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Azetidine ring, salicylic acid, and salicylic acid bioisosteres as determinants of the binding characteristics of novel potent compounds to Stat3

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Introduction

We analyzed in depth the novel Signal transducer and activator of transcription 3 (Stat3) pharmacophore of our recent potent small molecule inhibitors of Stat3. The impact of the structural variations of the class of potent glycine- and azetidine-based small molecule Stat3 inhibitors on their Stat3 binding characteristics and inhibitory potencies was investigated. Of these two broad classes of inhibitors, there are salicylic acid and benzo-fused-*N*-heterocyclic-containing sub-groups. Glycine-based salicylic acid compounds, such as BP-1-102, azetidine-based salicylic acids, such as H098, and the azetidine-based benzo-fused-*N*-heterocyclics, such as H182, bind covalently and irreversibly to Stat3 at different sites, despite all sharing the same pharmacophore. The azetidine salicylic acids bind irreversibly to Stat3 DNA-binding domain (DBD) Cys426, the azetidine-benzo-fused-*N*-heterocyclics irreversibly bind to Stat3 DBD Cys468, and the glycine-based compounds bind irreversibly somewhere else from the Stat3 DBD and linker domain. Glycine-based salicylic acid (BP-1-102) and azetidine-based salicylic acid (H098) compounds, but not the azetidine-based benzo-fused-*N*-heterocyclics (H182), also bind reversibly at the Stat3 SH2 domain. While not directly binding the Src Homology (SH2) domain, co-immunoprecipitation studies showed H182 blocks the Stat3 association with epidermal growth factor receptor (EGFR), Janus Kinases (JAK2) and Interleukin-6 receptor (IL-6R)/gp130 in human pancreatic

Abbreviations: Stat, signal transducer and activator of transcription; PBST, phosphate-buffered saline tween-20; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; EGFR, epidermal growth factor receptor, JAK, Janus kinase, SH2, Src homology 2, MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; FBS, fetal bovine serum.

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cancer cells, likely due to its strong covalent and irreversible binding (IC_{50} 0.38 μM, one hour incubation) with the Stat3 DBD Cys468 that might allosterically affect the protein-protein interaction of Stat3 with EGFR, JAK2 or gp130. This would explain the reported inhibition by H182 of Stat3 tyrosine705 phosphorylation in human tumor cells, including pancreatic cancer.

Signal transducer and activator of transcription (Stat) proteins are a family of cytoplasmic transcription factors that are induced in response to stimulation by cytokines and growth factors [1]. The Stat proteins are modular in structure, with *N*-terminal domain, Coiled-coil domain, DNA-binding domain (DBD), Linker domain (LD), SH2 domain, and Transcriptional activation domain at the C-terminus [1–3]. Stats are activated via phosphorylation on a critical tyrosine residue (Tyr, Y; Y705 for Stat3) by Tyr kinases, including JAKs or Src family kinases. This event induces the dimerization of two Stat monomers (Stat:Stat) through a reciprocal phospho-Tyr-SH2 domain interaction, and the translocation of the Stat:Stat dimers into the nucleus where they bind to specific DNA-response elements in the target gene promoters to induce gene transcription. In this way, the Stat proteins regulate growth factor and cytokine-induced cellular processes.

In contrast to normal cells, many human solid and hematological malignancies harbor constitutively-active Stat3, a member of the Stat protein family. Constitutively-active Stat3 serves as an important mechanism to support the progression of cancer [4–6]. Therefore, Stat3 is an important target for the development of new anticancer therapeutic modalities [6–9]. However, the clinical development of suitable small molecule Stat3 inhibitors has proven to be a significant challenge, and no drug is available yet in the market. With a rather flat surface, strong binding to Stat3 by regular inhibitors has proven difficult. Most of the previously developed Stat3 inhibitors over the last fifteen to twenty years have all been micromolar in potency [6,10], except for the recently reported PROTAC-Stat3 inhibitors, including SD-36 [11,12]. As known for PROTAC typical compounds [13], SD-36 has a large molecular weight (MW 1158), and therefore pharmacokinetics (PK) may become an issue, including a likely compromised membrane cell permeability. Despite its high *in vitro* potency (IC_{50} 10–50 nM), SD-36 was administered at relatively high doses for *in vivo* mouse efficacy experiments (25 mg/kg) and only by intravenous (IV) [11,12] route of administration.

Recently, potent azetidine inhibitors were disclosed with Stat3-inhibitory potency (IC_{50}) of 0.38–0.98 μM in Stat3 DNA-binding activity/electrophoretic mobility shift assay (EMSA) [14,15]. The azetidine-based compounds selectively inhibit constitutive and ligand-induced Stat3 activation through a covalent irreversible binding mechanism of action, and block Stat3 nuclear accumulation. Lead compound, H182 inhibited growth of human breast tumors in mice, and it enhanced the response to radiation and prolonged survival in mice bearing breast tumors [15]. By site-directed mutagenesis studies, it was determined that H182′s pentafluorophenylsulfonamide *para*-fluorine is replaced mainly by the sulfur atom of Cys468 in the Stat3 DNA-binding domain (DBD) forming an irreversible covalently-bound complex [15].

