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Protein phosphatase inhibitory activity of tautomycin photoaffinity probes evaluated at femto-molar level

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Abstract—Herein we describe the further improvement of our in-house developed firefly bioluminescence assay system for the determination of inhibition of protein phosphatase (PP). The advantage with the new system is higher sensitivity as well as being time and sample efficient. The inhibition activity of tautomycin with PP1γ was determined using the upgraded test system and K_i was found to be 4.5 nM, which compare favorably with the activity reported previously by others using different methods. The test system was then used in order to determine the activity of nine tautomycin (TTM) photoaffinity probes. One of the TTM photoaffinity probes (*anti*-**10**) was found to possess higher activity than the natural product itself with a K_i of 3.4 nM, while the remaining photoaffinity probes were found to possess K_i in the range of 8.0–213 nM.

1. Introduction

Tautomycin (TTM, **1**, Scheme 1) was isolated in 1987 by Isono and co-workers from *Streptomyces spiroverticillatus* found in a soil sample collected in Jiangsu Province, China.[1] Three years later the fully assigned structure of TTM was reported to be as depicted in Scheme 1.[2] Tautomycin was found to have strong activity against *Sclerotinia sclerotiorum*[1] as well as induce morphological exchange in human leukemia cells K562.[3] However, more interesting is TTM's ability to inhibit type 1[4] and type 2A[5] protein phosphatase (PP1 and PP2A),[6,7] and in particular the fact that it inhibits PP1 more selectively than PP2A.[8] The dissociation constant for the PP1-TTM interaction compared to that of the PP2A-TTM interaction (viz. PP1/PP2A ratio) is in the range of 0.01–0.03.

As indicated in Scheme 1, TTM exists in a pH dependent equilibrium between the dicarboxylic acid form**1b** and the anhydride form **1a**. Under mild alkaline conditions (CH_3CN, 3% $NaHCO_3$, pH 8) TTM (**1**) predominantly exists as the diacid (TTMDA, **1b**).[10] However, under alkaline conditions (MeOH, 20% $CsCO_3$, pH 9) TTM (**1**) is unstable and undergoes trans-esterification followed by β-elimination resulting in the formation of acid **2** and alcohol **3** (Scheme 1),[2,10] neither of which shows any inhibitory activity toward PP1 or PP2A.[11] It was found that the active inhibitor is the dicarboxylic acid form of TTM (TTMDA, **1b**),[10,12] and indeed, the anhydride form (**1a**) possesses no inhibitory activity toward PP1 or PP2A. The hydroxyl groups at C22 and C3′ are also essential for biological activity[9,12] as well as the hydrophobic spiroketal moiety (see red boxes in Scheme 1).[9]

Based on computer calculations using Biograf and NMRgraf package programs utilizing NOESY data, we found that one of the stable conformations of TTMDA (**1b**) is U-shaped in D_2O and has a flexible structure from carbon C20 to C7′.[10] Recently this folded conformation was proven through special fluorescence-quenching experiments of the excited states of two chromophores attached at each end of TTM (**1a**).[13] However, when TTMDA (**1b**) is bound to the active site of PP1γ, it is expected that it will change conformation to the active one. A possible conformation of compound

Keywords: Tautomycin; Photoaffinity probe; Protein phosphatase 1; Firefly bioluminescence assay; Luciferine phosphate.
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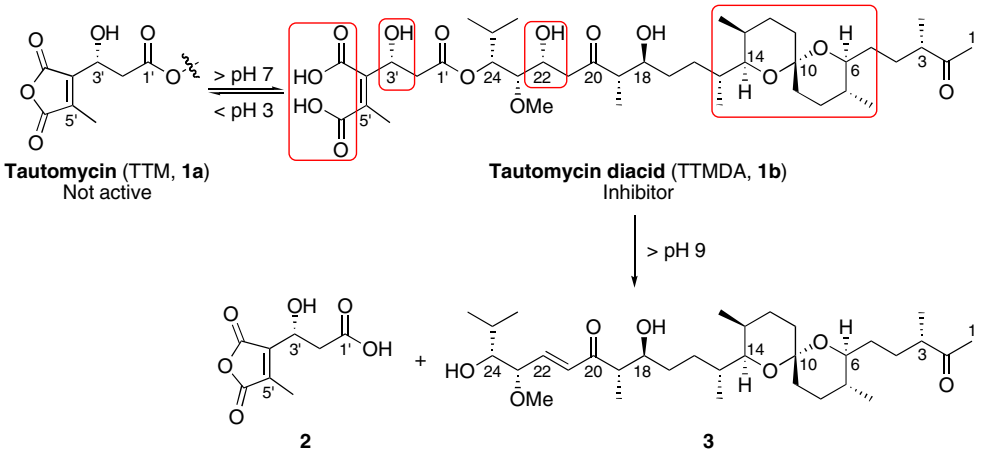


Scheme 1. Tautomycin (TTM, **1a**) and tautomycin diacid (TTMDA, **1b**). The red boxes indicate functional groups that are important for the biological activity.

