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Ligand binding affinity determined by temperature-dependent circular dichroism: Cyclin-dependent kinase 2 inhibitors

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Abstract

To support drug discovery efforts for cyclin-dependent kinase 2 (CDK2), a moderate-throughput binding assay that can rank order or estimate the affinity of lead inhibitors has been developed. The method referred to as temperature-dependent circular dichroism (TdCD) uses the classical temperature-dependent unfolding of proteins by circular dichroism (CD) to measure the degree of protein unfolding in the absence and presence of potential inhibitors. The midpoint of unfolding is the T_m value. Rank ordering the affinity and predictions of the dissociation constant of compounds is obtained by measuring the increase in T_m for different protein–inhibitor complexes. This is the first time an extensive characterization of the TdCD method has been described for characterizing lead inhibitors in a drug discovery mode. The method has several favorable properties. Using the new six-cell Peltier temperature controller for the Jasco 810 spectropolarimeter, one can determine the affinity of 12–18 compounds per day. The method also requires only 20–40 μg protein per sample and can be used to estimate the affinity of compounds with dissociation constants of picomolar to micromolar. An important property of the method for lead discovery is that dissociation constants of approximately 5 μM can be estimated from a single experiment using a low concentration of compound such as 20 μM, which is generally low enough for most small molecules to be soluble for testing. In addition, the method does not require labeling the compound or protein. Although other methods such as isothermal titration calorimetry (ITC) can provide a full thermodynamic characterization of binding, ITC requires 1–2 mg protein per sample, cannot readily determine binding constants below nanomolar values, is most versatile with soluble compounds, and has a throughput of two to three experiments per day. The ITC method is not usually used in a high-throughput drug discovery mode; however, using the thermodynamic information from several ITC experiments can make the TdCD method very robust in determining reliable binding constants. Using the kinase inhibitors BMS-250595, purvalanol B, AG-12275, flavopiridol, and several other compounds, it is demonstrated that one can obtain excellent comparisons between the K_d values of binding to CDK2 obtained by TdCD and ITC.

Keywords: CDK2; Inactive kinase; Inhibitors; Thermodynamics; T_m; Temperature; Circular dichroism; Isothermal titration calorimetry

In cancer therapy, one way of controlling the high rate of cellular proliferation is to regulate the cell cycle pathway. Stimulation of the cell cycle is promoted from several signal pathways that activate serine/threonine kinases called cyclin-dependent kinases (CDKs)[1] [1–4]. These kinases are activated through phosphorylation of specific serine/threonine residues on a flexible region referred to as the activation loop or T-loop and by forming a heterodimer with a highly helical cyclin protein. Cyclin-dependent kinase 2 (CDK2) regulates the cell

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[1] *Abbreviations used:* CDK, cyclin-dependent kinase; CDK2, cyclin-dependent kinase 2; ITC, isothermal titration calorimetry; TdCD, temperature-dependent circular dichroism; DSC, differential scanning calorimetry; CD, circular dichroism; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; SAR, structure–activity relationship.

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cycle at the point of entry into the G1 to S phase (DNA synthesis). The nonphosphorylated CDK2 monomer is not enzymatically active. CDK2 is activated by phosphorylation of Thr160 and association with either cyclin A or cyclin E [5]. The binding of cyclin A to CDK2 has been shown to cause a shift and rotation of helix C in the N-terminal domain of CDK2 [6–10]. This realignment brings the catalytically important residues in proper position to bind to the gamma phosphate of ATP and to promote phosphotransfer to a hydroxyl acceptor substrate.

Several CDK2 inhibitors have been synthesized and shown to successfully inhibit cellular proliferation. The thermodynamics of binding for BMS-250595, purvalanol B, AG-12275, flavopiridol, and several other compounds to CDK2 (uncomplexed with cyclin A or cyclin E) were evaluated in this report using isothermal titration calorimetry (ITC) [11–13]. Although the ITC method provides valuable thermodynamic information (free energy of binding, ΔG; enthalpy of binding, ΔH; entropy of binding, ΔS; and stoichiometry, N), only two to three of these experiments can be performed per day [14–17]. Another limitation is that the method requires milligram amounts of protein and is most versatile when soluble ligand concentrations exceed 100 μM, a condition that is often not possible for early lead compounds in drug discovery.

