CATALYTIC ACYL TRANSFER MODIFICATION OF NUCLEAR RECEPTORS AND DETECTION BY IN-GEL LABELING

by

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ABSTRACT

Androgen receptor (AR) signaling plays a central role in prostate development and homeostasis as well as the progression of prostate cancer (PCa). Upon hormone binding, AR enters nucleus and induces down-stream expression of specific genes. Post-translational modification of AR could alter this signaling process and serve as an alternate approach in PCa treatment.

In this study, we prepared N-acetylcysteamine (NAC) mimicking a charged CoA as an alkyne group substrate. As the structure of SAMT has been reported to act as an acyl transfer catalyst, we combined its structure with a ligand for AR and perform proximity-directed protein labeling to modify a lysine residue on AR. Efficiency and specificity of the labeling are evaluated with fluorogenic click reaction *in vitro* and in cell. The in-solution and in-gel detection limit are assessed, against small molecule reference and Bovine Serum Albumin (BSA) reference, respectively.

Our result indicated that with the method discussed, the proximity-directed labeling has not been able to deliver a satisfactory efficiency and selectivity. The methods still show great potential and needs further diagnosis and optimization.

Chapter 1

INTRODUCTION

Second to lung cancer, prostate cancer (PCa) is the second leading cause of death among men worldwide, especially in more developed countries. [1]. The number of new prostate cancer cases for 2015 in the United States is 220,800 (740 in Delaware) and estimated deaths are approximately 27,540 (100 in Delaware). Prostate cancer is often a very survivable disease. Close to 100% of men are still alive 5 years after diagnosis, and nearly 2.8 million prostate cancer survivors are alive today. [2] PCa is a heterogeneous disease, and the best treatment option depends on the patient's specific condition (such as stage, grade, age, and other health conditions etc.). Major treatment options for PCa are prostatectomy (surgery), radiation therapy, chemotherapy, hormonal therapy or Androgen Deprived Therapy (ADT), vaccine treatment, and bone-direct treatment, separately or combined. Androgen deprivation therapy (ADT), a commonly used treatment for PCa, has a relatively low mortality rate, but the disease can always progress to an advanced, resistant stage and become androgen-independent (AI) after prolonged treatment. This condition is named castration-resistant prostate cancer (CRPC). Many studies have been done on the progression of PCa becoming AI, and shows that it could be caused by androgen receptor (AR) mutations or AR over expression.

AR is a steroid hormone receptor that regulates androgen-dependent genes [3] and steroid hormones such as testosterone activate it. AR is located in the cytoplasm in the absence of ligand and is associated with heat shock proteins. It undergoes

conformational change upon ligand binding that causes AR dissociation from heat shock proteins and exposure of a nuclear localization sequence. This allows the binding of importin proteins and AR to translocate into the nucleus. In the nucleus, AR binds to DNA hormone response elements, recruits coactivators and leads to target gene transcription [4]. Certain modifications of AR will have some impact on its translocation and gene activation process, so we are exploring ways to modify AR and test their function. In the following sections, proximity directed protein labeling and click reaction for protein labeling would be discussed as basic ideas in designing the experiments scheme.

1.1 Acetylation of AR

Post-translational modifications of proteins play an important role in protein function regulation. They include phosphorylation, acetylation, methylation, ubiquitination, biotinylation, palmitilation, sumoylation, mesitylation and glycosylation. [5] Of these, acetylation of lysine has been discovered to occur to histone proteins and plays a role in transcription regulation. It is regulated by lysine acetyltransferase (KATs) and histone deacetylases (HDACs), of which the former transfer acetyl group from acetyl-CoA to the amino group of lysine while the latter did deacetylation [6, 7].

There is a hinge region in AR between its DNA binding domain (DBD) and ligand binding domain (LBD), and it's a major part of nuclear localization sequence (NLS) [8]. It has been reported that Lysine 630, 632, 633 are important for importin-αbinding and AR localization. A single L to A mutation reduces affinity to importin-αthus slows down AR nuclear import; triple mutation completely abolishes importin binding and impairs nuclear import. [8] Those lysine residues are subjected to

acetylation by transcription coactivator p300 and Tip60, which is esteemed to affect AR localization (even bound with DHT) and transcription activity. [8,9,10,11,12] Acetylation of the NLS seems to promote AR nuclear localization and promote cell proliferation. [13]

1.2 Proximity-Directed Protein Labeling

Nature utilizes proximity effects with enzymes to accomplish good specificity and high reaction rates. Affinity labeling was introduced by Singer at al. in the early 1960s [14]. It's about placing a chemically or photochemically reactive group on a substrate or ligand to covalently modify a target protein upon specific recognition (Figure 1.1) [15]. In proximity-directed protein labeling, a protein binding ligand and a functional group are connected by a linker. Similar to enzymatic reactions, the local concentration of labeling reagent is raised after specific binding.

Different from gene manipulation and molecular biology based modifications, which are also valuable to study the structure, dynamics, function, and cellular localization of protein by protein labeling and live cell imaging, chemical approaches in studying proteins hold the advantage of relatively small probe size and less perturbations within the complexed biophysical environment. Synthetic chemistry based approaches has overcome limitations that existed in other techniques and have been widely used since first reported in the early 1960s. Great efforts have been done to fine-tune the chemical structures of both the reactive group and ligand, which have led to protein labeling with excellent reactivity and target selectivity in the whole proteome of living cells. The chemical probes used for protein labeling may inactivate target proteins, but more research is being done to resolve this problem to make it a

more valuable tool for the labeling and imaging of endogenous proteins in living cells and even in living animals.

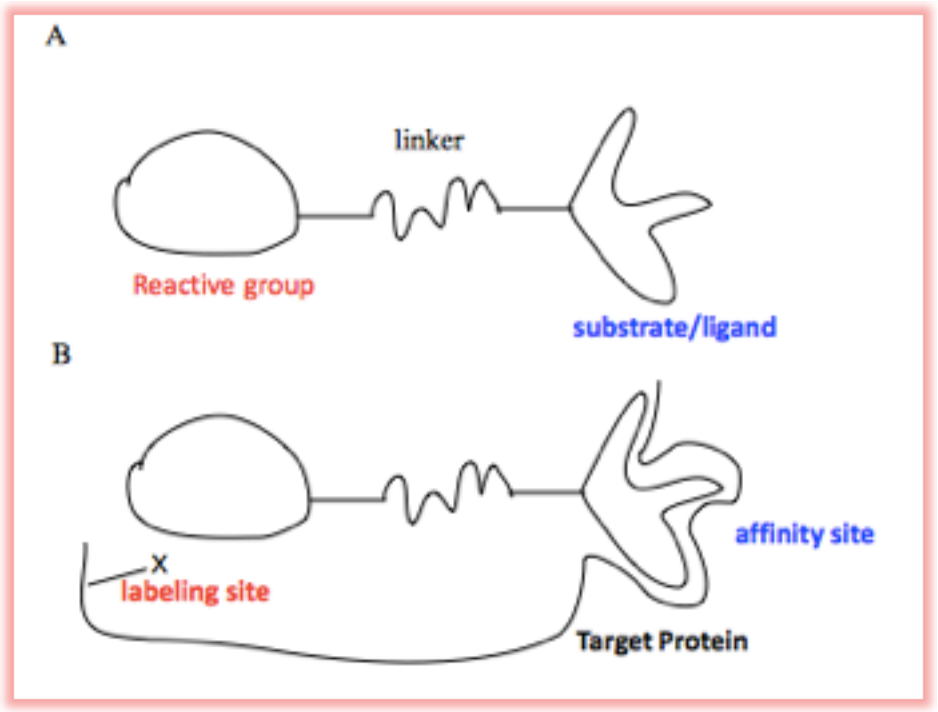

Figure 1.1: A. Affinity probe; B. Affinity interaction between the probe and target protein

Hamachi group has worked on chemically affinity labeling since their first report that was performed in test tubes [16], and they have developed several new ways (like LDT and AGD) in affinity labeling and achieved labeling and monitoring labeled proteins in cell lysates, living cells, and ultimately to living animals [17, 18]. They have also explored a traceless affinity labeling, which allow for dissociation of the ligand after labeling reaction so that the labeled protein retains its native function [15]. All these tudies proved that high specificity and biocompatibility could be

achieved with present techniques in protein labeling and monitoring even in miscellaneous native environments.

Proximity-directed labeling can be designed and tuned in different aspects. The length of the linker depends on the target site and the binding site. A longer linker means more flexibility and lower local concentration. The reactive site is essential to the labeling both in efficiency and specificity. An over reactive functional group will lead to a considerable amount of nonspecific labeling and possibly rapid hydrolysis. As with YZ06 that will be discussed later, a less reactive functional group that is more selective [19, 20]. For binding sites, the distance between they and target sites is important in the probe design. There are three binding sites in the LBD of AR (Figure 1.2): coactivator binding site called AF-2, hormone binding pocket and a surface allosteric site named BF-3 [21, 22]. The allosteric change that AR undergoes upon BF-3 binding was proposed to prevent its association with coactivators. Also, the BF-3 site is close to the lysine in the coactivator binding interface and hinge region [22]. All these rendered BF-3 a good choice for the binding part of the proximity directed reagent. Of the binding small molecules included are triiodothyronine (T3), flufenamic acid, tolfenamic acid, 3,3', 5-triiodo thyroacetic acid (TRIAC) [22]. These small molecules were ruled out as they do not have specificity for AR binding. Tolfenamic acid was found to have better potency and also inactive in other pathways in cells [22]. In Koh group lab, we found the tolfenamic amide to be a potent AR antagonist and conjugated it to a small catalytic acyl transfer molecule to transfer acyl groups to proximal lysines on the BF-3 domain of AR [19].