Herein, we have characterized how the structural differences between the glycine-based and azetidine-based compounds defined their interactions with Stat3 and their Stat3-inhibitory modes of action. These studies demonstrate that the glycine-based compounds bind differently from the azetidine-based, and within the latter group, a salicylic acid group-dependency for the choice of Stat3 site interactions is additionally observed.

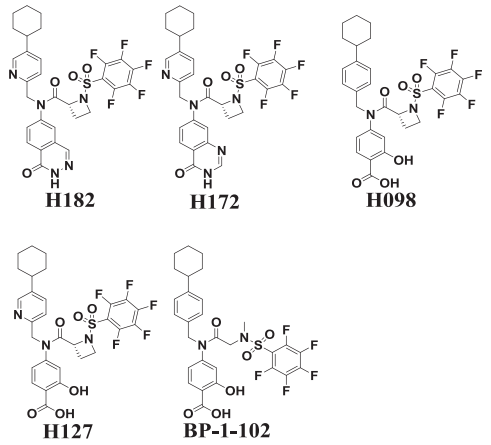
First, we focused on Stat3 DNA-binding activity/EMSA analysis and extended the previously reported time-course studies of the azetidine-based inhibitors [15]. In these studies, nuclear extract samples of equal total protein were each incubated at 30 °C with 0–10 μM H172, H127, H098 (azetidine-based compounds) or BP-1-102 (glycine-based compound) for 10 min, 30 min, and 60 min, prior to incubation with the radiolabeled hSIE probe that binds Stat3 and subjected to gel shift analysis [14–17]. All compounds feature the same pharmacophore, and are represented by azetidine-salicylic acids, e.g., H098 and H127 [14], azetidine-benzo-fused-*N*-heterocyclics, e.g., H182 and H172 [14,15], and glycine-salicylic acids, e.g., BP-1-102 [17], (Fig. 1A). Results showed a time-dependent inhibition of Stat3 DNA-binding activity for all the compounds (Fig. 1B). However, we observed that not all the azetidine- and glycine-based compounds behaved with the same pattern. A marked weaker inhibitory activity was observed for the glycine-based compound, BP-1-102, compared to any of the azetidine-based inhibitors (Fig. 1B). The inhibitory potencies and the time-dependency together are consistent with the model in which the azetidine-based and the glycine-based compounds covalently bind to Stat3 and inhibit its activity. We deduce that the stronger inhibitory impact on the Stat3 DNA-binding activity of the azetidine-based inhibitors is presumably due to the rigidity of the azetidine ring, which might induce the right structural conformation for the S_NAr reaction to take place (*i.e.*, displacing, by close proximity, of *para*-fluorine by the Cys sulfhydryl group) ensuing efficiently the covalent irreversible modification.

In the recently reported nano-LC/MS/MS proteomics studies, the azetidine-salicylic acid, H098 was found to bind irreversibly to Stat3 at Cys328 and Cys426 [15], while the azetidine- phthalazinone H182 was observed to bind irreversibly at Cys468 and Cys542 [15]. These results were intriguing especially because the EMSA potency for both compounds, H098 and H182, were very similar (IC_{50} 0.37 and 0.38 μM, respectively, after one hour incubation with Stat3, Fig. 1B). The main structural difference between these compounds is the salicylic acid group in H098 versus the phthalazinone in H182. Site-directed mutagenesis was used to follow up with H182, which suggested that Cys468, is the main residue that binds irreversibly with H182 [15]. As of yet, the critical Stat3 Cys residues required to mediate the irreversible binding to the other azetidine-based and glycine-based compounds are not determined. Therefore, to understand more comprehensibly, we extended the site-directed mutagenesis study to the compounds that show time-dependency of activity in the DNA-binding assay/EMSA, such as H172, H098, H127 and BP-1-102 (Table 1).