1b complexed with PP1γ would be the one depicted in Fig. 1. This model was generated so that each functional group that is important for activity can have maximum interaction with one of the amino acid residues Arg96, Tyr134, Arg221, and Tyr272.[14] In particular interaction with Tyr272 seems to be important for activity. This fact was highlighted by Lee et al. who showed that a mutation in the protein at this amino acid lowered the activity of tautomycin and calyculin A (another natural product with activity toward PP1) by three orders of magnitude compared with the natural protein.[15]

Previously we have reported that the in-house developed firefly bioluminescence assay system is excellent for determining the inhibition activity of PP2A[16] and PP1γ[17] by tautomycin and other inhibitors of PP, such as okadaic acid, calyculin A, and microcystin-LR. Protein phosphatase 1γ is one of totally three isoforms of the PP1 catalytic subunit in mammalian cells with PP1α and PP1β (also called PP1δ) being the remaining two isoforms.[18]

The principle for the test system is outlined in Scheme 2. In the presence of PP1γ, luciferin phosphate (**4**) is dephosphorylated to give luciferin (**5**), which reacts with luciferase, adenosine triphosphate sodium salt (ATP), and Mg^{2+} to yield oxyluciferin (**6**) and light. The light emitted is detected by a single photon counter and converted to a digital signal, which can be integrated. However, with an inhibitor present the conversion of luciferin phosphate (**4**) to luciferin (**5**) will be hampered and the process eventually stops. As also shown in the Scheme, luciferin phosphate (**4**) does not react with luciferase, ATP, and Mg^{2+} to give light, eliminating the possibility of a false result.

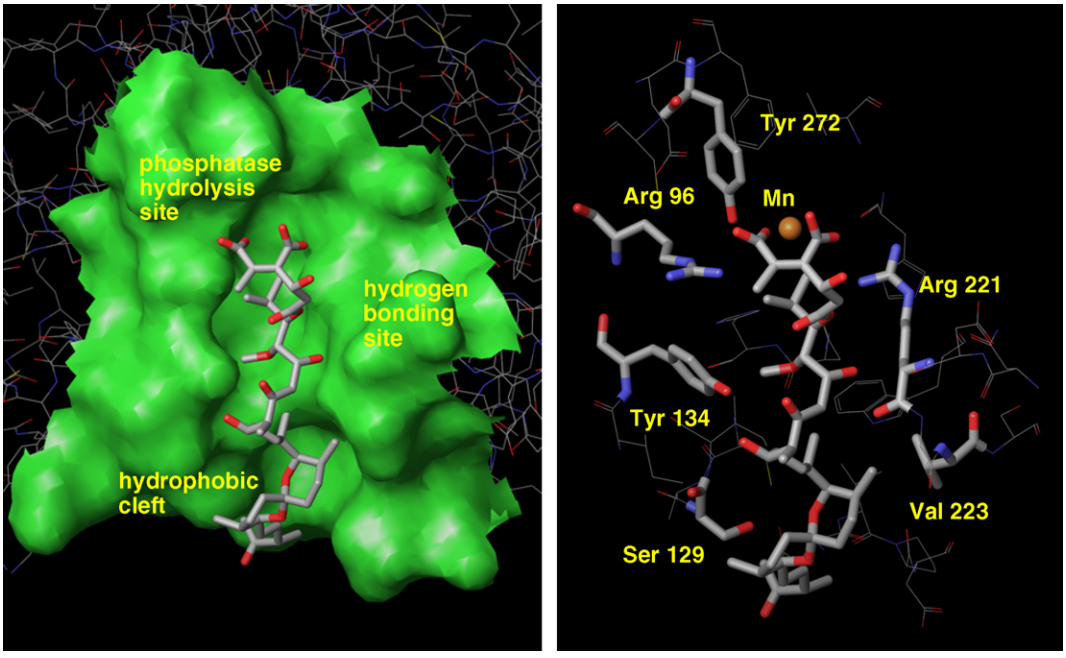
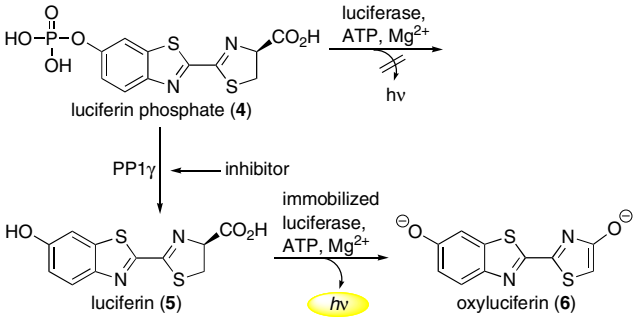


Figure 1. Proposed structure of PP1γ complexed with TTMDA (1b) (TTMDA was placed in the active site of PP1γ instead of calyculin A). The model is generated and minimized using MacroModel 9.1 (force field MMFFs and OPLS2001, using water as solvent).



Scheme 2. Principle for the firefly bioluminescence system.

