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Fluorescence Polarization for the Evaluation of Small-Molecule Inhibitors of PCAF BRD/Tat-AcK50 Association

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A fluorescence polarization competitive assay was developed to efficiently screen and evaluate inhibitors of PCAF bromodomain/Tat-AcK50 protein–peptide interaction. A series of pyridine 1-oxide derivatives were synthesized and evaluated. Some of the novel compounds, 2-(3-aminopropylamino) pyridine 1-oxide derivatives, could be effective inhibitors of PCAF bromodomain/Tat-AcK50 association. Specifically, 2-(3-aminopropylamino)-5-(hydroxymethyl)pyridine 1-oxide hydrochloride (**15**) and the 5-((3-aminopropylamino)methyl) derivative (**20**) were found to be effective ligands for the PCAF BRD pocket. First preliminary cellular studies indicate that these small-molecule inhibitors have lower cytotoxicities and are potential leads for the anti-HIV/AIDS therapeutic strategy by targeting host-cell protein PCAF BRD to block HIV replication.

Human p300/CBP associated factor bromodomain (PCAF BRD), a relatively conserved host-cell protein, can selectively bind to Tat-AcK50 (lysine 50 acetylated trans-activator). Former research showed that the transcription of the integrated HIV provirus required the interaction of HIV Tat and human co-activator PCAF BRD.[1,2] PCAF BRD adopts a highly conserved structural fold of a left-handed four-helix bundle (αZ, αA, αB, and αC). The ZA and BC loops are at one end of the bundle, forming a hydrophobic pocket, which is suitable for the binding of Tat-AcK50.[3] Previously, we developed a series of N^1-aryl-propane-1,3-diamine compounds, which could bind to the hydrophobic pocket and inhibit the interaction of PCAF BRD and Tat-AcK50.[4] Based on this former research, we are attempting to discover novel small molecules that bind to PCAF BRD. Targeting an essential host-cell protein for HIV-1 replication rather than enzymes or proteins encoded in the viral gene, we assumed that this target could minimize drug resistance resulting from rapid mutations of the HIV gene.

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In preceding research, 2D NMR and enzyme-linked immunosorbent assay (ELISA) were applied for the evaluation of ligand binding affinity towards PCAF BRD in vitro.[4,5] However, as these methods are rather inefficient and time consuming, it is necessary to develop new assays for inhibitor screening and evaluation. Fluorescence polarization (FP) is a technique especially applied to study molecular interactions. It gives a direct and nearly instantaneous measurement of the ratio of bound to free tracer.[13] In recent years, as a homogeneous assay, the fluorescence polarization assay (FPA) was widely applied in systems that involve binding of ligands to receptors, in which fluorescently labelled ligands are used as a tracers. Because of its simplicity, sensitivity and speed, FPA is readily suitable for probing biological associations such as ligand–protein,[6] enzymatic activity,[7] peptide–protein binding,[8] DNA hybridization,[7,9] DNA–protein binding,[10] and even for Hg^{2+} ion determination.[11] In addition, outstanding advances of FPA, showing the measurement of DNA wrapping, the close contact involved in protein–DNA binding and the application in immunoassays, have been reported.[12] Recent developments in the field indicate that FP is a useful tool in high-throughput screening (HTS) and in small-molecule drug discovery.[8c,13] Inspired by former research and using the FP principle, we finally developed a novel FPA involving the interaction between PCAF BRD and fluorescein isothiocyanate (FITC)-labelled Tat peptide. More importantly, a series of pyridine 1-oxide derivatives were synthesized and evaluated.

A schematic illustration of the FP competition assay principle is presented in Figure 1. In FP competitive binding assays, the

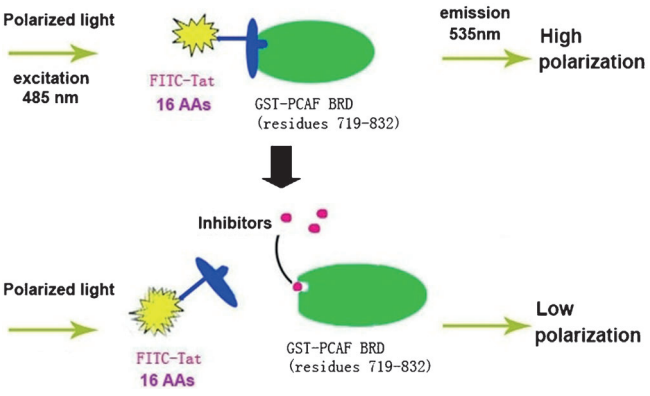


Figure 1. Schematic illustration of the FP competition assay. When the fluorescence tracer binds to the target protein, high FP signal is observed. When small-molecule inhibitors completely disrupt the peptide–protein interactions, the FP signal is shifted to low values.

