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STAT3 Differential Scanning Fluorimetry and Differential Scanning Light Scattering Assays: addressing a missing link in the characterization of STAT3 Inhibitor interactions

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Abstract

STAT3 protein is an established target for the development of new cancer therapeutic agents. Despite lacking a traditional binding site for small molecule inhibitors, many STAT3 inhibitors have been identified and explored for their anti-cancer activity. Because STAT3 signaling is mediated by protein-protein interactions, indirect methods are often employed to determine if proposed STAT3 inhibitors bind to STAT3 protein. While established STAT3 inhibition assays (such as the fluorescence polarization assay, electrophoretic mobility shift assay and ELISAs) have been used to identify novel inhibitors of STAT3 signaling, methods that directly assess STAT3 protein-inhibitor interactions could facilitate the development of novel inhibitors. In this context, we herein report new STAT3 binding assays, based on differential scanning fluorimetry (DSF) and differential scanning light scattering (DSLS) to characterize interactions between STAT3 protein and inhibitors. Several peptide and small molecule STAT3 inhibitors have been evaluated, and new insight into how these compounds may interact with STAT3 is provided.

Introduction

Signal transducer and activator of transcription 3 (STAT3) protein is a widely explored target for anti-cancer drug development.[1] This protein possesses several biological characteristics that make it an

attractive target for therapeutic intervention in cancer. Overactive STAT3 signaling drives proliferation, survival and immune system evasion in cancer cells, but healthy cells have transient STAT3 activation and can survive in the absence of STAT3 function.[2-4] While the biology of STAT3 suggests it is a good anti-cancer target, the protein itself is notoriously difficult to target with small molecule inhibitors. STAT3 does not possess a typical enzyme active site, and its activity is mediated by protein-protein and protein-DNA interactions that involve large, relatively flat areas of the protein surface. Selectively disrupting these interactions with small, drug-like molecules remains an evasive scientific challenge. In spite of this, many STAT3 inhibitors have been identified and new inhibitors continually flow into the scientific literature.[2] While early inhibitors, such as STATTIC,[5] S3I-201[6] and peptide inhibitors,[7] failed to progress into clinical testing, more recently identified STAT3 inhibitors have reached this prestigious goal, including STA-21,[8] STAT3 decoy-oligonucleotides,[9] and OPB-51602[10, 11].

While the development of STAT3 inhibitors has continued at a rapid pace, the implementation of new techniques to evaluate these inhibitors has fallen behind. There are several established assays that are commonly used to measure STAT3 inhibition *in vitro* and in tumor models.[2] While these assays are often used to characterize and optimize the activity of STAT3 inhibitors, there are still gaps in technology that limit the understanding of how proposed STAT3 inhibitors interact with STAT3 protein.

Consequently, after two decades of research, an interesting trend has formed. Many reported STAT3 inhibitors have the propensity to act as electrophilic alkylating agents. This has recently been highlighted using mass spectrometry[13, 14] and fluorescence tagging[15] techniques with some of the most widely used STAT3 inhibitors. The majority of published STAT3 inhibitors are reported as selective Src Homology 2 (SH2) domain antagonists, however, the assays that are used to support SH2 domain binding may be also sensitive to compounds that can alkylate STAT3. As outlined in **Figure 1A**, inhibition of STAT3 may produce a protein-inhibitor complex that is energetically

favorable, where the protein-inhibitor complex has a lower free energy than the inhibitor and protein apart. In this case, STAT3 is inhibited (exemplified by a dimmed color compared to the brightly-colored native STAT3), and the complex is themodynamically stable. Alternatively, a reactive inhibitor may covalently modify residues on the surface of STAT3 and induce conformational changes that alter or destabilize STAT3's tertiary structure. In these cases, STAT3 would also be inhibited (represented by the dimmed color in **Figure 1A**), however the modified tertiary structure may not bind to traditional STAT3 binding partners, or the induced instability may cause STAT3 to denature and precipitate (as depicted by the mesh surface representation). This may be especially important for *in vitro* STAT3 assays because recombinant STAT3 protein is known to be poorly soluble, unstable and difficult to work with.[16] In commonly used STAT3 inhibition assays, (including the fluorescence polarization (FP) assay, electrophoretic mobility shift assay (EMSA) and ELISAs), reactive compounds that chemically modify STAT3 to impair its stability or binding interactions would induce the same response as potent but non-reactive STAT3 inhibitors (described in **Figure 1B**).[12]

When used in cell-based assays, these reactive compounds may non-specifically alkylate cellular components to induce toxicity. A particularly hazardous manifestation of this would be in cancer cell proliferation assays where reactive compounds (that show inhibitory activity in traditional *in vitro* STAT3 assays) would inhibit cancer cell proliferation and could modify cell signaling networks because of their inherent toxicity, and not necessarily because they bind selectively to STAT3 or another protein of interest. Thus, differentiating between selective STAT3 binders and compounds that may non-specifically react with STAT3 in biochemical assays is an important challenge within the current STAT3 inhibitor development landscape.