We have for some time been involved in work aiming at elucidating the binding site of TTM with PP1γ using photolabeling followed by analysis using various MS techniques. This has to date resulted in the synthesis of various TTM photoaffinity probes.[19] Recently we also reported the synthesis of ^{13}C-labeled tautomycin and our efforts to study the interactions with PP1γ by using ^{13}C NMR.[20] However, the signals of the bound form of ^{13}C-labeled TTMDA with PP1γ could not be discerned due to line broadening occurring upon strong binding with PP1γ. The work described herein is part of our ongoing effort toward the ultimate target of pinpointing the binding site of TTM with PP1γ. Herein, we disclose the modifications made to the bioluminescence assay system; we also report the biological activity of our synthetic photoaffinity probes.

2. Results and discussion

2.1. Firefly bioluminescence system

The first generation firefly bioluminescence assay with PP1γ and inhibitors was operated by measuring the total light production during the dephosphorylation reaction (0–15 min).[17] The data obtained was then plotted as light intensity vs. time for each concentration of inhibitors [see Figure S1 in supplementary material (SM)]. The slope of these curves corresponds to the activity of luciferase at the tested concentration. The relative activities were then derived by comparing the absence of inhibitors to the presence of inhibitor at the different concentration of inhibitors. This data was then plotted in order to afford the IC_{50} value for the inhibitors.

For our advanced assay system we have further developed a new protocol for the measurement of the PP1γ activity (see Fig. 2). Protein phosphatase 1γ (10 μL of a 40 nM solution) and inhibitors with various concentration (5 μL of solutions with concentration 0–40 μM, which equals a test concentration of 0–10 μM after addition of PP1γ and luciferin phosphate solution) were premixed in sample vials to promote the formation of PP1γ-inhibitor complex. These vials containing the resulting 15 μL of sample mixtures were caped and placed in the sample-stage rack in the auto-sampler. Luciferin phosphate (5 μL of a 40 nM solution) was then injected into the first vial and mixed by applying a 2 μL air purge. After 6 min of reaction time an aliquot (1 μL)

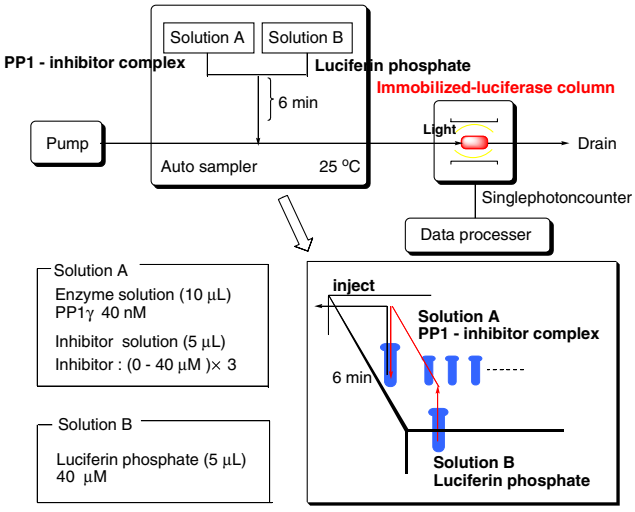


Figure 2. Schematic outline of the new assay protocol.

of the resulting solution was injected into the luciferase column (see Figs. 2 and 3) with a flow rate of 0.5 mL/min in order to measure the light yield. This procedure was then repeated three times for each sample at each concentration of inhibitor. The light intensity is directly correlated to the total amount of luciferin present in the sample, which is produced from luciferin phosphate upon hydrolysis by PP1γ. Based on the light yield for each sample [value found by integrating the signals in the PowerChrom chart (for an example of a PowerChrom chart see Figure S2 in SM)], the mean light yield at each concentration of inhibitor was obtained. Plotting this data gives the inhibitory curve (for an example of a inhibitory curve, see Figure S3 in SM), which enables us to obtain the IC_{50} value for the inhibitor tested. The K_i value was then obtained by using the function (Eq. 1) as outlined in Section 4.

Tautomycin (TTMDA, **1b**) was measured to have a K_i of 4.5 nM with our new assay protocol (Table 1). This value matched well with the data reported previously by others using different test methods [K_i = 0.425 nM,[8]

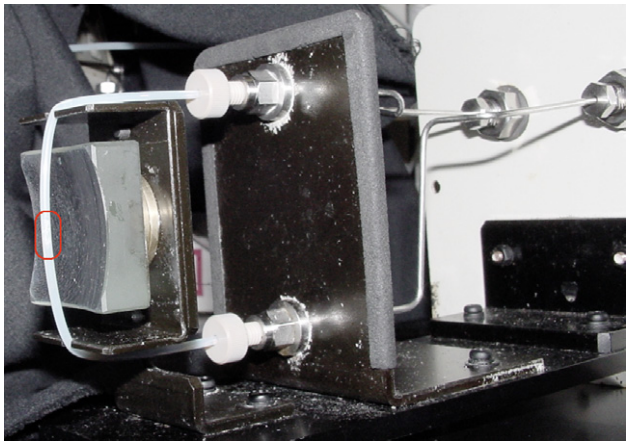


Figure 3. Photo of the column containing biotinylated luciferase placed in front of the mirror in the single photon counter. The immobilized luciferase is in the middle of the column (in the center of the red box).

