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Channel Interactions and Robust Inference for Ratiometric β -lactamase Assay Data: a Tox21 Library Analysis.

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Abstract

Ratiometric β -lactamase (BLA) reporters are widely used to study transcriptional responses in a high-throughput screening (HTS) format. Typically, a ratio readout (background/target fluorescence) is used for toxicity assessment and structure-activity modeling efforts from BLA HTS data. This ratio readout may be confounded by channel-specific artifacts. To maximize the utility of BLA HTS data, we analyzed the relationship between individual channels and ratio readouts after fitting 10,000 chemical titration series screened in seven BLA stress-response assays from the Tox21 initiative. Similar to previous observations, we found that activity classifications based on BLA ratio readout alone are confounded by interference patterns for up to 85% (50 % on average) of active chemicals. Most Tox21 analyses adjust for this issue by evaluating target and ratio readout direction. In addition, we found that the potency and efficacy estimates derived from the ratio readouts may not represent the target channel effects and thus complicates chemical activity comparison. From these analyses we recommend a simpler approach using a direct evaluation of the target and background channels as well as the respective noise levels when using BLA data for toxicity assessment. This approach eliminates the channel interference issues and allows for straightforward chemical assessment and comparisons.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website and includes further data description, detailed data tables, and assay-specific response information.

Explicit analysis of all readouts produced by HTS bioassays may help chemical comparison and design of safer alternatives.

Keywords

β -lactamase; qHTS; *in vitro*; concentration-response; Tox21

Introduction

In vitro reporter assays are efficient and cost-effective tools for chemical toxicity assessment. Although numerous reporter technologies are available, β -lactamase (BLA) based assays have increased in popularity over the past decade due to their sensitivity, versatility, and user-friendly format.¹⁻³ BLA assays are capable of detecting as few as 100 or 15,000 BLA molecules following 16 h or 1 h incubations, respectively.⁴ Neither BLA nor any of the associated assay reagents is toxic at concentrations below 100 μ M.^{2,5} In addition, BLA reporters can be easily customized, miniaturized, automated, and standardized for high-throughput screening (HTS).⁶⁻⁸ Therefore, many BLA assays were developed for HTS and quantitative high-throughput screening (qHTS) formats.^{2,5,9-12} These qHTS data are widely used for chemical assessment, prioritization, and toxicity model development.¹³⁻¹⁶

The β -lactamase reporter system relies on truncated Temoneira-1 β -lactamase enzymes that can efficiently cleave β -lactam-containing molecules.^{1,3} The cell lines express the ligand binding domain (LBD) of the protein under investigation fused with Galactin 4 (GAL4) DNA binding domain and contain a BLA reporter gene under the transcriptional control of an upstream activator sequence (UAS). If a chemical binds to the LBD of the protein under investigation, then the GAL4-DNA-protein-LBD translocates to the nucleus where it binds to the UAS and causes BLA transcription.¹⁷ Thus, conditions that activate the protein of interest should induce BLA transcription. Thus, conditions that induce target gene transcription should induce BLA transcription. The system makes it possible to monitor BLA-coupled transcription (please refer to Figure 1), localization, or protein binding with the help of β -lactam-containing 7-hydroxycoumarin-3-carboxamide and fluorescein dye bridged by cephalosporin (CCF2/4) and its acetoxymethylated analogue (CCF2/4-AM). The CCF2/4-AM is lipophilic and non-fluorescent. It readily traverses cell membranes without

damaging cells. Once CCF2/4-AM enters the cell, endogenous esterases cleave CCF2/4-AM to form negatively charged CCF2/4. CCF2/4 is trapped inside the cell and can be detected by fluorescence resonance energy transfer (FRET) at $\lambda = 530$ nm (green, channel 1, i.e., the background readout). When present in cytosol, β -lactamase cleaves CCF2/4 into two fluorophores, replacing the green FRET with blue fluorescence ($\lambda = 460$ nm, channel 2, i.e., the target gene readout). The ratio of blue fluorescence over green fluorescence is typically used to control for assay interference signals, such as well-to-well variations in cell number, cell size, substrate loading, and fluorescence signal intensity.^{2,4,5} Thus the accepted methodology is to infer chemical activity from the ratio readout after controlling for cytotoxicity, and auto-fluorescence interference with appropriate counter screens.¹⁸

