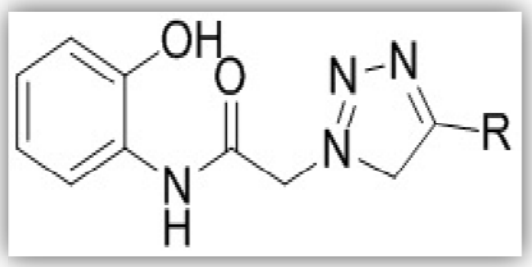
Annexure 1

Aniline and Triazole Adducts



Abstract:

In this part of work strategy was designed to conjoin two pharmacophores triazole and *o*-hydroxy aniline adducts. Triazole possessing three nitrogen atoms in a five membered heterocyclic ring is an important pharmacophore. Synthetic strategy for novel triazole and *o*-hydoxy aniline adducts were designed and then synthesis was carried out. Synthesis starts with condensation of chloro acetylchloride with *o*-hydroxy aniline. The next step is the azide formation followed by *in situ* click reaction to give the desired product. Overall yields of 30-40% were achieved. Single crystal of one of the intermediate compound was developed and solved. Present study concludes that N-(2-hydroxyphenyl)-2-(4-phenyl-1H-1,2,3-triazol-1-yl)acetamide can be synthesized in less time, with high yields using above strategy and can help in developing novel drug candidates for anticancer activity.

I.1 Introduction

A molecule like triazole possessing three nitrogen atoms in a five membered heterocyclic ring is an important pharmacophore and shows various clinical properties such as: antiHIV, antimicrobial, antiparasitic, anti-inflammatory, anticancer, antimalarial and antiviral (*Figure I.1*) [1-4].

Another clinically important substructure is benzoxazole, which is widely used as a starting material for the synthesis of bioactive structures and forms a part of important drugs such as flunoxaprofen and oxazolamine [5-6].

The benzoxazole derivatives have been investigated for their inhibitory activity on eukaryotic DNA topoisomerase and are known to exhibit antibacterial, antitubercular, anticancer, antiparasitic and antiHIV activity [7-8].

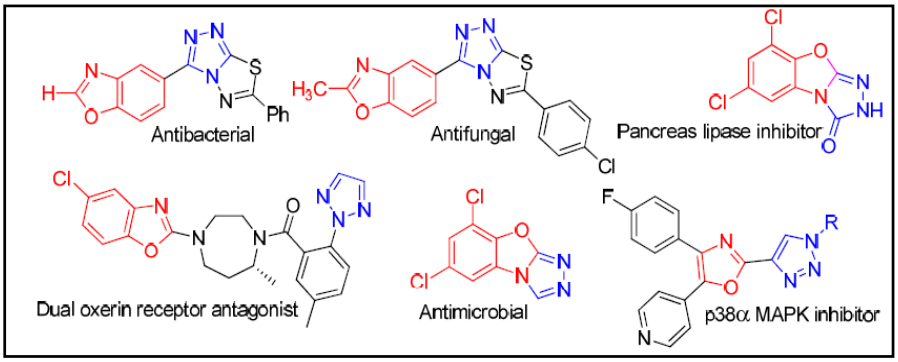


Figure I.1: Marketed drug with benzoxazole and triazole

I.2 Our Strategy:

Keeping above literature survey in mind, our efforts were focused on the synthesis of o-hydroxy aniline and triazole adducts (*Figure I.2*).

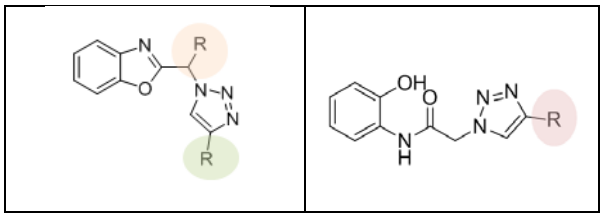


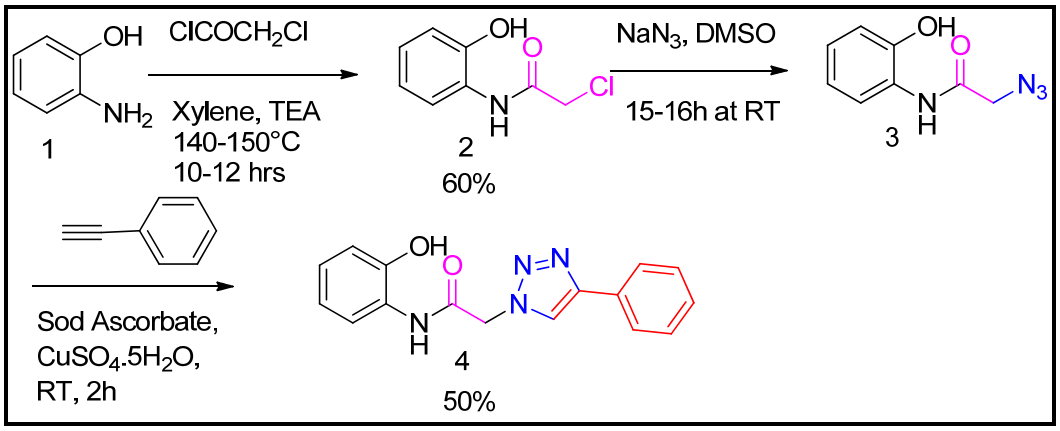
Figure I.2: Strategy of designing

I.3 Results and Discussion:

I.3.1 Synthesis:

Our efforts in this section were focused on synthesis of aniline and triazole adducts, keeping Pan Assay Interference Compounds (PAINS) structure in mind. The designing of compounds, see *Figure I.2*, was driven by three basic principles: (i) derivatising triazole at C_4 position with rigid/flexible group and (ii) using aniline derivatives.

