FUNCTION ORIENTED ORGANIC COMPOUNDS DESIGN;
A CATALYTIC ENZYME INHIBITOR AND A CNS ACTIVE REACTIVATOR

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry and Biochemistry

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A CATALYTIC ENZYME INHIBITOR AND A CNS ACTIVE REACTIVATOR

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In chapter one, a new concept of catalytic acetyl transfer enzyme inhibitor design was explored. Thiol salicylamide analogues were recently used to transfer acetyl group in vitro and in vivo to lysine/serine of target proteins. The ability of thiol salicylamide compounds to re-acetylate make them ideal to catalytically transfer acetyl groups. Here this approach is used to make a catalytic cyclooxygenase (COX) inhibitor. A catalytic COX inhibitor would have unique inhibition kinetics.

Based on known inhibitors and the crystal structure of COX-1/2, we initially synthesized several compounds and tested their catalytic inhibition. The IC50 and kinetics of pre-acetylated thiol salicylamide compounds were tested.

Using molecular dynamics simulation, the binding affinity of several proposed inhibitors were assessed. The potential mean force (PMF) from bound acetylated compounds to access COX’s Ser530 were evaluated. The combined results of these two properties were used to select several candidates to be synthesized. Candidates were then tested in vitro and in vivo for their COX inhibition efficiency and kinetics. The top hit, WB3/AcWB3 proved to be an efficient catalytic inhibitor that had a sustained effect in COX inhibition in cells.

In chapter two, a new approach was explored to modify positively charged pyridinium oxime (PAM) reactivator drugs in a manner that would facilitate their ability to cross the blood brain barrier (BBB).

PAM is currently used to reactivate organophosphorus inhibited acetylcholinesterase. However, PAM has very low CNS activity due to its low BBB
permeability. The high abundance of glucose transporter 1 (GLUT1) in human BBB could potentially be exploited to facilitate transportation of PAM to across the BBB. Glucose conjugated sugar-oximes (SOx) were synthesized and tested in vitro. The reactivity of SOx was characterized using an in vitro enzyme kinetics assay. To assess the permeability of BBB, a cell model was used combining with LC-MS quantification.

A complete molecular dynamics model of SOxs’ GLUT1 transportation was constructed. Three stages of GLUT1 (outward facing state, ligand binding state, inward facing state) were built from crystal structures in an explicit membrane system. Adaptive steered MD was used to evaluate the SOx’s transportation through GLUT1.
Chapter 1

CATALYTICAL COVALENT INHIBITOR, A NEW PARADIGM FOR INHIBITOR DESIGN

1.1 Introduction

1.1.1 Acetylation in nature

Acetylation is one of many naturally occurring post translational modifications (PTMs). By acetylating protein, the cell can modify the activity and the function of certain proteins; acetylation has effects on the protein level as well as on the metabolome level. Histone acetylation was first discovered, it was regulated by the levels of lysine acetyltransferase (KATs) and lysine deacetylases (KDACs). The regulation of acetylation and deacetylation controls a lot of crucial cellular processes.

A newly discovered PTMs is acetylation of serine residues, Orth et al. found that bacterial pathogens, protein YopJ and VopA, acetylate eukaryotic kinase MKK at Ser207, Lys210 and Thr211 on its catalytic loop and inhibit MKK activity. YopJ and VopA function as acetyltransferases, these two enzymes first load acetyl group from Ac-CoA and transfer the acetyl group to target kinase. By inhibiting MAPK signaling pathways, YopJ and VopA cripple the immune response system. Like lysine acetylation, serine and threonine acetylation can be reversed by a deacetylase.

1.1.2 Artificial acyl transfer

Hamachi et al. developed an affinity-guided 4-Dimethylaminopyridine (DMAP) acyl transfer system. They used DMAP as an acyl transfer catalyst to
transfer exogenous acyl group to a specific protein. With DMAP linked to a protein binding ligand, an acyl group was transferred to DMAP. DMAP then transferred acyl group to nucleophiles (lysines) on the protein’s surface by proximity; an acyl group with fluorescent label was hence covalently linked to target protein as a probe (Figure 1.1).

Figure 1.1. Affinity-guide-catalyst-mediated acyl transfer.

Kanai et al. developed another system using DMAP with ortho methyl thiol (DSH) to transfer acetyl group from endogenous Ac-CoA to lysines on target protein (histone). In their design, the DMAP catalyzed the acetyl transfer from Ac-CoA to the thiol group (Figure 1.2). The binding domain (LANA) would bind to a specific region of the histone, a thioester would then transfer endogenous acetyl group to histone’s lysine.
Apella et al. discovered a novel thiol phenol compound, MT-1 that can be acetylated in cells and then transfer the acetyl group to the gag protein. They suggested that their compound operated catalytically in cells through thioester exchange between Ac-CoA and MT-1 followed by transfer acetyl group to the target protein. The unacetylated MT-1 showed similar efficiency as the pre-acetylated compound SAMT-237 in cells (Figure 1.3).

In the Koh lab, Yuchen Zhang et al. developed another proximity directed acetyl transfer catalyst based on the thiol-phenol containing compound, designed to target surface lysines of the androgen receptor (AR). In this design, the compound contains two parts, an AR binding ligand and an acetyl transfer/thiol-phenol end. The
ligand binds to the surface of the protein, and thiol-phenol can catalyze the acetyl transfer from Ac-CoA to the protein’s surface lysines. By controlling the length of the compound, lysines were selectively acetylated based on the proximity to thioester and lysines’ reactivity (Figure 1.4).

![Diagram](image)

Figure 1.4. Proximity directed catalytic acetylation of androgen receptor.

1.1.3 Utilizing small molecule to catalyze acetyl transfer from Ac-CoA to a specific enzyme

Mammalian cells contain large pools of Ac-CoA. The concentration of Ac-CoA is different in different cell compartments and is tissue specific. The cytoplasmic concentration has been calculated to be ~100 µM, and in mitochondria it can be ~20-30 fold higher. This high concentration of Ac-CoA provides high level of reactive acetate that is sufficient for small molecules to be spontaneously acetylated without the need of an acetyl transfer enzyme. The fast reaction rate of thiol-thioester exchange gives the advantage of thiol-phenol containing compounds to transfer acetyl
Fast, proximity directed S-O and S-N acetyl transfer can ensure the acetyl transfer from designed compounds to a lysine or serine of the target protein.\(^{11}\)

Targeting an enzyme using a small molecule acetyl transfer catalyst has never been done before. Incorporation of such a catalytic system to enzyme inhibitor design would provide a novel aspect. The regeneratable acyl properties of an acetyl transfer catalyst could provide sustaining effect in enzyme inhibition. Long term but low dosage of covalent inhibitor, such as aspirin, shows benefits in cardiovascular disease prevention.\(^{12}\)

However, it would need very different strategy to design a catalytic inhibitor for enzymes. Most enzymes’ active sites are buried inside a binding pocket which is selective for substrate. Simply adding an acyl transfer group to enzyme’s natural substrate or a known inhibitor would disrupt its binding affinity. The binding configuration of an inhibitor affects acyl transfer since the acyl transfer needs a specific orientation and distance between acyl transfer group and the target residue.

### 1.1.4 Catalytic acetyl transfer inhibitor for cyclooxygenase (COX)

Aspirin as a covalent inhibitor has been found to be able to reduce pain during inflammations. Aspirin transfer acetyl group to Ser530 of cyclooxygenase. The structure of aspirin shows resemblance to SAMT-237, COX is an excellent model to establish catalytic acetyl transfer inhibitor design.

COX is an important therapeutic target involved in psychological response that includes inflammation response, platelet aggregation, cardiovascular disease, and tumor development. Compounds with different inhibition kinetics could help to address selective biological functions (Figure 1.15). For example, in diabetic patients, the Ac-CoA level is elevated, and patients have a higher risk of cardiovascular disease.
Aspirin has proven to be able to reduce the risk of cardiovascular disease by inhibiting COX and hence limiting TxA2 production. However, high doses of aspirin or other COX inhibitors can cause bleeding risk. A catalytic acyl transfer COX inhibitor could take advantage of endogenous Ac-CoA and slowly produce an acyl transfer inhibitor with sustaining effect. But since it is self-regenerating, it would need a lower dosage and could potentially lower the bleeding risk.

![Diagram](image)

Figure 1.5. Three types of COX inhibition kinetics. A) Noncovalent (competitive) inhibitor. B) Covalent (irreversible) inhibitor. C) Catalytic covalent inhibitor.

### 1.1.5 Cyclooxygenase (COX) and NSAID

COX is involved in the inflammatory response. COX converts arachidonic acid to PGH2 (Figure 1.6), and PGH2 can then be converted to different prostaglandins, such as PGE2, TXA2, PGI2, PGD2 etc.13 There are two main forms of COX in human cells, COX-1 and COX-2. COX-2 is inflammation-inducible. During inflammatory response, COX-2 expression is upregulated; as a result, PGE2 and some other
prostaglandins levels are also elevated. Elevated level of PGE2 in neurons will cause fever responses and other aspects of sickness syndrome. COX-2 selective inhibitors are often used for treatment of those conditions. In platelets, besides constitutively expressed COX-1, elevated levels of COX-2 will produce more TXA2 and induce platelet aggregation and thrombus formation. Aspirin, a nonselective NSAID with sustained inhibition of platelet aggregation, is currently used for cardiovascular disease prevention with long-term/low-dosage dosage intake.
The role of cyclooxygenase in prostaglandin synthesis.\textsuperscript{13}

Crystal structures of COX have been solved with bound inhibitors. COX-1 and COX-2 shares very similar active site. COX is a homodimer and is associated with the endoplasmic reticulum.\textsuperscript{17} Tyr385 of COX forms a tyrosyl radical by HEME when oxidant exists. The tyrosyl radical oxidizes arachidonic acid to a carbon-centered radical, it is then converted to PGG2 by oxidant.\textsuperscript{18} Acetylation of Ser530 of COX will block substrate binding and any further reaction with Tyr385 (Figure 1.7).

Figure 1.7. A) Homodimer form of COX-1. B) Active site of COX-1

1.1.6 Ac-CoA, COX related to malignancies

Tumor cells often increase Ac-CoA concentrations as a result of rapid cell proliferation.\textsuperscript{19} This is due to increased glycolysis and fatty acid metabolism in many cancer cells. Inhibition of Ac-CoA synthesis has been shown to inhibit tumor growth.\textsuperscript{20}
Additionally, chronic inflammation has been discovered to be related to cancer and cell proliferation.\textsuperscript{21} COX-2 was found to promote and modulate cancer cell proliferation while NSAIDs were found to effectively reduce tumor growth.\textsuperscript{22}
1.2 Results and Discussion

1.2.1 The scheme of COX catalytic covalent inhibitor design

This new type of inhibitors should contain following properties (Figure 1.8): first, they can be acetylated by endogenous Ac-CoA efficiently; second, the acetylated inhibitors should have high binding affinity to COX, the binding affinity decides the bound inhibitor-enzyme ratio; third, acetylated inhibitors need to efficiently transit from binding state to acetyl transfer state, this may need to overcome some binding energy as they need to move close enough to target residue and also the overcome the energy barrier of transition state of chemical reaction.

![Diagram of COX catalytic inhibitor](image)

Figure 1.8. COX catalytic inhibitor.

1.2.2 AcWB1, the first COX catalytic inhibitor

From previous research, we know that a thiophenol ortho to an amide is a better nucleophile for acetyl transfer from Ac-CoA comparing to a thiol-phenol without electron withdrawing amide. This is because that an ortho amide group can lower the pKa of thiol phenol. The nature substrate of COX, arachidonic acid, has a
long hydrophobic alkyl chain. This is important for any compound to bind into COX active site. To demonstrate the concept of catalytic inhibitor, pre-acetylated compound AcWB1/free thiol WB1/methylated MeWB1 were synthesized and tested.

