

Use of a fluorescent polarization based high throughput assay to identify new Calmodulin ligands

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Abstract

In order to develop a fluorescence polarization (FP) assay for calcium binding proteins, a fluorescent peptides based library of 1328 compounds has been synthesized. The use of this library has been validated by setting up a FP-high-throughput screening (FP-HTS) assay for calmodulin using the synthetic gene product (synCaM). With this assay, a set of 880 FDA approved compounds was screened. Besides the promazine class, we discovered two new classes of compounds that interact with calmodulin in a calcium dependent manner. One class has compounds with anti-histaminic/spasmodic activities, and the other one are detergents with antibacterial activities.

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1. Introduction

Calmodulin (CaM) is a fundamental calcium-modulated protein that is ubiquitous among eukaryotes. It has no inherent enzymatic activity but it mediates the intracellular Ca^{2+} signal to the degree of activation of a large number of regulatory proteins, including kinases, phosphatases and ion channels. As it plays important roles in many critical physiological processes, such as inflammation, metabolism, apoptosis, muscles contraction, intracellular movement, and short-term and long-term memory [1,2], it is of great importance to develop highly potent and specific inhibitors of CaM which may become a key site for pharmacological intervention.

Drugs that modulate calmodulin action offer tools for dissecting and investigating CaM-dependent pathways. Some small molecules have been previously reported to bind to CaM with high affinity [3,4]. However, a substantial number of them tend to do so with low specificity and uncertain stoichiometry [5,6]. The aim of our study is to develop a quantitative assay to

identify a diverse set of small molecules that disrupt the regulatory function of CaM.

High-throughput screening (HTS) based on fluorescence polarization (FP) measurements has acquired popularity as it is a highly sensitive method, easily miniaturizable, homogeneous in that no separation of bound and free species is necessary, and with a simple readout [7]. The fluorescence polarization technique is a very useful tool for binding studies at equilibrium when the free and bound species involved in the equilibrium have different rotational rates. When fluorescent molecules in solution are excited with polarized light, the degree of polarization depends on the extent to which the fluorescent molecule rotates during the interval between excitation and emission. The rapid rotation of small fluorescent molecules in solution results in substantial loss of polarization. If such small molecules bind to larger molecules, their rotational diffusion is reduced and the polarization degree is correspondingly increased. We therefore need to find at least one small fluorescent molecule that interacts with CaM.

The aim of our work was to find fluorescent lissamine-tagged peptides that would exhibit good calmodulin binding

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activity so that they could serve as probes in competition assays with unknown compounds.

We have developed a FP-high-throughput screening (FP-HTS) assay for calmodulin, using synthetic CaM (synCaM) [8,9] and a new fluorescent ligand synthesized in the laboratory. With the assay in hands, we have screened a collection of FDA approved drugs (The Prestwick library). The characterization of three classes of compounds that interact with calmodulin are reported here.

2. Experimental section

2.1. Materials

All chemicals were obtained from commercial suppliers and used without further purification. DMSO, KCl, HEPES and EDTA were purchased from Sigma. Tris was from ALDRICH and CaCl₂ from FLUKA. Lissamine sulfonyl chloride was purchased from Acros, Rink Amide resin (100–200 mesh) and *N*-[(1*H*-benzotriazolyl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) from Novabiochem and Fmoc amino acids were from Advanced Chem Tech. Ultra pure water (Milli Q instrument from Millipore Corp. MA, USA) was used for the aqueous solutions.

Solid phase parallel synthesis of libraries was performed on a FlexChem apparatus (Robbins Scientific) on a 7 μ mol scale in FlexChem polypropylene Reactor Blocks (96-well, 2.4 mL/well medium, 50–90 μ m polyethylene frits).

The assays were carried out with synthetic CaM (synCaM). When necessary Ca²⁺ was removed from synthetic CaM using trichloroacetic acid precipitation as described previously [10].

2.2. Characterization of the fluorescent peptides

The RP-HPLC analyses were performed on a C18 Symmetry Shield (4.6 \times 150 mm) column using water/acetonitrile linear gradient (0–100% B in 30 min, 1 mL/min, 220 and 254 nm). The following buffers were used: (eluent A) water containing 0.1% TFA by volume; (eluent B) acetonitrile containing 0.1% TFA by volume.