We used the same recombinant Stat3 construct that contains the core fragment of human Stat3 protein from amino acids 127 to 711 [15,18]. Mutations in Stat3 Cys-to-Ala (Stat3CA) or Cys-to-Ser (Stat3CS) were created for specific Cys residues, and the inhibitory activities of azetidine-based H098, H127 and H172, and the glycine compound, BP-1-102, were assessed on the mutant protein and compared to the wild-type (wt) Stat3 protein versions. Purified, phosphorylated wtStat3 (wtpYStat3), and the phosphorylated mutant Stat3C328A, Stat3C426A, Stat3C468A, and Stat3C542S protein samples of equal total protein were pre-incubated with increasing concentrations of the compounds for 30 min at room temperature prior to incubation with the radiolabeled hSIE probe that binds Stat3 and subjecting to EMSA analysis. Results showed that comparing to the potencies against the DNA-binding activity of the wtpYStat3, the inhibitory potencies of the azetidine-salicylic acid compounds, H098 and H127 were mostly impacted with respect to the DNA-binding activity of the mutant pYrStat3C426A (IC_{50} 23 and 20 μM, respectively, Table 1), whereas the potencies of the two azetidine-benzo-fused-*N*-heterocyclic compounds, H182 [15] and H172, were mostly affected with respect to the DNA-binding activity of the mutant pYrStat3C468A (IC_{50} 40 and 47 μM, respectively, Table 1). Unlike the azetidine-based compounds (H098, H127, H172, H182), the glycine-based salicylic inhibitor, BP-1-102 showed similar inhibitory potencies against the DNA-binding activities of the wtpYrSTAT3 and all the mutant Stat3 protein versions, pYrStat3C328A, pYrStat3C426A, pYrStat3C468A and pYrStat3C542S (Table 1). These findings suggest that BP-1-102 may not bind irreversibly at the Stat3 DBD Cys328, Cys426 or Cys468, or the LD Cys542, and instead it appears to exhibit a different site requirement for binding, despite similarly presenting time-dependency of inhibition of the Stat3 DNA-binding in the EMSA that suggests it binds covalently to Stat3 (Fig. 1B and Table 1).

Once we obtained a better understanding of the covalent interaction of the azetidine-salicylic and glycine-salicylic compounds with Stat3, we then focused on how these compounds might additionally interfere with

A)



B)

ID	10 min IC_{50} (μM)	30 min IC_{50} (μM)	60 min IC_{50} (μM)
H182	1.5*	0.66*	0.38*
H172	2.2	1.2	0.87
H127	1.0	0.35	0.27
H098	1.2	0.50	0.37
BP-1-102	16.7	6.2	5.2

*From Ref 15

Fig. 1. Compounds H182, H172, H098, H127 and BP-1-102, and their time-dependent inhibitory effects against *in vitro* Stat3 DNA-binding activity/EMSA. (A) Structures of H182, H172, H098, H127, BP-1-102; (B) EMSA analysis of Stat3 DNA-binding activities in nuclear extracts of equal total protein containing activated Stat3 pre-incubated with H182, H172, H098, H127 or BP-1-102 for 10, 30, or 60 min at room temperature prior to incubation with the radiolabeled hSIE probe that binds Stat3.

Table 1
Inhibitory potencies of azetidine-based compounds against wild-type and mutant Stat3 in DNA-binding activity/EMSA analysis.

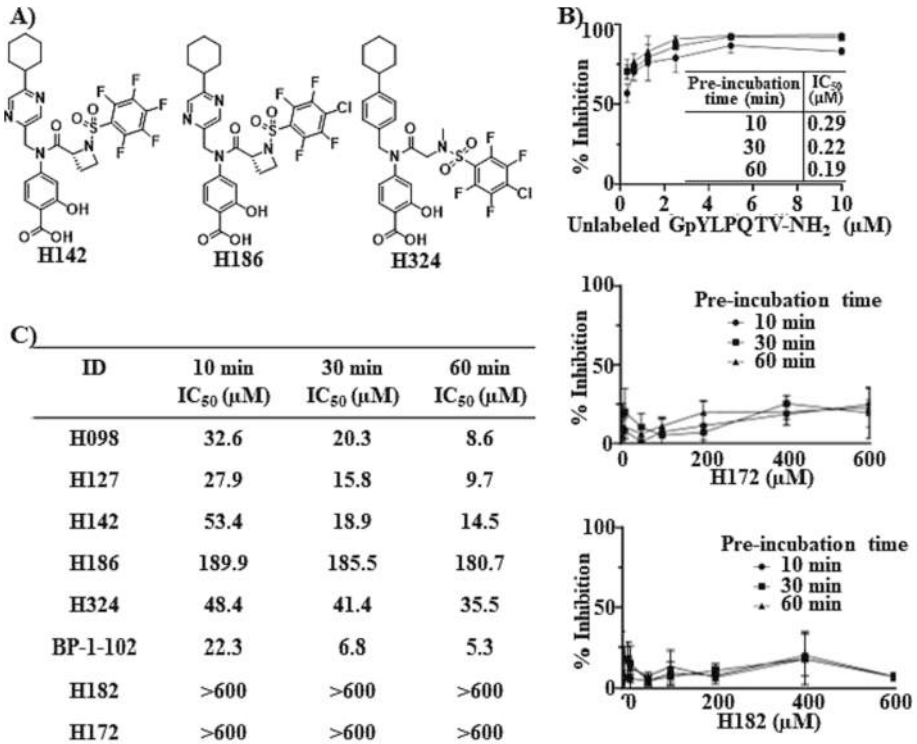
ID	pYrSTAT3 IC_{50} (μM)	pYrSTAT3C328A IC_{50} (μM)	pYrSTAT3C426A IC_{50} (μM)	pYrSTAT3C468A IC_{50} (μM)	pYrSTAT3C542S IC_{50} (μM)
H182	4.8*	8.8*	6.0*	40*	5.5*
H172	5.2	8.2	7.6	47	6.7
H098	2.2	6.2	23	0.8	6.8
H127	0.6	4.8	20	2.2	1.2
BP-1-102	13.6	11.2	10.6	20.1	22.7