![AcWB1, WB1, MeWB1](image)

Figure 1.9. WB1 was synthesized to demonstrate acetyl transfer from Ac-CoA to COX.

1.2.3 AcWB1 can inhibit ovine COX-1, *in vitro*

WB1 was designed to be a catalytic covalent inhibitor; the acetylated form is referred as AcWB1. AcWB1’s ability to inhibit ovine COX-1 was first assayed as it is the product of WB1’s acetylation. Ovine COX-1 was incubated with different concentrations of AcWB1 at 37 °C for 1 hr, and excess arachidonic acid was added and incubated for another 20 min. The reactions were terminated by adding 1 N HCl to make the reaction solution’s pH <2. PGE2, the COX-1’s enzymatic product, was assayed using LC-MS/MS quantification with deuterated labeled PGE2 as internal standard.

PGE2 concentration was used as assessment of COX-1 activity (Figure1.10). AcWB1 was indeed an inhibitor for COX-1 with IC50 at 50.3 µM.
The commonly used value IC50 to measure the potency of a covalent inhibitor might not be very accurate and not entirely appropriate, because the covalent modification of the enzyme active site happens during and after non-covalent binding equilibration. The length of incubation would change the apparent IC50 significantly. This change in apparent IC50 can be used to indicate a covalent modifying mechanism.

A more accurate evaluation of covalent inhibition is to calculate both non-covalent binding concentration and covalent modification rate.

$$E + I \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} [EI] \overset{k_{\text{inact}}}{\longrightarrow} EI^*$$

Figure 1.11. Parameters of covalent enzyme inhibition.
The $k_1/k_{-1}$ ratio, which represents $K_i$, is a measurement of non-covalent inhibition while $k_{\text{inact}}$ is a component of overall the covalent modification rate. The $K_i$ and $k_{\text{inact}}$ values can both be calculated from time and dose dependent enzyme inhibition assay. Ovine COX-1 was incubated with different concentrations of AcWB1 at different time points (Figure 1.12). The observed rate constant of inhibition, $k_{\text{obs}}$ was determined from the slope of a semi-logarithmic plot of inhibition versus time (10 min, 20 min, 30 min, 40 min) at different inhibitor concentrations. Then $k_{\text{obs}}$ values were re-plotted against inhibitor concentrations and fitted to a hyperbolic equation, $k_{\text{obs}}=k_{\text{inact}}[I]/(K_i+[I])$. $K_i$ and $k_{\text{inact}}$ values were obtained from the fitted equation.23

![AcWB1](image)

**Figure 1.12. AcWB1’s kinetics of ovine COX-1 inhibition.**

From this analysis, the $K_i$ is calculated to be $33.9\pm2.6 \, \mu M$ and $k_{\text{inact}}$ is $2.1\pm0.3 \times 10^{-2} \, \text{min}^{-1}$ for AcWB1, as Aspirin’s $K_i$ is $27.9\pm5.1 \, \mu M$ and $k_{\text{inact}}$ is $0.74\pm0.13 \times 10^{-2} \, \text{min}^{-1}$. 
Even though AcWB1 has higher $K_i$ value (which means the non-covalent binding affinity is lower than aspirin), the $k_{inact}$ of AcWB1 is 3-fold faster than aspirin. This means thioester tested can be transferred faster than aspirin’s oxygen-ester. In principle, this thiol-phenol ester with an ortho-amide can better stabilize the leaving group, and should be more reactive than aspirin. This is an agreement with the experimental data.

With these data, it is a viable design to make AcWB series compounds with similar structures to AcWB1 and make them better covalent inhibitors than aspirin. To increase potency, one strategy is to increase the non-covalent binding affinity. The alkyl chain of AcWB1 is obviously the easiest part of molecule to be modified. Another strategy is to lower the energy barrier for the inhibitor transiting from binding
state to acetyl transfer state (increase $k_{\text{inact}}$). This is to lower the work for AcWB’s thiol ester compounds moving from binding site to Ser530.

### 1.2.4 WB1 can be acetylated by Ac-CoA to form AcWB1

![Figure 1.14. WB1 and MeWB1](image)

AcWB1 has been shown to work as an ovine COX-1 covalent inhibitor and has the advantage of being a better acetyl transfer agent since it has a better leaving group than aspirin. However, the goal of the project is to make a catalytic covalent inhibitor. So, not only does AcWB1 need to be a good covalent inhibitor, but WB1 must also be capable of getting acetyl group from Ac-CoA efficiently.

Thiol-phenol is known to be more nucleophilic than O-phenol; with ortho amide to lower its pKa, it could be even more efficient acetylated by Ac-CoA. Therefore, an assay was designed to quantify the rate of the inhibitor’s acetylation by Ac-CoA. This assay uses HPLC-MS to calculate the rate of acetyl transfer from an Ac-CoA mimic (N, S-Diacetylcysteamine, AcSNAc).
The calculated $k_1$ is $6 \times 10^{-2} \text{M}^{-1} \cdot \text{min}^{-1}$, the calculated $k_2$ is $1 \times 10^{-6} \text{M}^{-1} \cdot \text{min}^{-1}$ under physiological condition. WB1 is efficiently acetylated by AcSNAc, a frequently used Ac-CoA mimic. Compared to salicylic acid, the WB1 acetylation is ~60,000 times faster.

### 1.2.5 WB1, incubated with AcSNAc, can inhibit COX, in vitro and in cells

To demonstrate the concept of catalytic inhibition, WB1’s acetylation effect was tested. Generally, COX-1 was incubated with WB1 with or without AcSNAc at 37 °C for 3 h in a pH 7.4 Tris buffer (Figure1.14) with TCEP (50 µM). Then, excess arachidonic acid and excess hemin were added to reaction mixture. The working concentrations of arachidonic acid and hemin were 100 µM and 1mM respectively. The reaction was incubated at 37 °C for 10 min and terminated by adjusting to pH <1. The d4-PGE2 was added and mixed thoroughly. The reaction was extracted with EtOAc/Hexane (1:1 v/v) and re-dissolved in water/methanol (1:1 v/v). PGE2 concentrations were quantified by LC-MS/MS. COX-1 activities were normalized based on PGE2 concentration.
From this assay, it is clear that in vitro WB1 was acetylated by AcSNAc and WB1/AcSNAc was inhibiting COX-1 activity. Note that the observed inhibition is a combination of non-covalent binding and covalent modification. TCEP was added to keep the thiol group reduced without removing acetyl group from thioester. Excessive hemin was added to avoid TCEP’s interference of COX-1 redox activity.

A cell based assay was performed to see if this catalytic inhibitor design is applicable to the cellular environment. Human cell line A549 was cultured and stimulated by LPS; then compounds were added and incubated at 37 °C for 12 hrs. PGE$_2$ in the media; which is the product of COX, was measured by LC-MS/MS to evaluate the activity of cellular COX activity as the in vitro assay.
Combining the in vitro and in cell based assay results, WB1 can be acetylated by Ac-CoA and form AcWB1; it then inhibits COX activity in vitro and in cells.

1.2.6 AcWB1 acetylates ovine COX-1 at Ser530 as found with aspirin

With careful analysis of the active site of COX, there is no obvious acetylation site for aspirin or AcWB1 other than Ser530. However, another confirmatory assay was developed to demonstrate that AcWB1 is indeed acetylating Ser530.

Ovine COX-1 was incubated with 40 µM of AcWB1 for 6 hrs at 37°C. Then the enzyme was digested by trypsin and Glu-C protease as described in elsewhere.26 In the active site, the digested peptide MGAPF (Ser530) LK was identified. The Ser530 was found to be acetylated by AcWB1 as well as aspirin.
Figure 1.18. LC-MS/MS analysis of the digested Ser530 peptide of COX-1 incubated with AcWB (A)/Aspirin (B).
1.2.7 Sustained effect of AcWB1 vs. Aspirin

One possible benefit of a catalytic COX inhibitor is that it would continuously acetylate the target enzyme by reloading acetyl group from Ac-CoA. The hypothesized result of this property is that AcWB1 should have sustained effect of inhibition compared to aspirin. To test this sustaining effect, AcWB1 and aspirin were added to A549 cells and the activity of COX was tested over a period of time.

![A549 Cell activities](image)

Figure 1.19. Time dependent COX activity with aspirin and AcWB1 in A549 cell line.

AcWB1 shows longer inhibition compared to aspirin. Both at 40 µM, AcWB1 can keep COX activity at a lower level after 36 hrs. AcWB1 showed a sustaining inhibition effect due to its reacetylation.

Aspirin, as a covalent inhibitor, shows a relative long term inhibitory effect compared to noncovalent inhibitors. Aspirin’s inhibition remains even after aspirin itself is consumed or degraded\(^\text{12}\). Due to this advantage over noncovalent inhibition,
aspirin is commonly used for anti-platelet treatment which helps prevent cardiovascular disease.\textsuperscript{12, 15, 27} Analogues of AcWB1 that have longer sustaining effect than aspirin may also be used in similar treatment.

1.2.8 Optimization of AcWB compounds based on computational design

AcWB1 was shown to work as a COX catalytic covalent inhibitor. The $K_i$ of AcWB1 is $33.9 \pm 2.6 \mu \text{M}$, and aspirin is $27.9 \pm 5.1 \mu \text{M}$. To make AcWB1 more potent, the strategy comes in two parts. First, the analogue should be a more potent binder. This is because the inhibitor must first form an inhibitor-enzyme complex before transferring the acetyl group to the active site residue Ser530. Higher binding affinity means lower $K_i$. Second, the highest binding affinity configuration for non-covalent inhibition is not necessarily the same as that configuration needed for acetyl transfer to occur. The hydroxyl group of Ser530 has to be close enough to thiol ester carbonyl to react. Binding too tightly in a non-reactive conformation may increase non-covalent inhibition but can be detrimental to covalent inhibition.

Using molecular dynamics simulation, we can estimate the non-covalent binding energy to the active site with MM-PBSA/GBSA. As all compounds we proposed contain a similar thioester as the acetyl transfer group, the $\Delta G$ differences of O-S ester transfer reaction are negligible. The free energy differences between thioester analogs can be estimated by calculating the work needed for thioester to move within reactive distance to the hydroxyl group of COX. Using steered molecular dynamics simulation, we calculated the Potential Mean Force (PMF) for inhibitors to move from their highest binding affinity energy conformation in the active site to a conformation compatible with acetyl transfer.
Marnett et al. made a series of aspirin analogues that showed much improved IC50 values, we here used similar designs elements and proposed additional structures for virtual analysis (Figure 1.21). The candidates were optimized by DFT/b3lyp with 6-311G basis. RESP charges were assigned before subjecting to Amber14 for MD simulation.

Figure 1.20. Proposed AcWB compounds for MD simulation.
As shown in Figure 1.21, compounds were plotted based on two calculation results, binding enthalpy ($\Delta H$), from free solution to the active site and PMF, from the binding state in the active site to a reactive confirmation. $\Delta H$ was directly correlated to binding affinity which can be used to estimate $K_i$. PMF in this simulation is correlated to the work needed of inhibitions to move from their bound configuration to a configuration capable of acetyl transfer. This can be used to compare the differences in the apparent acetyl transfer energy barrier between AcWB analogs. Top candidates should have a low PMF and more negative $\Delta H$. AcWB2, AcWB3, AcWB5 were chosen for further experimental analysis.
1.2.9 Time-dependent IC50 of AcWB compounds

For aspirin, the rate limiting step of ovine COX-1 inhibition is acetylation of Ser530. However, S-O ester transfer is theoretically faster than O-O ester transfer. Without assuming the rate limiting step is the acetyl transfer step for our thioester series, time-dependent IC50 assays were performed to identify the efficiency of AcWB compounds.

