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Miscellaneous

Development of a differential scanning fluorimetry based high throughput screening assay for the discovery of affinity binders against an anthrax protein

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ABSTRACT

The anthrax protein protective antigen (PA) is responsible for cell-surface recognition and aids the delivery of the toxic anthrax enzymes into host cells. By targeting PA and preventing it from binding to host cells, it is hoped that the delivery of toxins into the cell will be inhibited. The current assay reported for PA is a low throughput functional assay. Here, the high throughput screening method using differential scanning fluorimetry (DSF) was developed and optimized to screen a number of libraries from various sources including a selection of FDA-approved drugs as well as hits selected by a virtual screening campaign.

DSF is a rapid technique that uses fluorescence to monitor the thermal unfolding of proteins using a standard QPCR instrument. A positive shift in the calculated melting temperature (T_m), of the protein in the presence of a compound, relative to the T_m of the unbound protein, indicates that stabilization of the protein by ligand binding may have occurred. Optimization of the melting assay showed SYPRO Orange to be an ideal dye as a marker and lead to the reduction of DMSO concentration to <1% (v/v) in the final assay. The final assay volume was minimized to 25 μL with 5 μg protein per well of 96-well plate. In addition, a buffer, salt and additive screen lead to the selection of 10 mM HEPES–NaOH pH 7.5, 100 mM NaCl as the assay buffer. This method has been shown here to be useful as a primary method for the detection of small-molecule PA ligands, giving a hit rate of ~7%. These ligands can then be studied further using PA functional assays to confirm their biological activities before being selected as lead compounds for the treatment of anthrax.

1. Introduction

Anthrax, judged to be one of the most serious of the potential biowarfare agents, is caused by Bacillus anthracis, a gram-positive, spore-forming bacterium that is naturally found in soil. The disease chiefly affects herbivorous mammals, although other animals and humans can also contract the disease. Cases of infection in humans from handling animal products have been known, but are relatively rare and usually limited to those working in close contact with animal products [1]. Deliberate release events can expose people to higher concentrations of anthrax spores and an increased chance of infection, such as the 2001 anthrax attacks in the US where envelopes containing spores were mailed to news media offices and two US senators, resulting in the deaths of 5 people. Anthrax spores are able to survive for decades in extreme environmental conditions. They are resilient to heat, gamma radiation, UV light or disinfectants. Infection can occur via three different modes of entry: ingestion, inhalation or subcutaneous infection. The latter produces the most obvious symptoms of skin lesions and is therefore usually the simplest to diagnose and treat. Inhalational anthrax, however, has a mortality rate approaching 100%. This is due to the lack of distinctive initial symptoms and a long incubation period, often resulting in the disease remaining undetected until infection has progressed beyond the point where current antibiotics could cure it. Infection leads to tissue decay, hypotension, shock and finally death [2].

There is currently no known anthrax-specific treatment. After exposure to anthrax, the recommended treatment consists of a 60-day course of the broad-spectrum antibiotic ciprofloxacin [3]. Side effects of ciprofloxacin, a quinolone, are unpleasant [4] and this, coupled with the fact that the initial symptoms of anthrax may not be noticeable, leads to poor patient compliance. There is currently an unmet need for an orally available, rapid acting, anthrax-specific treatment with few side effects.

Pathogenesis occurs via the germination of anthrax spores to form bacteria, which release the anthrax toxin. The toxin is composed of three separate components: two toxic enzymes known as edema factor (EF) and lethal factor (LF), plus protective antigen (PA). PA is responsible for cell-surface recognition and mediates the delivery of LF and EF into host cells, where they then exert their toxic effects [5]. One strategy to prevent this is to block the interaction between PA and the host cell-surface receptors (anthrax toxin receptors, or ATRs) by targeting

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the ATR recognition site on PA. Currently, there are no known small-molecule inhibitors of the PA–ATR interaction and work to block this interaction has mainly involved the use of soluble receptor decoys [6,7]. The current assay reported for PA is a low throughput functional assay [6,8] and there is no assay available for the high throughput screening of compound libraries against PA.