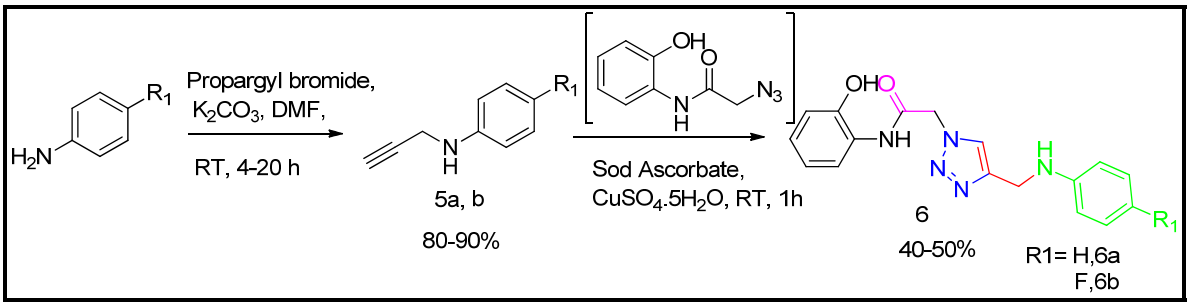
Strategy 1: *o*-hydroxy aniline was made to react with chloro acetyl chloride (acetylation) (*Scheme I.1*) [9]. We envisaged ortho hydroxyl aniline for the stepwise construction of N-(2-hydroxyphenyl)-2-(4-phenyl-1H-1,2,3-triazol-1-yl)acetamide and analogs. The chemo selective attack of aniline N over the hydroxyl O depicts better donor property of N over O. The reaction condition, especially solvent selection, was optimized to get the best yields. The concept here was to get the cyclised product leading to benzoxazole. Cyclisation couldn't be achieved and every time we got non cyclised product [10]. The next step was the azide formation. Without isolating compound **3**, *in situ*, alkyne derivative was added to obtain final compound **4** [10]. Overall yield 30%.



Scheme I.1: Synthesis of *N*-(2-hydroxyphenyl)-2-(4-phenyl-1H-1,2,3-triazol-1-yl)acetamide

Strategy 2: Our aim in this strategy is to derivatize triazole ring at C_4 position using the N-(prop-2-yn-1-yl) aniline derivatives. Substituted aniline derivatives were allowed to react with propargyl bromide in the presence of K_2CO_3 base and DMF as solvent (*Scheme I.2*), as we have already mentioned in chapter 2.2. Terminal alkynes are known to provide

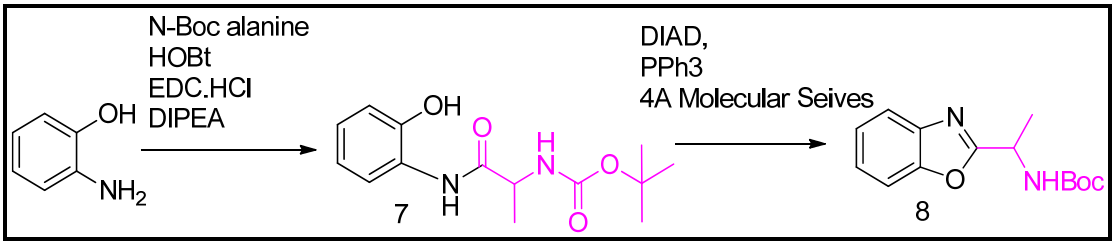
an efficient method for the synthesis of triazole. Krim and co-workers reported (Prop-2-ynyloxy) benzene as an intermediate, for the synthesis of triazole via Huigen dipolar cycloaddition method using click chemistry, which is followed in the present study [10].



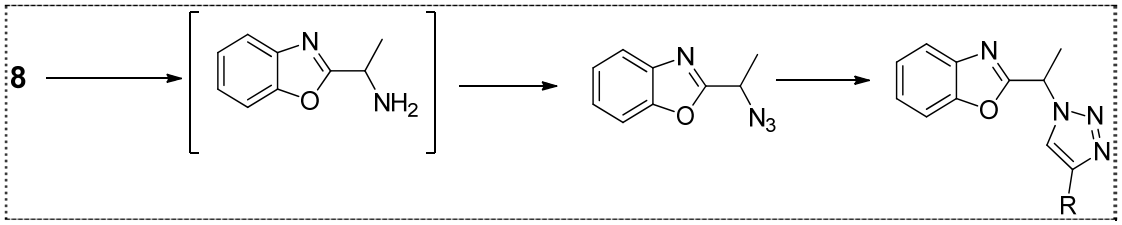
Scheme I.2: Synthesis of *N*-(2-hydroxyphenyl)-2-(4-((phenylamino)methyl)-1H-1,2,3-triazol-1-yl)acetamide

Attempted Synthesis for 2-(1-(4-phenyl-1*H*-1,2,3-triazol-1-yl)ethyl)benzo[*d*]oxazole:

Scheme 3 was designed to prepare novel benzoxaole derivatives but unfortunately we couldn't complete this strategy [11]. Molecule **8** is achieved in overall 60% yield.



Scheme I.3: Synthesis of tert-butyl (1-(benzo[d]oxazol-2-yl)ethyl)carbamate



Scheme I.4: Attempted synthesis

In the initial step N-Boc alanine is activated by 1-Ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC.HCl). The next step is the attack of

hydroxybenzotriazole (HOBt). After activating alanine in it aniline derivative was added and coupling in good yield was achieved. Diisopropyl ethyl amine (DIPEA) is used as base. The step is cyclisation step, here diisopropyl azodicarboxylate (DIAD) is initially activated by triphenyl phosphine and then cyclisation reaction starts. Molecular sieves are added to remove the extra water produced *in-situ* during the reaction. The overall yield achieved is good. The next cyclisation was tried but required product was not obtained.

I.3.2 Characterization:

Spectral Analysis

New compounds were characterized by ^{1}H NMR. Spectra analyses were consistent with the assigned structures.

Single Crystal Analysis

Single crystal of one of the intermediate **8** was developed and solved (*Figure I.3 and Table I.1*). Crystal of compound **8** was harvested from solution in ethylacetate. Compound **8** got crystallized in monoclinic system with $P2_1$ space group.

