Published in final edited form as:

Mol Biosyst. 2009 September ; 5(9): 1039–1050. doi:10.1039/b902021g.

A Quantitative High-Throughput Screen for Modulators of IL-6 Signaling: A Model for Interrogating Biological Networks using Chemical Libraries

Ronald L. Johnson^{1,3}, Ruili Huang¹, Ajit Jadhav¹, Noel Southall¹, Jennifer Wichterman¹, Ryan MacArthur¹, Menghang Xia¹, Kun Bi², John Printen², Christopher P. Austin¹, and James Ingles¹

¹ NIH Chemical Genomics Center, National Institutes of Health, Bethesda, MD 20892

² Invitrogen Discovery Sciences Madison, WI 53719

Abstract

Small molecule modulators are critical for dissecting and understanding signaling pathways at the molecular level. Interleukin 6 (IL-6) is a cytokine that signals via the JAK/STAT pathway and is implicated in cancer and inflammation. To identify modulators of this pathway, we screened a chemical collection against an IL-6 responsive cell line stably expressing a beta-lactamase reporter gene fused to a sis-inducible element (SIE-*bla* cells). This assay was optimized for a 1536-well microplate format and screened against 11,693 small molecules using quantitative high-throughput screening (qHTS), a method that assays a chemical library at multiple concentrations to generate titration-response profiles for each compound. The qHTS recovered 564 actives with well-fit curves that clustered into 32 distinct chemical series of 13 activators and 19 inhibitors. A retrospective analysis of the qHTS data indicated that single concentration data at 1.5 and 7.7 μ M scored 35 and 71% of qHTS actives, respectively, as inactive and were therefore false negatives. Following counter screens to identify fluorescent and nonselective series, we found four activator and one inhibitor series that modulated SIE-*bla* cells but did not show similar activity in reporter gene assays induced by EGF and hypoxia. Small molecules within these series will make useful tool compounds to investigate IL-6 signaling mediated by JAK/STAT activation.

Keywords

IL-6; small molecule; HTS; STAT; assay

Introduction

Exploration of the interface between chemistry and biology has been greatly augmented with the implementation of automated methodologies¹. High throughput screening (HTS) of small molecule libraries has enabled rapid identification of chemical modulators of biological activity, principally for enzymes. Engineered cellular assays designed for HTS, which are both sensitive and scalable to highly miniaturized formats, offer a rich source of targets and activities for compound library screening. There are however, particular challenges to successfully identifying small molecule modulators of a cellular pathway that are exacerbated by traditional screening methods. For example, cell-based assays have relatively high backgrounds arising in part from heterogeneity of the cell population, low amounts of target components within

³Address correspondence to Ron Johnson, rjohnso2@mail.nih.gov.

cells, and signal amplification of biological cascades and networks². In addition, biologically active molecules can display complex pharmacology brought about by cytotoxic effects or interactions with multiple cellular targets. In many cases, the cellular components targeted by active compounds are not known.

Quantitative high-throughput screening (qHTS) is a method where chemical libraries are screened at multiple concentrations to generate titration-response curves for all library compounds³. qHTS enables pharmacological assessment of each library member's potency and efficacy as well as nascent structure-activity relationships (SAR) among analogs. Application of qHTS to cell-based screens can greatly improve the efficiency of recovering biologically active compounds and delineate pharmacologically complex responses⁴. The assembly of data generated from multiple qHTS experiments across numerous cellular assays will provide an unprecedented opportunity to interpret the chemical activity of compound libraries, assign mechanism of action, and establish novel relationships among biological pathways.

The collection and analysis of data from large-scale chemical screens of cellular assays will require an efficient and automated process for qHTS and subsequent data processing. Identifying the appropriate cellular assay formats that will maximize the information content, screening efficiency and data quality is a key practical consideration in developing this chemical biology paradigm. The reporter gene technology using the beta-lactamase enzyme and fluorescent substrates⁵ has been widely employed in HTS^{6–10}. In this study, we have examined the activity of a cytokine signaling pathway against a compound library including biologically annotated small molecules and uncharacterized combinatorial and synthetic molecules selected for chemotype diversity.

The interleukin-6 (IL-6) signaling pathway is important for mobilizing the immune response against foreign agents and when aberrantly activated, contributing to inflammation and cancer¹¹. IL-6 signals by binding to the IL-6 receptor and recruiting the transmembrane protein gp130 into a signaling complex^{12, 13}. Formation of this receptor complex activates Janus kinase (JAK) family members to phosphorylate tyrosines in the gp130 cytoplasmic domain that in turn, recruit signaling components such as signal transducer and activator of transcription (STAT) proteins. Phosphorylation of STAT proteins causes dimerization and subsequent translocation to the nucleus where they bind interferon-gamma activation sequences (GAS) or GAS-like elements to control gene expression¹⁴.

To identify small molecule modulators of the IL-6 signaling pathway, we assayed ME-180 cervical carcinoma cells stably expressing a beta-lactamase reporter under the control of a sis-inducible element (SIE), a GAS-like sequence that recruits certain STAT proteins after IL-6 stimulation¹⁵. ME-180 cells respond to IL-6 by activating STAT1 and STAT3¹⁶ and inducing proliferation¹⁷. IL-6 has a role in cervical biology, as normal and neoplastic cervical cells express IL-6 *in vitro*¹⁸ and *in situ*¹⁹ as well as proliferate in response to IL-6 stimulation^{17, 18}. Here we screened this IL-6 signaling assay in a qHTS of 11,693 compounds and identified 32 distinct chemical series comprising known and novel bioactive molecules. In following counter screens to eliminate fluorescent and nonselective compounds, we found four activator and one inhibitor series that modulate IL-6-stimulated signaling but do not show similar activity in cell-based beta-lactamase reporter assays for EGF- or hypoxia-stimulated pathways.