ESI-TOF (electrospray time of flight) spectra were recorded on a Perseptive Biosystem Mariner 5155 spectrometer. The *m/z* range 200–2100 was scanned using an ionspray voltage of 4500 V. The nozzle was ranged between 30 and 60 V.

2.3. Synthesis of the fluorescent peptides based library: general procedure

The library was synthesized on an acid-labile Rink resin [11] (0.7 mmol/g, 0.15 mmol scale) using standard Fmoc/*tert*-butyl protocols [12] Coupling reactions were performed with the appropriate amino acid (4 eq.) in the presence of HBTU (4 eq.) and diisopropylethylamine DIEA (12 eq.) in DMF for 30 min. The Fmoc group was removed using an 80/20 (v/v) DMF/piperidine solution for two 10-min periods. Lissamine Rhodamine fluorophore was introduced using its corresponding sulfonyl chloride reagent (mixture of para/ortho isomers) in the presence of DIEA in CH₂Cl₂ for 24 h. Final cleavage and deprotection were performed by treating the resin with TFA/triisopropylsilane(TIS)/H₂O (95/2.5/2.5, v/v/v) mixture for 3 h. The resin was washed with CH₂Cl₂ and DMF and the filtrate collected in 96 well plates which was subsequently placed in a Genevac DD4 for 3 h to remove solvents. Resulting purple powders were dissolved with DMSO prior to biological testing.

2.4. Resynthesis of Lissamine Rhodamine B peptides 17F7-para/ortho and 16B5-para/ortho

Compounds were synthesized following the above general procedure. Lissamine Rhodamine B peptides were isolated by precipitation with Et₂O, dissolved in a 1:1 acetonitrile/H₂O solution and lyophilised to red foams. Both ortho and para Lissamine Rhodamine B peptide isomers were isolated using a semi-preparative HPLC on a C₁₈ Symmetry Shield column from Waters (19 \times 300 mm,

7 μ m) at a flow rate of 10 mL/min. All Lissamine Rhodamine B peptides were obtained with purity greater than 95% as assessed by RP-HPLC at 220 nm.

17F7-ortho isomer: 13.1 mg, 6% based on the loading of Rink Amide resin, *t_R* = 16.06 min, LRMS (EI) calcd for C₆₁H₇₉N₁₀O₁₂S₂ (M+H)⁺, 1207.5 found *m/e* 604.4 (M+2H)²⁺, 1207.5 (M+H)⁺, 1230.8 (M+Na)⁺. 17F7-para isomer: 35 mg, 16%, *t_R* = 16.31 min, LRMS (EI) calcd for C₆₁H₇₉N₁₀O₁₂S₂ (M+H)⁺, 1207.5 found *m/e* 604.6 (M+2H)²⁺, 1207.5 (M+H)⁺. 16B5-ortho isomer: 17 mg, 7%, *t_R* = 15.99 min, LRMS (EI) calcd for C₆₅H₉₁N₁₂O₁₁S₂ (M+H)⁺, 1279.6 found *m/e* 640.5 (M+2H)²⁺, 651.5 (M+H+Na)²⁺, 1279.8 (M+H)⁺. 16B5-para isomer, 49 mg, 22%, *t_R* = 16.58 min, LRMS (EI) calcd for C₆₅H₉₁N₁₂O₁₁S₂ (M+H)⁺, 1279.6 found *m/e* 640.5 (M+2H)²⁺, 1279.9 (M+H)⁺.