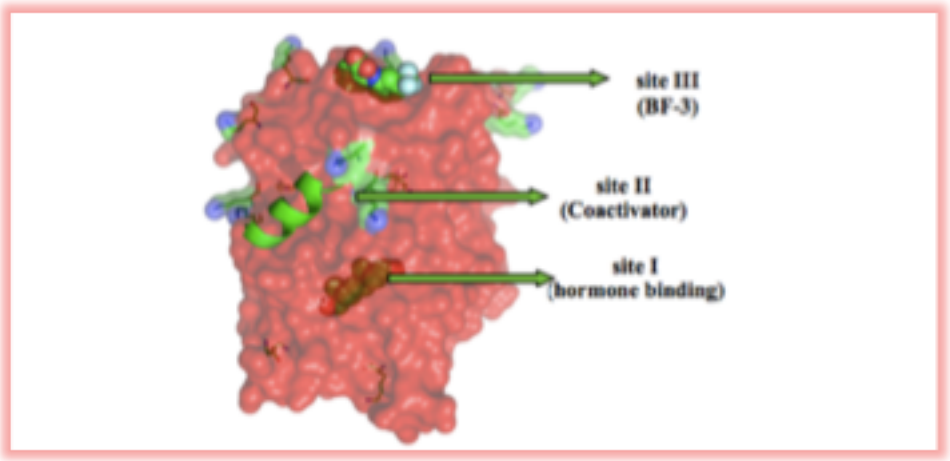

Figure 1.2: Three binding sites in AR LBD domain

1.3 Catalytic Small Molecule in Acyl Transfer

If AR nuclear localization can be reduced or even prevented by AR NLS modification, a catalytic modification reagent directed by proximity labeling is of great potential in PCa treatment.

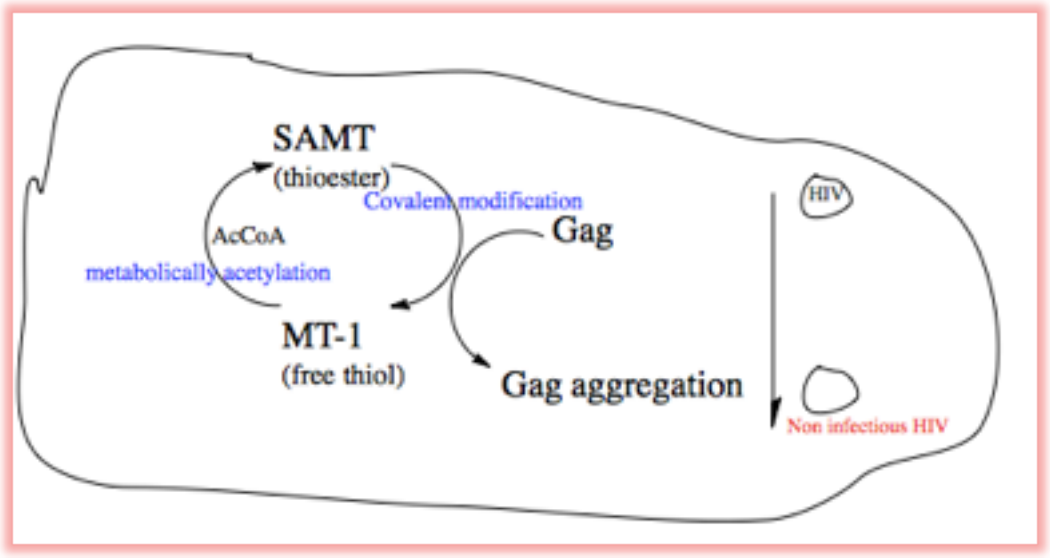

Figure 1.3: HIV inhibition process with SAMT

1.3.1 SAMT

It was shown that S-acyl-2-mercaptobenzamide thioester (SAMT) chemotypes could inhibit HIV by irreversibly unfolding the NCp7 region, the zinc finger motifs of the HIV-1 nucleocapsid protein Gag, in infected cells and reduce viral activity [23]. The thiol left after SAMT transfers its acyl group to NCp7, and was then acetylated by cellular enzymes to regenerate active SAMTs via a recycling mechanism unique among small-molecule inhibitors of HIV（Figure 1.3）[24]. *In vitro* experiments show that irreversible intramolecular transfer of the acyl group rapidly follows the reversible acyl transfer between the SAMT and the sulfur side chain of Cysteine to one of several proximal lysine residues [25]. Further experiments with SAMT247 show that after its reaction with NCp7 (the MT-1 form) render it inactive *in vitro*, but MT-1 is activated upon entering cells or when incubated with acetyl-CoA *in vitro* to regenerate SAMT-247 (Figure 1.4 A) [23, 24].

Thus, **MT-1** (Figure 1.4 B) is a small molecule with a structure that can be metabolically acetylated and then directed them to a new protein target. We envisioned that the structure of thiosalicylic acid amide can be conjugated to specific ligand to target AR as a way to enhance antagonist function [19].

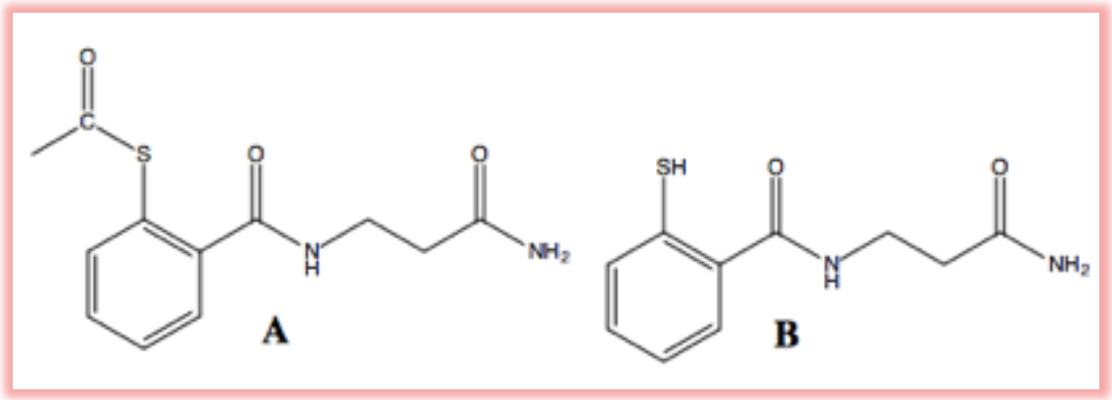

Figure 1.4: A. SAMT-247, a simple SAMT with good antiviral activity and low cellular toxicity in cells; B. MT-1, Mercaptobenzamide thiol

1.3.2 Hamachi DMAP

DMAP (4-dimethylaminopyridine) is a well-established acyl transfer catalyst in organic synthesis, which can activate an acyl ester for transfer to a nucleophilic residue [27]. Hamachi group has developed Affinity-Guided DMAP (AGD) method using ligand-directed acyl transfer catalyzed by DMAP (Figure 1.5)[26]. In the presence of appropriate acyl donors (basically thioesters), the conjugate of DMAP and an affinity ligand will facilitate the acyl transfer to a nucleophilic amino acid residue in the proximity of target site of the protein. DMAP act not only as an activator but also as a base, thus accelerate the acyl transfer greatly, and the rate of acyl transfer with di- and tri-DMAP was enhanced by 4.7 and 11.5 fold respectively [18].

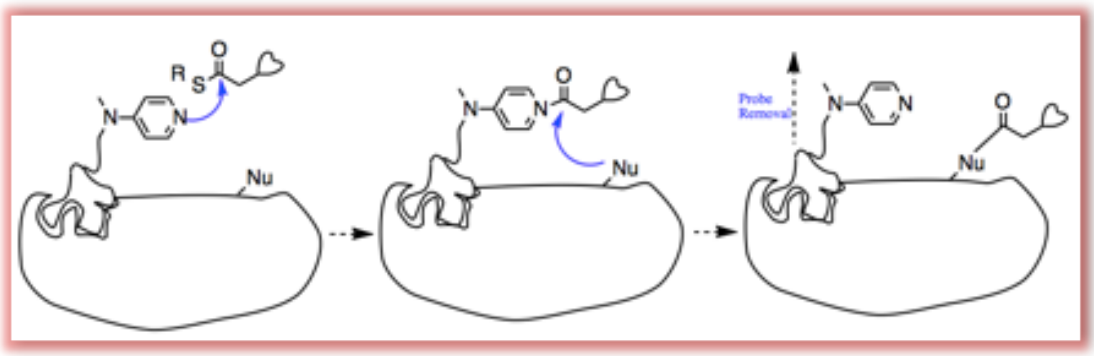

Figure 1.5: Ligand-directed acyl transfer catalyzed by DMAP

1.3.3 YZ03 and YZ06

Coupling MT1 analog with tolfenamic amide by click reaction, Ethan Zhang in our group synthesized YZ03 (Figure 1.6). Upon YZ03 treatment, AR acetylation was observed in comparison to the negative control in which separate ligand and catalytic molecule was used. It was proved by Orbitrap that the acetylated lysine 720 and several other lysine residues are proximal to the BF-3 binding pocket [19]. The conjugate is metabolically charged as acylthioesters and can direct acyl group to target AR and then get recharged like an endogenous acyltransferase.