Results

Assay optimization and qHTS

To screen for small molecule modulators of IL-6 signaling, we assayed SIE-*bla* cells, a ME-180 line stably expressing a SIE beta-lactamase reporter fusion²⁰. This cell line likely reports

signaling through STAT1 and STAT 3 as these transcription factors bind the SIE upon IL-6 stimulation¹⁵ and ME-180 cells respond to IL-6 by transiently activating STAT1 and STAT3 hetero- and homodimers¹⁶. Treatment of SIE-*bla* cells with increasing concentrations of IL-6 resulted in a dose-dependent, saturable increase of beta-lactamase activity with a half-maximal concentration of activity (AC₅₀) of about 50 pM (0.9 ng/mL, Fig. 1A), similar to previously-reported values for IL-6-induced gene expression^{21, 22}. This response was inhibited by a small molecule pan-JAK antagonist with an AC₅₀ of 20 nM (Fig. 1A), a value similar to that reported for JAK1 inhibition²³.

The SIE-*bla* cells were screened in 1536-well plate format against a collection of 11,693 small molecules that included known bioactives, natural products, and synthetic combinatorial and diversity compounds (Fig. 1B). To identify both activators and inhibitors, the cells were stimulated at 40 pM IL-6, near the AC₅₀ level of activity for the SIE reporter. Using qHTS, each compound was assayed at seven or more concentrations³, the highest concentration starting at 9 μ M for most compounds (17 μ M for the LOPAC and Prestwick collections, Figure 1). The 132 plate qHTS performed well with an average signal to background of 5.3 and Z' of 0.76 for the AC₅₀ IL-6 and inhibitor control wells (Fig. 1C).

Classification of Actives

Beta-lactamase activity in the SIE-*bla* assay was detected by CCF4-AM²⁰, a substrate that contains coumarin and fluorescein coupled by a cephalosporin moiety²⁴. Excitation of the coumarin donor at 405 nm results in fluorescein acceptor emission at 530 nm via fluorescence resonant energy transfer (FRET). Following uptake of CCF4-AM by cells and hydrolysis of the AM ester, cleavage of the CCF4 cephalosporin ring by beta-lactamase results in liberation of fluorescein and direct detection of the resultant coumarin derivative emission at 460 nm. Fluorescence detection at 530 nm measures the amount of uncleaved CCF4 substrate within cells. While beta-lactamase enzymatic activity is detected by 460 nm emission, the ratio of cleaved (460 nm) to uncleaved (530 nm) substrate is frequently used, as this ratiometric approach helps normalize the well-to-well variation in volume and cell number⁵.

To determine compound activity in the SIE-*bla* qHTS, the titration-response data from the 460 nm emission (460), 530 nm emission (530) and the 460/530 ratio (Ratio) measurements for each sample was plotted and modeled by a four parameter logistic fit. Curve-fits were then classified by the criteria described in³. In brief, Class 1.1 and 1.2 were full curves containing upper and lower asymptotes with efficacy $\geq 80\%$ and $<80\%$, respectively. Class 2.1 and 2.2 were incomplete curves having only one asymptote with efficacy $\geq 80\%$ and $<80\%$, respectively. Class 3 curves showed activity at only the highest concentration or were poorly fit. Class 4 curves were inactive having a curve-fit of insufficient efficacy or lacking a fit altogether.

Fluorescence from the uncleaved beta-lactamase substrate can be used as a measure of viability because living cells are required to de-esterify and retain the substrate within cells. A decrease in the 530 nm reading may indicate cell cytotoxicity, or alternately, may denote a high level of beta-lactamase enzyme that has consumed much of the substrate within cells. In the SIE-*bla* qHTS, only 19 of 1,330 actives showed decreases at 530 nm. This very low incidence of activity suggests the five-hour incubation time is insufficient for cytotoxic molecules to manifest substantial cell killing. Increases in the 530 nm reading suggest compound fluorescence. Indeed, of the 64 activators at 530 nm, 55 were tested on parental ME-180 cells (see below) and all were positive, indicating fluorescence. Overall, of the 83 compounds that showed activity 530 nm, 78 showed activity at 460 nm as well. Hence, few compounds displayed effects solely by decreasing cell viability or fluorescing only at 530 nm.

To identify active compounds, the 460 and Ratio measurements were used. The qHTS identified 907 compounds (Class 1-3) active in both the 460 and Ratio determinations (Table 2). However, 211 additional compounds were active only at 460 while another 120 compounds were active only by the Ratio determination. Examination of Class 1 and 2 curves from these two subsets indicated well-fit titration curves ($r^2 > 0.8$, $P \leq 0.05$). However, the efficacies were near the 30% threshold defined for activity which led to their inconsistent classification as active or inactive between the 460 and Ratio determinations (Fig. 2). As most of these Class 1 and 2 curves comprised acceptable curve fits, they were considered as active. Likewise, Class 3 curves from these groups were included as actives, though the curve fits were of lower quality and in some cases, spurious (data not shown), as was expected for this class. An additional 92 Class 4 compounds were reassigned as Class 3, because these curves had good curve fit metrics ($P \leq 0.05$, $\leq 5\%$ zero activity and ≤ 2 masked data points) in both 460 and Ratio readings but the efficacies were within the range considered as background (Fig. 2). For compounds that were Class 1-3 in both 460 and Ratio determinations, the Ratio curve class was used and AC_{50} s were averaged. From this process, 1,330 compounds were identified as active (Table 2).

The qHTS indicated that 11% of the screened compounds displayed some level of activity, composed of an almost equal number of activators and inhibitors (Table 3, Fig. 3). Class 1.1 and 1.2 curves each comprised 0.5% of the screened collection, totaling 55 and 63 compounds, respectively. Class 2 curves totaled almost 4% of the screened library and 91% had AC_{50} s between 1 and 10 μ M. Of the 171 Class 2.1 samples, 139 were activators and only 32 were inhibitors, while of the 275 Class 2.2 compounds, 121 were activators and 154 were inhibitors. Compounds associated with Class 3 curves constituted 6.5% of the library and 58% of the identified actives with 339 activators and 427 inhibitors. As this category of actives had lower quality curve-fits based on fewer points of activity, the biological activity of these compounds in this qHTS was less certain. While the potencies of the actives spanned over three orders of magnitude, most actives displayed μ M potencies; 73% were 1–10 μ M AC_{50} and 19% were 0.1–1 μ M AC_{50} , while 3% <0.1 μ M AC_{50} and 5% >10 μ M AC_{50} .

