology.
For the two compounds (2-19 and 2-22) where condensation of the benzaldehyde with the hydantoin failed to afford the 5-benzylideneimidazolidine-2,4-dione, we used the alternative methodology shown in (Scheme 2-7).

**Scheme 2-7.** Synthesis of α-keto-acids and oximes from 1,4-diacetylpyrrolidine-2,5-dione
This alternative method utilized a condensation of benzaldehydes \(3-18a\) and \(3-19a\) with 1,4-diacetylpirperazine-2,5-dione to afford the 1-acetyl-3-benzylidenepiperazine-2,5-diones \(3-18b\) and \(3-19b\). These intermediates were then hydrolyzed to afford the phenylpyruvic acid analogues \(3-18c\) and \(3-19c\), followed by condensation with hydroxylamine to form the hydroxyimine analogues \(2-19\) and \(2-22\). We were unable to synthesize compounds \(2-7\) and \(2-8\).

**Second generation analogues**

![Scheme 2-8. Synthesis of second generation analogues](image)

For the three compounds (\(2-28\), \(2-29\) and \(2-30\)), where condensation of the benzaldehyde with both hydantoin and 1,4-diacetylpirperazine-2,5-dione failed to afford the corresponding condensed intermediates, we used the alternative methodology shown in (Scheme 2-8). This alternative method utilized a condensation of benzaldehydes \(3-20a\), \(3-21a\) and \(3-22a\) with N-acetylglycine to afford the condensed intermediates \(3-20b\), \(3-21b\) and \(3-22b\). These intermediates were then hydrolyzed to afford the phenylpyruvic acid analogues \(3-20c\), \(3-21c\) and \(3-22c\), followed by condensation with hydroxylamine to form the hydroxyimine analogues \(2-28\), \(2-29\) and \(2-30\).
2.4.2. Biological Assays – Recombinant CtBP and Cellular assays

All the compounds synthesized above or purchased were tested against recombinant CtBP enzyme and also in cellular growth/viability assays (HCT-116 p53−/− colon cancer cells). While most of the compounds were inactive, three compounds 2-14, 2-21 and 2-22 showed good inhibitory activity against CtBP.

2.4.3 LDH Assay to Determine Off-target Toxicity

Lactate Dehydrogenase is closely related to CtBP in structure and function. We wished to test if our compounds are specific against CtBP. Thus, three compounds (most potent in our series) – HIPP, 3-Cl HIPP and 4-Cl HIPP were tested for their ability to inhibit L-Lactate Dehydrogenase (LDH from rabbit muscle). Sodium oxamate (a known inhibitor of LDH) was used as the standard. Stock solutions of the compounds in DMSO were added to a reaction mixture containing phosphate buffer (pH 7.2), LDH, pyruvate and NADH. The enzyme inhibition was measured by monitoring change in levels of NADH (absorbance) over a period of 30 min. The minimal concentration of compounds leading to 50% inhibition of LDH activity (IC₅₀) was calculated using non-linear regression of the experimental data, using Prism software (GraphPad).

The average IC₅₀ (µM) values are reported below:

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIPP</td>
<td>42.53 ± 0.11</td>
</tr>
<tr>
<td>3-Cl-HIPP</td>
<td>94.86 ± 7.51</td>
</tr>
<tr>
<td>4-Cl-HIPP</td>
<td>181.9 ± 12.87</td>
</tr>
<tr>
<td>oxamate</td>
<td>45.80 ± 1.89</td>
</tr>
</tbody>
</table>

Detailed experimental procedure is reported in 6.4d.
2.4.4. HPLC Study to Determine Stability of the Oxime Compounds

As the \textit{in vitro} activity and the \textit{in vivo} activity of the oxime compounds did not show proper correlation, we wanted to test if the oxime compounds are unstable under the assay conditions, as instability of the compounds would explain the complex SAR between in vitro and in vivo activities. We tested the stability of three most potent (HIPP, 3-Cl HIPP and 4-Cl HIPP) compounds by HPLC. These compounds were found to be stable.

2.5. Discussion

2.5.1. Inhibitory activity against Recombinant CtBP

Starting from MTOB (2-1, IC\textsubscript{50} = 300 μM), phenylpyruvic acid (2-2) was found to be a ~3-fold better inhibitor than MTOB (Table 2-1) with an IC\textsubscript{50} of 116 μM. Compounds 2-3, 2-4, 2-5 and 2-6 were less active than phenylpyruvic acid (we were unable to synthesize compounds 2-7 and 2-8). Compounds 2-9 through 2-13 were less active than phenylpyruvic acid itself (Table 2-1), whereas hydroxyimine 2-14 inhibited CtBP with an IC\textsubscript{50} of 0.24 μM, a ~480-fold improvement over PPA.

Hydroxyimine analogues 2-15 through 2-27 were tested for their ability to inhibit the functional dehydrogenase activity of CtBP in a NADH disappearance assay. Relative to the parent hydroxylamine 2-14, we observed that ortho-substitutions on the ring were detrimental to inhibitory activity [2-17 (2-Me), 2-20 (2-OMe), 2-23 (2-Cl), Table 2-2]. Para- and meta-substitution by electron-donating groups [2-18 (4-OMe), 2-19 (3-OMe), 2-24 (4-OH), 2-25 (3-OH)] resulted in a large decrease (4-OH) or a small decrease (4-OMe, 3-OMe, 3-OH) in inhibitory activity. Para- and meta-substitution by the electronically neutral methyl group [2-15 (4-Me), 2-16 (3-Me)] resulted in only a
small decrease in inhibitory activity. However, para- and meta-substitution by an
electron-withdrawing chlorine group [2-21 (4-Cl), 2-22 (3-Cl)] resulted in analogues
that were more active as inhibitors than parent hydroxyimine 2-9. Interestingly, this
effect seems specific to chlorine as para-substitutions with other electron-withdrawing
groups [2-26 (4-F), 2-27 (4-CN)] resulted in no increase in inhibition (2-26) or a
decrease in inhibitory activity (2-27).

We are yet to test the activity of our second generation analogues.

2.5.2. Inhibitory activity against Cellular CtBP (HCT-116 Colon Cancer Cells)

To determine whether inhibition of the dehydrogenase activity of CtBP by
hydroxyimine 2-14 and analogues 2-15 through 2-27 is effective at inhibiting cancer
cell growth, we carried out a short-term cell growth/viability assay (Table 2-2). HCT-
116 p53⁻/⁻ colorectal cancer cells were treated with the compounds for 72 hours and
cell growth and viability was determined by MTT assay. Compounds 2-17, 2-20, 2-21,
2-22 and 2-27 were found to inhibit the growth of HCT-116 cells.

2.5.3. SAR – In vitro vs In vivo Activity

A complex structure/activity relationship was observed, between enzyme inhibition
and cell inhibitory activity, shown by the plot in Figure 2-12. A number of compounds
appeared to have cytotoxic effects out of proportion to their enzymatic activity,
consistent with off-target toxicity 2-18 (4-OMe), 2-27 (4-CN), while others had lower
than expected cellular inhibitory effects consistent with instability or poor intracellular
penetration 2-19 (3-OMe), 2-25 (3-OH). However, a series of compounds exhibited a
relatively consistent correlation of enzymatic and cellular inhibitory activity 2-15 (4-
Me), 2-16 (3-Me), 2-21 (4-Cl), 2-22 (3-Cl), 2-26 (4-F) with 2-21 (4-Cl) and 2-22 (3-Cl)
demonstrating the strongest enzymatic and cellular inhibitory activity of this subset of the compounds.

![Enzymatic vs cellular activity of CtBP inhibitors](image)

**Figure 2-12.** Enzymatic vs cellular activity of inhibitors

### 2.5.4. Docking of Inhibitors in the Active Site of CtBP

More detailed computational docking with this crystal structure model were performed to support the SAR studies. While no clear trend in docking scores vs. protein or cellular inhibition IC\textsubscript{50}'s were found, the docked poses of designed analogues at CtBP active site revealed factors important for binding and inhibition. Most importantly, the phenyl rings of the ligands fit well in a hydrophobic region primarily formed by Try318, Tyr76 and Met327. Compounds 2-15 and 2-21 form stronger hydrophobic and aryl-X interactions in the pocket, compared to 2-24, where its polar (OH) substitution results in unfavorable hydrophobic-polar interactions. These results will inform our design of next-generation analogues.

### 2.5.5. Off-target Toxicity

The three most potent compounds in our series – HIPP, 3-Cl HIPP and 4-Cl HIPP showed no inhibition against LDH, a closely related enzyme to CtBP. This shows that
our inhibitors are highly specific for CtBP. The aryl ring (complementary to Trp318 of CtBP), and the α-keto acid portion of the molecule (in the hydrophilic cavity of CtBP) might be conferring selectivity towards CtBP vs other dehydrogenases which lack these two key features present in CtBP. So, the compounds 2-17, 2-18, 2-20, 2-23 might be targeting some other proteins resulting in their cytotoxic effects (poor in vitro activity but good in vivo activity).

2.5.6. Stability of Oximes

As the in vitro activity and the in vivo activity of the oxime compounds did not show proper correlation, we wanted to test if the oxime compounds are indeed stable under the assay conditions, as instability of the compounds would explain the complex SAR between in vitro and in vivo activities. We tested the stability of three most potent (HIPP, 3-Cl HIPP and 4-Cl HIPP) compounds by HPLC (detailed procedure in section 2.7.8). These compounds were found to be stable. Thus, it is highly possible that compounds 2-19 and 2-25 might be having cell permeability issues (good in vitro activity but poor in vivo activity).

2.5.7. Hypothesis and Model

There is concrete evidence in the literature that targeting CtBP could be a highly tumor-selective and effective strategy for treating cancer. CtBP is activated as a transcriptional co-regulator only under conditions that are unique to tumor cells (hypoxia and/or high NADH concentration – Warburg effect). Disrupting the transcriptional co-regulatory function of CtBP in a tumorigenic environment has wide ranging effects on multiple cellular mechanisms, as was seen in the genomic study by Gardner and coworkers,70 and can restore the natural, endogenous function of tumor suppressor genes.
While genetic inhibition of CtBP (with siRNA) and pharmacological inhibition with peptides are effective tools for studying the role of CtBP in cancer biology, small molecule inhibitors of CtBP are needed for further clinical drug development. Here we report the first small molecule inhibitors for CtBP, and have optimized inhibition of the dehydrogenase function of CtBP to a submicromolar level. While these compounds are effective inhibitors of recombinant CtBP, high concentrations are still required to inhibit growth in cells, with the \textit{in vivo} cell growth inhibitory activity of the best compounds disappointingly falling at just below 1 mM. Earlier work with the parent compound MTOB, however, suggests that in long term colony formation assays, GI\textsubscript{50} values are usually 10-fold lower than those seen in short term assays, and those studies are ongoing with the hydroxyimine derivatives.

Even taking the possible 10-fold lower GI\textsubscript{50} of CtBP inhibitors in long-term assays into account, the cellular inhibitory constants for the current series of CtBP inhibitors remain 2-3 orders of magnitude higher than the in vitro IC\textsubscript{50}’s. A possible rationale for this effect is that in cancer cells, CtBP has already formed transcriptional co-repression complexes with NADH, transcription factors, and repressor enzymes, and is not available for effective inhibitor binding. Binding of the inhibitor to CtBP either must break up the transcriptional co-repression complex or wait until the complex dissociates naturally in order to bind. This mechanistic model is consistent with our data that the compounds effectively bind to and inhibit the dimeric form of CtBP generated in the \textit{in vitro} assay. We are currently working to investigate this mechanistic model and develop analogues with improved activity in the cellular assays. We are also using these new CtBP inhibitors as tools for studying the role of
CtBP in cell survival and migration, as well as the impact of restoring tumor suppressor gene expression and inhibiting oncogenes in tumor cells.

2.6. Computational Approach towards Design of Diverse CtBP Inhibitors

Arriving at lead compounds quickly and efficiently (both time and cost-wise) is best achieved by using a combination of different approaches. We used computational approaches to design more CtBP inhibitors complementary to the traditional medicinal chemistry SAR approach discussed above. Through computational approaches, a large chemical space can be explored and diverse scaffolds of lead compounds could be identified in less time, which is not always possible with the traditional medicinal chemistry approach.

Virtual screening is a computational process in which a large set of compound libraries can be screened to identify compounds that could potentially bind in a particular proteins binding site. It is a process in which we can virtually filter compounds that need to be synthesized/purchased and tested, thereby saving on the time and resources. It can either be ligand-based or structure-based. In ligand-based virtual screening, the features of a pharmacophore (from a known ligand of a protein) are used to screen libraries of compounds. In structure-based virtual screening, compounds are docked into the active site of the protein, and scored using scoring functions to determine which compounds are likely to bind to the protein.

We carried out virtual screening of the ZINC database using UNITY in SYBYL using a combination of both ligand-based and structure-based methods. We utilised the co-crystal structure of CtBP-NADH-HIPP. The binding pocket with HIPP is shown in the figure below (Figure 2-13).

UNITY query features: We defined the following features as queries in UNITY.
a. Aromatic feature – 1.5 Å radius – complementary to Trp318 (pi-stacking)
b. Aromatic feature – 1.0 Å radius – complementary to Tyr76 (pi-stacking)
c. Negative center – 1.0 Å radius – complementary to Arg266 (electrostatic)
d. Acceptor site – 1.0 Å radius – at His 315 (H-bond)
e. Exclusion spheres – 0.75 Å radius – receptor cavity (to avoid clashes)
f. Bond path constraint – 3-5 bonds – between negative center and aromatic feature.

We performed a UNITY Flex search to screen the ZINC database of clean drug-like subset (~10 million compounds). We obtained 289 hits.

![Figure 2-13. UNITY features based on HIPP](image)

**Docking** is a process in which the compounds are docked in the binding site of a protein to see which conformations the compound can possibly bind in. We performed docking of the 289 hits using GOLD. The binding cavity was defined as 10 Å radius around Arg266, the number of GA runs was 50.

**Scoring** is a process in which the different docked poses are ranked based on the favorable and unfavorable interactions. Higher score means more favourable
interactions usually, although the poses have to be manually visualized to make sure that the interactions are possible, and are not errors. We used HINT scoring function to score all the solutions obtained from GOLD. Initial top HINT scores of best ligand conformations 1290-1926. We then performed energy minimization of top scored 50 protein-ligand complexes (to mimic actual binding of compound to the protein) followed by HINT rescoring of ligands. The final HINT scores were around 1362-2294.

We evaluated binding modes of these compounds, an example (ZINC 02586210, final HINT score 2294) is shown below (Figure 2-14). There are two phenyl rings (yellow) which show pi-stacking interactions with Tyr76 and Trp318. The carboxylate group (red) shows electrostatic interactions with Arg266 and Arg97. The amine (blue) has a H-bonding interaction with His315.

**Figure 2-14.** Binding mode of compound ZINC02586210

Based on the HINT scores, we ordered the following 10 hits, and are yet to test their activity against CtBP (Figure 2-15).
Figure 2-15. Hits obtained through virtual screening to be tested
2.7. Experimental Procedures

2.7.1. General Chemical Methods

Reagents/chemicals, catalysts, solvents were purchased from Sigma-Aldrich, Fisher and Alfa-Aesar. Analytical Thin Layer Chromatography (TLC) was performed using silica gel GHLF plates (Analtech Inc.). Flash chromatography was performed on TELEDYNE ISCO CombiFlash® Rf instrument using RediSep Rf Normal-phase Flash Columns (4-gm, 12-gm, 24-gm or 40-gm). $^1$H NMR and $^{13}$C NMR experiments were recorded on BRUKER 400MHz NMR instrument in deuterated solvents - chloroform (CDCl$_3$), acetone ((CD$_3$)$_2$CO), dimethyl sulfoxide ((CD$_3$)$_2$SO) or methanol (CD$_3$OD). All chemical shifts are reported in parts per million (ppm) with reference to chloroform, acetone, DMSO and methanol residual peaks at 7.26, 2.05, 2.50 and 3.31 respectively ($^1$H NMR spectra); 77.16, 29.84, 39.52 and 49.00 respectively ($^{13}$C NMR spectra). The data is reported as: chemical shifts (ppm), multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant(s) (Hz) and integral values.

2.7.2. Rationally Designed and Deconstruction Analogues

The following compounds were purchased from commercial sources and tested for CtBP inhibition without further purification: 4-methylthio-2-oxobutyric acid (MTOB, 2-1, Sigma-Aldrich), phenyl pyruvic acid (2-2, Sigma-Aldrich), 2-benzylacrylic acid (2-10, TCI America), benzylmalonic acid (2-11, Sigma Aldrich), anilino(oxo)acetic acid (2-12, Sigma-Aldrich), pyruvate (2-3, Sigma-Aldrich), hydrocinnamic acid (2-4, Sigma Aldrich), and phenylglyoxylic acid (2-6, Sigma-Aldrich).
3-Phenyl-2-thioxopropanoic acid (2-9). Rhodanine 3-4 (1.25 g, 9.4 mmol), anhydrous sodium acetate (2.16 g, 26.4 mmol), and benzaldehyde 3-3 (1.0 g, 9.4 mmol) were added to a vial followed by glacial acetic acid (17.8 mL, 0.53 M). The reaction was heated to 115 °C for three hours, upon which time the reaction had turned orange and developed a precipitate. The reaction mixture was cooled to room temperature and poured into ice water, which caused additional precipitate to form. Upon warming to room temperature, the precipitate was filtered and then dried in a oven for 36 hours to afford a dry powder 3-5 (1.66 g, 80% yield). This intermediate was dissolved in water (10 mL) and 6N sodium hydroxide (10 mL) and heated to 95 °C for 1 h. The reaction mixture was cooled to room temperature, water (10 mL) was added, and 6N HCl was added to crash out a white precipitate. This precipitate was filtered and purified by flash chromatography (silica gel, 5% MeOH/DCM) to afford the product (0.86 g, 63% yield). \textsuperscript{1}H NMR (400 MHz, (CD\textsubscript{3})\textsubscript{2}SO) \textit{δ} 7.74 (s, 1H), 7.68 (d, \textit{J} = 7.4 Hz, 2H), 7.48 (t, 2H), 7.42-7.36 (m, 1H). HRMS C\textsubscript{9}H\textsubscript{7}O\textsubscript{2}S [M-H]: Expected: 179.0167, Found: 179.0164.

