Profiling inhibitor selectivity for PARP protein family members

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Inhibitors of PARP1, a member of the family of human poly-ADP-ribose polymerases, are presently in clinical trials for the treatment of cancer. Here, interactions between PARP enzymes and a panel of potential low molecular weight inhibitors from a focused compound library were studied using Biacore T200 (GE Healthcare), firstly for screening, confirmation and ranking of hits based on binding selectivity and affinity. This was then followed by detailed kinetic profiling of the selected leads on Biacore T200 to help explain differences in affinity and to provide information on binding mechanisms. Data are compared with results from analyses of the same interactions using differential scanning fluorimetry (DSF), a fluorescence-based binding assay used to identify low molecular weight compounds that interact with proteins. In assays performed using Biacore T200, it was possible to differentiate potential inhibitors of target proteins based on their interaction profiles despite binding to highly similar active sites. Further, weak binders selective for specific target proteins were easily identified. DSF, while suitable for the identification of high affinity compounds, was unable to reliably identify weak binders in this study, or to accurately rank compounds based on affinity.

Fig 1. A model of the interaction between the catalytic site of the enzyme, TNKS2 and the inhibitor, XAV939 (here designated Compound 1). The interaction is characterized in detail for affinity and kinetics using Biacore T200.

Introduction

PARP proteins comprise a family of 17 enzymes. PARP1, the most comprehensively studied member of the family, is implicated in the response of cells to DNA damage via poly-ADP-ribosylation. Other members of the PARP family, although less well understood, are also attracting attention. The tankyrases (TNKS1/2, also known as PARP5a/5b), for example, were recently identified as positive regulators of the Wnt-signaling pathway, and inhibition of tankyrase has been reported to induce apoptosis in cell lines deficient in the protein encoded by BRCA, an important human tumor suppressor gene. PARP1, tankyrases, and possibly other members of the PARP family, are thus interesting potential targets for anticancer drugs (Fig 1). Comprehensive knowledge of the underlying molecular basis for ligand selectivity may greatly accelerate the development of inhibitors of PARP enzymes. The Structural Genomics Consortium (SGC) – a public-private partnership in Canada, Sweden, and Great Britain that aims to determine the three dimensional structures of proteins of medical interest – is systematically mapping the structure of the entire PARP protein family and to date has determined the structures of 9 different catalytic domains.

Biacore

DSF and surface plasmon resonance (SPR) were used to profile interactions between PARP enzymes and a panel of ligands from a focused compound library.

At each stage of the process, from screening to detailed kinetic characterization of the most promising candidates, the high sensitivity of Biacore T200 enabled conclusions to be confidently drawn, based on relevant information. The instrument is sufficiently sensitive that in practice, no molecule is too small to be detected and a true kinetic analysis of the simplest organic compounds can thus be carried out.

Materials and methods

A library of 184 compounds based on patent literature (PARP1) and structures (PARP1 and PARP3) was tested. Twenty-five percent of the compounds were known PARP inhibitors or analogs thereof and the remainder was selected by structure-based virtual screening. Following an initial screening process, selected compounds were subjected to detailed kinetic characterization. All stages of the selection process and final kinetic analysis were performed using SPR or DSF.

Target proteins were generated at the SGC using recombinant DNA technology (1).

Prior to screening on Biacore T200, the quality of the proteins was assessed using calorimetry. For TNKS1 and TNKS2, this was done in a series of isothermal calorimetric (ITC) titrations with a known inhibitor using a MicroCal™ VP-ITC instrument (GE Healthcare).

TNKS1 and TNKS2 proteins were titrated with compound XAV939 (2) as a potent inhibitor. The low nanomolar affinity of the compound to TNKS1 and TNKS2 was confirmed. Both proteins were found to be almost 100% competent for ligand binding.