IC50s of each compound were assessed at 1 hr, 3hrs and 5hrs incubation times at 37 °C. The results are shown below in figure 1.22:
AcWB1

AcWB3

Relative activity (%) vs. Log (μM) for AcWB1 and AcWB3 at different time points (1h, 3h, 5h).
The results of our time dependent inhibition assays are compiled in Table 1.1. We calculated COX-1 activity drop from 1 hr to 5 hrs at different concentrations. Picking the concentration of every compounds at IC50@1 hr, we can directly compare every compounds time dependent activity drop at similar inhibitor-enzyme occupancy.

Table 1.1 IC50 at 1h and activity drop from Time dependent IC50 results

<table>
<thead>
<tr>
<th></th>
<th>Aspirin</th>
<th>AcWB1</th>
<th>AcWB3</th>
<th>AcWB5</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 1h (µM)</td>
<td>92.8±33.6</td>
<td>52.6±15.3</td>
<td>7.2±4.8</td>
<td>54.5±20.2</td>
</tr>
<tr>
<td>Activity Drop (%, 1 h-&gt;5 h) at IC50 (1 h)</td>
<td>34.0</td>
<td>33.2</td>
<td>35.6</td>
<td>36.8</td>
</tr>
<tr>
<td>ΔH (Kcal/mol)</td>
<td>-21.3±2.1</td>
<td>-29.7±2.5</td>
<td>-32.8±2.3</td>
<td>-29.3±1.84</td>
</tr>
<tr>
<td>PMF (Kcal/mol)</td>
<td>10.8±0.1</td>
<td>31.3±0.2</td>
<td>27.8±0.2</td>
<td>13.9±0.1</td>
</tr>
</tbody>
</table>

Figure 1.22. Time dependent IC50 of Aspirin, AcWB1, AcWB3, AcWB5.
The trends observed from the time-dependent IC50 results agree with computational results. AcWB3 has the high binding affinity (enthalpy) and should be the best non-covalent COX-1 inhibitor. From the observed drop in activities over 5h, one can conclude that similar to aspirin, the rate limiting step for the AcWB inhibitors is also the acetylation step.

At 1h, AcWB2 had an IC50 1.86 ± 0.43 µM, however, AcWB2 started to precipitate at any concentration above 25 µM. This is likely due to the lack of amide or other additional hydrogen bonding moiety. The electron withdrawing effect of the amide group conjugated to the free thiophenol of WB1, WB3, and WB5 lowers the pKa’s of their thiol groups which increases the percentage of deprotonated thiol. although AcWB2 showed the best IC50 at 1 hr, AcWB2 reactivity and solubility precluded its further study.

![Graph](image)

Figure 1.23. IC50 of AcWB2 inhibition of ovine COX-1 at 1 hr, 37 °C incubations.
1.2.10 **Kinetics of AcWB compounds’ COX acetylation**

AcWB1, AcWB3 and AcWB5 were selected for further COX inhibition kinetics analysis. Inhibitors and ovine COX-1 were incubated at 37°C for different times and concentrations. Kinetics of covalent inhibition were analyzed by fitting hyperbolic equations for $k_{obs}$ previously described for AcWb1.23
Figure 1.24. Kinetics plot of covalent inactivation of COX-1.
Table 1.2 COX inhibition kinetics vs. computational prediction

<table>
<thead>
<tr>
<th></th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (µM)</th>
<th>k&lt;sub&gt;inact&lt;/sub&gt; (10&lt;sup&gt;-2&lt;/sup&gt; • min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;inact&lt;/sub&gt;/K&lt;sub&gt;i&lt;/sub&gt; (10&lt;sup&gt;-3&lt;/sup&gt; • min&lt;sup&gt;-1&lt;/sup&gt;•µM&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Binding ΔH (Kcal/mol)</th>
<th>PMF (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>27.9±5.1</td>
<td>0.74±0.13</td>
<td>0.27±0.11</td>
<td>-21.3±2.11</td>
<td>10.8±0.1</td>
</tr>
<tr>
<td>AcWB1</td>
<td>33.9±2.6</td>
<td>2.1±0.3</td>
<td>0.62±0.03</td>
<td>-29.7±2.53</td>
<td>31.3±0.2</td>
</tr>
<tr>
<td>AcWB3</td>
<td>8.9±0.8</td>
<td>2.2±0.4</td>
<td>2.5±0.3</td>
<td>-32.8±2.29</td>
<td>27.8±0.2</td>
</tr>
<tr>
<td>AcWB5</td>
<td>15.3±1.3</td>
<td>2.5±0.3</td>
<td>1.66±0.6</td>
<td>-29.3±1.84</td>
<td>13.9±0.1</td>
</tr>
</tbody>
</table>

The k<sub>inact</sub> and K<sub>i</sub> were determined from hyperbolic fitting (Figure 1.24). The k<sub>inact</sub>/K<sub>i</sub> evaluates the time-dependent inactivation kinetics of COX’s inhibition by covalent inhibitors (Table 1.2).<sup>29</sup> The experimental results are consistent with computational results. PMF results accurately reflect the observed trend for the rates of inactivation noting that aspirin is an oxygen ester whereas the rest are thioesters. Binding enthalpies, which only approximate binding free energies, correctly predicted the tightest binder AcWB3 but had the rank order of WB1 and WB5 similar but reversed. To make more accurate computational prediction, entropy calculations were needed to calculate binding free energies. Current normal mode analysis of entropy has its limitations and is very time consuming.<sup>30</sup> Enthalpy calculation using MM-PBSA in this project is accurate enough and time-efficient, ΔH instead of ΔG was used as an evaluation of computational binding affinity.

AcWB3 shows the best overall parameters for covalent inactivation. The mode of covalent and non-covalent inactivation of AcWB3 and the ability for WB3 to act as catalytic covalent inhibitor were further investigated.
1.2.11 AcWB3 also acetylates ovine COX-1 at Ser530

![LC-MS/MS spectrum of AcWB3 acetylated ovine COX-1 at Ser530](image)

Figure 1.25. LC-MS/MS of digested ovine COX-1 acetylated at Ser530 by AcWB3.

To better understand how AcWB3 inactivates COX, ovine COX-1 was incubated with AcWB3 as well as Aspirin and AcWB1. The inhibited protein product was digested by trypsin and Glu-c, analyzed using Orbi-trap LC-MS/MS. The peptide containing Ser530 was found to be acetylated at Ser530 in AcWB3 treated samples (Figure 1.25).

1.2.12 Critical micelle concentration (CMC) of AcWB3

Aggregation and micelle formation of compounds is known to cause nonspecific enzyme inhibition. The CMC was determined using the fluorescent dye N-Phenyl-1-naphthylamine (NPN). Briefly, NPN fluorescence was determined in assay buffer (0.1 M Tris-HCl, pH 7.5) in the presence of different concentrations of compounds. Micelle formation is determined as a sharp increase in fluorescence with
increasing concentration of compound. The CMC is determined as the sharp turning point in fluorescence.

The SDS CMC concentration was determined to be 8mM, which agrees with literature. AcWB3’s CMC was not detected and was determined to be greater than 256 µM (Figure 1.26), therefore the observed inhibition of COX-1 by AcWB3 is not due to compounds aggregation.

Figure 1.26. Critical micelle concentration of A) AcWB3 B) SDS
1.2.13 Covalent inhibition analysis by dialysis

To further demonstrate AcWB3 is acts through irreversible covalent inhibition, dialysis was used to recover enzyme activity from reversibly inhibited (noncovalently bound) COX. Ovine COX-1 was incubated with 200 µM AcWB3, 200 µM MeWB3 or 200 µM aspirin at 37°C for 6 hrs. The high concentration of inhibitor ensured similar active site occupancy by the three compounds. The samples were then dialyzed for 8h to reactivate COX by removing non-covalently bound inhibitors (Figure 1.27).

![Dialysis scheme for COX covalent inhibition analysis](image)

- AcWB series compounds
- WB series compounds (de-acetylated)
- Active Enzyme
- Inactive Enzyme (acetylated)

Figure 1.27. Dialysis scheme for COX covalent inhibition analysis.
Like aspirin, AcWB3 permanently inhibited COX (Figure 1.28). COX incubated MeWB3 which has an inactive methylsulfide in place of the thioacetate group, recovered 83% activity after dialysis. Together this shows that AcWB3, like aspirin, is a covalent inhibitor of COX, and that the thioester is the functional group required for covalent inhibition of COX.

1.2.14 WB3 incubated with AcSNAc catalytically inhibits COX via covalent modification

It is proposed that the free thiol WB3 can act in a catalytic manner to transfer acetyl groups from Acetyl-CoA (AcSNAc) to covalently inactivate COX. Using the post-incubation dialysis assay protocol (Figure 1.27), we can detect the level of
covalent modification of ovine COX-1 without interference from non-covalent inhibition by WB3 or AcWB3.

Figure 1.29. WB3’s catalytic inhibition of ovine COX-1. In this assay, WB3 is 10µM, AcSNAc is 200µM. The reaction was incubated at 37°C for 16h before another 16h dialysis.

In presence of the Acetyl-CoA mimic (AcSNAc) as acetyl source, WB3 was acetylated to from AcWB3 in situ which in turn can covalently inhibit COX. WB3 or AcSNAc could not covalently inhibit COX (Figure 1.29).

1.2.15 WB3 catalytic inhibits COX in A549 cells

The ultimate goal of this project is to develop a catalytic compound that can perform multiple turnover acetylation in cells without an exogenous acetyl source. Acetyl-CoA, is sufficiently chemically reactive and of sufficient concentration for
WB3’s to achieve a practical acetyl exchange rate and function as an inset acyl transfer catalyst.

To demonstrate that unacetylated WB3 can act as catalytic covalent inhibitor, WB3 was added to adenocarcinomic human alveolar basal epithelial cells (A549), without addition of an exogenous acetyl source, COX (COX-1/2) activity was assayed over time. A549 cells were cultured in 6 well plates, when they achieved 80% confluence, bacterial Lipopolysaccharide (LPS) was added to give final 1µg/ml concentration in media. COX levels within LPS pretreated cells remain stable for an extended period of time. WB3 (15 µM), aspirin (15 µM) or vehicle were added to LPS-pretreated cells. PGE2 concentrations were assayed every 3 hrs for 24 hrs. COX activities were normalized to the activity of the vehicle control at each time point.

Cells treated with WB3 and aspirin initially showed similar ~60% COX activities at 3 hrs. Over time, aspirin became less as it is consumed through acetylation of COX, thorough hydrolysis, or loss of its acetyl group by reaction with cellular nucleophiles. WB3, which can be acetylated in cells, shows a sustained and additive COX inhibitory effect after 12 hrs which continues to increase in efficacy through the 24 hrs experiment (Figure 1.30).
Figure 1.30. WB3 and aspirin time dependent COX inhibition assay. WB3 and aspirin supplied at 15 µM at time 0 hr. COX activity was detected every 3 hrs. A549 cells were plated in 12 well plate, every detection was an end point assay with no reuse of cells. All value of activities at certain time point were normalized by blank (vehicle) value.

To ensure that the observed decrease in COX activity was not the result of WB3’s cell toxicity, treated cells were separately assayed by MTT assay for cell proliferation. A549 cells were incubated with WB3/AcWB3 and aspirin for 24 hrs (Figure 1.31).
Cell proliferation under aspirin and WB3/AcWB3 were very similar (Figure 1.31), there is no significant cellular toxicity differences between the two treatment methods. These results support the notion that the observed activity differences from cellular COX activity assay (Figure 1.30) are directly due to COX inhibition.
1.3 Experimental

1.3.1 General in vitro COX inhibition assay procedure

The procedure was adapted from a published article and optimized as follows: in 0.1 M pH 7.5 Tris buffer, Ovine COX-1 enzyme was added to make an enzyme concentration of ~5 units/188 μL. The enzyme solution, 188 μL, was added to each Eppendorf tube, to which was added 2 μL of 100 μM hematin at room temperature. 10 μL of 20x compound stock solution or vehicle was added and the solution fully mixed by pipetting. The reactions tubes were then incubated at 37 °C for the specified time.

