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A fluorescence polarization-based assay for the identification and evaluation of calmodulin antagonists

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ABSTRACT

A fluorescence polarization (FP) assay was developed to identify calmodulin (CaM) antagonists. A fluorescent tracer was newly designed by covalently labeling *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), which is a well-known CaM antagonist, with the Cy5 dye. In the FP assay, the tracer (Cy5–W-7) was bound to CaM with a dissociation constant (K_d) of 6.5 μM and demonstrated efficient competitive activity with other CaM antagonists, including W-7, chlorpromazine, trifluoperazine, W-5, and clozapine, indicating that Cy5–W-7 binds to the ligand-binding site of CaM in a specific manner. The inhibitory activities of Cy5–W-7 and CaM antagonists were subsequently measured by the CaM-dependent calcineurin phosphatase assay, and the results were confirmed with those of the FP assays. In addition, assay optimization for high-throughput screening was performed, and a *Z′* factor of 0.7 was achieved in a 1536-well format. The FP assay was found to be a simple and reliable alternative to conventional assays for evaluating CaM antagonists.

Calmodulin (CaM)[1] is a ubiquitous protein highly conserved in eukaryotic cells as a primary intracellular mediator [1,2]. Two pairs of Ca^{2+} binding to the helix–loop–helix motifs (called the EF hands) expose the hydrophobic regions that provide interaction sites for target proteins such as enzymes, ion channels, and signal transduction proteins [3–6]. Despite its wide range of physiological substrates, CaM itself has no enzymatic activity; therefore, its activity is usually detected indirectly through the activity of CaM-dependent enzymes [7,8]. Although such enzyme assays are well suited for investigating the functional aspects, the complexity of their mechanisms usually obscures the results when multiple molecules are involved in regulation.

The molecular interaction of a protein and a ligand is the most primitive and essential biological event for indicating the presence of biological activity. Fluorescence polarization (FP) is one of the few methods that can detect molecular interactions without the use of radioisotopes. Other methods include surface plasmon resonance, calorimetry, frontal chromatography, and two-dimensional nuclear magnetic resonance (NMR) spectroscopy [9–12]. The FP assay is based on the physical property of small molecules, which rotate more rapidly than large molecules in solution [13,14]. Therefore, a small fluorescent molecule produces a high FP when it binds to a large molecule. The unique features of FP allow the development of simple and homogeneous assays in the microplate format [15,16].

We have developed a new assay to identify CaM antagonists and to determine their binding affinity using the FP technology. A previous FP study used a CaM-binding fluorescent peptide that was obtained by screening a peptide library [17]. Although the peptide probe bound to CaM in the presence of calcium ions, it was unclear whether the binding was specific, which can be determined by a competition experiment with a nonlabeled peptide. Furthermore, it was unknown whether the peptide had an antagonistic effect against CaM. In our study, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), which is a well-known CaM antagonist, was labeled with the Cy5 dye and used as a fluorescent tracer (Cy5–W-7) [8]. A previous structural study by multidimensional NMR spectroscopy suggested that the primary amine moiety of W-7 is not essential for binding to the active site of CaM [18]. In addition, it has been indicated that the derivatives of W-7 with different carbon chain lengths showed more potent activity [19]. These findings suggest that W-7 conjugated to Cy5 still maintains its specific activity to CaM. In fact, the polarization shift was observed in a dose titration study. A competition assay was subsequently performed with nonlabeled W-7 and other known CaM antagonists. The functional activities of these compounds were determined by stimulating calcineurin phosphatase with CaM [20]. FP assays can be readily

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[1] *Abbreviations used:* CaM, calmodulin; FP, fluorescence polarization; NMR, nuclear magnetic resonance; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; HTS, high-throughput screening; NHS–Cy5, *N*-hydroxysuccinimide ester-functionalized Cy5; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; mP, millipolarization units; MDCC–PBP, MDCC-labeled phosphate-binding protein; EGTA, ethyleneglycol-tetraacetic acid.

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adapted for high-throughput screening (HTS) in drug discovery research due to their simple and homogeneous format. The FP assay developed was further optimized and validated for HTS application. The results and conclusions are presented in this article.

Materials and methods

Materials

W-7 hydrochloride and W-5 hydrochloride were purchased from Wako Pure Chemical (Osaka, Japan). Clozapine and bovine serum albumin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human recombinant CaM, calcineurin, and RII phosphopeptide were purchased from Biomol (Plymouth Meeting, PA, USA). A phosphate-binding protein (phosphate sensor) was obtained from Invitrogen (Carlsbad, CA, USA). *N*-Hydroxysuccinimide ester-functionalized Cy5 (NHS–Cy5) was obtained from GE Healthcare (Piscataway, NJ, USA). Trifluoperazine and chlorpromazine were prepared in our lab. All other reagents were of analytical grade.