Recently, BLA technology was used in the U.S. Federal Tox21 collaboration to screen thousands of chemicals for cell stress and nuclear receptor effects.^{17,19,20} The data are widely used for chemical prioritization and assessment.^{19,21-30} However, in a large chemical library the ratio readout may be confounded by channel interference patterns as over 40% of chemicals classified as active in ratio readout were not considered as active after investigating the effect in the target channel.¹⁸ To account for this issue, most Tox21 analyses adjusted the activity classification (i.e., active $\rightarrow$ inconclusive) if the target channel effect does not respond in the same direction as the ratio readout.^{17,18,31} The results of the analysis are available online at <https://tripod.nih.gov/tox21/assays/>, and <https://sandbox.ntp.niehs.nih.gov/tox21-activity-browser/>. However, the overall direction of response can be obscured when responses are not monotonic. The relationship between potency and efficacy (AC_{50} , E_{MAX}) estimates in target and ratio readouts have not been reported. Since these estimates and activity designations are commonly used in chemical assessment, a better understanding of the channel discrepancies would further help with the use *in vitro* data in chemical assessment.

In this paper, we investigate relationships between concentration-response patterns observed in the three readouts of stress response BLA reporter assays from the Tox21 qHTS data set. Specific attention is given to the effects that background signal changes have on the ratio readout. The AC_{50} and E_{MAX} parameter estimates and activity classification based on ratio and target channel are compared; the differences in these estimates and reasons for the differences are explored in detail. Since the ratio and target channel readouts are shown to frequently produce substantially different inference results, we suggest explicitly considering channel 2 and channel 1 concentration response curves (CRC) when comparing chemicals. These considerations can help avoid inconsistent chemical comparisons and retain majority of the data in diverse *in vitro* data sets.

Methods

Assay Data

Tox21 partners developed and miniaturized a series of BLA *in vitro* assay to assess chemicals' effect on cell's stress defense system.^{17,19,20} This qHTS robotic platform was used to assess stress-related effects of a large chemical library that included plasticizers, pesticides, food additives, antimicrobials, and discontinued pharmaceuticals.^{13-16,32} Normalized data for seven Tox21 stress-response BLA assays (Table 1) and CellTiter-Glo

(Promega) viability counter screen⁶ were obtained from the NIH web portal: <https://tripod.nih.gov/tox21/assays/> (assessed by February 15, 2017). The final data set contained 9,451 – 10,444 titration series experiments per assay (Table 2). All titration series included at least 3 replicates with 15 concentrations each. However, triplicate titration series for some substances were repeated in the database because multiple Tox21 partners provided the same chemical for screening.¹⁸ Since this study focused on comparing the responses in multiple channels for a triplicate set of titration series, triplicate titration series for the same chemical from different sources were analyzed separately. The data were standardized with two-fold normalization algorithm to correct for cross-plate and between-plate differences.³¹ The standardized data were normalized to positive control (PC) and vehicle control (VC) as described by Equation 1 (1)

$$\% \text{ Activity} = \frac{F_{CD} - F_{VC}}{F_{PC} - F_{VC}} \times 100 \quad (1)$$

For all assays, F_{CD} is fluorescence reading in the well with compound of interest. For ratio and channel 2 readouts, F_{PC} is the median readout from the wells exposed to the positive control chemical and F_{VC} is the median fluorescent reading from the DMSO-only wells. For channel 1 and viability readouts, F_{VC} is the median fluorescence from the DMSO-only wells and F_{PC} is set to zero to account for the full range of possible fluorescence values.

Background variability for BLA assays was estimated as the median absolute deviation (MAD) across DMSO control plates (Table 1). The data for DMSO control plates was obtained directly from NIH (the data are too large for supplementary materials but are available upon request). Results of auto-fluorescence counter screens for BLA assay were also obtained from the literature.¹⁸ A chemical was considered auto-fluorescence at blue or green wavelengths corresponding to channel 2 and channel 1 activity if it produced fluorescence readings above 20% of positive control in the appropriate counter screen, and was active in at least one assay.