Derivation of structure-activity relationships

To identify chemical series for further characterization, structure-activity relationships (SAR) were derived from the qHTS data. Selected compounds (Class 1 and 2 by Ratio determination, Class 1-3 at 460 nm and Class 4 at 530 nm) were grouped using Leadscope to yield clusters of three or more actives. For each cluster, maximal common substructures (MCS) were extracted and used to search the entire screening collection to find all analogs including inactives. From each MCS, scaffolds were extracted, each containing 2 or more actives, to form a SAR series. This process was applied separately for activators and inhibitors. For the SAR analysis of activators, 209 actives were grouped yielding 14 clusters and 13 series (Supp Table 1 and Fig. 4A) while for inhibitors, 209 actives were grouped yielding 35 clusters and 19 series (Supp Table 2 and Fig. 4B).

Counter screen of selected actives to find fluorescent artifacts

Because detection of the beta-lactamase substrate and the cleavage product is fluorescence based, fluorescent compounds that penetrate cells may be incorrectly scored as positive. As many fluorescent compounds that populate typical heterocyclic collections emit in the blue-green spectrum of light^{25–27}, such molecules may emit light in the 460 nm and 530 nm ranges and be identified as active in the SIE-*bla* assay. To find fluorescent artifacts, the 3,000-member combinatorial library and selected follow-up compounds were counter screened on parental ME-180 cells, which do not contain the beta-lactamase reporter gene. The library was screened as a qHTS of seven five-fold dilutions beginning at 8 μ M while the follow-up compounds were tested at 24 two-fold dilutions in duplicate beginning at 40 μ M. Compounds were considered

fluorescent if they were Class 1-3 activators by the 460, 530 or Ratio determination whereas inhibitory Class 1-3 curves in either the 460 nm or 530 nm reading were scored as inactive. Though molecules fluorescent only at 530 nm would appear as inhibitors by the Ratio determination, none were observed. Of the 663 qHTS actives counter screened, 374 scored as positive by virtue of compound fluorescence. This result indicated the activity of these compounds arose from fluorescence instead of induction of the SIE beta-lactamase reporter. Therefore, these compounds were considered artifacts.

Each of the 32 SAR series was examined for fluorescence as determined by activity in parental cells. The counter screen of parental cells indicated activator series **4**, **8**, **11** and **12** as fluorescent because $\geq 50\%$ of the actives within each series scored were positive (Table 4). A 6,8-dimethylpteridinone series (**8**) from the combinatorial library contained 322 positives, accounting for 82% of the positives recovered from the ME-180 counter screen. Activator series **2**, **3**, **5**, **6** and **10** contained $\leq 40\%$ actives that scored positive in ME-180 cells, indicating as inconclusive whether the activity of the series arose from fluorescence, induction of the reporter, or a combination of both. Only 10 compounds from the inhibitor series were positive in the parental cells (Table 4) and none was active at 530 nm (data not shown). This ruled out the possibility of artifact compounds showing strong fluorescence at 530 nm and scoring as inhibitory by the Ratio determination. Two inhibitor series, methylquinazolinamine (**18**) and 2-(methylamino)pteridinone (**30**), each contained five actives that were positive in the parental cells and comprised 8% and 38%, respectively, of actives tested (Table 4). The fluorescent compounds in these series may have caused underestimation of actual biological potency and efficacy because compound fluorescence at 460 nm would offset the decrease in 460 nm fluorescence brought about by lowered production of the beta-lactamase reporter enzyme.

Retest of selected compounds to confirm activity

To confirm the action of selected qHTS actives, 55 activators and 58 inhibitors were chosen for retesting (113 total retests). These compounds were tested on SIE-*bla* cells as a titration series of 24 two-fold dilutions in duplicate beginning at 38 μ M in two independent experiments. The activity of 13 qHTS actives did not confirm upon retest; seven were inactive while six showed opposite activity between the qHTS and follow-up or between the two follow up experiments. Mass spectrometry of these compounds indicated that ten had the expected molecular weight (MW) while for three samples the MW could not be determined. For most of these apparent non-confirmations, the qHTS or follow-up titration-response curves were either poorly fit or of low efficacy, thus calling into question the initial curve classification assignment, for example (see below). One hundred of the 113 (88%) compounds confirmed activity upon retest on SIE-*bla* cells (47 activators and 53 inhibitors, Table 4, Supp Table 1 and 2). Of the retested activators, 16 were positive in the parental cell counter screen and were likely fluorescent artifacts. Thus, 84 samples showed confirmed biological activity.

Plotting of the qHTS and follow-up AC₅₀s of all confirmed actives but two indicated that the qHTS derived potencies correlated but were lower overall in the follow-up assay (Fig. 5). The two excluded actives were outliers where the discrepancy in potency were explained by the automated curve-fit of the qHTS titration data, whereby incorrect masking of a data point resulted in a higher potency curve.

Identification of chemical series selective for IL-6 signaling

To identify series that were selective or biased toward modulation of IL-6 signaling, we examined the activity of each series in two other cell-based assays: AP-1-*bla*, which detected EGF signaling via an AP-1 reporter gene²⁸, and HRE-*bla*, which measured cobalt chloride-induced hypoxia by a hypoxia response element (HRE) reporter²⁰. Like SIE-*bla* cells, both assays use stably transfected ME-180 cells and a beta-lactamase reporter gene, thereby

controlling for differences in activity that might arise from use of different cell types or reporter enzymes. Of the two assays, AP-1-*bla* was more closely comparable to SIE-*bla*, as both assays were incubated with ligand and compounds for five hours before assaying, while for HRE, inducer and compounds were incubated overnight. The AP-1-*bla* and HRE-*bla* assays were screened using qHTS³ (PubChem AIDs 257, 914 and 915).