In this context, thermal stability assays, such as differential scanning fluorimetry (DSF) or differential scanning light scattering (DSLS), can differentiate between stabilizing and destabilizing interactions between a protein of interest and potential inhibitors.[16] The traditional DSF assay relies on a polarity sensitive fluorescent dye (such as Sypro Orange™), which increases its fluorescence when exposed to

hydrophobic environments. Thus, thermal denaturing of a recombinant protein can be monitored by measuring Sypro Orange™ fluorescence while increasing the temperature. Capillary DSF or so called nanoDSF experiments typically use a tryptophan (Trp) fluorescence ratio to track protein unfolding. When Trp residues are buried in the hydrophobic core of the protein, their fluorescence maxima occurs around 330 nm, however when they are exposed at the protein surface, this is shifted to 350 nm. Thus, by tracking the ratio of $F_{350}/_{F330}$ one can also monitor protein unfolding. Major advantages of the nanoDSF technique include low sample volume and no exogenous dye is added to the samples. Finally protein aggregation can also be measured by turbidometric scattering of the sample by differential scanning light scattering (DSLS). Using the same set up as for nanoDSF (nanoDSLS), a capillary tube is loaded with an inhibitor and the protein of interest, and thermal denaturing can be simply monitored by measuring absorbance as a representation of the turbidity of the solution.

In thermal stability assays, interactions between the recombinant protein and inhibitory small molecule may alter the melting temperature (T_m) of the protein (the temperature at which half-maximal fluorescence or light scattering is reached). Generally, non-covalent binding of an inhibitor to a recombinant protein will increase the T_m in thermal stability assays, while covalent modifications to the protein tend to alter the tertiary structure and destabilize the protein, resulting in a lower T_m.

Recent efforts have demonstrated that commonly used STAT3 inhibitors BP1-102[14], STATTIC[13] and S3i-201[15] can alkylate STAT3 protein *in vitro*. It is proposed that these covalent modifications may induce conformational changes which impair interactions between STAT3 and its binding partners in biochemical assays. Thus, unlike the typically employed STAT3 assays, a STAT3 DSF assay could distinguish between the stabilizing or destabilizing interactions of proposed STAT3 inhibitors. Therefore, we aimed to generate STAT3 thermostability assays and focused on testing established STAT3 SH2 domain binders, some of which have also been implicated as possible alkylating agents (**Figure 1C**). Peptide-based STAT3 inhibitors, Ac-pYLPQTV (gp130), Ac-pYKPQMH (LIFR) and pYLKTK

(STAT3 consensus sequence, or STAT3c), were also evaluated as these represent validated SH2 domain binders that do not possess reactive groups.[7]

Materials and Methods

Protein Production and Purification

BL21 DE3(T1R) pRARE2 cells were transformed with the $STAT3^{127-688}$ and $STAT3^{127-465}$ constructs and 1.5l TB (supplemented with 8 g/L Glycerol, 50 µg/mL Kanamycin, 34 µg/mL Chloramphenicol) cultures were started from overnight cultures (grown in the same medium at 30°C). The cultures were grown using a LEX bioreactor (Epiphyte3) at 37°C until an OD (600nm) of approximately 2 was reached. The temperature was then reduced to 18°C and after a further hour the cultures were induced with IPTG (0.5 mM). After incubation overnight the cultures were harvested by centrifugation at 4500g for 10min. The cell pellets were resuspended in Lysis buffer (100 mM HEPES, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 0.5 mM TCEP, pH 8.0, 5 µL Benzonase Nuclease (Sigma), Complete EDTA free protease inhibitor cocktail tablet (Roche)) and then frozen at -80°C. The thawed suspended cells were lysed by sonication on ice in ~40 mL samples (Sonics, Vibra Cell 1:45min, 4 sec on, 12 sec off) and the cell debris removed by centrifugation at 49000g x 20 min before filtration of the supernatant through 0.45 µm filters. Chromatography was carried out using an Äkta Xpress (GE HEALTHCARE) at 8°C. 5 mL HisTrap (GE HEALTHCARE) columns equilibrated with Wash Buffer 1 (20 mM HEPES, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 0.5 mM TCEP, pH 7.5) were loaded with the filtered supernatant and then washed with Wash buffer 1 and Wash Buffer 2 (20 mM HEPES, 500 mM NaCl, 10% glycerol, 50 mM imidazole, 0.5 mM TCEP, pH 7.5) before being eluted with Elution Buffer (20 mM HEPES, 500 mM NaCl, 10% glycerol, 500 mM imidazole, 0.5 mM TCEP, pH 7.5). The eluate was loaded on to a HiLoad 16/60 Superdex 75 (GE HEALTHCARE) column which had been equilibrated with gel Filtration buffer (20 mM HEPES, 500 mM NaCl, 10% glycerol, 0.5 mM TCEP, pH 7.5, 2 mM TECP) and eluted using the same buffer. After SDS-PAGE analysis,

fractions containing the desired protein were pooled and concentrated using Vivaspin concentrators (Sartorius). The protein was frozen in aliquots using liquid nitrogen and stored at -80°C.

