Supplementary Material (ESI) for Molecular BioSystems

A combinatorial approach to characterize the substrate specificity of protein arginine methyltransferase 1

Supporting Information

Supporting Information Contents

Experimental Methods

Chemicals. FITC and ethyl acetimidate hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Fmoc-protected amino acids and O-Benzotriazole-N,N,N',N'- tetramethyl-uronium-hexafluoro-phosphate (HBTU) were purchased from Novabiochem Corp (Gibbstown, NJ). TentaGel resin was acquired from Rapp Polymere (Tübingen, Germany). All other chemicals were purchased from Acros Organics (Morris Plains, NJ).

Library Synthesis

The peptide library was synthesized via a split-and-pool based combinatorial approach using the Fmoc strategy. The sequence of this library was Ac-XXX**O***XXXBBRM-resin, where **O*** denotes the placement of a warhead modified ornithine, X is a randomized amino acid, and B is β-alanine. To begin, 1.0 g of amino terminated TentaGel resin (0.25 mmol/g substitution; 320 μm diameter) was pre-swollen in dimethylformamide (DMF) for 30 min. After removing the DMF, a solution of Fmoc-Met-OH (401 mg; 1.08 mmol; 4 eq) activated with HBTU (410 mg; 1.08 mmol; 4 eq) in 25 mL of 0.4 M N-methylmorpholine (NMM) in DMF was added to the resin and the mixture was tumbled for 45 min. After removing the solution, and washing with DMF and methanol (MeOH), the resin was tumbled twice in 20% piperidine in DMF (25 mL) for 10 min to afford removal of the Fmoc protecting group. The resin was then washed again with DMF and MeOH, and these steps were repeated in order to couple Fmoc-Arg(Pbf)-OH, Fmoc-β-Ala-OH, and the second Fmoc-β-Ala-OH. At this point the resin was divided equally into 17 reaction vessels. To each vessel was added 4 eq (0.0635 mmol) of each of 17 different amino acids activated with HBTU. The amino acids used were Fmoc protected Ala, Gly, Leu, Phe, Thr(tBu), Ser(tBu), Tyr(tBu), Val, Asn(Trt), Gln(Trt), Asp(OtBu), Glu(OtBu), His(Trt), Arg(Pbf), Lys(Boc), Trp(Boc), and Pro. Note that all of these amino acids use acid labile side

chain protecting groups. After one round of coupling, the resin was combined back into one reaction vessel and the Fmoc group removed using 20% piperidine in DMF, as before. This split-and-pool procedure was repeated twice more. At this point Fmoc-Orn(Dde)-OH (519 mg; 1.08 mmol; 4 eq) was activated with HBTU (410 mg; 1.08 mmol; 4 eq) and coupled to the resin. After subsequent deprotection of the Fmoc groups, the split-and-pool procedure was employed three more times to construct the remainder of the library. After removing the final Fmoc group, the N-terminus was acylated by tumbling in acetic anhydride (1 mL), and pyridine (1 mL) in dichloromethane (DCM; 25 mL) for 30 min.

After washing with DCM and DMF, the Dde group was removed by tumbling the resin in 30 mL of 2% hydrazine monohydrate in DMF for 1 h. The resin was then washed with DMF and MeOH. Attachment of the mixed warhead system was achieved by tumbling the resin in a 1:1 mixture (0.54 mmol:0.54 mmol) of ethyl chloroacetimidate hydrochloride and ethyl acetimidate hydrochloride in 30 mL of DMF with 300 μL (8 eq) of triethylamine for 18 h at 25 ºC (**Scheme 1**). This step was repeated and the resin washed thoroughly with DMF and MeOH. Ethyl chloroacetimidate hydrochloride was prepared as previously described.[1] At this point, the acid labile side chain protecting groups were removed by treating the resin with 25 mL of 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIS). Subsequent washing with DCM, DMF, and MeOH afforded the completed peptide library. Acylated resin was also synthesized, to be used as a control, by treating amino terminated TentaGel resin with acetic anhydride (1 mL), and pyridine (1 mL) in dichloromethane (DCM; 25 mL) for 30 min, followed by washing with DCM, DMF, and MeOH.

