Dynamic Ligation Screening

DOI: 10.1002/anie.200901647

Selective Identification of Cooperatively Binding Fragments in a High-Throughput Ligation Assay Enables Development of a Picomolar Caspase-3 Inhibitor**

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Fragment-based lead discovery has become popular over the past years, allowing for a more efficient sampling of the chemical space and thus resulting in higher hit rates than that for the screening of non-fragment chemical libraries.[1] Since the concept of fragment-based lead discovery was first proposed,[2] various detection methods have been used to identify the binding of fragments to protein templates, including HPLC,[3] NMR spectroscopy,[4] X-ray crystallography,[5] and mass spectrometry.[6] Recently, dynamic ligation screening has been introduced to detect reversibly ligated fragments in a biochemical assay through their competition with a fluorogenic enzyme substrate.[7]

Reported detection methods for protein-binding fragments, however, do not answer the most challenging question in fragment-based ligand discovery: How can a primary ligand as the starting point be extended optimally by a secondary fragment? While "second-site screening"[8] by NMR spectroscopy or crystallography delivers fragment hits for different binding sites, it does not provide information regarding the optimal, bioactive combination of fragments.

Evidently, for a thermodynamically optimized extension of a primary ligand (**A**) by a secondary fragment (**B**), cooperative binding of both components is required. Cooperative binding in this context results if the binding of **A** is amplified by the binding of **B**. Cooperative binding therefore is distinct from additive binding (in which **A** and **B** bind independently without mutual influence) and competitive binding where the secondary fragment **B** inhibits the binding of **A**. Cooperatively binding fragments result in strongly increased binding affinities if the two components are connected by a suitable covalent linkage. Consequently, a method for the selective detection of cooperatively binding fragments should be powerful in the development of potent protein ligands.

We envisioned that the selective detection of cooperative fragments should be possible through the combination of dynamic ligation screening (DLS)[7] with fluorescence polarization (FP) experiments.[9] Using a fluorescent directing probe for reversible fragment ligations would allow differentiation between additive, cooperative, and competitive binding of fragments to a protein target, as detected by unchanged, enhanced, or reduced FP, respectively (Figure 1).

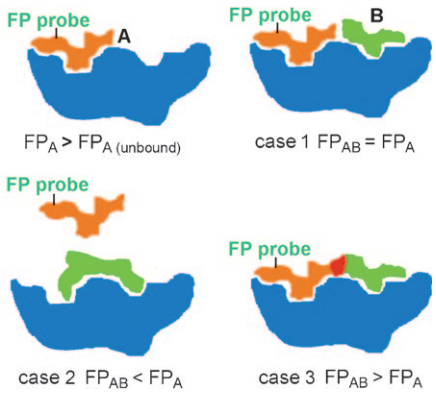

Figure 1. Selective detection of cooperatively binding fragments in a dynamic ligation assay. Binding of fragment **A** (orange) is determined by fluorescence polarization. Three alternative cases of fragment interactions can be distinguished in the assay: Additive binding of fragment **B** (green) has no effect on the observed polarization (case 1). Competitive binding leads to decreased polarization (case 2). Finally, cooperative binding of fragments **A** and **B** results in an extended correlation time τ_{AB} and, thus, an increase in FP (case 3).

Caspase-3 (cysteinyl-aspartyl-specific protease 3) was selected as protein target to test the feasibility of this approach for the identification of cooperative fragments. The protein has been identified as the cellular switch towards for apoptosis.[10] Therefore, it is considered as a drug target for clinical indications involving overregulated cell death, such as traumatic brain injury, status epilepticus, amyotrophic lateral sclerosis (ALS), and Parkinson's disease,[11] and potent inhibitors of caspase-3 have been reported.[12]

The fluorescent ligation probe was designed on the basis of the native substrate consensus sequence DEVD.[12c] An α-ketoaldehyde peptide was selected, as it enables the clear separation of the protein-interacting keto functionality from