STAT3 Circular Dichroism (CD) Spectroscopy

Far ultraviolet (UV) CD spectra of STAT3 $^{127-688}$ & STAT3 $^{127-465}$ were recorded on a Jasco J-810 spectrometer (Jasco Spectroscopic Company, Japan) at 20 ˚C in a buffer containing (2 mM HEPES, 50 mM NaCl, 1 % glycerol, 0.05 mM TCEP, pH 7.5, 0.2 mM TECP). The CD Spectra were recorded over a wavelength range of 260-190 nm, with a step size of 1.0 nm, a bandwidth of 1 nm and an averaging time of 2.0 sec. Measurements were performed in a 2 mm path length quartz glass cell using a 0.1 mg/ml concentration of proteins. Five scans were applied continuously and the data were averaged. The CD spectra were smoothed and processed after baseline subtraction using Pro-Data Viewer software (Applied Photophysics, UK).

STAT3 Fluorescence Polarization (FP) Assay

The STAT3 FP assay was carried out as previously described with subtle modifications.[17, 18] A corning 384 well black flat bottom plate was loaded with serial dilutions of STAT3 protein or truncated variants and 10 nM of FP assay probe (5-FAM-GpYLPQTV) in buffer containing 50 mM NaCl, 10 mM HEPES, 1 mM DTT at pH 7.5. After 10 minutes of incubation, polarized fluorescence was measured using a HidexSense reader ($\boldsymbol{\lambda}_{ex}$ = 492 nm, $\boldsymbol{\lambda}_{em}$ = 535 nm, medium lamp intensity, 10 flashes). Experiments with phosphopeptide inhibitors were carried out with 150 nM STAT3 protein. IC_{50} values were determined by plotting concentration of probe *versus* polarized fluorescence and fitting the data to a one site binding curve.

For competition experiments, data points were plotted using GraphPad Prism and curves were fit using non-linear regression analysis for competitive binding according to the formula below. Experiments were performed in duplicate or triplicate and repeated in at least two independent experiments.

$$y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(x - Log(IC_{50}))}}$$

Standard error for IC_{50} values were transformed from the standard error in the Log(IC_{50}) values, by adding or subtracting the standard log(IC_{50}) errors from the log(IC_{50}) value and then transforming those into upper and lower limits for the IC_{50} error values. The larger of these errors was reported for the error in IC_{50} value.

Hydrogen-Deuterium Exchange (HDX) Mass Spectrometry

Binding-interface characterization of the STAT3-gp130 complex was performed by differential HDX MS. First, the complex was prepared in ≈ 1:4 protein/ligand molar ratio by mixing 25 µL of 5.8 mg/mL STAT3 protein in 50 mM HEPES, 1 mM EDTA, 50 mM NaCl at pH 7.5 with 0.9 µL of 10 mM peptide stock solution in 100 % DMSO. Each individual HDX labeling reaction was initiated by mixing 2 µL of complex solution with 18 µL of deuterated buffer at room temperature (22°C). All the experiments were done in triplicates using a 10 min HDX labeling reaction time. For labeling, the deuterated buffer contained the same ionic composition than the protein solution, but water was replaced by heavy water (D_2O). The control experiment was prepared by mixing 25 µL of of 5.8 mg/mL STAT3 protein with 0.9 µL of DMSO, and labeled in the same manner than the STAT3-gp130 complex. After 10 min HDX labeling, each reaction was stopped by adding 30 µL of an ice-cold 100 mM phosphate buffer pH 2.3 containing 100 mM TCEP, 3.3 M Urea, and snap-frozen in liquid nitrogen.