Differential scanning fluorimetry (DSF), also called Thermofluor®, is a technique that is used to determine conditions that stabilize proteins, such as buffers, salts and additives [9,10], but also can detect ligands that bind to and stabilize the native form of the protein [11,12]. The unfolding of the protein is monitored by fluorescence as temperature is increased. A dye that is fluorescent in a non-polar environment is added to a solution containing the target protein. At low temperature the dye is quenched by the aqueous solution, resulting in a low fluorescence intensity measurement. As the temperature increases, the protein begins to unfold due to the decrease in the temperature-dependent Gibbs free energy of unfolding (ΔG_u). At equilibrium ΔG_u becomes zero, where the concentrations of folded and unfolded protein are equal. This is known as the melting temperature (T_m). A compound that binds to the protein generally causes an increase in the ΔG_u, which results in a positive shift in the T_m relative to the unliganded protein. The assay can be carried out on a standard RT-PCR instrument and results are produced in the form of a graph of fluorescence intensity of the dye measured against temperature. A sigmoidal curve is generated, where the point of inflection gives T_m and this can be calculated using the Boltzmann equation. Studies have shown that the stabilization of the protein due to ligand binding is proportional to the affinity and the concentration of the ligand [13–15].

Development of many high throughput methods can be hindered by the need to covalently modify the protein of interest. DSF can be used to screen any soluble protein against potential binders [16]. Other advantages of the method include the possibility of miniaturization to 384-well format and the rapid generation of data (roughly an hour to scan one plate, based on a scan rate of 1 °C per minute from 25 to 95 °C).

In the present work a DSF high throughput screening (HTS) assay for the discovery of potential PA ligands was developed. The method was successfully optimized for use with the protein of interest, and then an initial screen of 657 compounds from a range of sources was carried out. In order to produce a concentration–response curve, the hits were then re-screened at varying concentrations.

2. Experimental

2.1. Chemicals

HEPES, Tris, sodium acetate, sodium chloride, potassium phosphate, glycine, EDTA, DMSO, Nile Red were purchased from Sigma (St. Louis, MO, USA), SYPRO Orange was purchased from Invitrogen (Paisley, UK).

Chemicals for HTS: 226 chemicals were selected from an in-house database of US Food and Drug Administration (FDA) approved drugs and the publically available ZINC database [17] based on a docking study carried out against PA. The compounds were purchased from a variety of sources. Alongside this, a total of 431 in-house compounds that had purity of over 95% were prepared for screening. All compounds were dissolved in DMSO to make a 10 mM stock solution, which was then further diluted in DMSO immediately before screening took place.

2.2. Protective antigen expression

rPA_{83} was provided by the Health Protection Agency, Porton Down, UK. The protein was expressed in and purified from *E. coli* and supplied frozen as 6.8 mg/mL aliquots at >97% purity, as determined by SDS-PAGE. Before storage the protein was further aliquoted into the volume required to fill one 96-well plate, in order to minimize the number of freeze/thaw cycles.

2.3. DSF optimization

DSF was carried out using a Stratagene Mx3005P RT-QPCR system (Agilent Technologies, La Jolla, CA, USA) fitted with custom filter sets. The data was recorded in MxPro version 4.10 QPCR software. Initially, the general protocol outlined by Niesen et al. was followed [10]. Conditions of the assay were then optimized for use with PA before HTS was carried out to find ligands from an initial list of 657 compounds, 431 of which were from the in-house library and 226 remaining compounds were shortlisted by in silico methods. Parameters of the assay that were optimized included choice of dye, dye concentration, choice of buffer, salt concentration, protein concentration, incubation times, DMSO concentration, temperature range and total well volume. Each parameter was tested in triplicate on a white, non-skirted 96-well PCR plate (Starlab, Milton Keynes, UK), sealed with transparent foil (Starlab) using a rubber roller. The concentration of protein used was 0.2 mg/mL in all optimization trials, except for the determination of optimum rPA_{83} concentration where it was varied over a range from 0 to 5 μM. Melting temperature changes were monitored with use of a reporter dye and the filter sets were varied according to the appropriate wavelengths required for the dye in use. For SYPRO Orange, the wavelengths used for excitation and emission were 492 and 610 nm respectively, whilst for Nile Red wavelengths used were 585 nm (excitation) and 665 nm (emission). Raw data was exported into Microsoft Office Excel 2007. T_m values were calculated from the melting curves using the Boltzmann sigmoidal non-linear regression function in GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