Concentration-Response Analysis

The titration series qHTS format allowed for the derivation of concentration-response relationships, which increase confidence in HTS results, and reduces false positive and false negative rates.²⁰ In order to adequately capture the diverse array of concentration-response patterns, monotonic and non-monotonic concentration-response curves were fit to all titration series in the seven Tox21 stress response assays. The analysis was conducted in R statistical environment³³ using a likelihood based optimization routine similar to the TCPL (ToxCast Pipeline for High-Throughput Screening Data).³⁴ Briefly, three distinct models were fitted to each titration series, namely, 1) a constant model, 2) a constrained four-parameter Hill model and 3) a constrained seven-parameter gain-loss model. The hill and gain-loss models were fit in positive and negative directions, producing a maximum of five models. Hill and gain-loss models were fit only if 2 consecutive median activity responses exceeded an assay specific activity threshold (Table 1) or if a single median activity response in a titration series exceeded twice that same threshold in the direction of model fit. The approach allowed to capture small responses while minimizing model overfitting. The

optimization routine was allowed to extrapolate the E_{MAX} parameter up to 33% above the maximum observed median response and extend the CRC beyond the concentration range as long as the AC_{50} estimate was within the concentration range. The algorithm used T-distribution loss function that is robust to noisy data.³⁴ Once all models allowed by the threshold requirements were fit, the model that fits each readout of the titration series best was selected with sample-size corrected Akaike information criteria (AIC_C).³⁵ A chemical was considered an activator in a readout if its titration series was best described by hill or gain-loss model. Since BLA activity measured by target and ratio readouts was designed to identify gene activation as signal increase, all constant or decreasing CRC shapes were considered inactive in these readouts. While other responses in target and ratio readouts are possible, their biological significance is less clear and should be studied separately from the channel interference question. However, since background and viability readouts can indicate both increase or decrease in cell number and function, both increased and decreased signals in these readouts were considered meaningful.¹⁸ Thus, in addition to regular activator classification, titration series with background or viability readouts best described by an inverted hill or gain-loss mode were considered repressors.¹⁸ All titration series best described by a constant model were considered inactive. The concentration-response models are fit on a logarithmic concentration scale. Thus, AC_{50} refers to base 10 log of AC_{50} ($\log_{10}[AC_{50}]$) in $\log_{10}$ M units throughout the paper.

Comparing Channel Output

The best concentration-response models for each of the four readouts from a titration series were selected based on AIC_C , as described above. Thus, each titration series-assay combination produced 4 CRCs. The chemical used to generate that titration series was then classified as activator, repressor or inactive in each of the 4 readouts (target channel, background channel, target/background ratio, and viability counter-screen) based on the winning CRC model as described above. The discrepancies between target and ratio CRC, and the effects of background CRC on these discrepancies, were analyzed. Specifically, the differences in activity classification (hitcall) and common CRC parameters (AC_{50} and E_{MAX}) were calculated. Herein, the chemicals that were activators in the ratio readout but inactive in the target readout are termed ratio false positive (RFP); chemicals concluded to be inactive in the ratio readout but active in the target readout were termed ratio false negatives (RFN). The RFN and RFP categories should not be interpreted as false positive or false negative results; they merely indicate that the titration series for that chemical appeared inactive or active in the ratio channel with or without corresponding target channel signal, respectively. The conventional signal interference sources such as auto-fluorescence are assessed separately. Chemicals that are classified as active in both target and ratio readouts, are referred to as channel concordant active (CCA). RFP channel 2 titration series and RFN ratio titration series were refit with a lower threshold (9% activity) to avoid labeling chemicals as RFP or RFN due to minor activity difference. In this way, RFN and RFP designations are conservative and are largely independent of the selected activity cutoff. In addition, CCA titration series were assessed for large differences between target and ratio potency (AC_{50}) and efficacy (E_{MAX}) estimates (see Figure S1 for details). Chemicals with ratio CRCs that overestimated or underestimate channel 2 efficacy by over 30 % of positive control, were termed “efficacy underestimation of concern” (EUC) and “efficacy

overestimation of concern” (EOC), respectively. Chemicals with ratio CRC overestimated or underestimate channel 2 potency by over 0.5 log concentration units, were termed “potency overestimation of concern” (POC) and “potency underestimation of concern” (PUC), respectively. It might be important to note that higher potency corresponds to lower AC_{50} value. All non-constant CRC fit results and associated chemical classifications are available in Table S6.