Sample Analysis – LC MS

Each labeled and quenched sample was analyzed in a semi-automated HDX-MS system (Biomotif AB, Stockholm, Sweden) in which manually injected samples were automatically digested, cleaned and separated at 2°C. Deuterated samples were digested using an in-house packed immobilized pepsin column (2.1×30 mm from ACE HPLC Columns packed with pepsin-agarose from porcine gastric mucosa obtained from Sigma-Aldrich) by a 75 seconds at 70 µL/min flow protocol, followed by an on-line desalting step using a 2 mm I.D x 10 mm length C-18 pre-column (ACE HPLC Columns, Aberdeen, UK) using 0.05% TFA at 350 µL/min for 3 min. Peptic peptides were then separated by a LC MS gradient method using an aqueous Solution A containing 5% of acetonitrile (ACN) in 0.3% formic acid

and an organic Solution B containing 95% of ACN and 0.3% of formic acid. The LC MS gradient profile consisted in 2-20 % ACN in 5 min, 20-30 % ACN from 5 to 20 min, 30-95% ACN from 20 to 23 min, followed by 95% ACN for 1.5 min, and 95 to 2 % ACN B in 1.5 min. The analytical column was a 2 mm I.D x 50 mm length HALO C18/1.8 µm operated at 100 µL/min flow. An Orbitrap XL mass spectrometer (Thermo Fisher Scientific) operated at 60,000 resolution at *m/z* 400 was used for analysis. Peptic peptide identification was performed by 3 independent LC MS/MS analysis of an undeuterated protein sample using the same methodology than for the deuterated samples. The HDExaminer software (Sierra Analytics, USA) was used to process all HDX-MS data.

STAT3 Differential Scanning Fluorimetry Assay

The DSF assay was optimized according to procedures outlined previously.[19] Optimal conditions were achieved using buffer containing 100 mM Tris-Cl, 40 mM NaCl, 10 mM $MgCl_2$ at pH 7.4. STAT3 proteins were used at a final concentration of 1 µM and Sypro Orange™ at "5x" (from a stock concentration of "5000x"). Inhibitor solutions were added to protein prior to the addition of Sypro Orange™. Experiments were conducted on a Biorad C1000 Thermal Cycler with CFX96 Real Time System. Heating was conducted from using gradiants from 25-95 °C or 30 to 80°C (increasing 1°C per minute). Collected data was normalized to maximum and minimum values then fit using GraphPad Prism non-linear regression to a Boltzman sigmoidal curve with the formula:

$$y = Bottom + \frac{(Top - Bottom)}{1 + e^{\left(\frac{T_m - x}{Slope}\right)}}$$

Data sets were trimmed for curve fitting to include 3 additional points from minimal and maximal fluorescence values of the melt curve (which were set to 0 and 100 % relative fluorescence for STAT3 truncations, the 30 °C fluorescence value was set as the baseline for full-length STAT3 experiments). Full melt curves are shown in **Supplementary Figures 1** and **2**. K_d values were calculated from dose-response curves of the T_m values as previously described.[20] Briefly T_m values were plotted against ligand concentration and fitted to the following equation to find the Kd value:

$$y = Bottom + (Top - Bottom) \times \left(1 - \frac{P - K_d - x + \sqrt{(P + x + K_d)^2 - 4Px}}{2P}\right)$$

where P represents the protein concentration, x represents the ligand concentration and y is the experimentally determined T_m.[20]

STAT3 nanoDSF and nanoDSLS Assays

NanoDSF standard glass capillaries were filled with the truncated STAT3 variants (with or without compound) in 100 mM Tris-HCl buffer, pH 7.5, containing 40 mM NaCl and 10 mM $MgCl_2$. Using a Prometheus NT.48 (Nanotemper) instrument, the mixtures were subjected to a thermal gradient from 20 to 95°C at a heating rate of 1° C per minute. For nanoDSF experiments, Trp fluorescence at 350 and 330 nm was recorded. T_m values were determined by plotting normalized fluorescence ratio (F_{350}/F_{330}) *versus* temperature and fitting to a Boltzman Sigmoidal curve as above. Data sets were trimmed to include points from 35-75 °C prior to fitting. Simultaneously, the intensity of the back-reflected light was analyzed to assess protein aggregation by nanoDSLS which served as a secondary output for this method. Again T_m values were determined from a plot of relative scattering *versus* temperature (from 35-75 °C) and fitting to a Boltzman Sigmoidal curve.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 using. 1way ANOVA was used to generate p values which are displayed as ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** = $p \leq 0.0001$.

Chemical Reagents

All reagents and inhibitors were purchased from commercial suppliers and used without further modifications. Fluorescent and non-labeled phosphopeptide sequences were purchased from Innovagen AB (Lund, Sweden) or Biomatik (Cambridge, Canada) and diluted in DMSO or H_2O prior to use. Full-length STAT3 protein (amino acids 1-770) was purchased from NordicBioSite (Täby,

Sweden). Cloning and production of truncated STAT3 proteins was conducted at the Karolinska Institute Protein Science Facility (Solna, Sweden).