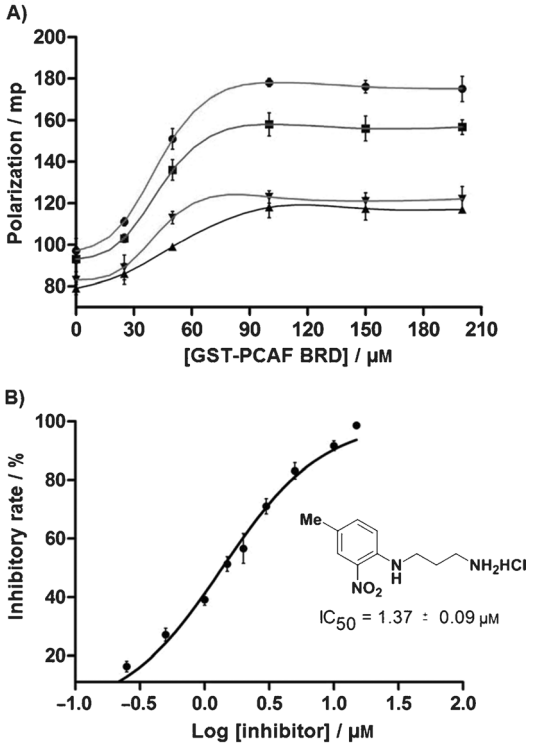
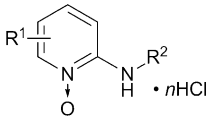


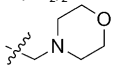
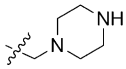
Figure 2. A) Binding test: FITC-Tat 3 nM, GST-PCAF BRD concentration: 0 nM, 25 nM, 50 nM, 100 nM, 150 nM, 200 nM, 4 °C, 6 h in either 125 mM Tris, pH 6.85 (●); 125 mM Tris, pH 7.2 (■); 100 mM PBS, pH 6.85 (▲); 100 mM PBS, pH 7.2 (▼). Excitation: 485 nm, emission: 535 nm. B) Competitive FP assay for IC_{50} calculation of NP1. *Assay conditions*: 3 nM FITC-Tat, 100 nM GST-PCAF BRD, 125 mM Tris, pH 6.85, 4 °C, 6 h. Excitation: 485 nm, emission: 535 nm.

presence of unlabelled small-molecule inhibitors result in the displacement of FITC-labelled Tat peptide, thereby increasing their tumbling motion, which in turn can be detected as a decrease in FP value. Figure 2A shows the effects for the typical variation of the concentration of glutathione S-transferase (GST)-tagged PCAF BRD, and satisfactory FP signals (in mP) could be achieved. To verify the conditions for the FP competition assay, we performed the assay in 125 mM Tris, pH 6.85 (●, Figure 2A) to assess the inhibitory activity of the known small-molecule ligand NP1. The obtained IC_{50} value (1.37 ± 0.09 μM, see Figure 2B) was consistent with the previous IC_{50} value assessed by ELISA in vitro (1.60 ± 0.10 μM). The FP competitive assay results of other compounds also coincide with our in vitro anti-HIV-1 activity (Table 1.). These results indicate that our FP competition assay could be an effective evaluation method and could be applied for inhibitor screening and evaluation for PCAF BRD.