Synthesis of Fluorescein-PRMT1

The methods for the expression and purification of N-terminal hexa-His tagged human PRMT1 have previously been described.[2] Fluorescently labeled PRMT1 (F-PRMT1) was prepared by mixing 77.1 μL of a stock solution of hPRMT1 (32 μM) in 100 mM HEPES, 200 mM NaCl, 2 mM EDTA, 1 mM DTT and 10% glycerol (pH 8.0) with 37.0 μL of fluorescein isothiocyanate (FITC) (54 μg/mL) dissolved in carbonate buffer (500 mM, pH 9.5). This mixture was incubated at 37 ºC for 1 h and then transferred to a 3.5 kDa cut-off Slide-A-Lyzer cassette and dialyzed against 500 mL of 100 mM HEPES, 200 mM NaCl, 2 mM EDTA, 1 mM DTT and 10% glycerol (pH 8.0), exchanging the buffer three times. F-PRMT1 was subsequently collected and the final protein concentration determined using the Bradford assay. The steady state kinetic parameters of F-PRMT1 were determined using a previously described gel-based discontinuous assay that monitors the transfer of a methyl group from ^{14}C-methyl-SAM to AcH4-21, a peptide substrate.[2] Briefly, a reaction mixture of 50 mM HEPES pH 8.0, 1 mM EDTA, 50 mM NaCl, 0.5 mM dithiothreitol, 15 μM ^{14}C-methyl-SAM, and varying concentrations of AcH4-21 (0-1000 μM final) was incubated at 37 °C for 10 min. F-PRMT1 (200 nM final) was then added, and the reaction was allowed to proceed for 15 min, at which point it was quenched with 6X tris-tricine gel loading dye. The standard deviation of the duplicate measurements agreed within ≤ 20%. The gels were dried and then quantified using a phosphorimager (Molecular Dynamics). The steady state kinetic parameters were obtained by fitting the initial rate data to Eq1

$$v = V_{max}[S]/([S] + K_m) \qquad \text{Equation 1}$$

using GraFit version 5.0.11.[3]

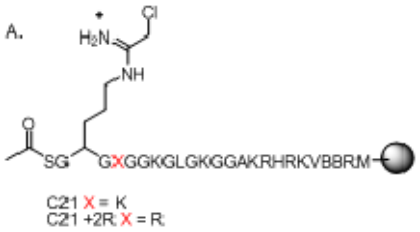
Library Screening

To begin, 10 mg of dried library resin was weighed out into a 1.5 mL centrifuge tube. Subsequently, 1 mL of Screening Buffer (50 mM HEPES, 50 mM NaCl, 1 mM EDTA, and 0.5 mM DTT; pH 8.0) was added to pre-swell the beads. This solution was removed and then the resin was treated with 1% bovine serum albumin (BSA) in Screening Buffer for 15 min to prevent non-specific protein binding. This solution was removed and the resin mixed with the desired concentration of F-PRMT1 in Screening Buffer containing 1% BSA for the prescribed time at 37 ºC. After incubation, the resin was washed three times with Screening Buffer and the beads were observed under a Leica MZ16F stereoscope using an EL6000 light source and a GFP3 filter set (Ex. 450-490 nm; Em. 500-550 nm). Images were acquired using a QImaging Micropublisher 5.0 RTV digital camera and analyzed using Adobe Photoshop to obtain luminosity values. Good binders, or "hits" (beads whose luminosity was 30% higher than the library average), were picked manually using surgical forceps.

Peptide Cleavage and Sequencing

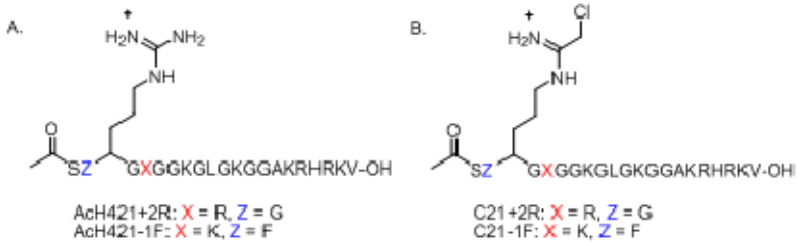
Isolated hits were heated to 100 ºC in 1% SDS in water for 1 h to wash the resin and remove non-covalently bound protein. This solution was removed and the beads washed with water three times. Beads were then separated into individual 0.5 mL centrifuge tubes and treated with 30 μL of 40 mg/mL cyanogen bromide in 0.1 M HCl in the dark for 18 h at 25 ºC to cleave the peptide from the bead. This solution was removed using a SpeedVac, and the peptide resuspended in 0.1% TFA in water. These samples were then analyzed using a Bruker Ultraflex MALDI-TOF/TOF, utilizing α-cyano-4-hydroxycinnamic acid as the matrix.