Often in drug discovery, it is equally valuable to be able to rank order the affinity of lead inhibitors. One such method that has been developed to routinely support drug discovery is temperature-dependent circular dichroism (TdCD). TdCD is a method that measures protein unfolding by increasing the temperature of the protein sample and detects changes in secondary structure by measuring changes in the ellipticity in the far UV wavelength range. The midpoint temperature of unfolding for the unliganded protein is referred to as T_m. The addition of inhibitors to the protein sample will increase the T_m because compounds bound to the protein stabilize the native state. The higher the affinity of a compound, the higher the apparent T_m because it requires more energy to dissociate higher affinity compounds. Thus, rank ordering the affinity of related compounds can be performed by evaluating the increase or decrease in T_m. The theory developed to describe the correlation between protein stability and ligand binding affinity was described in detail by Brandts and Lin [18] for the related method of differential scanning calorimetry (DSC) and was recently elaborated by Waldron and Murphy [19]. Although other thermal unfolding methods have been described to obtain T_m values in the presence of ligands (e.g., DSC, fluorescence, absorbance) this is the first time a detailed description has been presented on how to perform and evaluate experiments using circular dichroism as the detection method to determine the affinity of ligands [18,20–22].

This article describes the use of TdCD measurements to determine the affinity of seven CDK2 inhibitors. In particular, TdCD measurements were performed using a new six-cell Peltier temperature-controlled Jasco 810 spectropolarimeter. Optimization of the unfolding experiments demonstrated that less than 40 μg of protein sample and inhibitor concentrations of approximately 10–20 μM could enable nanomolar to micromolar leads to be determined. Using the K_d values obtained from ITC, we were able to develop a correlation between T_m and $\log([L_{free}]/K_d)$ for each control compound. We used this correlation to determine the K_d values of other compounds after the T_m values were measured by TdCD. The routine use of the TdCD method provides a relatively rapid analysis for estimating the affinity of lead compounds binding to soluble proteins in general and is especially useful for evaluating the affinity to both inactive and active states of protein kinases.

Materials and methods

Protein purification and compound preparation

CDK2 was expressed in Sf9 insect cells using a recombinant baculovirus encoding human CDK2 and was purified following a slight modification to the published method [23]. Briefly, Sf9 cells adapted to serum-free medium (Invitrogen) were maintained in shake flasks at 27 °C. Cells (2.0×10^6) were infected with human CDK2 baculovirus at a multiplicity of infection of 10. At 72 h postinfection, the cells were harvested and lysed in lysis buffer (10 mM Tris–HCl [pH 7.5], 25 mM NaCl, 1.0 mM ethylenediamine tetraacetic acid [EDTA], 1.0 mM dithiothreitol [DTT], 1 ml/L of protease inhibitor cocktail III [EMD Biosciences]). After microfluidizing, the lysate was clarified by ultracentrifugation (100,000*g* for 1 h at 4 °C) and loaded onto a 20-ml DEAE–Sepharose Fast Flow HiPrep column (GE Healthcare) preequilibrated in lysis buffer. The NaCl concentration of the flow-through was adjusted to 50 mM prior to loading onto a 25-ml SP-Sepharose column (GE Healthcare). CDK2 eluted in the flow-through and was further purified using an ATP–agarose column (Sigma) preequilibrated in 10 mM Hepes (pH 7.5), 25 mM NaCl, 1.0 mM EDTA, and 1 mM DTT. CDK2 was eluted with an increasing NaCl gradient in the presence of 10% glycerol. Peak fractions were pooled based on SDS–PAGE. The concentration of CDK2 was determined using 6 M GndHCl by UV spectrophotometry and an extinction coefficient at 280 nm of 35,680 $M^{-1}\,cm^{-1}$. Mass spectrometry confirmed that the mass of CDK2 was 33,975 Da. The purified CDK2 was shown to be fully active following the addition of cyclin A and activation with CAK1.

Compounds BMS-250595, AG-12275, Cpd#1, Cpd#2, and Cpd#3 were synthesized at Schering–Plough.