Due to their structural similarity to NP1 and low toxicity, 2-(3-aminopropylamino)pyridine 1-oxide derivatives, a novel class of compounds, were specifically designed and synthesized as PCAF BRD ligands. Initially, compound **3** was synthesized as an analogue of NP1. In the primary FPA screening, **3** showed promising inhibitory potency against the PCAF BRD/Tat-AcK50 interaction. The IC_{50} value was assessed by the competitive assay against FITC-labelled Tat-AcK50 binding to PCAF BRD described above. Surprisingly, an IC_{50} value of 4.62 ± 0.23 μM was obtained. Intrigued by this promising lead, a series of analogues of **3** were synthesized, and their binding affinities with PCAF BRD were evaluated using the developed FPA. The assay results indicated several candidate compounds to be effective inhibitors of the PCAF BRD/Tat-AcK50 protein–peptide interaction, as shown in Table 1. According to the FPA results, a primary structure–activity relationship (SAR) study was performed. First of all, the pyridine-oxide moiety of the compounds was very important for binding to PCAF BRD. The binding affinity was largely decreased without this moiety in the molecules (**3** versus **22**, **6** versus **23**). Secondly, the 1,3-diamine moiety on the 2-position also played a crucial role for activity retention. Lengthening or shortening the carbon chain weakened the binding affinity (**3** versus **2** and **4**). Changing the 1,3-diamine moiety to other chain moieties decreased the inhibitory potency, and most of

Table 1. 2-Aminopyridine 1-oxide derivatives as inhibitors for PCAF BRD/Tat-AcK50 interaction.



Compd[a]	R_1	R_2	IC_{50} [μM][b]	EC_{50} [μM][c]
1	5-Me	$-(CH_2)_2CH_3$	38.24 ± 1.82	94.51
2	5-Me	$-(CH_2)_2NH_2$	21.28 ± 1.11	52.11
3	5-Me	$-(CH_2)_3NH_2$	4.62 ± 0.23	91.52
4	5-Me	$-(CH_2)_4NH_2$	10.69 ± 0.26	112.55
5	5-Br	$-(CH_2)_3NH_2$	20.70 ± 0.67	87.28
6	5-CF_3	$-(CH_2)_3NH_2$	11.73 ± 0.27	13.94
7	5-Ph	$-(CH_2)_3NH_2$	86.66 ± 2.31	61.85
8	4-Me	$-(CH_2)_3NH_2$	9.31 ± 0.26	180.68
9	5-Me	–Bn	> 200	> 200
10	5-Me	$-(CH_2)_2NH(CH_2)_2OH$	5.51 ± 0.19	100.12
11	5-Me	$-(CH_2)_2NH(CH_2)_2NH$	10.25 ± 0.31	78.83
12	5-Me	$-(CH_2)_2NHCH_2CH(OH)CH_3$	31.43 ± 1.21	75.28
13	5-Me		> 200	> 200
14	5-Me		> 200	> 200
15	5-CH_2OH	$-(CH_2)_3NH_2$	1.18 ± 0.09	11.92
16	5-CH_2NH_2	$-(CH_2)_3NH_2$	8.90 ± 0.31	66.1
17	5-CH_2OH	$-CH_2CH(OH)CH_2NH_2$	> 200	> 200
18	5-CH_2NHCH_2Ph	$-(CH_2)_3NH_2$	162.61 ± 3.68	> 200
19	5-$CH_2NH(CH_2)_2NH_2$	$-(CH_2)_3NH_2$	2.26 ± 0.19	71.85
20	5-$CH_2NH(CH_2)_3NH_2$	$-(CH_2)_3NH_2$	0.93 ± 0.12	11.52
21	5-$CH_2NH(CH_2)_4NH_2$	$-(CH_2)_3NH_2$	9.76 ± 0.37	40.64
22[d]	5-Me	$-(CH_2)_3NH_2$	> 200	> 200
23[d]	5-CF_3	$-(CH_2)_3NH_2$	145.20 ± 2.89	181.28

[a] Compounds bearing primary amine groups were transformed to their hydrochloride salts for assays. [b] Determined using FP competition assay described above. Data are the mean ± SD of three experiments performed in triplicate. [c] Determined using human T lymphocyte cell line C8166 and HIV-1IIIB. Data are the mean of three triplicate experiments, and EC_{50} values were determined by nonlinear regression analysis using GraphPad Prism 5.0. [d] Without *N*-oxide on the pyridine ring.