Synthesis and On Bead Screening of C21 and C21+2R



C21 and C21+2R were synthesized on amino terminated TentaGel resin (320 μm) using the Fmoc strategy described above. These resin bound inhibitors were then screened against F-PRMT1 as described previously and their average luminosities calculated.

Synthesis of Solution Phase Substrates and Inhibitors



The AcH421+2R and AcH421-1F peptides were synthesized using solid phase Fmoc strategies. To begin, 0.5 g of Val pre-loaded Wang polystyrene resin (100-200 mesh; 0.53 mmol/g substitution) was pre-swollen in DMF for 30 min. After removal of the solvent, the resin was treated twice with 20% piperidine in DMF (15 mL) for 10 min each. This treatment was followed by washing with DMF and DCM. Next, Fmoc-Lys(Boc)-OH (0.495 g; 1.06 mmol; 4 eq) was activated with HBTU (0.402 g; 1.06 mmol; 4 eq) in 0.4 M NMM in DMF for 10 min. This solution was added to the resin and tumbled for 45 min followed by washing with DMF and DCM. This cycle of deprotection and amino acid coupling was continued until the first 14 amino acids were coupled (up to the +3 position). To generate the AcH4-21 and C21 analogues, small portions of resin (~50 mg) were removed from the larger batch and complete sequences generated using the Fmoc strategies described above. The amino acids used for this synthesis

were Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Orn(Dde)-OH, and Fmoc-Ser(tBu)-OH. After completing peptide synthesis, the N-terminus was capped with acetic anhydride as described above. Attachment of the Cl-acetamidine warhead to the C21 derivatives was done by first removing the Dde protecting group, as described above, and then tumbling the resin in 4 mL of DMF with 40 μL (10 eq) of triethylamine and 25 mg (6 eq) of ethyl chloroacetimidate hydrochloride for 18 h at 25 ºC. The resin was washed with DMF and DCM and then tumbled in 95% TFA, 2.5% water, and 2.5% TIS for 6 h to cleave the peptides from the resin and remove acid labile protecting groups. TFA was blown off gently with N_2 gas and the peptides precipitated from ether. Peptides were purified by HPLC and lyophilized and their structures confirmed by MALDI-TOF MS (**Table S1**).

Table S1: Mass confirmation of synthesized peptides.

Peptide	Sequence	Expected Mass (Da)	Observed Mass (m/z)
AcH4-21+2R	Ac-SGRGRGGKGLGKGGAKRHRKV-OH	2161.48	2161.68
AcH4-21-1F	Ac-SFRGKGGKGLGKGGAKRHRKV-OH	2223.59	2223.78
C21+2R	Ac-SG**O***GRGGKGLGKGGAKRHRKV-OH	2194.93	2194.39
C21-1F	Ac-SF**O***GKGGKGLGKGGAKRHRKV-OH	2257.04	2257.47

IC_{50} Assays

The IC_{50} values for C21+2R and C21-1F were determined using the gel-based methyltransferase assay described above according to previously established methodologies.[4] Briefly, 25 μM AcH4-21 substrate peptide was used to initiate the reaction. The concentrations of ^{14}C-Methyl-SAM (15 μM) and PRMT1 (0.2 μM) were held constant, while the concentration of added inhibitor was varied. PRMT1 was pre-incubated with the reaction buffer for 10 min prior to the addition of the peptide substrate. The methyltransferase reaction was run for 15 min

at 37 °C, then quenched with 6X tris-tricine loading dye and separated on a 16.5% tris-tricine gel. Reactions were performed in duplicate and the standard error of the measured velocities was typically less than 20%. Radioactive product was quantified by a phosphorimager and the IC_{50} values were determined by fitting the data to Eq2

$$\text{Fractional Activity of PRMT1} = 1/(1+([I]/IC_{50})) \qquad \text{Equation 2}$$

using GraFit version 5.0.11.[3]