Results

Full-length STAT3 protein ($STAT3^{Full}$) is known to be difficult to produce, store and handle.[16] Thus, it was suspected that $STAT3^{Full}$ might have problematic instability at the elevated temperatures needed for thermal stability assays. More stable STAT3 variants had previously been reported for protein crystallography experiments, where N- and C-terminal truncations afforded a STAT3 variant that could be crystalized.[16]

Because many reported STAT3 inhibitors claim to bind to the SH2 domain, truncated STAT3 variants were designed so that SH2 domain binding could be assessed. Several truncated proteins were developed in matched pairs to include or exclude the SH2 domain. Of the possibilities analyzed in test expressions, $STAT3^{127-465}$ (containing the coiled-coil domain (CCD) and DNA binding domain (DBD)) and $STAT3^{127-688}$ (CCD to SH2 domain) gave high expression of soluble protein (schematics are shown in **Figure 2A**). The corresponding variant from CCD to Linker domain ($STAT3^{127-578}$) was not soluble and could only be detected at low levels in test expressions. $STAT3^{Full}$ could not be isolated under these conditions and instead was purchased from a commercial supplier. $STAT3^{127-465}$ and $STAT3^{127-688}$ were analyzed by circular dichroism (CD) to determine if they possessed appropriate secondary structures (**Supplementary Figure 3**). Indeed, both truncations had well-defined CD spectra with mostly alpha helical character indicating that these truncated STAT3 variants still formed folded structures.

$STAT3^{127-688}$ was furthre analyzed for SH2 domain integrity using the STAT3 FP assay[17] and hydrogen-deuterium exchange (HDX) experiments.[21] The STAT3 FP assay utilizes fluorescently tagged peptide probe (5-aminofluorescein-GpYLPQTV, referred to herein as 5-FAM-gp130) to assess SH2 domain binding.[17] Typically, the FP assay is used to assess the ability of proposed SH2 domain binders to displace 5-FAM-gp130 from the SH2 domain of STAT3. In this case, $STAT3^{127-688}$, $STAT3^{127-465}$ and

STAT3^{Full} were titrated against 5-FAM-gp130 and polarized fluorescence was measured to ensure that the SH2 domain of STAT3$^{127-688}$ was intact. As shown in **Figure 2B**, STAT3$^{127-688}$ (which contains the SH2 domain) retained its ability to bind 5-FAM-gp130, as increasing concentrations of STAT3$^{127-688}$ resulted in greater polarized fluorescence output. As well, STAT3$^{127-688}$ gave greater FP signal compared to full-length STAT3 and had a lower K_d value (61 ± 6 nM *versus* 550 ± 230 nM for STAT3^{Full}). As anticipated, STAT3$^{127-465}$, which lacks the SH2 domain, showed no binding to 5-FAM-gp130.

To further assess the integrity of the SH2 domain of STAT3$^{127-688}$, known SH2 domain-binding peptide sequences from LIFR, gp130 and STAT3c were also assessed by FP assay (**Figure 2C**).[7] As expected, 5-FAM-gp130 binding was inhibited by these peptides with IC_{50} values of 0.7 ± 0.15 μM, 0.66 ± 0.09 μM and 130 ± 30 μM for gp130, LIFR and STAT3c, respectively.

As another confirmation that the SH2 domain of STAT3$^{127-688}$ was intact, HDX mass spectrometry was used to identify the interaction site between STAT3$^{127-688}$ and gp130 (**Figure 2D**). Indeed, exposure of STAT3$^{127-688}$ to gp130 decreased the incorporation deuterium within the STAT3 SH2 domain, indicating binding. Thus, the truncations employed to generate STAT3$^{127-688}$ did not affect its ability to interact with known STAT3 SH2 domain binders in biochemical assay settings.

STAT3^{Full} and the truncated variants were then assessed for their suitability for use in thermal stability assays (**Figure 3A-E**). Under the described DSF conditions, STAT3^{Full} was quite unstable towards elevated temperatures and gave a T_m of just 37.2 °C (**Figure 3A**). Moreover, gradual increased fluorescence emission at sub-physiological temperatures (30-37 °C) was detected which may reflect the inherent instability of the recombinant STAT3^{Full} protein. Both STAT3 truncations were much more stable towards thermal degradation (STAT3$^{127-465}$ T_m = 53.8 °C and STAT3$^{127-688}$ T_m = 53.5 °C, representative curves shown in **Figure 3B** and **C**, respectively and multiple experiments are summarized in **Figure 3D** and **E**).

To determine if thermal stability could be affected by SH2 domain binding, the truncated and full-length proteins were analyzed by DSF in the presence of 1 mM gp130. Unexpectedly, the T_m of full-length STAT3 was not shifted in the presence of gp130 (**Figure 3A**). This may reflect the presence of unstable regions within N- and C-terminal domains that may instigate the thermal denaturing process which cannot be stabilized by SH2 domain binding. The gp130 sequence also did not stabilize $STAT3^{127-465}$ which lacks the SH2 domain (**Figure 3B**), however gp130 induced a positive T_m shift (ΔT_m) of 8.2 °C with $STAT3^{127-688}$, indicating a direct stabilizing interaction (**Figure 3C**).