Table 1. K_i values for the PP1γ inhibitory activity of photoaffinity probes *syn*-**7**–*anti*-**11**[a]

Compound	K_i (nM)	SD[b]
TTMDA (**1b**)	4.5	±0.1
syn-**7**	32.5	±2.3
anti-**7**	8.0	±0.2
syn-**8**	49.7	±4.1
anti-**8**	213	±6.1
syn-**9**	8.4	±0.3
anti-**9**	17.3	±1.7
anti-**10**	3.4	±0.4
syn-**11**[c]	23.9	±0.3
anti-**11**[c]	19.6	±1.2

[a] Each measurement was repeated three times.
[b] SD = standard deviation.
[c] The assay was conducted under the same way except the omission of dithiothreitol (DTT) in the Tris buffer in order to protect the S–S bonds (see Section 4 for details).

K_i = 0.16 nM,[21] IC_{50} = 0.19 nM,[22] and IC_{50} = 22–32 nM][23] and represents a significant improvement from the old assay system (IC_{50} = 610 nM).[17] Thus the results acquired with the new assay protocol were confirmed to be consistent with the results obtained previously by others. One of the merits with the new method is time-saving due to the simplified assay protocol. Since the reaction time between PP1γ and luciferin phosphate is fixed to 6 min, there is no longer a need to plot the light intensity versus time. By just measuring the amount of luciferin produced from the phosphatase reaction mixtures, the inhibitory curve can easily be obtained. The new method also has the advantage that we can measure the activity of many inhibitors at once, since the autosampler has 196 wells. With the high accuracy of the sample loader, we have been able to decrease the total sample usage to half of the volume needed for the previous method. Furthermore, as evident from the calibration data, the system can detect concentrations of 0.2 fmol/μL (0.2 nM) luciferin (see Figure S4 in SM) and shows a linear calibration curve with increasing concentration of luciferin (see Fig. 4). The system also has a very good reproducibility with only a 0.7% fluctuation for every integrated light yield at the same concentration of luciferin.

The assay system described herein offers advantages over other methods used to determine PP1 inhibition activity. The ^{32}P-labeled glycogen phosphorylase method, a commonly used method for such assaying,[21] uses radioactive phosphorous atom and therefore generates radioactive waste that requires special treatment post assaying. Compared with the *p*-nitrophenyl phosphate (*p*NPP) method, another commonly used method, the bioluminescence assay is much more sample efficient.[9] The *p*NPP method normally requires a sample concentration of 200–20000 μM while our assay system only requires a sample concentration of 10 μM.

2.2. Analysis of the photoaffinity probes of tautomycin

The photoaffinity probes of tautomycin (*syn*-**7**–*anti*-**11**) analyzed in this study possess three different photolabeling units, namely benzophenone (*syn*-**7** and *anti*-**7**), diazirine (*syn*-**8** and *anti*-**8**), and azide (*syn*-**9**-*anti*-**11**) (see Fig. 5). The synthesis of these compounds has recently been reported.[19] Tautomycin and the photoaffinity probes are stored as the anhydride and must therefore be converted to the active diacid form prior to testing. This was accomplished by treating the anhydride in acetonitrile/water 4:1 with base ($NaHCO_3$ aq solution) according to our previously reported method (see Section 4 for details).[19a] After a simple purification by HPLC, we obtained the active diacid form of the photoaffinity probes, viz. compounds *syn*-**7**–*anti*-**11**, which were subjected to activity testing using our improved bioluminescence assay system.

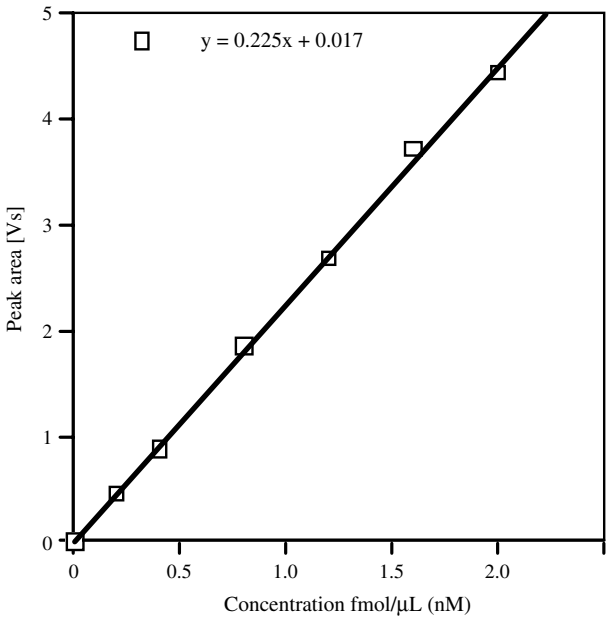


Figure 4. Calibration curve for luciferin at concentrations 0.2–2.0 fmol/μL (0.2–2.0 nM).

The assaying of the TTM photoaffinity probes was conducted in two rounds spread out in time with two different lots of protein using TTMDA (**1b**) as internal standard in both rounds.[24] The results from these analyses are summarized in Table 1 and show that photoaffinity probe *anti*-**10** has a K_i of 3.4 nM, which implements that this compound has higher activity than the natural product itself, making it an exceptional good inhibitor of PP1γ (for a proposed structure of PP1γ complexed with photoaffinity probe *anti*-**10**, see Fig. 6). Compounds *anti*-**7** and *syn*-**9** also possess strong activity toward PP1γ with K_i in the same order of magnitude as TTMDA (**1b**) (K_i = 8.0 and 8.4 nM, respectively). Photoaffinity probes *syn*-**7**, *syn*-**8**, *anti*-**9**, *syn*-**11**, and *anti*-**11** possesses moderate activity toward PP1γ, while compound *anti*-**8** is a rather weak inhibitor.