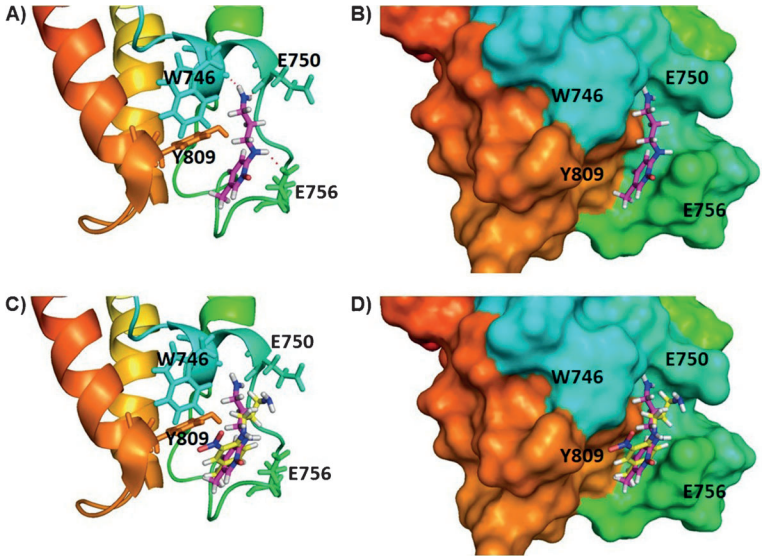


Figure 3. Structural basis of ligand recognition of PCAF BRD. A) A docking model of the BRD/compound **3** complex, showing the binding site of **3** (magenta). B) GRASP view of the binding pocket of **3**. C) A docking model of the BRD/**3** (magenta) and BRD/NP1 (yellow) complex, showing the binding sites of **3** and NP1. D) GRASP view of **3** and NP1 binding pocket.

the changes greatly impaired the binding affinity (**3** versus **1**, **17**, **9**–**14**). Moreover, it was noted that large substitution groups at the 2-position of the pyridyl ring abolished activity, with $IC_{50} > 200$ μM (**9**, **13** and **14**), which implied that big volume of the substituent at the 2-position of pyridine *N*-oxide would block the pocket recognition of PCAF BRD to the small molecule. Thirdly, the substituent at the 5-position had a great influence on the biding affinity. For instance, when 5-Me (**3**) was changed to 4-Me (**8**), the corresponding IC_{50} value decreased to 9.31 ± 0.26 μM. The bioactivity was almost disrupted with an IC_{50} value of more than 80 μM when 5-Me (**3**) was replaced with 5-Ph (**7**). In other cases, the variation of the 5-position substitution could also have a positive influence on the bioactivity, as shown with **15**, **19** and **20**. In particular, when a CH_2OH group was located at the 5-position of pyridine *N*-oxide (**15**), the corresponding IC_{50} value was increased nearly fourfold compared to that of 5-Me substitution (**3**). Similarly, with 5-$CH_2NH(CH_2)_2NH_2$ (**19**) and 5-$CH_2NH(CH_2)_3NH_2$ (**20**), which differ in the number of methylene groups only, the binding affinity was increased remarkably. The bioactivity of **20** was the best compound with an increased IC_{50} value of nearly fivefold compared to that of **3**. Finally, when a chiral center in the central carbon of the 1,3-diamine moiety of **15** was introduced by adding a hydroxy group (**17**), the binding affinity was abolished with an $IC_{50} > 200$ μM after this minor modification. This indicates that the 1,3-diamine moiety at the 5-position of pyridine *N*-oxide played an important role in the binding affinity of these small molecules.

In order to find how pyridine *N*-oxide 1,3-diamine derivatives bound to PCAF BRD, we attempted to analyze the interaction by molecular docking (Figure 3). It was found that the docked molecule took a better scoring pose with a higher level of sampling than the previous binding pocket of NP1. Based on the structure of complex PCAF BRD/NP1,[4] we obtained the docking structure (DOCK software)[14] of PCAF BRD with **3** and found that **3** interacts with the PCAF BRD side chain residues of W746, E750 and E756, similar to the interaction with NP1.

In conclusion, we developed an inhibitor screening and evaluation method for PCAF BRD using a FP competitive assay. Furthermore, a series of pyridine *N*-oxide 1,3-diamine small molecules were synthesized and screened by this FP competitive assay. Some effective lead compounds were selected to inhibit the PCAF BRD/Tat-AcK50 association in vitro, and the corresponding SAR was investigated. Among these compounds, **20** and **15** were found to be effective ligands for the PCAF BRD pocket. Importantly, the primary SAR will facilitate our efforts to design new inhibitors to recognize the PCAF BRD pocket. First preliminary cellular studies indicate that these small-molecule inhibitors have lower cytotoxicities and are potential leads for the anti-HIV/AIDS therapeutic strategy by targeting a cellular protein PCAF BRD to block HIV replication.

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Keywords: fluorescence polarization · HIV Tat · PCAF · protein–peptide interactions · small-molecule inhibitors

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