In order to better understand the nature of chemicals with discrepancies between channel 2 and ratio readouts, we clustered these chemicals using Tanimoto similarity coefficient.³⁶ A relaxed cutoff value of 0.7 was selected to allow for complete or inclusive clusters.^{37,38} The structural clustering was performed with ChemmineR package in R statistical environment.³⁹ The results of chemical cluttering are available in Table S5.

Results

Agreement between Channel 2 and Ratio Readouts.

CRCs were fit to all titration series in the seven BLA stress-response assays. The inference from target and ratio readouts for every chemical and assay were compared. Across all 7 assays, 2087 RFP, 545 EUC, 539 EOC, 371 PUC, 68 POC, and 190 RFN chemicals were identified. Together these six categories identified chemical with potentially concerning differences between channel 2 and ratio CRCs in every assay. To give an intuitive description of the CRCs that fall into each of the six categories, Figure 2 illustrates a representative curve for each (Figure 2 A-F), as well as representative examples of CRCs with minor differences between ratio and channel 2 CRCs (Figure 2 G-H). These six curve classifications are important because they identify chemicals with large discrepancies between target and ratio readouts. Chemicals in RFP, EOC, and POC categories that make up 35, 9, and 1 % of all active chemicals (Table 2) and will appear less active in the ratio readout than target channel readout. Similarly, chemicals in RFN, EUC, and PUC categories that make up 3, 9, and 6 % of all active chemicals (Table 2) appeared more active in the ratio than target channel readouts. When comparing chemical activity, responses that differed in target channel activities appeared similar in the ratio readout (e.g. Figure 2A and 2E). Alternatively, chemicals with very similar target readout CRCs appeared different in the ratio readout (e.g. Figures 2C and 2D).

It was clear from Table 2 that many substances in the qHTS data sets produce different results in the target channel and ratio readouts. Furthermore, it was not possible to distinguish RFN or RFP chemical from channel concordant one based on AC_{50} and E_{max} estimates alone (Figure S2). Consequently, background channel, cytotoxicity, and chemical-specific effects target-ratio readout concordance was explored next.

Background Channel Effects on Ratio Readouts

The direction of background readout response plays a large role in the discrepancies between channel 2 and ratio CRCs. Ninety nine percent of RFP, 98% of EOC, and 86% of PUC chemical-assay pairs were classified as channel 1 repressors (Figure 3). The EUC, POC, and RFN show increased frequency in both activator and repressor background

channel responses as compared to CRCs not in any of the categories of potential concern (Figure 3).

RFP cases appear when a chemical produces ratio signal in the absence of channel 2 increase (Figure 2A). Since the phenomenon is associated with a repression signal in the background readout, it is not surprising that background and ratio CRC parameters were associated. In general, channel 1 potencies were correlated with ratio potency in RFP chemicals (Figure S3A, $R=0.78$). This correlation was high for all assays, with *ap1* assay showing lowest R of 0.55, followed by *p53* ($R=0.67$), *are* (0.71), and the correlation coefficient for the remaining 4 assays exceeded 0.83 (Table S2). The relationship between background and ratio efficacies is more complex. The raw correlation is only 0.43 and the relationship is clearly not linear. However, after accounting for assay-specific positive control values and log-transforming the data, all but two assays (*nfkb* & *esre*) showed correlations between background and ratio efficacy between 0.71 and 0.86 (Table S2, Figure S3B).

EOC (e.g. Figure 2C) and PUC (e.g. Figure 2D) examples arise when a chemical represses background activity at concentrations above those that induce target channel activation. In these cases, the ratio readout may be amplified by the decrease in background signal (Figure 2C). As a result, the ratio readout is shifted and amplified with respect to the target channel readout. In the extreme case (Figure 2D), a 2nd ratio increase created purely by the decreasing channel 1 fluorescence dominates the ratio readout and is picked up at the major CRC. It is clear that the EOC and PUC categories are not mutually exclusive just like AC_{50} and E_{MAX} estimates from the hill equation are not independent. An increase in E_{MAX} shifts AC_{50} estimated in the ratio readout to the right compared to the corresponding target channel readout. Not surprisingly, 217 titration series are flagged in both EOC and PUC categories.