From the test results it seems that both the benzophenon and azide containing photoaffinity probes fit well in the active site of PP1γ while the methoxy group associated with the diazirine photolabeling unit prevents the photoaffinity probe to adapt an ideal position for binding. Furthermore, from the azide containing

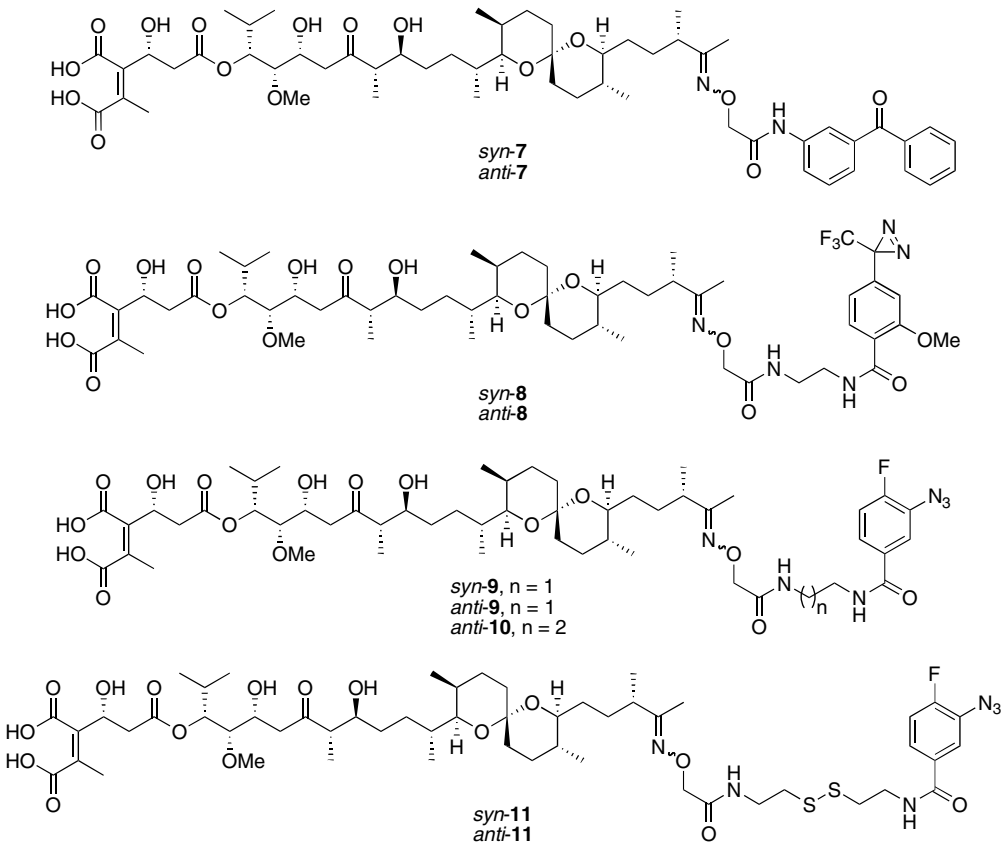


Figure 5. Tautomycin with photoaffinity probes.