2.5. FP-high throughput screening (FP-HTS)

Fluorescence polarization assays were performed using a Victor3 apparatus (Perkin-Elmer Life and Analytical Sciences, Boston, MA). The assay was optimized with respect to the concentration of lissamine-tagged compounds, and incubation conditions to fulfill HTS requirements. Assays were conducted in Corning Costar 96-well black polystyrene Xat-bottomed plates (Model 3686; Corning, Acton, MA). To each well, lissamine-labeled compounds (ranging from 20 to 100 nM) were added to a volume of 50 μ L in the assay buffer (50 mM HEPES, 150 mM KCl, pH 7.5) and 0.02% DMSO. The plate was mixed and incubated for 15 min at room temperature. The polarization degrees (FP) were measured with an excitation wavelength set at 530 nm (bandwidth 7 nm) and an emission wavelength set at 610 nm (bandwidth 10 nm). These values termed FP_{free} correspond to the polarization of free lissamine-tagged compounds. The binding assays were realized by adding a CaM solution at a final concentration of 2 μ M and 10 μ M CaCl₂ in the same buffer in a total volume of 100 μ L. The polarization values FP_{bound} corresponding to CaM-bound lissamine-tagged compounds, were determined under the same conditions. For each plate, FP measurements were performed with control wells containing no compounds.

For assay stability testing, a plate was measured before and after conservation for 24 h at 4 °C. Ca²⁺-dependence was tested by adding EDTA at a 1 mM final concentration to the wells. To determine the best concentration ratio (synCaM/fluorescent compounds), binding experiments were repeated with concentrations of fluorescent molecules varying from 10^{−8} to 10^{−6} M. Assays were usually done in a final volume per well of 100 μ L.

2.6. Fluorescence polarization degree

Fluorescence polarization is defined by the equation $FP = (I_{//} - I_{\perp}) / (I_{//} + I_{\perp})$ where FP is the fluorescence polarization degree, *I_{//}* and *I_⊥* are the fluorescence intensities of the vertically (*//*) and horizontally (*⊥*) polarized emission, when the sample is excited with vertically polarized light.

In FP-HTS experiments, background correction was done by subtracting blank parallel and perpendicular components (means of 8 wells) from the respective intensities.

2.7. Spectroscopic measurements

Steady-state absorption spectra were recorded on a Cary 4000 (Varian) spectrophotometer to determine the protein and fluorescent compounds concentrations. Extinction coefficients of 1500 and 88,000 M^{−1} cm^{−1} were used for synCaM and lissamine-tagged compound, respectively. All spectra were corrected for lamp intensity variations and background.

Fluorescence polarization measurements were performed in a 4 \times 10 mm quartz cuvette under continuous illumination using a T-format device on an SLM-Aminco 8000 spectrofluoropolarimeter. All measurements were carried out at 20 °C, the excitation wavelength was 540 nm, and the emitted light was monitored at 580 nm (interference filters (Schott) with a bandpass of 8.0 \pm 0.5 nm).

2.8. CaM-ligand binding assays

Polarization titrations were performed by adding increasing amounts of a fluorescent compound to a fixed concentration of synCaM (2 \pm 0.5 μ M) in 50 mM HEPES, 150 mM KCl, and 10 μ M CaCl₂, pH 7.5. Experiments were also performed in the presence of 1 mM EDTA.

During titration, for each fluorescent ligand, the equilibrium ratio of free over total ligand concentration was monitored by fluorescence polarization. The equilibrium association constant ($K_{\text{ass}}=1/K_d$) was calculated by fitting the sigmoidal dose-dependent FP change as a function of fluorescent probe concentrations using Microsoft Excel solver and the following equations, taking into account the fluorescent intensity change for the probe from the bound to the unbound form.

$$[P] + [L][PL]$$

$$K_{\text{ass}} = \frac{[PL]}{[P] \cdot [L]} \quad (1)$$

$$[P]_0 = [P] + [PL] \quad (2)$$

$$[L]_0 = [L] + [PL] \quad (3)$$

$[P]$, $[L]$, and $[PL]$ are, respectively, the equilibrium concentrations of the free protein, of the free ligand, and of the protein–ligand complex. $[L]_0$ is the total ligand concentration (X input), $[P]_0$ is the total protein concentration (constant), and K_{ass} is the association constant (variable to be fitted).