Supplemental Results

Time Dependence

Based on the fact that C21 is a time dependent enzyme inactivator, we hypothesized that the binding between F-PRMT1 and the peptide library would also be time dependent. Therefore, we initially identified an optimal screening time, i.e., an incubation period that would result in the majority of the beads being dark (i.e., low background fluorescence), while a very small percentage of beads are brighter. To identify an optimal incubation time, resin was incubated with 0.01 mgmL F-PRMT1 for 0, 15, 30, 60, or 120 min intervals. Acylated resin was also incubated with F-PRMT1 for 60 min to act as a control. It was observed that the average luminosity increased as time increased, however, very little library differentiation was observed until 60 min (**Figure S1**).

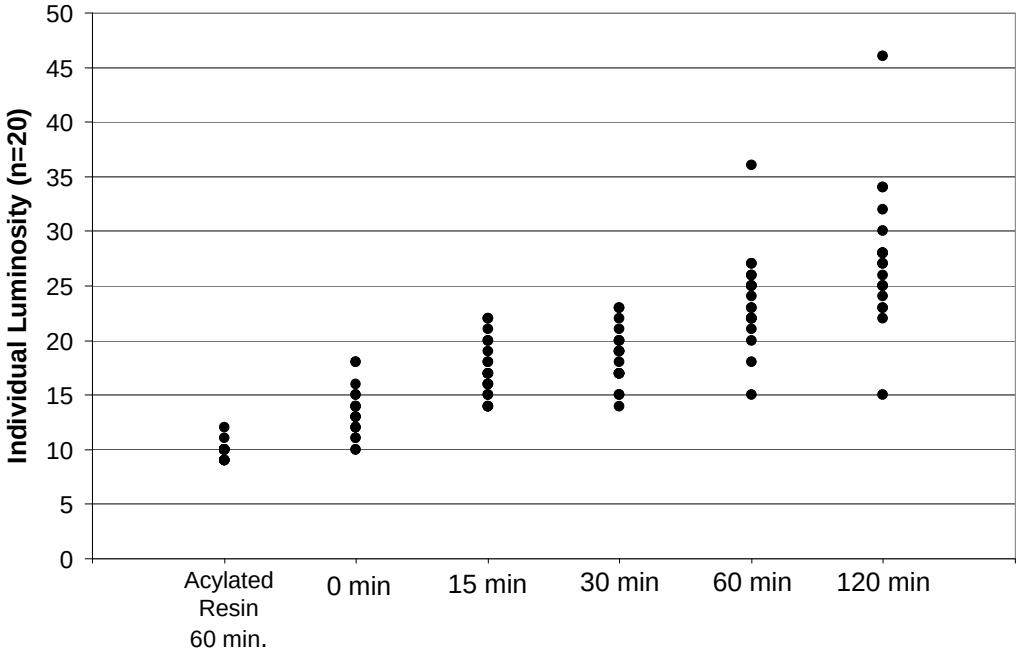


Figure S1. Binning chart showing individual bead luminosities for the screening of FITC-PRMT1 over various times.

By differentiation we mean that the large majority of beads in the screening were dark, indicating little or no binding, while a very small subset of beads would be brighter and

presumably good binders of F-PRMT1. This data indicated that incubating the library with F-PRMT1 for 60 min would provide sufficient time for the enzyme to interact and equilibrate with the library and covalently react with the warhead.

Concentration Dependence

To study the concentration dependence of F-PRMT1 reaction with the library, resin was prepared as described above, and subsequently one tube of resin was treated with 0.01 mg/mL F-PRMT1 and another tube of resin with 0.001 mg/mL F-PRMT1 for 1 h. After subsequent washing and analysis, we observed that there was essentially no differentiation in the resin screened with 0.001 mg/mL enzyme (**Figure S2**). However, excellent differentiation was observed at a concentration of 0.01 mg/mL, indicating that at this concentration we are able to identify selective binders for F-PRMT1.