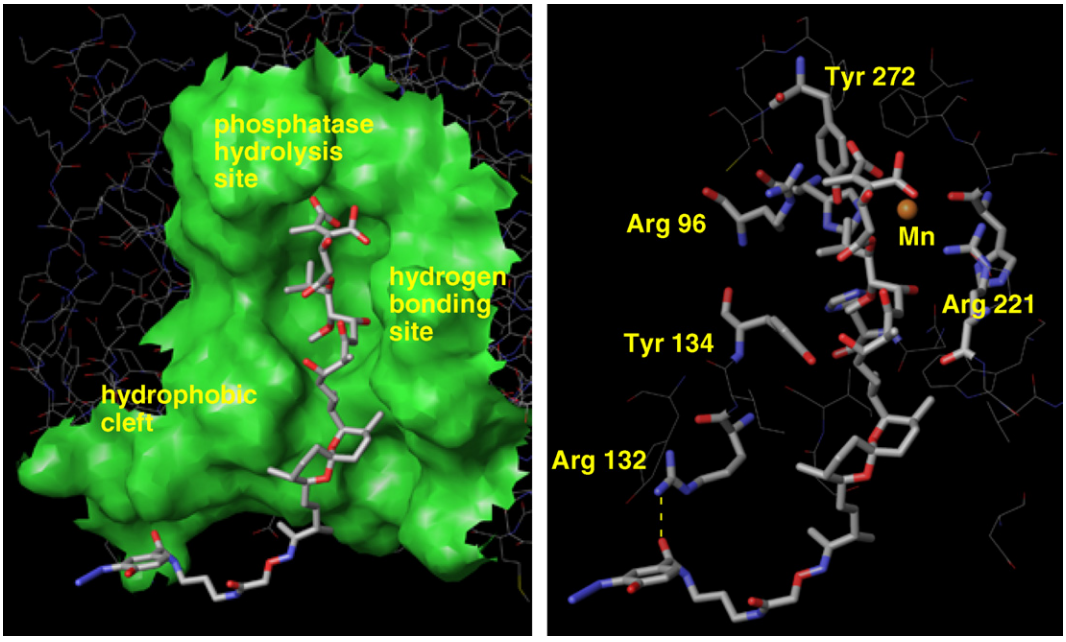


Figure 6. Proposed structure of PP1γ complexed with photoaffinity probe *anti*-**10** (compound *anti*-**10** was placed in the active site of PP1γ instead of calyculin A). The model is generated and minimized using MacroModel 9.1 (force field MMFFs and OPLS2001, usign water as solvent).

photoaffinity probes we see that the length of the linker also has some influence on the activity with the three-carbon spacer being the ideal length. The high activity for compound *anti*-**10** is thought to be due to the beneficial hydrogen bonding with Arg132 (see Fig. 6), an interaction which is not possible for the remaining photoaffinity probes.

Interestingly, compound *anti*-**7** possessed higher activity toward PP1γ than photoaffinity probe *syn*-**7**, while the

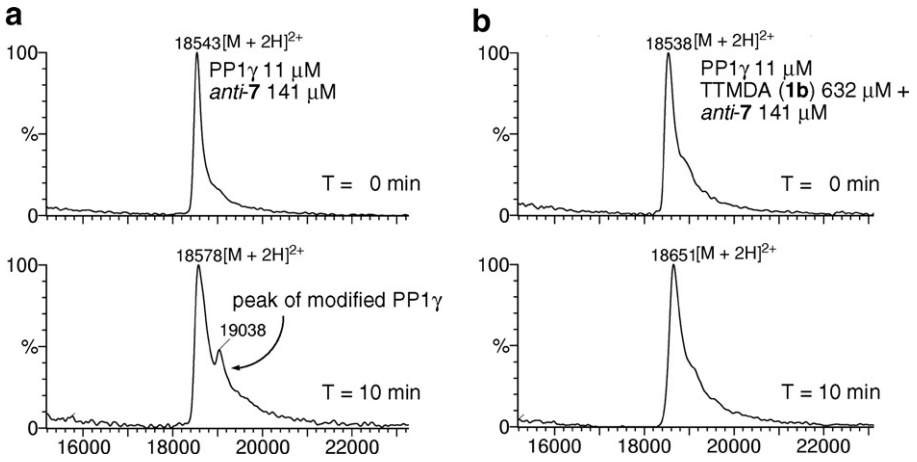


Figure 7. Preliminary results obtained by MALDI–TOF–MS analysis from photolabeling experiments utilizing photoaffinity probe *anti*-**7**. (a) Successful photolabeling of PP1γ. (b) Control experiment.

result was opposite for the two isomers of compound **8** with the *syn* isomer being the most active of the two. A similar phenomenon is also seen for photoaffinity probes **9** and **11**; however, the difference in K_i is much smaller in this case. The reason for this phenomenon is most likely connected with the spacial constraints present in the active site of PP1γ.

Despite the rather low activity for photoaffinity probe *anti*-**8**, we still anticipate that it will be useful for photolabeling studies. It could even be possible that the compounds with lower activity toward PP1γ could be the best substrates for photolabeling due to closer interaction with the protein surface.

With these active photoaffinity probes in hand, work is now focused on utilizing them for photolabeling studies. Preliminary results from these studies show that after 10 min irradiation (using a high-pressure mercury lamp) of a solution containing the complex between TTMDA (**1b**) and photoaffinity probe *anti*-**7** resulted in an increased mass of the protein (Fig. 7a). On the other hand, when the control experiment was conducted (Fig. 7b), which implies irradiating a sample of PP1γ, TTMDA (**1b**), and compound *anti*-**7**, no mass increase was observed. The latter result demonstrates that the labeling of PP1γ shown in Figure 7a is not due to random labeling of the protein, but the result of specific labeling after the photoaffinity probe has been bound to the active site. These encouraging results are now the basis for our further efforts toward establishing the specific point that has been labeled.

3. Conclusion

We have succeeded in improving the performance of our in-house developed firefly bioluminescence assay system. The new assay system has higher sensitivity [0.2 fmol/μL (0.2 nM)] and is time and sample efficient compared to the old version providing us with a powerful analytical method for the evaluation of our photoaffinity probes. The inhibitory activity of TTMDA with PP1γ measured with the new assay system matched well with the data reported in the literature. The nine TTM photoaffinity probes were found to possess activity toward PP1γ in the range of 3.4–213 nM. In particular, compound *anti*-**10** was found to be a very good inhibitor of PP1γ with activity greater than the natural product (K_i = 3.4 nM). With these active photoaffinity probes in hand, work is now focused on using them in order to pinpoint the binding site of TTM with PP1γ. Results from these studies will be reported in due course.