EUC, POC, and RFN, chemical show mixture of background channel response patterns. However, 88, 79, and 73 % of titration series in these categories show minimum background channel CRC activity above that of positive control used in these assays (Table 1). It is obvious that if the sample channel 1 fluorescence is higher than the channel 1 fluorescence of the positive control, the ratio readouts will be lower than the corresponding target readouts (e.g. Figure 2B, 2E), or disappear entirely (e.g. Figure 2F). Furthermore, the concentration at which the changes in background fluorescence are observed again important. While the remaining 125 cases exhibit repressor background activity with efficacy below the channel 1 controls, the decrease in channel 1 fluorescence occurs at higher concentration than the increase in channel 2 fluorescence for 114 of them. Thus, compared to channel 2 signal, the ratio signal is still damped at concentration associated with target channel activity (EUC category). Consequently, both magnitude of the channel 1 response and its location relative to target channel response may influence the magnitude of the ratio readouts. When the difference between ratio and target channel readouts is large or the target channel signal is weak, the titration series will appear as RFN instead of EUC. As pointed out above, the efficacy and potency estimates are not independent. Thus, the decrease in ratio efficacy leads to increase in ratio potency relative to the corresponding target channel estimates (e.g. Figure 2E). Consequently 63 % of POC titration series are also classified as EUC.

Channel 1 and Cytotoxicity

Not surprisingly, the analysis so far suggested that the background channel activity has a strong influence on the relationship between target and ratio CRCs and on the activity classifications. It is thus important to properly interpret change in background readouts. Concentration-dependent changes in background fluorescence are often associated with cytotoxic effects because CCF2/4 reagents rapidly leak from impaired cells.^{4,22} However, previous studies suggested that the relationship between the loss of background signal and the loss of cell viability is not always clear.^{18,22} To assess the relationship, we compared the background activities with the multiplexed Cell Titer Glo viability counter that measures the intracellular ATP levels that are proportional to the number of functional cells¹⁷. Overall, only 50.7 % (3482/6866) of background repressors are considered cytotoxic based on the Cell Titer Glo viability screen. While, multiple cytotoxicity and cell viability screens can be used together to capture different pathways and markers of cytotoxicity, a review of BLA assay-development literature suggested that BLA activity may decrease background fluorescence by converting green cells to blue without affecting the cell number.⁴⁰⁻⁴² As BLA breaks down CCF2/4, coumarin concentration increases, and the cell changes color from green to blue.^{40,41} The change in color corresponds to increase in 460 nm fluorescence, and may lead to decreased background fluorescence because fewer cells are now green, while the total number of cells remains constant.^{40,41} Thus, strong BLA upregulation is expected to turn many cells blue and may decrease fluorescence at 530 nm without cytotoxicity. Indeed, 67.6 % of chemicals that induce channel 2 response with E_{MAX} above 50 % of positive control activity, and AC_{50} below $-4.5 \log_{10} M$ units repress background activity. In addition, all positive control chemicals repress background activity (Table 1). In essence, for these chemicals, the mathematical ratio of target to background fluorescence counts the same effect twice – once in the numerator and once in the denominator.

Chemical Auto-fluorescence, Structure, and Activity

We evaluated chemicals in each of the 6 categories of potential concern for auto-fluorescence and structural similarities. Overall, 43 and 19 were found to be auto-fluorescent in blue and green auto-fluorescence counter screens, respectively. Of the 43 blue auto-fluorescent chemicals, 13 were active in all 7 assays and 19 were active in 3 or fewer only. Only 1 of the 19 green auto-fluorescent chemicals showed activity in more than 3 assays. Every category of potential concern contained a 0-4 % CRCs produced by auto-fluorescent chemicals (Table S3). However, 21.3 % CRCs classified as EUC, and 23.5 % of CRCs classified as POC were auto-fluorescent in channel 2. Auto-fluorescent chemicals induce strong positive target response and constant or positive background response as well. Thus, as described in the readout interaction section above, ratio efficacy estimates from ratio CRCs appear lower and potency efficacy from ratio tend to appear higher than the corresponding target channel estimates. In addition, RFN CRCs has the highest fraction of green auto-fluorescent compounds (6.8 %). Increase in green (background) fluorescence created by these substances decreases the ratio readout compared to the target readout leading to higher likelihood of inactive classification in the ratio readout.

While auto-fluorescent chemicals do not appear active in every assay, they do appear active in both ratio and channel 2 readouts. Thus, counter screens should be used to identify auto-

fluorescent chemicals. However, when the auto-fluorescent counter-screen is not available, auto-fluorescent chemicals may be flagged by ubiquitous increase in all readouts. That is, unlike BLA-inducing substances that must turn green cells blue to produce strong target channel signals, auto-fluorescent chemicals do not need to affect the green cells at all.