Solving Eqs. (1)–(3), we obtain $[L]$ as a function of K_{ass} , $[P]_0$, and $[L]_0$.

$$[L] = \frac{-1 - K_{\text{ass}}[P]_0 + K_{\text{ass}}[L]_0 + \sqrt{(1 + K_{\text{ass}}[P]_0 - K_{\text{ass}}[L]_0)^2 + 4K_{\text{ass}}[L]_0}}{2K_{\text{ass}}} \quad (4)$$

From Eqs. (1) and (2), omitting $[P]$, we obtain:

$$[LP] = \frac{K_{\text{ass}}[L] \cdot [P]_0}{K_{\text{ass}} \cdot [L] + 1} \quad (5)$$

Fluorescence polarization as a function of $[L]$ is described as follow:

$$FP = FP_L \frac{[L]}{[L]_0} + FP_{LP} \frac{[LP]}{[L]_0} \quad (6)$$

where FP_L is the FP value of free ligand, FP_{LP} is the FP value of protein–ligand complex.

Substituting Eq. (5) into Eq.(6) we obtain

$$FP = FP_L \frac{[L]}{[L]_0} + FP_{LP} \frac{K_{\text{ass}}[L] \cdot [P]_0}{(K_{\text{ass}}[L]_0 + 1) \cdot [L]_0} \quad (7)$$

3. Results and discussion

To find new compounds that interact with synCaM, our strategy consisted in four steps:

- designing and synthesizing the fluorescent bis-cations based library,
- screening of the fluorescent chemical library by synCaM to select interacting probes,
- resynthesizing of these probes and characterization by anisotropy titration,
- screening of the Prestwick chemical library by displacement with the probe of highest affinity.

3.1. Design and synthesis of the fluorescent bis-cations based library

In order to have a high hit rate, we designed our library around a “frequent hitter” pharmacophore, the bis-cation motif formed by two basic groups protonated at physiological pH

(Fig. 1). Indeed, the presence of charged residues at the hydrophilic surface of proteins increases the likelihood to bind organic bis-cations with some affinity in the neighbourhood of important binding and functional sites. The nature of the charged building blocks, the additional uncharged building blocks, the spacers as well as the fluorophore itself were expected to modulate the affinity and the specificity of these compounds. Two fluorescent peptides based libraries L1 and L2 [13] were designed around the bis-cation platform allowing the synthesis of a total of 1328 compounds. Chemical structures of library L1 bearing 4 points of diversity (R_1 to R_4) are depicted in Fig. 1.

The Lissamine Rhodamine B as a mixture of ortho and para isomers was selected as fluorescent dye. In addition to its suitable spectral properties within this FP based assay, this dye was found cheap, chemically stable through the synthesis and readily incorporated into the peptide backbone by using its sulfonyl chloride precursor. R_1 and R_3 groups have been chosen for their potency to be protonated at physiological pH to form the bis-cation structure. R_2 moieties have been carefully selected to cover all possible intermolecular interactions. Finally, the linker R_4 has the simple role of varying the distance between the core of the molecule and the fluorescent dye. Among the 612 possible combinations for each sub-library, only 560 molecules have been actually prepared in order to take into account the 80 well architecture of our synthesis device. The synthesis was performed in parallel on 96 well plates by using a traditional Fmoc/*tert*-butyl solid phase approach [11] on a Rink amide resin [12]. Following the above strategy, a total of 7 plates containing 80 compounds each were obtained.

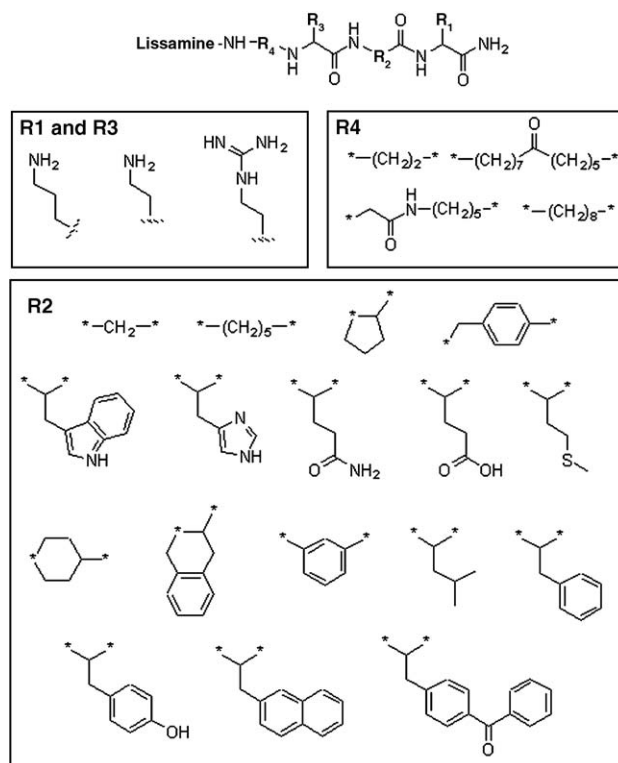


Fig. 1. Chemical structures of fluorescent peptides based library L1.