2-hydrazono-3-phenylpropanoic acid (2-13): Phenylpyruvic acid 3-6 (0.1 g, 0.61 mmol) is dissolved in ethanol (0.4 ml). Hydrazine (0.02 mL, 0.61 mmol) is added and the reaction mixture is stirred overnight at room temperature. Sodium carbonate (0.03 g, 0.31 mmol) is added and the reaction mixture is stirred for 2 h at room temperature. The precipitated solid is filtered and dried to give the title compound in 62% yield (0.07
g, 0.38 mmol). $^1$H NMR (400 MHz, DMSO) $\delta$ 7.26 (d, $J = 7.5$ Hz, 2H), 7.19 (t, $J = 7.7$ Hz, 2H), 7.10 (t, $J = 7.5$ Hz, 1H), 6.26 (s, 2H), 3.78 (s, 2H); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 168.60, 146.52, 138.01, 128.68, 127.88, 125.37, 30.21. HRMS C$_9$H$_{10}$N$_2$O$_2$ [M+Na]$^-$ Expected: 201.0634, Found: 201.0622.

![2-(hydroxyimino)-3-phenylpropanoic acid (2-14)](image)

2-(hydroxyimino)-3-phenylpropanoic acid (2-14): Phenyl pyruvic acid 3-6 (3.0 g, 18.28 mmol) was dissolved in a solution of NaOH (2.2 g, 54.83 mmol) in water (1 mL). Hydroxylamine hydrochloride (1.9 g, 27.41 mmol) was added to the reaction and stirred overnight at room temperature. 1N HCl was added to the reaction, the precipitated product is filtered and dried. The crude product is purified using flash chromatography (silica gel, 5% MeOH/DCM). Yield: 76% (2.50 g, 13.95 mmol). $^1$H NMR (400 MHz, DMSO) $\delta$ 12.28 (s, 1H), 7.27 (m, 2H), 7.19 (m, 3H), 3.82 (s, 2H); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 165.14, 150.13, 136.68, 128.52, 128.32, 126.13, 29.85. HRMS C$_9$H$_9$NO$_3$ [M-H]$^-$ Expected: 178.0509, Found: 178.0494.

![1-phenylpropan-2-one (2-5)](image)

1-phenylpropan-2-one (2-5): Phenylacetic acid 3-1 (0.5 g, 3.7 mmol) was dissolved in DCM (9.2 mL). To this, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (0.8 g, 4.4 mmol), 4-methylmorpholine (0.4 g, 3.7 mmol), N,O-Dimethylhydroxylamine hydrochloride (0.4 g, 4.0 mmol) were added. The reaction mixture was stirred at room temperature for 4 h. It was concentrated and diethyl ether was added. The organic layer was washed with brine, dried over Na$_2$SO$_4$ and evaporated. Purification of the
residue on silica gel (5:1 hexanes/EtOAc) afforded the product $N$-methoxy-$N$-methyl-2-phenylacetamide 3-2 in 87% yield. 0.6 g (3.2 mmol) of this product was dissolved in ether (10.6 ml) under nitrogen at 0 °C. Methylmagnesium bromide solution (3.4 mL, 4.8 mmol, 1.4 M in THF:toluene 1:3) was added over 30 min. The reaction was stirred at 0 °C for 1 h and at room temperature for 30 min. It was quenched at 0 °C with the slow addition of 1 M HCl. The reaction was extracted with ether. The organic layer was washed with brine, dried over $Na_2SO_4$ and evaporated. Purification of the residue on silica gel (5:1 hexanes/EtOAc) afforded the product 1-phenylpropan-2-one in 89% yield (0.38 g, 2.83 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.21-7.33 (m, 5H), 3.75 (s, 2H), 2.15 (s, 3H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 209.24, 136.00, 130.58, 129.67, 127.97, 51.37, 29.27. HRMS C$_9$H$_{10}$O [M-H] Expected: 133.0659, Found: 132.0813.

2.7.3. Synthesis of Hydroxyiminophenylpyruvic acid Analogues

2.7.3.1. Synthesis of keto-acid intermediates

**General Procedure 1:** Hydantoin (15.0 mmol) was dissolved in water (15.0 mL) at 70 °C. The pH of the solution was adjusted to 7 using saturated sodium bicarbonate solution. Ethanolamine (1.4 mL) was added and the temperature was raised to 90 °C. The corresponding aldehyde (15.0 mmol) in ethanol (15.0 mL) was added drop wise to the reaction mixture. The reaction was refluxed at 120 °C for 5-10 h. After being cooled to room temperature, the product was filtered, washed with alcohol/water (1:5) and dried to give the corresponding benzalhydantoin intermediate. This corresponding benzylhydantoin (8.98 mmol) was dissolved in 20% aqueous NaOH solution (28.4 mL) and refluxed at 100 °C for 3 h. After being cooled to room temperature, 12N HCl (11.8 mL) was added. Sodium bicarbonate was added to bring the pH to 7. The reaction mixture was extracted with ether until the ether layer was clear. This layer was discarded. To the aqueous layer, 12N HCl (7.1 mL) was added. It was extracted with
ether until no more acid was obtained. The ether layer was dried to give the crude keto acid. It was recrystallized in water to give pure product.

\[
\text{\text{Me}} \quad \text{\text{OH}}
\]

2-oxo-3-\text{\text{p}}-tolylpropanoic acid (3-7c): Procedure 1 was followed using 4-methylbenzaldehyde. Yield 54\% (1.0 g, 5.61 mmol). \text{\text{\text{H}}} NMR (400 MHz, MeOD) \delta 7.64-7.66 (d, \text{\text{J}} = 8.1 \text{~Hz}, 2\text{H}), 7.12-7.14 (d, \text{\text{J}} = 8.1 \text{~Hz}, 2\text{H}), 6.46 (s, 1\text{H}), 2.32 (s, 3\text{H}); \text{\text{\text{\text{C}}} NMR (125 MHz, MeOD) \delta 168.46, 141.49, 138.44, 133.49, 130.72, 129.87, 111.79, 21.28. HRMS C_{10}H_{10}O_{3} [M-H]^{-} Expected: 177.0557, Found: 177.0562.

\[
\text{\text{Me}} \quad \text{\text{OH}}
\]

2-oxo-3-\text{\text{m}}-tolylpropanoic acid (3-8c): Procedure 1 was followed using 3-methylbenzaldehyde. Yield 57\% (0.3 g, 1.68 mmol). \text{\text{\text{H}}} NMR (400 MHz, MeOD) \delta 7.57 (d, \text{\text{J}} = 4.1 \text{~Hz}, 1\text{H}), 7.55 (s, 1\text{H}), 7.18-7.22 (t, \text{\text{J}} = 7.5 \text{~Hz}, 1\text{H}), 7.03-7.05 (d, \text{\text{J}} = 7.9 \text{~Hz}, 1\text{H}), 6.45 (s, 1\text{H}), 2.32 (s, 3\text{H}); \text{\text{\text{\text{C}}} NMR (125 MHz, MeOD) \delta 170.0, 143.64, 140.38, 137.85, 132.89, 130.72, 129.55, 113.34, 23.07. HRMS C_{10}H_{10}O_{3} [M-H]^{-} Expected: 177.0557, Found: 177.0565.

\[
\text{\text{Me}} \quad \text{\text{OH}}
\]

2-oxo-3-\text{\text{o}}-tolylpropanoic acid (3-9c): Procedure 1 was followed using 2-methylbenzaldehyde. Yield 58\% (0.08 g, 0.45 mmol). \text{\text{\text{H}}} NMR (400 MHz, (CD_{3})_{2}CO) \delta 8.18 (d, \text{\text{J}} = 8.2 \text{~Hz}, 1\text{H}), 7.85 (s, 1\text{H}), 7.11-7.23 (m, 4\text{H}), 6.74 (s, 1\text{H}), 2.38 (s, 3\text{H}); \text{\text{\text{\text{C}}} NMR (125 MHz, (CD_{3})_{2}CO) \delta 167.10, 141.15, 137.11, 133.96, 130.84, 130.64,
3-(4-methoxyphenyl)-2-oxopropanoic acid (3-10c): Procedure 1 was followed using 4-methoxybenzaldehyde. Yield 88% (1.8 g, 9.27 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.71-7.73 (d, $J = 8.8$ Hz, 2H), 6.88-6.90 (d, $J = 8.9$ Hz, 2H), 6.46 (s, 1H), 3.80 (s, 3H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 168.61, 160.57, 140.55, 132.24, 129.08, 114.76, 111.83, 55.69. HRMS C$_{10}$H$_{10}$O$_3$ [M-H]$^-$ Expected: 177.0557, Found: 177.0549.

3-(2-methoxyphenyl)-2-oxopropanoic acid (3-11c): Procedure 1 was followed using 2-methoxybenzaldehyde. Yield 56% (0.23 g, 1.29 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 8.20-8.22 (m, 1H), 7.19-7.23 (m, 1H), 6.90-6.95 (m, 3H), 3.86 (s, 3H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 168.64, 158.20, 141.79, 131.72, 129.78, 124.98, 121.41, 111.46, 105.03, 56.11. HRMS C$_{10}$H$_{10}$O$_4$ [M-H]$^-$ Expected: 193.0506, Found: 193.0511.

3-(4-chlorophenyl)-2-oxopropanoic acid (3-12c): Procedure 1 was followed using 4-chlorobenzaldehyde. Yield 62% (1.1 g, 5.54 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.74-7.76 (d, $J = 8.8$ Hz, 2H), 7.30-7.32 (d, $J = 8.5$ Hz, 2H), 6.45 (s, 1H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 168.57, 158.21, 141.80, 131.71, 130.20, 129.78, 124.98, 121.41, 111.46, 105.02, 56.11. HRMS C$_{10}$H$_{10}$O$_4$ [M-H]$^-$ Expected: 193.0506, Found: 193.0496.
3-(2-chlorophenyl)-2-oxopropanoic acid (3-13c): Procedure 1 was followed using 2-chlorobenzaldehyde. Yield 56% (0.3 g, 1.51 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 8.32-8.35 (d, $J = 7.9$ Hz, 1H), 7.38-7.40 (d, $J = 8.1$ Hz, 1H), 7.26-7.30 (t, $J = 7.6$ Hz, 1H), 7.19-7.21 (t, $J = 7.8$ Hz, 1H), 6.89 (s, 1H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 167.97, 143.77, 134.42, 134.04, 132.20, 130.31, 129.41, 127.73, 105.93. HRMS C$_9$H$_7$O$_3$Cl [M-H]$^-$ Expected: 197.0011, Found: 197.0016.

3-(4-hydroxyphenyl)-2-oxopropanoic acid (3-14c): Procedure 1 was followed using 4-hydroxybenzaldehyde. Yield 74% (1.3 g, 7.22 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.62-7.64 (d, $J = 8.6$ Hz, 2H), 6.75-6.77 (d, $J = 8.8$ Hz, 2H), 6.45 (s, 1H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 168.74, 158.19, 139.97, 132.41, 127.94, 116.15, 112.35. HRMS C$_9$H$_8$O$_4$ [M-H]$^-$ Expected: 179.0350, Found: 179.0354.

3-(3-hydroxyphenyl)-2-oxopropanoic acid (3-15c): Procedure 1 was followed using 3-hydroxybenzaldehyde. Yield 88% (0.7 g, 3.89 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 7.29 (s, 1H), 7.07-7.14 (m, 2H), 6.63-6.66 (m, 1H), 6.29 (s, 1H); $^{13}$C NMR (125 MHz,
3-(4-fluorophenyl)-2-oxopropanoic acid (3-16c): Procedure 1 was followed using 4-fluorobenzaldehyde. Yield 71% (0.5 g, 2.74 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.78-7.81 (m, 2H), 7.02-7.07 (t, $J$ = 8.9 Hz, 2H), 6.47 (s, 1H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 168.26, 164.52, 162.07, 142.02, 132.57, 132.65, 115.88, 116.10, 110.42. HRMS C$_9$H$_8$O$_4$ [M-H]$^-$ Expected: 179.0350, Found: 179.0348.

3-(4-cyanophenyl)-2-oxopropanoic acid (3-17c): Procedure 1 was followed using 4-cyanobenzaldehyde. Yield 70% (0.05 g, 0.26 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) $\delta$ 8.62 (d, $J$ = 1.7 Hz, 1H), 8.01 (d, $J$ = 8.6 Hz, 2H), 7.76 (d, $J$ = 8.6 Hz, 2H), 6.60 (s, 1H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$CO) $\delta$ 167.49, 145.32, 141.54, 133.02, 132.69, 132.65, 131.03, 120.00, 110.87, 108.92. HRMS C$_{10}$H$_7$NO$_3$ [M-H]$^-$ Expected: 188.0353, Found: 188.0346.

**General Procedure 2:** 1,4-diacetyl-piperazine-2,5-dione$^{119}$ (0.5 mmol) was dissolved in DMF (1 mL). Triethylamine (0.5 mmol) was added followed by corresponding aldehyde (0.5 mmol). The reaction mixture was stirred for 12 h at room temperature. It was extracted with DCM, washed with NH$_4$Cl solution. The organic layer was dried over Na$_2$SO$_4$ and evaporated. Purification of the residue on silica gel afforded the product (benzylidene)-piperazine-2,5-dione intermediate. This intermediate (0.1 mmol) was refluxed in 6 N HCl (4.0 ml) for 4 h. The reaction was cooled to room
temperature, extracted with ether, dried over Na$_2$SO$_4$ and evaporated to give the crude keto acid. It was recrystallized in water to give pure product.

![Image of keto acid structure]

3-(3-methoxyphenyl)-2-oxopropanoic acid (3-18c): Procedure 2 was followed using 3-methoxybenzaldehyde. Yield 56% (0.5 g, 2.57 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.44 (t, $J = 2$ Hz, 1H), 7.20-7.29 (m, 2H), 6.79-6.82 (m, 1H), 6.46 (s, 1H), 3.79 (s, 3H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 168.32, 161.04, 142.44, 137.66, 130.11, 123.52, 115.80, 114.39, 111.51, 55.62. HRMS C$_{10}$H$_{10}$O$_4$ [M-H]$^-$: Expected: 193.0506, Found: 193.0495.

![Image of keto acid structure]

3-(3-chlorophenyl)-2-oxopropanoic acid (3-19c): Procedure 2 was followed using 3-chlorobenzaldehyde. Yield 56% (0.12 g, 0.60 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) $\delta$ 7.96 (t, $J = 1.96$ Hz, 1H), 7.76 (dt, $J = 7.8$ Hz, 1H), 7.25-7.41 (m, 3H), 6.54 (s, 1H), 4.28 (s, 0.6H), 4.05 (d, $J = 6.2$ Hz, 0.5H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$CO)) $\delta$ 166.54, 137.85, 134.59, 130.74, 130.67, 129.80, 128.83, 127.99, 109.31. HRMS C$_9$H$_7$O$_3$Cl [M-H]$^-$: Expected: 197.0011, Found: 197.0005.

**General Procedure 3**: Substituted benzaldehyde (2 mmol) was dissolved in acetic-anhydride (0.25 M). N-acetyl glycine (2.4 mmol) was added followed by sodium acetate (10 mmol). The reaction mixture was refluxed for 4 h at 140 °C. It was poured into crushed ice, stirred for 30 min. Precipitate was separated, washed with cold water, and dried under vacuum to afford the oxazolyl intermediate. This intermediate (0.4
mmol) was refluxed in 3 N HCl (4.0 ml) for 24 hours at 100 °C. The reaction was cooled to room temperature, extracted with ether, washed with 6 N HCl, dried over Na₂SO₄ and evaporated to give the crude keto acid. It was recrystallized in water to give pure product.

3-(3,4-dichlorophenyl)-2-oxopropanoic acid (3-20c): Procedure 3 was followed using 3,4-dichlorobenzaldehyde. Yield 71% (0.06 g, 0.28 mmol). ¹H NMR (400 MHz, (CD₃)₂CO) δ 8.1 (d, J = 1.89 Hz, 1H), 7.76 (dd, J = 8.49 Hz, 1H), 7.56 (d, J = 8.44 Hz, 1H), 6.53 (s, 1H); ¹³C NMR (125 MHz, (CD₃)₂CO) δ 166.39, 143.09, 136.55, 132.63, 131.78, 131.29, 131.11, 130.27, 108.38. HRMS C₉H₆O₃Cl₂ [M-H]⁻ Expected: 230.9627.

3-(2,4-dichlorophenyl)-2-oxopropanoic acid (3-20c): Procedure 3 was followed using 2,4-dichlorobenzaldehyde. Yield 77% (0.35 g, 1.5 mmol). ¹H NMR (400 MHz, MeOD) δ 8.34 (d, J = 8.79 Hz, 1H), 7.29-7.53 (m, 3H), 6.82 (s, 1H); ¹³C NMR (125 MHz, MeOD) δ 167.62, 144.44, 134.76, 133.96, 132.96, 131.83, 130.41, 129.95, 129.78, 128.75, 128.35, 128.04, 127.61, 104.58. HRMS C₉H₆O₃Cl₂ [M-H]⁻ Expected: 230.9627.
2-oxo-3-(4-(trifluoromethyl)phenyl)propanoic acid (3-20c): Procedure 3 was followed using 3-trifluoromethylbenzaldehyde. Yield 90% (0.09 g, 0.41 mmol). ¹H NMR (400 MHz, (MeOD) δ 7.93 (td J = 8.15 Hz, 2H), 7.59 (d, J = 8.15 Hz, 2H), 6.51 (s, 1H); ¹³C NMR (125 MHz, MeOD) δ 167.71, 144.46, 140.44, 132.22, 130.82, 126.01, 125.97, 125.94, 125.64, 125.61, 109.28. HRMS C₁₀H₇O₃F₃ [M-H]⁻ Expected: 231.0227.

