Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 802–808

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Miscellaneous

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

Fiona J. Sorrell, Gemma K. Greenwood, Kristian Birchall, Beining Chen*

*Department of Chemistry, University of Sheffield, Sheffield S3 7HF, UK*

ARTICLE INFO

*Article history:*
Received 15 November 2009
Received in revised form 12 February 2010
Accepted 18 February 2010
Available online 15 March 2010

*Keywords:*
Differential scanning fluorimetry (DSF)
Thermal shift assays
Thermofluor
Fluorescence
Temperature

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

* Corresponding author. Tel.: +44 0114 22 29467.
*E-mail address:* b.chen@sheffield.ac.uk (B. Chen).

0731-7085/$ – see front matter
doi:10.1016/j.jpba.2010.02.024

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 ($\Delta G_u$). At equilibrium $\Delta G_u$ 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 $\Delta G_u$, 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 $\Delta T_m$. A hit was defined as a compound that caused a positive shift in the $\Delta T_m$ of the protein greater than three times the standard deviation (~0.3 °C). Following initial screening