*From Ref 15

Stat3 dimerization (Stat3:Stat3), which occurs via pTyr peptide:SH2 domain interaction [2]. To study this, we employed the Stat3 fluorescence polarization (FP) assay [17,19,20], which is designed to model the Stat3:Stat3 dimerization [21–23] and uses 5-carboxy fluorescein-labeled high affinity pTyr peptide, GpYLPQTV-NH_2 to bind to the SH2 domain of pure recombinant Stat3 (5-fl-GpYLPQTV-NH_2:Stat3 complex). The FP assay has been used to evaluate and identify classical disruptors of Stat3:pTyr peptide interaction, which are classified as dimerization disrupting inhibitors (dimerization disruptors) [21,22,24–29]. The GpYPQTV-NH_2 peptide is derived from the interleukin-6 receptor (IL-6R)/gp130 [19,30] and binds with high affinity (K_D 150 nM) to the Stat3 SH2 domain [30].

For the FP assay, in addition to the compounds already introduced above, we included the azetidine-salicylic acid compounds, H142, H186 (Fig. 2A) [14] and H215 (Fig. 3A), and the glycine-based salicylic acid compounds, H324 (Fig. 2A) [14] and S3I-201-1066 [16] (Fig. 3A) to increase the coverage of compounds. In the FP assay, as expected, the incubation with the unlabeled GpYLPQTV-NH_2 peptide led to a dose-dependent inhibition of the FP signal in a time-independent manner indicative of reversible disruption of pTyr:SH2 domain (Fig. 2B, left panel). By contrast, the presence of up to 600 μM of the azetidine-benzo-*N*-heterocyclic compounds, H182 or H172, led to no substantial change in the FP signal (Fig. 2B, H172, H182), suggesting that these two compounds at concentrations up to 600 μM are unable to displace the labeled pTyr peptide from the Stat3 SH2 domain. These results are in sharp contrast to their strong inhibitory potencies against Stat3 DNA-binding activity in the EMSA analysis (H182, IC_{50} 0.38 and H172, IC_{50} 0.87 μM at 60 min incubation). On the other hand, salicylic acid-containing analogs, whether the azetidine-based, such as H098, H127 (Fig. 1A), and H142 and H186 (Fig. 2A) [14], or the glycine-based, such as BP-1-102 (Fig. 1A) [17] and H324 (Fig. 2A) [14] disrupted the Stat3 SH2:pTyr peptide interaction, with the typical dimerization disruption profile (Fig. 2C), with the implication that they reversibly bind to the SH2 domain. We surmise that the key determinant for the binding of the latter group of inhibitors to the Stat3 SH2 domain is the salicylic acid motif, which mimics the crucial pTyr moiety and specifically binds reversibly to the pTyr-binding pocket of the SH2 domain [17].

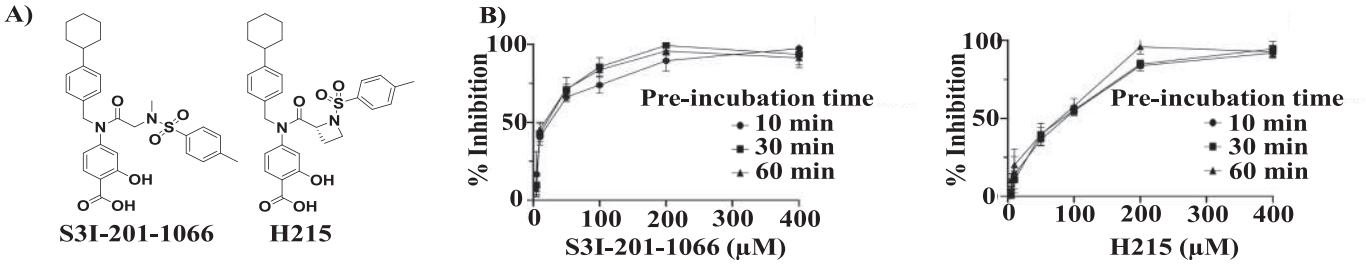
The salicylic acid-containing compounds that have the pentafluorophenyl group in the molecule regardless of whether azetidine-based or glycine-based show a pattern of inhibition in the FP assay that is time-dependent, as exemplified by H098 (IC_{50} 32.6, 20.6 and 8.6 μM), H127 (IC_{50} 27.9, 15.8 and 9.7 μM), H142, (IC_{50} 53.4, 18.9 and 14.5 μM), and BP-1-102 (IC_{50} 22.3, 6.8 and 5.3 μM, at 10-min, 30-min, and 60-min incubation, respectively, Fig. 2C). The time-dependency of binding to the SH2 domain for these compounds likely reflects their covalent interactions via their respective pentafluorophenyl group with Stat3 cysteine residues [15] somewhere else from the SH2 domain, as the latter domain lacks cysteine residues. This is further supported by the results showing that salicylic acids lacking the pentafluorophenyl group, such as H186 (IC_{50} 189.9, 185.5 and 180.7 μM, for 10-min, 30-min, and 60-min incubation, respectively) and H324 (48.4, 41.4 and 35.5 μM, for 10-min, 30-min, and 60-min incubation, respectively) [14] (Fig. 2C) show no time-dependent inhibition in the FP assay. Accordingly, the FP assay results for BP-1-102 and H142 (IC_{50} 5.3 and 14.5 μM, 60 min incubation) suggest they bind stronger to Stat3 than their corresponding *para*-