Several series that were selective for SIE-*bla* showed little or no activity in AP-1-*bla* and HRE-*bla* assays. Of the activators, three spirocycle series (**2**, **6** and **7**, Fig. 4A), which share a diazaspiropropanone core, were active in SIE-*bla* at submicromolar concentrations (Table 4). Of the 29 compounds in series **2** that were activators in SIE-*bla*, five compounds were inhibitors in AP-1-*bla* and four other compounds were activators of lower potency in HRE-*bla*. For the SIE-*bla* actives in series **6** and **7**, none was active in AP-1-*bla* and only one activated HRE-*bla* cells with a 10 μ M AC₅₀ (Table 4, data not shown). Six of seven anthracycline actives in series **13** behaved as activators in SIE-*bla* but were inhibitors or inactive in both AP-1-*bla* and HRE-*bla* indicating these compounds were selective in their directionality of action. For inhibitors, all four SIE-*bla* actives in series **24**, which contained a xanthene-4,5,6-triol core, were inactive in AP-1-*bla* and HRE-*bla*.

Known bioactive compounds modulate IL-6-stimulated activity in SIE-*bla* cells

The qHTS identified a number of compounds with known biological activity that target components involved in IL-6-mediated signaling thereby biologically validating the SIE-*bla* assay. Of note were actives that modulate protein kinases or phosphatases. The tyrosine kinase inhibitor emodin, which blocks IL-6-stimulated JAK2 activation in multiple myeloma cells²⁹, inhibited SIE-*bla* at 10 μ M IC₅₀, as did ZM 39923, a naphthyl ketone that inhibits *in vitro* JAK1 kinase activity at 20 μ M IC₅₀³⁰, and SD1008, a JAK2 inhibitor³¹, blocked SIE-*bla* expression with a 3 μ M IC₅₀. The tyrosine phosphatases SHP2 and CD45 are important in IL-6 signal transduction; IL-6 can mobilize the MAP kinase pathway via JAK phosphorylation of SHP2¹³ and CD45, a JAK phosphatase³², is required for IL-6-induced proliferation of multiple myeloma cells³³. Indeed, dephostatin and Me-3,4-dephostatin, small molecule inhibitors of tyrosine phosphatases including CD45^{34, 35}, antagonized IL-6 signaling in SIE-*bla* cells at ~5 μ M AC₅₀ (Supp Table 2, data not shown). SU6656, U0126, and SB 202190 are inhibitors of the IL-6-activated kinases Src³⁶, MEK³⁷ and p38³⁸, respectively and these were identified in the qHTS as antagonists of 1–5 μ M AC₅₀. These inhibitors may block transcription of the SIE-*bla* reporter gene by preventing STAT3 phosphorylation, as IL-6 requires MEK or Src activity to activate STAT3 in some cell types^{36, 37}.

Several SAR series identified from the qHTS contained known bioactives and displayed selectivity for SIE-*bla*. Series **13**, which contained the anthracycline antibiotics idarubicin, doxorubicin and daunomycin, activated SIE-*bla* but inhibited AP-1- and HRE-*bla* cells (Table 4). This selective modulation suggested that anthracyclines were specifically activating the IL-6 pathway. Indeed, in the absence of IL-6, these compounds did not induce the SIE-*bla* reporter (data not shown), indicating they were IL-6-dependent potentiators. While IL-6 has been implicated in conferring drug resistance in breast carcinoma and osteosarcoma cells^{39, 40}, addition of exogenous IL-6 did not increase resistance of ME-180 cells to cell killing by doxorubicin, daunomycin, paclitaxel, or camptothecin, among others (data not shown). Na, K-ATPase inhibitors (series **21**) and retinoic acids (series **32**) inhibited SIE- and HRE-*bla* reporters but not AP-1-*bla* (Table 4). This inhibition of SIE-*bla* is not likely caused by cytotoxicity because AP-1-*bla* activity was not affected, even though both lines were incubated with compound for five hours. However, the inhibition of HRE-*bla* by series **21** and **32** could arise from cytotoxicity because compounds were incubated with cells overnight in this assay.

Retrospective comparison of actives identified by qHTS and at single concentrations

Traditional HTS involves assaying the library at one concentration and scoring compounds that exceed a threshold of activity as active, typically 3σ above the background activity of the assay⁴¹. To compare how well traditional single-concentration screening would identify actives in the SIE-*bla* qHTS, we conducted a retrospective analysis of the qHTS data using compound activities at individual concentrations to identify actives. The qHTS recovered 564 compounds associated with Class 1 or 2 curves and these compounds were considered high-quality actives for use in the retrospective analysis. The 766 compounds associated with Class 3 curves, that were poorly-fit or showed activity at only the highest concentration, were excluded from the comparison because their activity could not be ascertained reliably. Using the 1.5 μ M screening concentration and applying a 3σ threshold (40% activity), 180 positives were identified, of which 10% were qHTS inactives and therefore false positives (Table 5). This concentration failed to identify 403 qHTS actives as positive, indicating 71% false negatives. At 7.7 μ M, the highest concentration where all compounds were tested, and a 3σ cutoff, 365 positives were recovered, of which all but 2 were qHTS actives, demonstrating 1% false positives. False negatives were 36% where 201 qHTS actives were not scored as active. The 17 μ M concentration showed false positive and false negative percentages similar to 7.7 μ M. Examination of the fitted curves of these false negatives indicates that though the percent activity at 7.7 μ M was within the cutoff thresholds, the percent activities at 1.5 and/or 17 μ M concentrations exceeded the thresholds (Fig. 6). These results indicate for the SIE-*bla* assay, use of a single concentration yielded a low percentage of false positives but a significant percentage of false negatives, ranging from 35% to 71% at the three highest concentrations assayed.

Discussion

We took several approaches to identify actives in the SIE-*bla* qHTS. Use of both the 460 and Ratio readings were instrumental in determining active compounds. About 70% of the 1330 declared actives displayed Class 1-3 curves in both the measurements. An additional 25% showed activity in either reading (Table 2). In many of these cases, particularly for Class 1 and 2 curves, the discrepant activity assignments corresponded to compounds of low efficacy, near the threshold level assigned for background (Fig. 2). Having two means to measure activity allowed us to recover actives that would not have been possible using either measurement alone. Furthermore, 92 samples (7% of actives) were originally scored as inactive by our automated curve-fit program but showed high-quality curve fits of low efficacy and were therefore reassigned as Class 3 actives. While these approaches helped ensure recovery of many biological actives, some declared actives, especially compounds associated with Class 3 curves, may be false positives.

