Dynamic Combinatorial Chemistry

Detection of Ligands from a Dynamic Combinatorial Library by X-ray Crystallography**

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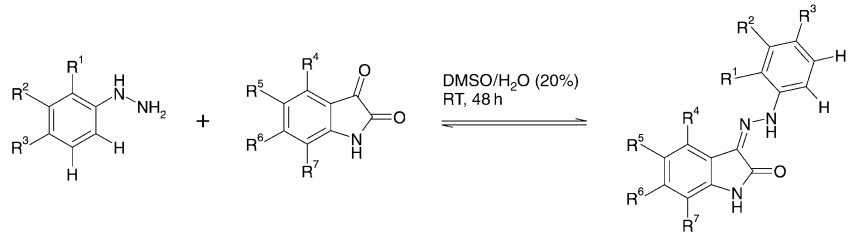
Dynamic combinatorial chemistry (DCC) is an approach to molecular recognition in which specific members of a combinatorial library are selected and amplified with the use of a template.[1–6] The principle difference between DCC and traditional combinatorial chemistry is that the reaction linking the building blocks together in DCC is reversible and there is an ongoing interchange between the different members of the dynamic combinatorial library (DCL) under thermodynamic control.[7] A DCL is therefore able to respond to molecular recognition events owing to the presence of a template, such as a protein, which can stabilize a particular member of the library and induce a shift in the equilibrium, favoring the formation of the selected species. A drawback of the method is that it usually requires excess quantities of protein for an effect on the equilibrium to be observed. Also, the effect can only be detected by comparison of identical libraries, generated with and without the protein component present, using either mass spectrometric analysis or HPLC chromatograms as a fingerprint. We now report a complimentary approach in which ligands are observed directly by X-ray crystallography by interpretation of electron-density maps from crystals exposed to a dynamic combinatorial library mixture. We call this technology dynamic combinatorial X-ray crystallography or DCX. This approach was used to detect rapidly potent inhibitors of the cyclin-dependent kinase 2 protein. DCX has key advantages over previously reported DCC technologies[8,9] in that direct identification of the ligand is possible from the mixture of components in the DCL, and its detailed binding mode is defined from the electron-density maps. Furthermore, only very small amounts of protein are required for each individual DCX experiment compared with previously reported DCC protocols.

It has been established with both X-ray crystallography and NMR spectroscopic techniques that very small molecules or "fragments" ($M_W = 100$–200) are capable of binding to proteins in a reproducible and specific manner, even though their intrinsic potency, as determined with an in vitro biological assay, is very weak (IC_{50} μM–mM).[10–14] We postulated that fragments bound to adjacent pockets within the active site of a protein could, in principle, self-assemble to generate larger, more potent ligands if they had complimentary chemical reactivities.[15] Such a method would have key advantages, as the time-consuming and expensive practice of conventional synthesis, purification, and testing of combinatorial libraries designed to discover potent inhibitors might be obviated.

To explore the potential of DCX, we used cyclin-dependent kinase 2 (CDK2) for an initial proof-of-principle study. This target has been the subject of intense investigation by many groups, with the aim of developing inhibitors for the treatment of a number of human cancers.[16–17] We chose an inhibitor series developed for CDK2 based on an oxindole template.[18] These inhibitors (Table 1) present substituents in adjacent lipophilic binding pockets within the ATP-binding site of the kinase and can be disconnected to reactive fragments (termed monomers) of approximately equal size and complexity (hydrazines **A** and isatins **B**; Table 1). Before attempting to perform DCX with crystals of CDK2, we first established the suitability of the chemistry for synthesizing a

Table 1: Combinatorial array of oxindole compounds synthesized by reaction of hydrazines **A** and isatins **B**.[a]



A **B** **A1–6 B1–5**

A × B		A1 –[b]	A2 R^1 = Cl	A3 R^2 = Cl	A4 R^3 = Cl	A5 $R^3 = SO_2NH_2$	A6 R^1 = Cl; $R^3 = SO_2Me$
B1	$R^5 = NO_2$	10–25	60–95	60–95	30–50	60–95	30–50
B2	R^5 = Cl	60–95	60–95	60–95	60–95	60–95	30–50
B3	$R^5 = SO_3H$	10–25	60–95	30–50	10–25	60–95	30–50
B4	$R^7 = CF_3$	30–50	60–95	60–95	60–95	60–95	30–50
B5	$R^5 = OCF_3$	30–50	60–95	60–95	60–95	60–95	10–25

[a] Values indicate the extent to which the reaction occurred in aqueous DMSO after 48 h at room temperature as assessed by percentage purity by peak area of the product by LC/MS (10–25%, 30–50%, or 60–95% of total peaks excluding solvent front). [b] R groups = H unless indicated otherwise. DMSO = dimethyl sulfoxide.