It was not possible to obtain meaningful ITC data for PARP15 protein due to the low affinity and insufficient solubility of the few known inhibitors. Instead, differential scanning calorimetry using a MicroCal VP-DSC instrument was used to assess the effect of a generic PARP inhibitor, 3-aminobenzamide (3-AB), on the thermal stability of PARP15. The protein was found to be thermally stable and was further stabilized by the presence of a 10-fold excess of the inhibitor.

Biacore assays

For the initial screen of all compounds, and the first kinetic analysis of compounds selected from the screen, a modified method was used for Ni^{2+}-mediated immobilization of the histidine-tagged proteins TNKS1, TNKS2 or PARP15 on the surface of Sensor Chip NTA, available from GE Healthcare.

This chip has the advantage of allowing the orientation of the immobilized target protein to be controlled and standardized.

Compounds from the library were injected over the prepared surface. Experiments were performed on a Biacore T200 instrument at 25°C. The assay buffer was 20 mM PBS, 0.05% surfactant P20, and 4% DMSO, pH 7.4.

Screen of focused library

PARP15 and TNKS1 (5 µg/ml in assay buffer without DMSO) were captured on Sensor Chip NTA via a Ni^{2+} histidine tag interaction and further stabilized by amine coupling to a level of approximately 5000 resonance units (RU) (Fig 2). Here, the aim of the experimental design was to ensure correct orientation of the immobilized protein and also to prepare a sensor surface sufficiently stable for repeated analyses, without consuming target protein unnecessarily.

All 184 tested compounds were diluted to 30 µM in assay buffer, injected over the prepared surface for 60 s and allowed to dissociate for 10 min. A positive control compound was analyzed at intervals to ensure that the protein remained active during the run.
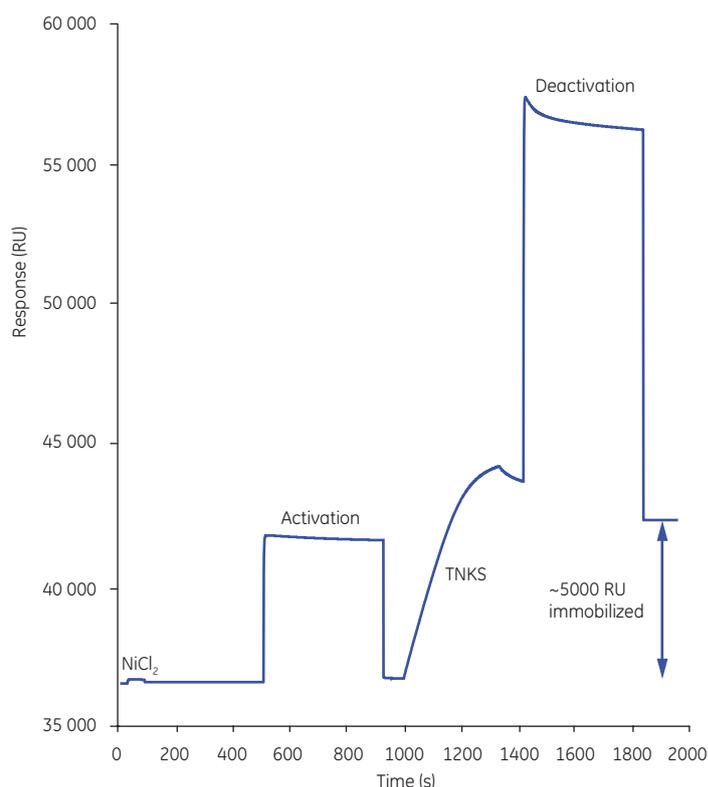

Fig 2. For the initial screen and first kinetic analysis, 500 µM $NiCl_2$ was first injected to enable capture of the histidine tag, ensuring correct orientation of the protein.

Kinetic and steady state characterization

Immobilization was performed as for the initial screen. The binding kinetics of 38 compounds, selected from the initial screen, were analyzed. Compounds were simultaneously injected over immobilized PARP15 and TNKS1 for 60 s, and were allowed to dissociate for 10 min. A similar concentration series (0.78 to 200 µM) was used for all compounds (not shown).