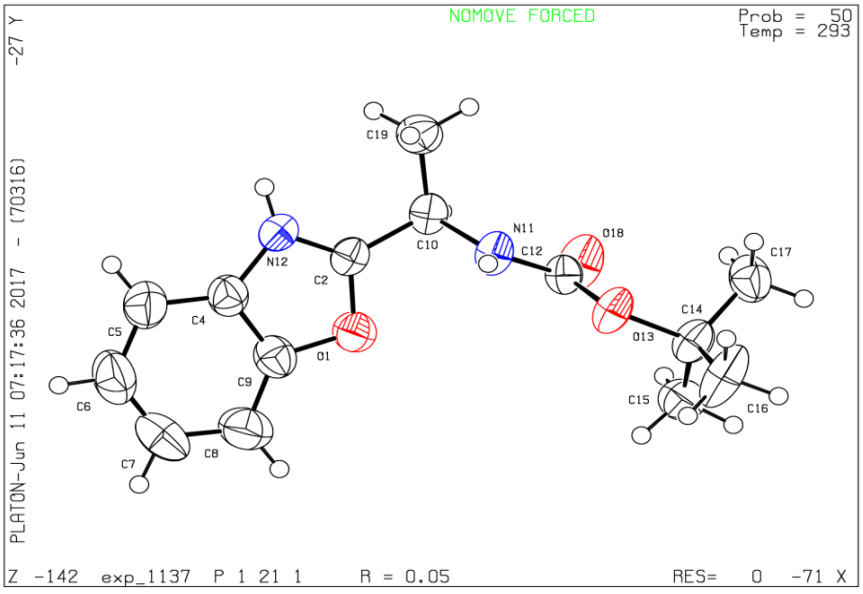


Figure I.3: Molecular view of compound 8 having thermal ellipsoid are shown with 50% probability

Table I.1: Crystallographic data and structure refinements for compound 8

	8
CCDC	1555340
Empirical formula	$C_{14}H_{19}N_2O_3$
Formula weight	263.32
Temperature/K	293(2)
Crystal system	monoclinic
Space group	$P2_1$
a/Å	9.6432(12)
b/Å	5.1056(5)
c/Å	14.883(3)
α/°	90
β/°	105.649(15)
γ/°	90
Volume/Å^3	705.60(17)
Z	2
ρ_{calc}g/cm^3	1.2393
μ/mm^{-1}	0.088
F(000)	282.1
Crystal size/mm^3	0.3 × 0.25 × 0.2
Radiation	Mo Kα (λ = 0.71073)
2Θ range for data collection/°	8.06 to 57.82
Index ranges	$-7 \le h \le 13$, $-6 \le k \le 3$, $-20 \le l \le 12$
Reflections collected	2897
Independent reflections	2220 [R_{int} = 0.0263, R_{sigma} = 0.0474]
Data/restraints/parameters	2220/0/175
Goodness-of-fit on F^2	0.927

I.4 Conclusion

Synthesis for conjoining two pharmacophores *o*-hydroxy aniline and triazole was tried. Designing of the molecules were done so as to have the rigid and flexible linker in the molecules. Single crystal of one of the intermediate was obtained. Synthetic strategy was designed so as to achieve the maximum yield.

I.5 Experimental

I.5.1 Materials and Methods:

All the compounds were purified using column chromatography (2000- 400 mesh silica) before characterization. TLC analysis was done using pre-coated silica on aluminum sheets. Melting points were recorded in Thiele's tube using paraffin oil and are uncorrected. The NMR spectra were obtained on a Bruker AV-III 400 MHz spectrometer using TMS as an internal standard. The chemical shifts were reported in parts per million (ppm), coupling constants (J) were expressed in hertz (Hz) and signals were described as singlet (s), doublet(d), triplet(t), broad (b) as well as multiplet (m). Single crystal data was collected with Xcalibur, EoS, Gemini.

I.5.2 Synthesis of compounds:

Synthesis of 2-chloro-*N*-(2-hydroxyphenyl)acetamide 2:

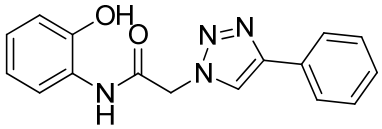
The title compound was synthesized according to the literature procedure. Commercially available o-hydroxyaniline (5.4 g), chloroacetic acid (7.1 g), triethylamine (10ml) and xylene (50 ml) were heated at 120-130°C for overnight. After completion of the reaction it was partitioned between water and xlene layer. Column purified to obtain the pure product. Yield: 70%.

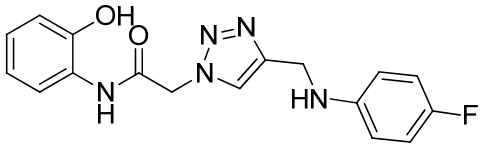
Melting point: 158-160°C (literature melting point: 160-161°C)

^{1}H NMR (400 MHz, DMSO-d6) δ: 7.56(m, 2H), 7.21(m,2H), 4.92(s, 2H)

Synthesis of *N*-(2-hydroxyphenyl)-2-(4-phenyl-1*H*-1,2,3-triazol-1-yl)acetamide 4:

Synthesis of compound **4** was synthesized in a single step. A mixture of compound **2** (0.500g, 3mmol) and sodium azide (0.195g, 3mmol) was charged in DMSO (5mL) at room temperature for 15-16hrs. Reaction completion was monitored using thin layer chromatography (TLC, EtOAc:PET, 7:3). After *in situ* formation of compound **3**, the respective alkyne derivative (0.321g, 3.14mmol) was added and mixture of $CuSO_4.5H_2O$ (0.112g, 0.4mmol) and sodium ascorbate (0.267g, 1.35mmol) in water (0.5mL) was added as shown in *Scheme 1*. The reaction was stirred at room temperature and its completion was monitored by TLC. Compound **4** was then isolated and further purified by column chromatography using various concentrations of ethyl acetate and petroleum ether to afford the desired compound **4** as a yellow colored semi solid.

 4	Yellow colored semi solid (Jelly) **Yield:** 50% **^{1}H NMR (400MHz,DMSO-d6) δ:** 9.97(s, 1H), 9.72 (s, 1H), 8.59(s, 1H), 7.88-7.84(m, 3H, *ortho* H and proton near NH), 7.47-7.44 (t, 2H, *J*=7.2Hz, *meta* proton of phenyl ring), 7.36-7.32 (t, 1H, *J*=7.6Hz), 6.97-6.88(m, 2H), 6.78-6.74 (t, 1H, *J*=7.6Hz), 5.47 (s, 2H, $-CH_2$) ppm **^{13}C NMR (100MHz, DMSO-d6) δ:** 164.8 (C=O), 148.2, 146.6, 131.1, 129.4, 128.3, 126.0, 125.5, 125.3, 123.5, 122.4, 119.3, 115.6, 52.7 ppm (*Figure I.4, I.5*)