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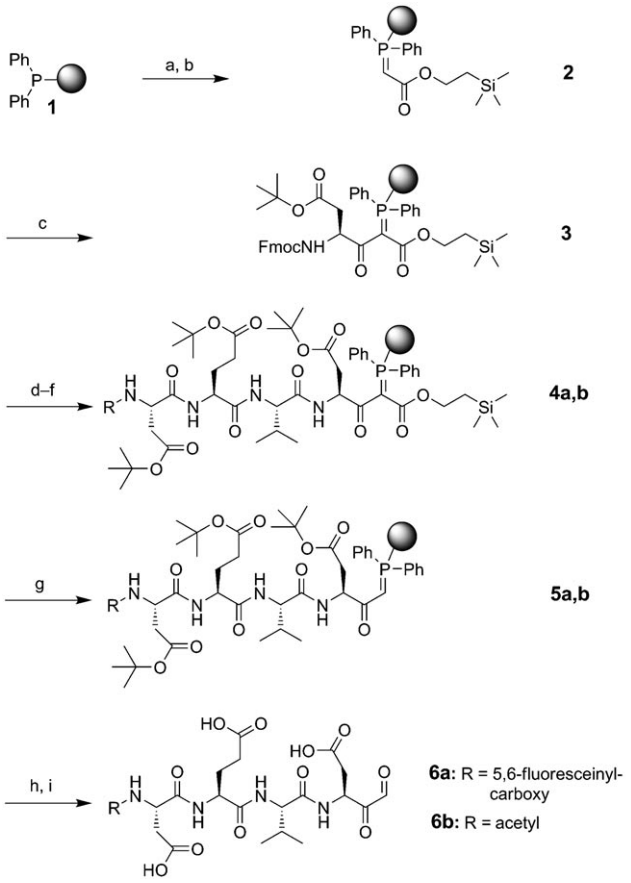
[**] We thank Christoph Erdmann, Franziska Hinterleitner, and Dagmar Krause for technical support and Dr. Samuel Beligny, Jörn Saupe, and Dr. Peter Schmieder for helpful discussions. Work at the Leibniz Institute of Molecular Pharmacology was supported by the DFG (FOR 806, SFB 765, Ra 895/2-5). J.R. thanks the Fonds der Chemischen Industrie for continuous support.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200901647.

the fragment-interacting aldehyde group. α-Ketoaldehydes have been reported to be fully hydrated in water, and reactions with amine nucleophiles are disfavored (endergonic) in aqueous solvents, the equilibrium being largely on the side of the hydrate and free amine.[13]

Reporter probes **6a** and **6b** were prepared by C-acylation of polystyrene-supported trimethylsilylethyl(TMSE)-phosphoranylidene acetate (Scheme 1).[14] The TMSE protecting group was removed by employing the mild fluoride reagent tris(dimethylamino)sulfonium difluorotrimethylsilicate (TAS-F), leading to instantaneous decarboxylation of the phosphorane on the resin. Oxidative cleavage with dimethyldioxirane (DMD) and acidic removal of the side-chain protecting groups yielded compounds **6a** and **6b**.



Scheme 1. Synthesis of the peptidyl α-ketoaldehydes CF-DEVD-CHO (**6a**) and Ac-DEVD-CHO (**6b**) from triphenylphosphane polystyrene, which was alkylated and acylated as reported earlier.[14] Reaction conditions: a) Trimethylsilylethyl bromoacetate (5 equiv), toluene, 15 min, microwave, 100 °C; b) Et_3N (5 equiv), in CH_2Cl_2, 2 h, RT; c) Fmoc-Asp-(O*t*Bu)-OH (5 equiv), MSNT (5 equiv), lutidine (4.9 equiv) in CH_2Cl_2, 12 h, RT; d) 20% piperidine/DMF, 6 min. e) Fmoc-AA-OH (5 equiv), DIC (5 equiv), HOBt (5 equiv) in DMF, 3 h (steps (d) and (e) were repeated *n* times); f) 5,6-carboxyfluorescein (10 equiv), DIC (10 equiv), HOBt (10 equiv) in DMF, 3 h, RT or Ac_2O (4 equiv) in DMF, 20 min, RT (two times); g) TAS-F (3 equiv), in DMF, 3 h, RT; h) DMD (3–4 equiv)/acetone, in CH_2Cl_2, 30 min, 0 °C; i) TFA/CH_2Cl_2/H_2O (50:45:5, v:v:v). Fmoc = 9-fluorenylmethyloxycarbonyl, MSNT = 1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole, DIC = *N*,*N*′-diisopropylcarbodiimide, HOBt = 1-hydroxy-1*H*-benzotriazole, TFA = trifluoroacetic acid.