2.4. High throughput screening

For HTS, each of the 226 compounds selected by virtual screening (FDA-approved and ZINC database compounds) was screened at 20 μM in triplicate. Prior to addition of the compound to the protein solution, a 2 mM sub-stock solution of each compound was made from a 10 mM stock solution in DMSO. This was added to a solution of rPA_{83} in 10 mM HEPES–NaOH pH 7.5, 100 mM NaCl and incubated for 10 min at room temperature to allow the compound to bind to the protein. After incubation, a 1:500 dilution of SYPRO Orange dye in buffer was added to the protein solution to give a final concentration of 0.2 mg/mL protein and 1:1000 dilution of dye. Each solution of protein and compound was added in triplicate to a white, non-skirted 96-well PCR plate (Starlab). Three of the wells were used as references and were identical to the experiment wells except that DMSO was used in place of the compound solution. The plate was then sealed with transparent foil before placement in the PCR instrument. DSF was carried out from 25 to 95 °C in increments of 1 °C per minute. Data was recorded using the MxPro software, as above, and then exported into an Excel worksheet (ftp://ftp.sgc.ox.ac.uk/pub/biophysics) [10] for visualisation and processing. Accurate T_m values were calculated from the Boltzmann equation using GraphPad Prism 5 software, as above. T_m values for each compound were compared to the T_m of the reference well in order to find the ΔT_m. A hit was defined as a compound that caused a positive shift in the ΔT_m of the protein greater than three times the standard deviation (~0.3 °C). Following initial screening

of the set of 226 ZINC and FDA compounds at 20 μM concentration, these compounds were re-screened using identical assay conditions at 10 μM compound concentration, from addition of a 1 mM sub-stock diluted from the original 10 mM stock in DMSO. 431 in-house compounds that were of purity greater than 95% were then also screened at 10 μM concentration using the same procedure. All hits were then re-screened in triplicate at least twice more, to confirm the melting temperature shift and check reproducibility.

2.5. Concentration–response curves

Using the 10 mM stock of compound in DMSO, dilutions of each hit compound were made ranging from 7 to 0.01 mM in DMSO. 0.8 μL of diluted compound was then incubated at room temperature with 39.2 μL of protein in buffer. Addition of 40 μL of the 1:500 dye solution in buffer resulted in further dilution to give final compound concentrations ranging from 0.1 to 70 μM, and protein concentration of 0.2 mg/mL. Each solution was then pipetted in triplicate onto a 96-well PCR plate. DSF was carried out via the optimized procedure and ΔT_m was calculated in the usual way. ΔT_m was then plotted against the log of concentration of the ligand using GraphPad, and a sigmoidal dose–response curve (variable slope) was fitted to the data.

3. Results and discussion

3.1. Protein melting temperature determination

A detailed description of the analysis of protein unfolding curves to determine the melting temperature is given in the literature [11,13]. When fluorescence intensity of the probe is reported as a function of the temperature of the solution, a sigmoidal curve is generated. The point of inflection can be calculated from the equation shown:

$$y(T) = \text{Max} + \frac{(\text{Min} - \text{Max})}{1 + \exp(T_m - x/a)}$$

where Max signifies the maximum fluorescence intensity of the melting transition, Min is the minimum intensity and a is the slope of the curve within T_m.

In the literature, the T_m of other proteins as determined by DSF has been found to correlate with values obtained by other methods, such as differential scanning calorimetry (DSC), circular dichroism (CD) and turbidity measurements [9]. In this study, the T_m calculated using DSF was compared with that published previously for protective antigen found by CD and DSC. Chalton et al. used a thermal ramp of 1 °C per minute for each method to study the unfolding transitions of PA_{83} in 30 mM PIPES pH 7.0, calculating T_m to be 49.9 °C by DSC, and around 49 °C by far-UV CD_{220} [18]. This is in good agreement with the values calculated here by DSF, using identical buffer conditions and thermal ramp rate, with an average T_m of 49.8 °C (SD ~ 0.3 °C) over six wells (data not shown).

3.2. Optimization of assay conditions

It is possible to monitor protein unfolding using an intrinsic fluorescence probe such as tryptophan or tyrosine residues, although in this case it was felt to be advantageous to use an external probe. Fluorescence of an external probe such as ANS, SYPRO Orange or Nile Red would be more likely to remain unaffected by changes brought about when a compound binds to the folded protein.