While qHTS is well suited to determine comprehensively the activity of a chemical library, it is critical to consider how, and with what level of confidence, activity is scored. This is important when profiling a compound's activity across many assays as inaccurate assignments may lead to incorrect assessment of target selectivity. As shown here, the scoring of compounds as inactive can be less certain in cell assays because compounds of insufficient efficacy but well-fit titration-response curves may not be scored as active (Fig. 2). We found that a combination of curve-fit parameters (P value <0.05 , x-intercept between -5 and 5 % activity and ≤ 2 masked points) applied to both 460 and Ratio readings were useful in recovering some of these low efficacy compounds.

The SIE-*bla* qHTS identified 11% of the screened compounds having some degree of activity. However, this percentage falls to ~ 2.5 % when only actives associated with high confidence curve classes (Class 1 and 2.1) are counted (Table 3). Because our screen was configured to recover actives in two modes, agonist and antagonist, the high confidence percentage decreases

to 1.6% and 0.9% of the library for the agonist and antagonist modes, respectively. A substantial component of library activity was derived from compounds displaying lower potency and lower curve classes. The low potency actives were recovered because our qHTS included 9 μ M and 17 μ M library concentrations. Such actives are valuable for evaluating nascent SAR of chemical series. An additional factor contributing to the overall yield of actives was the occurrence of fluorescent compounds within the library. The follow up counterscreen identified 374 as fluorescent, thus indicating that at least 28% of library actives were fluorescent artefacts.

Having identified these compounds as active, what level of confidence should be applied to the activity assignments? Curve Classes 1 and 2 provide reasonable confidence of indicating a compound as active. Such curves show activity above background at two or more concentrations and have goodness of fit values (r^2) of at least 0.8. Indeed, the highest quality curves, Class 1, 2.1 and sometimes Class 2.2, are all used for the initial clustering of actives for our SAR analysis^{3, 4}. Class 3 curves are less certain indicators of an assay response, as these curves display activity at only one concentration, typically the highest tested, or have two or more points of activity but poor curve-fits ($r^2 < 0.8$). As well, the reclassified inactives mentioned above were categorized as Class 3. For the SIE-*bla* qHTS, 24 Class 3 compounds, all of which showed two or more points of activity, were retested and 83% confirmed as active compared to 90% of Class 1 and 2 actives. This result indicated that this category of Class 3 was a reliable predictor of activity, though not as well as Class 1 and 2. For profiling purposes, compounds associated with Class 3 curves are conservatively viewed as inconclusive until subsequent retesting confirms the initial result and/or the activity is supported by SAR derived from Class 1-2.1 actives.

Our retrospective analyses have shown that titration-based screening identifies more actives than by use of a single concentration and therefore decreases the number of false positives and false negatives^{3, 42}. Indeed, the retrospective analysis of the SIE-*bla* qHTS indicated that 35% to 71% of Class 1 and 2 qHTS actives were scored as false negatives when the 1.5, 7.7 or 17 μ M concentration and a 3 σ threshold of activity were used (Table 5).

Several factors contributed to the high percentage of false negatives. First, while sub-threshold activity may be detected at one concentration, other concentrations can show activity that exceed the threshold, particularly when multiple concentrations are fit to a curve (Fig. 6). Second, qHTS data can use activity thresholds lower than 3 σ and generate well-fit curves; for this assay, 30% was used as the activity threshold whereas 3 σ of background activity was 40%. Importantly, the qHTS method can make use of titration curves derived from the 460 and 530 readings in addition to ratiometric determination. This allowed us to identify compounds that showed sub threshold titration-response curves in the Ratio determination but exceeded threshold activity in the 460 measurement as well as assess potential cytotoxicity. In contrast, the individual 460 and 530 readings are difficult to use with single concentration data because of well-to-well variations in cell number and reagent dispenses, leaving only the ratiometric data to assess activity.

The SIE-*bla* qHTS did not identify two reported JAK inhibitors as active; Tyrphostin AG 490⁴³ and SD-1029⁴⁴ were screened but determined as inactive. However, the SD-1029 sample that was screened failed QC, having an incorrect mass, indicating it was likely degraded or a different compound. While it is difficult to estimate what percentage of SIE-*bla* qHTS was false negative without rescreeing the collection, we expect a value of less than one percent. Indeed, if the 92 'rescued actives' are considered as false negatives, the percentage of false negatives from the screen is about 0.8%.

A general degree of connectivity among the STAT, AP-1 and hypoxia signalling pathways can be estimated by comparing the number of SIE-*bla* actives shared with or distinct from AP-1-

bla and/or HRE-*bla* actives. Actives were defined as having curve class 1, 2.1 or 2.2 (with >50% efficacy) in the ratio reading and class 1-3 in the 460 nm reading. Using these criteria, 20% of the SIE-*bla* actives were active in both AP-1-*bla* and HRE-*bla* assays, 45% were active in either the AP-1-*bla* or HRE-*bla* assay, and 35% were active in neither. This result indicates significant overlap between the three assays, possibly because of the commonality in the assay technology used (beta-lactamase gene reporter), common components utilized in the pathways, or cross-talk among signalling components between pathways. In addition, many of the small molecule actives likely modulate multiple targets, whether closely related members within a protein family (for example, kinases) or structurally unrelated targets.

The SIE-*bla* qHTS found a number of selective compounds that will provide useful reagents for studying STAT signaling mediated by IL-6 and perhaps the biology of cervical cancer as well. Of the 32 active series identified from the qHTS, six were selective for SIE-*bla*, showing opposite or no activity in AP-1-*bla* and HRE-*bla* assays. Three other series were active in SIE-*bla* and AP-1-*bla* assays while five were active in SIE-*bla* and HRE-*bla* assays. While our comparative approach identified these series as STAT-selective, this interpretation should be held with some caution. Some selective actives may in fact be nonselective, because the promoter-gene reporters in the AP1-*bla* and HRE-*bla* may be less sensitive or efficient in recording transcriptional activation. Additional evidence of STAT selectivity can be sought using secondary assays involving JAK or STAT activation such as those described in ⁴⁵. The molecules reported here are likely to be important tool compounds for investigating this important pathway.