Kinetic profiling at low, stable capture levels

TNKS1 and TNKS2 were captured at low density on Sensor Chip NTA, with no supplementary amine coupling. Prior to kinetic profiling, proteins were diluted to 5 µg/ml in assay buffer and injected for 30, 60, 90, 120, and 180 s (Fig 3). This was done to establish a suitable capture level for stable binding of the protein on the sensor surface. The surface of Sensor Chip NTA can be simply regenerated, if necessary, by an injection of EDTA to remove the nickel ions.
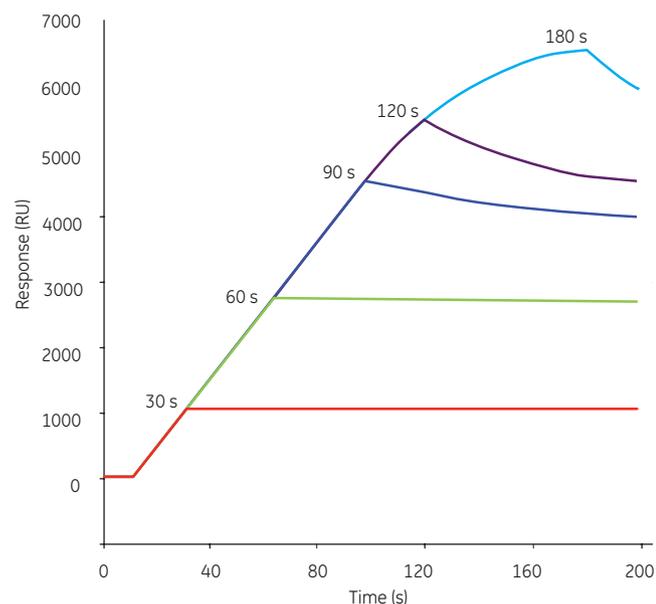

Fig 3. TNKS1 (5 µg/ml) was injected for 30 s, as levels of 1000 RU or less result in stable binding of the protein to Sensor Chip NTA. Calculations of theoretical R_{max} for this capture level gave R_{max} of 10 RU (1:1 binding of a compound with relative molecular mass [M_r] 300).

An injection time of 30 s was selected, giving about 1000 RU of stably bound protein. Fresh protein was captured for each new interaction analysis. Kinetic profiling was performed using single cycle kinetics, a method that significantly reduces time-to-results. The same flow rate (15 µl/min) was used for protein capture and sample injections to maintain stable capture. In single cycle kinetic analyses, increasing sample concentrations are injected consecutively in the same analysis cycle. For capture assays, this typically reduces reagent consumption by a factor of two to four, thus reducing costs. Compounds were diluted to 5 different concentrations in the range 0.06 to 5 µM, injected for 30 s, and allowed to dissociate for 10 min after the last injection. Results were fitted either to a 1:1 binding model or to a steady state affinity fit.

DSF assay

DSF is a fluorescence-based binding assay used to identify low-molecular weight compounds that interact with proteins. As the temperature is increased, the protein unfolds, exposing hydrophobic patches and making them available for interaction with dyes. The difference in temperature required to reach the midpoint of the unfolding process in the presence and absence of compound (ΔT_m) is related to the binding affinity of the compound.
A solution containing protein in the presence of a potentially interacting compound was added to wells in a 96-well plate. Protein (0.2 mg/ml) was mixed with compound at a concentration of 50 µM in PBS containing 2% DMSO, pH 7.4. Protein in the absence of the compound under investigation, and in the same buffer, was included as a control. The fluorescent dye, SYPRO™ Orange (Invitrogen) was used (a 5000-fold dye concentrate in DMSO was diluted 1:1000 in the well). Plates were scanned from 20°C to 90°C.