The COX activity was determined discontinuously by measuring its reaction with arachidonic acid. Reactions were initiated by adding 10 μL of 200 μM arachidonic acid and incubated for 10 min. The conversion was then terminated by adding 10 μL of 1N HCl plus 10 μl of 50 μg/L d4-PGE2 which was added as an internal standard.

To each assay tube was added 800 μL hexane/ethyl acetate (1:1, v/v) and the mixture vortexed to extract PGE2 (and d4-PGE2). The organic layer was transferred to another tube and dried under vacuum before being reconstituted in 200 μL methanol/water (1:1, v/v) which was then used for LC-MS/MS analysis.

LC-MS/MS analysis was carried following article protocol and optimized as follows: all the samples were subjected to Thermo Q-Exactive Orbitrap with autosampler. Separation of PGE2 was carried out using a ACE Excel C18 column (30 x 2.1 mm) with a 10min linear gradient from 20-80% acetonitrile in water. PGE2 and d4-PGE2 were detected in negative ion mode. For MS-MS analysis, PGE2/d4-PGE2 were subjected to HCD (higher energy collisional dissociation) induced fragmentation.
For quantification, 351/271 mass transition was used for PGE2, 355/275 was used for internal standard d4-PGE2. d4-PGE2 was purchased from Cayman Chemicals.

A standard curve of PGE2 versus PGE2/d4-PGE2 ratio was determined (Figure 1.32). PGE2 concentrations from each assay were then calculated from the first order equation. IC50s were plotted inhibitor concentration versus PGE2 concentration using GraphPad Prism, with nonlinear regression least squares method fitting to dose-response sigmoid equation.

![Figure 1.32. PGE2 standard curve for quantification. PGE2/d4-PGE2 ratio was determined with MS/MS fragments from HCD.](image)

1.3.2 General procedure for cellular COX activity assay

COX activity was determined in the human lung carcinoma cells, A549 (ATCC) as follows. A549 cells were cultured with DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin plus streptomycin. Cells were incubated under 5% CO₂ at 37 °C. Cells were seeded into 6-well plates and grown to approximately 80% confluency at which point, lipopolysaccharide (LPS)
were added to a final concentration of 10 µg/mL. After 12 hrs incubation with LPS, inhibitors or vehicles were then added to and the cells incubated at 37 °C for specific time.

After incubation with inhibitors, the media was removed and the cells were treated with 10 µM of arachidonic acid in PBS buffer (pH 7.4) and incubated at 37 °C for 20 min. The media was then transferred into tubes for PGE2 concentration determination following the same procedure as in vitro assay.

1.3.3 Molecular dynamics simulation of COX-AcWB complexes

The ovine COX-1 crystal structure (PDBID:1EQG) was used to construct an in-silico protein model. Solvent and small molecules were removed and only protein dimer was retained. Protonation of protein residues were assigned using H++ server. Force field of small molecules were constructed as following: RESP charges and configuration optimization were calculated by Gaussian09 using DFT/6-31(G)d basis set. The topology parameters of small molecules were generated with general Amber force field (GAFF) using ANTECHAMBER. Small molecules were docked into the binding site using Autodock vina. Protein topology parameters were generated using Amber 14SB force field. The protein-ligand system was then solvated with the TIP3PBOX water model in a cubic periodic unit cell. Each side of the unit cell is at least 10 Å from the nearest solute atom. The system the was neutralized by adding sodium cations. The Amber14 package was used to perform all MD simulations. The system was first minimized by 50000 steps of steepest descent minimization followed by another 50000 steps conjugated gradient minimization. The system was then heated from 0K to 300 K over 500 ps with NVT ensemble, with a 10 Kcal•mol⁻¹•Å⁻² restraint on the protein-ligand complex followed by an unconstrained 5
ns equilibrium MD of the NPT ensemble. SHAKE algorithm was employed to constrain bonds containing hydrogen atoms. 2 fs steps were used for all MD simulations. The Particle Mesh Ewald method was used to calculate electrostatic interactions with a nonbonded cutoff distance of 10 Å. The complexes were then run for another 50-ns productive MD with the NPT ensemble at 300 K. The last 1000 (1ps per snapshot) snapshots of MD trajectories was extracted and analyzed using MMPBSA.py to calculate binding enthalpy.39

1.3.4 Using Umbrella sampling to calculate PMF of binding configuration to acetyl transfer configuration

Umbrella harmonic potential with a force constant of 1.0 Kcal/mol·Å² was applied at center of mass of AcWB compounds’ aromatic ring and center of mass Ser530 of COX. 10 Kcal/mol·Å² restraint was applied on the backbone of residue Ser530. The 6 Å (binding state to acetyl transfer state) reaction coordinate was split into 6 windows with 1 Å interval. Each window was simulated independently for 5 ns. All windows are combined with Grossfield WHAM (version 2.0.9) to construct PMF. 500 Monte Carlo data sets were generated for each sampling point of the sampling coordinate to perform bootstrapping error analysis.40 Histograms of Frequency vs. Distance were checked to make sure the umbrella overlapping, if not, more simulation at a particular distance would be added to ensure sufficient sampling (Figure 1.33).
1.3.5 Hept-2-yn-1-yl 4-methylbenzenesulfonate synthesis (1)

To a solution of hept-2-yn-1-ol (10.00 g, 88.73 mmol, 1 eq) in ethyl ether (500 mL) on ice bath, Ts-Cl (2 eq) was added. Kept stirring on ice, potassium hydroxide powder (3 eq) was added portion wise. The reaction mixture was stirred for 3 hrs, then organic layer was washed with ice water (3 x 500 mL). The combined organic layer was dried over MgSO₄, concentrated by rotary evaporation, and dried by high vacuum to give product as clear oil. ¹H NMR (600 MHz, CDCl₃) δ 7.82 (d, J = 8.3 Hz, 2H),
7.34 (d, J = 8.3, 2H), 4.70 (t, J = 2.2 Hz, 3H), 2.45 (s, 3H), 2.14 – 1.99 (m, 2H), 1.39 – 1.33 (m, 2H), 1.33 – 1.27 (m, 2H), 0.87 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 144.94, 133.63, 129.85, 128.26, 90.71, 71.94, 58.95, 30.26, 21.97, 21.79, 18.49, 13.65.

1.3.6 Hept-2-yn-1-amine synthesis (2)

To a solution of 1 (2.00 g, 7.51 mmol, 1 eq) in DMF (50 mL), NaN$_3$ (1.2 eq) was added. The reaction mixture was stirred at 40 °C. After 5 hrs, reaction mixture was cooled to RT. DMF was removed by high vacuum rotary evaporation. The residue was resuspended in water (300 mL). The suspension was extracted by ethyl acetate (3 x 300 mL). The combined organic layer was then dried over MgSO$_4$ and concentrated with rotary evaporation. The residue was re-dissolved in THF (50 mL). Triphenyl phosphine (2 eq) was added followed by 5 mL H$_2$O, the reaction mixture was stirred for another 5 hrs. Solvent was then removed by rotary evaporation. Then the residue was washed with hexane (3 x 100 mL) and dried by high vacuum to give crude product as yellow oil. It was used without further purification. $^1$H NMR and $^{13}$C NMR were consistent with report in the literature.  

\[
\begin{align*}
\text{H}_2\text{N} & \equiv \\
\end{align*}
\]
1.3.7 Scheme for synthesis of AcWB compounds

2,2'-disulfanediylbis (N-hexylbenzamide) (3) synthesis:

To a solution of 2,2'-dithiobenzoic acid (5.00 g, 16.3 mmol, 1.00 eq) in DCM (100 mL), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (7.50 g, 39.12 mmol, 2.40 eq) was added, stirred under RT for 20 min. Then DMAP (398 mg,
3.26 mmol, 0.20 eq) and hexan-1-amine (3.63 g, 35.9 mmol, 2.20 eq) were added to reaction mixture at RT. The reaction mixture was then stirred at RT for 12 hrs. 1N HCl (100 mL) was then added to reaction mixture. The combined mixture solution was then extracted with ethyl acetate (3 x 100 mL). The combined organic layer was then washed with saturated NaHCO₃ (100 mL) and brine (100 mL). Then organic mixture was then dried over magnesium sulfate and filtered. The organic mixture was then concentrated by rotary evaporation and purified by normal phase silica chromatography (50% Hexane/EtOAc) to give 3 as yellow solid. \(^1\)H NMR (600 MHz, CDCl₃) δ 7.74 (d, J = 8.1 Hz, 2H), 7.46 (d, J = 7.6 Hz, 2H), 7.34 (t, J = 7.7 Hz, 2H), 7.22 (t, J = 7.5 Hz, 2H), 6.11 (s, J = 6.0 Hz, 2H), 3.52 – 3.27 (m, 4H), 1.65 – 1.58 (m, 4H), 1.44 – 1.36 (m, 4H), 1.36 – 1.29 (m, 8H), 0.94 – 0.85 (m, 6H). \(^1^3\)C NMR (151 MHz, CDCl₃) δ 167.95, 136.85, 135.24, 131.23, 128.40, 127.61, 126.73, 40.40, 31.71, 29.74, 26.90, 22.79, 14.24.

![Chemical Structure](image)

**2,2'-disulfanediylbis(N-(hept-2-yn-1-yl) benzamide) (4):**

Prepared via procedure of compound 3 synthesis. Synthesized using hept-2-yn-1-amine (3.99 g, 35.9 mmol, 2.20 eq), purified by flash column chromatography using hexane/ethyl acetate (50:50) to give 4 as white solid. \(^1\)H NMR (600 MHz, CDCl₃) δ 8.03 (d, J = 7.8, 1.1 Hz, 2H), 7.61 (t, J = 8.2, 7.0, 1.2 Hz, 2H), 7.56 (d, J = 8.1 Hz, 2H), 7.42 – 7.37 (m, 2H), 4.66 (t, J = 2.3 Hz, 4H), 2.24 (ddd, J = 7.1, 4.7, 2.3 Hz, 4H), 1.56 – 1.48 (m, 4H), 1.47 – 1.38 (m, 4H), 0.92 (t, J = 7.3 Hz, 6H). \(^1^3\)C NMR (151 MHz,
S-(2-(hexylcarbamoyl) phenyl) ethanethioate (5, AcWB1):

Compound 3 (2.36 g, 5.00 mmol, 1.00 eq) were then dissolved in methanol and cooler in iced bath to 0 °C. NaBH₄ (0.757 g, 20.0 mmol, 4.00 eq) powder was then added into reaction mixture portion wise. The reaction mixture was further stirred in ice bath for 2 h until starting material 3 was consumed while monitored by TLC. 1 N HCl was then added to the reaction mixture until the mixture’s pH<2. Then methanol was removed by rotary evaporation. The aqueous residue was extracted with ethyl acetate (3 x 50 mL). Then combined organic extraction was then dried over MgSO₄ and concentrated by rotary evaporation, and dried in vacuo.