The binding affinity of compound **6a** was determined by FP titration with caspase-3. The FP titration curve indicated a dissociation constant (K_D) of 15 nM. The FP assay was adapted to 384-well microtiter plates and used to test 7397 fragments including 4019 nucleophilic, primary amines from the ChemBioNet library. For the high-throughput screening 20 nM of caspase-3, 10 nM of **6a**, and 10 μM of each fragment was incubated in a total volume of 10 μL.

In agreement with the rationale behind our approach (Figure 1), fragments tested in this dynamic ligation assay were clustered into three different classes. First, no change in the FP signal was observed for most fragments, indicating noncooperative, additive binding of **A** and **B** or, more likely, no binding of **B** at all (Figure 1, case 1). Second, for 78 fragments a decrease in the FP signal was observed, suggesting a negative effect of **B** on the binding of **A**. One reason could be competitive inhibition of the binding of the fluorescent reporter probe **A** by fragment **B** (Figure 1, case 2). All 78 fragments were tested in an enzymatic assay using Ac-DEVD-AMCA **7** (see the Supporting Information) as the fluorogenic substrate of caspase-3.[12] Indeed, 21 of the negatively cooperative case 2 ligands were active at a concentration of 10 μM as competitive inhibitors. Four of them, **10**–**13**, could be identified as competitive inhibitors with K_I values in the low micromolar range (for structures, see Scheme 2 in the Supporting Information), making them some of the best nonpeptidic inhibitors reported to date.[12] Third, for 176 fragments the FP signal more than 20% stronger than that of the controls. This observation indicated positively cooperative binding of **A** and **B** to the protein, possibly through the formation of a ligation product with increased affinity. Fifty of the cooperatively binding fragments were validated in the enzyme assay. Fragment **8** (see Figure 4 and Scheme 3 in the Supporting Information) was identified as the most potent FP enhancer among the 176 tested compounds and displayed a K_I value of 120 μM.

To better understand the experimental data obtained, we had to rationalize the binding and interaction of compounds **6a** and **8** and quantify the degree of cooperativity (Figure 2). For this purpose, FP data obtained by titrating 10 nM **6a** in the presence of various concentrations of fragment **8** with caspase-3 (Figure 3a) were interpreted in terms of a thermodynamic model assuming either merely additive or cooperative binding of **6a** and **8** to caspase-3 (Figure 3b). In the first scenario, we assumed no interaction between **6a** and **8** by taking the ligation equilibrium constant as $K_C = 0$ (Figure 2). In this purely additive, noncooperative case, the presence of **8** is predicted to have no influence on the binding of **6a** (Figure 3b, black line). Indeed, the experimental data in the absence of **8** (black symbols) or in its presence at concentrations of 100 nM (blue symbols) and 1 μM (green symbols) were found to be in reasonable agreement with this scenario (Figure 3b).

The FP data at 10 μM **8** (red symbols), however, revealed that the stronger binding of **6a** is not in agreement with the noncooperative model. Therefore, in the second scenario, we explicitly implemented cooperative binding by allowing the formation of the ligation product from protein-bound **6a** and **8**, as reflected by $K_C > 0$ (Figure 2). Using this model, the FP