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DCL based on these oxindole inhibitors. Hydrazone chemistry, used for assembly of the library products, has previously been shown to be highly suitable for dynamic combinatorial library syntheses.[19–20] A range of hydrazines (**A1–6**) and isatins (**B1–5**) were selected that would present a variety of functional groups to the lipophilic pockets in the ATP-binding site. The selection of these monomers was aided by knowledge of the features of the active site. A trial of each of the 30 possible reactions was then carried out under aqueous conditions in the presence of 20% of the cosolvent dimethylsulfoxide (DMSO) over a 48-hour period, and the results showed that each product was successfully synthesized (Table 1). Further monomer competition studies were then carried out and these indicated that all the possible products could be formed as part of the DCL.[21]

Once the suitability of the chemistry was established, the reactions were then carried out in the presence of protein crystals. A summary of these experiments is given in Table 2.

Table 2: Summary of X-ray crystallography experiments and biological assay results.[a]

Mixture composition	Product	IC_{50} [nM]
A5 + B1	YES	30
A5 + B2	YES	30
A5 + B3	YES	30
A5 + B4	NO	inactive
A5 + B5	YES	30
B2 + (A1,A2,A3,A4)	NO	N/A
B2 + (A1,A2,A3,A4,A5,A6)	YES (**A5B2**)	N/A
(B1,B2,B3,B4,B5) + (A1,A2,A3,A4,A5,A6)	YES (**A5B2**)	N/A

[a] Table indicates if electron density consistent with a reaction product from mixtures of isatins and hydrazines was detected upon exposure to CDK2 protein crystals. The biological activity subsequently measured of the corresponding purified product of the reaction is also given when synthesized. Full details of the X-ray data collected and biological assay conditions are described in the Supporting Information.

The first studies were performed with individual crystals of CDK2[22–24] soaked in reaction solutions containing monomer **A5** and each of the isatin monomers (**B1–5**) in turn. In all but one case (**A5 + B4**) the resulting electron density in the ATP pocket indicated that the corresponding ligand had bound (Table 2, Figures 1a–d). To correlate these findings with the biological activity, each of these ligands was then synthesized as purified single compounds, and the activity determined in an assay for CDK2. Each of the ligands that had bound in the protein crystal was shown to be a potent enzyme inhibitor. Analogue **A5B4**, which did not bind in X-ray studies, was biologically inactive (Table 2).[25] These data are broadly consistent with the published structure–activity relationships for this series of inhibitors.[18]

Studies were then carried out to investigate the use of our approach to detect ligands from a dynamic combinatorial library mixture. Firstly, two "cocktails" of reaction solutions were added to CDK2 crystals to explore the potential of the method to discover potent inhibitors from a mixture of nonbinders. Comparison of the cocktail containing (**A1–4**) + **B2** with the cocktail that contained (**A1–6**) + **B2** revealed electron density that was consistent with ligand binding only in the latter case (Table 2). Furthermore, the electron density was consistent with the expected potent ligand **A5B2** (Figure 1e). Next, the degeneracy of the DCL was increased to 30 possible ligands using a reaction cocktail that contained hydrazines (**A1–6**) and isatins (**B1–5**). Difference electron density consistent with the reaction product **A5B2** was again observed, clearly demonstrating that the method is useful for the identification of ligands from a DCL mixture (Figure 1f).

The detection of ligands formed in this study does not rely on an observed perturbation of the reaction equilibrium in the way that is conventionally used in the screening of dynamic combinatorial libraries. Indeed, since the total amount of protein in the crystals is very small compared to the total amount of the monomers present in solution, one would not expect to be able to measure any gross changes in the outcome of the reaction caused by binding of product ligands to the crystals. However, it is still possible that the protein influences the outcome of the chemical reactions occurring directly in the crystals. In this study the reaction products are formed reversibly, and each product might be formed in situ within the protein crystal, under thermodynamic control. A consideration of the environment within the protein crystal would lead one to suggest that the monomers may tend to compete for occupancy of the ATP-binding groove, allowing "templating" to occur and helping to drive selection of the most potent ligands in the active site. Indeed, this effect has been previously shown in studies of reactions that occur in the active site of a protein, but under kinetic control.[26] However, it cannot be discounted that the effect observed in this case might be driven by equilibration of the ligands themselves into the CDK2 crystals, preformed by reaction of monomers present in the aqueous solution. Experiments designed to distinguish between these two possible situations are underway in our laboratories.

In conclusion, we have demonstrated that X-ray crystallography can be used to detect small-molecule ligands generated in situ and bound to a target protein. These findings provide the basis for a new drug discovery technology in which dynamic combinatorial libraries can be used to identify novel, potent ligands. We believe this approach could be broadly applicable to the discovery of inhibitors of therapeutically useful proteins within drug discovery programs.