Conclusion

In a qHTS of SIE-*bla* cells, we identified small molecule modulators that enhance or inhibit an IL-6-activated reporter gene. From the primary screening data, we found 564 samples associated with high-quality Class 1 or 2 curves. SAR analysis partitioned these actives into 13 activator and 19 inhibitor series. Counter screens for activity in parental cells and activity in other beta-lactamase gene reporter assays indicated five series as selective for IL-6-stimulated STAT activity.

Experimental

Reagents

CellSensor[®] SIE-*bla* ME-180, CellSensor[®] AP-1-*bla* ME-180 and CellSensor[®] HRE-*bla* ME-180 cells were provided by Invitrogen. ME-180 cells were purchased from ATCC, pan Jak inhibitor (2-(1,1-Dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinolin-7-one, ²³ was obtained from Calbiochem, and recombinant human IL-6 was obtained from R & D Systems.

Cell culture

SIE-*bla*, AP-1-*bla* and HRE-*bla* cells were cultured in DMEM medium with 10% dialyzed fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 1mM sodium pyruvate, 25 mM HEPES pH 7.3, 100 U/ml penicillin, 100 ug/ml streptomycin, and 5 ug/ml of blasticidin at 37°C and 5% CO₂. ME-180 cells were cultured in McCoy's 5a Medium with 1.5 mM L-glutamine, 2.2 g/L sodium bicarbonate supplemented with 10% FBS, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 ug/ml streptomycin at 37°C and 5% CO₂.

Assay and qHTS

The protocol is detailed in Table 1. Cells were dissociated with 0.05% trypsin/EDTA and then washed and suspended in assay medium (OPTI-MEM[®] medium with 0.5% FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES pH 7.3, 100U/ml penicillin, and 100 ug/ml streptomycin). Cells were dispensed into 1536-well black clear bottom plates (Grenier Bio-One) at 5 ul/well using a Flying Reagent Dispenser (Beckman Coulter)⁴⁶ at either 2500 (AP-1-*bla* and HRE-*bla*) or 3000 cells/well (SIE-*bla*). Approximately 23 nL of compounds in DMSO were transferred to columns 5 to 48 of each 1536 well plate by a pin tool (Kalypsys)⁴⁷ either immediately (HRE-*bla*) or after overnight incubation (AP1-*bla* and SIE-*bla*) at 37 C. Following transfer, 1 uL of medium with or without agonist or agonist plus inhibitor was dispensed in the following format. Column 1, AC₉₀ of agonist- 100 uM CoCl₂ (HRE-*bla*), 160 pM EGF (AP1-*bla*), 300 pM IL-6 (SIE-*bla*). Column 2, AC₅₀ of agonist alone or with AC₉₀ of inhibitor: 50 uM CoCl₂ (HRE-*bla*), 30 pM EGF plus 100 nM PD153035, an EGF receptor antagonist, (AP1-*bla*), 40 pM IL-6 plus 125 nM pan JAK inhibitor (SIE-*bla*). Column 3, Medium only. Columns 4 to 48, AC₅₀ of agonist: 50 uM CoCl₂ (HRE-*bla*), 30 pM EGF (AP1-*bla*), 40 pM IL-6 (SIE-*bla*). The cells were centrifuged at 1000 rpm for 1 minute and incubated at 37 C for 5 hr (AP-1-*bla*, SIE-*bla*) or 17 hr (HRE-*bla*). One uL of LiveBLazer[™] FRET B/G detection mix (Invitrogen) was then added, the plates incubated at ambient temperature for 2.5 hrs, and fluorescence intensity was measured at 405 nm excitation and 460 and 530 nm emission using an Envision (Perkin Elmer) or Safire II (Tecan) detector. Screening was performed over several days in batches of 20 to 35 plates a day. To control for changes in activity during a run, each screen included several interspersed control plates where DMSO was transferred to the compound area of the plate (columns 5 to 48) by a pin tool.

For the pan-JAK inhibitor titration, sixteen 2-fold dilutions starting at 19 uM were made in duplicate, transferred to 1536 well plates by pin tool, stimulated with 27 pM IL-6 and assayed as above. For the IL-6 titration, 10,000 cells per well in 20 uL were dispensed in a 384-well black clear plate and treated with different concentrations of ligand in 20 uL and assayed as above. IL-6 was titrated in ten 3-fold dilutions beginning at 15 nM in 8 replicates.

Preparation of compounds

The assays were screened against 11,693 compounds collected from the following sources: Sigma-Aldrich LOPAC collection (1,280), Prestwick Chemical (1,117), TimTec (280), Pharmacopeia (3,000), Boston University Center for Chemical Methodology and Library Development (718), the NIH Molecular Libraries Small Molecule Repository (3,319), and the National Cancer Institute (1,979). Preparation and titration-response screening of compound libraries was performed as described⁴⁸. Follow-up compounds were plated as 24 two-fold dilutions beginning at 10 mM.

Data Analysis

Raw plate reads were normalized to the median values of the AC₅₀ agonist and AC₁₀₀ antagonist controls (460 nm/530 nm ratio and 460 nm readings) and the DMSO only wells (530 nm reading) present on each plate, and then corrected by applying a pattern correction algorithm using DMSO-only control plates at the beginning and end of each plate stack. For each compound, a titration response series was generated, curve-fit, and categorized as described³. Data were deposited in Pubchem (AID 446 and 357)

Derivation of SAR

Compounds with Class 4 curves in the 530 nm reading, Class 1-3 in the 460 nm reading, and Class 1 and 2 in the ratio determination were selected for SAR analysis. To identify active scaffolds, selected compounds were clustered using Leadscape[®] fingerprints and subjected to

hierarchical clustering using an average linkage method with a similarity threshold of 0.7. Maximal common substructures were then extracted from each cluster containing at least three compounds, which were used to search the entire screening collection to find all analogs including inactives. Compounds sharing a common scaffold formed a series. For SAR of activators, 209 actives were grouped yielding 14 clusters, 13 series and 25 singletons. For SAR of inhibitors, 209 actives were grouped yielding 35 clusters, 19 series and 72 singletons with $AC_{50} < 5 \mu M$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge Sam Michael and Carleen Klumpp for automation assistance, Paul Shinn and Adam Yasgar for compound management, Anton Simeonov for critical reading of the manuscript and Dr. Craig Thomas for helpful discussions. This research was supported by the NIH Roadmap for Medical Research and the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health.