Then the dried crude intermediate oil was then re-dissolved in dry DCM (50 mL). DIPEA (1.95 g, 2.26 mL, 15.0 mmol) and acetyl chloride (1.18 g, 1.06 mL, 15.0 mmol) were added to the DCM solution. The reaction mixture was further stirred for 2h. The reaction mixture was then concentrated by rotary evaporation, and subject to flash-column chromatography (30% DCM/EtOAc) to yield final compound 5 as white solid. ¹H NMR (600 MHz, CDCl₃) δ 7.54 (dt, J = 6.6, 1.5 Hz, 1H), 7.49 – 7.42 (m, 3H), 5.95 (s, 1H), 3.42 – 3.34 (m, 2H), 2.43 (s, 3H), 1.57 – 1.50 (m, 2H), 1.39 – 1.27 (m, 6H), 0.93 – 0.81 (m, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 195.34, 168.27, 142.30, 136.56, 130.22, 128.25, 124.54, 39.95, 31.51, 30.25, 29.53, 26.63, 22.58, 14.04. HRMS (ESI) calculated for [M+H]⁺ 280.1366, found at 280.1371.
S-(2-(hept-2-yn-1-ylcarbamoyl) phenyl) ethanethioate (6, AcWB3):

Prepared via synthesis method of compound 5. $^1$H NMR (600 MHz, CDCl$_3$) δ 7.60 – 7.54 (m, 1H), 7.51 – 7.42 (m, 3H), 6.12 (s, 1H), 4.26 – 3.99 (m, 2H), 2.44 (s, 3H), 2.23 – 2.09 (m, 2H), 1.52 – 1.45 (m, 2H), 1.44 – 1.37 (m, 2H), 0.91 (t, J = 7.3 Hz, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 195.23, 167.81, 141.39, 136.74, 130.64, 130.31, 128.55, 125.03, 84.43, 75.02, 30.81, 30.40, 30.28, 22.06, 18.51, 13.71. HRMS (ESI) calculated for [M+H]$^+$ 290.1209, 290.1213 found.

S-(2-(hept-2-yn-1-ylthio) phenyl) ethanethioate (7, AcWB2):

To a solution of benzene-1,2-dithiol (1.00 g, 7.03 mmol, 1 eq) in nitrogen purged THF (70 mL), compound 1 (1 eq) was added in a solution of THF. Then DIPEA (2 eq) was added to the reaction mixture. The reaction mixture was stirred under N$_2$ for 3 hrs at RT. Then acetyl chloride (2 eq) was added to reaction mixture, and stirred for another 1h. Reaction mixture was then poured into 100mL ice water. The mixture was extracted with ethyl acetate (3 x100 mL). The combined organic layer was then washed with brine (100 mL), dried over MgSO$_4$ and concentrated by rotary evaporation. The crude product was then purified by normal phase silica chromatography (30% DCM/EtOAc) to give clear oil as final product, with 42% yield. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.54 – 7.50 (m, 1H), 7.49 – 7.40 (m, 2H), 7.28 – 7.22
(m, 1H), 3.65 (t, J = 2.4 Hz, 2H), 2.46 (s, 3H), 2.26 – 2.12 (m, 2H), 1.50 – 1.40 (m, 2H), 1.40 – 1.31 (m, 2H), 0.89 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 193.12, 141.85, 136.56, 130.47, 127.97, 127.24, 126.22, 84.48, 74.66, 30.63, 30.34, 22.20, 21.88, 18.51, 13.62. HRMS (ESI) calculated for [M+H]$^+$ 279.0872, found at 279.0878.

![Chemical structure](image)

S-(2-(prop-2-yn-1-ylcarbamoyl) phenyl) ethanethioate (8, AcWB5):

Prepared via synthesis procedure of compound 7. Synthesized with prop-2-yn-1-amine and purified with normal phase silica chromatography (30% DCM/EtOAc), to give final product as white solid, yield 64%. 1H NMR (600 MHz, CDCl$_3$) δ 7.59 – 7.56 (m, 1H), 7.52 – 7.44 (m, 3H), 6.22 (s, 1H), 4.19 (dd, J = 5.4, 2.5 Hz, 2H), 2.46 (s, 3H), 2.26 (t, J = 2.6 Hz, 1H). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 195.39, 167.84, 141.16, 136.63, 130.66, 130.30, 128.44, 124.78, 79.10, 71.73, 30.38, 29.48. HRMS (ESI) calculated for [M+H]$^+$ 234.0583, found at 234.0589.

**1.3.8 Scheme for synthesis of WB compounds**

![Scheme diagram](image)
N-hexyl-2-mercaptobenzamide (9, WB1):

Compound 5 (500 mg, 1.79 mmol, 1 eq) was dissolved in 20 mL 1:1 Methanol:1 N HCl (aq.). Mixture was heated to 50 °C for 1 hr. The reaction mixture was put under rotary evaporation to remove methanol. The aqueous residue was extracted with ethyl acetate (3 x 20 mL). The combined organic layer was dried over MgSO₄. Then dried organic layer was concentrated with rotary evaporation. The residue was further dried in vacuo to yield final product as white solid without further purification. ¹H NMR (600 MHz, CDCl₃) δ 7.42 (dd, J = 7.7, 1.4 Hz, 1H), 7.32 (dd, J = 7.9, 1.2 Hz, 1H), 7.25 (td, J = 7.6, 1.5 Hz, 1H), 7.14 (td, J = 7.5, 1.2 Hz, 1H), 6.04 (s, 1H), 4.76 (s, 1H), 3.43 (td, J = 7.2, 5.8 Hz, 2H), 1.61 (p, J = 7.3 Hz, 2H), 1.43 – 1.36 (m, 2H), 1.36 – 1.28 (m, 4H), 0.94 – 0.87 (m, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 168.59, 133.66, 132.70, 130.97, 130.44, 127.78, 125.17, 40.10, 31.47, 29.55, 26.64, 22.55, 13.99. HRMS (ESI) calculated for [M+H]⁺ 238.1260, found at 238.1254.

2-(hept-2-yn-1-ylthio) benzenethiol (10, WB2):

Prepared via synthesis procedure of compound 9. ¹H NMR (400 MHz, CDCl₃) δ 7.47 (dd, J = 7.5, 1.7 Hz, 1H), 7.43 (dd, J = 7.5, 1.7 Hz, 1H), 7.25 – 7.17 (m, 2H), 4.35 (s, 1H), 3.60 (t, J = 2.4 Hz, 2H), 2.21 – 2.07 (m, 2H), 1.47 – 1.38 (m, 2H), 1.38 – 1.28 (m, 2H), 0.87 (t, J = 7.2 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 137.75, 135.08,
HRMS (ESI) calculated for [M+H]$^+$ 237.0766, found at 237.0763.

\[
\begin{array}{c}
\text{N-(hept-2-yn-1-yl)-2-mercaptobenzamide (11, WB3):} \\
\text{Prepared via synthesis procedure of compound 9.} \\
\text{\textsuperscript{1}H NMR (600 MHz, CDCl}_3\text{)} \\
\delta 7.47 (dd, J = 7.8, 1.5 Hz, 1H), 7.33 (dd, J = 7.9, 1.2 Hz, 1H), 7.27 (td, J = 7.7, 1.5 \\
Hz, 1H), 7.16 (td, J = 7.5, 1.3 Hz, 1H), 6.10 (s, 1H), 4.76 (s, 1H), 4.21 (dt, J = 4.8, 2.3 \\
Hz, 2H), 2.19 (tt, J = 7.2, 2.3 Hz, 2H), 1.53 – 1.46 (m, 2H), 1.45 – 1.37 (m, 2H), 0.91 \\
t, J = 7.3 Hz, 3H). \text{\textsuperscript{13}C NMR (151 MHz, CDCl}_3\text{)} \delta 168.01, 133.30, 132.50, 131.10, \\
130.79, 127.95, 125.19, 84.75, 74.94, 30.65, 30.43, 21.95, 18.37, 13.58. HRMS (ESI) \\
calculated for [M+H]$^+$ 248.1104, found at 248.1108. \\
\end{array}
\]

1.3.9 Scheme for synthesis of MeWB compounds
**N-hexyl-2-(Methylthio) Benzamide (12, MeWB1)**

To a solution of 2-(methylthio) benzoic acid (1.00 g, 5.94 mmol, 1.00 eq) in DCM (20 mL), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1.25 g, 6.53 mmol, 1.10 eq) was added. The reaction mixture was stirred under RT for 20 min. DMAP (73 mg, 0.59 mmol, 0.10 eq) and hexan-1-amine (661 mg, 6.53 mmol, 1.1 eq) were added to reaction mixture at RT. The reaction mixture was further stirred at RT for 12 h. The reaction mixture was then poured into 1 N HCl (100 mL). The mixture was then extracted with ethyl acetate (3 x 20 mL). The combined organic layer was then washed with saturated NaHCO$_3$ (20 mL) and brine (20 mL). Then combined organic layer was then dried over MgSO$_4$ and filtered. The organic layer was concentrated by rotary evaporation. The resulting crude residue was purified by normal phase silica chromatography (20% Hexane: EtOAc) to give final compound 12 as white solid. $^1$H NMR (600 MHz, CDCl$_3$) δ 7.53 (d, J = 7.6 Hz, 1H), 7.35 (t, J = 7.6 Hz, 1H), 7.29 (d, J = 8.0 Hz, 1H), 7.17 (t, J = 7.5 Hz, 1H), 6.46 (s, 1H), 3.43 (m, 2H), 2.45 (s, 3H), 1.69 – 1.53 (m, 2H), 1.42 – 1.36 (m, 2H), 1.35 – 1.28 (m, 4H), 0.94 – 0.85 (m, 3H). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 168.12, 136.74, 135.64, 130.59, 128.65, 127.32, 125.35, 40.19, 31.62, 29.60, 26.84, 22.70, 16.83, 14.15. HRMS (ESI) calculated for [M+H]$^+$ 252.1417, found at 252.1411.
N-(hept-2-yn-1-yl)-2-(methylthio) Benzamide (13, MeWB3):

Prepared via synthesis procedure of compound 12. Synthesized using hept-2-yn-1-amine (725 mg, 6.53 mmol, 1.10 eq). The product was purified by column flash chromatography (Hexane: EtOAc 30:80) to afford product. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.68 – 7.54 (m, 1H), 7.44 – 7.37 (m, 1H), 7.37 – 7.33 (m, 1H), 7.26 – 7.19 (m, 1H), 6.58 (s, 1H), 4.25 (dt, J = 4.9, 2.3 Hz, 2H), 2.49 (s, 3H), 2.21 (tt, J = 7.0, 2.3 Hz, 2H), 1.57 – 1.46 (m, 2H), 1.46 – 1.37 (m, 2H), 0.93 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 167.43, 137.06, 134.39, 130.86, 128.72, 127.51, 125.34, 84.44, 75.06, 30.66, 30.30, 21.94, 18.38, 16.86, 13.63. HRMS (ESI) calculated for [M+H]$^+$ 262.1260, found at 262.1265.