Choice of dye was limited to those that are quenched in aqueous solution and that give a large gain in fluorescence intensity when exposed to a hydrophobic environment, such as the hydrophobic core of an unfolded protein. It was also important that the compounds themselves would not be able to interfere with the signal

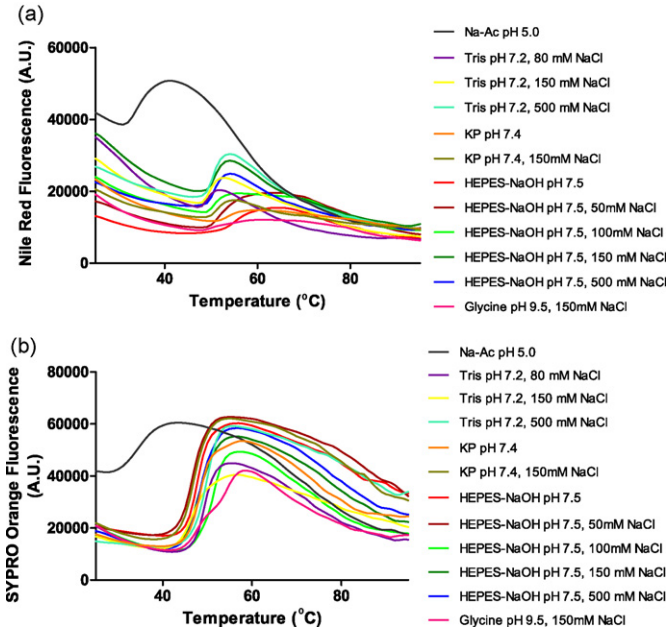

Fig. 1. Melting temperature curves produced for protective antigen in 12 different buffer conditions using two different fluorescent probes (a) Nile Red and (b) SYPRO Orange.

of fluorescence, so a dye with a high wavelength would be preferable. Both Nile Red and SYPRO Orange were promising candidates, since both have a relatively high excitation wavelength (554 and 492 nm, respectively) compared to other potential dyes, for example the ANS series of dyes (350 nm excitation). After trials using both Nile Red (Fig. 1(a)) and SYPRO Orange (Fig. 1(b)) to monitor the unfolding of the protein, each in the same 12 buffer conditions, the latter was chosen as the most suitable probe due to its much greater gain in fluorescence intensity. In nine of the 12 different buffer conditions tested with Nile Red the fluorescence gain was too weak to accurately determine a T_m. Furthermore, the melting curve of the protein exhibited high initial fluorescence intensity with all the buffer conditions tested when monitored by Nile Red, as seen in Fig. 1(a). This indicates that the dye binds to exposed hydrophobic areas of the protein in its native state at low temperatures [10], making it less suitable for use to monitor the thermal unfolding of PA than SYPRO Orange, where it was possible to determine a T_m under all 12 tested conditions. Using SYPRO Orange as the probe, only one out of the 12 buffer conditions resulted in a high initial intensity (sodium acetate buffer, pH 5.0; see buffer optimization below).

SYPRO Orange was tested at three dilutions: 1:500, 1:1000 and 1:1500. The structural formula and exact concentration of the dye is undisclosed by the manufacturer. The dye is supplied as a 5000× concentrated solution dissolved in 100% (v/v) DMSO so concentration should be kept as low as possible in order to prevent DMSO damage to the protein. 1:1000 was found to give a large gain in fluorescence without damaging the protein. The dye was diluted in buffer prior to the addition of the protein as an extra precaution, to prevent exposure of the protein to localised high concentrations of DMSO. This is in agreement with the protocol suggested by Niesen et al. [10].

The stabilizing or destabilizing effect on rPA_{83} of various buffers and salts was tested (Table 1). Salt concentration in the buffer screen varied from 0 to 500 mM, and pH varied from 4.8 to 9.5. A range of different buffers was tested including HEPES, PIPES, Tris, sodium acetate, glycine and potassium phosphate. Each condition was tested in triplicate and an average T_m was calculated.

Table 1
Buffer and salt screen results showing average melting temperature of protective antigen in various conditions, in order of highest to lowest mean T_m.