2.7.3.2. Synthesis of Hydroxyimino (Oxime) Analogue

General Procedure 4: The corresponding keto-acid (0.50 mmol) is dissolved in a solution of NaOH (1.51 mmol) in water (1 mL). Hydroxylamine hydrochloride (0.76 mmol) is added to the reaction and stirred overnight at room temperature. 1 N HCl is added to the reaction, the precipitated product is filtered and dried. The crude product is purified using flash chromatography (silica gel, 5% MeOH/DCM) as necessary.

2-(hydroxyimino)-3-p-tolylpropanoic acid (2-15): Title compound was prepared following general procedure 4. Yield 55% (0.03 g, 0.16 mmol). ¹H NMR (400 MHz, MeOD) δ 7.05-7.14 (m, 4H), 3.86 (s, 2H), 2.27 (s, 3H); ¹³C NMR (125 MHz, MeOD) δ 166.90, 152.35, 136.96, 134.84, 129.96, 129.89, 30.51, 21.03. HRMS C₁₀H₁₁NO₃ [M-H]⁻ Expected: 192.0666, Found:192.0673.
2-(hydroxyimino)-3-\textit{m}-tolylpropanoic acid (2-16): Title compound was prepared following general procedure 4. Yield 74\% (0.04 g, 0.21 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.03-7.13 (m, 3H), 6.98 (d, $J$ = 7.31 Hz, 1H), 3.87 (s, 2H), 2.28 (s, 3H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 166.88, 152.22, 139.03, 137.84, 130.61, 129.26, 128.02, 127.02, 30.85, 21.42. HRMS C$_{10}$H$_{11}$NO$_3$ [M-H]$^-$ Expected: 192.0666, Found: 192.0656.

2-(hydroxyimino)-3-\textit{o}-tolylpropanoic acid (2-17): Title compound was prepared following general procedure 4. Yield 92\% (0.12 g, 0.62 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) $\delta$ 7.01-7.10 (m, 4H), 3.88 (s, 2H), 2.35 (s, 3H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$CO) $\delta$ 167.77, 153.63, 137.10, 136.37, 130.67, 129.10, 126.82, 126.60, 28.37, 19.93. HRMS C$_{10}$H$_{11}$NO$_3$ [M-H]$^-$ Expected: 192.0666, Found: 192.0652.

2-(hydroxyimino)-3-(4-methoxyphenyl)propanoic acid (2-18): Title compound was prepared following general procedure 4. Yield 71\% (0.08 g, 0.36 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.18 (d, $J$ = 8.6 Hz, 2H), 6.79 (d, $J$ = 8.8 Hz, 2H), 3.86 (s, 2H), 3.74 (s, 3H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 167.48, 159.71, 153.20, 131.07, 130.09, 114.79, 55.67, 30.16. HRMS C$_{10}$H$_{11}$NO$_4$ [M-H]$^-$ Expected: 208.0615, Found: 208.0601.
2-(hydroxyimino)-3-(3-methoxyphenyl)propanoic acid (2-19): Title compound was prepared following general procedure 4. Yield 72% (0.08 g, 0.37 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.13-7.17 (t, $J = 8.1$ Hz, 1H), 6.83 (m, 2H), 6.74 (dd, $J = 8.3$ Hz, 1H), 3.89 (s, 2H), 3.75 (s, 3H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 166.87, 161.21, 152.07, 139.41, 130.29, 122.35, 115.73, 112.86, 55.56, 30.95. HRMS C$_{10}$H$_{11}$NO$_4$ [M-H]$^-$ Expected: 208.0615, Found:208.0612.

2-(hydroxyimino)-3-(2-methoxyphenyl)propanoic acid (2-20): Title compound was prepared following general procedure 4. Yield 56% (0.07 g, 0.33 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) $\delta$ 7.17 (t, $J = 7.8$ Hz, 1H), 7.00 (d, $J = 7.6$ Hz, 1H), 6.93 (d, $J = 8.5$ Hz, 1H), 6.83 (t, $J = 7.6$ Hz, 1H), 3.90 (s, 2H), 3.82 (s, 3H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$CO) $\delta$ 165.17, 158.28, 151.76, 129.60, 28.34, 125.57, 121.06, 111.32, 55.72, 25.53. HRMS C$_{10}$H$_{11}$NO$_4$ [M-H]$^-$ Expected: 208.0615, Found:208.0607.

3-(4-chlorophenyl)-2-(hydroxyimino)propanoic acid (2-21): Title compound was prepared following general procedure 4. Yield 56% (0.06 g, 0.28 mmol). $^1$H NMR (400
MHz, DMSO) δ 12.34 (br s, 1H), 7.33 (d, J = 8.5 Hz, 2H), 7.20 (d, J = 8.5 Hz, 2H), 3.79 (s, 2 H); 13C NMR (125 MHz, MeOD) δ 166.71, 151.68, 136.88, 133.20, 131.63, 129.40, 30.39. HRMS C9H8NO3Cl [M-H]− Expected: 212.0120, Found: 212.0102.

3-(3-chlorophenyl)-2-(hydroxyimino)propanoic acid (2-22): Title compound was prepared following general procedure 4. Yield 66% (0.15 g, 0.70 mmol). 1H NMR (400 MHz, DMSO) δ 7.24-7.34 (m, J = 8.8 Hz, 3H), 7.17 (d, J = 7.3 Hz, 1H), 3.82 (s, 2H); 13C NMR (125 MHz, DMSO) δ 165.16, 139.26, 132.81, 130.14, 128.32, 127.32, 126.14, 29.64. HRMS C9H8O3NCl [M-H]− Expected: 212.0120, Found: 212.0108.

3-(2-chlorophenyl)-2-(hydroxyimino)propanoic acid (2-23): Title compound was prepared following general procedure 4. Yield 54% (0.02 g, 0.07 mmol). 1H NMR (400 MHz, DMSO) δ 7.41-7.44 (m, 1H), 7.21-7.28 (m, 2H), 7.03 (m, 1H), 3.88 (s, 2H); 13C NMR (125 MHz, DMSO) δ 164.97, 149.01, 134.08, 132.83, 129.22, 129.11, 127.96, 127.11, 27.93. HRMS C9H8O3NCl [M-H]− Expected: 212.0120, Found: 212.0134.
2-(hydroxyimino)-3-(4-hydroxyphenyl)propanoic acid (2-24): Title compound was prepared following general procedure 4. Yield 91% (0.10 g, 0.51 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.08 (d, J = 8.5 Hz, 2H), 6.66 (d, J = 8.5 Hz, 2H), 3.80 (s, 2H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 166.93, 156.93, 152.63, 131.06, 128.70, 116.13, 30.03. HRMS C$_9$H$_9$NO$_4$ [M-H]$^-$ Expected: 194.0459, Found:194.0459.

2-(hydroxyimino)-3-(3-hydroxyphenyl)propanoic acid (2-25): Title compound was prepared following general procedure 4. Yield 65% (0.07 g, 0.36 mmol). $^1$H NMR (400 MHz, DMSO) $\delta$ 7.04 (t, J = 8.0 Hz, 1H), 6.55-6.62 (m, 3H), 3.72 (s, 2H); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 165.18, 157.22, 137.89, 129.09, 119.24, 115.44, 113.07, 48.52, 29.74. HRMS C$_9$H$_9$NO$_4$ [M-H]$^-$ Expected: 194.0459, Found:194.0442.

3-(4-fluorophenyl)-2-(hydroxyimino)propanoic acid (2-26): Title compound was prepared following general procedure 4. Yield 74% (0.08 g, 0.41 mmol). $^1$H NMR (400 MHz, DMSO) $\delta$ 7.20-7.24 (m, 2H), 7.10 (t, J = 8.8 Hz, 2H), 3.79 (s, 2H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 165.06, 161.94, 159.54, 150.03, 132.82, 132.79, 130.38, 130.30, 115.12, 114.91, 29.06. HRMS C$_9$H$_8$NO$_3$F [M-H]$^-$ Expected: 196.0415, Found:196.0398.
3-(4-cyanophenyl)-2-(hydroxyimino)propanoic acid (2-27): Title compound was prepared following general procedure 4. Yield 64% (0.09 g, 0.44 mmol). $^1$H NMR (400 MHz, DMSO) $\delta$ 7.74 (d, $J = 7.6$ Hz, 2H), 7.37 (d, $J = 8.1$ Hz, 2H), 3.89 (s, 2H); $^{13}$C NMR (125 MHz, DMSO) $\delta$ 164.93, 149.11, 142.74, 132.26, 129.52, 118.77, 109.06, 30.20. HRMS C$_{10}$H$_8$N$_2$O$_3$ [M-H]$^-$ Expected: 203.0462, Found: 203.0452.

3-(3,4-dichlorophenyl)-2-(hydroxyimino)propanoic acid (2-28): Title compound was prepared following general procedure 4. Yield 66% (0.01 g, 0.04 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.38-7.43 (m, 2H), 7.20 (dd, $J = 8.34$ Hz, 1H), 3.89 (s, 2H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 166.80, 151.37, 138.94, 132.96, 132.01, 131.31, 131.14, 129.98, 30.22. HRMS C$_9$H$_7$Cl$_2$N$_2$O$_3$ [M-H]$^-$ Expected: 245.

3-(2,4-dichlorophenyl)-2-(hydroxyimino)propanoic acid (2-29): Title compound was prepared following general procedure 4. Yield 66% (0.02 g, 0.08 mmol). $^1$H NMR (400 MHz, MeOD) $\delta$ 7.42 (d, $J = 2.04$ Hz, 1H), 7.21 (dd, $J = 8.25$ Hz, 1H), 7.09 (d, $J = 8.34$ Hz, 1H), 3.98 (s, 2H); $^{13}$C NMR (125 MHz, MeOD) $\delta$ 166.88, 150.90, 135.71, 134.68, 133.73, 131.82, 129.90, 128.07, 28.72. HRMS C$_9$H$_7$Cl$_2$N$_2$O$_3$ [M-H]$^-$ Expected: 245.97.
2-(hydroximino)-3-(4-(trifluoromethyl)phenyl)propanoic acid (2-30): Title compound was prepared following general procedure 4. Yield 48% (0.01 g, 0.03 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) δ 7.63 (d, $J = 8.32$ Hz, 2H), 7.52 (d, $J = 8.32$ Hz, 2H), 4.04 (s, 2H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$CO) δ 166.89, 151.58, 142.85, 130.62, 129.84, 129.52, 127.16, 126.20, 126.16, 124.46, 31.01. HRMS C$_{10}$H$_8$F$_3$NO$_3$ [M-H]$^-$ Expected: 246.04.

**2.7.4. Protein Production and Purification of CtBP2**

Truncated CtBP2 (amino acids 31-384), which lacks both the N- and C-terminal domains, was expressed as a His$_6$-tagged protein in BL21-CodonPlus®(DE3)-RIL competent cells (Stratagene). Protein expression was induced in cultures with 200 μM IPTG for 4 hours and cells were homogenized and incubated in the presence of NiNTA beads (Thermo Scientific). CtBP2(31-384) was eluted with 300 mM imidazole after several washes and further purified by overnight dialysis and passage through a 6-8,000 kDa MWCO filter (Fisher Scientific). Purity was assessed by Coomassie stain (Invitrogen).

**2.7.5. Inhibition of Dehydrogenase Activity of Recombinant CtBP (NADH inhibition assay)**

Purified CtBP2 in 50% glycerol was added to 150 μM NADH, 48 μM MTOB and various concentrations of inhibitor in buffer containing 25 mM HEPES, pH 7.1, 25 mM potassium chloride, and 1 mM DTT. The final concentration of CtBP2 was 40 μg/mL (986 nM) per reaction. Inhibitors dissolved in DMSO constituted 1% of the total
volume. For compounds 4-13, inhibitor concentrations ranging from 300 μM to 10 μM and a no-drug (DMSO) control were tested. For 9 and analogues 14a-m, inhibitor concentrations of 5 μM, 2.5 μM, 1 μM, 500 nM, 100 nM, 50 nM, 10 nM, and 0 nM (DMSO control) were used. Three triplicate runs were performed for each inhibitor.

Reaction components were added to 96-well UV-Star Microplates (Greiner Bio-One) and upon addition of CtBP, reactions were mixed vigorously and immediately read by a Synergy H1 microplate reader (BioTek). Absorbance was recorded at A=340nm every 30 sec for 15 min at 25 °C to measure CtBP2 dehydrogenase function (NADH, but not NAD⁺ absorbs light at 340 nm). Change in absorbance was plotted after 15 minutes and IC₅₀ concentrations were determined using Prism (Graphpad,Version 5.04). Representative IC₅₀ curves for 2-14 (HIPP), 2-21 (4-Cl HIPP), and 2-22 (3-Cl HIPP) are shown below.
2.7.6 Inhibition of Cell Growth (MTT Assay)

HCT-116 p53⁺ colorectal cancer cell (~1,000 cells) in 100 μL media were plated in a 96 well plate. After a 24 hour incubation, inhibitors in DMSO were further diluted in NaHCO₃ (0.8% diluted DMSO final volume/well) and added to plates at 4, 2, 1, and 0.5 mM concentrations. 72 h after addition of inhibitor, 20 μL of MTT solution (Alfa
Aesar) was added to each well and cells were incubated a further 4 h. Media was then aspirated and the MTT metabolic product formazan was resuspended in 200 μL DMSO. Optical density was measured at 560 nm (subtracting background at 670 nm) using a microplate reader and IC₅₀ concentrations were determined using Prism (Graphpad, Version 5.04).

2.7.7. LDH Assay

Three compounds – HIPP, 3-Cl HIPP and 4-Cl HIPP were tested for their ability to inhibit LDH (which is closely related to CtBP in function). Sodium oxamate (a known inhibitor of LDH) was used as the standard. Commercially available L-Lactic Dehydrogenase (from rabbit muscle) was purchased from Sigma-Aldrich (USA). Stock solutions of the compounds (15.36 mM in DMSO) were prepared. They were added (in amounts of 0, 3, 6, 12, 24, 96 and 192 μM final concentrations) to a reaction mixture containing 100 mM phosphate buffer (pH 7.2), LDH (0.5 U/mL – solution in 50% glycerol), 200 μM pyruvate and 300 μM NADH at 0 °C. The final DMSO concentration in all the wells was kept constant at 6.25%, and the final volume was 80 μL in each well. The enzyme inhibition was measured by monitoring change in levels of NADH over a period of 30 min. Absorbance was measured at 340 nm every 10 sec. The assay was performed three times in 96-well-clear bottom plates (in triplicate each time) using FlexStation plate reader (Molecular Devices) at 28 °C. The minimal concentration of compounds leading to 50% inhibition of LDH activity (IC₅₀) was calculated using non-linear regression of the experimental data, using Prism software (GraphPad).
2.7.8. Oxime Stability Studies

To study the stability of oximes under assay conditions, we ran HPLC samples (HIPP, 3-Cl HIPP and 4-Cl HIPP) to check if the compounds hydrolyze during assay runs. In order to see if the compounds hydrolyze to parent phenylpyruvic acids, we ran controls of PPA, 3-Cl PPA and 4-Cl PPA. All the samples were dissolved in DMSO. Final concentration was 4 mM (15.36 mM DMSO stock was diluted in 25 mM phosphate buffer pH 7.2 to give final conc. of 4 mM). The samples were incubated at room temperature without shaking for 72 h. 20 µL aliquots were run on the HPLC at time points 0 min, 24 h, 48 h and 72 h. The graphs of the runs are shown below.

Solvents used – Water (with 0.1% formic acid), ACN (with 0.1% formic acid)

Conditions: Initial 0 min 5% ACN/95% water,
0-10 min 5% → 95% ACN/5% water,
10-12 min 95% → 5% ACN/95% water
Column Flow: 1.000 mL/min
Stoptime: 12.00 min
Injection Volume: 20.0 µL
Wavelengths observed: 254 nm and 280 nm.
As seen in the graphs above, there is no significant change in the retention times between 0 min vs 24 h, 48 h and 72 h of HIPP, which shows that the oxime is stable up to at least 72 h. This retention time is different from that of phenylpyruvic acid,
which shows that the oxime is not getting hydrolysed to the parent keto acid. Similar results were obtained with 3-Cl HIPP and 4-Cl HIPP (shown below).
2.7.9. HINT Scoring

Analogues of hydroxyimine 2-14 were modeled with SYBYL-X 2.1 (Tripos Inc.); Gasteiger–Hückel charges were assigned and models were energy minimized with the Tripos forcefield (10,000 iterations, termination gradient of 0.01 kcal/mol-Å). The crystal structure of CtBP1/HIPP/NAD⁺ was used as the target for docking studies using GOLD v5.2, with the binding defined as all the residues within 10 Å of the bound ligand 2-14. Since the ligands are analogues of 2-14, it is reasonable to expect that they will adopt binding modes very similar to that of the parent compound. Thus, the binding pose of 2-14 defined a scaffold match constraint, i.e., the common substructure forced the corresponding atoms of ligands to lie in the exact, or very close, position within the binding site. Default GOLD genetic algorithm parameters were used and a total of 50 solutions per compound were generated. The generated conformations were re-ranked using the free-energy based HINT force-field, that quantifies all non-bonded interactions in a biological environment, including hydrogen-bonding, electrostatic interactions, hydrophobic interactions as well as desolvation energy. The ligand in its most energetically favorable binding conformation and the CtBP1/NAD+ complex were subjected to minimization (Powell, 2500 iterations, termination gradient 0.01 kcal/mol-
Å), to remove steric clashes and optimize the protein-ligand interactions within the active site. HINT scores for the thus optimized complex were then recalculated.
3. C–H Activation

3.1. Introduction

Organic molecules comprise chains or rings of carbon atoms, capped with hydrogen atom(s), and with or without heteroatoms (mostly nitrogen, oxygen, halogens, sulfur, and phosphorous). These form simple hydrocarbons, natural products, proteins, pharmaceuticals, plastics and agrochemicals. Carbon–hydrogen bonds are present ubiquitously in nature (Figure 3-1).\textsuperscript{121}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig31.png}
\caption{Ubiquitous nature of C-H bonds}
\end{figure}

Organic chemistry involves transformation of functional groups or highly reactive structural features. C–H bonds are not considered as functional groups generally. In traditional approaches, the presence of a heteroatom (halogen, oxygen) or unsaturation is essential for the installation of a new bond, i.e. functional groups are installed only on pre-functionalized substrates for the purpose of reactivity and to achieve selectivity (Scheme 3-1).\textsuperscript{121} This forms the basis of all syntheses. Synthesis of pre-functionalized substrates itself adds additional steps to the synthetic scheme of the final product (Scheme 3-2).\textsuperscript{121} This is inefficient as it adds many steps to the overall synthetic route.