 6b	Yellow colored semi solid (Jelly) **Yield:** 50% **^{1}H NMR (400MHz,DMSO-d6) δ:** 8.15 (s, 1H), 7.75-7.71 (m, 2H), 7.42-7.38 (m, 2H), 6.92-6.88 (m, 2H), 6.65-6.61(m, 2H), 6.05 (s, 2H), 4.29-4.27(s, 2H) ppm **^{13}C NMR (100MHz, DMSO-d6) δ:** 173.4, 160.9, 156.0, 153.7, 150.7, 146.5, 145.4, 140.6, 126.2, 125.3, 124.4, 120.4, 115.7, 115.5, 113.6, 113.5, 111.4, 64.0, 46.8 ppm

Synthesis of tert-butyl (1-((2-hydroxyphenyl)amino)-1-oxopropan-2-yl)carbamate 7:

N-Boc alanine (2.0 mmol, 1.0 eq) in dry MDC was charged in a round bottom flask. Reaction was performed under nitrogen atmosphere at RT. Added HOBt (4.0 mmol, 1.0 eq) and EDC.HCl (3.0mmol, 1.5eq) in it. Stirred reaction mass for 15-20 mins. Now, DIPEA (3eq) and *o*-aminophenol (2.4mmol, 1.2eq) was added in one lot. After completion the reaction mass was diluted with MDC and washed with 1M HCl (50ml), saturated aqueous $NaHCO_3$ (25ml) and brine (25ml). Column chromatography was performed to obtain the pure compound **7**. Yield: 85%

Synthesis of tert-butyl (1-(benzo[*d*]oxazol-2-yl)ethyl)carbamate 8:

Compound **7** was dissolved in dry THF (20ml). Triphenylphosphine (2.2eq) and freshly activated 4Å molecular sieves were added into it. Reaction mass was stirred at 0°C for 1h under nitrogen atmosphere. Solution of DIAD (2.2eq) in THF was slowly added to the reaction mass. Reaction mass was then stirred at room temperature for 8h. After reaction completion solvent was removed and column purified to obtain the pure compound **8**. Yield: 70%.

I.6 Selected Spectra:

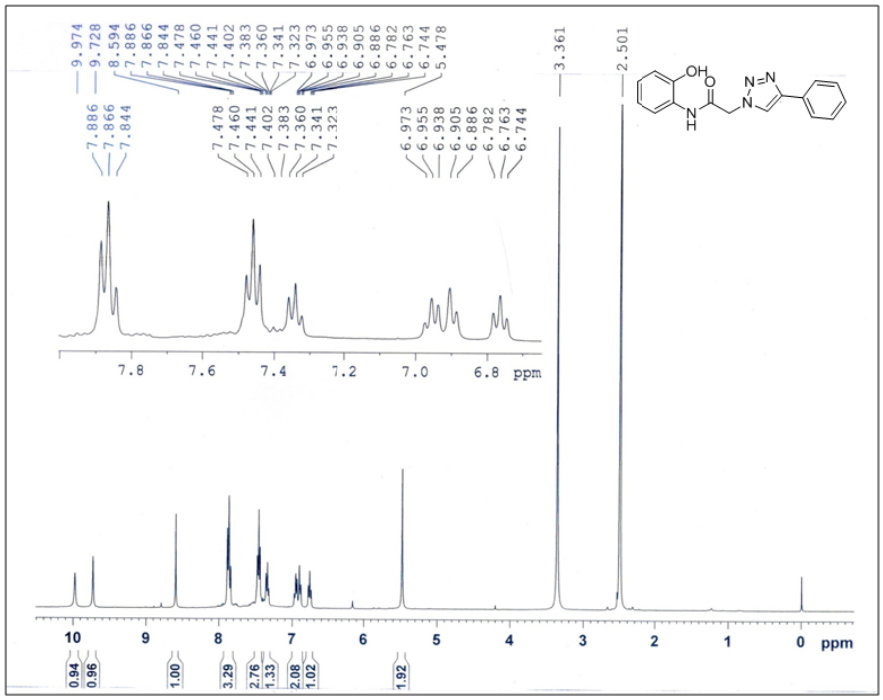


Figure I.4: ^{1}H NMR of 4

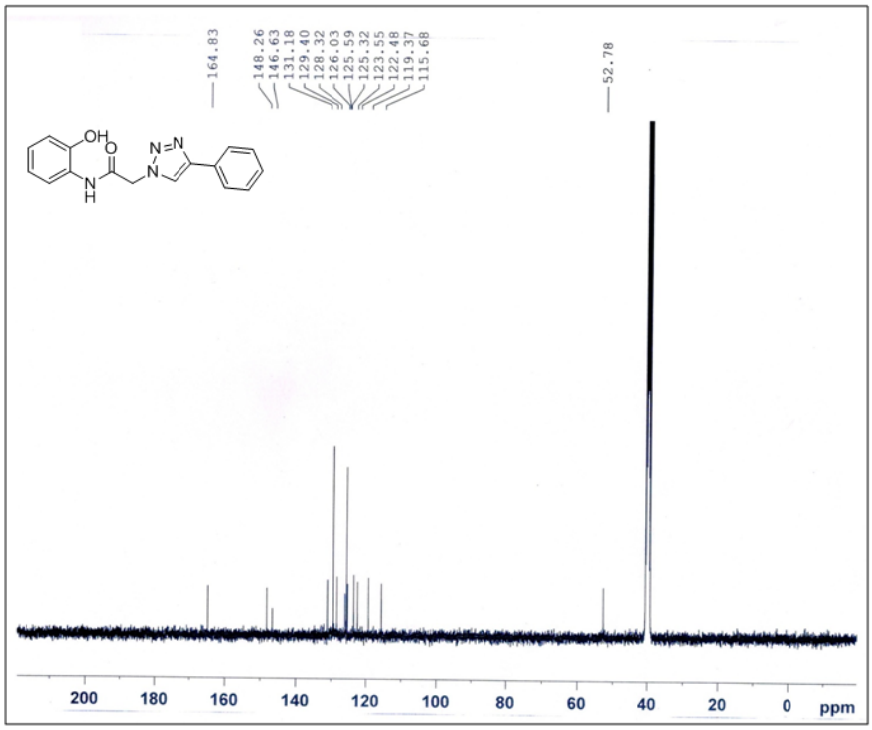


Figure I.5: ^{13}C NMR spectra of 4

I.7 Reference:

[1] Mohammed, I.; Kummetha, I. R.; Singh, G.; Sharova, N.; Lichinchi, G.; Dang, J.; Stevenson, M.; Rana, T. M. *Journal of medicinal chemistry*, **2016**, *59*, 7677-7682.