Buffer condition	pH	Mean T_m (°C ± SD)	RSD (%)
10 mM HEPES–NaOH, 100 mM NaCl	7.5	50.92 ± 0.06	0.11
10 mM Glycine, 150 mM NaCl	9.5	50.87 ± 0.01	0.03
30 mM PIPES	7.0	49.82 ± 0.38	0.76
10 mM Tris–EDTA, 500 mM NaCl	7.2	49.53 ± 0.35	0.71
10 mM Tris–EDTA, 80 mM NaCl	7.2	49.02 ± 0.13	0.27
10 mM HEPES–NaOH, 150 mM NaCl	7.5	48.90 ± 0.01	0.01
10 mM Tris–EDTA, 150 mM NaCl	8.9	48.77 ± 0.07	0.14
10 mM Tris–EDTA, 150 mM NaCl	7.2	48.75 ± 0.27	0.55
10 mM HEPES–NaOH, 500 mM NaCl	7.5	48.50 ± 0.31	0.63
10 mM KP	7.4	48.29 ± 0.10	0.22
10 mM HEPES–NaOH	7.5	47.87 ± 0.03	0.05
30 mM PIPES	6.5	47.62 ± 0.37	0.78
10 mM HEPES–NaOH, 50 mM NaCl	7.5	46.94 ± 0.19	0.41
10 mM KP, 150 mM NaCl	7.4	46.93 ± 0.06	0.14
30 mM PIPES	6.1	44.37 ± 0.24	0.54
10 mM Na–Ac	4.8	34.60 ± 0.19	0.54

The buffers were then ranked according to the average T_m value. A higher T_m value indicates that the buffer induces structural changes in the protein to a more ordered conformation, whereas a lower T_m value may be an indication of destabilization [9]. It has been reported that there is little change in the structure of PA between pH 6 and 10, whereas aggregation of the protein occurs between pH 4.0 and 5.0 as monitored by ANS binding [18]. This is consistent with our findings that T_m measured under all buffer conditions with pH between 6 and 9.5 was at least 10 °C higher than with the sodium acetate buffer at pH 4.8. Not only was the T_m significantly shifted in the destabilizing direction at pH 4.8, but also a large gain in initial fluorescence intensity was always observed, indicating that the protein was in a denatured state, where dye could bind to exposed hydrophobic sites. Of the fourteen other buffer conditions tested, 10 mM HEPES–NaOH pH 7.5, 100 mM NaCl was found to be the buffer with the highest mean T_m shift (50.9 °C), i.e. the buffer that most stabilized the folded state of the protein. This buffer was selected for use in the assay to encourage binding of ligands to the native state of PA.

After an appropriate buffer, salt concentration and dye had all been selected; protein concentration was varied to identify a suitable compromise between maximizing fluorescence gain and minimizing the amount of protein used, in order to conserve supplies. Around 0.2 mg/mL was the lowest suitable concentration of protein that was found to be sufficient to give an increase in fluorescence intensity that would allow accurate determination of T_m.

Due to the fact that the compounds would themselves be dissolved in DMSO, it was necessary to determine the effect of DMSO concentration on the stability of the protein prior to deciding what concentration to screen the library of compounds. The assay was repeated using the optimized conditions and with the addition of 1% up to 20% (v/v) DMSO (Table 2). T_m was calculated relative to a reference containing buffer, with no DMSO. It was clear from the

Table 2
Effect of increasing DMSO concentration on the melting temperature of protective antigen in 10 mM HEPES–NaOH, 100 mM NaCl, pH 7.5.

[DMSO] (%)	Mean T_m (°C ± SD)	RSD (%)
0	50.85 ± 0.13	0.26
1	47.74 ± 0.22	0.46
2	47.20 ± 0.25	0.52
5	46.78 ± 0.11	0.24
7	46.86 ± 0.06	0.12
10	46.54 ± 0.22	0.47
15	47.20 ± 0.17	0.37
20	46.87 ± 0.07	0.15

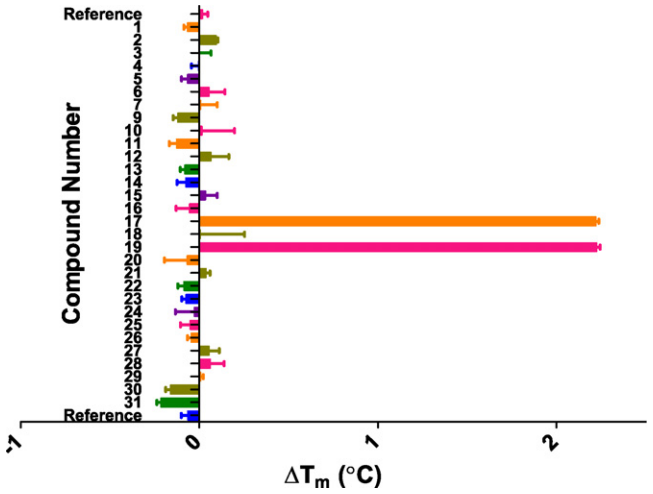

Fig. 2. Example of processed data from one plate of the initial screen of 657 compounds, showing two reference wells and 30 compounds at 10 μM from the ZINC database. Melting temperature (T_m) shifts show two compounds, screening numbers 17 and 19, leading to protective antigen ΔT_m above 1 °C.