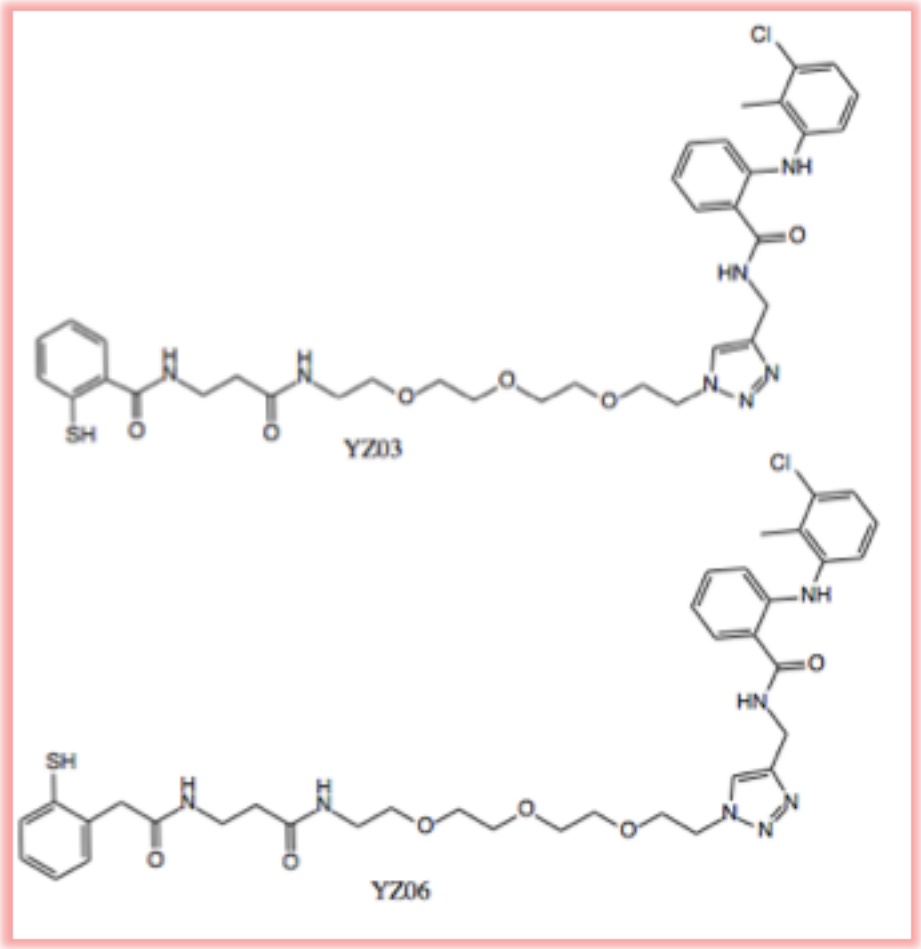

Figure 1.6: YZ03 and YZ06

As the less reactive version of YZ03, YZ06 (Figure 1.6) has an extra methylene group to break amide-thiophenol conjugation and shows higher efficiency and lower background labeling [19]. Besides showing the irreversible covalent acyl labeling is it is as fast as the the acyl labeled conjugate. This also suggests that the reactivity of the thiol group could be fine-tuned by modifying the structure to improve specific acyl transfer. Overall, the labeling efficiency is affected by the thiol reactivity, ligand proximity and affinity.

1.4 Click Reaction for Protein Labeling

Click reaction has a good biocompatibility as it tolerates a broad range of reaction conditions (salt, buffer, detergent concentration, pH, temperature etc.). In the click reaction, Cu(II)SO4 serves as the precursor to Cu(I) that catalyzes the 1,3 dipolar cycloaddition between azide and terminal alkyne to form a stable triazole. Tis-carboxyethyl phosphine (TCEP) or ascorbic acid could be used to reduce Cu ion in the fresh mixture. Tris (benzyltriazolylmethyl) amine (TBTA) is a poly triazole ligand that stabilizes the Cu(I) ion and enhances its catalytic activity in the solution [28]. The click reaction has been used in bio conjugation of small reporter groups and large biomolecules, and a variety of azide- and alkyne- reporters have been designed [29] or are commercially available. The reporter groups are clicked after covalent modification of the probe to the target protein, and thus minimize the perturbation of adding a labeled group while allow detection or monitoring of target. Compared with radiolabels, the click reaction costs less, require less in operation and equipment while maintain high sensitivity. In addition, the addition of a chemical group like biotin or TAMRA could be utilized in affinity purification.

1.4.1 Fluorogenic Reactions

The fluorogenic click reactions could be used in bioorthogonal conjugation probes for tracking and imaging a biological process with low or no background. Nonfluorescent 3-azidocoumarins and terminal alkynes can undergo click reaction to give fluorescent 1,2,3-triazole products（Figure 1.7）. Coumarin is biocompatible and easy to manipulate. Coupling an azide to the position 3 of coumarin could quench the fluorescence; while with the removal of the azide group upon triazole formation, the whole thing becomes fluorescent [30]. The reaction goes to completion under 0℃,

which can be taken advantage of to slow down protein degradation or other similar process. The Wang group has built a large library of pure fluorescent coumarin dyes and tested their fluorogenic properties. Of which some of the pairs could be directly used in our experiment.

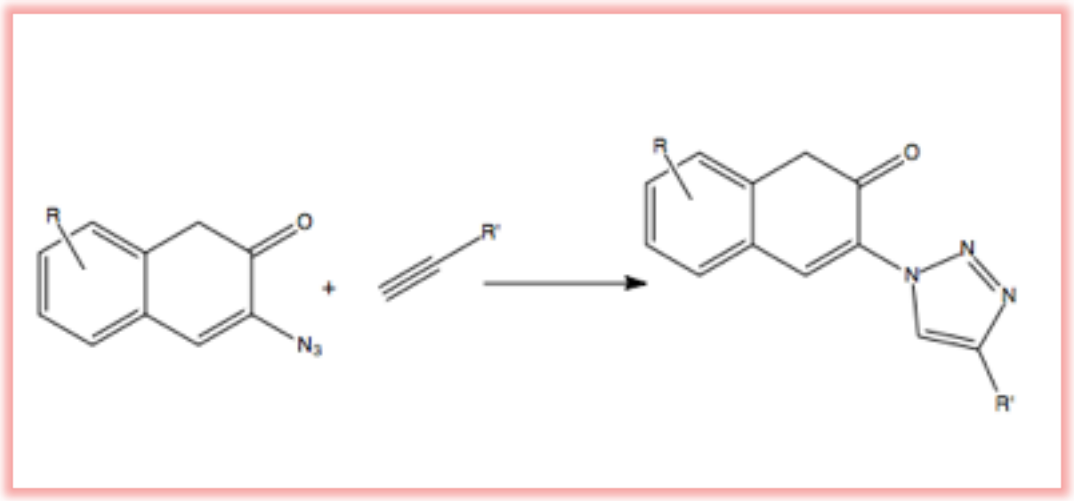

Figure 1.7: Fluorogenic click reaction with Nonfluorescent 3-azidocoumarin

1.4.2 TAMRA-azide Reporter

If the fluorescent signal of the fluorogenic click reaction is not strong enough, a linked fluorophore like rhotamine could be considered. TAMRA azides are commercially available and could be used in alkyne tagged protein detection (Figure 1.8)[31]. To reduce the background, protein samples could be treated with dialysis, filtration or precipitation. Furthermore, affinity purification with anti TAMRA antibody could be performed to pull down even the fragments of the protein to detect the efficiency of the prior labeling step [32].

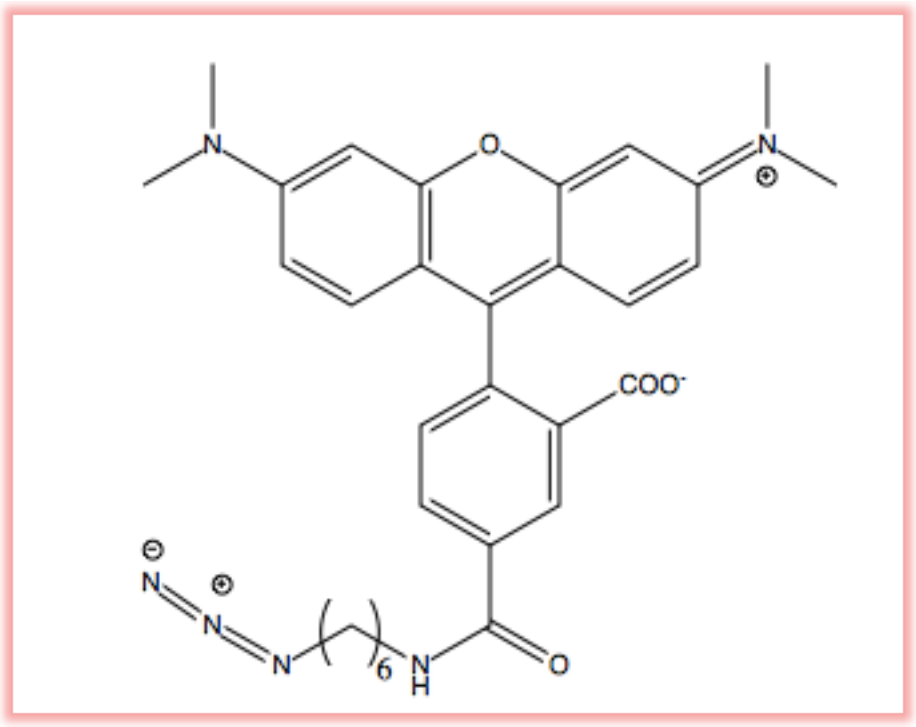

Figure 1.8: TAMRA(ThermoFisher)

1.5 Thesis Overview

The catalytic acetylation activity has been confirmed by Ethan Zhang in our group and detected with radioactive labeled acetyl substrate and western blot [19]. The work described in this thesis is to evaluate a proposed better detection method and the click reaction pairs could be utilized in future work to optimize the specificity of labeling.

For the design of the click reaction, the reaction pairs were prepared as the 'acetyl substrate' with alkyne group and pre-fluorescent probe small molecule azide in the fluorogenic click or TAMRA azide in the nonfluorogenic click. We used 4-pentynoic acid (4PY) or its thioester as the alkyne part, as 4PY-CoA was mentioned a relatively good substrate of p300 [33]. The scheme is drawn as Figure 1.9 shows.