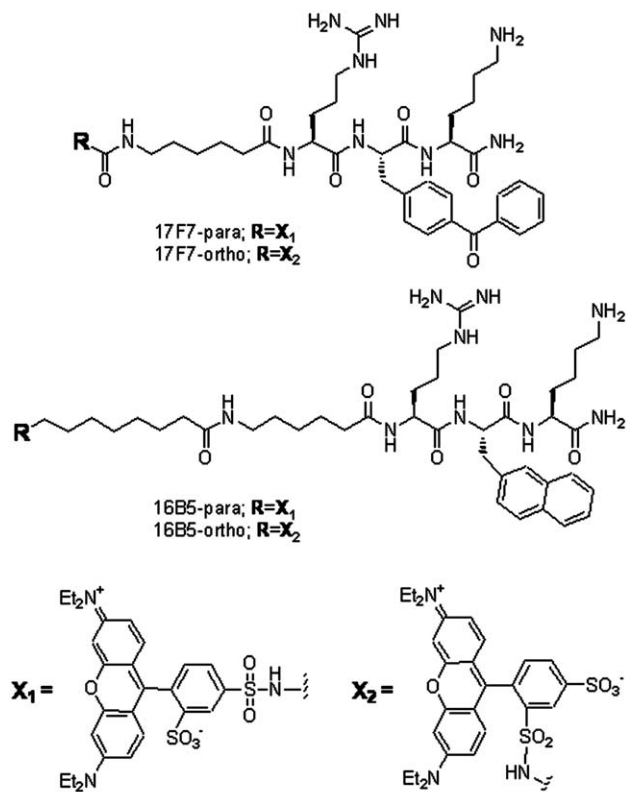


Fig. 2. Chemical structures of fluorescent peptides derived from HTS screening.

Compounds located on the diagonal of each plate were analyzed by both RP-HPLC (at 220 and 254 nm) and ES-MS to get a statistical representation of both the purity and identity of the library.

3.2. SynCaM FP screening assay of the fluorescent chemical library

The library of the 1328 lissamine-tagged compounds was screened with 2 μ M synCaM in the presence of 10 μ M Ca^{2+} . The assays were based on a change in fluorescence polarization (excitation at 535 nm and emission at 610 nm) upon binding of the fluorescent compound to synCaM. While the free fluorescent compounds show a low polarization, the bound fluorescent compounds give high fluorescence polarization values. Thus, by measuring the relative intensity of emitted light in the planes normal and orthogonal to the plane of the incident polarized light, the extent of rotation of a target molecule, and inferentially, the extent of binding of the target molecule to CaM can be calculated.

The number of compounds determined to interact with synCaM in a Ca^{2+} -dependant manner (the “hit rate”) was high. Initially, approximately 10% of the compounds from the library showing a polarization degree higher than 190 mP in their free state were discarded. The standard deviation (SD) was calculated on the 90% remaining compounds.

The performance assay was measured by the analysis of statistical quality parameter, such as the Z' factor [14]. The

Z' factor quantifies the amount of separation in measured signals, that is the fluorescence polarization degree between bound and free-ligand in our FP-screening assay, while accounting for the observed variability. A Z' value of 0.69 was calculated from our data. Typically, an assay in which $0.5 \leq Z' \leq 1$ is considered as excellent because it reflects a large separation band and dynamic range. Hits were selected when $\frac{FP_{\text{bound ligand}} - FP_{\text{free ligand}}}{SD} \geq 26$. They constitute 1% of

the lissamine-tagged compounds and belong to two main families sharing structural analogies. Among these, we have chosen 2 molecules with easily synthesis procedure and high affinities: compounds 16B5 and 17F7 belonging to the naphthalen and benzophenon families, respectively.