Finally, we attempted to find common structures among chemicals identified in any of the potential concern categories. Across all assays, 1318 distinct chemicals produced the 3534 titration series that fall in any of the 6 categories of potential concern (Table S6). Of these, 716 chemicals produce a CRC that falls in a category of potential concern in one assay only and 302 produce CRCs of potential concern in 4 or more assays (Table S6). Structural clustering showed that the 1318 chemicals were structurally diverse and formed 1019 distinct clusters (Table S5). Only 2 clusters contained 10 or more chemicals. The largest cluster (n=15) was composed of imidazolium salts. All but 2 imidazolium salts on the list were flagged in the RFP category. The majority of the flags came from *hre*, *hes*, and *p53* assays (31/45 CRCs). The 2nd largest cluster (n=10) contained quaternary ammonium salts. The ammonium salts were again primarily flagged as RFP chemicals and all 10 of them were designated as RFP CRCs in *p53* assay. These salts induce cytotoxicity and a channel 1 fluorescence decrease at high concentration, creating a phantom ratio spike. Their effects can be easily identified with cytotoxicity counter-screen

Discussion

This investigation of the relationships between three different readouts from the BLA stress response assays observed in the Tox21 collaboration data showed that a) the response in target and ratio readouts could differ substantially for a large number of chemicals in the data set and b) the direction of background response has a substantial effect on the response differences seen in the target channel and ratio readouts. Commonly observed decreases in background fluorescence are associated with a) apparent ratio activity in absence of any target channel activity, b) underestimated potency, and/or c) overestimated efficacy CRC parameters. Thus, for large chemical collections, such as the Tox21 library, the potency and efficacy estimates based on the ratio readouts from BLA assays cannot be easily compared without explicitly accounting for target and background channel activities.

The analysis of channel 2 / channel 1 ratio is the gold standard for BLA assays as the differences in channel 1 fluorescence may be used to control for the well-to-well differences in cell and substrate loading.^{2,4} in the BLA-CCF2/4 system, channel 1 fluorescence indicates that the CCF2/4-AM substrate has been successfully absorbed into cytosol and cleaved to CCF2/4. The well-to-well differences in cell and dye loading are expected to be random; while concentration-dependent channel 1 trends are often interpreted as cytotoxic effects.^{4,22} However, previous analysis of Tox21 BLA assays⁴³, and our analysis of channel 1 and Cell Titer Glo viability counter screen shows that, in the case of Tox21 data, loss of channel 1 fluorescence did not correlate well with loss of cell viability as indicated by ATP concentration. Furthermore, literature review suggested that the channel 1 fluorescence may decrease due to BLA activity and without cytotoxic effects.⁴⁰⁻⁴²

The potential for co-dependence between BLA-activity and the background fluorescence complicates chemical comparisons when background readouts do not share a common CRC shape. In the diverse Tox21 library, ratio CRC parameters often indicate that two chemicals with very different target readouts are similar or show large differences between chemicals with similar target readout behaviors. Thus, when researchers aim to incorporate as much data as possible, as is usually the case in chemical assessment, prioritization, and modelling projects, additional processing is necessary to ensure that the similar target responses are indeed grouped together. For example, Tox21 partners NCATS and NTP adopted an approach to active calling by adjusting the activity classification (i.e., active -> inconclusive) if the target channel effect does not respond in the same direction as the ratio readout.^{18,31,43} We suggest comparing chemicals based on the target channel readouts when background readouts are not similar across the chemicals under investigation. Previous analysis preferred fitting CRCs to the ratio readouts due to their relatively low noise levels.^{18,31,43} However, the robotics platform⁶ and extensive pattern correction procedures³¹ employed in Tox21 pipeline result in a final data set with low well-to-well variability in ratio and target readouts (Table 1). The authors did not experience any meaningful difference in the quality of CRC fits between target and ratio readouts. While cytotoxicity interference may remain a concern, especially in inhibition-type assays, cytotoxic compounds can be screened with the multiplexed viability counter screen¹⁸ or through a burst effect analysis.²⁴ Given the diversity of background and target responses in Tox21 data set, explicit analysis of target readouts helps identify similar response patterns in the data. More generally, direct comparison of CRC parameters is meaningful only when the overall CRC shapes are similar.⁴⁴ It is then natural to compare parameters from the compound ratio readouts only when the shapes of all component CRCs are similar.