Results

Biacore T200 assay

Screen of focused library

All compounds were screened at a single concentration by injecting each compound simultaneously over TNKS1 and PARP15. Binding responses (Fig 4) and the shapes of the sensorgrams were used to identify selective binders and high affinity binders. From this hit selection, 38 compounds were chosen for further kinetic characterization.
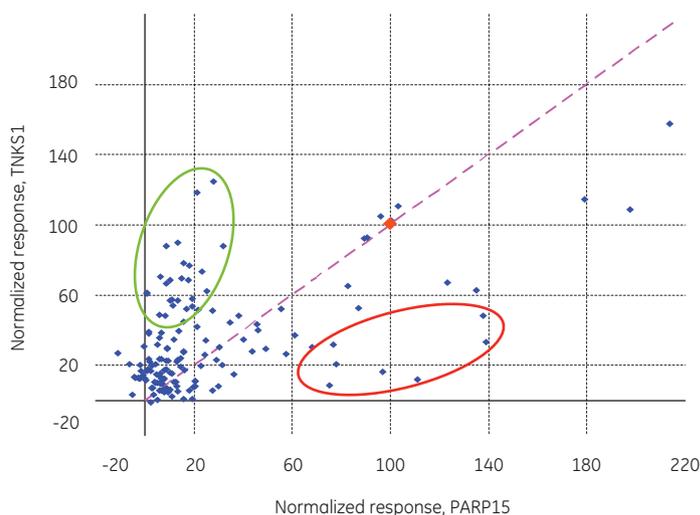

Fig 4. Display of binding responses from the hit selection screen. Responses were adjusted for molecular weight and normalized against a positive control (red), set to 100. The compounds displaying selectivity towards PARP15 or TNKS1 are marked by red and green circles, respectively.

Kinetic and steady state characterization

Each of the 38 selected compounds were kinetically analyzed by allowing them to interact with PARP15 and TNKS1 immobilized in two flow cells on a single sensor surface, using a standardized compound concentration series (0 to 200 µM). Data were fitted to a 1:1 binding model (not shown). Compounds with less than 15% uncertainty in the computation of kinetic rate constants were selected for further binding characterization.

Kinetic profiling at low, stable capture levels

Eight compounds were finally chosen for more detailed kinetic and selectivity characterization. The binding profiles of four of those compounds to TNKS1 and TNKS2 were compared. The high sensitivity of Biacore T200 allowed precise kinetic and affinity data to be obtained from very low densities of captured proteins, providing stable binding (Fig 5).

Although the active sites of TNKS1 and TNKS2 are nearly identical, a trend toward higher compound affinity for TNKS2 was seen. This was due to significantly slower off-rates in several cases, which is of particular interest in the pharmaceutical development of leads, where slow off-rates are often desirable.
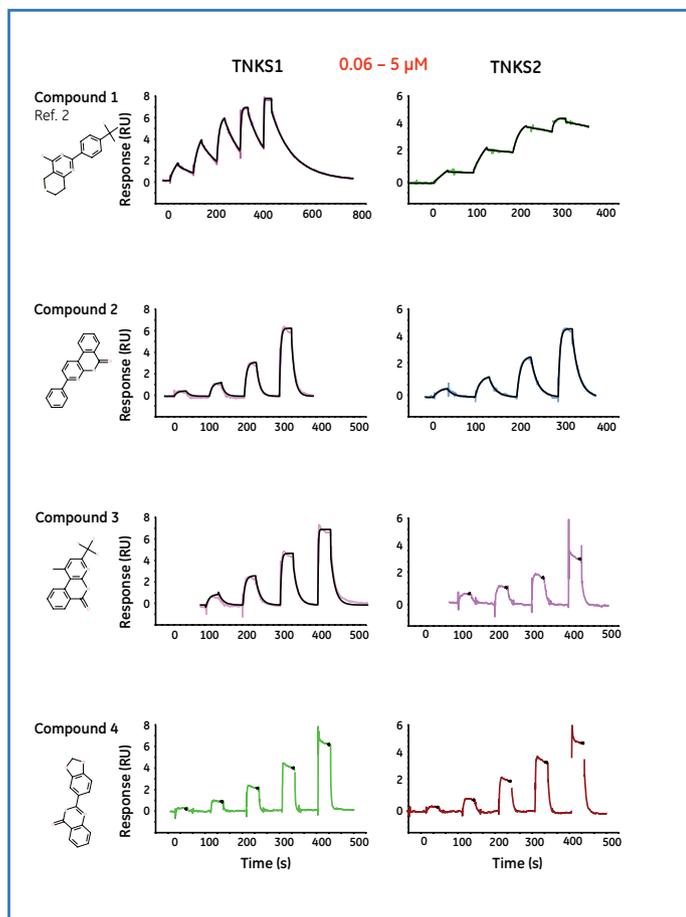