**Scheme 3-1.** Traditional organic synthesis (A) vs C–H functionalization (B)

**Scheme 3-2.** Comparison of traditional and C-H activation approaches
Although this is not an expensive approach on small scale reactions, it is very cost-ineffective in the case of large scale reactions, especially in industries. Thus, solving this issue will be very atom-economical and time-efficient.

\[
\begin{align*}
\text{(C)–H} & \quad + \quad \text{R–X} \quad \xrightarrow{\text{catalyst}} \quad \text{(C)–R} \\
\text{C} & \text{ - } \text{sp}^2 \text{ or sp}^3 \text{ hybridized carbon} \\
\text{R} & \text{ - } \text{carbon or heteroatom} \\
\text{X} & \text{ - } \text{functional group or hydrogen}
\end{align*}
\]

**Scheme 3-3. C–H activation**

One of the major challenges in organic chemistry has been the direct conversion of carbon–hydrogen bonds into carbon–halogen, carbon–sulfur, carbon–nitrogen, and carbon–carbon bonds using mild and selective methods. These transformations are very useful as C–H bonds are ubiquitous in nature. Overcoming this challenge would be of great use in the field of organic chemistry with wide applications in synthesis of pharmaceutical products, natural products, polymers, and agrochemicals. Direct transformation of a C–H bond to introduce a new functional group is the key to overcome the above challenges. For example, the final product in scheme 3-2 can be obtained by displacing just one hydrogen atom. This direct and selective functionalization of a C–H bond by an organometallic catalyst is termed “C–H Activation” (Scheme 3-3). This approach is atom- and step-economical, and can be used to make synthetic connections that are not possible by traditional methods.

The four main challenges in direct C–H functionalization reactions are: reactivity, chemoselectivity, regioselectivity and stereoselectivity.
Reactivity challenges arise because of the inert nature of most of the carbon-hydrogen bonds (pKa >30-35, BDE 85-105 kcal/mol). Though many oxidation reactions are thermodynamically favorable, the cleavage of a C–H bond has a high kinetic barrier. This challenge has been overcome by many groups of researchers by the use of transition metals. Transition metals (M) react with C–H bond to give C–M bonds, which are much more reactive than the corresponding C–H bonds. These C–M bonds can then be functionalized into various functional groups in a single step.124

Chemoselectivity challenges arise because of the difficulty in stopping reactions at the required oxidation state, over-oxidized products are generally thermodynamically favored. This challenge has been overcome by using excess substrate relative to oxidant, low conversion of reactions.

Regioselectivity challenges arise because organic compounds invariably contain several C–H bonds in different environments. This challenge has been overcome through different approaches. The most common approach has been the use of “directing groups” or “coordinating ligands” that are basic and coordinate to the metal center, and deliver the catalyst to a proximal C–H bond. The intramolecular directing groups are basic groups containing nitrogen such as pyridine or oxazole. This “ligand-directed C–H activation” has been reported at several transition metal centers such as Pd, Pt, Ru and Rh. Other approaches such as using substrates containing weaker C–H bonds, having five- or six-membered cyclometalated species, and variation of ligand-catalyst systems have been employed to achieve selectivity.

Stereoselectivity challenges are involved in the difficult process of generating new stereocenters in a dia- or enantio- selective manner. This has not been explored as much as other challenges. Approaches such as use of stereo center containing
substrates, or chiral catalyst-ligand systems have been used to overcome this challenge.

3.1.1. Brief Examples of Use of C-H Activation in Synthesis

3.1.1.1. C–H activation in natural product synthesis

![Figure 3-2. C-H activation steps in Teleocidin B-4 core synthesis](image)

Above figure reprinted with permission from "C-C Bond Formation via C-H Bond Activation: Synthesis of the Core of Teleocidin B4." Brian D. Dangel, Kamil Godula, So Won Youn, Bengü Sezen, and Dalibor Sames. JACS 2002, 124, 11856-11857. Copyright 2002, American Chemical Society.

C–H activation has been used in natural product synthesis. Dangel et al reported the synthesis of a complex natural product fragment Teleocidin B-4 core by employing four C–C bond forming steps (alkenylation of alkyl group, Friedel-Crafts reaction, carbonylation and alkenylation of phenol, Figure 3-2).125

Starting from Schiff base 1, cyclometallation with PdCl₂ gives the palladacycle 2, which undergoes reaction with boronic acid to afford 4. Treatment with MeSO₃H catalyzes a Friedel-Crafts reaction to afford intermediate 5, which undergoes cyclopalladation to
afford 7 and 8 by carbonylation and hydrolysis of the Schiff base (Scheme 3-4). Intermediate 7 is then alkylated to afford intermediate 10, which undergoes Pd-catalyzed alkenylation to afford the final product Teleocidin B-4 core (Scheme 3-5).²²⁵

Scheme 3-4. Synthesis of intermediate 7

Above figure reprinted with permission from "C-C Bond Formation via C-H Bond Activation: Synthesis of the Core of Teleocidin B4." Brian D. Dangel, Kamil Godula, So Won Youn, Bengü Sezen, and Dalibor Sames. JACS 2002, 124, 11856-11857. Copyright 2002, American Chemical Society.

Scheme 3-5. Synthesis of Teleocidin B-4 core

Conditions: a). t-BuOK, THF; b). BBr₃, DCM; c). Pd(OAc)₂, P(t-Bu)₃, Cs₂CO₃, DMA.

Above figure reprinted with permission from "C-C Bond Formation via C-H Bond Activation: Synthesis of the Core of Teleocidin B4." Brian D. Dangel, Kamil Godula, So Won Youn, Bengü Sezen, and Dalibor Sames. JACS 2002, 124, 11856-11857. Copyright 2002, American Chemical Society.
3.1.1.2. C–H activation in late-stage diversification

Development of new drugs requires the identification of lead compounds, and design of analogs with improved activities. Thus, methods to quickly form analogs from a core structure is highly desirable. In efforts towards this, C–H activation has also been applied in “structural core diversification” in which various C–H bonds in a complex molecule are selectively transformed to produce series of analogues from a common substrate (Scheme 3-6). This is highly advantageous over traditional approaches where multi-step and usually different synthetic routes are needed to arrive at series of analogs.

Scheme 3-6. Structural core diversification

Late-stage diversification has been used in the synthesis of drug candidates, organic light-emitting diodes (OLEDs), perylene tetracarboxylic acid bisimide (PBI), metal organic frameworks (MOFs) and polymers (Figure 3-3).
Figure 3-3. C-H activation in late-stage diversification applications


Yu et al reported the late-stage diversification of celecoxib, an anti-inflammatory drug (Scheme 3-7). Sulfonamide group is an essential part of the pharmacophore for these compounds, converting it to perfluoroaryl sulfonamide allowed for its use as a directing group. Using Pd catalysis, ortho-directed C–H activation led to the synthesis of various ortho-substituted analogues in acceptable yields by olefination, carboxylation, carbonylation, iodination, arylation and alkylation reactions. This is advantageous over traditional approaches as it is more time-saving, and can install groups that could be unfeasible by non C–H activation reactions.
Itami and Wunsch reported the late-stage diversification of $\sigma_1$ receptor ligands containing a spirocycle (Scheme 3-8). Based on the hypothesis that the aryl residue on the $\alpha$-position of thiophene would lead to better activity and selectivity, they carried out Pd-catalyzed C–H activation (arylation) reactions. Introduction of aryl groups on the thiophene ring at various positions afforded rapid synthesis of analogues which were used in receptor-binding studies.

Late-stage diversification has applications outside of medicinal chemistry as well. It can be used where ‘lead optimization’ is required. It has been used in synthesis of compounds for optical applications in electronic devices with digital displays.
Triscyclometallated Ir (III) complexes are useful as they have high emission-quantum yields and longer lifetimes. Late-stage diversification has been used to synthesize several analogues of such complexes.

3.2. C–H Activation by Palladium Catalysts

Homogenous transition metal catalysts have been extensively studied in C–H activation reactions to produce aryl or alkyl halides, alcohols, amines and aryl compounds. These transformations can occur through two general mechanisms – “organometallic” and “coordination.”

In the “organometallic” mechanism, there are two steps (Scheme 3-9). The first step involves cleavage of a C–H bond to afford a C–M bond (M is a transition metal). The second step involves functionalization of a C–M bond by an external reagent to afford the product. The structural and electronic nature of the organometallic intermediate dictates the regio-selectivity of the functionalization, which usually occurs at the less hindered C–H bonds in the molecule.

Scheme 3-9. Organometallic mechanism

In the “coordination” mechanism, higher oxidation state metal complexes that contain activated ligand (X, metal -oxo/imido/carbene) are formed (Scheme 3-10). This is followed by reaction of X with the C–H bond, either through direct insertion or H-atom abstraction, to finally afford the product. In this mechanism, the substrate undergoing C–H activation does not interact directly with the transition metal, but only interacts with the ligand (X).
This dissertation focuses on Pd-catalyzed C–H activation reactions. Palladium has several advantages over other transition metal catalysts.\textsuperscript{124}

Palladium is much cheaper than Pt and Rh (Figure 3-4). Pd centers can be used to install a variety of bonds, such as C–O, C–halogen, C–N, C–S and C–C. Only few other catalysts can catalyse such bond formations. This is because Pd is compatible with a variety of oxidants. Pd can be utilized to achieve C–H activation not only at sp\textsuperscript{2} centers, but also at sp\textsuperscript{3} centers. Pd catalysed C–H activation reactions are not sensitive to air and moisture; this makes it an attractive catalyst for industrial applications.

\textbf{Scheme 3-10.} Coordination mechanism of C–H activation
3.3. Mechanism of C–H activation

3.3.1. N-Chelation-Directed C–H activation

Coordination-directed metalation is one of the approaches for C–H activation reactions. It can be applied to both sp² and sp³ carbons. This approach utilizes heteroatoms such as oxygen or nitrogen (which are in proximity to the C–H to be activated) to coordinate with the transition metal. This gives rise to a five- or six-membered metallocycle, which is very reactive and subsequently forms the products.

In this dissertation, we will focus on N-chelation-directed C–H activation. Examples of selective C–H to C–O,¹³⁰⁻¹³⁶ C–Halogen,¹³⁰,¹³⁷,¹³⁸ C–C,¹³⁹,¹⁴⁰ C–N,¹⁴¹,¹⁴² C–F,¹⁴³,¹⁴⁴ and C–CF¹⁴⁵ transformations using N-chelation-directed palladium catalysis have been reported in the literature.
The mechanism is explained in detail in the scheme below (Scheme 3-11):

In the first step, Pd(II) catalyst undergoes insertion into the proximal C–H bond to give a five- or six-membered palladacycle. The selectivity in this “chelation-directed” step is driven by directing groups such as nitrogen or oxygen that are basic in nature and direct the catalyst to a specific C–H bond. In the second step, usually an oxidant containing a functional group oxidizes Pd(II) to Pd(IV).\textsuperscript{146, 147} This is in contrast to the traditional coupling reactions that undergo Pd(0)/Pd(II) cycle. In the last step, reductive elimination causes Pd(II) to be regenerated, and the functional group is transferred to the activated C–H bond.

\textbf{Scheme 3-11.} Pd(II)/Pd(IV) catalytic cycle for N-chelation-directed C–H activation reactions

In the past two decades, C–H activation has been reported by several groups to make C–O, C–halogen, C–N, C–S and C–C bonds. This chapter focuses on the oxygenation, halogenation and arylation reactions catalyzed by palladium catalysts. This section focuses on homogenous palladium catalysts. Solid-supported and heterogenous catalysts will be discussed in section 3.5.

3.4.1. Carbon–Oxygen Bond Forming Reactions

Pd-catalyzed ortho-oxygenation reactions use various oxidants. Reactions that utilize Pd(OAc)$_2$ catalyst and PhI(OAc)$_2$ have been reported. In these reactions, nitrogen containing groups such as pyridine$^{130,148}$, pyrazole$^{130}$, isoxazoline$^{148}$, imine$^{130}$, oxime ether$^{149}$ function as directing groups to direct the acetoxylation to the ortho- position (Scheme 3-12). Ketones and aldehydes are poor directing groups, and hence do not undergo acetoxylation under these conditions.

Scheme 3-12. Palladium-catalyzed N-chelation-directed oxygenation reactions

The acetoxylation reactions are carried out under ambient air and moisture conditions. Many functional groups in the molecules are well tolerated such as: aryl halides, nitro
groups, ketones, oximes. These features make Pd-catalyzed oxygenation reactions very attractive.

When a substituent is present at the meta- position, the less hindered ortho- position undergoes acetoxylation.

![Scheme 3-13. Effect of solvent on oxygeantion reactions](image)

Switching the solvent leads to the installation of various alkoxy groups in the substrates. For example, MeOH solvent installs -OMe and EtOH solvent installs -OEt (Scheme 3-13). PhI(OAc)$_2$ might be forming PhI(OR)$_2$ in situ. The following mechanism has been proposed for C-H acetoxylation (Scheme 3-14).

![Scheme 3-14. Mechanism of C-H acetoxylation](image)
Other oxidants that have been used in these reactions are oxone, IOAc, and dioxygen (Scheme 3-15).\(^{132}\)

![Scheme 3-15. C-H acetoxylation by IOAc](image)

IOAc is generated \textit{in situ} by reacting I\(_2\) with Phl(OAc)\(_2\) or AgOAc. The mechanism at work when IOAc is utilised is: chelation-directed C–H activation, oxidation of Pd\(\text{II}\) to Pd\(\text{IV}\), reductive elimination forming C–I bond, displacement of I\(^-\) by OAc\(^-\).

### 3.4.2. Carbon–Halogen Bond Forming Reactions

Pd catalyzed C–H activation is an efficient method to install halogens such as Cl, Br and I. This was first reported in 1970, using Pd for the chlorination of azobenzene (Scheme 3-16).\(^{152}\) Although this reaction used Cl\(_2\), and gave rise to a mixture of isomers, there have been a lot of improvements since then in the use of more practical oxidants and improvement of selectivity.

![Scheme 3-16. Pd-catalyzed chlorination of azobenzene](image)
Halogenation reactions catalyzed by Pd(OAc)$_2$ using chlorinating agent N-chlorosuccinimide (which also acts as an oxidant) gives ortho-chlorinated benzo[h]quinoline product in excellent yields. In a similar reaction, using NBS gives the brominated product (Scheme 3-17).$^{130,138}$ On the other hand, the more reactive PhICl$_2$ gives the 5-chlorinated product. This reaction occurs without N-chelation in the presence or absence of Pd catalyst (Scheme 3-17). Thus, Pd catalysts can be used to afford products that are complementary to the products obtained by electrophilic substitution reactions. Below are some more examples. In the presence of Pd, N-chelation-directed ortho-halogenation occurs. In the absence of Pd, electrophilic aromatic substitution occurs (Scheme 3-18).$^{138}$
The halogenation reactions using N-halosuccinimides and Pd catalysts has been applied to a wide variety of substrates with basic directing groups such as pyridines, oxime ethers, isoxazolines, isoquinolines (Scheme 3-19). Using 2.5 equivalents of N-halosuccinimide gives the di-ortho-halogenated product. In the presence of a meta-substituent, the less hindered ortho-position is halogenated.

Scheme 3-18. Pd-catalyzed N-chelation-directed and non-catalyzed halogenation reactions

Scheme 3-19. Pd-catalyzed N-chelation-directed halogenation reactions
Mechanism of halogenation: There are two proposed models of halogenation. Pd(II)/Pd(IV) cycle (Scheme 3-20). NCS is a strong oxidant that oxidizes Pd(II) to Pd(IV).\textsuperscript{147}

![Scheme 3-20. Mechanism of halogenation by Pd catalyst – Pd(II)/Pd(IV) cycle](image)

Or, Pd(III)-Pd(III) dimer cycle which occurs in the presence of PhICl\textsubscript{2} (Scheme 3-21).\textsuperscript{153}

![Scheme 3-21. Mechanism of halogenation by Pd catalyst – Pd(III)/Pd(III) cycle](image)

Other halogenating agents that have been used in C-H activation reactions are CuCl\textsubscript{2}, CuBr\textsubscript{2} (Scheme 3-22).\textsuperscript{137}

![Scheme 3-22. Pd-catalyzed halogenation reaction by CuCl\textsubscript{2}](image)
Yu et al have reported the use of Suarez-type reagents (XOAc) for halogenation of sp$^2$ and sp$^3$ C-H bonds (Scheme 3-23).\(^{154}\)

Carbon-fluorine bond formation reactions have been carried out using Pd catalysts and electrophilic fluorinating reagents (Scheme 3-24).\(^{143}\) This is a very important transformation as it can be applied to PET imaging using radioactive fluorine. Instead of re-developing synthetic routes to incorporate radioactive fluorine, it can be easily installed through C-H activation reactions such as below in the final stages of the synthetic routes. This would be a time- and cost-efficient process.
3.4.3. Carbon–Carbon Bond Forming Reactions

C–C bond formation is one of the most important reactions in organic chemistry. Traditional carbon–carbon bond forming reactions include coupling reactions such as Heck, Suzuki and Stille coupling reactions that utilize the Pd(0)/Pd(II) cycle, which have led to an immense progress in the synthesis of organic molecules. But there is still a need for improvement in the atom-economy, and cost-efficient processes. One major disadvantage of these coupling reactions is that all of them require two pre-functionalized substrates. This adds additional steps in the synthesis of the final product. In efforts to overcome these disadvantages, C–H activation chemistry has been developed as an alternative, in which carbon–carbon bond formation is achieved using one or zero pre-functionalized substrates.