3.3. Characterization of the fluorescent ligands

Both probes were re-synthesized following the solid phase strategy described in the experimental section. For each compounds, 16B5 and 17F7, their para and ortho isomers were carefully separated by RP-HPLC (Fig. 2).

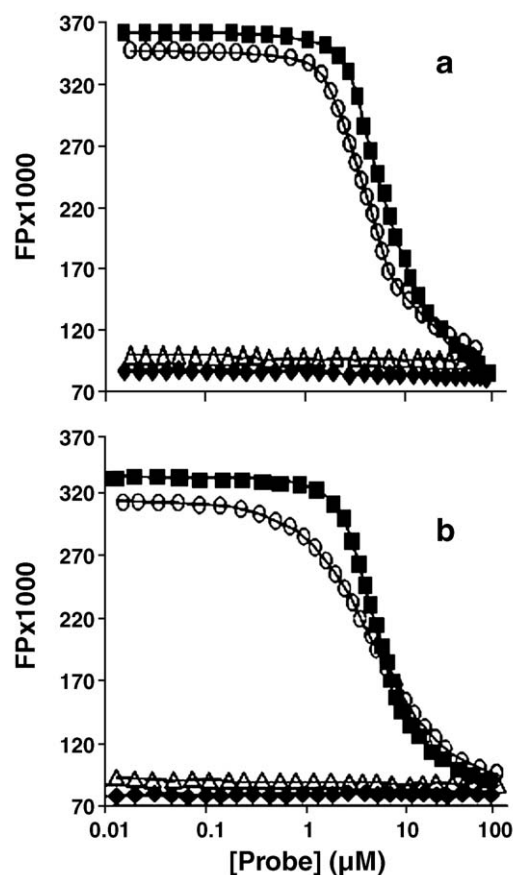


Fig. 3. Fluorescence polarization titration in the direct binding of probes to SynCaM (2 μ M) by 16B5 (a) and 17F7 (b) compounds with Ca^{2+} (■) para and (○) ortho isomers or without Ca^{2+} (◆) para and (△) ortho isomers. Each data point was measured in duplicate. The polarization degree FP was plotted against the log of the test compound concentration and nonlinear regression analysis was performed to determine the K_d of each compound.

Then synCaM was titrated with the isomeric forms of both compounds in the absence and the presence of Ca^{2+} in order to determine K_d values in the binding assay. Representative experiments are shown in Fig. 3. Data points consist of an average of two independent experiments. In the absence of calcium (EDTA 1 mM) the FP-values remain very low and do not vary with the probe concentration. In the presence of calcium, addition of low concentrations of either fluorescent compound resulted in an increase in the fluorescence polarization degree, indicating that the probe binds to synCaM in a calcium-dependent manner. As the concentration of each compound was increased the amount of binding decreased after a plateau; hence the degree of polarization correspondingly decreased. Binding data were fitted to a one-site binding equation using nonlinear regression curve fitting (see Experimental section). Analysis of the binding curves allows determining K_d values of 0.144, 1.45, 0.067 and 0.1 μM for compounds 17F-para and ortho isomers and 16B5-para and ortho isomers, respectively. We then developed the assay using the compound with the highest affinity, namely 16B5-para isomer, for synCaM in the presence of saturating calcium concentration.

3.4. Screening of the Prestwick chemical library

Using the assay, we then screen the Prestwick chemical library ($Z'=0.84$ for this assay). This library contains compounds already on the market to treat specific pathologies. The study was performed by FP-based competitive binding assay. When there are no competing compounds present, the lissamine-tagged product will be bound to synCaM resulting in a high FP value. In the presence of competitors, the lissamine-tagged compound is displaced from synCaM, resulting in a decreased FP value. About 880 compounds were screened with synCaM in a competitive polarization assay. Thirteen hits were found and characterized by polarization titration ($\frac{\text{FP}_{\text{bound ligand}} - \text{FP}_{\text{displaced ligand}}}{\text{SD}} \geq 12$). Analysis of the titration curves leads us to identify three structural classes of compounds corresponding to three classes of affinity: three compounds belonging to the already known class of promazine [4], seven compounds with antihistaminic/spasmolytic activities, and three compounds with antibacterial activities [15]. As an example, curves obtained with compounds 9F4, 11A8 and 10F8 which belong to each class (mentioned above) were presented in Fig. 4.