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[9] (a) Hou, J.; Li, Z.; Fang, Q.; Feng, C.; Zhang, H.; Guo, W.; Wang, H.; Gu, G.; Tian, Y.; Liu, P.; Liu, R.; Lin, J.; Shi, Yi-K.; Yin, Z.; Shen, J.; Wang, P. G. *J. Med. Chem.***2012**, *55*, 3066. (b) Chen, P. J.; Yang, A.; Gu, Y. F.; Zhang, X. S.; Shao, K. P.; Xue, D. Q.; He, P.; Jiang, T. F.; Zhang, Q. R.; Liu, H. M. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 2741. (c) Gu, S. J.; Lee, J. K.; Pae, A. N.; Chung, H. J.; Rhim, H.; Han, S. Y.; Min, Sun-J.; Cho, Y. S. *Bioorg. Med. Chem.Lett.***2010**, *20*, 2705.

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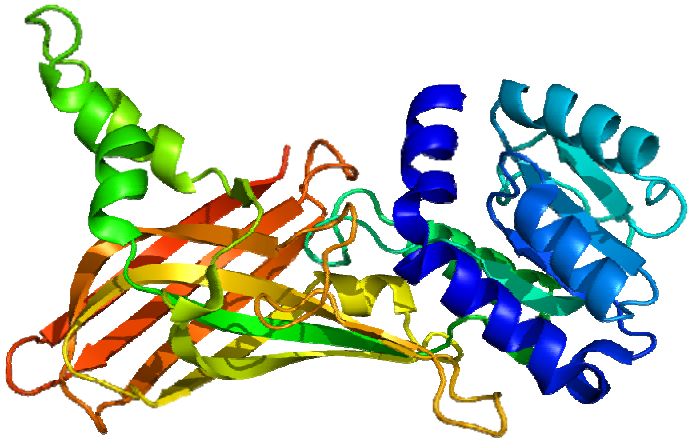
[11] Hou, J.; Li, Z.; Fang, Q.; Feng, C.; Zhang, H.; Guo, W.; Wang, H.; Gu, G.; Tian, Y.; Liu, P.; Liu, R. *Journal of medicinal chemistry*, **2012**, *55*, 3066-3075.

Annexure II

Protein Isolation



Abstract:

Protein Arginine Methyl Transferase Type 1 (PRMT1) is an enzyme that post-translationally methylates various proteins regulating several homeostatic cellular processes. This has been realized and researched as a potential therapeutic target in cancers. To test the binding of a novel compound synthesized in this work with PRMT1, this collaborative work was carried out. From lung cancer cell-line A549, PRMT1 transcription variant 1 transcript was amplified and cloned in Eschrichia coli bacterial expression system and assessed for expression of the PRMT1v1 protein.

II.1 Introduction

As we have already discussed the importance of working with cancer in chapter 1 and introduction of PRMT1 (Protein Arginine Methyl Transferase Type 1) in chapter 2.1, Protein arginine methylation (PAM) is a common post-translational modification (PTM) that regulates numerous cellular processes, including gene transcription, mRNA splicing, DNA repair, protein cellular localization, cell fate determination and signaling [1,2]. Interestingly, it was shown that about 2% of the total arginine residues isolated from rat liver nuclei are dimethylated [3], this led to aggressive studies on arginine methylation. The methylation of arginine is mainly controlled by methyl transferases protein family, PRMTs. Currently, there are ten PRMT's annotated in the human genome [4].

PRMT's as a therapeutic targets plays an important role not only in cancer but for other diseases such as cardiovascular disease, virus related diseases and endothelial cell (EC) inflammatory responses [5]. These findings not only unveil the critical functions of PRMTs in oncogenesis but also make the PRMT family of enzymes promising therapeutic targets in drug discovery [6].

Arginine methylation includes mono-methylation, asymmetric di-methylation and symmetric di methylation in mammalian cells (Figure II.1). The protein arginine methyl transferases are responsible for arginine methylation. PRMT's have been shown to be over expressed in multiple types of cancers including breast cancer [7] and lung cancer [8]. Therefore PRMT's are potential targets for cancer therapy. Among the PRMT family, PRMT1 is a major asymmetric arginine N-methyl tranferase in mammalian cell. PRMT1 catalyzes asymmetric dimethylation of arginine siting on the tail of histone H4 [9].

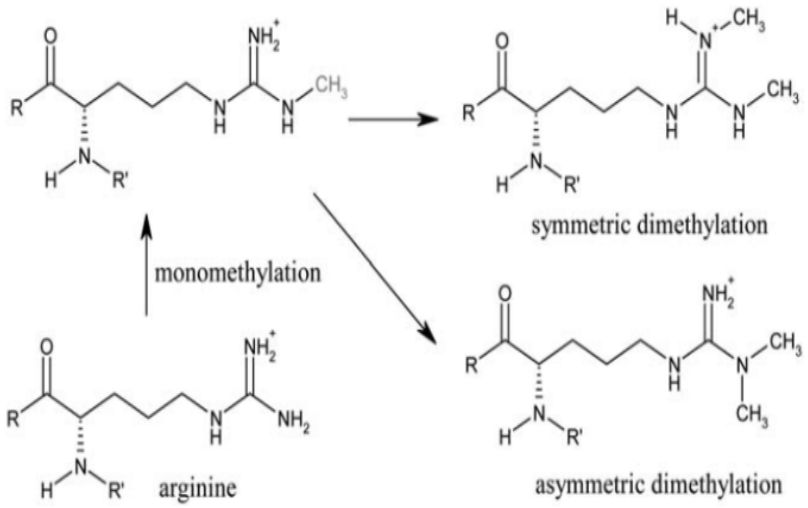


Figure II.1: Mechanism of process of methylation by PRMT1 (reference 10)

One strategy to control anti-proliferative activity is to design PRMT inhibitors with a varying pharmacophore. In literature, these inhibitors are divided into two groups. The first group consists of peptide derivatives, means having critical role of 'amide' pharmacophore [11-14].The second class consists of inhibitors derived out of small organic molecules, which are normally obtained from random or target based screening such as AMI-1, stilbamidine, allantodapsone, RM-65 and SAM derivatives [15-17]. Most of these targeted compounds have diamidine structure in them.