C)

ID	10 min IC_{50} (μM)	30 min IC_{50} (μM)	60 min IC_{50} (μM)
H098	32.6	20.3	8.6
H127	27.9	15.8	9.7
H142	53.4	18.9	14.5
H186	189.9	185.5	180.7
H324	48.4	41.4	35.5
BP-1-102	22.3	6.8	5.3
H182	>600	>600	>600
H172	>600	>600	>600

Fig. 2. Compounds H098, H127, H142, H186, H324, BP-1-102, H182 and H172, and their effects against *in vitro* interactions of Stat3 SH2 domain with its cognate phospho-tyrosine peptide in fluorescent polarization (FP) assay. (A) Structures of H142, H186 and H324; (B, C) Time-course FP assay of the binding of recombinant His-tagged Stat3 (rHis-Stat3 (127–711)) to its cognate fluorescently-labeled peptide, 5-carboxy-fl-GpYLPQTV-NH_2 probe for effects of (B) unlabeled GpYLPQTV-NH_2 peptide, H182, and H172, insert, IC_{50} values at 10, 30, and 60 min incubation; and (C) the inhibitory potency (IC_{50}) values for the different azetidine and glycine inhibitors at 10, 30, and 60 min of incubation with the Stat3 protein. Control (0) represents incubation with 0.1 % DMSO. Values, mean ± SD. Data are representative of 3–4 independent determinations.



C)

ID	10 min IC_{50} (μM)	30 min IC_{50} (μM)	60 min IC_{50} (μM)
S3I-201-1066	22.4	21.6	20.1
H215	86.4	83.6	82.5

Fig. 3. Fluorescent polarization assay of the Stat3 SH2 domain active site binding to the cognate phospho-tyrosine peptide probe and competition with the reversible Stat3 inhibitors. (A) Glycine-based S3I-201–1066 and azetidine-based H215 structures; and (B and C) Time-course FP assay of the binding of recombinant His-tagged Stat3 (rHis-Stat3 (127–711)) to its cognate fluorescently-labeled peptide, 5-carboxy-fl-GpYLPQTV-NH_2 probe for effects of S3I-201–1066 and H215 (B), and IC_{50} results at different times (C). Control (0) represents incubation with 0.1 % DMSO. Values, mean ± SD. Data are representative of 3–4 independent determinations.

chlorine-containing compounds, H324 and H186 (IC_{50} 35.5 and 180.7 μM, 60 min incubation, Fig. 2C), respectively. Presumably, BP-1-102 and H142, but not H324 and H186, have a dual mode of action, and simultaneously bind reversibly to the Stat3 SH2 domain, and irreversibly to a Stat3 cysteine residue, which would explain the stronger potency (IC_{50}) in the FP assay for these two compounds.

It was noted above that azetidine-based compounds were more potent than their glycine-based counterparts in the Stat3 DNA-binding activity/EMSA analysis (Fig. 1). However, in the phospho-tyr-peptide: SH2 domain focused FP assay, it is just the opposite, *i.e.*, glycine-based compounds are more potent than azetidine-based compounds. Thus, glycine compounds BP-1-102 and S3I-201-1066 [16] (FP IC_{50} 5.3, and 20.1 μM at 60-min incubation, respectively, Fig. 2c and Fig. 3) are superior to the azetidine-based analogs H098 and H215 [14], respectively (FP IC_{50} 8.6, and 82.5 μM at 60-min incubation, respectively, Fig. 2c and Fig. 3). These findings support the assertion that salicylic acids with a

glycine linker (as in BP-1-102 and S3I-201-1066) promote better interaction with the Stat3 SH2 domain than salicylic acids with an azetidine ring (as in H098 and H215).