It could be assumed that each class of compounds interacts differently with synCaM. Based on previous observations that mutations in the calmodulin sequence induce different conformational changes that differentially modulate the interaction with CaM target enzymes [16], one may expect that a differential binding of small molecules to CaM may also lead to alternative structures in which a given interaction or a given set of interactions between CaM and target enzymes is disrupted. Such molecules would constitute valuable tools in deciphering Ca^{2+} /CaM signalling. To further characterize the interaction of these compounds, we will now use our

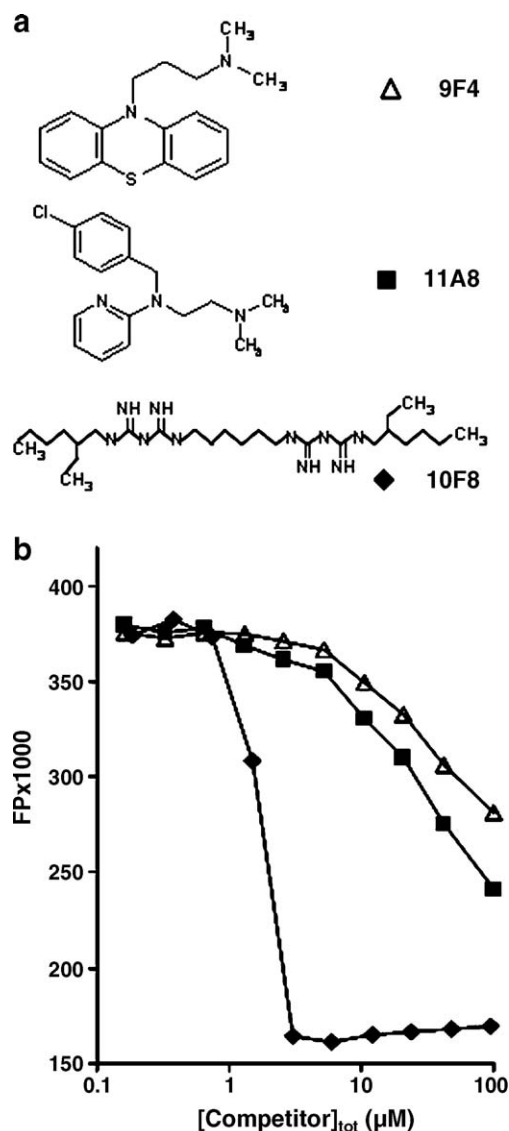


Fig. 4. (a) Molecular structures of the selected Prestwick chemical compounds. (b) The affinities of the Prestwick chemical compounds 9F4 (Δ), 11A8 (\blacksquare) and 10F8 (\blacklozenge) were determined by their ability to displace the fluorescent ligand 16B5 para isomer (0.1 μM) using FP-based competition assay. SynCaM concentration was 1 μM . Each data point was measured in duplicate. The polarization degree FP was plotted against the log of the concentration of the total competitor added. The order of affinities is 10F8 > 11A8 > 9F4.

collection of isofunctional synCaM Trp-mutants [17] to decipher the thermodynamical and the structural properties of the synCaM-small molecules complexes for each class of compounds.

