qPCR as a primary screen in drug discovery

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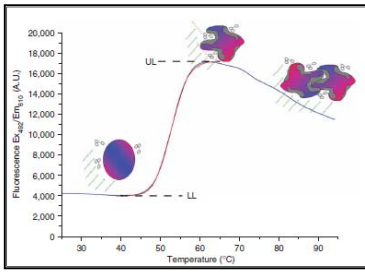
Summary

We report the use of qPCR technique to follow the thermal unfolding of proteins by the binding of the dye SYPRO Orange, and exploit its potential as a robust and high-throughput primary screen for small molecule drug discovery. A small in-silico screened library of 80 compounds was tested using qPCR on destabilized mutant p53 Y220C. 11 hits out of these were found to raise the melting temperature of the mutant p53 Y220C. All of these were confirmed by $^{1}H/^{15}N$-HSQC nuclear magnetic resonance spectroscopy, which produced significant changes in chemical shifts induced by binding to the protein. One of the compounds, a carbazole derivative (PhiKan083), bound to mutant p53 with a dissociation constant of ~150 µM. Small molecules that bind to these destabilized p53mutants and stabilize them could be effective anti-cancer drugs. PhiKan083 raised the melting temperature of the mutant and slowed down its rate of denaturation. We demonstrate that qPCR is an effective technique to be used as primary drug screening for lead compounds and estimating their dissociation constants.

Introduction to the technique

Using qPCR as a differential scanning fluorimetry technique is a rapid and inexpensive screening method to identify low- molecular-weight ligands that bind and stabilize purified proteins. The temperature at which a protein unfolds is measured by an increase in the fluorescence of a dye with affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds. A simple fitting procedure allows quick calculation of the transition midpoint; the difference in the temperature of this midpoint in the presence and absence of ligand is related to the binding affinity of the small molecule,. Results can be obtained in a single day.



Typical recording of fluorescence intensity versus temperature for the unfolding of protein (citrate synthase) in the presence of SYPRO orange dye (three-ring grey aromatic molecule). In the presence of a globular protein, a basic fluorescence intensity is excited by light of 492 nm . Through unfolding of the protein, hydrophobic patches become exposed, and strong fluorescent light of 610 nm is emitted by the dye molecules bound to them. Following the peak in the intensity, a gradual decrease is observed, which is mainly explained by protein being removed from solution owing to precipitation and aggregation.

Relating T_m with the dissociation constant

Increase in *Tm of a protein on addition of ligand.*

In absence of ligand (L), suppose the protein melts at a temperature *Tm. In the presence* of [L], the protein appears to melt at temperature *T, where the concentration of D, the* denatured state, is 50% of the total protein and the sum of the concentrations N (Native state) and NL complex constitute the other 50%.

Suppose D is in equilibrium with N
with equilibrium constant $K_{ND} = [N]/[D]$, *and* $Kd = [N][L]/[NL]$.
Then, at *T*, $[D] = [N] + [NL] = [N](1 + [L]/Kd)$. *That is:* $([D]/[N])T = (1 + [L]/Kd)$

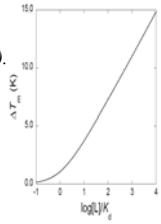
Also, $([D]/[N])T = \exp(-\Delta GD\text{-}N(T)/RT)$, *where* $\Delta GD\text{-}N(T)$ *is the free energy of denaturation* at *T*. *Therefore,* $\Delta GD\text{-}N(T) = -RT \ln(1 + [L]/Kd)$.

We can relate the observed changes in *T to* $\Delta GD\text{-}N(T)$ *using the equation* $d\Delta GD\text{-}N/dT = -\Delta SD\text{-}N$.

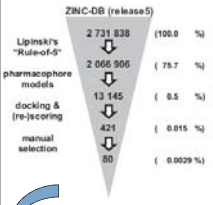
For small changes in temperature, $\Delta GD\text{-}N(T) \sim -\Delta SD\text{-}N(Tm)(T\text{-}Tm)$.
So that, $\Delta S\ D\text{-}N(Tm)(T\text{-}Tm) = RTln(1 + [L]/Kd)$,

which rearranges to:
$T = Tm /(1 - (R/\Delta S\ D\text{-}N(Tm))ln(1 + [L]/Kd))$

(To a further approximation, $\Delta Tm = Tm\ (R/\Delta S)ln(1 + [L]/Kd))$.