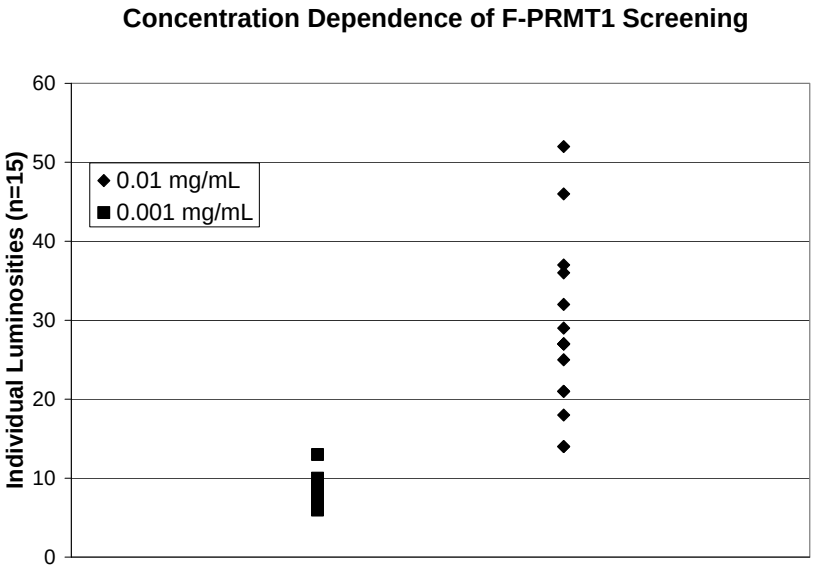


Figure S2. Binning chart of individual bead luminosities for the screening of 0.001 mg/mL (■) and 0.01 mg/mL (♦) F-PRMT1.

Peptide Sequencing

During sequencing we observed that there are two distinct peaks in the MALDI-TOF mass spectrum, corresponding to peptide that is lacking the warhead, as well as peptide containing the H-warhead (+41 Da); degradation of the Cl-warhead occurs during cyanogen bromide cleavage. The presence of these two peaks, which are separated by 41 Da, are a useful diagnostic, and both masses were analyzed by MS/MS methods to confirm the sequence of the peptide. A representative MALDI-TOF MS and MS/MS spectrum are shown in **Figures S3** and **S4** respectively. Note that in the MS/MS spectrum the analysis of *y* ions begins at 400 m/z, which corresponds to the mass of the initial BBRh sequence found on each peptide, where 'h' is the homoserine lactone formed from Met during cyanogen bromide cleavage.

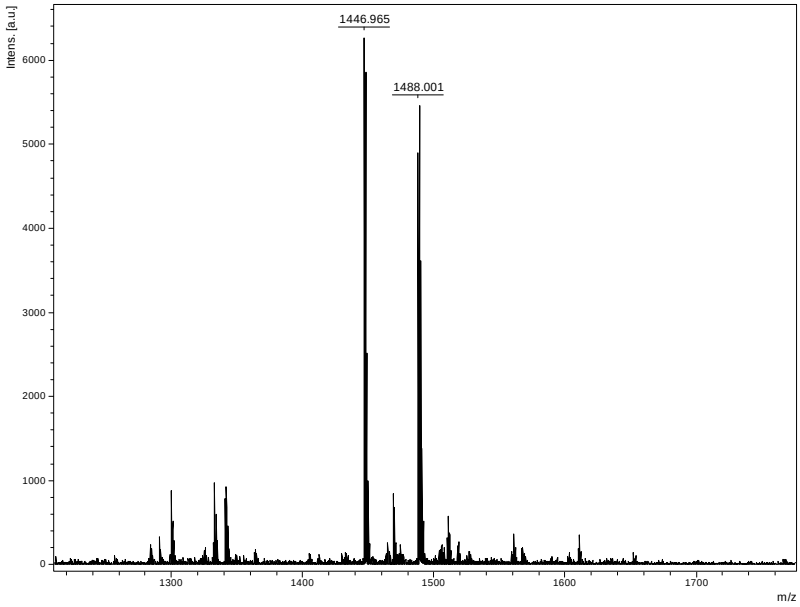


Figure S3. MALDI-TOF MS of F-PRMT1 hit 34.