Hept-2-yn-1-yl(2-(methylthio) phenyl) sulfane (14, MeWB2) synthesis:

To solution of 2-(methylthio) benzenethiol (1.00 g, 6.41 mmol, 1.00 eq) in THF (50 mL), compound 1 (1.20 eq) was added, followed by DiPEA (1.00 eq). The reaction mixture was stirred under N$_2$ at RT for ~5 hrs until 2-(methylthio) benzenethiol was consumed by TLC monitoring. The reaction mixture was concentrated and then subjected to normal phase silica chromatography (20:80 Hexane:EA) to give final product as clear oil. $^1$H NMR (600 MHz, CDCl$_3$) δ 7.81 – 7.74 (m, 1H), 7.29 – 7.25 (m, 1H), 7.25 – 7.16 (m, 2H), 3.53 (t, J = 2.4 Hz, 2H), 2.47 (s, 3H), 2.22 – 2.03 (m, 2H), 1.46 – 1.28 (m, 4H), 0.88 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ
138.07, 136.37, 129.01, 128.19, 127.95, 126.30, 86.03, 74.60, 30.74, 27.99, 22.06, 18.71, 17.10, 13.77. HRMS (ESI) calculated for [M+H]$^+$ 251.0923, found at 251.0929.
REFERENCES


Chapter 2

GLYCOCONJUGATION OF ACETYLCHOLINESTERASE REACTIVATORS FOR ENHANCED CNS ACTIVITY BY FACILITATED TRANSPORT ACROSS BLOOD BRAIN BARRIER

2.1 Introduction

2.1.1 Acetylcholinesterase (AchE) and its inhibition

\[
\begin{align*}
\text{Acetylcholine} & = \text{Acetylcholinesterase (AchE)} \\
\text{Acetylcholine} & = \text{Choline}
\end{align*}
\]

Acetylcholine is one of the neurotransmitters found in muscle, brain and the central nerve system. It plays an important role in autonomic nervous system which includes the control of memory formation, digestion and cardiac function as well as motor neurons activate in muscles. Acetylcholinesterase (AchE) is an enzyme that catalyzes the hydrolysis of acetyl choline to choline. This enzymatic hydrolysis terminates the neural signal transmission by acetylcholine\(^1\). Organophosphorus compounds (Figure 2.1) inhibit AchE by covalently phosphorylating the active site serine of AchE. The covalent inhibition of AchE will lead to accumulation of acetylcholine. If untreated, inhibition of AChE leads to a disruption of respiratory and cardiac function and eventually death.\(^1\)\(^-\)\(^2\)
2.1.2 Current treatments and previous approaches to make them CNS active reactivators

Current treatment of phosphorylated AchE uses nucleophilic compounds such as 2-PAM and HI6 (Figure 2.2). The mechanism of reactivation of phosphorylated AchE is the oxime group nucleophilic attacks phosphorylated serine (Figure 2.3). This treatment is effective in the peripheral nervous system but is not effective in reactivating inhibited AChE in CNS, because pyridinium oxide reactivators compounds (Figure 2.2) do not effectively penetrate the BBB to enter the CNS.\textsuperscript{3}
2.2. Traditional AchE reactivators.

![Chemical structures of 4-PAM, 2,4-DIPAM, and 2-PAM, Hi6, and MMB4](image)

Figure 2.2. Traditional AchE reactivators.

2.3. Oxime compounds to rescue covalently inhibited AchE.

![Diagram of AchE enzyme structure with oxime compounds](image)

Figure 2.3. Oxime compounds to rescue covalently inhibited AchE.
There are several examples of enhancing the BBB permeability of small-molecule drugs through glucose conjugation. Glucose conjugates can enhance permeability by using the glucose transporter, GLUT1, which is highly expressed in the microvascular endothelial cells. Inspired by early work which suggested that glucose linked oxime reactivators had enhance CNS activity.\textsuperscript{3-6} Carparis et al. suggests the glucoconjugation through the 6-position of glucose would have greater BBB permeability.\textsuperscript{5-6}

2.1.3 GLUT1 structures and MD simulation

GLUTs belong in Major Facilitator Superfamily (MFS), they transport glucose across cell membrane. Human GLUT1 had been crystalized, however the for GLUT1, only glucose binding configuration structure was available.\textsuperscript{7} The structure of the \textit{E. coli} D-xylose transporter (XylE) had also been crystalized and solved in both the outward facing and the inward facing ligand-bound configurations.\textsuperscript{8} GLUT1 and XylE have ~60% homology, several articles have reported using XylE to model GLUT1 to obtain structural models for the GLUT1 transportation process.\textsuperscript{8-9} Min-Sun Park\textsuperscript{9} modeled all three configurations of glucose transportation using steered molecular dynamics simulation and targeted molecular dynamics simulation, they profiled ~7 Å long of glucose transporting of GLUT1 with analysis of potential mean force (PMF).
2.2 Results and Discussion

2.2.1 Synthesis of Sugar linked pyridinium oxime compounds

To synthesize pyridinium oxime analogs linked to glucose at 6-position, several protecting group strategies were tried. Acetyl or benzoyl protecting groups are base/nucleophile sensitive, and benzyl or PMB protecting groups would require hydrogenation or strongly reducing steps for deprotection which would not be compatible with the pyridinium ring. We chose acetal protecting groups to protect hydroxyl groups. As 3,4 hydroxyl groups of glucose are on opposite sides of the ring in its pyranose form, we chose the glucofuranose form for protection, as the two hydroxyl groups can then be protected by dimethoxy propane (DMP).

Two linkers have been chosen as tethers between the glucose and the oxime. Ether linkages are one of most bio stable groups and the synthesis of it was developed.
first (Figure 2.4). Azide-alkyne click chemistry to form a hydrophilic triazole group was also used as conjugation method (Figure 2.5), as it may increase the affinity to GLUT1 during GLUT1 facilitated transport.

The triazole-linked series is more easily synthesized than the ether-linked series is that the propargyl ether can be made directly from tosylated 5 and the synthesis does not require the 3,4 hydroxyl groups to be protected during synthesis (Figure 2.5).

The azide-alkyne pairing can be reversed by displacing tosylate 5 with azide and coupling it to the N-propargyl-pyridinium analog 10.

Figure 2.5. Triazole linked SOx synthesis.

### 2.2.2 SOx retained reactivity after linked with glucose

The SOx compounds were subjected in vitro to reactivity testing by Dr. Cadeaux at USAMRICD. Purified AchE was first inhibited by different organophosphorus compounds. The enzyme was purified away from the agent and
were subsequently incubated with SOx reactivators. The amount of reactivated AChE was determined discontinuously by incubating aliquots with thioacetylcholine and DTNB. The liberated enzyme activities can be used to determine an AchE reactivation rate.\(^1\)

As our goal was to demonstrate if glucose conjugation would still retain a significant amount of the parent oxime’s reactivation rate, SOx analogs that retained more than 15% of the reactivation rate of parent PAMs were considered to have “passed” the selection criteria (Table 2.1).

<table>
<thead>
<tr>
<th>SOx</th>
<th>GA</th>
<th>GB</th>
<th>GF</th>
<th>VX</th>
<th>VR</th>
<th>PX</th>
<th>CPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN-30</td>
<td>ND*</td>
<td>Fail</td>
<td>Fail</td>
<td>Fail</td>
<td>ND</td>
<td>Fail</td>
<td>ND</td>
</tr>
<tr>
<td>RN-46</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
</tr>
<tr>
<td>RN-47</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
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<tr>
<td>RN-86</td>
<td>Pass</td>
<td>Fail</td>
<td>Fail</td>
<td>Fail</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>W2-127</td>
<td>ND</td>
<td>Fail</td>
<td>ND</td>
<td>Pass</td>
<td>ND</td>
<td>Pass</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND: not detectable; Pass: ratio higher than 0.15; Fail: ratio lower than 0.15

2.2.3 Blood Brain Barrier (BBB) permeability of SOx

The permeabilities of SOx reactivators were tested in a human induced pluripotent stem cells (hPSCs) model.\(^10\) Compared to a traditional MDCK BBB model, hPSCs model have two advantages. First, the transepithelial electrical resistance (TEER) value of MDCK cell model is relatively low, which means that the integrity of barrier is not high and the model has significant nonspecific permeability. The hPSCs cell model, by contrast, has TEER values as high as \(~2500\ \Omega \cdot \text{cm}^2\)
compared to ~200 Ω·cm² for MDCK cell models. Second, unlike the human BBB, GLUT1 abundance in MDCK cell model is significantly lower than hPSCs model.¹⁰

Collaborating with the Kelvin Lee Lab at Delaware Biotech Institute, we used hPSCs cell model to assay each compounds’ BBB permeability. We determined the apparent permeability ($P_{\text{app}}$) of top active SOx analogs based on reactivity tests (Table 2.1). Once the hPSCs forms a tight junction on a supporting membrane, SOx compounds at a donor concentration of 0.5mM, were added at luminal (blood facing) or abluminal (brain facing) sides of the trans-wells. The SOx concentrations on the opposite side of the donor were quantified by LC-MS at 20min, 40min and 60min time points. All samples from permeability assays were added with 1µM RN53 as internal standard, as RN53 samples were added with RN47 as internal standard. All samples were subjected to Thermo Orbitrap LC-MS with positive detection mode. All SOxs were separated in a C18 column with 30 mm x 2.1 mm dimension with 5%-60% gradient acetonitrile in water. The first order equations of SOx concentration versus MS signal of SOx/internal standard ration were plotted to calculated SOx concentrations in samples. $P_{\text{app}}$ was then calculated for each compound to give luminal and abluminal transporting rate (Table 2.2).

$$P_{\text{app}} = \frac{C_R * V_R}{t * S * C_D}$$

Figure 2.6. $P_{\text{app}}$ calculation. $C_R$: concentration of receiver, $V_R$: volume of receiver, $t$: time, $S$: surface area of inserts, $C_D$: initial concentration of donor.
Table 2.2 Compounds permeability**

<table>
<thead>
<tr>
<th>Compound</th>
<th>P_{app} (10^{-6} \text{ cm/s})</th>
<th>efl.ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-&gt;B*</td>
<td>B-&gt;A</td>
</tr>
<tr>
<td>W2-109</td>
<td>2.1±0.1</td>
<td>9.4±1.0</td>
</tr>
<tr>
<td>W2-219</td>
<td>3.3±0.2</td>
<td>3.7±0.3</td>
</tr>
<tr>
<td>RN-53</td>
<td>3.7±0.6</td>
<td>2.0±0.7</td>
</tr>
<tr>
<td>RN-46</td>
<td>7.2±2.3</td>
<td>5.6±0.4</td>
</tr>
<tr>
<td>RN-47</td>
<td>5.6±1.9</td>
<td>1.2±0.12</td>
</tr>
</tbody>
</table>

*A:luminal side B:abluminal side. **Triplicated tests for each

From $P_{app}$ results, compounds with 5-8 carbon linkers are the most permeable SOx analogs (Figure 2.7). The triazole linker slightly decreases BBB permeability. To confirm their transportations through GLUT1, RN47 was incubated with and without the GLUT1 inhibitor STF-31, and compared its parent compound 4PAM (Figure 2.8).

![Chemical structures of RN-46, RN-47, RN-53, w2-109, and w2-219](image)

Figure 2.7. Two different types of active SOx.
RN47 was also compared to its parent oxime 4PAM. Glucose conjugation increased luminal to abluminal (blood to brain) transportation rate by 6.1-fold; and decreased abluminal to luminal transportation rate by 3.9-fold (Figure 2.8). The combined effect of glucose conjugation increased RN47’s efflux ratio of transportation by 23-fold. STF-31 inhibited BBB cell model significantly lowered the transportation of RN47, indicating that RN47 indeed used GLUT1 or other glucose transporters to facilitate its BBB permeability.
2.2.4 Constructing GLUT1-Ligand complex for MD simulation

To understand the relationship between SOx structure and BBB transport, a MD simulation of the entire GLUT1 transportation process was constructed. RN47, W2-109, STF-31 and glucose were constructed with GAFF\textsuperscript{11} force field with RESP charges assigned.

The ligand binding state of GLUT1 was extracted from crystal structure (PDB id:4PYP), the outward facing and inward facing structures are homology modelled based on the x-ray structures of xylose symporter (PDB ids:4GBZ,4QIQ) using EasyModeller.\textsuperscript{12} POPC lipids, water and salt were added to the system using CHARMM-GUI membrane builder.\textsuperscript{13} SOx were placed near the entrance of GLUT1, and the system was heated to 300 K and equilibrated for 1ns with NPT ensemble (Figure 2.9).