When difference in well-to-well cell and substrate loading present a larger concern, a sequential regression analysis can be applied to separate the concentration-dependent effects on background from the random well-to-well differences. The random differences are captured by first regressing the concentration dependent effects from the titration curve and then creating a new ratio metric that normalizes target readouts to the residuals of the background CRC. While the approach is straight-forward and has been implemented in microarray analysis with lowess regression⁴⁵, it is not directly applicable to Tox21 data. The current Tox21 data sets are normalized to positive and negative controls before plate correction algorithms are applied. Thus, the pattern corrected fluorescence data needed to separate the concentration-dependent channel 1 changes from the ratio readouts are not readily available.

Conclusion

The BLA technologies and Tox21 data provide a robust and rich chemical-induced transcriptional factor activation/inhibition data set useful to further elucidate chemical activity pathways. However, the combination of non-monotonic concentration-response patterns in the target readouts and the potential for BLA-dependent background effects convolute ratio readout interpretation and effect comparisons. Specifically, large fractions of chemicals found active in the ratio readout of BLA assays analyzed here did not show any target channel activity. In addition, chemicals with similar target readout activity differed

substantially in their ratio CRCs due to the effects of the background readout. The ratio readout remains the gold standard for activity assessment when both target and background readouts similar CRC shapes for all substances under investigation. In practice, large scale analysis of diverse chemical libraries, such as Tox21, require explicit consideration of target and background readouts, when BLA assay results are intended to be used directly for chemical toxicity evaluation. The direct analysis of target readout in combination with appropriate cytotoxicity, auto-fluorescence, and background noise counter screens makes it easier to identify chemicals with similar biological response patterns. Since chemical assessment and modelling efforts usually rely on the CRC parameters and activity classifications for accuracy, target channel analysis aims to help clarify and simplify toxicity modeling based on *in vitro* qHTS data from BLA assays. The analysis in the manuscript are limited to Tox21 β -lactamase stress-response assays. Thus, while channel interference patterns should be considered in other ratiometric assays, the conclusions may differ for other types of β -lactamase assays (e.g., nuclear receptor assays) and other ratiometric technologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AC₅₀	log ₁₀ of the activity concentration that corresponds to 50% of maximum % activity for the chemical CRC; a.k.a. potency.
BLA	β -lactamase
CC	channel concordant
CCA	channel concordant active
CCF2/4	β -lactam-containing 7-hydroxycoumarin-3-carboxamide and fluorescein dye bridged by cephalosporin
CRC	concentration-response curve
E_{MAX}	the maximum response estimated from the CRC for a chemical; a.k.a. efficacy
EOC	efficacy overestimation of concern
EUC	efficacy underestimation of concern

GAL4	Galactin 4
LBD	ligand binding domain
POC	potency overestimation of concern
PUC	potency underestimation of concern
RFN	ratio false positive
RFP	ratio false positive
qHTS	quantitative high throughput screening
UAS	upstream activator sequence

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Figure 1:

Set up and mechanism of β -Lactamase (BLA) assays. Broadly, the left panel shows the cell before BLA transcription is upregulated, while the right panel shows the BLA activity when stimulated by chemical exposure. Specifically, A) Cell culture is grown and exposed to test chemical. B) Chemical (“the star”) enters the cell and activates target transcription factors (TF). C) TF activates BLA transcription through target promoter and thus results in BLA production. D) CCF2/4-AM reagent is added. E) CCF2/4-AM is absorbed into the cell. F) CCF2/4-AM is converted to CCF2/4 by the cytoplasmic esterases and is trapped in the cell. G) Esterase activity is assessed by CCF2/4 green FRET fluorescence at 530 nm. H) BLA cleaves CCF2/4. G) BLA activity is measured by fluorescence at 460nm. In summary, the chemical effect on the target TF is quantitatively measured by 460nm fluorescence (channel 2) that indicates BLA activity and substrate loading is measured by FRET fluorescence at 530 nm (channel 1). The cell color represents the expected fluorescence effects.