Fig 5. The interaction profiles of four of the eight selected compounds with TNKS1 and TNKS2 were compared. A concentration series (0.06 to 5 µM) was injected over a prepared sensor surface. The exceptional sensitivity of Biacore T200 allowed kinetic and affinity data to be obtained at very low protein capture levels, providing stable binding at neutral conditions. A trend toward higher affinities for TNKS2, driven by slower dissociation rates, was observed in these experiments.
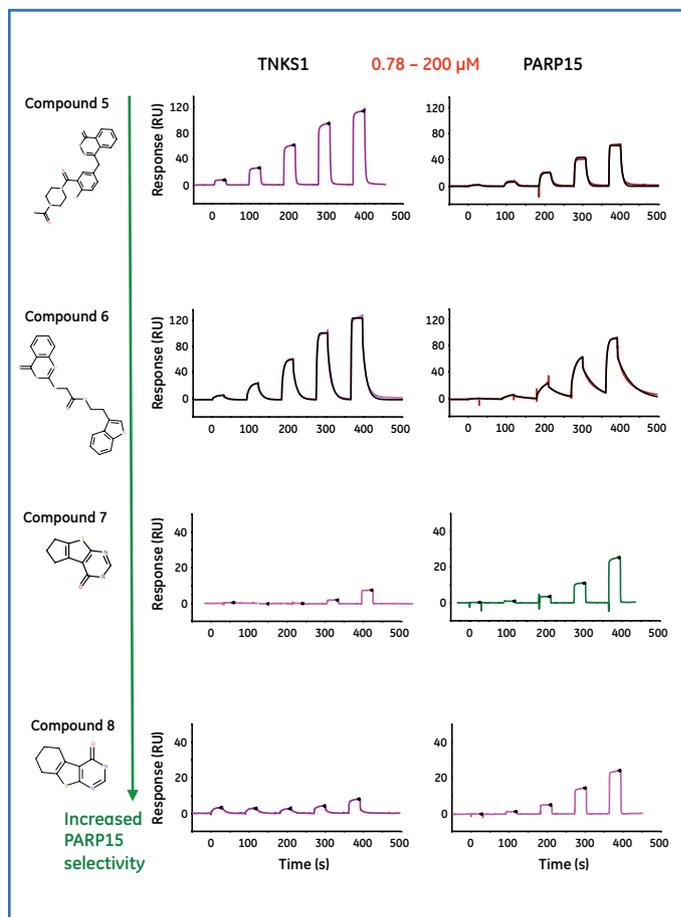
Fig 6. Compound selectivity. Four compounds in a concentration series (0.78 – 200 µM*) were injected simultaneously over immobilized TNKS1 and PARP15. Weak ligands with selectivity for PARP15 were identified.
* Compound 8 was injected at concentrations up to 300 µM.

To assess selectivity, a concentration series of 4 compounds were injected over immobilized TNKS1 and PARP15. The interaction profiles are shown in Figure 6 and identify weak ligands selective for PARP15 in comparison to TNKS1. Binding of Compounds 7 and 8, for example, was detected at lower concentrations for PARP15. Although Compounds 5 and 6 both had a slightly higher affinity for TNKS1, they dissociated from PARP15 at a slower off-rate. A list of suppliers of the compounds used in this study is provided in Table 1.