A further observation is that in both of the FP and EMSA studies, the glycine-based BP-1-102 showed nearly the same potencies and a similar time-dependency pattern of potencies (IC_{50} 22.3 and 16.7 μM at 10 min; 6.8 and 6.3 μM at 30 min; and 5.3 and 5.2 μM at 60 min incubation, respectively, for FP and EMSA, Fig. 2C and Fig. 1B). This feature is not observed for the azetidine-based compounds. For the latter, the azetidine-salicylic acids, H098 and H127 are much more potent in the EMSA studies against Stat3 DNA-binding activity than against the phospho-tyr:SH2 domain interaction in the FP assay (H098, IC_{50} 8.6 and 0.37 μM at 60 min; and H127, IC_{50} 9.7 and 0.27 μM at 60 min, respectively, for FP and EMSA) (Fig. 2C and Fig. 1B).

Informed by the site-directed mutagenesis of Stat3 and its interactions with the compounds, which demonstrated Stat3Cys468 and Cys426 are the major residues that are needed for the covalent, irreversible binding to the azetidines, H182 and H127, respectively, we developed a minimized modeling structure using Molecular Operating Environment (MOE) software (MOE 2020.09, Chemical Computing Group, Montreal, Quebec, Canada (2021)) and crystal structure of Stat3 (PDB code 1BG1 [31]. Results for H182 show the covalent binding to Cys468 (Fig. 4A, upper and bottom views), in addition to hydrogen bonds of Lys574 NH_3^+ to the amide oxygen, as well as three water molecules to the phthalazinone system, and lipophilic interactions of the cyclohexyl ring with Val563 and Lys615 with distances under 4.0 Å (Fig. 4A, bottom). As for H127, results show the covalent binding to Cys426 (Fig. 4B, upper and bottom views), a hydrogen bond interaction of the salicylate carboxyl group with backbone Asp427 NH (Fig. 4B), and lipophilic interactions of the cyclohexyl ring with Thr494 methyl and Lys495 side methylene groups under 4.2 Å (Fig. 4B). To understand the binding preference of structurally similar H182 and H127 to different Stat3 DBD cysteine sites we performed MOE energy calculations. Briefly, the Stat3-inhibitor complexes were minimized to convergence using default parameters built-in MOE with and without the inhibitors to derive the change in the binding energy. Results show that minimized Stat3 H127-Cys426 adduct has lower relative potential energy than that from H127-Cys468 complex (180 kcal/mol difference, Supplemental Table S1). On the other hand, Stat3 H182-Cys468 adduct has very similar relative potential energy to H182-Cys426 complex (only a difference of about 5 kcal/mol in favor of H182 bound to Cys468, Supplemental Table S1). For the latter, the H182 ligand energy contribution (to binding energy) was then calculated by removing it from each Stat3 H182-cysteine complex. It was found that the Stat3 H182-Cys468 complex is more energetically favored when H182 is bound compared to H182-Cys426 complex, which *is energetically less favored and therefore less stable* when H182 is bound (Supplemental Table S2).

Present FP results suggest only salicylic acid-containing compounds can directly disrupt Stat3 binding to its cognate IL-6R/gp-130-derived GpYLPQTV-NH_2 phospho-Tyr peptide in the FP assay, while the benzo-fused-*N*-heterocyclic compounds, such as H182 appeared incapable of directly disrupting Stat3 binding to the labeled 6-amino acid peptide. This could lead to expectation that H182 would not be able to affect the Stat3 Tyr phosphorylation (pY705Stat3) by growth factor receptor Tyr kinases, JAKs or Src family kinases. However, as we have previously reported, H182 and related benzo-fused-*N*-heterocyclic