The general reaction conditions for coupling reactions include homogenous Pd catalyst, base, ligand, and a solvent. Because the C–H bonds are inert (high dissociation energy), usually high temperatures (>100 °C) and Pd catalyst loading (5-20 mol%) are required for the reactions to take place. In heterocyclic structures where the C–H bond is activated due to the presence of a heteroatom, the coupling reactions can be run at lower temperatures\textsuperscript{155,156} and catalyst loading.\textsuperscript{157,158} Pd(OAc)\textsubscript{2} has been shown to catalyze arylation of substrates using diphenyliodinium salts as the arylating agents. This reaction is driven by $N$-chelation of Pd. Aryl pyridines, quinolines, oxazolidinones can undergo arylation reactions as they possess basic nitrogen chelating group which directs arylation to the ortho- position (Scheme 3-25).\textsuperscript{139,159-161}
To install substituted aryl groups, asymmetrical arylating agents [Mes-I-Ar]BF₄ can be used. Due to sterics, the bulky mesityl group is not transferred, and only the Ar group gets transferred selectively (Scheme 3-26).

The proposed mechanism of these arylation reactions is slightly different from that of the oxygenation and halogenation reactions. The catalyst resting state is Pd(II) monomeric species. It forms the cyclometalated dimer, which is oxidized by the arylating agent to give Pd(IV)/Pd(II) adduct (in some cases it is called as Pd(III)-Pd(III) species). This undergoes reductive elimination to afford the arylated product (Scheme 3-27).
Another example of sp² C–H arylation that utilizes Pd(OAc)₂ and AgOAc has been reported. Amide⁶² and oxime ether⁶³ containing substrates undergo arylation with aryl iodides to afford the coupled product fluorenones (Scheme 3-28). These reactions are tolerant to various functional groups.

**Scheme 3-27.** Mechanism of Pd-catalyzed C–H arylation

**Scheme 3-28.** Fluorenone synthesis by C–H arylation
Arylation of unactivated sp$^3$ C–H bonds has also been achieved under similar conditions. Pyridines$^{164}$ and aminoquinolines$^{165}$ function as the directing groups (Scheme 3-29).

The above reactions that utilize Ag have been proposed to go through Pd(II)/Pd(IV) cycle. Ag salt is necessary for regenerating the Pd catalyst. Mechanistic studies have been conducted on benzoic acids (Scheme 3-30)$^{166}$.

![Scheme 3-30. Mechanism of Pd-catalyzed arylation with AgOAc](image)

**C–H Arylation without Prefunctionalized Arylating Reagents:**
Reactions involving Pd-catalyzed ligand-directed arylations have been reported which do not require the presence of prefunctionalized substrates. As an example, the dimerization of arylpyridine in the presence of Pd(OAc)$_2$ using oxone as oxidant (Scheme 3-31)$^{167}$. Meta substituents on the aryl ring resulted in low site selectivity of arylation, in contrast to C–H arylation at Pd(II) centers.
So, the following pathway has been proposed for this type of arylation reactions.\textsuperscript{167} Cyclometalation at Pd(II) followed by oxidation of Pd(II) to Pd(IV) by oxone, cyclometalation at Pd(IV), and reductive elimination to give the arylated product and release of Pd(II) (Scheme 3-32).

More recently, a general $N$-chelation directed Pd(OAc)$_2$ catalyzed oxidative cross coupling reaction on benzo[h]quinoline has been reported. This reaction uses Ag$_2$CO$_3$
as the oxidant, benzoquinone as the promoter, DMSO to protect the catalyst. This reaction was shown to have a wide scope (Scheme 3-33).

Scheme 3-33. Oxidative cross coupling reaction on benzo[h]quinoline

These reactions are proposed to occur through the following mechanism: cyclopalladation, Ar-H activation, reductive elimination to afford the arylated product, oxidation of Pd(0) to Pd(II). Benzoquinone helps in the reductive elimination step (Scheme 3-34).

Scheme 3-34. Mechanism of oxidative cross coupling reaction on benzo[h]quinoline
3.5. Pd-MWCNT Catalyst

Homogenous catalysts such as palladium acetate show high conversion of substrates to products because of easy access to all catalytic sites, but it is very difficult to remove the catalyst from the reaction mixture due to its high solubility. Thus, commercialization of homogenous catalyzed reactions has been rare. This has led to the development of heterogeneous and solid-supported catalysts in which the catalyst is in a different physical state from the solvent, and hence can be easily removed from the reaction mixture. This makes the process of purification less tedious. Such catalysts can also be reused/recycled, which makes the process very economical.

In heterogeneous catalysis, reactants are adsorbed on the solid-supported catalyst surface, undergo reaction catalyzed by transition metal catalyst such as palladium, products get desorbed from the catalyst surface, and are extracted from the reaction mixture. Another mechanism of solid-supported catalysis is the "leaching-redeposition" mechanism in which solid-supported Pd(0) nanocatalyst is oxidized to Pd(II) and leaches from the support, catalyzes the conversion of substrate to product in the reaction mixture, undergoes reduction to Pd(0) and is redeposited on the support. This can be tested by two experiments – hot filtration and three-phase test. In the hot filtration test, a portion of the reaction mixture is filtered midway through the reaction, and fresh reagents are introduced. Further conversion indicates presence of soluble Pd species. Absence of conversion indicates absence of soluble Pd species, but it could also mean that there is a fast leaching-redeposition of the Pd species, which cannot be measured. Three-phase test is more reliable as a test for heterogeneity – one substrate is immobilized on a solid-support, and the reaction is
carried out using a solid-supported catalyst and a soluble substrate. Product formation indicates soluble Pd species catalyzing the reaction.

New supports and catalysts are being continuously developed. Several materials such as polymers,\textsuperscript{175} dendrimers,\textsuperscript{176} metal oxides\textsuperscript{177} and carbonaceous materials\textsuperscript{178} have been used as solid supports for Pd and other transition metal catalysts. Ideal properties for the solid supports are – high surface area, chemical inertness, thermal and mechanical stability.

Palladium metal has been shown to have a good catalytic activity in the form of nanoparticles. Optimization of shape and size of these nanoparticles is essential for improving catalytic activity.\textsuperscript{179} Metal-decorated carbon nanotubes (CNTs) – single-walled and multi-walled (Figure 3-5)\textsuperscript{180} – are increasingly being used for many applications such as optics, electronics and catalysis. They have high chemical stability and surface area which makes them very efficient as catalysts. Graphene is a 2D mono-layered structure of graphite packed into a honeycomb-like lattice. It has excellent properties of thermal and mechanical stability. Reduction of graphene oxide by hydrazine was an early method to synthesize graphene sheets.\textsuperscript{181} 3D derivatives of graphene are single walled carbon nanotubes (SWCNT) and multi-walled carbon nanotubes (MWCNT). These have been shown to have excellent catalytic activity. SWCNT consists of a single layer of graphene wrapped into a cylindrical column. The synthesis of SWCNT is very difficult, and is achieved by chemical vapor deposition. MWCNT have multiple sheets of graphene wrapped up into cylinders of varying diameters. These can be prepared by chemical vapor deposition as well. MWCNT have better stability compared to SWCNT owing to their multiple layers.
Scheuermann et al have reported the synthesis of graphene supported palladium nanoparticles.\textsuperscript{182} The synthesis of carbon nanotubes is complex and expensive, is achieved through lasers, vapor deposition and other techniques.

Palladium is deposited on the nanotubes by the solvent-free methods of mortar-pestle or ball-milling, or with a solvent-based method. A schematic representation of of Pd decorated on MWCNT is shown in Figure 3-6.\textsuperscript{183}
The Gupton lab has provided us with Pd-MWCNT catalyst for carrying out C–H activation reactions. Multi-walled nanotubes have an outer diameter of 6-9 mm, length 5 µm and 95% carbon. In the *mortar-pestle method*, the carbon nanotubes are mixed with palladium acetate and ground using mortar and pestle until homogenous mixture of Pd-MWCNT is obtained. In the *ball-mill method*, carbon nanotubes and palladium acetate along with zirconia balls are placed in a ball mill and shaken mechanically (back-and-forth 5.9 cm, side-to-side 2.5 cm, 115 volts, 1060 cycles/min) to afford Pd-MWCNT.

The major advantages of solid-supported catalysts over that of homogenous catalysts are: recyclability, less/no contamination of products by residual metal, and ease of filtration and removal of catalyst from the reaction mixture.

The first arylation catalyzed by heterogenous Pd was reported in 1982 by Nakamura et al.\textsuperscript{184} Arylation on muscimol was achieved using Pd/C catalyst on substituted isoxazoles (Scheme 3-35).
Fagnou et al used Pd(OH)$_2$ supported on activated charcoal (Pearlman’s catalyst) for the arylations (intra- and inter-molecular) of arenes (Scheme 3-36).\textsuperscript{185}

Zhang et al reported a ligandless and additive-free Suzuki reaction using Pd-CNT as the catalyst (Scheme 3-37).\textsuperscript{186} Excellent yields were achieved with various aryl bromides (both electron withdrawing and electron donating).
Siamaki et al reported the use of solid-supported Pd(0) on carbon nanotubes (Pd-MWCNT 1-3 nm) as a highly efficient catalyst in the Suzuki cross-coupling reaction (Scheme 3-38).\textsuperscript{187} Coupling reactions using these solid-supported nanoparticle catalysts displayed remarkable catalytic activity with a high turnover number (TON 7250) and turnover frequency (TOF 217500 h\(^{-1}\)). The reactions generally were reported to be complete within 10 min, run at lower temperature (\(\sim\) 80 °C) using either conventional or microwave heating, and run in either batch or flow format. They were able to control the Pd(0)/Pd(II) ratio based on the method of preparation and the type of carbon nanotube used as the solid support.

Tang et al reported the arylation of thiophenes and benzo[b]thiophenes using Pd/C as the catalyst under ligand-free and additive-free reaction conditions (Scheme 3-39).\textsuperscript{188} This reaction was proved to be heterogenous in nature.
Zhang et al reported ortho-directed C–C coupling reaction between 2-aryl pyridines and aldehydes catalyzed by palladium nanoparticles via Pd\(^{0/II/IV}\) cycle.\(^\text{189}\) Aluminum oxide was used as support for palladium, and oxidant used was TBPB (Scheme 3-40).

### 3.6. Scope of this dissertation

The second project in my dissertation focuses on the C–H to C−OMe/OAc, C−Cl, C−Br and C−C functionalizations using solid-supported Pd(II) multi-walled carbon nanotube catalyst. The turn-over frequencies of solid-supported catalyst were higher than the reported homogenous catalyst.
4. C-H Halogenation and Alkoxylation reactions catalyzed by Pd(II)-MWCNT Catalyst

We wished to test the hypothesis that a solid-supported nanoparticle catalyst containing predominantly Pd(II) can be used to catalyze oxidative N-chelation-directed C–H activation reactions that undergo the Pd(II)/Pd(IV) catalytic cycle. As the catalyst, we have prepared nanoparticles containing predominantly Pd(II) supported on multi-walled carbon nanotubes (Pd(II)/MWCNT) by a modification of our previous procedure. We have evaluated the ability of this catalyst to carry out oxidative C–H to C–OMe/OAc, C–Cl, and C–Br functionalizations.

4.1. Results and Discussion

We initially chose substrates that have been previously reported to undergo the C–H functionalization reactions so that we could compare the results of our solid-supported Pd(II)/MWCNT catalyst to that of the known homogeneous Pd(II) system (Pd(OAc)₂). We first explored the C–H to C–O transformation, which utilizes Phl(OAc)₂ as the oxidant (Table 4-1).

Treatment of 8-methylquinoline with Pd(II)/MWCNT and Phl(OAc)₂ in acetic acid at 120 °C for 10 min (Table 4-1, entry 1) afforded the desired 8-(acetoxyethyl)-quinoline in 90% yield, comparable to the previously reported yield of 88% with the homogeneous catalyst. However, treatment of benzo[h]quinoline (entry 2) and 2-phenylpyridine (entry 3) failed to give any of the desired acylation products, even at extended reaction times and higher temperatures. Only starting material was recovered in these reactions. Having confirmed that solid-supported Pd(II)/MWCNT can catalyze a reaction that undergoes the Pd(II)/Pd(IV) catalytic cycle and
encouraged by the initial success of the acylation reaction with 8-methylquinoline, we elected to evaluate whether other oxygen containing functional groups could be installed using our catalyst. We repeated the reaction of 8-methylquinoline with Pd(II)/MWCNT and PhI(OAc)$_2$, except this time using methanol as a solvent (Table 4-1, entry 4). We obtained a near-quantitative yield (99%) of the desired 8-(methoxymethyl)quinoline in 10 min at 100 °C, which is a significant improvement over the reported yield of 77% with the homogeneous catalyst system. Application of these conditions to benzo[h]quinoline (entry 5) afforded a 90% yield of product, again comparable to the reported yield with the homogeneous catalyst (95%). Finally, treatment of 2-phenylpyridine with Pd(II)/MWCNT/PhI(OAc)$_2$/MeOH (entry 6) did result in formation of the product after an extended reaction time, albeit in much lower yield (25%).
Table 4-1. C-H to C-O functionalizations catalyzed by Pd(II)-MWCNT

<table>
<thead>
<tr>
<th>Entry&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Product</th>
<th>Solid-Supported Pd(II)/MWCNT</th>
<th>Yield and time with Pd(OAc)&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Image" /></td>
<td>120 °C 0.2 h 90%</td>
<td>88%, 22 h</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2.png" alt="Image" /></td>
<td>120 °C 1 h 0%</td>
<td>86%, 12 h</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3.png" alt="Image" /></td>
<td>120 °C 1 h 0%</td>
<td>52%, 12 h</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Image" /></td>
<td>100 °C 0.2 h 99%</td>
<td>77%, 18 h</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Image" /></td>
<td>100 °C 0.2 h 90%</td>
<td>95%, 22 h</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6.png" alt="Image" /></td>
<td>100 °C 5 h 25%</td>
<td>25% &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Solvent for entries 1-3: AcOH; entries 4-6: MeOH. <sup>b</sup> Previous reported results with homogeneous Pd(OAc)<sub>2</sub>. <sup>c</sup> Yield with homogeneous Pd catalyst has not been reported.

We next sought to determine whether alcohols other than methanol could be installed in substrates using the solid supported catalyst. Treatment of benzo[h]quinoline with Pd(II)/MWCNT and Phl(OAc)<sub>2</sub> in alcohol solvents (Table 4-2) demonstrated that methoxy (from methanol, entry 1) and ethoxy (from ethanol, entry 2) functional groups can be installed in moderate to high yields with our catalyst. However, as the size of the alcohol increased, incorporation of the functional group into the product decreased.
(with isopropyl, entry 3) and eventually failed (in the tert-butyl case, entry 4). Previously reported data for the homogeneous catalyst system show only a moderate decrease in yield as the size of the alcohol increases.

**Table 4-2.** Steric Trend in C-H to C-O Alkyl Functionalizations Catalyzed by Pd(II)/MWCNT

<table>
<thead>
<tr>
<th>Entry</th>
<th>ROH</th>
<th>Product</th>
<th>Yield Pd-MWCNT</th>
<th>Yield Pd(OAc)₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MeOH</td>
<td>![C-H to C-O functionalization] MeO</td>
<td>90%</td>
<td>95%</td>
</tr>
<tr>
<td>2</td>
<td>EtOH</td>
<td>![C-H to C-O functionalization] EtO</td>
<td>73%</td>
<td>80%</td>
</tr>
<tr>
<td>3</td>
<td>iPrOH</td>
<td>![C-H to C-O functionalization] iPrO</td>
<td>33%</td>
<td>72%</td>
</tr>
<tr>
<td>4</td>
<td>tBuOH</td>
<td>![C-H to C-O functionalization] tBuO</td>
<td>0%</td>
<td>-a</td>
</tr>
</tbody>
</table>

a. Yield with homogeneous Pd catalyst has not been reported.

Having demonstrated that solid-supported Pd(II)/MWCNT can catalyze the C–H to C–O functionalization reaction, we next turned our attention to halogenation reactions (Table 4-3). Treatment of benzo[h]quinoline with Pd(II)/MWCNT and N-chlorosuccinimide (NCS) in acetonitrile at 100 °C for 5 h (Table 4-3, entry 1) afforded the desired 10-chlorobenzo[h]quinoline in 92% yield, comparable to the previously
reported yield of 95% with the homogeneous catalyst. It should be noted that the reaction with the Pd(II)/MWCNT is much faster than with homogeneous Pd(OAc)$_2$ (5 h vs 3 days).

Chlorination of (E)-3,4-dihyronaphthalen-1-(2H)-one O-methyloxime (entry 2), 2-phenylpyridine (entry 3), and 3-methyl-2-phenylpyridine (entry 4) in either acetic acid (entries 2 and 4) or acetonitrile (entry 3) all afforded the desired chlorinated products in moderate yields in reaction times from 10 min to 6 h. While the yields for these transformations are lower than those for the comparable reaction with the homogeneous catalyst, the reaction times are much shorter, pointing to faster reaction kinetics (vide infra). The 2-phenylpyridine substrates (entries 3 and 4) required additional catalyst loading to reach this level of conversion and product isolation. For all of these substrates, further increasing the number of equivalents of NCS led to the formation of other products.