Table 1. Suppliers and article identification numbers of compounds used in the study

Compound ID	Compound Name	Supplier	Article ID
Compound 1	XAV939	Maybridge	RF03920
Compound 2		ENAMINE	T5974626
Compound 3		ENAMINE	T5989689
Compound 4		INTERBIOSCREEN	STOCK5S-79756
Compound 5	Olaparib ('AZD2281, KU-0059436)	JS Research Chemicals Trading	
Compound 6		INTERBIOSCREEN	STOCK6S-43755
Compound 7		ENAMINE	T0514-1628
Compound 8		Chemdiv	1306-0033

DSF assay

While it was possible to identify high affinity TNKS1 binders with ΔT_m above 1.2˚C using DSF, reliable ranking in terms of affinity could not be made. Further, only one of the four weak PARP15 binders identified using SPR was identified as a binder using DSF (Fig 7).
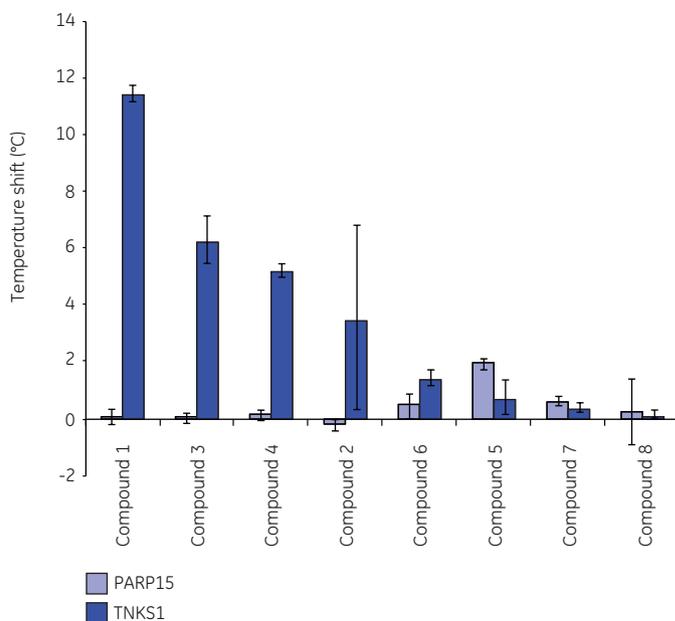

Fig 7. Overlay of the temperature shift data collected for PARP15 and TNKS1. The concentration for both proteins was 0.2 mg/ml. The compound concentration was 50 µM. A temperature shift of 1.2ºC was regarded as the statistical cut-off in both screens.

Discussion

Biacore T200

The work described in this study covers screening, confirmation and ranking of small molecule hits based on binding selectivity and affinity followed by detailed kinetic and selectivity profiling of the selected leads. The aims of each of these processes were successfully accomplished using Biacore T200.

At each stage, the high sensitivity of the instrument made it possible to confidently draw conclusions based on relevant information. The sensitivity of Biacore T200 is such that in practice no molecule is too small to be detected and accurate kinetic analyses can thus be performed for very simple organic compounds.

As one approaches the detection limit of any assay or instrument, precision, and thus confidence in the data, is reduced. With improvements in sensitivity, Biacore T200 extends the range of kinetic rate constants that can be precisely determined so that previously borderline data may now be confidently measured.

The accurate detection of small molecules that interact weakly with a target protein places very high demands on the biosensor system used. As sensitivity of detection increases, lower quantities of target proteins may be immobilized on the sensor surface and lower concentrations of interacting partners in solution may be used. The quality of the output is thereby improved, and the risk for artifacts caused by phenomena such as compound crowding or aggregation, is minimized. During early screening, the high quality of data provided a strong basis for informed decisions on which hits to advance.

Despite possessing almost identical active sites, when the binding profiles of five selected compounds with TNKS1 and TNKS2 were compared, there was a trend toward higher affinity for TNKS2, due to significantly slower off-rates in several cases. In the hit verification assays, precise kinetic rate constants were derived from maximum binding responses of as low as 6 to 8 RU, enabling the identification of weak ligands that were selective for PARP15 in comparison to TNKS1. In addition to immobilizing small amounts of target protein, the Biacore T200 assay required minimal consumption of compounds injected in solution.