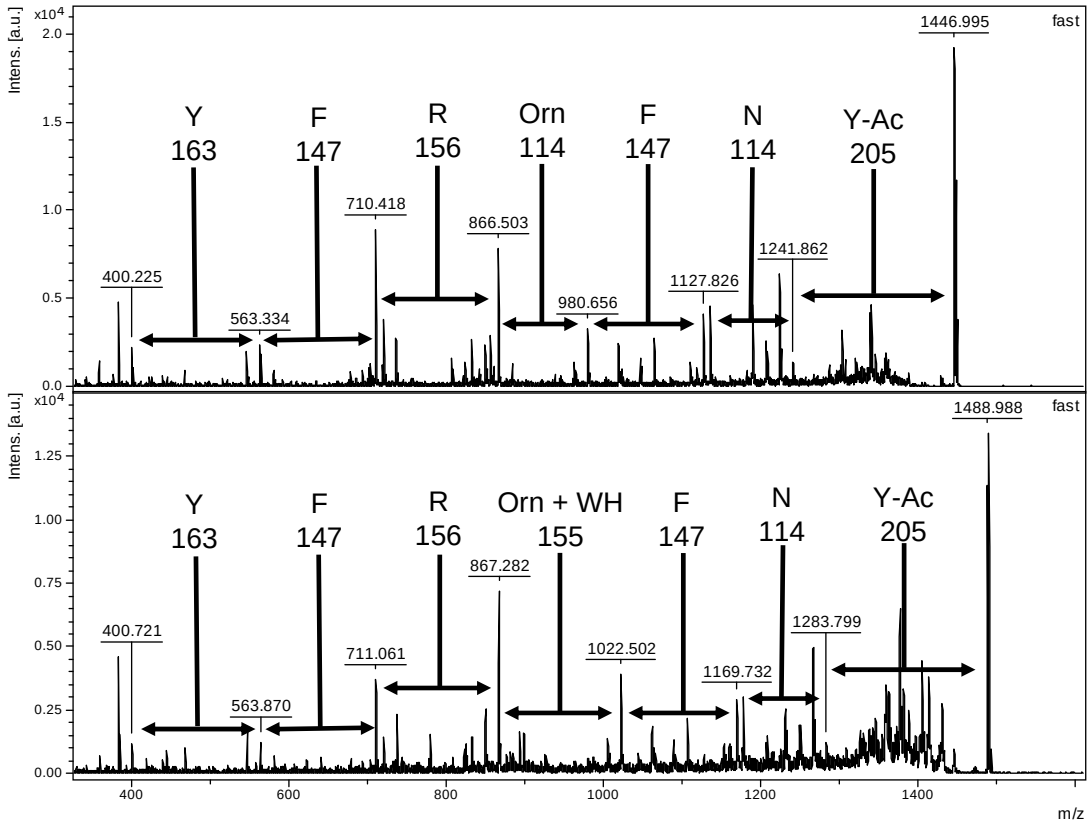


Figure S4 – MS/MS spectra of F-PRTM1 hit 34 (sequence 34 = Ac-YNF**O***RFYBBRh). All of the labeled peaks in the MS/MS spectra relate to *y* ions. The top spectrum is the MS/MS of the 1446.965 m/z peak in **Figure S3** and corresponds to sequence 34 where **O*** is free ornithine. The bottom spectrum is the MS/MS of the 1488.001 m/z peak in **Figure S3** and corresponds to sequence 34 where **O*** is ornithine coupled with H-amidine.

Determination of Sequence Homology

In total, 57 F-PRMT1 hits were isolated and sequenced. Of these, 45 complete sequences and, nine partial sequences were obtained; the sequences of three peptides were indeterminable. The sequences of these hits are shown below in **Table S2**. These sequences were aligned using WebLogo,[5] available online (http://weblogo.berkeley.edu/logo.cgi), to generate the sequence homology plot (**Figure 3**).