The core structure of PRMTs is conserved with one or two methyltransferas (MTase) domain (Figure II.2) [18]. Methylation of arginine residues is catalysed by the PRMT's. These isozymes transfer a methyl group from S-adenosylmethionine (SAM) to the guanidinium moiety of arginine residues in proteins. This reaction produces a mono methylated product that is monomethyl arginine rsidue (ω-MMA), which further gets methylated either to produce symmetrically dimethylated arginine (SDMA) or asymmetrically dimethylated arginine (ADMA).

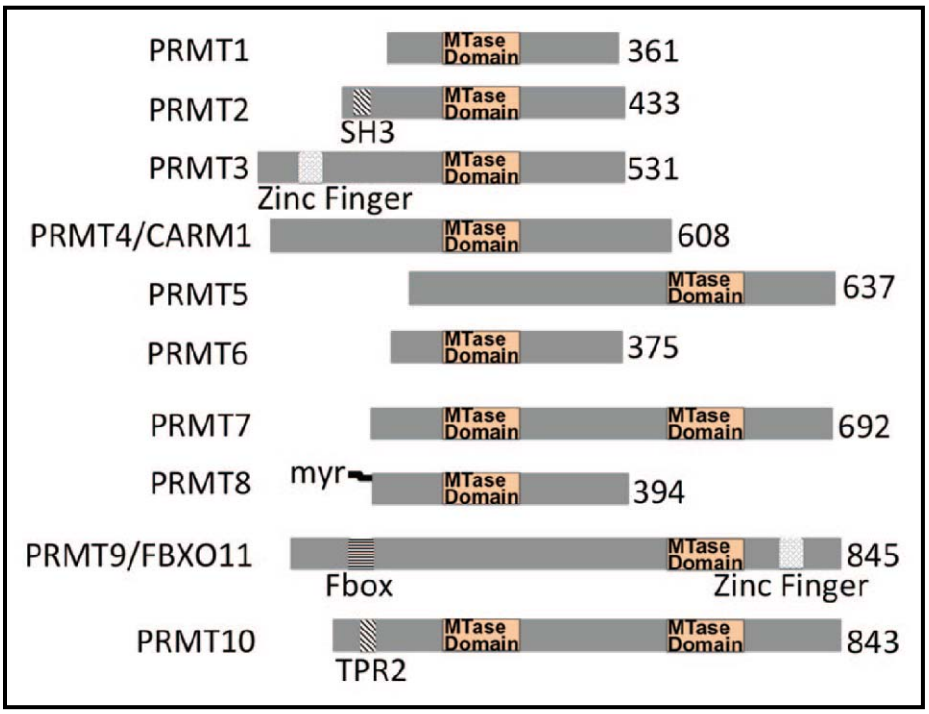


Figure II.2: Structure of PRMT family members. PRMT family members universally have 1-2 methyltransferase(MTase) domains, SAM binding domain and one motif in their unique PRMT domain. Eachisozyme has a distinct N-terminus with some containing common protein domains such as a SH_3 or a Zn finger domain figure adapted from ref [18]

Alternative splicing of PRMT1 exists with seven distinct isoforms (v1–v7) with each isoform having a unique N-terminal sequence and a slightly different molecular weight [19]. Examination of expression of PRMT1 v1-v3 identified a strong correlation of variant 1 in breast cancer samples and poor patient prognosis in v2 and v3 [20]. Survival curves revealed that PRMT1v1 status low expression related to longer disease free survival.

II.2 Why is cloning and expression of PRMT1 required?

Knowing the drug interaction with the target protein is very essential in order to build structure activity relationship (SAR).This approach aids in targeted drug discovery which is the demand in this time as to have the minimum side effects of the drug to the patient.

II.3 Protein arginine N-methyltransferase 1

Details of PRMT1 is briefed in Table II.1

Table II.1 General information regarding PRMT1 ~~adopted~~adapted from Uniprot

Protein names	Protein arginine N-methyltransferase 1 Short name=PRMT1
Sequence length	347 AA.
Catalytic activity	S-adenosyl-L-methionine + arginine-[histone] = S-adenosyl-L-homocysteine + N(omega)-methyl-arginine-[histone].
Sequence similarities:	Belongs to the protein arginine N-methyltransferase family.
Enzyme regulation:	By BTG1, BTG2 and ILF3.
Subunit structure	Homodimer and heterodimer with PRMT8. The dimer can then associate to form a homohexamer. Interacts with ILF3, BTG1, BTG2, SUPT5H and interferon-alpha/beta receptor 1. Interacts with NFATC2IP .
Subcellular location:	Nucleus. Cytoplasm › cytosol
Tissue specificity	Widely expressed.
Biophysicochemical properties:	Kinetic parameters: K_M=1 µM for AdoMet K_M=4.2 µM for H4 V_{max}=1.2 nmol/min/mg enzyme toward AdoMet V_{max}=1.24 nmol/min/mg enzyme toward H4

X-ray crystal structure for human PRMT1 is not yet available, but for rat PRMT1 it is available. They share a sequence identity of 95.1%. The substrate binding surface of hPRMT1 is expected to be acidic, because most substrates for PRMT1 contain one or multiple arginine residues. The substrate binding pocket is cone shaped with an opening of 8x6 Å and a depth of 8 Å. The residues lining the substrate pocket are negatively charged at the bottom and hydrophobic in the middle. This fits well with the asymmetric polarity of an arginine residue with three hydrophobic methylene groups and the basic guanidine group [21].

PRMT1 is the most prevalent PRMT isozyme and it is responsible for 85% of the total activity amongst PRMTs. PRMT1 gene is found in all eukaryotes and is highly conserved. The sequence identity is over 90% among mammals [21]. The PRMT1 is the smallest member of the PRMT family and has three major human splice variants (v1 to v3) [22], that translates into proteins ranging from 353-371 amino acids in length. Other variants of PRMT1 are also reported but from literature we found that variant 1 was highly expressed in the many cancers [20].