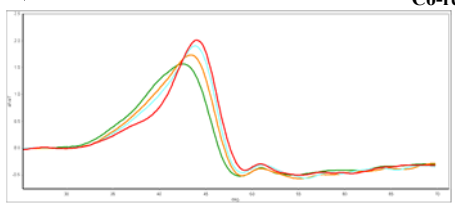
Screening of compound library



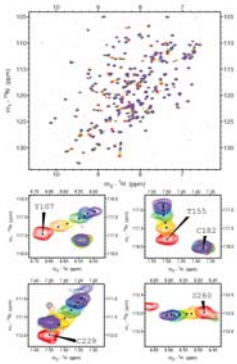
*(i) structure-based pharmacophore mode*ls in MOE, *(ii) ligand* docking into the crystal structure with GOLD and rescoring of the best poses to form a consensus score, and *(iii) manual selection according* to criteria of medicinal chemistry and crystallography

In-silico screening overview

qPCR – NMR Co-relation



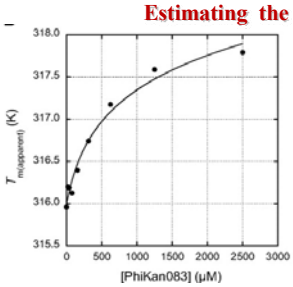
qPCR for monitoring Thermal Stabilization



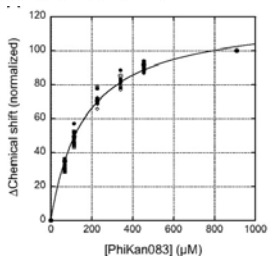
1H/15N-HSQC spectrum/ residues of p53C-Y220C in the presence of various concentrations of PK083

PhiKan083 stabilized *T*-p53C-Y220C in a concentration-dependent manner. The T_m is raised nearly 2 C from 316 K by 2.5 mM. PhiKan083, and the data fit the equation expected for stabilization by simple binding with an approximate K_d of 150 73 µM at 316–318 K. Denaturation is irreversible. However, at the very high heating rate of 270 K/h, the measured *Tm is close to the reversible value*

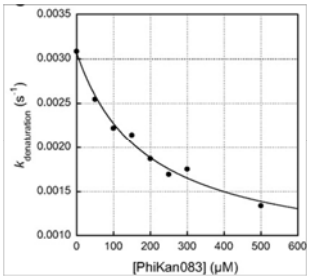
Estimating the dissociation constant



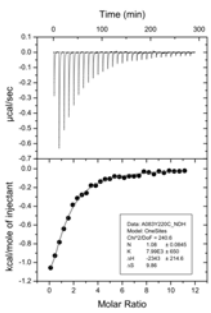
Plotting Tm values at increasing concentrations *[L] Kd of 150 µM*



Chemical-shift differences at higher concentrations *[L]* → *Kd of 167 µM*

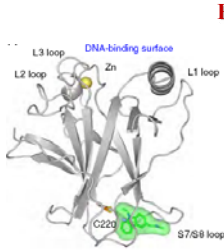


Effect of PhiKan083 on kinetics of thermal denaturation

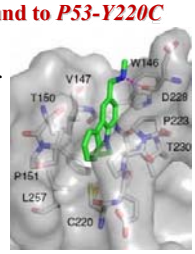


ITC of PK083 binding showing raw data → *Kd of 125 µM*

PhiKan 083 bound to *P53-Y220C*



Ribbon representation of the overall structure of *P53C-Y220C in complex with* PhiKan083 (green)



Stereoview of the PhiKan083-binding site. p53 residues within a 5-Å distance of the ligand shown as gray stick models. The protein surface highlighted in semitransparent gray

Conclusions

qPCR keeps a wide potential as a robust and high throughput primary screening technique for low molecular weight binders. It is a very rapid screening method and inexpensive in terms of the amount of protein needed. Also the dissociation constants calculated using the Tm shift s in qPCR are in agreement with the ones estimated by other established methods.

References

1) F.M. Boeckler, A.C. Joerger, G.Jaggi, T.J. Rutherford, D.B. Veprintsev, and A.R. Fersht, "Targeted rescue of a destabilized mutant of p53 by an in silico screened drug", *Proc Natl Acad Sci U S A 105:10360-10365* (2008).
2) F. H. Niesen, H. Berglund, and M. Vedadi , "The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability" *Nature Protocols*. (2007).