Table S2: PRMT1 hit sequences															
Hit Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Hit Number**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	T	R	Y	Orn	R	H	N	30		D	R	Orn	A	W	G
2	L	F	F	Orn	H	R	V	31	T	V	R	Orn	R	R	Y
3	D	G	A	Orn	V	F	G	32	T	N	V	Orn	Y	V	A
4	H	A	A	Orn	Y	P	R	33	P	R	R	Orn	W	F	W
5	-	-	H	Orn	A	G	P	34	Y	N	F	Orn	R	F	Y
6	-	S	P	Orn	A	-	-	35	H	W	F	Orn	S	F	Y
7	-	-	-	Orn	-	-	-	36	T	W	Y	Orn	R	N	E
8	W	E	H	Orn	A	R	H	37	G	H	A	Orn	W	F	F
9	-	-	-	Orn	-	-	-	38	G	R	Y	Orn	A	F	G
10	A	K/G	F	Orn	N	R	G	39	R	R	R	Orn	F	R	V
11	-	-	-	Orn	-	-	-	40	E	K/Q	L	Orn	L	F	F
12	-	-	-	Orn	Y	W	F	41	D	T	Y	Orn	S	Y	K/Q
13	-	-	-	Orn	-	Q/K	W	42	T	L	A	Orn	R	L	W
14	-	-	-	Orn	F	R	Y	43	T	N	L	Orn	H	F	R
15	V	P	R	Orn	Y	P	G	44	E	V	W	Orn	F	G	-
16	-	-	-	Orn	G	S	R	45	-	-	-	Orn	-	D	G
17	S	P	K/G	Orn	R	L	E	46	W	D	N	Orn	R	Y	F
18	T	V	R	Orn	Q/K	R	F	47	G	G	N	Orn	R	F	F
19	-	-	-	Orn	H	F	F	48	W	F	P	Orn	L	R	F
20	-	Y	F	Orn	R	Y	G	49	V	K/Q	Y	Orn	P	V	W
21	G	A	F	Orn	H	D	G	50	G	F	N	Orn	P	S	D
22	T	H	A	Orn	R	V	Y	51	V	P	Y	Orn	Y	Y	F
23	D	F	A	Orn	A	K	F	52	W	E	K/G	Orn	R	T	F
24	G	T	E	Orn	Y	F	F	53	H	N	R	Orn	W	V	K/Q
25	A	P	E	Orn	F	K	F	54	K/Q	L	H	Orn	R	V	V
26		E	F	Orn	R	N	F	55	H	G	H	Orn	R	S	Y
27	V	A	R	Orn	S	V	W	56	-	-	-	Orn	-	P	F
28	T	T	F	Orn	D	F	Y	57	R	L	S	Orn	F	L	G
29	F	R	Y	Orn	N	A	P								

On Bead Screening of C21 and C21+2R

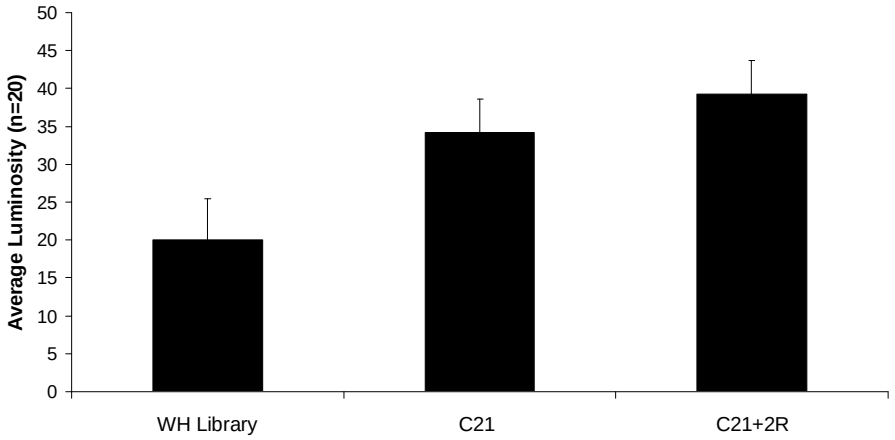


Figure S5. Bar graph showing the average luminosity of the C21 and C21+2R inhibitors and the warhead library after incubating with F-PRMT1.

Both C21 and C21+2R exhibited a higher average luminosity than the warhead library, which was used as a control in this study, indicating above average binding to F-PRMT1 (**Figure S5**).

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