To expand on these initial results, LIFR and STAT3c were also analyzed by DSF with $STAT3^{127-688}$. Like gp130, these inhibitors also stabilized $STAT3^{127-688}$. The T_m shifts for these inhibitors were in accordance with reported inhibitory values[7] (representative curves are shown in **Figure 3F** and multiple experiments are summarized in **Figure 3G**). Dose response experiments with the inhibitory peptides (from 2.4 μM to 5 mM at 2-fold dilution steps) were also performed to generate K_d values from the DSF assay. The observed K_d values also corresponded to known inhibitory constants for STAT3 with these peptide inhibitors (**Figure 3H**).[7] No interactions were detected between these peptides and Sypro Orange™ when run without STAT3 proteins in control experiments (**Supplementary Figure 4**).

Having demonstrated that our DSF assay could be used for assessing direct interactions between the STAT3 SH2 domain and known peptide inhibitors, established small molecule STAT3 inhibitors were also tested. Of the plethora of small molecule STAT3 inhibitors present in the scientific literature, STATTIC,[5] S3I-201,[6] BP1-102[22, 23] and STA-21[24] were selected for analysis. All four of these agents are proposed STAT3 SH2 domain binders.[5, 6, 22-24] Furthermore, STATTIC, S3I-201 and BP1-102 have been identified as probable covalent modifiers of STAT3 protein,[13-15] adding more interest to their evaluation in the DSF assay. Binding of BP1-102 to $STAT3^{127-688}$ was also confirmed by FP assay (**Supplementary Figure 5**).

Unexpectedly, no overt stabilizing (or destabilizing) interactions were detected between $STAT3^{127-688}$ S3I-201 or STA-21 (**Figure 4A**). BP1-102 and STATTIC caused dose-dependent decrease in the T_m of $STAT3^{127-688}$. This fits with theories suggesting that these agents may function *via* covalent modification of STAT3 *in vitro*, which could result in decreased overall stability.

To assess if these destabilizing effects were mediated by specific binding to the STAT3 SH2 domain, these same inhibitors were assessed for binding to $STAT3^{127-465}$ which lacks the SH2 domain. Like $STAT3^{127-688}$, dose-dependent destabilization of $STAT3^{127-465}$ was observed with BP1-102 or STATTIC (**Figure 3B**). This indicates that these compounds may interact with STAT3 at other locations than only its SH2 domain. Only very small T_m shifts (< 1 °C) were observed upon treatment of $STAT3^{127-465}$ with S3i-201 or STA-21, and although some variation proved to be statistically significant (likely do to the high level of reproducibility between experiments), it is believed that the fraction of a degree difference in $STAT3^{127-465}$ T_m values between S3i-201 and STA-21 versus the DMSO control would not have major biological implications.

Surprisingly, in control experiments where the small molecule inhibitors were incubated with Sypro Orange™ alone, BP1-102 was found to interact with the dye and alter its fluorescence properties in a temperature dependent manner (**Supplementary Figure 6**). This may help to explain the high degree of variability with the BP1-102 samples in these experiments which contributed to a lack of statistical significance upon analysis with $STAT3^{127-688}$. Thus, to further clarify if the observed T_m shifts were due to interactions with Sypro Orange™, thermal denaturing curves were generated using the intrinsic fluorescence of Trp residues and by turbidometric scattering to monitor protein aggregation (so-called nanoDSF and nanoDSLS experiments, respectively). $STAT3^{127-465}$ has only 3 Trp residues, which prevented accurate analysis of thermal denaturing based on Trp fluorescence ratios (**Supplementary Figure 7a**). Therefore melt curves for $STAT3^{127-465}$ could only be generated by measuring scattering from the capillary solution with increasing temperature (**Supplementary Figure 7b**). $STAT3^{127-688}$

melting curves were measured using Trp fluorescence ratios or scattering as shown in **Supplementary Figure 7a-b**.

Using these additional methods, interactions between truncated STAT3 proteins and inhibitors were assessed (representative melting curves are shown in **Supplementary Figure 8** and **Figure 4C-F** summarizes multiple experiments). NanoDSF and nanoDSLS methods confirmed the results from the DSF assay with Sypro Orange™, showing that peptide inhibitors (gp130, LIFR and STAT3c) stabilized $STAT3^{127-688}$ but not $STAT3^{127-465}$ towards thermal degradation. As well, nanoDSF and nanoDSLS experiments confirmed that BP1-102 and STATTIC destabilized both $STAT3^{127-688}$ and $STAT3^{127-465}$ suggesting that these agents might function differently than the peptide inhibitors and may interact with STAT3 at sites beyond its SH2 domain. The nanoDSF experiments could also detect a very subtle destabilizing effect of DMSO on $STAT3^{127-688}$, again it is suspected that this likely would not have biological significance.