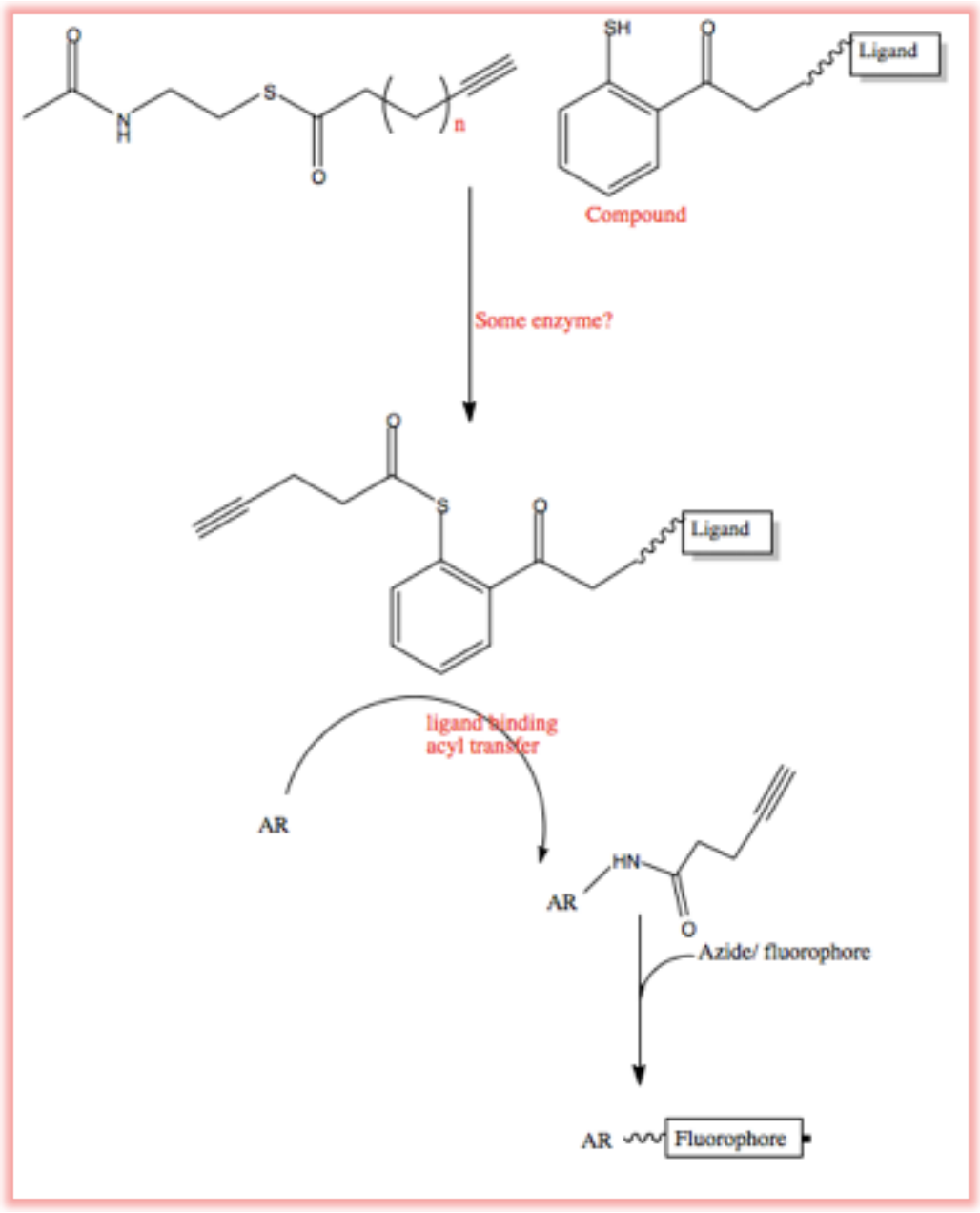

Figure 1.9: General design and scheme (The metabolically acetylated process has not yet been proved to be an enzymatic process)

The detection process is first performed with small molecule reference and BSA reference, and then was performed with labeled cell lysate. We hope to see selective labeling on SDS PAGE showing our expected labeled AR bands with relatively low background. But we are not surprised to see the following possible problems.

First, since AR abundance is relatively low and may not standout on the gel and may not be able to be detected directly from the background, thus enrichment of AR (like subcellular fractionation, acetone precipitation, affinity purification et. al) could be performed to raise signal.

Also, the background noise could be very high due to the high concentration of probes, total protein (non-specific labeling) or even high levels or degradation. Thus proper storage and optimal experiment conditions are to be explored.

Additionally, in Hang's paper, they performed proteomic study of alkyne labeled CoA as the substrate for acetylation and found a wide range of proteins get labeled by throwing in the salt form of the alkynyl-acetate analogs [33]. The labeling process could probably be conducted by different KATs or even some chemical acetylation. So we probably would see multiple bands light up including AR. Also, they showed that the acetylation is analog-specific and cell type-specific. So in future work, we will try different carbon chainlengths or even different structures to make the probe more exclusive utilized by our labeling method.

In total, this thesis provides a non-radioactive way to detect the efficiency and selectivity of our catalytic proximity affinity-labeling probe. The next step could be modifying our probes to make the method more specific and efficient. And we will also look into the details of the labeling, like the percentage of AR labeled, identify

which lysines get labeled and the downstream effect, and possible applications towards improved PCa treatment development.

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Chapter 2

CLICK REACTION IN ACYLATION DETECTION

2.1 Coumarin-azide and Thioester Alkyne

As the previous chapter mentioned, 4-pentynoyl-CoA could be good substrate for p300-KAT, and could be utilized for protein acylation. Also, 4-pentynoic acid could be metabolically charged onto CoA and thus be utilized for acyl transfer catalysis. The Hang group showed that they are good chemical reporters for protein acylation [1]. NAC analogue, a CoA mimic that can be coupled with 4-pentynoic acid to be the alkyne-acyl transfer substrate. It's much more simple to synthesize than CoA, and it's relatively small so that may be easier to be taken up by cells.

The synthesis of coumarin-azide was performed by Wei in our group following the procedure published [2]. The one that was chosen (3-Azido-7-hydroxycoumarin) showed strong intensity of fluorescence that's within 490-499nm after click coupling with several alkynes.

2.2 Characterization of Small Molecule Click Product and Standard Curve

Organic compound are easy to synthesize, quantify, purify and could be a good reference in solution. Series dilution of small molecule click product was set for a standard curve, so that the percentage of labeled groups could be calculated.

The synthesis and click reaction setup was described in chapter 3. The small molecule click product was prepared as a saturated solution and test its featured wavelength on the fluoremeter. The fluoremeter test result showed that the highest

excitation is around 425nm, the emission peak at 470nm, while UV absorption is around 400nm (figure 2.1).

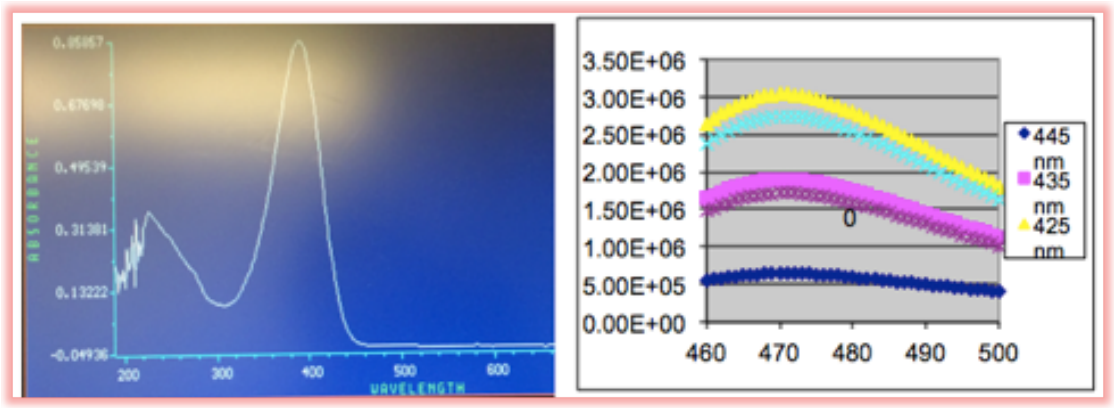


Figure 2.1: A UV absorption Spectra of small molecule click product; B Excitation Spectra of small molecule click product (fluoremeter)

The small molecule click product was prepared as a stock of 181.52µM, and was diluted each time by half. All the samples were excited at 396nm and the emission value were recorded for 470nm. As the sample was diluted, the peak become less sharp and finally wavy and difficult to distinguish from noise when the concentration is around 0.02µM. This shows that the detection limit on the fluoremeter can go down to that value. The standard curve shows different linear relationships within different ranges, the range close to the test value was graphed separately.

Table 2.1: Small molecule click standard curve (fluoremeter)

Sample#	Molar concentration (μM)	CPS
1	181.52	5654870
2	90.76	6943055
3	45.38	6786970
4	22.69	4259425
5	11.345	1705380
6	5.673	1452255
7	2.836	910370
8	1.418	425800
9	0.709	221320
10	0.3545	111900
11	0.1773	50900
12	0.0886	23360
13	0.0443	18235
14	0.0222	9020(wavy)

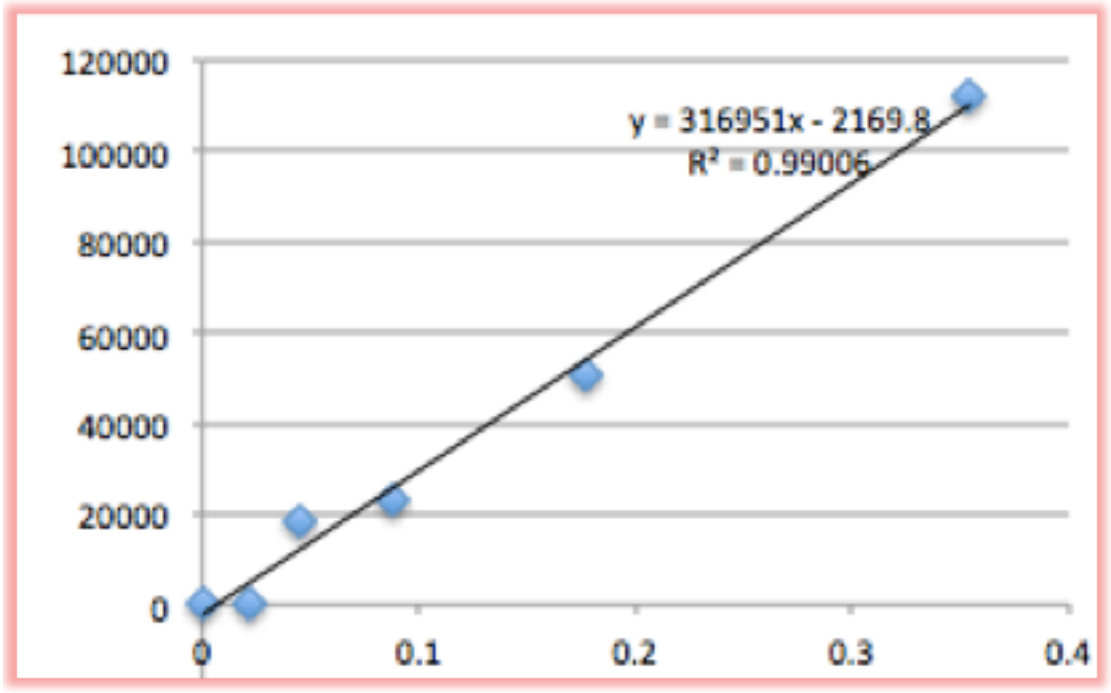


Figure 2.2: Standard curve-test range

2.3 BSA-click for Gel Reference

BSA is soluble, stable and easy to handle. Labeling alkyne groups onto BSA before click reaction would provide a reference for on-gel scanning detection. Fitting the BSA click reference into the mentioned standard curve above, and then running both BSA-click and AR-click on gel, would facilitate the calculation on relative AR-click level (or acylation level) when projected onto the standard curve of solution reference. Study was focused on the detection limit, optimal reaction condition (ratio, concentration, incubation time etc.) as reference for the following experiments.