4. Experimental

4.1. General experimental

Tautomycin (**1**) used for this study was purified according to our previously published procedure[25] and luciferin phosphate (**4**) used for the assay was freshly prepared using our well-established method.[26,27] Unless otherwise mentioned, chemical reagents were of analytical grade. Immobilized biotinylated luciferase (BLU-Y) on avidine-beads (conjugated form)[28] was kindly provided by Kikkoman Company and was kept in the refrigerator prior to use. The immobilized luciferase retains its activity for a prolonged period of time,[29] and after more than 70 h at 25 °C it still has an activity greater than 60%. Protein phosphatase type 1γ expressed in *Escherichia coli* was donated by Dr. P.T.W. Cohen[30] and was purified according to our previously reported method.[20] Protein phosphatase 1γ was then stored in buffer containing 20 mM Tris (hydroxymethyl) aminomethan hydrochloric acid (Tris–HCl), 4 mM ethylendiamine tetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), and 50% (v/v) glycerol (pH 7.4 at 25 °C) at −20 °C until use. After measuring the concentration of PP1γ by using the titration method described by Takai and Mieskes,[31] PP1γ was diluted with the Tris–HCl buffer to give a 40 nM solution used for the assay. Tautomycin and the photoaffinity probes are stored as the anhydride and must therefore be converted to the active diacid form prior to testing. The progress of this reaction was monitored by HPLC using a Develosil C30-

UG-5 column (4.6 × 250 mm id), CH_3CN/H_2O 4:1 with a flow rate of 1.0 mL/min, detected at 254 nm.

4.2. Instrumentation

The luminescence detector consisted of a JASCO PU-980 pump, JASCO AS-1559® auto-sampler, JASCO single photon counter equipped with a HP53131 digital counter and an ADC R6142® voltage/current generator, and PowerChrom® software for digital sampling of the photon counting. The counted photons were integrated using the PowerChrom software on a Macintosh LC 630. The mobile phase was kept in a bottle cooled in an ice-bath and was heated to 25 °C through a 20 cm long loop placed in a water bath (25 °C) before flowing into the luciferase column (see Figure S5 in SM).

4.2.1. Preparation of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer. 40 mM Hepes, 2 mM $MgCl_2{\cdot}6H_2O$, and 0.4 mM EDTA were dissolved in distilled water and pH was adjusted to 7.76 (at 25 °C) by adding 1 M NaOH aq solution. The resulting solution was kept cool in an ice-bath.

4.2.2. Preparation of mobile phase. ATP (0.1% w/v) and DTT (0.01 mM) were added to the Hepes buffer and the resulting solution was kept in a bottle cooled in an ice-bath.

4.2.3. Preparation of buffer for activation of immobilized luciferase. ATP (0.5% w/v) and DTT (0.01 mM) were added to the Hepes buffer and the resulting solution was kept in a bottle cooled in an ice-bath.

4.2.4. Preparation of Tris (hydroxymethyl) aminomethan–HCl (Tris–HCl) buffer. 40 mM Tris, 30 mM $MgCl_2{\cdot}6H_2O$, and 20 mM KCl were dissolved in distilled water and pH was adjusted to 8.43 (at 25 °C) by adding 1 M HCl aq solution. The resulting solution was kept cool in an ice-bath.

4.2.5. Preparation of Tris buffer for dilution of PP1γ (Tris-B). Bovine serum albumin (BSA)[32] (0.15% w/v) and DTT (4.0 mM) were added to the Tris–HCl buffer and the resulting solution was kept cool in an ice-bath. For photoaffinity probes *syn*-**11**, and *anti*-**11** the buffer was prepared without DTT.[33]

4.2.6. Preparation and mounting of luciferase column. The immobilized luciferase was packed as a 3 mm long band at the center of a transparent polytetrafluoroethylene tube (1.07 mm id × 15 cm) and both sides of the luciferase band were capped with Teflon wool. A polytetrafluoroethylene tube (1.07 mm od × 7.2 cm) was then inserted into each end until it was parallel with the outer tube (see Figure S6 in SM). The luciferase column was then attached in front of the single photon counter (single photon counter is beneficial for measuring weak light emission with high sensitivity) and the luciferase column was activated by flowing the activation buffer through for 1 h with a flow rate of 0.5 mL/min. Immediately after the activation of the luciferase column was finished the flow of mobile phase was started. The mobile phase was kept flowing into the column with a flow rate of 0.5 ml/min during the luminescence assay.

4.2.7. Procedure for calibration of the single photon counter. Calibration of the photon counter was performed with the reaction between immobilized luciferase and luciferin (0–20 nM solution in Tris–HCl buffer). Each aliquot (1 μL) of luciferin solution was injected into the column every 2 min by using the auto-sampler (JASCO AS-1559) with a flow rate of 0.5 mL/min. The resulting light emission from the column was detected by the single photon counter. The data was accumulated with a HP 53131® digital counter with high *S*/*N* ratio and was then converted to analogue signals with an ADC R6142® voltage/current generator. The resulting current was then sampled with the PowerChorm® software and transferred to a Macintosh LC 630 with a SCSI cable. Total light yield for each concentration of luciferin was integrated using the PowerChorm software on a Macintosh LC 630. The HP 53131® digital counter was controlled with the LabVIEW program modified by JASCO. The dynamic range of the photon counter was adjusted to 0.01 mV/count for measurement with the LabVIEW program.