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Fig. 1. SIE-bla assay response and qHTS performance

A) Dose-response of IL-6 and JAK inhibitor in SIE-bla cells. Cells were treated with shown concentrations of IL-6 (filled circles) or with 27 pM IL-6 with indicated concentrations of JAK inhibitor (unfilled circles) for 5 hrs at 37 C, loaded with detector dye for 2.5 hrs at ambient temperature, and fluorescence measured. The measured AC_{50} s of IL-6 and JAK inhibitor were 38 pM and 20 nM, respectively. B) Titration-response data for actives (red) and inactive (blue) samples from the qHTS. C) Signal to background ratio (S/B, filled circles) and Z' factor (unfilled circles) are shown for each plate of the qHTS.

Fig. 2. Determination of SIE-*bla* actives

Titration-response plots for 460 (upper graphs) or Ratio determinations (lower graphs) for compounds scored active in the 460 (left) or Ratio determinations (center), or originally scored inactive (right). Light blue planes indicate the threshold of activity, 30% above or below AC_{50} IL-6 stimulation. Dark blue lines indicate the curve fit of activity data (black) for each sample. Outlier data points (light grey) were masked and excluded from the curve-fit determinations.

Fig. 3. Titration-response plots of SIE-*bla* qHTS actives

Titration-response plots for Class 1 (A, D), Class 2 (B, E), and Class 3 (C, F) activators (A–C) and inhibitors (D–F) from the SIE-*bla* qHTS are shown. Blue lines indicate the curve fit of activity data (black) for each sample. Outlier data points (light grey) were masked and excluded from the curve-fit determinations.

Figure 4A

Activators

Figure 4B

Fig. 4. Structural classes of SIE-*bla* actives

Scaffolds for series of SIE-*bla* activators (A) and inhibitors (B) are shown. Grey shading indicate series that are likely fluorescent artifacts as detected by activity in parental cells lacking the beta-lactamase reporter.

Fig. 5. Comparison of qHTS and follow up potencies

Shown is a correlation plot of qHTS and follow up Log AC₅₀s of confirmed actives (filled circles) excluding two outliers (unfilled circles) marked 1 and 2 (top panel). The solid line indicates the fit calculated using linear regression while the dashed line indicates the axis of complete correlation. Graphs 1 and 2 (middle and bottom panels) indicate outlier compounds where inappropriate masking of qHTS data (circled filled squares) caused incorrect curve-fits of higher potencies compared to the follow up data (unfilled circles). Grey symbols are masked data excluded from the curve-fits.

Fig. 6. Titration response curves of qHTS actives scored as inactive at the 7.7 uM concentration Shown are fitted titration response curves (dark blue lines) of the qHTS actives (black filled circles) indicated as inactive using the 7.7 uM data (light blue filled circles) and a 3 σ activity threshold of 40% (red bars). Grey symbols are masked data excluded from the curve-fits.

Table 1
qHTS protocol for SIE-*bla*, AP-1-*bla* and HRE-*bla* assays.

Step	Parameter	Value	Description
1	Reagent	5 uL	Cells
2	Incubation	18 hrs	Cells adhere and acclimate
3	Library Compounds	23 nL	17 uM to 0.4 nM titration series
4	Reagent	1 uL	Medium +/- agonist +/- inhibitor
5	Incubation	5 hrs	Induce SIE reporter
6	Reagent	1 uL	Beta lactamase detection mix
7	Incubation	2.5 hrs	Cells load and cleave dye
8	Assay Readout	405/8 nm	EnVision

Notes

1 Black clear bottom plates, single tip dispense of 2500 (AP-1-*bla* and HRE-*bla*) or 3000 cells/well (SIE-*bla*) into all wells

2 37°C, 5% CO₂ incubator

3 Pintool transfer of library to columns 5–48

4 Single tip dispense for each reagent, Column 1, AC90 of agonist- 100 uM CoCl₂ (HRE-*bla*), 160 pM EGF (AP1-*bla*), 300 pM IL-6 (SIE-*bla*). Column 2, AC50 of agonist alone or with AC90 of inhibitor: 50 uM CoCl₂ (HRE-*bla*), 30 pM EGF plus 100 nM PD153035 (AP1-*bla*), 40 pM IL-6 plus 125 nM pan JAK inhibitor (SIE-*bla*). Column 3, Medium only. Columns 4 to 48, AC50 of agonist: 50 uM CoCl₂ (HRE-*bla*), 30 pM EGF (AP1-*bla*), 40 pM IL-6 (SIE-*bla*)

5 37°C, 5% CO₂ incubator

6 Single tip dispense of 0.6 uM CCF4-AM, 1 mg/ml Pluronic F127 surfactant, 3.5 % PEG 400, 2.6 % TR40, 2 mM probenecid, 0.1 % DMSO

7 Ambient temperature in the dark

8 460/25 nm and 530/20 nm emission filters

Table 2
Curve class distribution of qHTS actives identified by 460 and radiometric determinations.

	Class	460 & Ratio Active	460 Only Active	Ratio Only Active	Recovered Inactive	Total
Inhibitor	3	225	79	56	67	427
	2.2	116	26	12		154
	2.1	30	2			32
	1.2	32	4	1		37
	1.1	34				34
Activator	1.1	21				21
	1.2	21	1	4		26
	2.1	138	1			139
	2.2	85	31	5		121
	3	205	67	42	25	339
Total		907	211	120	92	1330

Table 3

Potency and curve class of qHTS actives.