Turning to the C−H to C−Br functionalizations, treatment of benzo[h]quinoline with Pd(II)/MWCNT and N-bromosuccinimide (NBS) in acetonitrile at 100 °C for 1.5 h (Table 4-3, entry 5) afforded the desired 10-bromobenzo[h]quinoline in 89% yield, comparable to the previously reported yield of 93% with the homogeneous catalyst. Again, it should be noted that the reaction with the Pd(II)/MWCNT is much faster than that with homogeneous Pd(OAc)$_2$ (1.5 h vs 1.5 days). Bromination of (E)-3,4-dihyronaphthalen-1-(2H)-one O-methyloxime (entry 6), 2-phenylpyridine (entry 7), and 3-methyl-2-phenylpyridine (entry 8) in either acetic acid (entries 6 and 8) or acetonitrile (entry 7) all afforded the desired brominated products in moderate yields in reaction times of 5−6 h. Again, the yields are lower than the comparable reaction with homogeneous catalyst but the reaction times are much shorter, pointing to faster reaction kinetics (vide infra).
**Table 4-3. C-H to C-Cl/C-Br Functionalizations Catalyzed by Pd(II)/MWCNT**

![Chemical Structure](image)

<table>
<thead>
<tr>
<th>Entry&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Product</th>
<th>Temp</th>
<th>Time</th>
<th>Yield&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Pd(OAc)&lt;sub&gt;2&lt;/sub&gt; Yield and time with Solid-Supported Pd(II)/MWCNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image" alt="Product Image" /></td>
<td>100 °C</td>
<td>5 h</td>
<td>92%</td>
<td>95%, 3 days</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="Product Image" /></td>
<td>120 °C</td>
<td>1.5 h</td>
<td>61%</td>
<td>88%, 12 h</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="Product Image" /></td>
<td>100 °C</td>
<td>6 h</td>
<td>55%&lt;sup&gt;+&lt;/sup&gt;</td>
<td>—&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td><img src="image" alt="Product Image" /></td>
<td>120 °C</td>
<td>0.2 h</td>
<td>50%&lt;sup&gt;+&lt;/sup&gt;</td>
<td>65%, 12 h</td>
</tr>
<tr>
<td>5</td>
<td><img src="image" alt="Product Image" /></td>
<td>100 °C</td>
<td>1.5 h</td>
<td>89%&lt;sup&gt;d&lt;/sup&gt;</td>
<td>93%, 1.5 days</td>
</tr>
<tr>
<td>6</td>
<td><img src="image" alt="Product Image" /></td>
<td>120 °C</td>
<td>5 h</td>
<td>30%</td>
<td>62%, 12 h</td>
</tr>
<tr>
<td>7</td>
<td><img src="image" alt="Product Image" /></td>
<td>100 °C</td>
<td>6 h</td>
<td>40%&lt;sup&gt;d&lt;/sup&gt;</td>
<td>63%, 12 h</td>
</tr>
<tr>
<td>8</td>
<td><img src="image" alt="Product Image" /></td>
<td>120 °C</td>
<td>5 h</td>
<td>51%&lt;sup&gt;e&lt;/sup&gt;</td>
<td>56%, 12 h</td>
</tr>
</tbody>
</table>
a. Oxidants for entries 1-4: NCS; entries 5-8: NBS. b. Solvents for entries 1, 3, 5, and 7: MeCN; entries 2, 4, 6, and 8: AcOH. c. 10 mol% Pd(II)/MWCNT was used. d. 1.5 eq of NBS was used. e. A directly analogous yield for the homogeneous catalyst has not been reported.

Both the benzo[h]quinoline and 2-phenylpyridine substrates (entries 5 and 7) required additional NBS (1.5 equiv vs 1.2 equiv) to reach this level of conversion and product isolation. Increasing the amount of NBS beyond 1.5 equiv led to the formation of other products. For the 3-methyl-2-phenylpyridine (entry 8), additional catalyst loading was required to reach this level of conversion and product isolation.

To characterize the solid-supported Pd(II)/MWCNT catalyst, we measured the composition of the palladium both before and after C−H activation reaction by X-ray photon spectroscopy (XPS, Figure 4-1). Prior to use in the C−H activation reactions, the solid-supported catalyst was found to be a mixture of Pd(II) (76.54%) and Pd(0) (23.46%), with Pd(II) being the predominant component. After the C−H to C−OMe functionalization of benzo[h]quinoline (Table 4-1, entry 5), XPS showed that the solid-supported catalyst contained only Pd(II), showing that all of the Pd(0) in the catalyst prior to the reaction is converted to Pd(II), and supporting the mechanism that these reactions use Pd(II) as the starting oxidation state.
Figure 4-1. Pd(II) and Pd(0) content in Pd(II)/MWCNT (a.) before and (b.) after a C-H Activation Reaction as measured by XPS

To demonstrate the ability of the Pd(II)/MWCNT catalyst to be recycled, we ran the C–H to C–OMe functionalization on 8-methylquinoline (Table 4-1, entry 4), recovered the catalyst by centrifugation, and iteratively repeated the reaction with the same batch of catalyst. We were able to recycle the catalyst a remarkable 16 times with minimal reduction in yield and no catalyst deactivation (Table 4-4). We terminated the experiment after 16 recycles and have yet to determine the limits of the recyclability of the catalyst. These data demonstrate that there must be good retention or highly efficient leaching/redeposition of Pd in these reactions. Otherwise, leaching would result in loss of catalytic activity after multiple recycles.
Table 4-4. Recycling experiments with Pd(II)/MWCNT

<table>
<thead>
<tr>
<th>Run</th>
<th>Conversion (%)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
</tr>
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<td>5</td>
<td>90</td>
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<tr>
<td>15</td>
<td>90</td>
</tr>
<tr>
<td>16</td>
<td>90</td>
</tr>
<tr>
<td>17</td>
<td>90</td>
</tr>
</tbody>
</table>

^a. Conversion was determined by GC/MS based upon the consumption of the starting material

For all of the C−H activation reactions, we observed that the reactions catalyzed by Pd(II)/MWCNT seemed to be faster than those with the homogeneous Pd(OAc)₂. To quantify this observation, we calculated turnover frequencies (TOFs) for one example reaction of each functionalization with both the solid-supported Pd(II)/MWCNT and homogeneous palladium catalysts (Table 4-5). For the C−H to C−OAc and C−OMe functionalization reactions, the turnover frequencies for the solid-supported Pd(II)/MWCNT catalyst were ~27-fold higher than that for the homogeneous catalyst. For the C−H to C−Cl and C−Br reactions, the turnover frequencies for the solid-supported Pd(II)/MWCNT catalyst were ~4-fold higher. This represents a significant
improvement over previously reported results, particularly with regard to the C–H to C–OMe functionalization reactions.

### Table 4-5. Comparison of Turn Over Frequencies in C–H to C–OAc, C–OMe, C–Cl, and C–Br Reactions Catalyzed by Solid-Supported Pd(II)/MWCNT and Homogeneous Pd(OAc)$_2$

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>Turn Over Frequency (h$^{-1}$)</th>
<th>Pd(II)/MWCNT</th>
<th>Pd(OAc)$_2$</th>
<th>Fold-increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>![Product Image 1]</td>
<td>106.44</td>
<td>4.02</td>
<td>26.48</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>![Product Image 2]</td>
<td>106.94</td>
<td>3.79</td>
<td>28.22</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>![Product Image 3]</td>
<td>3.60</td>
<td>0.73</td>
<td>4.90</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>![Product Image 4]</td>
<td>9.96</td>
<td>2.63</td>
<td>3.75</td>
<td></td>
</tr>
</tbody>
</table>

To determine whether the palladium metal from the solid supported Pd(II)/MWCNT catalyst leached into the C–H activation reactions and contaminated the products, we removed the catalyst by filtration over Celite from a C–H to C–OMe functionalization reaction on benzo[h]quinolone (Table 4-1, entry 5) and measured the palladium content in solution by ICP-MS. The palladium content of the reaction mixture was found to be <250 ppb, demonstrating that very little metal leached into the reaction medium. Combined with the ease of removing the catalyst from the reaction mixtures,
this low level of palladium in the reaction mixture is an improvement on the existing homogeneous catalyst for N-chelation-directed C–H activation reactions.

To demonstrate that the trace palladium in the reaction mixture is not the source of catalytic activity, a hot filtration experiment was performed with benzo[h]quinoline (Table 4-1, entry 5). After 10 min at 100 °C, the Pd(II)/MWCNT catalyst was removed by hot filtration over Celite. Fresh substrate and oxidant were added to the filtrate, which was reheated to 100°C. No further conversion to product or catalytic activity was observed in the filtrate in the absence of Pd(II)/MWCNT, showing that the <250 ppb of residual Pd that remains in solution is not adequate to catalyze the C–H activation reaction.

In conclusion, we have demonstrated that solid-supported Pd(II)/MWCNT can catalyze N-chelation-directed C–H to C–OAc, C–OMe, C–Cl, and C–Br activation reactions. For all of the C–H activation reactions examined, the solid supported catalyst demonstrated consistently higher turnover frequencies than the reported homogeneous catalyst. The solid supported Pd(II)/MWCNT also offers the advantages of ease of removal by filtration and low levels of residual palladium metal contamination in the products. We are currently working to optimize the reactions reported here as well as apply this catalyst to other N-chelation-directed C–H activation reactions.
Challenges faced in methoxylation reaction:

In order to apply the methoxylation reaction in pharmaceutically relevant compounds, we tried the methoxylation reaction on Imatinib, which is a tyrosine-kinase inhibitor used in the treatment of cancers (chronic myeloid leukaemia). Unfortunately, this reaction gave a mixture of products (seen as a single spot on TLC 5% MeOH-DCM) which could not be separated (Scheme 4-1). The presence of multiple nitrogen atoms (directing groups) might have resulted in the methoxylation at various positions. This reaction needs to be further optimized to give a single product.

![Scheme 4-1. Methoxylation on Imatinib catalyzed by Pd-MWCNT](image)

Challenges faced in fluorination and trifluoromethylation reactions:

First, we tried the fluorination reaction on 8-methylquinoline using the electrophilic fluorinating agent 1-Fluoro-2,4,6-trimethylpyridinium tetrafluoroborate (Scheme 4-2). The reaction did not yield any product even after various optimization attempts. This was not very surprising as fluorinating reactions are usually very difficult. The reported fluorination reactions using homogenous Pd catalysts use microwave heating to get the reactions to work. This shows that these reactions are thermodynamically difficult to achieve.

The next step is to try using nucleophilic fluorinating agents.
We next tried a trifluoromethylation reaction on 2-phenylpyridine (Scheme 4-3). A homogeneous catalyst has been reported to give a yield of 44% using (trifluoromethyl)dibenzothiophenium tetrafluoroborate as the trifluoromethylating reagent. We were able to get only 22% yield when we reproduced this reaction. Unfortunately, when we tried the reaction with Pd-MWCNT catalyst, we were unable to get any product.

We then tried the reaction on a different substrate – 8-methylquinoline to see if an \( sp^3 \) carbon would undergo trifluoromethylation with our Pd-MWCNT catalyst (Scheme 4-
4). This reaction did not afford any product. As this reaction utilizes Cu(OAc)$_2$, we also tried Pd-Cu nanocatalyst to see if the combined nanocatalyst would catalyze the reaction. Unfortunately, we did not observe any formation of product.

![Scheme 4-4. Trifluoromethylation on 8-methylquinoline catalyzed by Pd-MWCNT](image)

<table>
<thead>
<tr>
<th>Reaction Details</th>
<th>Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pd-MWCNT 10 mol%, Cu(OAc)$_2$ 1 eq., TFA 10 eq., DCE, thermal 110 °C for 48 h</td>
<td>not much conversion</td>
</tr>
<tr>
<td>2. Pd-Cu nanocatalyst 10 mol%, TFA 10 eq., DCE, thermal 110 °C for 48 h</td>
<td>not much conversion</td>
</tr>
</tbody>
</table>

These fluorination and trifluoromethylation reactions need to be further optimized in order to make them work. At present, the Pd-MWCNT catalyst does not appear to be a good catalyst for these reactions. Microwave heating of reactions and/or use of other solid-supported catalyst systems would be the next step in this direction.

### 4.2. Experimental Procedures

#### General Chemical Methods

All the reactions were carried out in vials sealed with Teflon lined caps under ambient atmosphere. Palladium (II) acetate (98% reagent grade) and multi-walled carbon nanotubes (cat. #: 724769) were purchased from Sigma-Aldrich. Benzo[h]quinoline and 3-methyl-2-phenylpyridine were purchased from TCI, 2-phenylpyridine was purchased from Chem-Impex. All solvents were purchased from VWR and other chemicals were purchased from Sigma-Aldrich and all were used without distillation or purification. Analytical Thin Layer Chromatography (TLC) was performed using silica gel GHLF plates (Analtech Inc., DE, USA). Flash chromatography was performed on TELEDYNE ISCO CombiFlash® Rf instrument using RediSep Rf Normal-phase Flash...
Columns (4-gm, 12-gm, 24-gm or 40-gm). NMR spectra were recorded on a Bruker 400 MHz instrument operating at 400 MHz for $^1$H and 125 MHz for $^{13}$C acquisitions. Electrospray ionization (ESI) mass spectra were obtained from Perkin Elmer Flexar UPLC/AxION2 TOF Mass Spectrometer. The X-ray photoelectron spectroscopy (XPS) analysis was performed on a Thermo Fisher Scientific ESCALAB 250 using a monochromatic Al KR X-ray. The Pd content in the Pd nanoparticles supported on carbon nanotubes before and after reaction was determined using an Inductively Coupled Plasma equipped with Mass Spectrometry (ICP-MS, Varian 820-MS).

**Synthesis of Pd(II) nanoparticles on Multi-Walled Carbon Nanotubes Catalyst [Pd(II)/MWCNT].**

Palladium (II) acetate (0.103 g, 4.6 mmol) and multi-walled carbon nanotubes (0.500 g) were loaded in a 45 ml zirconia grinding vial. Two 12.77 mm diameter zirconia balls are also placed in the vial before sealing. The container is then placed in an 8000 M Spex Mixer/Mill. The contents in the mixer were shaken back and forth 5.9 cm and side-to-side 2.5 cm for 10 minutes at room temperature at 115 volts (1060 cycles/minute). The resulting solid was collected and used directly in reactions.

**NOTE:** The shorter ball-milling time (10 minutes vs. 30 minutes used in the original method) resulted in a higher Pd(II)/Pd(0) ratio (76.54% Pd(II), 23.46% Pd(0)).

**Quinolin-8-ylmethyl acetate (1)**

![Quinolin-8-ylmethyl acetate](image)

PhI(OAc)$_2$ (89 mg, 0.28 mmol) and Pd-MWCNT (7 mg, 5 mol%) were added to a solution of 8-methylquinoline (20 mg, 0.14 mmol) in AcOH (1.2 mL). The reaction was
heated at 120 °C for 10 min. After cooling to room temperature, it was diluted with EtOAc and washed with brine. The organic layer was filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 20% EtOAc/n-hexane) to give the product as a white solid (25 mg) in 90% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.

8-(Methoxymethyl)quinoline (4)

PhI(OAc)₂ (89 mg, 0.28 mmol) and Pd-MWCNT (7 mg, 5 mol%) were added to a solution of 8-methylquinoline (20 mg, 0.14 mmol) in methanol (1.2 mL). The reaction was heated at 100 °C for 10 min. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 20% EtOAc/n-hexane) to give the product as yellow oil (24 mg) in 99% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.

10-Methoxybenzo[h]quinoline (5)

PhI(OAc)₂ (89 mg, 0.28 mmol) and Pd-MWCNT (7 mg, 5 mol%) were added to a solution of benzo[h]quinoline (25 mg, 0.14 mmol) in methanol (1.2 mL). The reaction was heated at 100 °C for 10 min. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na₂SO₄ and dried. The crude product was purified
by flash chromatography (silica gel, 5% MeOH/DCM) to give the product as a pale yellow solid (26 mg) in 90% yield. Analytical data (\(^1\)H NMR, \(^{13}\)C NMR, mass spec) matched the previously reported data.

2-(2-Methoxyphenyl)pyridine (6)

PhI(OAc)_2 (87 mg, 0.27 mmol) and Pd-MWCNT (10 mg, 5 mol%) were added to a solution of 2-phenylpyridine (30 mg, 0.19 mmol) in methanol (1.6 mL). The reaction was heated at 100 °C for 5 h. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na_2SO_4 and dried. The crude product was purified by flash chromatography (silica gel, 20% EtOAc/n-hexane) to give the product as a clear liquid (9 mg) in 25% yield. Analytical data (\(^1\)H NMR, \(^{13}\)C NMR, mass spec) matched the previously reported data.

10-Ethoxybenzo[h]quinoline (7)

PhI(OAc)_2 (89 mg, 0.28 mmol) and Pd-MWCNT (7 mg, 5 mol%) were added to a solution of benzo[h]quinoline (25 mg, 0.14 mmol) in ethanol (1.2 mL). The reaction was heated at 100 °C for 10 min. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na_2SO_4 and dried. The crude product was purified by flash chromatography (silica gel, 5% MeOH/DCM) to give the product as thick oil
(22.6 mg) in 73% yield. Analytical data (\(^1\)H NMR, \(^{13}\)C NMR, mass spec) matched the previously reported data.

10-Isopropoxybenzo[h]quinoline (8)

![Image](72x601 to 150x660)

Phl(OAc)\(_2\) (89 mg, 0.28 mmol) and Pd-MWCNT (7 mg, 5 mol\%) were added to a solution of benzo[h]quinoline (25 mg, 0.14 mmol) in isopropanol (1.2 mL). The reaction was heated at 100 °C for 3 h. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na\(_2\)SO\(_4\) and dried. The crude product was purified by flash chromatography (silica gel, 5% MeOH/DCM) to give the product as brown oil (11 mg) in 33% yield. Analytical data (\(^1\)H NMR, \(^{13}\)C NMR, mass spec) matched the previously reported data.