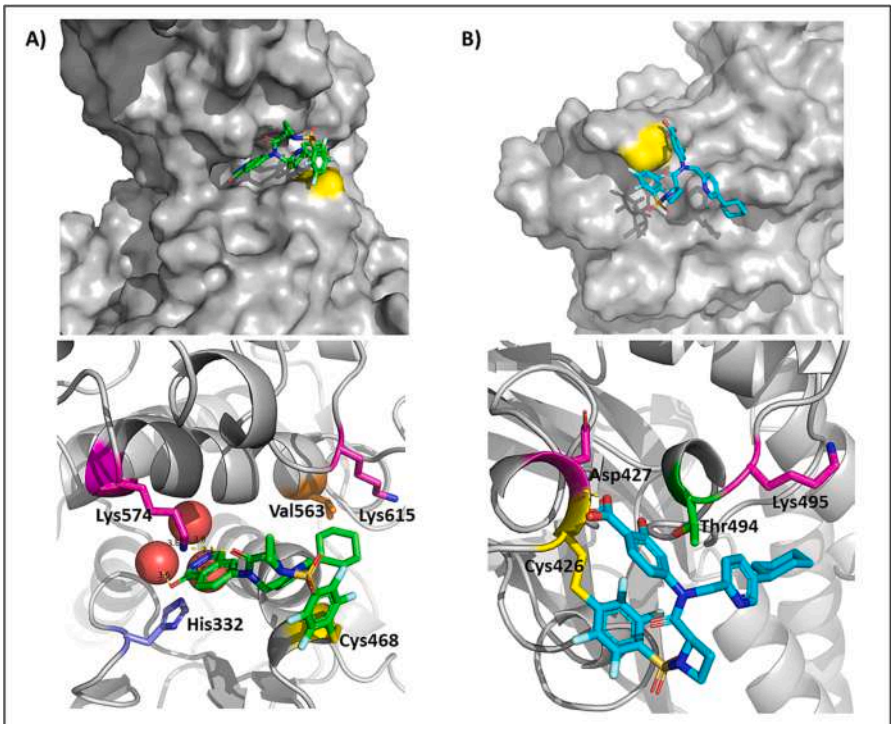


Fig. 4. Modeling structures of (A) H182 (green) bound covalently to Stat3 Cys468 (yellow), and (B) H127 (cyan) bound to Stat3 Cys426 (Stat3 PDB code 1BG1 [30]). Upper view shows monomeric Stat3 surface with (A) H182 (green) bound to Cys468 (yellow), and (B) H127 (cyan) bound to Cys426 (yellow). Bottom view shows active site residue close interactions with (A) H182, and (B) H127. Nitrogen is in dark blue, oxygen in red, sulfur in yellow, fluorine in light gray, and on the active site, interacting residues are highlighted as cysteines in yellow, lysines in magenta, valine in orange, histidine in purple, aspartic acid in red, and threonine in green. Modeling used Molecular Operating Environment (MOE) software (MOE 2020.09, Chemical Computing Group, Montreal, Quebec, Canada (2021)). MOE Dock was performed at Stat3 (PDB code 1BG1 [30]). MOE Site Finder result 4 utilizing the Triangle Matcher method (scored via Affinity dG) for the placement of the ligand at this site and refinement of docking utilizing the induced fit method (scored via London dG). Once the best docked pose was established at the corresponding site, according to results from site-directed mutagenesis, the covalent bond was created to the corresponding cysteine sulfur atom. The model was further minimized utilizing Amber rigid-body minimization to afford the final covalent docked model. Visualization was created using PyMOL software version 2.5.5 [31]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compounds did inhibit Stat3 Tyr phosphorylation in MDA-MB-231 and MDA-MB-468 cells [15]. Therefore, we sought to study further this apparent discrepancy and performed co-immunoprecipitation with immunoblotting analysis on cell lysates prepared from cells treated with the compounds.

Pancreatic cancer Panc-1 cells were treated with H182 for 8 h and whole-cell lysates were prepared, and the epidermal growth factor receptor (EGFR), gp130, Src, and JAK2 were immunoprecipitated and then immunoblotted for Stat3. Results showed unlike control (untreated) samples where Stat3 was co– immunoprecipitated with the EGFR, gp130, JAK2, or Src, the Stat3 levels that co-precipitated with the EGFR, gp130, or JAK2 protein were strikingly low, while the Stat3 levels that co-precipitated with Src were nearly unchanged (Fig. 5, compare lanes 5 vs 4 for EGFR, or lanes 4 vs 3 for gp130, JAK2, or Src). These findings suggest that the benzo-fused-*N*-heterocyclic compound, H182, has the ability to disrupt Stat3 binding to its cognate sites in the EGFR and IL6R/gp130 receptors or the JAK2 protein, but not Src. The non-immune IgG showed little co-precipitation of protein, and input shows equal amounts of proteins in the samples. Altogether, we deduce that an allosteric effect in Stat3 could be induced by the strong covalent irreversible binding at the Stat3 DBD Cys468 by H182 [18]. Although the recombinant Stat3:GpYLPQTV-NH_2 interactions in the FP assay appear to be unperturbed by the allosteric effect from the covalent binding of H182 to the DBD, likely due to the small size of the binding peptide, in the cells the Stat3 alteration is apparently sufficient to interfere with its association with the EGFR or JAK2 kinase, thereby inhibiting Stat3 Tyr705 phosphorylation, as evident in the pancreatic cancer cells treated with H182 (Fig. 5).

Although sharing the same basic pharmacophore, the glycine- and azetidine-based Stat3 small molecule inhibitors discussed in this work bind irreversibly to different sites in Stat3. For example, the azetidine-salicylic acids, H098 and H127 bind irreversibly to the Stat3 DBD C426 (EMSA IC_{50} 0.37 and 0.27 μM, respectively), and the azetidine-benzo-fused-*N*-heterocycles, H172 and H182 bind to the Stat3 DBD C468 (EMSA IC_{50} 0.87 and 0.38 μM, respectively), whereas for the glycine-based salicylic acid, BP-1-102, we will suggest that it does not bind to cysteine residue(s) located in the Stat3 DBD or LD. Only azetidine- and glycine-based groups of compounds that contain the salicylic acid group bind reversibly to the SH2 domain as deduced from the FP assay, albeit with low affinity. Although H182 does not bind the Stat3 SH2 domain (as supported by the lack of inhibition of Stat3 pTyr peptide:SH2 domain interaction in FP assay up to 600 μM), Co-IP results show it disrupts binding to EGFR, JAK2 or gp130. Presumably, the strong irreversible binding to the Stat3 DBD C468 allosterically affects Stat3 binding to these proteins, and hence, Stat3 tyr phosphorylation [15]. It is important to note that isothermal titration calorimetry (ITC) studies have derived the K_D of 1.97 μM for binding to Stat3 for H182 μM [15], apparently indicative of the first, reversible step given the timescale of ITC studies. Altogether, studies presented here give more in-depth knowledge as to the mechanism of action of the azetidine class of Stat3 inhibitors and the structural determinants for binding, especially compound H182, with the ultimate goal to advance the identification of Stat3 inhibitor clinical candidates.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