AC50 (uM)	Activity	Curve Class					Total
		1.1	1.2	2.1	2.2	3	
<0.1	Activator	0	2	0	1	11	14
	Inhibitor	0	3	1	4	18	26
>0.1 to 1	Activator	17	17	4	16	98	152
	Inhibitor	14	6	1	12	64	97
>1 to 10	Activator	4	7	134	104	206	455
	Inhibitor	20	28	30	138	302	518
>10 to 100	Activator	0	0	1	0	24	25
	Inhibitor	0	0	0	0	43	43
Subtotal	Activator	21	26	139	121	339	646
	% library	0.2%	0.2%	1.2%	1.0%	2.9%	5.5%
	Inhibitor	34	37	32	154	427	684
	% library	0.3%	0.3%	0.3%	1.3%	3.7%	5.8%
Total	Total	55	63	171	275	766	1330
	% library	0.5%	0.5%	1.5%	2.4%	6.6%	11%

Table 4

Curve class and potencies of STAT SAR series for qHTS and follow-up studies.

STAT qHTS										AP-1 qHTS		% Active of STAT		HRE qHTS		Parental Counterseen				STAT Retest	
Series I	% Total Active	# Class 1&2 Active	# Total Active	# Tested	AC50 (uM) ²	% Active of STAT	# Class 1&2Active	# Total Active	% Active of STAT	# Class 1&2Active	# Total Active	% Active of STAT	# Active	# Tested	% Active	# Active	# Tested	AC50 (uM) ³	Bioactive		
1	14%	3	8	58	3-4	75%	4	6	50%	3	4	0%	0	4	100%	4	4	5-30	adenosine		
2	20%	7	29	142	0.01-1	17%	1	5	17%	3	5	17%	5	29							
3	26%	5	9	35	3-6	44%	3	4	67%	5	6	25%	1	4	100%	4	4	6-40			
4	23%	3	3	13	4-10	33%	1	1	100%	3	3	100%	3	3	100%	3	3	10-20			
5	21%	5	7	33	1-10	57%	4	4	57%	2	4	25%	1	4	100%	4	4	4-20			
6	9%	3	7	74	0.1-3	0%	0	0	0%	1	0	14%	1	7							
7	10%	2	5	48	0.2-0.3	0%	0	0	0%	0	0	0%	0	5							
8	38%	219	335	887	0.03-4	83%	226	277	73%	225	245	96%	322	335							
9	24%	4	4	17	6-10	100%	4	4	100%	4	4										
10	63%	5	5	8	1-6	80%	1	4	60%	3	3	40%	2	5	100%	5	5	3-10	ellipticine		
11	50%	4	8	16	2-7	50%	0	4	75%	3	6	63%	5	8							
12	100%	3	3	3	5-10	100%	3	3	33%	0	1	100%	1	1	100%	1	1	30			
13	78%	7	7	9	2-5	57%	3	4	100%	6	7	0%	0	5	100%	5	5	3-10	doxo/dauno		
14	100%	2	2	2	0.6-3	50%	0	1	100%	1	2							protease inh			
15	100%	2	2	2	4-8	100%	2	2	100%	2	2	0%	0	2	100%	2	2	5-6	CDK inh		
16	100%	2	2	2	4	100%	1	2	100%	2	2	0%	0	1	100%	1	1	10	piperlongumine		
17	56%	3	5	9	3	20%	0	1	60%	1	3										
18	13%	26	68	509	0.3-9	50%	18	34	56%	25	38	8%	5	61	100%	1	1	20			
19	100%	2	2	2	4-8	100%	2	2	50%	1	1										
20	100%	5	5	5	0.2-1	100%	5	5	100%	5	5	0%	0	2	50%	1	2	0.6	emetine		
21	67%	3	6	9	0.09-1	0%	0	0	83%	5	5							ouabain			
22	45%	3	5	11	3-10	40%	2	2	60%	3	3	0%	0	1	100%	1	1	6	menadione		
23	36%	11	24	66	1-10	83%	10	20	46%	6	11	0%	0	1	100%	1	1	10	PK inh		
24	100%	4	4	4	3	0%	0	0	0%	0	0	0%	0	1	100%	1	1	10			
25	50%	3	4	8	1-10	50%	0	2	100%	2	4										
26	100%	5	10	10	0.1-3	60%	1	6	100%	5	10										
27	67%	3	4	6	8-10	50%	2	2	50%	1	2	0%	0	1	100%	1	1	20			

		STAT qHTS				AP-1 qHTS				HRE qHTS				Parental Counterseen				STAT Retest			
Series ¹	% Total Active	# Class 1&2 Active	# Total Active	# Tested	AC50 (uM) ²	% Active of STAT		# Class 1&2Active	# Total Active	% Active of STAT		# Class 1&2Active	# Total Active	% Active	# Active	# Tested	% Active	# Active	# Tested	AC50 (uM) ³	Bioactive
						Active	STAT			Active	STAT										
28	65%	11	13	20	1–8	69%		8	9	100%		13	13	0%	0	3	100%	3	3	10–20	
29	100%	5	5	5	4–8	80%		2	4	100%		5	5	0%	0	2	100%	2	2	20–30	
30	3%	8	13	436	1–4	100%		12	13	77%		7	10	38%	5	13					
31	83%	5	5	6	0.6–2	100%		5	5	100%		5	5	0%	0	1	100%	1	1	30	camptothecin
32	100%	3	6	6	0.04–0.4	0%		0	0	83%		3	5								RAR inh

¹Series 1–13 are activators and 14–32 are inhibitors.

²AC50 range for Class 1 and 2 compounds only.

³AC50 range for all Class 1–3 compounds. Grey shading indicates opposite activity

Actives Identified at a Single Concentration and 3 σ Threshold Compared to qHTS Class 1 and 2 Actives

Table 5

μ M	Total Positives	Correlated Positives ¹	False Negatives	% False Negatives	% False Negatives ²	False Positives	% False Positives ³
1.5	180	162	403	71%	71%	18	10%
7.7	365	363	201	36%	36%	2	1%
17	138	137	74	35%	35%	1	1%

¹Positives identified both by qHTS and at indicated concentration.

²Percentage of qHTS positives scored as inactive at indicated concentration.

³Percentage of total positives identified at the indicated concentration that were qHTS inactives.