2.3.1 BSA labeling

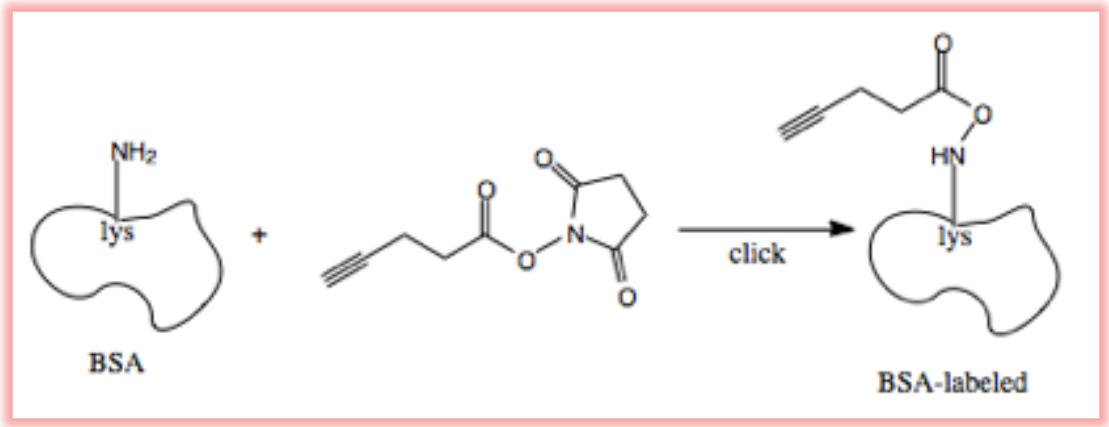


Figure 2.3: BSA labeling

Dissolve NHS ester in DMSO 2.5mg/ml. After reaction, it could be stored in solution for 1~2 months at -20℃. Dissolve BSA in sodium bicarbonate buffer (50ml, pH= 9.3, 0.05M) to 10mg/ml. Transfer 200µl NHS ester stock drop wise to the 1800µl protein stock solution in a 10ml vial upon stirring at room temperature, and stir for 3

hours. Purify BSA using Centrifugal Filter Units. Wash twice with 2ml Phosphate Buffered Saline (PBS) (1L, pH=7.5, 100mM) buffer then dissolve it in 2ml PBS buffer.

2.3.2 BSA-click

CuAAC the click reaction was carried out by adding 6µl of freshly pre-mixed click cocktail (100µM coumarin azide, 1mM sodium ascorbate, 100mM TBTA and 1mM $CuSO_4$) and 2µl labeled BSA to 42µl distilled H_2O at room temperature.

Table 2.2: Pre-mixed click cocktail

100µM azide	10mM in DMSO	1/100
1mM sodium ascorbate (or ascorbic acid)	50mM in deionized H_2O	1/50
100mM TBTA	10mM in DMSO	1/100
1mM $CuSO_4$	50mM in deionized H_2O	1/50

The reaction was shaken in darkness for 1h, and proteins were subsequently run on a 15% SDS-PAGE and in-gel fluorescence scanning was performed. For SDS-PAGE, serial dilution of the BSA click product was made by serial dilution for gel detect. The sample was mixed with equal amounts of loading buffer. Of the 50µl (25µl sample mix with 25µl buffer), only 20µl was loaded in each well. The lanes were loaded with: BSA-click with loading buffer; 1/2 concentration BSA-click with loading buffer; 1/4 BSA-click with loading buffer; 1/8 BSA-click with loading buffer and so on. On the resulting PAGE scan from the gel imager; the fourth lane gave the faintest band that could be seen. Calculation was as follows: 9mg/ml BSA* 2ml*(2/2000) *(20/50) *1000µg/mg=7.2µg for fist lane, so the 4^{th} one is about 900ng. This amount

is too high for one to be able to detect AR click from cell lysate. In the reaction system, the ratio of BSA to azide is about 1:2.22. To lower the detection limit, optimizing the reaction condition and scanning was considered.

2.3.3 BSA-click optimization and fit in standard curve

For the labeling step, if the pH is too low, the amine from lysine will show low reactivity; if too high, NHS ester will get hydrolyzed. We decided on a pH of 9.3. Since there're more than on lysine on the surface of BSA (with the pdb file, we counted around 60 amines on or close to he surface), we would like to see how different ratio of labeling reagent result on fluorescent intensity after click.

2.3.3.1 Plate reader detection

A plate reader was used as an alternate method of detection. The other methods are the fluoremeter and the gel imager. We chose the filters of excitation 440nm, and emission 480nm for the plate reader. Prepare Bicarbonate buffer and PBS, 2.5mg/ml NHS ester (in DMSO) and 10mg/ml BSA (in bicarbonate buffer). Add 200µl NHS ester drop wise to 1800µl BSA upon stirring, cap loosely, incubate one vial for 4h and the other for overnight for time length compare (after mixing, BSA become 9mg/ml). Purify BSA using Centrifugal Filter Units(30K, 50ml), set centrifuge to 4,000rpm and spin for 20min; wash twice with 2ml PBS, then dissolve in 20ml PBS buffer and transfer to a clean EP tube(5.42µM as final concentration). For the 4h labeled BSA, set 2 different conditions as A and B, and let click reaction go over night. A. 8ul BSA+16ul cocktail in 200µl(~1.5 eqv.) B. 8ul BSA+80ul cocktail in 200µl(~7.3 eqv.) Also for the small molecule, make it a saturated by diluting to around 1.25mg in 20ml distilled H2O (with 20µlDMSO), take 200ul into the 1st well in a

clear 96-well plate, then dilute with 20ml distilled H2O (with 20µlDMSO) each time by half from A-1 to C-10. Then take 100ul from 20ml distilled H2O (with 20µlDMSO) into C-12 as background. Put the overnight click of A and B in C-2 and C-1. The result is shown as the following table and graph (saturation is reached for the first few wells and was dismissed as out of range; for the small concentration samples, the readings are close to background as also dismissed).

Table 2.3: Plate reader result (the value close to protein click is highlighted)

Position	Reading	Concentration(µM)
A-6	187233	5.67
A-7	90153	2.84
A-8	52782	1.42
A-9	37621	0.71
A-10	24622	0.35
A-11	20624	0.18
A-12	19117	0.089
B-1	19334	0.044
B-2	16101	0.022
B-3	15269	0.011
C-1	35801	5.42
C-2	17918	5.42
C-12	15018	0

If counted as mono-labeling, the higher equivalent labeling looks like 13.1% of labeling, and much higher than the low equivalent labeling, which indicates the ratio could be even higher to make the labeling to near completion.

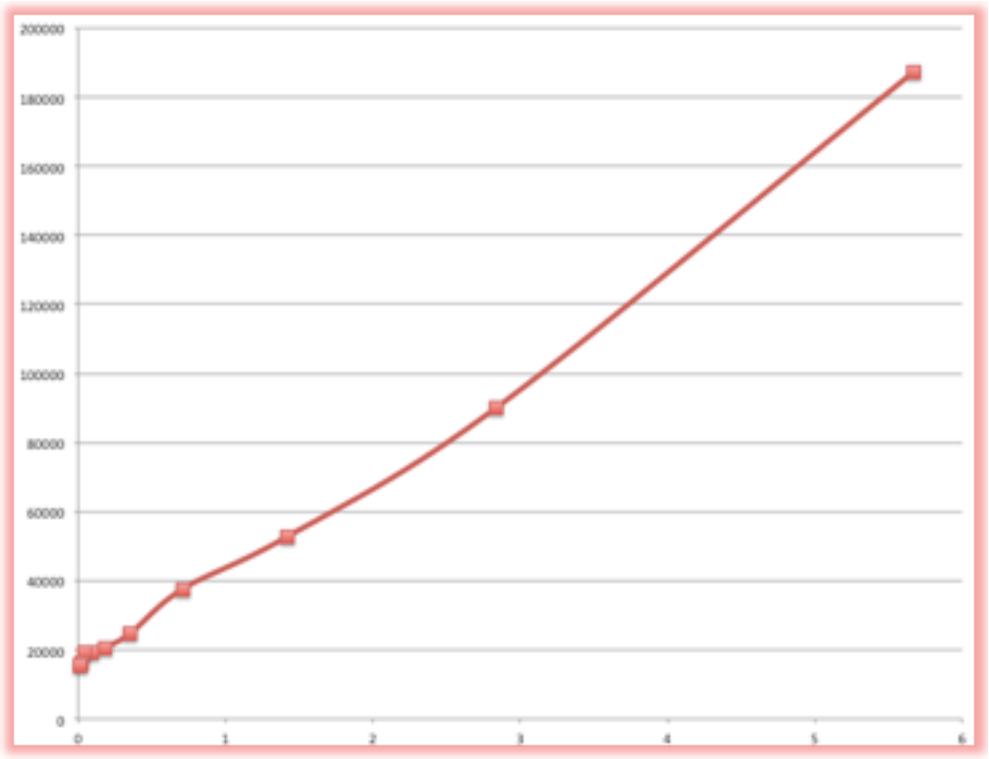


Figure 2.4: Plate reader result plot (from A-6 to B-3, C-12 background subtracted)

The experiment was later repeated with Costar opaque plate to lower the background. The results showed that the significant reading could go to lower concentration. The ratio of azide to protein also changes the reading, but since the molecule may absorb some excitation lights, the reading may not be as accurate for fluorescence intensity. The overnight labeling seems stronger, but not significantly. For the repeated experiment, the readings for the same sample varied from the previous experiment, suggesting the plate reader is not the ideal detecting method. Also, the signals increased as concentration of cocktail concentration increased but do not vary much as the protein concentration changed.

We're concerned about detection method range and limit and if it's good for our purpose. And we're also wondering about how many groups are being labeled and clicked for one protein molecule, also what percentage of the protein gets labeled. Drying a gel to reduce fluorescent quenching and raise signal is considered.

2.3.3.2 In-gel fluorescence scanning

The BSA click samples and the labeled BSA are subject to SDS-PAGE for further confirmation of the fluorogenic reaction. This would also facilitate the following in-gel fluorescence scanning experiments.