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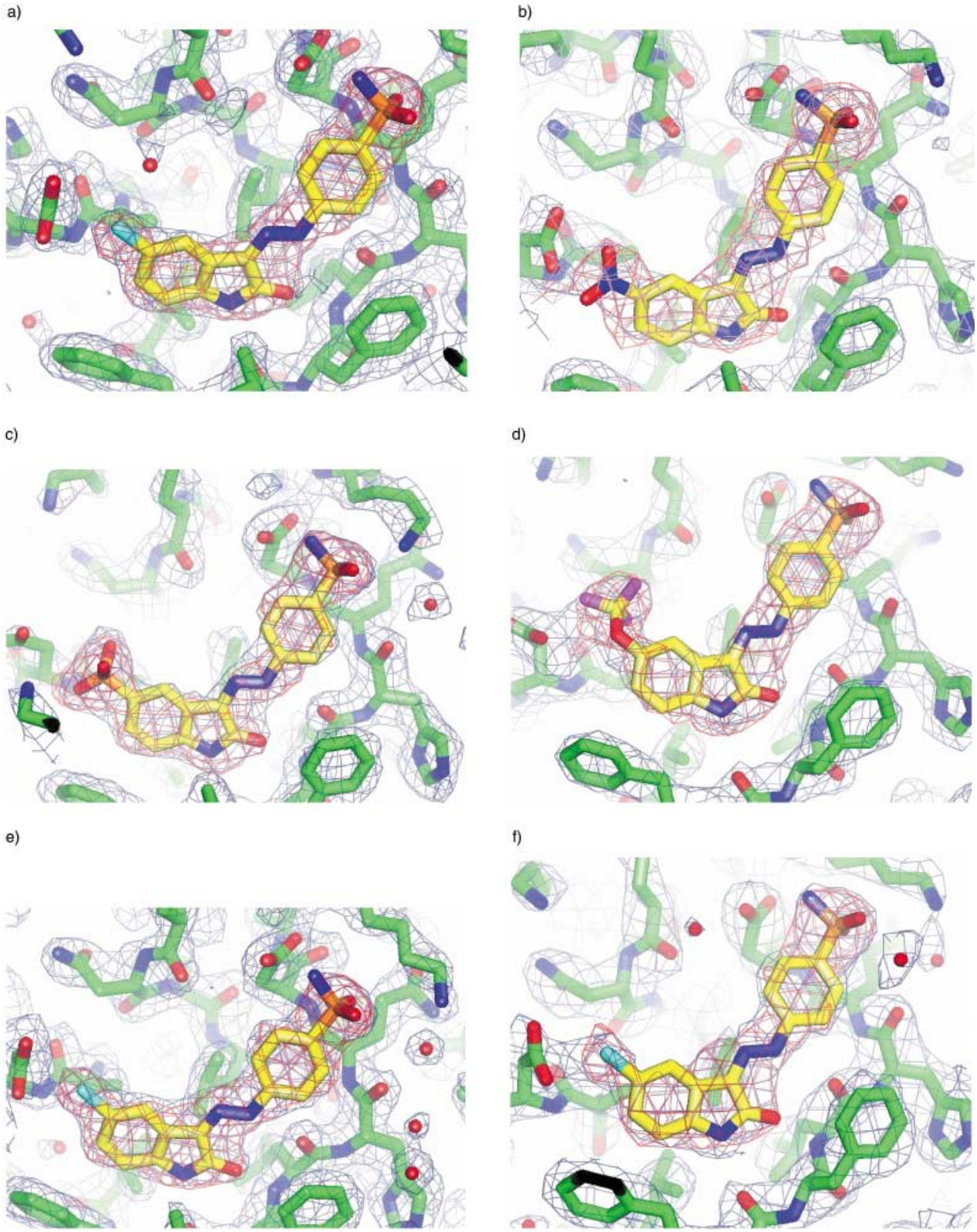

Figure 1. (F_o–F_c) and ($2F_o$–F_c) maps for the singlet and cocktail reactions. All (F_o–F_c) maps are colored red and trimmed to within 2.5 Å of the ligand. The difference maps were generated after segmented rigid body, positional, and isotropic B-factor refinements of the protein, in the absence of any ligand. The final refined protein–ligand structures and corresponding final ($2F_o$–F_c) maps (contoured at 1.0σ and colored gray) are shown in all six figures for reference. Elements in the ligands are colored as follows: yellow (C), blue (N), red (O), cyan (Cl), pink (F), and orange (S). Atoms in the protein are colored similarly, except that carbon atoms are green. a) 2.5σ (F_o–F_c) map for the reaction (**A5** + **B2**); b) 2.0σ (F_o–F_c) map for the reaction (**A5** + **B1**); c) 2.5σ (F_o–F_c) map for the reaction (**A5** + **B3**). d) 2.0σ (F_o–F_c) map for the reaction (**A5** + **B5**). e) 2.5σ (F_o–F_c) map for the reaction **B2** + (**A1**–**6**). f) 2.5σ (F_o–F_c) map for reaction (**A1**–**6**) + (**B1**–**5**). Maps for the reaction (**A5** + **B4**) are provided in the supplementary data. All figures were generated with AESOP (M.E.M. Noble, Oxford, unpublished results).

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