10-Chlorobenzo[h]quinoline (10)

![Image](72x281 to 150x339)

NCS (21 mg, 0.16 mmol) and Pd-MWCNT (7 mg, 5 mol\%) were added to a solution of benzo[h]quinoline (25 mg, 0.14 mmol) in ACN (1.2 mL). The reaction was heated at 100 °C for 5 h. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na\(_2\)SO\(_4\) and dried. The crude product was purified by flash chromatography (silica gel, 10% EtOAc/n-hexane) to give the product as a white solid (27 mg) in 92% yield. Analytical data (\(^1\)H NMR, \(^{13}\)C NMR, mass spec) matched the previously reported data.
(E)-8-Chloro-3,4-dihydronaphthalen-1(2H)-one O-methyl oxime (11)

NCS (24 mg, 0.18 mmol) and Pd-MWCNT (9 mg, 5 mol%) were added to a solution of (E)-3,4-dihydronaphthalen-1(2H)-one O-methyl oxime (30 mg, 0.17 mmol) in AcOH (1.4 mL). The reaction was heated at 120 °C for 1.5 h. After cooling to room temperature, it was diluted with EtOAc, washed with brine. The organic layer was filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 5% EtOAc/n-hexane) to give the product as clear oil (22 mg) in 61% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.

2-(2-Chlorophenyl)pyridine (12)

NCS (26 mg, 0.19 mmol) and Pd-MWCNT (17 mg, 10 mol%) were added to a solution of 2-phenylpyridine (25 mg, 0.16 mmol) in ACN (1.3 mL). The reaction was heated at 100 °C for 6 h. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 10% EtOAc/n-hexane) to give the product as a clear liquid (16.7 mg) in 55% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.
2-(2-Chlorophenyl)-3-methylpyridine (13)

NCS (28 mg, 0.21 mmol) and Pd-MWCNT (19 mg, 10 mol%) were added to a solution of 3-methyl-2-phenylpyridine (30 mg, 0.18 mmol) in AcOH (1.5 mL). The reaction was heated at 120 °C for 10 min. After cooling to room temperature, it was diluted with EtOAc, washed with brine. The organic layer was filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 10% EtOAc/n-hexane) to give the product as clear oil (18 mg) in 50% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.

10-Bromobenzo[h]quinoline (14)

NBS (37 mg, 0.21 mmol) and Pd-MWCNT (7 mg, 5 mol%) were added to a solution of benzo[h]quinoline (25 mg, 0.14 mmol) in ACN (1.2 mL). The reaction was heated at 100 °C for 1.5 h. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 10% EtOAc/n-hexane) to give the product as a white solid (27 mg) in 89% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.
(E)-8-Bromo-3,4-dihydronaphthalen-1(2H)-one O-methyl oxime (15)

NBS (106 mg, 0.60 mmol) and Pd-MWCNT (30 mg, 5 mol%) were added to a solution of (E)-3,4-dihydronaphthalen-1(2H)-one O-methyl oxime (100 mg, 0.57 mmol) in AcOH (4.7 mL). The reaction was heated at 120 °C for 5 h. After cooling to room temperature, it was diluted with EtOAc, washed with brine. The organic layer was filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 5% EtOAc/n-hexane) to give the product as yellow oil (43 mg) in 30% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.

2-(2-Bromophenyl)pyridine (16)

NBS (43 mg, 0.24 mmol) and Pd-MWCNT (8 mg, 5 mol%) were added to a solution of 2-phenylpyridine (25 mg, 0.16 mmol) in ACN (1.3 mL). The reaction was heated at 100 °C for 6 h. After cooling to room temperature, it was diluted with EtOAc, filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 10% EtOAc/n-hexane) to give the product as yellow oil (15.1 mg) in 40% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.
2-(2-Bromophenyl)-3-methylpyridine (17)

NBS (38 mg, 0.21 mmol) and Pd-MWCNT (19 mg, 10 mol%) were added to a solution of 3-methyl-2-phenylpyridine (30 mg, 0.18 mmol) in AcOH (1.5 mL). The reaction was heated at 120 °C for 10 min. After cooling to room temperature, it was diluted with EtOAc, washed with brine. The organic layer was filtered through celite, Na₂SO₄ and dried. The crude product was purified by flash chromatography (silica gel, 10% EtOAc/n-hexane) to give the product as clear oil (22.3 mg) in 51% yield. Analytical data (¹H NMR, ¹³C NMR, mass spec) matched the previously reported data.

Procedure for Recycling Experiment.

To a solution of 8-methylquinoline (20 mg, 0.14 mmol) in methanol (1.2 mL) in 10 mL reaction vial, was added Phl(OAc)₂ (89 mg, 0.28 mmol) and Pd(II)/MWCNT (7 mg, 5 mol%). The vial was sealed and the reaction mixture was heated at 100 °C for 10 min. Upon the completion of the reaction period, the mixture was diluted with 2 mL methanol and shaken. The entire mixture was centrifuged and the solvent above the Pd/MWCNT nanoparticles was decanted. The washing and centrifugation were repeated for two additional times to ensure the removal of the organic products from the surface of the catalyst. The Pd/MWCNT nanoparticles was then reused for the subsequent reaction using fresh reagents (8-methylquinoline, Phl(OAc)₂, and methanol). This procedure was applied for every recycling experiment and the percent conversion to the products was determined by means of GC-MS spectroscopy.
Procedure for Hot filtration Experiment.

Benzo[h]quinoline (25 mg, 0.14 mmol) in methanol (1.2 mL) was heated in the presence of Pd(II)/MWCNT (7 mg, 5 mol%) and PhI(OAc)₂ (89 mg, 0.28 mmol) in a 10 mL reaction vial at 100 °C for 10 min, resulting in 95% conversion to the product according to the GC-MS analysis. The reaction mixture was then hot filtered over celite and the filtrate solution was subjected to ICP-MS in which the amount of Pd content was determined to be <250 ppb. Fresh reagents (Benzo[h]quinoline (25 mg, 0.14 mmol), and PhI(OAc)₂ (89 mg, 0.28 mmol)) were added to the filtrate solution, the mixture was heated at 100 °C for additional 10 min. No further catalytic activity was observed in this mixture by GC-MS spectroscopy.
5. C-H Arylation Reactions Catalyzed by our Solid-Supported Pd(II)-MWCNT Catalyst

This chapter discusses C–H arylation reactions that were catalyzed by our Pd(II)-MWCNT catalyst.

5.1. Results and Discussion

Initially, we carried out reactions on the substrates that have already been reported to undergo N-chelation-directed C–H arylation reactions, so that we could compare the results with homogenous catalyst Pd(OAc)$_2$ (Table 5-1). First, we tried arylation reactions on 2-phenyl-3-methylpyridine (entry 1) using the symmetrical arylating agent [Ph$_2$I]BF$_4$. Reaction was carried out at 100°C, using 5 mol% of Pd (II)-MWCNT catalyst in the presence of solvent acetic-acid. We were pleased to find that the reaction was complete in 12 hours, and gave a yield of 90%. The turn-over frequency of this reaction was comparable to that reported for the homogenous palladium catalysed reaction. Motivated by this result, we carried out the same reaction on various substrates. There was a single isomeric product formed in entry 2, which suggests that this C-H arylation is dictated by steric factors, instead of dual chelation effects of Pd and ketone. In entries 3 and 4, where the substrates have amide type of nitrogen, the yields were very low – 27% and 32% respectively. This could be because the lone pair of electrons on amide nitrogen are not readily available, hence Pd chelation might be the rate limiting step. These reactions had lower TOFs than the homogenous Pd catalyst. The arylation reaction on benzo[h]quinoline (entry 5) gave the product albeit in low yield of 19%.
Table 5-1. C-H to C-C Functionalizations Catalyzed by Pd(II)/MWCNT

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>Yield and time with Pd(II)/MWCNT</th>
<th>Yield and time with Pd(OAc)$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Image" /></td>
<td>90%, 12 h</td>
<td>88%, 12 h</td>
</tr>
<tr>
<td>2$^b$</td>
<td><img src="image2.png" alt="Image" /></td>
<td>80%, 24 h</td>
<td>91%, 48 h</td>
</tr>
<tr>
<td>3$^c$</td>
<td><img src="image3.png" alt="Image" /></td>
<td>27%, 12h</td>
<td>49%, 12h</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4.png" alt="Image" /></td>
<td>32%, 24 h</td>
<td>75%, 24 h</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5.png" alt="Image" /></td>
<td>19%, 12 h</td>
<td>NR$^d$</td>
</tr>
</tbody>
</table>

a. Entries 1, 4, 5: [Ph$_2$I]BF$_4$ 1.2 eq. b. [Ph$_2$I]BF$_4$ 1.5 eq. c. [Ph$_2$I]BF$_4$ 2 eq, solvent AcOH:Ac$_2$O (1:1). d. not reported under similar conditions

We next wished to expand the scope of our transformations to incorporate other aryl rings (Table 5-2). In order to selectively transfer a specific aryl ring, we used asymmetrical arylating agents [Mes-I-Ar] as reported by Sanford et al.$^{139}$ The asymmetrical arylating agents consist of a mesitylene group (which cannot be transferred due to its high bulk) and a substituted aryl group (which would get transferred selectively). Both electronically neutral aryl methyl group (entry 1) and electronically poor aryl fluoro group (entry 2) were transferred with good yields (87% and 79% respectively) with reaction rates almost equal to that of the homogenous catalyst. In contrast, the electronically rich aryl methoxy group (entry 3) did not react...
very well, with a yield of only 33%. Increasing the reaction time did not improve the yield. This is in contrast to the homogenous catalyst which has been reported to have a yield of 81%. This effect was not seen in the ethoxy aryl group (entry 8), where the reaction gave a yield of 79%. The reason for this difference in the reactivity of methoxy- and ethoxy-substituted substrates is currently unknown.

We then carried out arylations that have not been reported yet with homogenous Pd catalyst. With the electron withdrawing nitro group, para/meta/ortho substitution position did not have any influence on the reaction. All three positions gave almost similar yields (entries 4-6). These transformations are significant as the products in entries 4 and 6 have not been reported to date. Thus, C-H arylation through Pd(II)/Pd(IV) can be used to give products that are not obtained through conventional coupling reactions.

In the case of aryl aldehyde, the ortho substituent gave a poor yield of 18% (entry 7), whereas the para substituent gave a good yield of 77% (entry 9). The huge difference between the reactivities of ortho nitro (entry 6) and ortho aldehyde (entry 7) could be explained by the difference in electronic nature of both the substituents. Nitro group is a much stronger electron withdrawing group compared to aldehyde. So, the nitro group makes the aryl ring highly reactive towards oxidative addition of Pd (II) to Pd (IV). Though it is in ortho position, the electronics might be dictating the reaction compared to sterics, which explains higher yield of the reaction. Whereas with ortho aldehyde, as it is not a strong electron withdrawing group, the sterics might be dictating the reaction, which explains lower yield.
Table 5-2. C-H to C-C Functionalizations Catalyzed by Pd(II)/MWCNT on 3-methyl-2-phenylpyridine

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>Yield and time with Pd(II)/MWCNT</th>
<th>Yield and time with Pd(OAc)$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>![Product Image]</td>
<td>87%, 12 h</td>
<td>84%, 12 h</td>
</tr>
<tr>
<td>2</td>
<td>![Product Image]</td>
<td>79%, 12 h</td>
<td>88%, 12 h</td>
</tr>
<tr>
<td>3</td>
<td>![Product Image]</td>
<td>33%, 12 h</td>
<td>81%, 12 h</td>
</tr>
<tr>
<td>4</td>
<td>![Product Image]</td>
<td>61%, 12 h</td>
<td>NR$^a$</td>
</tr>
<tr>
<td>5</td>
<td>![Product Image]</td>
<td>69%, 12 h</td>
<td>NR$^a$</td>
</tr>
<tr>
<td>6</td>
<td>![Product Image]</td>
<td>58%, 12 h</td>
<td>NR$^a$</td>
</tr>
<tr>
<td>7</td>
<td>![Product Image]</td>
<td>18%, 12 h</td>
<td>NR$^a$</td>
</tr>
<tr>
<td>8</td>
<td>![Product Image]</td>
<td>79%, 12 h</td>
<td>NR$^a$</td>
</tr>
</tbody>
</table>
We wished to expand C-H arylation catalysed by our Pd(II)-MWCNT catalyst to a more rigid and bulky substrate benzo[h]quinoline (Table 5-3). We were pleased to obtain the arylated product (entry 1) albeit in low yield of 19%. This transformation has been achieved with very simple reaction conditions. This transformation has been reported by Sanford et al, but using different conditions (89% yield using palladium acetate, benzoquinone oxidant, DMSO and Ag₂CO₃). Though the yield with our solid-supported catalyst is low, it is an attractive transformation due to its simplicity.

We then expanded this reaction to incorporate substituted aryl rings – electron withdrawing aryl fluoro group (entry 2) and electronically neutral aryl methyl group (entry 3). We were pleased to find that the fluoro substituent gave a yield of 35%, which is higher than the unsubstituted phenyl ring (entry 1). This could be due to the aryl ring being electron deficient due to the presence of fluoro group, thus making it more reactive towards oxidative addition of Pd(II) to Pd(IV). Methyl substituted aryl group gave a low yield of 15% (entry 3) comparable to that of unsubstituted phenyl ring. This type of reaction is very attractive because the products in entries 2 and 3 have been reported but using more complicated reaction conditions and/or using highly expensive catalysts such as Rh. We are in the process of optimizing our reaction conditions to improve the yields further.
Table 5-3. C-H to C-C Functionalizations Catalyzed by Pd(II)/MWCNT on benzo[h]quinoline

<table>
<thead>
<tr>
<th>Entry</th>
<th>Product</th>
<th>Yield and time with Pd(II)/MWCNT</th>
<th>Yield and time with Pd(OAc)$_2$</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td><img src="image" alt="Product 1" /></td>
<td>19%, 12 h</td>
<td>NR$^a$</td>
</tr>
<tr>
<td>2</td>
<td><img src="image" alt="Product 2" /></td>
<td>35%, 12 h</td>
<td>NR$^a$</td>
</tr>
<tr>
<td>3</td>
<td><img src="image" alt="Product 3" /></td>
<td>15%, 12 h</td>
<td>NR$^a$</td>
</tr>
</tbody>
</table>

a. not reported under similar conditions

**Arylation reaction challenges:**

Arylation on 8-methylquinoline using the symmetrical arylating agent gave a mixture of starting material and another isomer (which could not be identified, Scheme 5-1). The reaction did not go to completion. This mixture could not be purified on normal phase, so the yield of the product could not be determined. In order to optimize this reaction, when AcOH was used as the solvent, diarylated product was formed and the starting material was still not completely consumed.
Similar reaction on 2-phenylpyridine gave a mixture of product and another isomer, which could not be purified on normal phase. So, the yield was not calculated (Scheme 5-2).

We were able to successfully synthesize asymmetrical arylating agents – 4-bromomethyl and 2-cyano. But unfortunately, the coupling reactions of these arylating agents with 3-methyl-2-phenylpyridine did not afford the product (Scheme 5-3).
We were unable to synthesize some of the arylating reagents. The reactions to make 4-cyano and 4-dimethylamino arylating agent did not work at all, while 3-pyridyl arylating reagent was formed in very low yield of 4% (Scheme 5-4).
Arylation reaction between benzo[h]quinolone and 1,2-dichlorobenzene using benzoquinone promoter, Ag$_2$CO$_3$ oxidant, Pd-MWCNT catalyst, and DMSO (to protect catalyst) gave the product in good yield (68%, Scheme 5-5). In contrast, when the reaction was tried on 1,2-dimethoxy benzene, the reaction did not work (Scheme 5-6). This might be because methoxy is sterically bulkier than the chloro group. Sterics might be dictating this reaction.

Scheme 5-5. Arylation of benzo[h]quinolone with 1,2-dichlorobenzene

Scheme 5-6. Arylation of benzo[h]quinolone with 1,2-dimethoxybenzene
5.2. Experimentals

Diphenyliodonium tetrafluoroborate: Under nitrogen, phenylboronic acid (0.4 mmol) was dissolved in DCM (0.1 M), cooled to 0 °C. To this, BF₃OEt₂ (0.4 mmol) was added. The reaction mixture was stirred at 0 °C for 15 min. This was followed by the addition of diacetoxyiodobenzene (0.4 mmol) dissolved in DCM (0.1 M) to the above reaction mixture. It was stirred at 0 °C for 1.5 h under nitrogen. To this, saturated solution of aqueous NaBF₄ (1.5 mL) was added, the reaction was stirred for 30 min. The reaction mixture was extracted with DCM, washed with water and filtered over MgSO₄. The organic layer was dried, washed with hexanes : DCM (3:1). The precipitate was collected, washed with hexanes and dried to afford pure product in 84% yield (119.5 mg, 0.3 mmol). ¹H NMR (400 MHz, (CD₃)₂SO) δ 8.24 (d, J = 7.56 Hz, 4H), 7.66 (t, J = 7.45 Hz, 2H), 7.53 (t, J = 7.76 Hz, 4H); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 135.12, 132.02, 131.73, 116.44.