4.2.8. Method for converting tautomycin (1) and photoaffinity probe anhydrides (*syn*-7–*anti*-11) to diacid. An aqueous solution of $NaHCO_3$ (18 μL of a 20 mg/mL solution) was added to a stirred solution of the relevant anhydride (0.0025 mmol) in CH_3CN/water 4:1 (0.3 mL) at room temperature. The resulting reaction mixture was stirred for 3–4 h before being neutralized with HCl (0.1 N aq solution) and purified by HPLC [Develosil C30-UG-5 column (4.6 × 250 mm id), CH_3CN/H_2O 4:1, 1.0 mL/min, 254 nm] followed by concentration of the relevant fractions in vacuo. The resulting residue was then dissolved in DMSO/CH_3CN 4:1 in order to obtain a 4 mM stock solution of the inhibitor.

4.2.9. Method for sample preparation. The 4 mM stock solution of the inhibitors was diluted with Tris–HCl buffer in order to obtain samples with the following concentrations: 40.0 μM, 20.0 μM, 12.0 μM, 8.0 μM, 4.0 μM, 2.0 μM, 400 nM, 200 nM, 80 nM, 40 nM, 4.0 nM, 0.4 nM, 0.04 nM, and 0.0 nM. The sample solutions (5 μL) together with the PP1γ solution (10 μL of a 40 nM solution) were then put into sample vials and mixed prior to loading the vials into the sample-stage rack.

4.2.10. Activity test. Luciferin phosphate (5 μL of a 40 μM solution)[34] was injected into the sample vial followed by a 2 μL air purge. After 6 min 1 μL of the resulting mixture (final amount of sample 1.0×10^{-11}–0.0 mol, final amount of PP1γ 2.0×10^{-8} mol, final amount of luciferin phosphate 1.0×10^{-11} mol) was injected into the luciferase column with a flow rate of 0.5 mL/min. The resulting emitted light was then recorded as described in the procedure for calibration of the single photon counter (vide supra). This procedure was repeated three times for each sample at each concentration of inhibitors.

4.2.11. Dose–inhibition analysis; determination of K_i. In the previous experiments with tight-binding phosphatase inhibitors including okadaic acid, microcystins,

and calyculin A as well as TTMDA, we have shown that the steady-state dose–inhibition relationships at a fixed total enzyme concentration, E_t, is well described by a theoretical function

$$\phi(I_t) = \frac{E_t - I_t - K_i + \sqrt{(E_t - I_t - K_i)^2 + 4E_tK_i}}{2E_t} \times \phi(0) \quad (1)$$

where $\phi(I_t)$ denotes the enzyme activity at a given inhibitor concentration, I_t, and K_i stands for the dissociation constant.[8] This general model function takes into account the reduction of the free inhibitor concentration as a result of binding with the enzyme. In the present experiments the K_i values were estimated with the standard errors by non-linear least-squares regression of Eq. 1 to (I_t, $\phi(I_t)$) data obtained by dose–inhibition experiments. In the regression calculations, we used as the weight the reciprocal of the square of the standard error at each value of I_t.

4.2.12. Protein modeling. Docking simulations were performed following the method of Chamberlin et al.[22] Tautomycin and photoaffinity probes were overlaid on the bound conformation of calyculin A (from X-ray structure) and minimized by using MacroModel 9.1.

4.2.12.1. Photoaffinity labeling. A solution of photoaffinity probe *anti*-**7** (141 μM) and PP1γ (11 μM) in Tris–HCl buffer (10 mM) containing 0.4% DMSO and 3.4% CH_3CN was incubated at 0 °C for 30 min and then at 25 °C for 30 min. Five microliters of the resulting solution was loaded into a glass capillary tube (Ringcaps® 5/10 μL) and the tube was sealed with a gas burner. The sample was irradiated with a high-pressure mercury lamp for 10 min at room temperature and the resulting product mixture was subjected to MALDI–TOF–MS analysis.

4.2.12.2. Control experiment. A solution of TTMDA (**1b**) (632 μM), photoaffinity probe *anti*-**7** (141 μM), and PP1γ (11 μM) in Tris–HCl buffer (10 mM) containing 0.4% DMSO and 3.4% CH_3CN was incubated at 0 °C for 30 min and then at 25 °C for 30 min. Five microliters of the resulting solution was loaded into a glass capillary tube (Ringcaps® 5/10 μL) and the tube was sealed with a gas burner. The sample was irradiated with a high-pressure mercury lamp for 10 min at room temperature and the resulting product mixture was subjected to MALDI–TOF–MS analysis.

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Supplementary data

Figures S1–S6 and Supplementary material Figures 1 and 2 can be found in the supplementary material. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.11.034.

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34. The luciferin phosphate solution should be freshly made prior to use. If the luciferine phosphate solution is stored for a prolonged period of time, prior to use it is slowly dephosphorylated resulting in high background signals. Luciferin phosphate (solid) is stable for a long period of time when stored at −30 °C.