Figure 2.9. Assemble the initial outward facing GLUT1 complex.
2.2.5 Steer MD of compounds to GLUT1 glucose binding site

Adaptive steered MD\textsuperscript{14} was used to calculate the average work/PMF of compounds moving to the binding site of GLUT1. PMFs for the binding processes of glucose, ether link SOx (RN47), triazole link SOx (W2-109) and STF-31 (GLUT1 inhibitor) were calculated.

PMFs of each compound corresponds to the relative barrier to travel to access the binding site. Overall simulated PMF predicted the average work needed for different compounds to travel to GLUT1’s central binding site\textsuperscript{15}, SOxs (RN47 and W2-109) had higher PMF than glucose but lower than GLUT1 inhibitor STF-31 (Figure 2.10).

![SMD to GLUT1 binding pocket](image)

Figure 2.10. PMF of compounds from adaptive SMD simulation.

SMD accuracy depends on several of factors, pulling rate is one of the most important ones. Although a slower pulling rate could help to increase prediction
accuracy, one must balance the choice of accuracy based on optimal SMD pulling speed and external potential with the available computational resources. 1 Å/ns pulling speed with 1 Kcal•mol⁻¹•Å⁻² external potential was previously used successfully in a similar biological system⁹, therefore we tried both 0.1 Å/ns and 1 Å/ns pulling speeds with 1 Kcal•mol⁻¹•Å⁻² external potential and compared the PMF results of both conditions (Figure 2.11). With ten-fold longer simulation time, the simulation results converged to similar PMFs. A 1 Å/ns pulling rate was therefore chosen in the following SMD simulations.

![Adaptive SMD at different rate](image)

Figure 2.11. RN47 steered into GLUT1 binding pocket at two different rates.

### 2.2.6 Targeted MD for GLUT1 conformation change simulation

After compounds steered to central binding site, outward facing GLUT1 conformation were simulated to ligand bind state by 10 ns targeted MD. The whole structure then equilibrated for 10 ns before changed to inward facing conformation by
another 10 ns targeted MD. With another 10ns equilibration, the GLUT1-ligand complexes were in the states that allow the release of the compounds to abluminal side of the BBB (Figure 2.12).

Figure 2.12. Targeted MD simulations of GLUT1 conformation change.

**2.2.7 Steer compounds out of GLUT1 into abluminal side**

With GLUT1-Compounds configurations from targeted MD, ASMD simulations were used to simulate compounds releasing from GLUT1 to abluminal side. This process calculated the average work needed for releasing each compound. End to end PMFs of each compound from two ASMDs (Figure 2.10 and Figure 2.13) were consistent with results of the experimental $P_{app}$ assay.
Figure 2.13. ASMD of compounds releasing from binding site.
2.3 Experimental

2.3.1 General procedure for BBB cell model setup and compounds’ permeability assay

This method is adapted from prior publication. Human induced pluripotent stem cells (hPSCs) were maintained and differentiated as described by Mantle et al. Differentiated cells were plated on cell culture trans-well inserts (Corning #3470) to form monolayer cells with tight junctions. Transendothelial electrical resistance measurements (TEER) were obtained using an EVOM2 Epithelial Volt meter (World Precision Instruments). The resulting value was multiplied by the membrane surface area to get the TEER value in Ω•cm².

The trans-well inserts of cells with 2000 Ω•cm² or above were selected for measuring permeability of SOx analogs. All compounds were dissolved in Ringers-HEPES buffer (Sigma-Aldrich) with 5mM glucose to give final test concentration of 0.5mM. The test followed the same procedure as previous publication. Sample collections were repeated at 20, 40, 60 min. All Samples were stored at -20 °C before Orbitrap-LC/MS quantification.

Compounds were dissolved in MeOH and 2-fold serial dilutions of every compound was made to give samples of concentrations from 50 µM to 1.5 nM. Every concentration of every compound was injected to Orbitrap-LC/MS, and a first order standard curve between concentration and MS ion count was made for every compound. Correspond samples from P_app assays were quantified based on their MS standard curve.
2.3.2 Constructing GLUT1 membrane structures for MD simulation

Three states of human GLUT1 were constructed. Binding state: crystal structure of bind state GLUT1 was obtained from protein data bank (PDBID:4YPY). Inward-facing and outward-facing protein structures were homology modeled from inward-facing XylE (PDBID:4JA3) and outward-facing XylE (PDBID:4GBZ) using Easymodeller 4.0.12 Modelled structures were optimized by energy minimization and MD equilibration for 50ns, averaged structures of last 500 ps MD simulation were constructed to give final structures for membrane protein construction.

CHARMM-GUI membrane builder16 was used to constructed membrane-protein complexes. Proteins were added into 100 Å 100 Å POPC bilayer using replacement method and 20 Å high of water was added on both sides of lipid. 0.15 M NaCl was added into the system. Then three states of GLUT1-membrane complexes were constructed. Compounds’ force fields were constructed using Gaussian09 and antechamber as described in chapter one.

With initial protein configurations constructed, all configurations were minimized by 5000 steps of steepest descent minimization and then 5000 steps of conjugated gradient minimization with protein restrained. And then sample energy minimization steps were formed without protein restrained. The systems were then heated from 0 K to 300 K gradually with Langevin dynamics. Then 50 ns equilibration MD was performed. When added compounds (Glucose, SOx, STF-31), the configurations were equilibrated again with the same procedure unless otherwise noted.
2.3.3 Adaptive SMD of compounds into glucose binding site of GLUT1

Initial structures of membrane-protein complexes were obtained from conventional MD equilibrated structures.

Compounds were placed near the entrance of GLUT1 and equilibrated for 500 ps. All sugar contained compounds were placed with sugar group placed at the similar position and configuration near the entrance of outward faced GLUT1. ASMDs were performed with $1 \, \text{Kcal} \cdot \text{mol}^{-1} \cdot \text{Å}^{-2}$ external harmonic potential placed on center of mass of SOX’s sugar group (center of mass for STF-31) and center of mass of Trp388 of GLUT1. The backbone of Trp388 was constraint to keep stationary with $50 \, \text{Kcal} \cdot \text{mol}^{-1} \cdot \text{Å}^{-2}$ potential constant.

Using Rigoberto Hernandez et al. adaptive SMD method\textsuperscript{14, 17}, each simulation was run in NVT ensemble. Each step with 4 Å reaction path, 50 SMDs with random seeds were run at rate of 1 ns/Å. Then Jarzynski average (JA) was calculated through each stage. The one trajectory whose work value was closest to the JA value was chosen to initial next stage of SMD. When all stages of SMDs were done, all work files which were chosen at each stage are combined to construct PMF.\textsuperscript{17}

2.3.4 Targeted MD for configuration change of Ligand-GLUT1-membrane complexes

The outward facing ligand GLUT1 membrane complexes from last step were transformed into binding state with TMD. All $C_\alpha$ atoms were added with a force with force constant of $1 \, \text{Kcal} \cdot \text{mol}^{-1} \cdot \text{Å}^{-2}$ and 10ns of TMD was performed for the RMSD of two configurations decreased from $\sim 5 \, \text{Å}$ to less than 0.2 Å.

Then same TMDs were performed for configuration change from binding state to inward facing state.
2.3.5 Adaptive SMD simulation of compounds release from GLUT1

Same method as SMD to binding site, compounds were slowly pulled out of GLUT1 from the last step of TMD. Then PMF was calculated as described here. \(^{17}\)

2.3.6 General procedure one, synthesis of (E)-azido-alkyl-4-(hydroxyimino)methyl) pyridin-1-ium

\[
\text{HO-N} \underset{\text{N}}{\text{N}} \underset{\text{N}}{\text{N}} \underset{n}{\text{n}}
\]

To a solution of (E)-isonicotinaldehyde oxime (2.00 g, 16.4 mmol, 1 eq) in DMF (100 mL), dibromo alkane (4 eq) was added and stirred at 80 °C for 6 hrs. Then DMF was removed by rota-vap and the residue was washed by hexane (3 x 100 mL) to remove dibromo alkane. The residue was re-dissolved in DMF (100 mL), and NaN\(_3\) (3 eq) was added. The reaction mixture was further stirred at 50°C for 12 hrs. Then DMF was removed by high vacuum rotary evaporation. The residue was purified by reverse phase C18 chromatography to give final product as a salt.

\[
\text{OH} \overset{\text{N}}{\text{N}} \overset{\text{N}}{\text{N}} \overset{\text{N}}{\text{N}}
\]

(E)-1-(3-azidopropyl)-4-((hydroxyimino) methyl) pyridin-1-ium (1):

Prepared via general procedure one. \(^1\)H NMR (600 MHz, CD\(_3\)OD) \(\delta\) 8.90 (d, \(J = 6.8\) Hz, 2H), 8.33 (s, 1H), 8.22 (d, \(J = 6.8\) Hz, 2H), 4.69 (t, \(J = 7.2\) Hz, 2H), 3.55 (t, \(J = 6.3\) Hz, 2H), 2.39 – 2.23 (m, 2H). \(^13\)C NMR (151 MHz, CD\(_3\)OD) \(\delta\) 150.70, 144.46,
144.32, 123.48, 58.37, 47.70, 29.78. HRMS (ESI) calculated for [M+] 206.1036, found 206.1029.

(E)-1-(5-azidopentyl)-4-((hydroxyimino) methyl) pyridin-1-ium (2):
Prepared via general procedure one. $^1$H NMR (600 MHz, CD$_3$OD) $\delta$ 8.91 – 8.83 (m, 2H), 8.25 (s, 1H), 8.19 – 8.12 (m, 2H), 4.60 – 4.49 (m, 2H), 3.26 (t, J = 6.7 Hz, 2H), 2.04 – 1.92 (m, 2H), 1.63 – 1.55 (m, 2H), 1.46 – 1.34 (m, 2H). $^{13}$C NMR (151 MHz, CD$_3$OD) $\delta$ 149.88, 144.57, 144.25, 124.07, 60.88, 50.73, 30.43, 27.90, 22.99. HRMS (ESI) calculated for [M+] 234.1349, found 234.1340.

(E)-1-(6-azidohexyl)-4-((hydroxyimino) methyl) pyridin-1-ium (3):
Prepared via general procedure one. $^1$H NMR (600 MHz, CD$_3$OD) $\delta$ 9.06 (d, J = 6.8 Hz, 2H), 8.40 (s, 1H), 8.30 (d, J = 6.9 Hz, 2H), 4.73 – 4.68 (m, 2H), 3.36 (t, J = 6.9 Hz, 2H), 2.17 – 2.03 (m, 2H), 1.72 – 1.62 (m, 2H), 1.58 – 1.47 (m, 4H). $^{13}$C NMR
(151 MHz, CD$_3$OD) $\delta$ 149.71, 144.63, 144.29, 124.17, 61.04, 50.92, 30.84, 28.22, 25.83, 25.30. HRMS (ESI) calculated for [M+] 248.1506, found 248.1495.

(E)-1-(8-azidoctyl)-4-((hydroxyimino) methyl) pyridin-1-ium (4):
Prepared via general procedure one. $^1$H NMR (600 MHz, CD$_3$OD) $\delta$ 8.95 (d, J = 6.8 Hz, 2H), 8.32 (s, 1H), 8.22 (d, J = 6.8 Hz, 2H), 4.63 – 4.58 (m, 2H), 3.27 (t, J = 6.9 Hz, 2H), 2.09 – 1.96 (m, 2H), 1.65 – 1.55 (m, 2H), 1.51 – 1.28 (m, 8H). $^{13}$C NMR (151 MHz, CD$_3$OD) $\delta$ 149.77, 144.57, 144.20, 124.11, 61.14, 51.04, 30.92, 28.53, 28.44, 26.25, 25.65. HRMS (ESI) calculated for [M+] 276.1819, found 276.1811.