results that at increasing DMSO concentrations the stability of the protein became affected, leading to a negative shift in the T_m and a high initial fluorescence intensity reading. This indicates that the protein is destabilized at high DMSO concentrations, relative to the reference well. As a result, DMSO concentration in the protein solution was kept as low as possible, and was less than 2% throughout the preparation procedure and less than 1% in the final assay. It was also clear that in order to determine the contribution of the ligand to the change in T_m (ΔT_m) during compound screening the reference well must contain an equal volume of DMSO compared to the experiment wells so that changes in T_m brought about by DMSO could be accounted for.

After optimization of the other parameters, the total volume in each well of the plate could be reduced in order to minimize use of all the assay components and reduce wastage. The total well volume was scaled down from 50 to 25 μL, resulting in a total amount of protein used of less than 0.5 mg per 96-well plate or 5 μg per well. This volume could easily be further reduced with the use of robotic pipetting equipment and 364-well plates, if a larger screen was to be conducted.

3.3. High throughput screening

After optimization of the conditions of the assay, a selection of compounds shortlisted by vHTS was screened in triplicate in 96-well plate format, including 88 FDA-approved compounds, 138 compounds from the online ZINC database and 431 compounds from the in-house library.

Visualisation of the melting temperature shift was plotted as shown in Fig. 2 for each plate. In this example, two of the compounds (ZINC compounds "17" and "19") show a clear shift in melting temperature above 2 °C when screened at a concentration of 10 μM. The actual melting temperature curves for these two compounds are shown in Fig. 3, plotted against the reference.

Of the 226 FDA and ZINC compounds re-screened at 20 μM concentration, a total of 3 ZINC compounds (including ZINC 17 and 19) and 2 FDA-approved compounds gave rise to a protein melting temperature of more than 1 °C (Fig. 4(a)). ZINC 43 and ZINC 53 show borderline activity at this concentration.

Out of the 431 in-house compounds screened at a concentration of 10 μM, 46 were found to induce a positive shift in the T_m of the protein by 1 °C or more compared to the reference, indicating stabilization of the protein by ligand binding (Fig. 4(b)). These com-

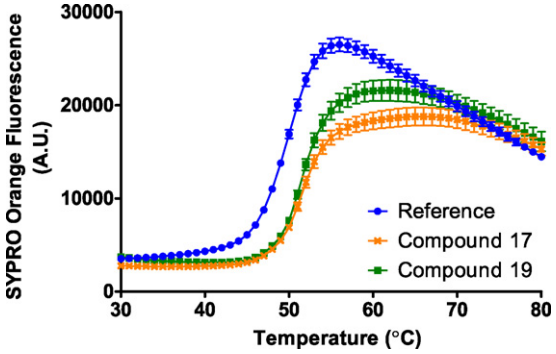

Fig. 3. Mean melting temperature curves of two compounds (screening number 17, orange crosses, and 19, green squares) that gave rise to an increased melting temperature of protective antigen compared to the reference (blue circles). Error bars indicate standard deviation.

pounds fell into five main structural classes. The positive hit rate at 10 μM concentration was 7%.

After determination of the hits in initial screening, the compounds were re-screened several times in triplicate to confirm the hits. Following on from this, titration of the compound at increasing concentrations was performed for each of the ZINC and FDA hits and for a hit compound in each structural class of the in-house compounds.

3.4. Concentration–response curves

Ligand concentration was varied as a function of melting temperature, demonstrating that the compounds bind to the protein in a concentration-dependent manner as shown for the in-house compound 765 in Fig. 5. The increasing concentration of compound causes an increasing shift in protein melting temperature coupled with a broadening of the curve and a large reduction in fluorescence intensity. For each compound, log of the ligand concentration was plotted against ΔT_m. In each case, a sigmoidal