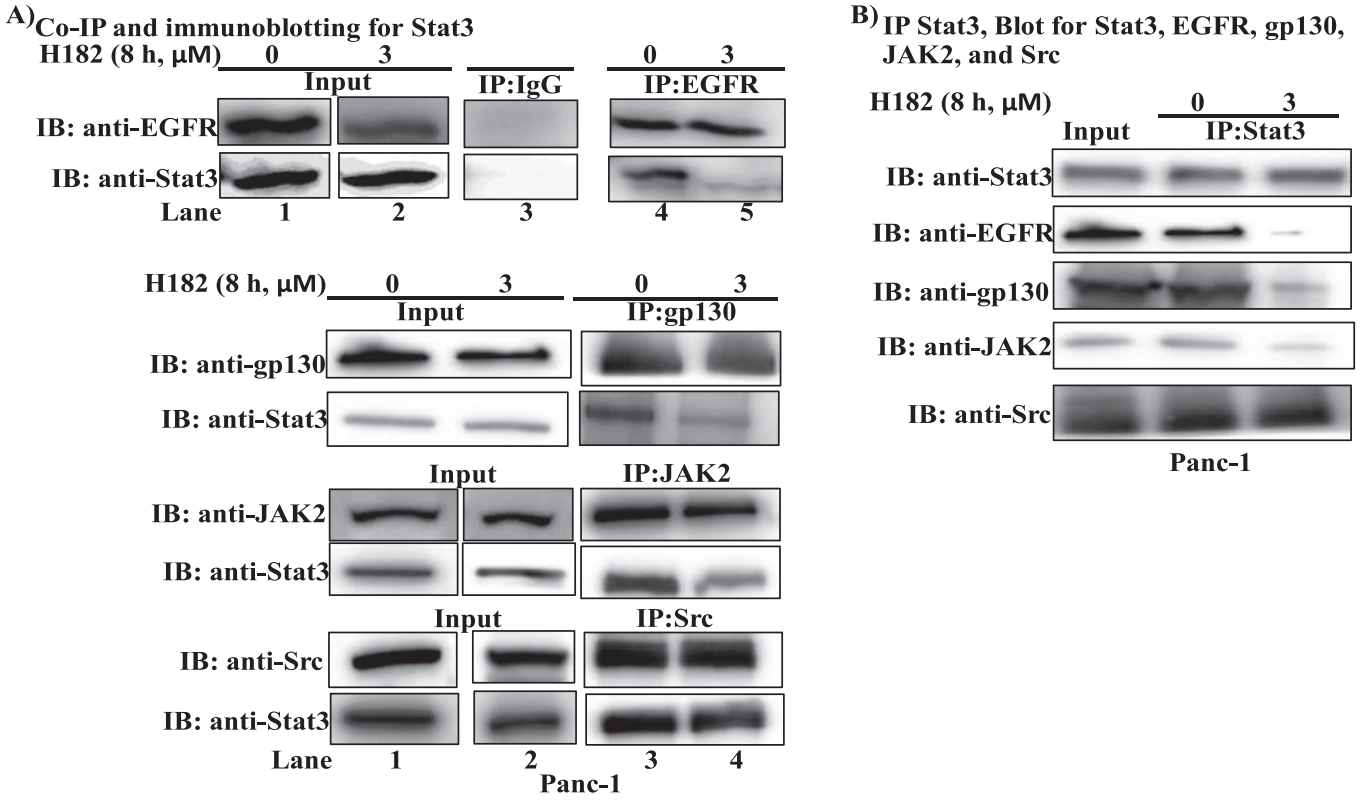


Fig. 5. Co-immunoprecipitation of receptors, kinases, or Stat3 with immunoblotting for Stat3, EGFR, JAK2, gp130, or Src and effect of H182. Immune precipitates (IP) of (A) EGFR, gp130, JAK2, or Src, and non-immune IgG samples, or (B) Stat3, or whole-cell lysate samples (input) of equal protein were immunoblotted (IB) for Stat3, EGFR, gp130, JAK2, or Src. Positions of proteins in gel are labeled; control lane (0) represents immune precipitates or whole-cell lysates prepared from 0.1% DMSO-treated cells. Data are representative of 2 independent determinations.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2023.129565.

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