Amino acid sequence of human PRMT1 (http://www.uniprot.org/uniprot/Q99873-2):

```
        10         20         30         40         50
MVGVAEVSCG QAESSEKPNA EDMTSKDYYF DSYAHFGIHE EMLKDEVRTL
        60         70         80         90        100
TYRNSMFHNR HLFKDKVVLD VGSGTGILCM FAAKAGARKV IGIECSSISD
       110        120        130        140        150
YAVKIVKANK LDHVVTIIKG KVEEVELPVE KVDIIISEWM GYCLFYESML
       160        170        180        190        200
NTVLYARDKW LAPDGLIFPD RATLYVTAIE DRQYKDYKIH WWENVYGFDM
       210        220        230        240        250
SCIKDVAIKE PLVDVVDPKQ LVTNACLIKE VDIYTVKVED LTFTSPFCLQ
       260        270        280        290        300
VKRNDYVHAL VAYFNIEFTR CHKRTGFSTS PESPYTHWKQ TVFYMEDYLT
       310        320        330        340
VKTGEEIFGT IGMRPNAKNN RDLDFTIDLD FKGQLCELSC STDYRMR
```

II.4 Steps Involved:

The steps involved in the process are as follows:

To identify the suitable cancer cell line as a source of PRMT transcript from where we can isolate the m-RNA

As shown in *Figure II.3*, the maximum expression of PRMT1 is observed in lung cancer cell line A549, and hence that particular cell line is used for RNA isolation in this study.

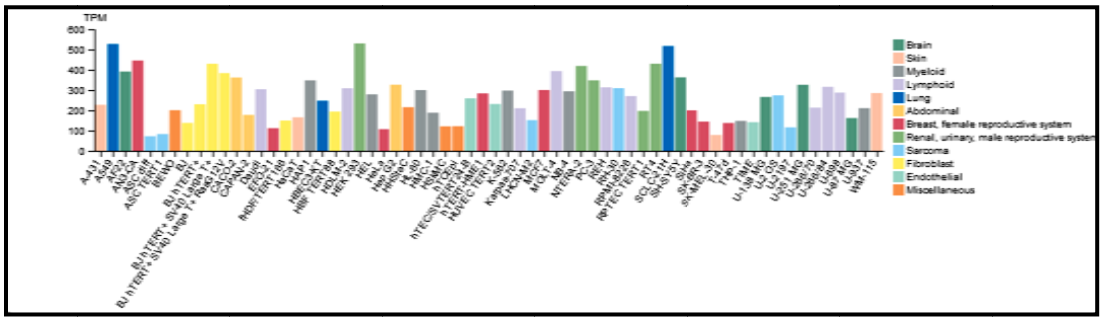


Figure II.3: The table showing the expression of PRMT1v1 in different cell lines. ~~Adopted~~Adapted from (http://www.proteinatlas.org/ENSG00000126457-PRMT1/cell/CAB022550)

1. Synthesizing cDNA to be used as template to amplify PRMT1v1 gene
2. PCR amplification of PRMT1v1 and cloning in intermediated vector
3. Subcloning of PRMT1v1 in the expression vector pET30a(+) and transformation of the construct in *E. coli* DH5α
4. Confirmation of the clone
5. Transferring the construct in the expression strain *E. coli*Rosetta Gami™2(DE3) and confirmation of clone
6. Isolation of proteins and SDS poly acrylamide gel electrophoresis analysis.

Figure II.4 gives the outline of the process of cloning and isolation.

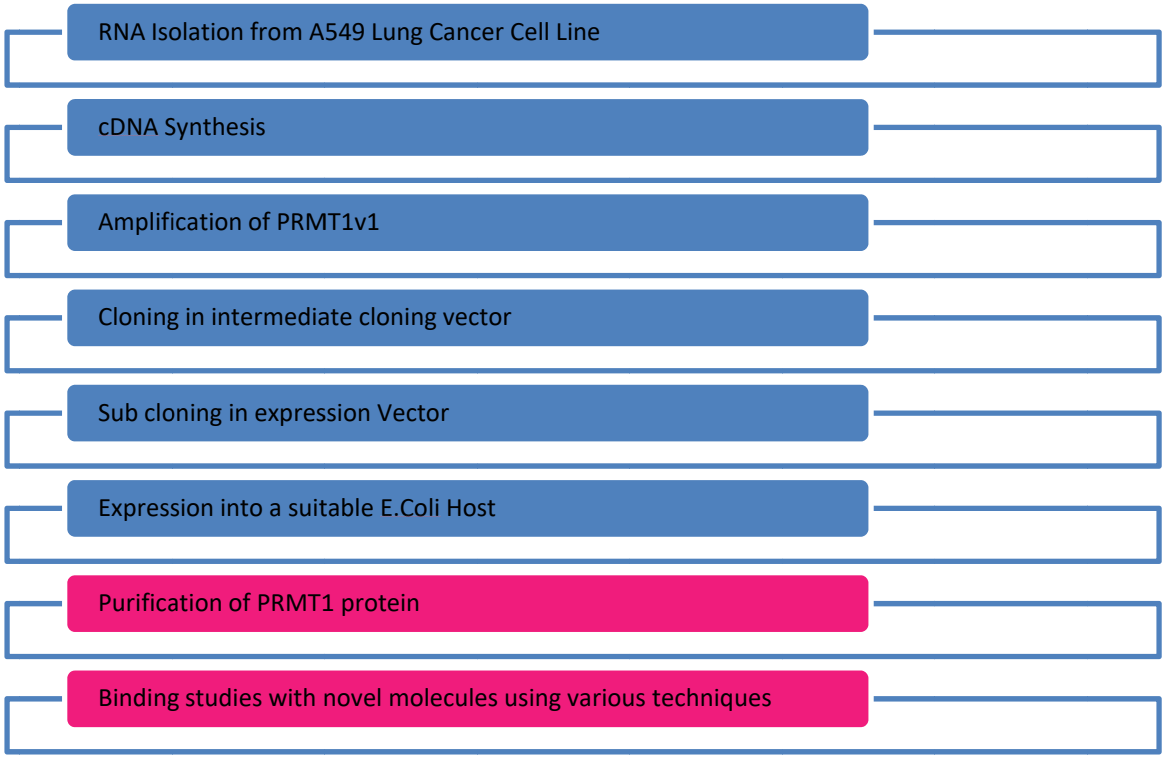


Figure II.4: An outline diagram showing the strategy of our work plan.

II.5 Experimental Data:

Materials and Methods

These methods are ~~adopted~~adapted from reference [23].

Bacterial strains, cell culture and Plasmids

All *E. coli* strains were grown at 37^0 C in Luria Bertani medium; liquid cultures were grown in shaking at 160 rpm. They were supplemented with appropriate antibiotics. *E. coli* DH5α was used for regular cloning and *E. coli* Rosetta Gami2 (DE3) was used for expression.