For labeled BSA, dilute 2ul into a 50ul solution system, and then take 10ul sample for loading so the concentration would be the same as BSA click samples. Coomassie blue staining was followed for further confirmation.

From the scan, we can observe faint signal for labeled BSA before click reaction, and the signal is strong for the ladder, which is not fluorescent. This indicated that the signals are not necessarily from fluorescence. The post- stain result indicated equal loading of samples, which confirmed that most signals were from fluorescence.

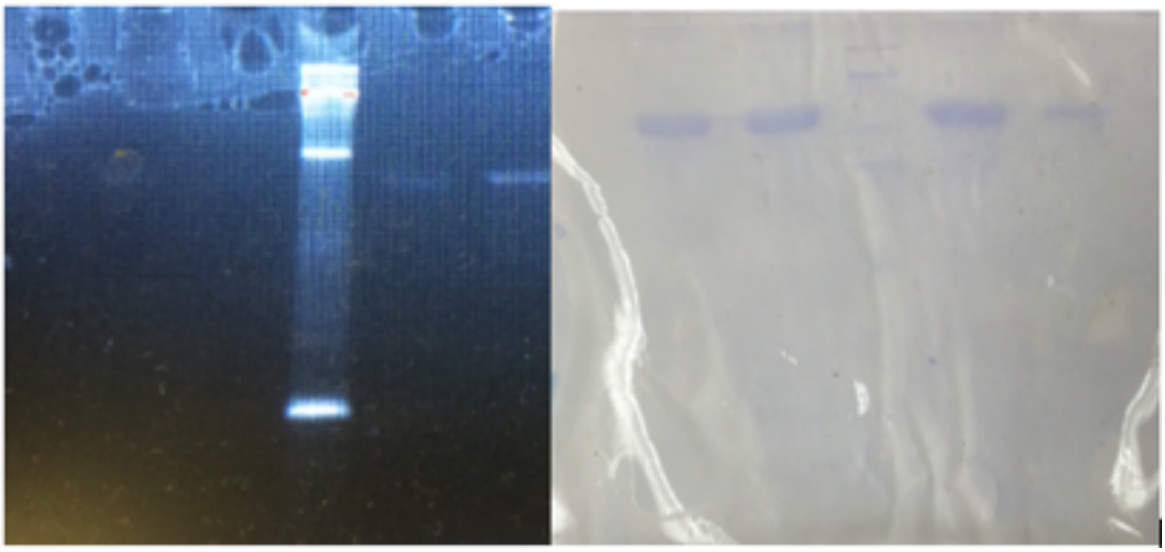


Figure 2.5: SDS-PAGE (before and after stain) From left to right: labeled BSA overnight; labeled BSA 4h; Novex R sharp pre-stained protein standards 2ul; click with 3eqv. cocktail; click with 10eqv. cocktail.

2.3.3.3 Fluoremeter

BSA samples were prepared as shown in Table 2.4. Both steps are allowed to go overnight. The BSA stock was 10mg/ml, 1.8ml BSA was put in a 3ml labeling system as a concentration of 6mg/ml. For click step, 20μl of previous step solution was put in a 1ml click reaction system. The BSA concentration was 120mg/L, or 1.8μM.

Peak shift from 460nm to 470nm was observed from sample A to C, also D to F. For high ratio click reaction, brown precipitate was seen at the step of using Amicon centrifugal unit dialysis, which may cause aggregation of proteins and inaccuracy in the test of sample G to I. BSA-label without click was tested as 7280 as a background. To fit sample F into the standard curve in Figure 2.2 B, 18% of mono labeling was obtained.

Table 2.4: BSA sample treated with different reaction ratio

1:1 label	1:10 label	1:40label	Samples and readings
Sample A 7490	Sample B 21060	Sample C 32870	1:2.5 click
Sample D 30000	Sample E 72700	Sample F 112070	1:10 click
Sample G 12020	Sample H 12220	Sample I 12130	1:50 click

The experiment above showed that the two-step label and click can get about 10~20% mono-site signal for BSA. We expected the same test level in the following experiment for AR.

2.4 AR Acyl Transfer *In Vitro* Assay

Plasmid containing AR-LBD was transformed into PET41a BL21 bacteria cell line and was kept at -80℃. The protein AR-LBD was expressed and purified for the *in vitro* acylation assay. The Bradford assay gave the concentration of AR-LBD as 341ng/μl, or 682μM. PBS buffer was adjusted pH to 7.4 and protease inhibitor was added.

Since the protein is much more diluted than BSA in previous labeling, the reaction rate would drop if the ratio were kept the same. The assay was set up as follows:

Table 2.5: *In vitro* acylation assay

	AR-LBD 341ng/μl stock	4-pentynoic acid 10mM	alkyne thioester 5mM	YZ03 1mM stock	PBS	click cocktail (20mM azide)	PBS
μl	20	1	/	1	28	5	45
μl	20	/	1	1	28	5	45
μl	20	1	/	/	29	5	45
μl	20	/	/	/	30	5	45
	Acylation labeling reaction					Click reaction	

Both steps were incubated overnight at 37℃. 20μl was taken out for SDS-PAGE, which is around 1.16μg of protein both after 3 hours and overnight. The resulting gel had smeared bands and showed little fluorescence. The next step is to concentrate the protein for noticeable signals and also set labeled BSA as positive control to run click reaction. Also the ladder showed too much signal and should reduce in loading amount and load in the lane far aside to avoid interference. The labeling step requires the whole LBD domain to proceed proximity-directed labeling, while the second step is all about testing the level of alkyne group on the protein, so that the protein could be denatured with SDS detergent to further expose the alkyne groups.

The fluorogenic click reaction may not give enough signals for the detection device we have. So TAMRA-azide is the next step for the *in vitro* assay detection.

2.5 In Cell Assay

HEK 293T cells were counted and seeded in 100mm dishes (1,000,000) using phenol red free media. Perform $Ca_3(PO_4)_2$ transfection the next day (20μg hAR), the cells were washed with DPBS and new media was added containing appropriate compound (in table 6) and incubate for 36 hours.

Table 2.6: Cell Assay

	4-pentynoic	alkyne thioester	YZ03	SAHA
A	100μM	/	20μM	5μM
B	/	100μM	20μM	5μM
C	/	100μM	/	5μM
D	/	/	/	5μM

Harvest with RIPA lysis buffer. Pour off media and keep the 100mm plate on ice all the time. Wash the cells gently with ice cold PBS buffer, and then add 0.2ml PIPA lysis buffer (with 1mM PMSF, 50μMDHT and protease inhibitor Minitab) to each plate. Swirl the plate and then use a cell scraper to scrape the cell layer off. Transfer the lysis buffer with cells into 1.5ml eppendorf tube, and then incubate on ice for 15 minutes. Run the whole thing through a 22-gauge needle several times to make it less viscous, and incubate 15 more minutes. At 4℃, centrifuge the lysate at

13,000rpm for 5 minutes and collect the supernatant. Determine the protein concentration with Bradford assay.

The cell lysate was separated into two parts; one part was subject to immunoprecipitation and the other part was adjusted to 0.5-10mg/ml of total protein for click. The protein samples were in 2% SDS buffer (pH7.4) to denature the protein and expose the terminal alkyne to the click reagents.

For immunoprecipitation, 100 µl lysate was added 1 µL anti-AR (N20, Santa Cruz) and was rock at 4℃ overnight. 10 µl prewashed protein A beads was added and the solution was rocked for another 4 hours. Place the tubes containing the beads in the magnetic separation rack and wait 10-15 seconds for the solution to clear before carefully removing the supernatant. Wash pellet 3 times with 500 µl cell lysis buffer. Resuspend the pellet with 20-40 µl 3X SDS sample buffer and vortex. Heat the sample to 95–100°C for 5 minutes and micro centrifuge for 1 minute at 14,000 rpm. Load the sample (15–35 µl) on SDS-PAGE gel to check result.

The protein samples were mixed with click cocktail with a ratio of protein to azide as 1:10 and 1:40. The reaction was incubated at 37℃ for 6 hours before they were put to SDS-PAGE. Run the protein sample on a 12% SDS-PAGE. Run the dye front off the gel to make sure the click reagents will make less effect on the imaging analysis.

The pulled down AR showed was confirmed on SDS-PAGE and fluorescence was observed against negative control (standards not shown). While the whole lysate showed smeared bands (Figure 2.6).

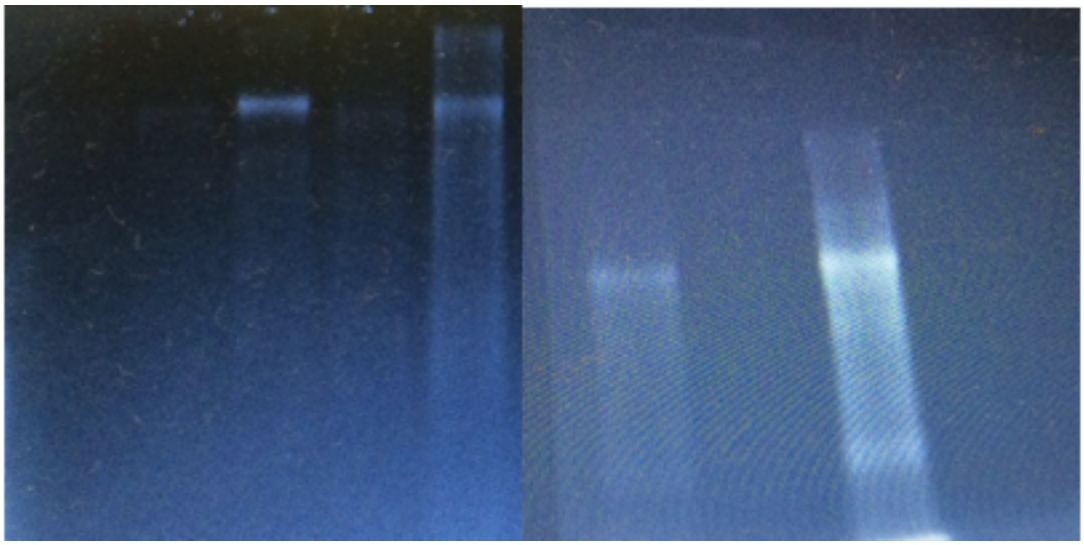


Figure 2.6: Cell assay result (left for IP sample; right for whole lysate)

The result showed that as the total protein concentration rises, the background labeling is raised. The whole lysate gel showed poor selectivity of acylation.