2.3.7 Synthesis of (3aR,3bS,7R,8aR)-7-(((7-bromoheptyl) oxy) methyl)-2,2,5,5-tetramethyltetrahydro-7H-[1,3]dioxolo [4',5':4,5]furo[3,2-d][1,3]dioxine (5)
To a round bottom flask with NaH (92 mg, 60% in mineral oil, 1.2 eq), a solution of ((3aR,3bS,7R,8aR)-2,2,5,5-tetramethyltetrahydro-7H-[1,3]
dioxolo[4',5':4,5]furo[3,2-d][1,3]dioxin-7-yl)methanol (500 g, 1.92 mmol, 1 eq) in THF (20 mL) was added with flask cooled on -78°C. Then 15-Crown-5 (1 eq) was added. The reaction mixture was then warmed to 0°C. 1,7-dibromoheptane (3 eq) was added to reaction mixture. Reaction mixture was then warmed to RT and stirred for another 5h. The reaction mixture was poured into ice water (50 mL). The suspension was extracted with ethyl acetate (3 x 50 mL). The combined organic solvent was dried over MgSO₄ and concentrated by rotary evaporation. The crude residue was purified with normal phase silica chromatography (EA:Hexane 1:4) to give final product as clear oil. ¹H NMR (600 MHz, CDCl₃) δ 6.00 (d, J = 3.7 Hz, 1H), 4.58 (d, J = 3.7 Hz, 1H), 4.38 – 4.30 (m, 1H), 4.21 (d, J = 3.8 Hz, 1H), 3.75 – 3.68 (m, 1H), 3.65 – 3.55 (m, 2H), 3.48 (td, J = 6.7, 2.6 Hz, 2H), 3.40 (t, J = 6.9 Hz, 2H), 1.85 (p, J = 7.0 Hz, 2H), 1.64 – 1.55 (m, 2H), 1.54 – 1.26 (m, 18H). ¹³C NMR (151 MHz, CDCl₃) δ 112.11, 106.36, 100.89, 84.05, 79.48, 74.96, 71.55, 71.44, 71.19, 33.89, 32.73, 29.36, 28.57, 28.10, 27.17, 26.56, 25.90, 24.09, 23.96.

2.3.8 General procedure two, synthesis of triazole linked sugar oxime
Correspond azido pyridinium (1 eq, 5.00 mmol) and (3aR,6S,6aR)-5-((R)-1-hydroxy-2-(prop-2-yn-1-yloxy) ethyl)-2,2-dimethyltetrahydrofuro[2,3-d][1,3] dioxol-6-ol (1eq) was dissolved in butanol: water (1:1 v/v) (50 mL), CuSO₄ (0.1 eq) and sodium ascorbate (1 eq) were then added. The reaction mixture was stirred for 24h. The butanol was removed by rota-vap. The aqueous residue was stirred with Dowex-Cl ion exchange resin for another 4 hrs. Then the resin was filtered away. The liquid residue was added with 20 mL 2 M HCl (aq.) and stirred at 50 ℃ for 1 hr. The crude product was purified by reverse phase chromatography to give final product as white solid.

4-((E)-(hydroxyimino) methyl)-1-(3-(((((2R,3S,4S,5R)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl) methoxy) methyl)-1H-1,2,3-triazol-1-yl)propyl)pyridin-1-ium (α:β=1:1) (6)

Prepared via general procedure two. ¹H NMR (600 MHz, CD₃OD) δ 8.06 – 7.99 (m, 2H), 7.91 (d, J = 3.5 Hz, 1H), 6.91 – 6.85 (m, 2H), 4.98 (d, J = 3.7 Hz, 0.5H), 4.56 – 4.53 (m, 2H), 4.42 (t, J = 6.5 Hz, 2H), 4.36 (d, J = 7.8 Hz, 0.5H), 4.13 (t, J = 7.0 Hz, 2H), 3.85 – 3.28 (m, 4H), 3.16-3.14 (m, 6H), 2.51-2.30 (m,2H). ¹³C NMR (151
MHz, CD$_3$OD) δ 168.87, 156.55, 145.05, 145.03, 141.66, 141.64, 123.90, 123.88, 107.66, 107.65, 96.87, 92.61, 76.69, 75.54, 74.89, 73.46, 72.45, 70.63, 70.44, 70.21, 69.70, 69.67, 63.92, 63.86, 54.64, 54.61, 46.69, 46.67, 38.94, 38.92, 30.32, 30.28.

HRMS (ESI) calculated for [M$^+$] 424.1827, found 424.1819.

4-((E)-(hydroxyimino) methyl)-1-(5-(4-(((2R,3S,4S,5R)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl) methoxy) methyl)-1H-1,2,3-triazol-1-yl)pentyl)pyridin-1-ium (α:β=1:1) (7)

Prepared via general procedure two. $^1$H NMR (600 MHz, CD$_3$OD) δ 8.99 – 8.83 (m, 2H), 8.66 – 8.30 (m, 2H), 8.23 (d, J = 6.3 Hz, 1H), 7.99 (d, J = 4.0 Hz, 1H), 5.10 (d, J = 3.7 Hz, 0.5H), 4.69 – 4.64 (m, 2H), 4.60 – 4.55 (m, 2H), 4.48 (d, J = 7.9 Hz, 0.5H), 4.47 – 4.44 (m, 2H), 3.95 – 3.40 (m, 4H), 3.37 (s, 2H), 2.15 – 1.95 (m, 4H), 1.48 – 1.30 (m, 2H). $^{13}$C NMR (151 MHz, CD$_3$OD) δ 149.90, 144.76, 144.69, 144.50, 144.23, 144.20, 126.94, 124.11, 124.09, 123.73, 123.70, 96.87, 92.60, 76.68, 75.50, 74.89, 73.46, 72.44, 70.60, 70.47, 70.24, 69.68, 69.63, 63.91, 63.88, 60.64, 49.41,
HRMS (ESI) calculated for [M$^+$] 452.2140, found 452.2136.

$4$-((E)-(hydroxyimino) methyl)-1-((6-(((2R,3S,4S,5R)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl) methoxy) methyl)-1H-1,2,3-triazol-1-yl)hexyl)pyridin-1-ium (α:β=1:1) (8)

Prepared via general procedure two. $^1$H NMR (600 MHz, CD$_3$OD) δ 8.95 – 8.88 (m, 2H), 8.34 (s, 1H), 8.23 (d, J = 6.8 Hz, 2H), 8.01 (d, J = 5.7 Hz, 1H), 5.10 (d, J = 3.7 Hz, 0.5H), 4.66 (d, J = 4.8 Hz, 2H), 4.59 (td, J = 7.6, 2.7 Hz, 2H), 4.47 (d, J = 7.8 Hz, 0.5H), 4.46 – 4.41 (m, 2H), 3.95 – 3.41 (m, 4H), 3.37 – 3.36 (m, 4H), 2.08 – 1.90 (m, 5H), 1.52 – 1.30 (m, 5H). $^{13}$C NMR (151 MHz, CD$_3$OD) δ 149.81, 144.62, 144.54, 144.27, 144.26, 124.07, 123.78, 123.73, 96.85, 92.59, 76.68, 75.48, 74.89, 73.46, 72.44, 70.60, 70.50, 70.27, 69.69, 69.64, 63.90, 63.88, 60.89, 49.69, 49.68, 48.47, 30.65, 30.63, 29.43, 29.38, 25.38, 25.36, 25.01, 25.00. HRMS (ESI) calculated for [M$^+$] 466.2296, found 466.2293.

$4$-((E)-(hydroxyimino) methyl)-1-((8-(((2R,3S,4S,5R)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl) methoxy) methyl)-1H-1,2,3-triazol-1-yl) octyl) pyridin-1-ium (α:β=1:1) (9)
Prepared via general procedure two. $^1$H NMR (600 MHz, CD$_3$OD) $\delta$ 8.96 (d, $J$ = 6.7 Hz, 2H), 8.35 (s, 1H), 8.28 – 8.13 (m, 2H), 8.01 (d, $J$ = 5.3 Hz, 1H), 5.11 (d, $J$ = 3.7 Hz, 0.5H), 4.69 – 4.65 (m, 2H), 4.63 – 4.58 (m, 2H), 4.49 (d, $J$ = 7.8 Hz, 0.5H), 4.44 – 4.40 (m, 2H), 3.96 – 3.42 (m, 4H), 3.38 – 3.36 (m, 4H), 2.08 – 1.99 (m, 2H), 1.96 – 1.86 (m, 2H), 1.44 – 1.25 (m, 8H).

$^{13}$C NMR (151 MHz, CD$_3$OD) $\delta$ 149.75, 144.57, 144.54, 144.30, 126.01, 125.52, 124.06, 123.75, 123.70, 96.86, 92.59, 76.69, 75.49, 74.90, 73.47, 72.44, 70.62, 70.52, 70.30, 69.71, 69.67, 63.95, 63.90, 61.08, 49.87, 30.93, 30.91, 30.86, 30.84, 29.70, 29.66, 28.36, 28.32, 28.29, 28.19, 28.14, 25.75, 25.71, 25.52, 25.48, 25.45. HRMS (ESI) calculated for [M$^+$] 494.2609, found 494.2601.
2.3.9 Synthesis of 4-((E)-(hydroxyimino) methyl)-1-(7-(((2R,3S,4S,5R)-3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-yl) methoxy) heptyl)pyridin-1-ium (10)

To a round bottom flask with AgoTf (347 mg, 1.5 eq), a solution of compound 5 (467 mg, 0.9 mmol, 1 eq) in chloroform (10 mL) was added. Then (E)-isonicotinaldehyde O-(tert-butyldimethylsilyl) oxime (1.5 eq) was added. The reaction mixture was then stirred under darkness for 3 days at 50 ℃. Then the precipitate was filter out and dissolved in MeOH (10 mL). DOWEX-Cl ion exchange resin was added to the methanol solution and stirred for 3h. The resin was filtered away and then MeOH was removed by rota-vap. The residue was re-dissolved in 20 mL 1M HCl(aq.) and stirred for 1 hr at 50℃. Then the crude product was purified by reverse phase chromatography to give final product as white solid. \(^1H\) NMR (600 MHz, CD\(\text{OD}\)) \(\delta\) 8.95 (d, \(J = 6.5\) Hz, 2H), 8.35 (s, 1H), 8.24 (d, \(J = 6.5\) Hz, 2H), 5.10 (d, \(J = 3.7\) Hz, 0.5H), 4.61 (t, \(J = 7.6\) Hz, 2H), 4.47 (d, \(J = 7.8\) Hz, 0.5H), 3.80 – 3.44 (m, 5H), 3.39 – 3.35 (m, 2H), 2.13 – 1.95 (m, 2H), 1.70 – 1.56 (m, 2H), 1.48 – 1.38 (m, 6H). \(^13C\) NMR (151 MHz, CD\(\text{OD}\)) \(\delta\) 149.80, 144.54, 144.22, 124.09, 104.99, 96.82, 92.57, 85.18,
80.52, 76.77, 75.50, 74.89, 73.68, 73.51, 72.45, 71.12, 71.10, 70.68, 70.55, 70.46, 70.03, 70.01, 67.61, 61.10, 54.59, 48.46, 30.84, 30.80, 29.00, 28.97, 28.36, 28.29, 25.71, 25.65, 25.62, 25.51, 25.05. HRMS (ESI) calculated for [M+] 399.2126, found 399.2125.
REFERENCES


Appendix A

1H NMR AND 13C NMR SPECTRUM
Appendix B

PERMISSION LETTER

Title: Antagonizing the Androgen Receptor with a Biomimetic Acyltransferase
Author: Yuchen Zhang, Pavan K. Mantriwadi, Soma Jobbagy, et al
Publication: ACS Chemical Biology
Publisher: American Chemical Society
Date: Oct 1, 2016
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