A549 cells were grown in Dulbecco's Modified Eagle's Medium and in the presence of 5% CO_2.

pJET (Thermo Scientific) was used as intermediate vector in *E. coli* DH5α and confers resistance to ampicillin (100ug/ml). pET30a(+) (Novagen) was used as expression vector and was shuttled from E. coli DH5α to E. coli Rosetta and it conferred resistance to Kanamycin (30ug/ml)

Isolation of RNA and first strand cDNA synthesis

Total RNA from confluent culture of A549 cells was isolated using TRIZOL method according to manufacturer's (TaKaRaBio Inc.) instructions. cDNA was synthesized using kit from the same manufacturer and was carried out according to its instructions.

Amplification of prmt1v1 from cDNA

Amplification of the specific, spliced coding sequence was achieved using polymerase chain reaction using pfu polymerase (Fermentas). The cycling parameters were as follows.

Initial denaturation: 95^0 C; 7 min,

Then 30 cycles of,

Denaturation: 95 ^{0} C; 30 sec

Primer annealing: 58^0 C; 45 sec

Extension: 72^0 C; 1 min 30 sec

Final extension: 72^0 C; 7 min.

The primer pair used was as follows.

ndeI and xhoI sites were included in the forward and reverse primer respectively (highlighted).

Forward primer

CATATGGCGGCAGCCGAGGCCGCGAAC

Reverse primer

CTCGAGGCGCATCCGGTAGTCGGTGGAGC

Intermediate cloning

PCR-amplicon was cloned in the suicide vector pJET (CloneJet, Thermo Scientific). Ligation of the blunt ended vector with the pfu polymerized-amplicon was performed according to the manufacturer's instructions. All subsequent molecular biology experiments including chemical transformation, clone selection via PCR and restriction digestion analysis were carried out as prescribed by Sambrook et al.[23] and the insert sequencing was outsourced to Europhins India. The resultant clone was pJET-PRMT.

Subcloning of the amplicon

Prmt1v1 amplicon was cleaved from the pJET-PRMTconstruct with ndeI and xhoI sites incorporated in the primers. pET30a(+) was digested with aforementioned REs. The resultant bands were extracted from the agarose gel and purified (Sambrook). The resulatant vector and insert fragments were ligated using DNA ligase (Fermentas) for 12 hours at 22^0C. This was followed by chemical transformation of the ligation mixture in DH5αand clone confirmed via Restriction digestion. This gave rise to the construct pET-PRMT. The construct was subsequently transformed into E. coli Rosetta.

Induction for protein expression

E. coli Rosetta Clone containing pET-PRMT was grown in LB broth at 37^0C at 160 rpm until the OD of the culture reached 0.6. E. coli Rosetta only containing the empty vector was used as negative control and grown in the same way as the pET-PRMT clone.1mM IPTG was added to the cultures at this stage followed by incubation at 12^0C for 12-15 hours. The cells were harvested thereafter.

Protein Extraction and SDS PAGE

Following the IPTG induction, culture was withdrawn from shaking and the cells were harvested. Harvested cells were either used immediately or freeze-stored at -80^{0}C.These cells were then lysed by boiling in presence of Sodium dodecyl sulphate and reducing agents as prescribed in Sambrook et al. Boil-lysed cells were pelleted at 4^{0}C and total protein amount was quantitated using Lowry's method. Equal amount of (25ug) protein of each sample was then loaded in the polyacrylamide gel and standard protocol was used for running as well as for Coomassie brilliant blue staining.

Results and discussions

Clone confirmation

Successful amplification of PRMT1 was obtained. The sequence was used to perform a BLAST and it was confirmed that the cloned gene was PRMT1.

SDS-PAGE analysis

PAGE analysis showed presence of a thicker band at ~39 kDa. However, this needs to further standardize.

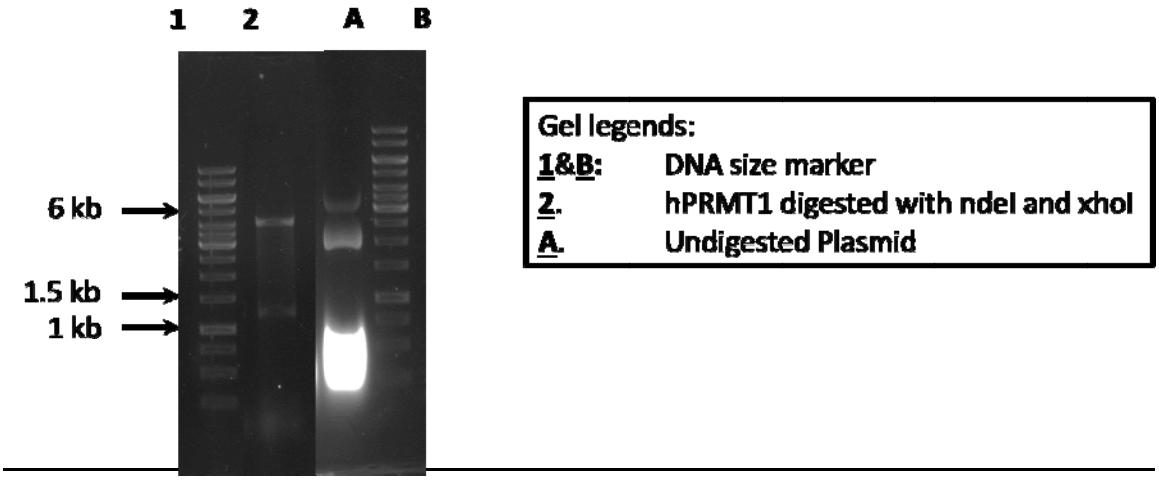


Figure II.5: Image of SDS page showing bright band of hPRMT1

II.6 Conclusions

PRMT1 is an enzyme that post-translationally methylates various proteins regulating several homeostatic cellular processes. It has been realized and researched as a potential therapeutic target in cancers. Owing to this, cloning of human PRMT was undertaken. It was amplified from the lung cancer cell line cDNA and cloned into pET30a(+). Protein expressed was assessed via SDS PAGE. Further confirmation of the protein expression and purification remains to be pursued. The purified protein is to be used for binding assays with the synthesized compounds.

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