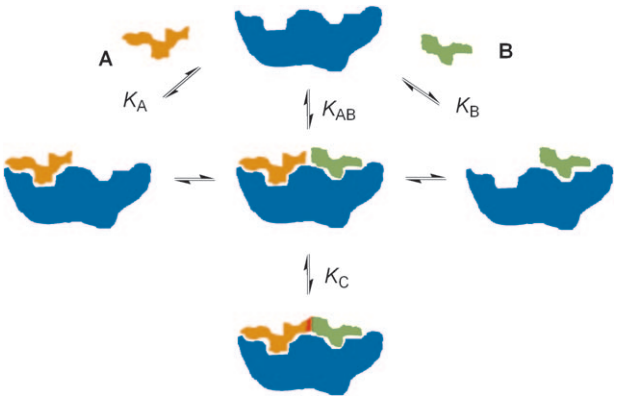


Figure 2. A model of additive and cooperative binding of molecules **A** (orange) and **B** (green) to a protein (blue). In the case of additive binding, **A** and **B** bind independently ($K_{AB} = K_A K_B$) and do not interact with each other ($K_C = 0$). Cooperative binding resulting from protein-templated ligation can be conceptualized as consisting of two stages: 1) additive binding of the two fragments as given by $K_{AB} = K_A K_B$ (upper part) and 2) formation of the ligation product as characterized by $K_C > 0$ (lower part).

data measured at all concentrations of **8** could be reproduced satisfactorily (Figure 3b, blue, green, and red lines). The best agreement with the experimental data was obtained assuming a ligation constant of $K_C = 20$. This indicates that the equilibrium concentration of the protein-bound ligation product is 20 times higher than that of protein carrying unligated **6a** and **8**, corresponding to a Gibbs free energy of ligation of $\Delta G° = -RT\ln K_C = -7\ \mathrm{kJ\,mol^{-1}}$. Thus, the protein-templated ligation reaction is exergonic, which starkly contrasts with the endergonic ligation reaction in aqueous solution, where no ligation product of α-ketoaldehyde and **8** is detectable.[13]

To confirm the cooperative binding of the reversibly formed ligation product, the covalent and irreversible ligation product of α-ketoaldehyde **6b** and the most active fragment **8** was synthesized by reductive amination (Figure 4). The obtained ketone **9** was tested in the functional caspase-3 assay. A K_I value of 80 pM was determined (Figure 3), making **9** the most active caspase-3 inhibitor reported to date.[12] Thus, compound **9** inhibits the enzyme more than 300 times more effectively than the α-ketoaldehyde precursor **6b** (K_I = 25 nM).

In summary we have developed and implemented the differential detection of cooperative and competitive fragments in one FP experiment operable in high-throughput format. Cooperative fragment binding can be verified and distinguished from merely additive effects. By employing a model for the ligation equilibrium we could estimate the ligation constant and the Gibbs free energy of the reversible ligation reaction on the protein surface. A chemically stable combination of the best fragment hits yielded the most potent inhibitor of caspase-3 reported to date. Thus, the described dynamic method for cooperativity screening can be used to dissect and analyze binding contributions of single fragments in a ligation system. As it is based on FP detection, the method is broadly applicable to enzyme targets as well as to protein-protein interactions. The information gained from this assay can be utilized directly for the construction of improved protein ligands by a rational approach.

Experimental Section

Synthetic procedures and analytical data of novel compounds are provided in the Supporting Information.

Fluorescence polarization (FP) assays were performed in untreated black 384-well microtiter plates (Corning B.V. Life Sciences, Schipol-Rijk, Netherlands) using the microplate reader Genios Pro, (Tecan, Crailsheim, Germany) (buffer conditions: 50 mM HEPES pH 7.4, 100 mM NaCl, 0.5 % CHAPS, and 1 mM EDTA). The peptidyl α-ketoaldehyde **6a** was titrated in a concentration of 10 nM against various concentrations of caspase-3 (0–100 nM) in a total volume of 10 μL. The excitation wavelength was 485 nm, and the emission wavelength was 535 nm. The measured FP data were analyzed in GraphPad Prism 4 for Windows (GraphPad, La Jolla, USA) by nonlinear regression (curve fitting).