Figure 2:

Titration series and concentration response curves (CRCs) representative of: A) an RFP chemical (e.g. CAS# 639-58-7; Tox21 ID: 303984); B) an EUC chemical (e.g. CAS# 207-08-9, Tox21 ID: 200721); C) an EOC chemical (CAS# 14866-33-2, Tox21 ID: 201028); D) a PUC chemical (e.g. CAS# 100-56-1, ; Tox21 ID: 300878); E) a POC chemical (CAS# 602-38-0, Tox21 ID: 202886); F) a RFN chemical (CAS# 50-65-7, Tox21 ID: 300749); G-H) CCA chemicals with non-monotonic and monotonic CRCs that are not flagged in any of the categories of potential concern (G: CAS# 1024009-92-7, Tox21 ID: 303340; H: CAS# 103577-45-3, Tox21 ID: 110184_1).

Figure 3:

The number of channel 1 activator, inactive, and repressor chemicals for ratio false positive (RFP), efficacy underestimation of concern (EUC), efficacy overestimation of concern (EOC), potency underestimation of concern (PUC), potency overestimation of concern (POC), ratio false negative (RFN), and all other active substances (All Other). The numbers above bars indicate % channel 1 activators, repressors, and inactive curves within each category.

Table 1:

Tox21 β -Lactamase stress response assays, their targets, positive control chemicals, and background variability.

Assay Information		Positive Control (Fold Change ^a)			Target Gene	DMSO Variability (% Activity)		Activity Cut-off (%) ^b
Name	PubChem AID	Name	Channel 2 effect	Channel 1 effect		Ch 2	Ratio	
ap1	1159528	Epidermal growth factor	2.69	0.76	JUN (Ap-1)	3.16	2.40	18
are	743219	5,6-Benzoflavone	1.41	0.77	NFE2L2	4.69	2.10	21
esre	1159519	Tanespimycin (17-AAG)	2.26	0.84	ATF6	1.49	1.28	15
hre	NA	Cobalt(II)chloride hexahydrate	4.15	0.76	HIF1A	0.51	0.34	12
hse	743228	Tanespimycin (17-AAG)	2.62	0.78	HSF11214	0.88	0.86	15
nfkb	1159518	TNF-alpha	6.21	0.55	NFKB1	0.41	0.25	15
p53	720552	Mitomycin C	2.35	0.75	TP53	1.41	1.06	15

^a - Fold change is the fraction of positive control fluorescence (F_{PC}) over vehicle control fluorescence (F_{VC}), calculated as the F_{PC}/F_{VC}.

^b - the same activity cut-off was used for all channels except in validation circumstances described in the methods section.

JUN (Ap-1) - Jun proto-oncogene, AP-1 transcription factor subunit; NFE2L2 - nuclear factor, erythroid 2 like 2; ATF6 - activating transcription factor 6; HIF1A - hypoxia inducible factor 1 alpha subunit; HSF11214 - heat shock transcription factors # 1,2 & 4; NFKB1 - nuclear factor kappa B subunit 1; TP53 - tumor protein p53.

Table 2:

Activity Classification Statistics for Seven BLA Assays

Assay	N CRC	Activators		RFP		EUC		EOC		PUC		POC		RFN	
		#	%	#	% ^a	#	% ^a	#	% ^a	#	% ^a	#	% ^a	#	% ^a
ap1	9802	1165	11.9	204	18	15	1	199	17	81	7	5	0.4	17	1
are	9451	1841	19.5	97	5	365	20	63	3	93	5	34	2	131	7
esre	9451	403	4.3	277	69	45	11	14	3	13	3	6	1	20	5
hre	9802	574	5.9	470	82	17	3	35	6	27	5	0	0	1	0
hse	9451	650	6.9	303	47	29	4	86	13	58	9	11	2	5	1
nfkb	9451	300	3.2	255	85	22	7	0	0	4	1	2	1	4	1
p53	10444	1043	10.0	481	46	52	5	142	14	95	9	10	1	12	1
Total	67852	5976	8.8	2087	35	545	9	539	9	371	6	68	1	190	3

^a - % is given as a percentage of the active compounds in the assay.

RFP – Ratio False Positive; EUC – Efficacy Underestimation of Concern; EOC – Efficacy Overestimation of Concern; PUC – Potency Underestimation of Concern; POC – Potency Overestimation of Concern. RFN – Ratio False Negative.