Fluorescent labeling

First, 5 mg of NHS–Cy5 and 4.8 mg of W-7 were dissolved in 630 μl of *N*,*N*-dimethylformamide to give concentrations of 10 and 20 mM, respectively. Next, the two solutions were mixed and the coupling reaction was initiated by adding 25 μl of 50% triethylamine in methanol. After incubation at 25 °C for 2 h, 630 μl of 50 mM ammonium bicarbonate (pH 9.0) was added to block the residual NHS ester groups.

Liquid chromatography purification

Cy5–W-7 was purified by a high-performance liquid chromatography system (Shimadzu, Kyoto, Japan) with a reverse phase column (Capcell Pak C18 UG80, 20 × 250 mm i.d., Shiseido, Tokyo, Japan). Mobile phase A consisted of 0.05% trifluoroacetic acid (TFA), and mobile phase B consisted of 0.05% TFA in acetonitrile. TFA (20 μl) was added to the reaction mixture obtained in the fluorescent labeling step. The mixture was fractionated with a linear gradient (35–45% B in 15 min) at a flow rate of 15 ml/min. The fractions containing Cy5–W-7 were gathered and evaporated under reduced pressure using a SpeedVac system. Dry powder was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM.

Saturation and competition binding assays

The FP assay was performed in 384-well low-volume black polystyrene plates (Corning, Corning, NY, USA) using a SpectraMax M5 plate reader (excitation 645 nm, emission 675 nm, Molecular Devices, Sunnyvale, CA, USA) or an Analyst GT plate reader (excitation 640 nm, emission 682 nm, Molecular Devices) coupled with a cutoff filter or dichroic mirror at 665 nm. Lyophilized CaM was dissolved in Tris/Ca^{2+} buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$). Cy5–W-7 was diluted with Tris/Ca^{2+} buffer supplemented with 0.02% (w/v) Triton X-100. A saturation binding assay was performed at concentrations ranging from 0.15 to 150 μM CaM with 1 μM Cy5–W-7, where 5 μl of Cy5–W-7 was added to an equivalent volume of CaM in each well. The concentration of Triton X-100 was maintained at 0.01%, which is below the critical micelle concentration, to avoid nonspecific binding of Cy5–W-7 to micelles of Triton X-100. After equilibration at room temperature for 1 h, FP was measured. Competition binding assays were performed in the presence of CaM antagonists W-7, chlorpromazine, trifluoperazine, W-5, and clozapine (Fig. 1), which were dissolved in DMSO. The compounds were serially diluted in Tris/Ca^{2+} buffer containing 0.02% (w/v) Triton X-100 and 2% DMSO. A 5-μl aliquot of the compound was added to 5 μl of premixed solution of CaM and Cy5–W-7 at final concentrations of 10 μM for CaM and 1 μM for Cy5–W-7. The same experiment was performed without CaM for measuring the polarization signal that was not due to protein binding. To minimize protein damage, the final concentration of DMSO was fixed at 1% (v/v). FP was measured after equilibration of competition for 1 h. The polarization degree was defined using the following equation:

$$P = 1000 \times \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + I_{\perp})},$$

where $I_{||}$ and $I_{\perp}$ represent the fluorescence intensities of parallel and perpendicularly polarized emission, respectively. These values were corrected by subtraction of the background fluorescence signal, which is derived from all assay components other than the fluores-

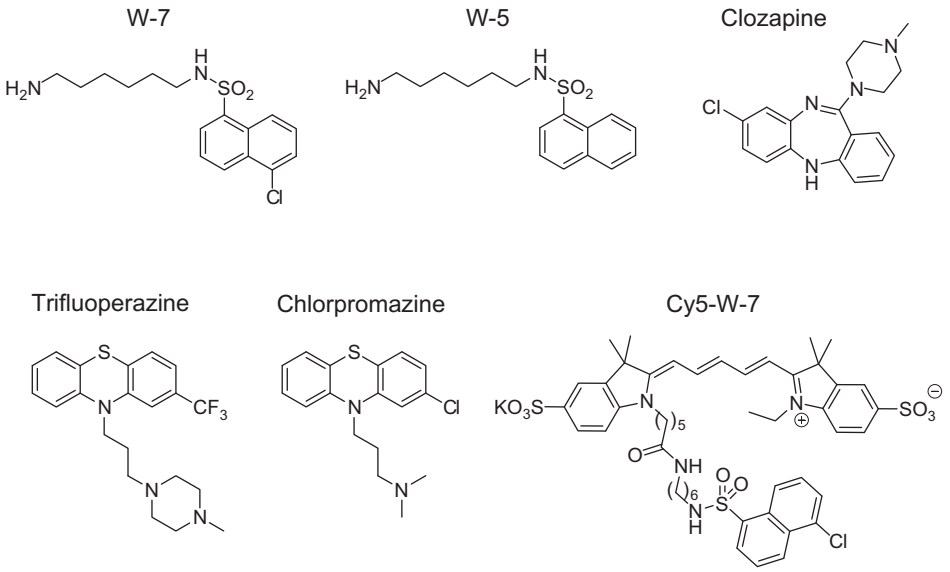

Fig. 1. Chemical structures of CaM antagonists.