2.6 Conclusion

The acyl transfer level was tested as around 5% in our group using other methods. So the fluorescent scanning detection method should efficiently provide signals strong enough for the low concentration level of AR. If AR is pulled down from the various other proteins in cell lysate, the selectivity is not given from the detection. Our next step is to use TAMRA-azide instead of fluorogenic click reaction to provide noticeable fluorescence. Since 4-pentynoic acid and its NAC analog derivative are good substrate for other KATs, the selectivity is not as good as the alkyne part could be utilized by acyl CoA synthetases in living cells. The future study is focused on two points: 1. adjusting the alkyne substrate structure to make it exclusive for our proximity-directed catalytic acyl transfer compound; 2. Improve the

detection conditions to raise signal to noise ratio (adjust filters of imager, get the two steps to higher yield, reduce any factors that quenches fluorescence).

REFERENCES

1. Yang Y Y, Ascano J M, Hang H C. Bioorthogonal chemical reporters for monitoring protein acetylation [J]. Journal of the American Chemical Society, 2010, 132(11): 3640-3641.

2. Sivakumar K, Xie F, Cash B M, et al. A fluorogenic 1, 3-dipolar cycloaddition reaction of 3-azidocoumarins and acetylenes [J]. Organic letters, 2004, 6(24): 4603-4606.

Chapter 3

EXPERIMENTAL

General Considerations

All compounds were purchased from Acros Organics (Morris Plains, NJ) or Aldrich Chemical Co. (Milwaukee, WI) unless otherwise specified. Tolfenamic acid was purchased from Cayman chemicals and ASDI, Newark, DE. 5(6) TAMRA PEG3 azide was purchased from Tenova Pharmaceuticals. THF was distilled from potassium; methylene chloride and toluene were distilled from calcium hydride. Silica gel (60Å) was purchased from Silicycle (Quebec, Canada) and TLC plates (60Å, 250 μM) were purchased from Merck (Whitehouse Station, NJ) and Dynamic Sorbent (Atlanta, GA). NMR spectra were recorded on a Bruker AVII 400 MHz and Bruker AV 600 MHz spectrometers at the University of Delaware. Chemical shifts are reported in δ and J values are reported in Hz. Mass spectrometry was performed at Liquid Chromatography/ Mass Spectrometry System (LC/MS) in the Delaware Mass Spectrometry Laboratory. Fluoremeter, SpectraMax Plus 384 Microplate Spectrometer and gel imager scanning was performed at Chemistry and Biochemistry Department in University of Delaware. HEK-293t cells were purchased from ATCC (American Type Tissue Collection, Manassas, VA). Plasmids of AR-LBD and hAR were generously provided by Kay from Zondlo's group. RPMI 1640, Dulbecco's modified Eagle Media (DMEM), Sodium pyruvate solution, and DPBS were purchased from Corning Cellgro. Phenol red free RPMI 1640 were Purchased from GIBCO. EDTA free protease cocktail mini tablets were purchased from Roche Applied science. GST

binding kits were purchased from Novagen. Amicon centrifugal 10k units were purchased from EMD Millipore. AR (N-20) Antibody was purchased from Santa Cruz Biotechnology. High capacity streptavidin beads were purchased from Thermo Scientific.

Expression and purification of AR-LBD

1M stock solution of DHT was prepared. LB broth with kanamycin (50ug/ml) was prepared for starter culture. 2X LB broth with kanamycin (50ug/ml) was prepared for use after starter culture.

Three 10ml starter culture was inoculated by scraping the solid -80℃ BL21 cell (transformed with PET41a AR-LBD plasmid) stock with an inoculation loop. One of the 10ml cell culture was added into 500ml 2X LB broth. The 500ml cell culture was put to shake under 37℃ for 5 h and then was moved to 16℃ incubator. Induction was performed when OD hits 0.7~0.8. 1ml of the non-inducted cell culture was put in a separate tube for reference in expression confirmation SDS PAGE. For induction 0.4mM IPTG and 50μM DHT was added, then the cell culture was put back to shake at 16℃ overnight. Cells were collected by spinning down at 5k/min for 20 min, the cell pellets could be stored at -20℃. The pellets were re-suspended with GST binding buffer (1/100-1/20 0f culture volume, recipe comes with the kit) with 50μM DHT and 0.2mM PMSF (or protease inhibitor cocktail, 1 tab in 10~20ml buffer). Sonication at 40 for 20s, 4 times (sample was kept on ice). Centrifuge for 20 min at 15k/min, then run the supernatant through a 0.2μm filter. Load the lysate to the pre-equilibrated GST column, wash with GST binding buffer, and elute with elution buffer (recipe comes with the kit). Check the presence of protein by Bradford assay. Dialysis with 10k

Amicon Centrifuge Units using buffer (DPBS with 5mM EDTA, 0.5mM DTT optional, and 10μM DHT) for 4 times. Quantify the protein with Bradford assay before assaying.

Cell culture

HEK293T cells were cultured in tissue culture flask in 1X DMEM media supplemented with 4.5g/L glucose, L-glutamine and sodium pyruvate, 10% Cosmic Calf Serum (CCS), and 500ug/ml streptomycin. Cells were incubated in a 5% CO2 humidified incubator at 37℃. The cells were passaged every four days and were diluted 1/20 each time for the passage.

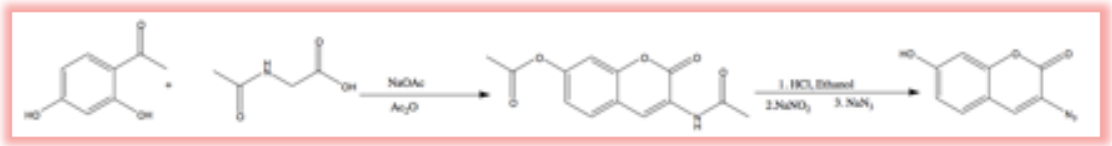
Transfection and Co-transfection

24 hours prior to transfection, HEK293T cells were seeded in 100mm cell culture dish using phenol red free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Dextran Charcoal stripped cosmic calf serum (DCC-CCS) (HyClone, Logan, UT) and 2 mM L-glutamine at a density of 1000,000 per dish. The cells were allowed to grow at 37℃ for 24 hours. The media was changed and the cells were allowed to grow to 40%-80% confluency. Then co-transfection of human AR (hAR) and pEGFP-N1 were performed using $Ca_3(PO_4)_2$ following the general protocol. 20μg of each DNA was mixed with 30μl 2.5M $CaCl_2$ solution and MilliQ H_2O to a volume of 0.5ml, then was added slowly by 200μl micro pipette into equal volume of 2×HEPES-Buffered Saline while vortexing. The solution was incubated at room temperature for 15min before added evenly to the culture dish, then was mixed by moving back and forth 3 times and put them back in 5% CO_2 incubator. The cells

were incubated for 6 hours. Replace the solution with complete growth medium. The expression of eGFP was monitored under epifluorescent microscope in the Koh cell culture lab to decide the efficiency of transient gene expression. Then cell assays are allowed to perform. There was transfection of only hAR performed later with a separate cell culture transfected with eGFP as the positive control to reduce the effects that may come with co-transfection.

SYNTHESIS

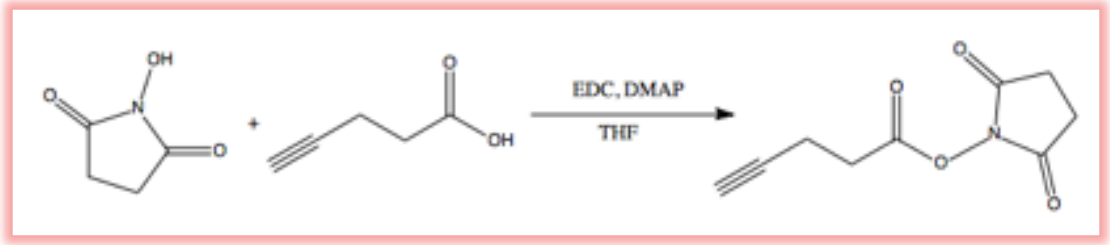
3-Azido-7-hydroxycoumarin [1]



Reagents were added in equimolar with 3x NaOAc in acetic anhydride, and the mixture was put to reflux upon stirring. product was checked with TLC. The reaction was cooled on ice to give product as a precipitate and was then filtered and washed with iced distilled water. Add concentrate HCl and ethanol to 2:1, dissolve the previous product in and reflux for an hour. Cool the reaction and mix 2x $NaNO_2$ in molarity into the solution and then add 3x NaN_3 slowly upon stirring. Filter out the precipitate and wash with cold water, dry under reduced pressure and obtain a brown solid powder. Store at -20℃ and avoid light.

^{1}H NMR(DMSO-d6, 600MHz) δ 7.56 (s, 1H), 7.44 (d, J=8.46Hz, 1H), 6.77 (dd, J= 8.52, 2.28, 1H), 6.72 (d, J=2.16, 1H). ^{13}C NMR (DMSO-d6, 600MHz) δ 160.75， 157.77， 153.24， 129.56， 128.34， 121.62， 114.26， 111.81， 102.52.

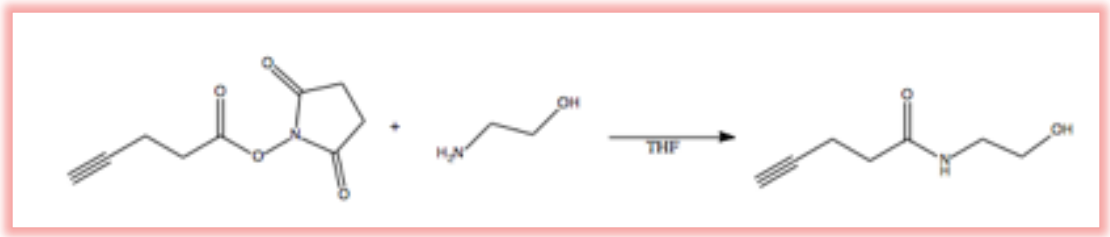
4-pentynoic acid succinimidyl ester



Dissolve 293.17 mg (1.53 mmol) EDC in 8ml-distilled THF in a 50ml clean and dry round bottom flask. Add 100mg (1.02mmol) 4-pentynoic acid and 311.34mg (2.548mmol) DMAP at 0 ℃ upon stirring. After 15 minutes, warm to room temperature, stir for 2 h to form intermediate compound. Add 117.3mg NHS (1.02mmol dissolved in 2ml THF) at 0 ℃ upon stirring, then warm to room temperature and stir overnight. This process should avoid any H_2O. Work up the reaction with 1:1 volume ratio of 0.1 M HCl, then extract with DCM twice and EA once. The mixture was washed with brine, dried over $MgSO_4$ and concentrated in vacuo. In 50 percent Hexanes/EA condition, the product shows an Rf of 0.57 upon $KMnO_4$ dye. Silica gel flash chromatography (50% Hexanes/EA) yielded product 4-pentynoic acid succinimidyl ester as a white solid (80.1 mg) in 40.23% yield.