The thermodynamic model used to distinguish between cooperative and additive binding is reported in detail in the Supporting Information. In short, raw FP and enzyme inhibition data (Figure 3a) were converted into binding isotherms (Fig-

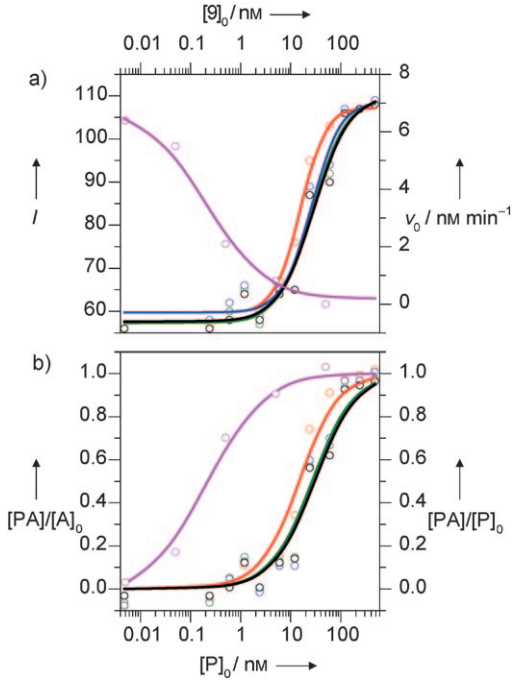


Figure 3. Titration experiments. a) Raw data (symbols) and sigmoidal fits (lines) according to Equation (1). FP was recorded upon adding caspase-3 (component P, lower abscissa) to 10 nM **6a** alone (black) or in the presence of **8** at concentrations of 100 nM (blue), 1 μM (green), and 10 μM (red). *I* is the FP signal intensity (left ordinate). Enzyme inhibition was performed by adding **9** (upper abscissa) to 3 nM caspase-3 in the presence of 5 μM Ac-DEVD-AMCA (magenta). v_0 is the initial rate of the enzyme reaction (right ordinate). b) Normalized titration data as obtained from the experimental FP data by employing Equation (2) (symbols) and best fits derived from Equations (3–9) (lines). The ordinates give the degree of binding, that is, the concentration of protein–ligand complex divided by the total concentration of **6a** (FP data, left ordinate) or caspase-3 (enzyme inhibition data, right ordinate). See the Supporting Information for equations and details.

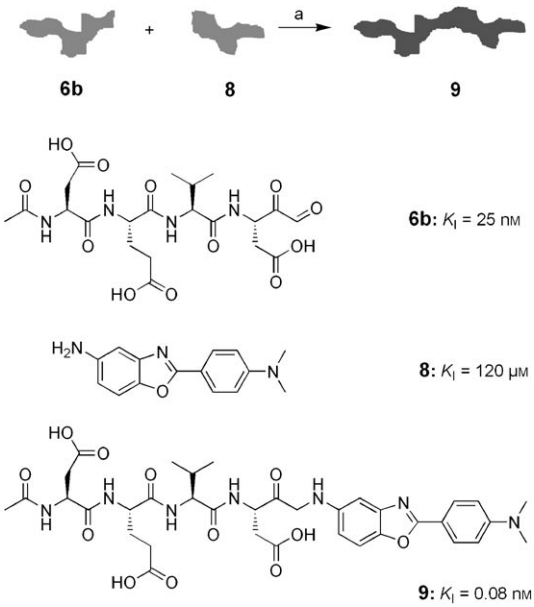


Figure 4. The α-ketoaldehyde inhibitor **6b** (component **A** in Figures 1 and 2; orange) was ligated with the active amine **8** (component **B** in Figures 1 and 2; green) by reductive amination, yielding the picomolar inhibitor **9** (red). Reaction conditions: a) $SiCl_3H$ (5 equiv), DMF/MeOH 1:1 (v/v) with 1% AcOH, 6 h, RT over molecular sieves (4 Å). Inhibition constants (K_i values) of **6b**, **8**, and **9** relative to caspase-3 were determined in a functional enzyme assay.

ure 3b) by using Equations (1) and (2) (for all equations, see the Supporting Information). Binding data for various concentrations of **8** were then fitted with the aid of a set of four equilibrium equations [Eqs. (3)–(6)] and three equations of mass conservation [Eqs. (7)–(9)] in order to obtain the ligation constant, K_C.

Received: March 25, 2009
Published online: July 23, 2009

Keywords: caspase-3 · drug discovery · fluorescence polarization · high-throughput screening · protease inhibitors

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