Discussion

Using the reported STAT3 thermal stability assays, stabilizing interactions were detected between peptide STAT3 inhibitors and $STAT3^{127-688}$. In contrast, small molecule STAT3 inhibitors failed to induce positive T_m shifts. Instead, BP1-102 and STATTIC decreased the T_m of $STAT3^{127-688}$, consistent with reports indicating that these agents may act *via* covalent modification of STAT3 protein.[13, 14] Surprisingly, S3i-201, which has also been implicated as a potential covalent modifier of STAT3,[15] did not decrease the T_m of $STAT3^{127-688}$. While STA-21 and S3i-201 induced minute shifts to the T_m of $STAT3^{127-688}$ (and $STAT3^{127-465}$) in the DSF assay, one cannot rule out that these agents may still bind directly to STAT3 protein, perhaps at regions beyond residues 127-688 or in a modality that does not affect the T_m of the protein.

Ideally, one would like to use $STAT3^{Full}$ in the DSF assay, however $STAT3^{Full}$ had a very low T_m which could not be appreciably shifted by high concentrations of gp130 (one of the most potent STAT3 inhibitors discovered) or by small molecule inhibitors BP1-102 and STATTIC (all $\Delta T_m < 1$ °C)

(**Supplementary Figure 9**). By truncating the N- and C-termini of the protein, more stable STAT3 variants were produced, as indicated by higher T_m values in the DSF assay. The T_m of $STAT3^{127-688}$ was shifted by peptide STAT3 inhibitors (gp130, LIFR and STAT3c), however they did not shift the T_m of $STAT3^{127-465}$, supporting their use as selective SH2 domain antagonists. Beyond the DSF assay, $STAT3^{127-688}$ was shown to be active in the STAT3 FP assay and could also be used in HDX experiments. ΔT_m values for $STAT3^{127-688}$ in the DSF assay were confirmed using nanoDSF and nanoDSLS to rule out possible interactions between Sypro Orange™ and the inhibitors. While BP1-102 demonstrated some interaction with the Sypro Orange™, the destabilizing nature of BP1-102 was confirmed using nanoDSF and nanoDSLS using intrinsic Trp fluorescence and protein aggregation in place of the exogenously added Sypro Orange™ dye.

Similar to these thermal stability assays, another thermofluorescence assay was recently reported for STAT proteins.[25] This assay used displacement of 5-FAM-gp130 from the STAT3 SH2 domain to track protein degradation by DSF. The authors demonstrated that upon heating, STAT proteins were denatured which resulted in the probe being unable to bind to the protein. When the probe was displaced, its fluorescence decreased due to solvent quenching effects. The authors contended that STAT3 inhibitors, including BP1-102, displaced the probe, leading to decreased fluorescence intensity as measured by area under the first derivative of fluorescence curve. While further exploration into this topic is recommended, our findings suggest an alternative explanation for the observed results. Instead of specifically displacing the probe, we suggest that BP1-102 destabilizes STAT3 and enhances its thermal degradation. This which prevent the probe from binding and could explain the observed decreases in fluorescence intensity.

Conclusions

Unlike many commonly used STAT3 inhibition assays, the described DSF assay can differentiate between inhibitors that form more stable complexes with STAT3 *versus* potentially reactive compounds that destabilize the protein or alter its tertiary structure. Advantageously, this assay can

identify compounds that directly interact with STAT3, providing important information about the binding region as well as the mechanism of action of such compounds. While developed for STAT3 inhibitors, the same platform can be applied to identify binders of other STAT proteins, becoming a valuable tool for the discovery of novel STAT inhibitors with a broad spectrum of applications.

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Figure Captions

Figure 1. A) Description of possible consequences of STAT3 inhibitor binding. Native STAT3 can be recruited to activated receptors and interact with binding partners to fulfill its cellular functions. A STAT3 inhibitor (STAT3i) may block STAT3 function through a variety of mechanisms. **i)** Selective non-covalent binding of an inhibitor preserves the tertiary structure and stability of STAT3 but inhibits STAT3 function (represented by the dimmed surface color). **ii)** Covalent modification to STAT3 may decrease the protein's stability (indicated by a mesh surface representation) or **iii)** could alter the tertiary structure of the protein and prevent interactions with binding partners (which would likely impact the protein's stability as well). **B)** Descriptions of commonly used STAT3 inhibition assays that show confounding results with compounds that destabilize or modify the tertiary structure of the protein. Non-specific binding or reactivity with STAT3 protein in these biochemical assays may masquerade as selective STAT3 inhibition. **C)** Established small molecule STAT3 inhibitors STATTIC, STA-21, S3i-201 and BP-1-102.