^{1}H NMR ($CDCl_3$, 600MHz) δ 2.05 (t, J=2.7Hz, 1H), 2.62 (td, J=6.6, 2.7Hz, 2H), 2.85 (s, 4H), 2.88 (t, J=7.14Hz, 2H). ^{13}C NMR δ 168.88, 167.01, 80.84, 70.04, 30.32, 25.58, 14.11.

N-(2-hydroxyethyl) pent-4-ynamide (Another scheme was published in [2])



Add NHS ester 20mg (0.102mmol) and 1ml distilled THF to a dried, clean 10ml round bottom flask upon stirring. Add 6.26µl ethanol amine (0.102mg, dissolved in 1ml THF; it's better to be purified and dried before the reaction) dropwise at 0℃ and see white precipitate forms. Allow the reaction to return to room temperature after 5 min, stir over night and follow the reaction with TLC. In 10% methanol/DCM condition, the product shows an Rf of 0.56 upon $KMnO_4$ dye. Put the reaction to reduced pressure and add silica for direct dry loading in column chromatography. The yield was 25% (3.6mg). Product may be volatile under high-reduced pressure.

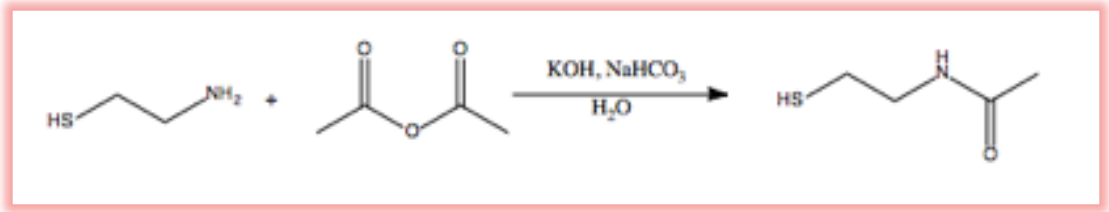
^{1}H NMR (600MHz, MeOD): δ 3.49(t, J=6Hz, 2H), 3.20 (t, J=6Hz, 2H), 2.36 (td, J=6.48, 2.4Hz, 2H), 2.30 (td, J=8.7, 1.62 Hz, 2H), 2.16 (t, J=2.64, 1H). ^{13}C NMR (600MHz, MeOD): δ 173.02, 82.09, 68.80, 60.18, 41.56, 34.60, 14.25.

Small molecule Fluorogenic click reaction

Add 3.6mg alkyne amide (0.0255mmol), 7.78mg coumarin azide (0.0383mmol in t-butanol), and 6.75mg ascorbic acid (0.0383mmol in H_2O), 12.8µl 1 M $CuSO_4$ (0.01275mmol in H_2O) and a stir bar into a 20ml reaction vial with aluminum foil covered, stir overnight at room temperature. Follow reaction with TLC. In 15% Methanol/DCM condition, the product shows an Rf of 0.44 under UV light. Work up with equal volume of H_2O to dissolve unwanted solute and extract with EA, dry and rotavap. Try solubility in DCM, and decide to dry-load and run Column Chromatography in 15% methanol/DCM. Since silica dissolves in methanol, use enough DCM to dissolve product then filter to get rid of trace amount of silica. The yield was low (36.5%) since the product has a relatively high solubility in water.

^{1}H NMR (600MHz, MeOD): δ 8.56 s 1H 8.28(s, 1H), 7.91 (s, 1H), 7.74 (d, 1H) 6.90 (dd, 1H), 6.84 (s, 1H), 3.38 (t, 2H), 3.12 (t, 2H), 2.94 (t, 2H), 2.49 (t, 2H). ^{13}C NMR (600MHz, DMSO-d6): 171.54, 162.91, 156.77, 155.05, 136.37, 131.34, 123.30, 119.85, 114.75, 110.81, 102.61, 60.36, 41.88, 35.04, 21.64. The expected Mass is 344.11 (or 367.1 as a Na^+ added), and detected was 367.4.

N-Acetylcysteamine [3]

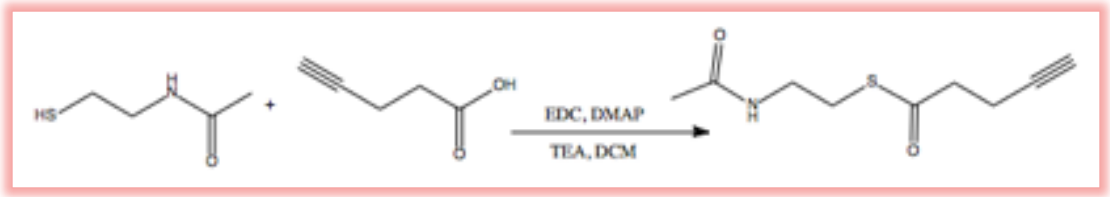


Weigh 1.14g 2-Aminoethanethiol hydrochloride (10mmol), 0.56g KOH (10mmol) and 2.52g $NaHCO_3$ (30mmol) into a clean round bottom flask, then add 50ml distilled water and stir to dissolve. Add 1ml acetic anhydride drop wise upon

stirring at 22℃. The solution became cloudy and oily. After 10 minutes, adjust the pH to 7.3 with NaOH and HCl solution. Extract 3 times with EA, 1 time with DCM, dry with $MgSO_4$, and concentrate under reduced pressure. 5% Methanol/DCM gives an Rf of 0.44. The product was 0.7484g with a 62.9% yield.

1H-NMR(600MHz, $CDCl_3$): 6.06 (bs, 1H), 3.44 (t, 2H), 2.69 (t, 2H), 2.02 (s, 3H), 1.38 (t, 1H). ^{13}C-NMR(600MHz, $CDCl_3$): 170.24, 42.43, 24.68, 23.33.

CoA mimic-thioester alkyne [4]



Weigh 145.2mg 4-pentynoic acid (1.48mmol) and 313.69mg EDC (1.628mmol) to a clean and dry round bottom flask. Add 15ml distilled DCM and stir to dissolve. Then add 230µl triethylamine (1.628mmol) and 91.37 mg DMAP (0.74mmol) upon stirring, and stir on ice for 10 minutes. Add 168.1mg N-Acetylcysteamine (1.41mmol) upon stirring. Heat the reaction to 30℃ and warm up back to room temperature, keep stirring for overnight. Follow reaction with TLC. 80% EA/DCM gives an Rf of 0.23. Work up the reaction by concentrating in vacuo and suspending the remaining oil in 10ml distilled H_2O, then extract with 30ml EA, wash with 2x10ml 0.1M HCl, and 10ml saturated sodium bicarbonate solution. Dry over $MgSO_4$ and concentrate in vacuo. A yield of 12.17% was obtained for the final product (34.2mg).

^{1}H NMR (400 MHz, CDCl3): δ 5.97 (bs, 1H), 3.44 (q, 2H), 3.07 (t, 2H), 2.81 (t, 2H), 2.55 (td, 2H), 2.01 (t, 1H), 1.97 (s, 3H). ^{13}C NMR (100 MHz, CDCl3): δ 197.77, 170.27, 81.76, 69.52, 42.39, 39.52, 26.64, 23.19, 14.67.

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Appendix

ABBREVIATIONS

ADT	Androgen Deprived Therapy
AF-2	AR Activation Function 2
AGD	Affinity Guided DMAP
AI	Androgen Independent
AR	Androgen Receptor
hAR	Human Androgen Receptor
BF-3	Binding Function 3
BSA	Bovine Serum Albumin
CRPC	Castration Resistant Prostate Cancer
CoA	Coenzyme A
CuAAC	Cu (I)-catalyzed Azide-alkyne Cycloaddition
DBD	DNA Binding Domain
DCM	Dichloromethane
DHT	Dihydroxytestostrone
DMAP	4-(dimethylamino) pyridine
DMEM	Dulbecco's modified Eagle Media
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's Phosphate-buffered Saline

DTT	Dithiothreitol
EA	Ethyl acetate
ER	Estrogen Receptor
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic Acid
GFP	Green fluorescent Protein
eGFP	Enhanced Green fluorescent Protein
GST	Glutathione S-transferases
HDACs	Histone Deacetylases
HRPC	Hormone Refractory Prostate Cancer
KAT	Lysine Acetyltransferase
LB	Lysogenic broth
LBD	Ligand Binding Domain
LDAI	Ligand Directed acyl imidazole
LDT	Ligand-Directed Tosyl
NAC	N-acetyl Cysteine
NES	Nuclear Export Sequence
NHS	N-hyrdoxysuccinimide
NLS	Nuclear Localization Sequence
NMR	Nuclear Magnetic Resonance
NR	Nuclear Receptor
PAL	Photo affinity labeling
PBS	Phosphate Buffered Saline

PCa	Prostate cancer
PMSF	Phenylmethane Sulfonyl Fluoride
SAHA	Suberoylanilide Hydroxamic Acid
SAMT	S-acyl-2-mercaptobenzamide thioesters
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel electrophoresis
TAMRA	Tetramethylrhodamine
TEMED	N, N, N', N', -tetramethyl-ethylenediamine
TCEP	Tris-carboxyethyl phosphine
THF	Tetrahydofuran
Tol	Tolfenamic acid
TBTA	Tris [1-benzyl-1H-1, 2,3-triazole-4-yl] methyl] amine
TRIAC	3,3', 5-triiodo thyroacetic acid
4-PY	4-pentynoic acid