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References

- [1] M. Zhang, T. Yuan, Molecular mechanisms of calmodulin's functional versatility, *Biochem. Cell Biol.* 76 (1998) 313–323.
- [2] D. Chin, A.R. Means, Calmodulin: a prototypical calcium sensor, *Trends Cell Biol.* 10 (2000) 322–328.
- [3] B.P. Orner, J.T. Ernst, A.D. Hamilton, Toward proteomimetics: terphenyl derivatives as structural and functional mimics of extended regions of an α -Helix, *J. Am. Chem. Soc.* 123 (2001) 5382–5383.
- [4] B. Sharma, S.K. Deo, L.G. Bachas, S. Daunert, Competitive binding assay using fluorescence resonance energy transfer for the identification of calmodulin antagonists, *Bioconjug. Chem.* 16 (2005) 1257–1263.
- [5] V. Harmat, Z. Böcskei, G. N  ray-Szab  , I. Bata, A.S. Csutor, I. Herme  cz, P. Ar  nyi, B. Szab  , K. Liliom, B.G. V  rtessy, J. Ov  di, A new potent calmodulin antagonist with arylalkylamine structure: crystallographic, spectroscopic and functional studies, *J. Mol. Biol.* 297 (2000) 747–755.
- [6] H. Yin, K.K. Frederick, D. Liu, A.J. Wand, W.F. Degrado, Arylamide derivatives as peptidomimetic inhibitors of calmodulin, *Org. Lett.* 8 (2006) 223–225.
- [7] G.J. Parker, T.L. Law, F.J. Lench, R.E. Bolger, Development of high throughput screening assays using fluorescence polarization: nuclear receptor–ligand-binding and kinase/phosphatase assays, *J. Biomol. Screen.* 5 (2000) 77–88.
- [8] D.M. Roberts, R. Crea, M. Malecha, G. Alvarado-Urbina, R.H. Chiarello, D.M. Watterson, Chemical synthesis and expression of a calmodulin gene designed for site-specific mutagenesis, *Biochemistry* 24 (1985) 5090–5098.
- [9] J. Haiech, M.C. Kilhoffer, T.J. Lukas, T.A. Craig, D.M. Roberts, D.M. Watterson, Restoration of the calcium binding activity of mutant Calmodulins toward normal by the presence of a Calmodulin binding structure, *J. Biol. Chem.* 266 (1991) 3427–3431.
- [10] J. Haiech, C.B. Klee, J.G. Demaille, Effects of cations on affinity of calmodulin for calcium: ordered binding of calcium ions allows the specific activation of calmodulin-stimulated enzymes, *Biochemistry* 20 (1981) 3890–3897.
- [11] H. Rink, Solid-phase synthesis of protected peptide fragments using a trialkoxy-diphenyl-methylester resin, *Tetrahedron Lett.* 28 (1987) 3787–3790.
- [12] G.B. Fields, R.L. Noble, Solid phase peptide synthesis utilizing 9 fluorenyl methoxycarbonyl amino acids, *Int. J. Pept. Protein Res.* 35 (1990) 161–214.
- [13] Design and synthesis of L2 library have been described into the 2 following publications: F. Renault, H. Chabane, D. Bonnet, M. Hibert, J. Haiech, H. Chneiweiss. Nouveaux compos  s interagissant avec PEA-15 Patent pending PB80200, 16/12/2005 and H. Chabane, F. Renault-Mihara, D. Bonnet, F. Guillier, P. Villa, M. Hibert, H. Chneiweiss, J. Haiech Intracellular FRET-based screening using a fluorescent library and GFP-tagged proteins: synthesis of a modulator of PEA-15 function. submitted for publication.
- [14] J.H. Zhang, T.D. Chung, K.R.A. Oldenburg, Simple statistical parameter for use in evaluation and validation of high throughput screening assays, *J. Biomol. Screen.* 4 (1999) 67–73.
- [15] P. Gilbert, L.E. Moore, Cationic antiseptics: diversity of action under a common epithet, *J. Appl. Microbiol.* 99 (2005) 703–715.
- [16] J. Haiech, M.C. Kilhoffer, T.A. Craig, T.J. Lukas, E. Wilson, K. Guerra-Santos, D.M. Watterson, Mutant analysis approaches to understanding calcium signal transduction through calmodulin and calmodulin regulated enzymes, *Adv. Exp. Med. Biol.* 269 (1990) 43–56.
- [17] M.C. Kilhoffer, M. Kubina, F. Travers, J. Haiech, Use of engineered proteins with internal tryptophan reporter groups and perturbation techniques to probe the mechanism of ligand–protein interactions: investigation of the mechanism of calcium binding to calmodulin, *Biochemistry* 31 (1992) 8098–8106.