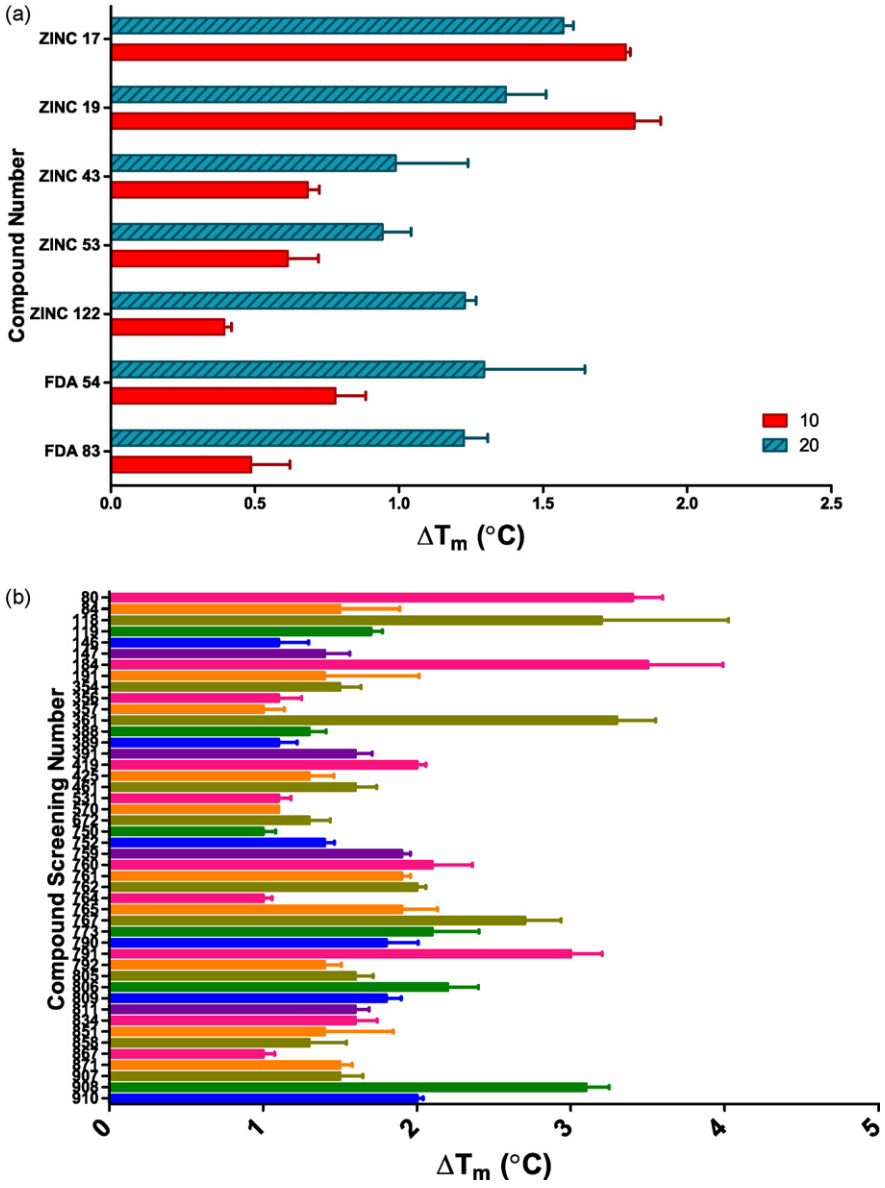

Fig. 4. Melting temperatures produced from compounds causing an increase in the melting temperature of protective antigen by 1 °C or more for (a) FDA-approved drugs and compounds selected from the ZINC database, screened at both 10 and 20 μM and (b) compounds selected from the in-house library, screened at 10 μM. Error bars indicate standard deviation.

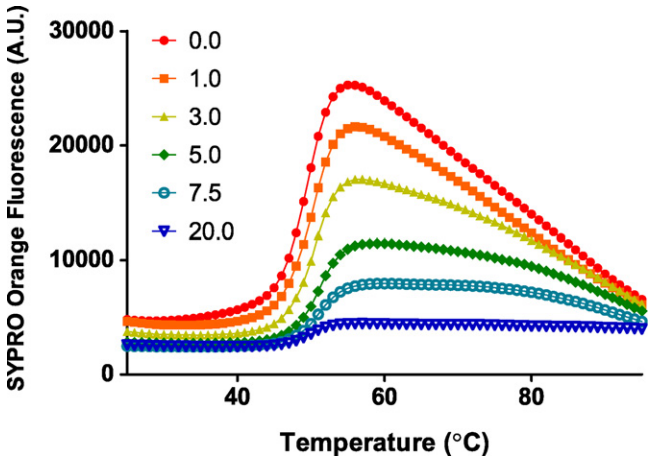

Fig. 5. Concentration dose–response of protective antigen (2.4 μM) with compound 765 at concentrations of 1, 3, 5, 7.5 and 20 μM (orange solid squares, green solid triangles, green solid diamonds, blue open circles, blue open triangles, respectively) compared to reference (red circles).