Figure 2. A) Representation of STAT3 proteins produced for thermal stability studies. Full length STAT3 ($STAT3^{Full}$) includes the N-terminal domain (ND), coiled-coil domain (CCD), DNA binding domain (DBD), linker domain, Src homology 2 domain (SH2) and the transactivation domain (TAD). $STAT3^{127-688}$ (CCD to SH2 domains) and $STAT3^{127-465}$ (CCD to DBD) were easily produced using traditional bacterial expression and purification techniques. **B)** Fluorescence polarization (FP) assay showing $STAT3^{Full}$, and $STAT3^{127-688}$ but not $STAT3^{127-465}$ can bind to 5-FAM-gp130 resulting in increased FP signal at higher concentrations of protein. **C)** $STAT3^{127-688}$ FP assay using peptide STAT3 inhibitors. $STAT3^{127-688}$ maintained strong binding affinity for known phosphopeptide sequences from gp130, LIFR and the STAT3 consensus sequence. **D)** Hydrogen-deuterium exchange assay with $STAT3^{127-688}$ and gp130. Relative change in deuterium incorporation per retrieved peptide after gp130 peptide binding to $STAT3^{127-688}$ is plotted against the amino acid sequence with functional domains indicated. Deuterium incorporation was significantly decreased in peptides extending below the red line, confirming that gp130 peptide binds specifically to the SH2 domain of $STAT3^{127-688}$. Data is an average of three independent replicates.

Figure 3. STAT3 Differential Scanning Fluorimetry Assays. **A)** A representative DSF assay showing $STAT3^{Full}$ has poor thermal stability (T_m = 37.2 °C) and it was not stabilized by the addition of 1 mM gp130. **B)** A representative experiment showing $STAT3^{127-465}$ is thermostable, T_m = 53.8 °C, and no stabilization of $STAT3^{127-465}$ was observed upon treatment with 1 mM gp130 as expected because $STAT3^{127-465}$ lacks the SH2 domain. **C)** A representative plot of melting curves, $STAT3^{127-688}$ was stabilized by treatment with the gp130 peptide sequence resulting in a large T_m shift (ΔT_m = 8.2 °C). **D)** Graphical summary of T_m shifts induced by gp130 from two independent experiments. Only the T_m for $STAT3^{127-688}$ was significantly shifted by the addition of gp130 compared to the DMSO control. **E)** Table of T_m values from **D**. **F)** Analysis of known peptide inhibitors of STAT3 protein: gp130, LIFR and

the STAT3 consensus sequence induced positive T_m shifts in $STAT3^{127-688}$, a representative experiment is shown. **G)** Graph and table summarizing three independent experiments investigating T_m shifts with peptide inhibitors. **H)** Dose-response analysis of T_m values with peptide STAT3 inhibitors (2.4 µM to 5 mM, 2 fold dilutions). Larger shifts in T_m were observed with increasing concentrations of inhibitors until saturation at high concentrations. K_d values derived from dose-response experiments are also shown.

Figure 4. A) Dose-response analysis of known STAT3 inhibitors in the DSF assay using $STAT3^{127-688}$. BP1-102 and STATTIC caused a dose-dependent decrease in the T_m of $STAT3^{127-688}$. S3I-201 and STA-21 did not shift the T_m of $STAT3^{127-688}$. The gp130 sequence caused a dose-dependent positive T_m shift with $STAT3^{127-688}$. **B)** STATTIC and BP1-102 also decreased the T_m $STAT3^{127-465}$ which does not contain the SH2 domain. STA-21, S3I-201 and the gp130 sequence did not affect the T_m of $STAT3^{127-465}$. **C-F)** NanoDSF and nanoDSLS experiments with $STAT3^{127-688}$ and $STAT3^{127-465}$. Inhibitory peptides (STAT3c, LIFR and gp130, 1 mM) stabilized $STAT3^{127-688}$ towards thermal degradation when analyzed by Trp fluorescence ratio (F_{350}/F_{330}) **C)** or by light scattering **D)**. Small molecule STAT3 inhibitors BP1-102 (80 µM) and STATTIC (80 µM) destabilized $STAT3^{127-688}$ towards thermal degradation. **E)** T_m values for $STAT3^{127-465}$ in the nanoDSLS assay monitoring protein denaturing by scattering. Trp fluorescence ratio could not by used to monitor $STAT3^{127-465}$ degradation due to the low number of Trp residues in $STAT3^{127-465}$. **F)** Table summarizing the results from the nanoDSF experiments depicted in **C-E)**.

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