DSF

Although the hit rate in the DSF assay for TNKS1 was high, it was considerably lower for PARP15 (Fig 7 and 8). This was partly expected due to the bias of the compound library to PARP1 and its closest relatives, such as tankyrases.

In this study, DSF was insufficient for comparative screening of interactions of members of a focused compound library with PARP proteins. The limitations of DSF in detecting low affinity binders ($K_D > 50$ μM in this study) and interference from interactions with partially or wholly unfolded proteins complicated the identification of binding compounds. In addition, although binders shown to be weak ($K_D > 50$ μM) using the Biacore T200 assay also affected the T_m of PARP15 and TNKS1 proteins in the DSF assay, the shifts produced by these compounds were either statistically insignificant or negative (Fig 8). Even for compounds that bound the target with K_D close to 1 μM, the spread in the T_m shift was rather high (from 1.5°C to 6.6°C).

DSF is a generic binding assay, well suited to the identification of low molecular weight ligands that stabilize proteins against thermally-induced unfolding. However, caution must be exercised in the application of the method to rank compounds according to affinity, or to compare binding profiles across several proteins. This is because DSF data describe interactions at elevated temperatures and there is no simple relationship between the magnitude of ΔT_m and affinity at physiological temperature. Different combinations of affinity, enthalpy, and change in heat capacity for interactions can produce identical temperature shifts. The more direct and sensitive binding assay performed on Biacore T200 is thus needed to acquire reliable data for accurate affinity ranking or comparisons between proteins.
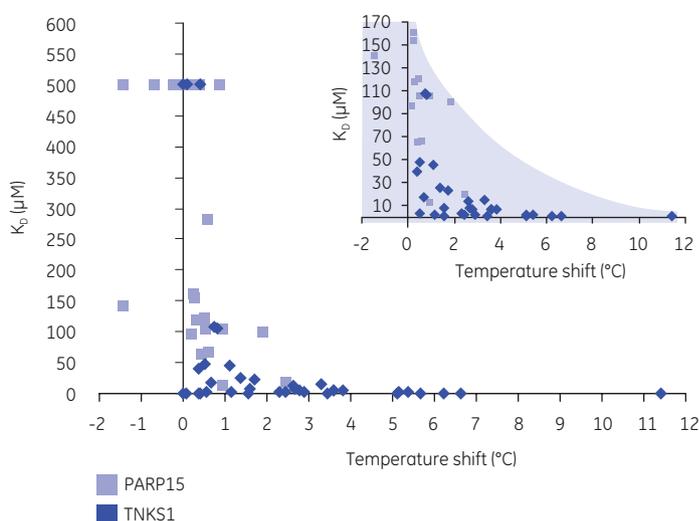

Fig 8. Correlation of data from experiments performed using Biacore T200 and DSF. Note that several binders that could not be resolved in the DSF assay were shown in the Biacore T200 assay to bind PARP15 with affinities between approximately 50 and 150 μM.

Conclusions

- Compounds displayed different affinities for TNKS1 and TNKS2 despite almost identical active sites

- Weak affinity binders selective for PARP15 compared with TNKS1 were easily identified using Biacore T200

- On- and off-rates determined using Biacore T200 help to explain differences in affinity and/or binding mechanisms

- DSF, while suitable for the identification of high affinity compounds, was unable in this study to reliably identify weak binders, or to accurately rank compounds based on affinity

- The high sensitivity of Biacore T200 enabled stable capture of proteins to Sensor Chip NTA under neutral conditions, allowing reliable kinetic data to be obtained

References

1. http://www.thesgc.org
2. Shih-Min A. *et al.* Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling. *Nature* **461**, 614-620 (2009).

Acknowledgements

Ordering information

Product	Code no.
Biacore T200 Processing Unit	28-9750-01
Series S Sensor Chip NTA	BR-1005-32

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