concentration–response curve was produced that showed a saturating effect on protein T_m at a concentration of ligand that was compound-dependent. Fig. 6 shows the concentration–response curve for compound 765. Theoretical models suggest that T_m should increase linearly with ligand concentration without reaching a point of saturation [12,13], although with many of the compounds screened here this was not the case. The saturation effect seen is in some cases probably due to ligand solubility issues, where the concentration of the ligand in solution becomes a limiting effect, and in other cases due to competing effects caused by the ligand binding to both the native and unfolded forms of the protein [12,13,19]. In the case of 765 (Fig. 6), the saturation effect may be linked to the reduction in fluorescence intensity seen at increasing molar ratio of the compound. At a 10:1 molar ratio of compound to protein (20 μM compound added, dark blue hollow triangles, Fig. 5) the fluorescence intensity is so reduced that it becomes extremely difficult to accurately determine a T_m for the protein. According to Matulis et al., the shape of the unfolding transition may become sharper if the unfolding of the protein is rapid and reversible or broader if the rate of unfolding is limited by the inhibitor [13]. The reduction in fluorescence intensity seen here at high molar ratio of compound may be an example of an extreme broadening of the curve indicating that the unfolding of the protein is kinetically limited by the binding of the compound. Alternatively, it could be speculated that the compound displaces the fluorescent probe as the molar ratio of compound increases.

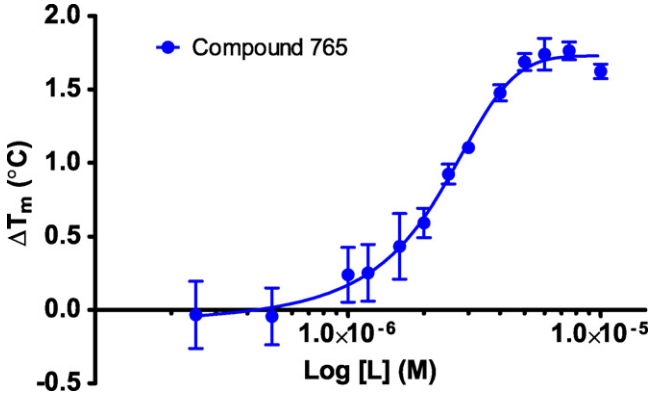

Fig. 6. Log [L] vs. change in melting temperature for compound 765 (L = Ligand). Solid line indicates sigmoidal dose–response curve fitted to data. Error bars indicate standard deviation.

Ligand binding to a protein increases its thermal stability by an amount proportional to the concentration and affinity of the ligand [20]. The dangers of ranking compounds by using their T_m alone is highlighted in the literature and determination of binding constants using thermodynamic data is not without its difficulties [10,12,13,21]. The binding constant cannot be directly determined from a thermal shift assay without knowing the relative contributions of enthalpy and entropy of the ligands. Binding constants determined at the T_m must be extrapolated to a common temperature, usually physiological temperature, in order to make comparisons [13]. In this case, differential scanning calorimetry experiments are required to determine the thermodynamics of protective antigen stability, which would then allow some estimation of ligand binding constant determination and enable a more accurate method for ranking of compounds. With methods such as DSF, there exists an equilibrium between the native and unfolded states of the protein, where the ligand may bind to the unfolded state(s) of the protein as well as to the native state. If the ligand binds significantly to the unfolded state of the protein as well as to the folded state, the binding constant may be underestimated, since stabilizing the unfolded state shifts T_m towards a lower temperature [12].

4. Conclusions

The DSF assay was successfully optimized for use with the protein of interest in order to carry out a high throughput screen of compounds that had been previously selected for screening using in silico methods. Around 8% of compounds tested were found to bind to the protein.

DSF is a rapid and relatively simple assay that can be used in conjunction with cell-based methods to shortlist promising candidates for further testing by more detailed, lower throughput methods. The data obtained here will be used to complement data obtained from a lethal-toxin (LF plus PA) cell-based survival assay, where the same set of compounds will be subjected to screening for anti-anthrax activity. It is hoped that compounds showing activity in both assays will become sustainable leads that can be developed further.

Crystallization trials are currently underway in order to determine the binding position of each of the compounds to the protein. Knowledge gained here by DSF has also been used to aid the design of these crystallization trials by revealing conditions likely to encourage protein stability and, therefore, result in successful crystallization.

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