General procedure A for the synthesis of asymmetrical arylating agents: Under nitrogen, substituted phenylboronic acid (0.7 mmol) was suspended/dissolved in DCM (0.1 M), cooled to 0 °C. To this, BF₃OEt₂ (0.8 mmol) was added. The reaction mixture was stirred at 0 °C for 15 min. This was followed by the addition of iodomesitylene diacetate (0.8 mmol) dissolved in DCM (0.3 M) to the above reaction mixture. It was warmed to room temperature, and stirred for 2 h under nitrogen. To this, saturated solution of aqueous NaBF₄ (3.0 mL) was added, the reaction was stirred for 30 min.
The reaction mixture was extracted with DCM, washed with water, filtered over MgSO₄. Organic layer was dried, washed with Et₂O to afford pure product.

\[(4\text{-fluorophenyl})(\text{mesityl})\text{iodonium tetrafluoroborate} :\] General procedure A was followed on substrate 4-fluorophenyl boronic acid to afford the product in 75% yield (229.6 mg, 0.54 mmol). \(^1\)H NMR (400 MHz, (CD₃)₂SO) δ 8.02-8.04 (m, 2H), 7.36 (t, J = 8.91 Hz, 2H), 7.21 (s, 2H), 2.60 (s, 6H), 2.29 (s, 3H); \(^{13}\)C NMR (125 MHz, (CD₃)₂SO) δ 143.16, 141.47, 137.21, 137.12, 129.77, 122.94, 119.31, 119.08, 108.62, 30.62, 26.22, 20.47.

\[\text{mesityl}(p\text{-tolyl})\text{iodonium tetrafluoroborate} :\] General procedure A was followed on substrate 4-tolyl boronic acid to afford the product in 70% yield (217.2 mg, 0.51 mmol). \(^1\)H NMR (400 MHz, (CD₃)₂SO) δ 7.85 (d, J = 8.36 Hz, 2H), 7.30 (d, J = 8.44 Hz, 2H), 7.20 (s, 2H), 2.59 (s, 6H), 2.32 (s, 3H), 2.29 (s, 3H); \(^{13}\)C NMR (125 MHz, (CD₃)₂SO) δ 142.99, 142.23, 141.44, 134.43, 132.45, 129.71, 122.68, 110.83, 26.24, 20.73, 20.45.

\[(4\text{-ethoxyphenyl})(\text{mesityl})\text{iodonium tetrafluoroborate} :\] General procedure A was followed on substrate 4-ethoxyphenyl boronic acid to afford the product in 95% yield (142.1 mg, 0.31 mmol). \(^1\)H NMR (400 MHz, (CD₃)₂SO) δ 7.66 (d, J = 9.35 Hz, 2H),
7.26 (s, 1H), 7.09 (s, 2H), 6.91 (d, $J = 9.39$ Hz, 2H), 4.02 (q, $J = 7.18$ Hz, 6.93 Hz, 2H), 2.65 (s, 6H), 2.35 (s, 3H), 1.39 (t, $J = 6.97$ Hz, 3H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 160.99, 142.85, 141.29, 136.48, 129.64, 123.04, 117.86, 103.15, 63.75, 26.19, 20.43, 14.30.

(4-formylphenyl)(mesityl)iodonium tetrafluoroborate: General procedure A was followed on substrate 4-formylphenyl boronic acid to afford the product in 69% yield (101 mg, 0.23 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 10.00 (s, 1H), 8.15 (d, $J = 8.48$ Hz, 2H), 7.95 (d, $J = 8.48$ Hz, 2H), 7.24 (s, 2H), 2.60 (s, 6H), 2.30 (s, 3H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 192.32, 143.27, 141.58, 137.74, 134.85, 131.85, 129.83, 122.91, 120.71, 26.24, 20.46.

mesityl(4-methoxyphenyl)iodonium tetrafluoroborate: General procedure A was followed on substrate 4-methoxyphenyl boronic acid to afford the product in 85% yield (251.2 mg, 0.57 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$SO) $\delta$ 7.91 (d, $J = 9.1$ Hz, 2H), 7.19 (s, 2H), 7.03 (d, $J = 9.25$ Hz, 2H), 3.78 (s, 3H), 2.60 (s, 6H), 2.29 (s, 3H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$SO) $\delta$ 161.69, 142.83, 141.27, 136.46, 129.62, 123.01, 117.47, 103.32, 55.63, 26.17, 20.41.
mesityl(2-nitrophenyl)iodonium tetrafluoroborate: General procedure A was followed on substrate 2-nitrophenyl boronic acid to afford the product in 79% yield (241 mg, 0.53 mmol). ¹H NMR (400 MHz, (CD₃)₂SO) δ 8.59 (d, J = 8.09 Hz, 1H), 7.79-7.90 (m, 2H), 7.41 (s, 2H), 7.03 (d, J = 8.04 Hz, 1H), 2.56 (s, 6H), 2.42 (s, 3H); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 145.93, 144.72, 142.95, 138.08, 132.23, 130.41, 130.22, 127.87, 120.58, 108.39, 26.15, 20.72.

(2-formylphenyl)(mesityl)iodonium tetrafluoroborate: General procedure A was followed on substrate 2-formylphenyl boronic acid to afford the product in 30% yield (86 mg, 0.2 mmol. ¹H NMR (400 MHz, (CD₃)₂SO) δ 10.32 (s, 1H), 8.43 (dd, J = 7.5 Hz, 1H), 7.91 (td, J = 7.28 Hz, 1H), 7.79 (td, J = 7.85 Hz, 1H), 7.39 (s, 2H), 6.90 (d, J = 8.29 Hz, 1H), 3.32 (s, 9H), 2.42 (s, 3H); ¹³C NMR (125 MHz, (CD₃)₂SO) δ 195.04, 144.41, 143.05, 138.48, 138.41, 132.97, 131.57, 130.05, 128.92, 116.99, 110.14, 25.99, 20.68.

mesityl(2-nitrophenyl)iodonium tetrafluoroborate: General procedure A was followed on substrate 3-nitrophenyl boronic acid. Product could not be isolated to get a pure NMR spectrum.
mesityl(4-nitrophenyl)iodonium tetrafluoroborate: General procedure A was followed on substrate 4-nitrophenyl boronic acid. Product could not be isolated to get a pure NMR spectrum.

General procedure B for arylation using [Ph₂]BF₄: Substrate (0.05 mmol) was added to acetic-acid (0.12 M) followed by the addition of arylating agent [Ph₂]BF₄ (0.08 mmol) and Pd(II)-MWCNT catalyst (5 mol%). The reaction mixture was heated at 100 °C for 3 h. It was then cooled to room temperature, diluted with DCM, washed with saturated aqueous NaHCO₃, brine, and filtered over celite and MgSO₄. The organic layer was dried and purified over silica using 20% EtOAc-hexanes unless otherwise noted.

1-(2-(pyridin-2-yl)-[1,1'-biphenyl]-4-yl)ethan-1-one: General procedure B was followed on substrate 1-(3-(pyridin-2-yl)phenyl)ethan-1-one to afford the product in 80% yield (11.1 mg, 0.04 mmol). ¹H NMR (400 MHz, (CD₃)₂CO) δ 8.60-8.62 (m, 1H), 8.27 (d, J = 1.8 Hz, 1H), 8.10 (dd, J = 8.1 Hz, 1H), 7.58 (d, J = 8.0 Hz, 1H), 7.52 (td, J = 7.7 Hz, 1H), 7.27-7.30 (m, 3H), 7.21-7.25 (m, 1H) 7.17-7.19 (m, 2H), 6.98 (dt, J = 7.9 Hz, 1H), 2.66 (s, 3H); ¹³C NMR (125 MHz, (CD₃)₂CO) δ 197.49, 159.32, 150.40,
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1-(7-phenylindolin-1-yl)ethan-1-one: General procedure B was followed on substrate 1-(indolin-1-yl)ethan-1-one, using a mixture of 1:1 CH$_3$COOH: (CH$_3$CO)$_2$CO as the solvent, and purified using 10% acetone-EtOAc to afford the product in 27% yield (6 mg, 0.03 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) $\delta$ 7.38-7.48 (m, 4H), 7.16-7.31 (m, 4H), 4.24 (t, $J$ = 7.6 Hz, 2H), 3.04 (t, $J$ = 7.5 Hz, 2H), 2.76 (s, 3H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$CO) $\delta$ 142.25, 141.63, 137.65, 129.75, 129.64, 128.17, 127.75, 125.98, 124.63, 51.16, 22.82.

1-([1,1'-biphenyl]-2-yl)pyrrolidin-2-one: General procedure B was followed on substrate 1-phenylpyrrolidin-2-one, and purified using 10% acetone-EtOAc to afford the product in 32% yield (7 mg, 0.03 mmol). $^1$H NMR (400 MHz, (CDCl$_3$) $\delta$ 7.31-7.42 (m, 9H), 3.21 (t, $J$ = 6.7 Hz, 2H), 2.41 (t, $J$ = 7.9 Hz, 2H), 1.83-1.90 (m, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 175.72, 139.87, 139.36, 136.58, 131.01, 128.71, 128.56, 128.17, 127.73, 50.35, 31.35, 29.85, 19.15.

2-([1,1'-biphenyl]-2-yl)-3-methylpyridine: General procedure B was followed on substrate 3-methyl-2-phenylpyridine, and purified using 5% EtOAc-DCM to afford the
product in 90% yield (19.5 mg, 0.08 mmol). $^1$H NMR (400 MHz, (CDCl$_3$) δ 8.49 (dd, $J$ = 4.9 Hz, 1H), 7.42-7.50 (m, 3H), 7.38-7.41 (m, 1H), 7.28-7.30 (m, 1H), 7.08-7.17 (m, 6H), 1.76 (s, 3H); $^{13}$C NMR (125 MHz, CDC$_3$) δ 159.63, 146.65, 141.28, 140.87, 139.58, 137.64, 131.82, 130.04, 129.87, 129.43, 128.51, 127.93, 127.57, 126.78, 122.24, 18.95.

**General procedure C for arylation using asymmetrical arylating agents:**
Substrate (0.05 mmol) was added to acetic-acid (0.12 M) followed by the addition of asymmetrical arylating agent [Mes-I-Ar]BF$_4$ (0.08 mmol) and Pd(II)-MWCNT catalyst (5 mol%). The reaction mixture was heated at 100 °C for 3 h. It was then cooled to room temperature, diluted with DCM, washed with saturated aqueous NaHCO$_3$, brine, and filtered over celite and MgSO$_4$. The organic layer was dried and purified over silica using 20% EtOAc-hexanes unless otherwise noted.

![3-methyl-2-(4'-methyl-[1,1'-biphenyl]-2-yl)pyridine](image)

**3-methyl-2-(4'-methyl-[1,1'-biphenyl]-2-yl)pyridine:** General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-I-p-Me-C$_6$H$_5$]BF$_4$ to afford the product in 87% yield (20 mg, 0.08 mmol). $^1$H NMR (400 MHz, (CD$_3$)$_2$CO) δ 8.43 (dd, $J$ = 4.9 Hz, 1H), 7.38-7.49 (m, 4H), 7.32-7.34 (m, 1H), 7.15 (q, 1H), 6.98-7.03 (m, 4H), 2.66 (s, 3H), 1.74 (s, 3H); $^{13}$C NMR (125 MHz, (CD$_3$)$_2$CO) δ 160.66, 147.33, 141.47, 140.83, 139.27, 138.04, 137.09, 132.10, 130.86, 130.35, 129.92, 129.36, 129.02, 127.82, 123.02, 20.99, 19.00.
2-(4'-fluoro-[1,1'-biphenyl]-2-yl)-3-methylpyridine: General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-I-p-F-C₆H₅]BF₄ to afford the product in 79% yield (18.5 mg, 0.07 mmol). ¹H NMR (400 MHz, (CD₃)₂CO) δ 8.43 (dd, J = 4.99 Hz, 1H), 7.42-7.52 (m, 4H), 7.34-7.37 (m, 1H), 7.13-7.19 (m, 3H), 6.92-6.97 (m, 2H), 1.78 (s, 3H), 1.74 (s, 3H); ¹³C NMR (125 MHz, (CD₃)₂CO) δ 163.94, 161.51, 160.26, 147.40, 140.88, 140.50, 138.43, 138.22, 132.12, 131.96, 131.88, 130.86, 130.39, 129.14, 128.23, 123.17, 115.51, 115.30, 18.97.

2-(4'-ethoxy-[1,1'-biphenyl]-2-yl)-3-methylpyridine: General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-I-p-OEt-C₆H₅]BF₄ to afford the product in 79% yield (20.3 mg, 0.07 mmol). ¹H NMR (400 MHz, CDCl₃) δ 8.50 (d, J = 4.85 Hz, 1H), 7.37-7.47 (m, 4H), 7.30 (d, J = 7.59 Hz, 1H), 7.10 (q, 1H), 7.01 (d, J = 8.64 Hz, 2H), 6.68 (d, J = 8.64 Hz, 2H), 3.96 (q, J = 6.9 Hz, 7.1 Hz, 2H), 1.74 (s, 3H), 1.38 (t, J = 7.01 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 159.85, 157.99, 146.66, 140.51, 139.44, 137.67, 133.60, 131.83, 130.46, 130.01, 129.67, 128.46, 127.12, 122.17, 114.01, 63.45, 18.93, 14.96. HRMS C₂₀H₁₉NO [M+H]⁺ Expected: 290.1540, Found: 290.1563.
**2'-(3-methylpyridin-2-yl)-[1,1’-biphenyl]-4-carbaldehyde:** General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-I-p-CHO-C₆H₅]BF₄ to afford the product in 77% yield (18.5 mg, 0.07 mmol). ¹H NMR (400 MHz, CDCl₃) δ 9.94 (s, 1H), 8.47 (dd, J = 4.90 Hz, 1H), 7.68 (d, J = 8.53 Hz, 2H), 7.42-7.52 (m, 4H), 7.33 (d, J = 7.86 Hz, 1H), 7.28 (d, J = 8.13 Hz, 2H), 7.12 (q, 1H), 1.80 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 192.07, 158.90, 147.65, 146.78, 139.58, 137.95, 134.78, 131.74, 130.26, 130.03, 129.75, 129.40, 128.72, 128.55, 122.58, 18.97. HRMS C₁₉H₁₅NO [M+H]⁺ Expected: 274.1227, Found: 274.1253.

**3-methyl-2-(4’-nitro-[1,1’-biphenyl]-2-yl)pyridine:** General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-I-p-NO₂-C₆H₅]BF₄ to afford the product in 61% yield (15.6 mg, 0.05 mmol). ¹H NMR (400 MHz, CDCl₃) δ 8.47 (dd, J = 4.96 Hz, 1H), 8.03 (dt, J = 8.95 Hz, 2H), 7.43-7.54 (m, 4H), 7.36 (d, J = 7.53 Hz, 1H), 7.26-7.29 (m, 2H), 7.14 (q, 1H), 1.83 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 158.49, 147.96, 146.81, 146.61, 139.58, 138.49, 137.85, 131.45, 130.16, 130.01, 129.56, 128.79, 128.64, 123.07, 122.56, 18.85. HRMS C₁₉H₁₄N₂O₂ [M+H]⁺ Expected: 291.1128, Found: 291.1110.
3-methyl-2-(3’-nitro-[1,1’-biphenyl]-2-yl)pyridine: General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-l-m-NO2-C6H5]BF4 to afford the product in 69% yield (17.7 mg, 0.06 mmol). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.48 (dd, \(J = 4.94\) Hz, 1H), 8.01-8.05 (m, 2H), 7.41-7.55 (m, 5H), 7.37 (d, \(J = 7.7\) Hz, 1H), 7.31 (td, \(J = 7.9\) Hz, 1H), 7.13 (q, 1H), 1.85 (s, 3H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 158.58, 148.10, 146.93, 142.88, 139.63, 138.42, 138.08, 135.45, 131.66, 130.23, 129.78, 128.92, 128.81, 128.76, 1224.17, 122.73, 121.78, 19.03. HRMS C\(_{18}\)H\(_{14}\)N\(_2\)O\(_2\) [M+H]\(^+\) Expected: 291.1128, Found: 291.1108.

3-methyl-2-(2’-nitro-[1,1’-biphenyl]-2-yl)pyridine: General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-l-m-NO2-C6H5]BF4 to afford the product in 58% yield (14.9 mg, 0.05 mmol). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.26 (dd, \(J = 4.7\) Hz, 1H), 7.72 (dd, \(J = 8.1\) Hz, 1H), 7.29-7.49 (m, 8H), 7.03 (q, 1H), 2.09 (s, 3H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 157.59, 148.95, 146.21, 138.33, 137.16, 136.06, 133.64, 132.41, 132.03, 129.92, 129.67, 128.44, 128.10, 127.96, 124.09, 122.31, 19.10. HRMS C\(_{18}\)H\(_{14}\)N\(_2\)O\(_2\) [M+H]\(^+\) Expected: 291.1128, Found: 291.1141.
2’-(3-methylpyridin-2-yl)-[1,1’-biphenyl]-2-carbaldehyde: General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-I-m-NO2-C6H5]BF4 to afford the product in 18% yield (2.8 mg, 0.01 mmol). ¹H NMR (400 MHz, CDCl₃) δ 9.94 (s, 1H), 8.34 (s, 1H), 7.82 (d, J = 7.75 Hz, 1H), 7.30-7.54 (m, 7H), 7.18 (d, J = 7.58 Hz, 1H), 7.00-7.03 (m, 1H), 1.91 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 192.02, 158.19, 146.63, 144.72, 140.62, 137.81, 137.04, 133.98, 132.82, 131.57, 131.41, 129.90, 128.50, 128.10, 127.69, 126.95, 122.43, 29.85, 19.14. HRMS C₁₉H₁₅NO [M+H]+ Expected: 274.1227, Found: 274.1246.

2-(4’-methoxy-[1,1’-biphenyl]-2-yl)-3-methylpyridine: General procedure C was followed on substrate 3-methyl-2-phenylpyridine, using [Mes-I-m-NO2-C₆H₅]BF₄ to afford the product in 33% yield (8.1 mg, 0.03 mmol). ¹H NMR (400 MHz, CDCl₃) δ 8.51 (dd, J = 5.09 Hz, 1H), 7.38-7.46 (m, 4H), 7.31 (dd, J = 8.1 Hz, 1H), 7.10 (q, 1H), 7.02-7.04 (m, 2H), 6.69-6.71 (m, 2H), 3.75 (s, 3H), 1.75 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 159.81, 158.63, 146.65, 140.46, 139.41, 137.74, 133.75, 131.86, 130.49, 130.03, 129.71, 128.50.
6. References

8. Riefler, G. M.; Firestein, B. L. Binding of neuronal nitric-oxide synthase (nNOS) to carboxyl-terminal-binding protein (CtBP) changes the localization of CtBP from the nucleus to the cytosol: a novel function for targeting by the PDZ domain of nNOS. *J Biol Chem* 2001, 276, 48262-8.


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