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Identification and profiling of environmental chemicals that inhibit the TGF β /SMAD signaling pathway

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Abstract

The transforming growth factor beta (TGF β) superfamily of secreted signaling molecules and their cognate receptors regulate cell fate and behaviors relevant to many developmental and disease processes. Disruption of TGF β signaling during embryonic development can, for example, affect morphogenesis and differentiation through complex pathways that may be SMAD (Small Mothers Against Decapentaplegic) -dependent or SMAD-independent. In the present study, the SMAD Binding Element (SBE)-beta lactamase (*bla*) HEK 293T cell line, which responds to the activation of the SMAD2/3/4 complex, was used in a quantitative high-throughput screening (qHTS) assay to identify potential TGF β disruptors in the Tox21 10K compound library. From the primary screening, we identified several kinase inhibitors, organometallic compounds, and dithiocarbamates (DTCs) that inhibited TGF β 1-induced SMAD signaling of reporter gene activation independent of cytotoxicity. Counter-screen of SBE antagonists on human embryonic neural stem cells demonstrated cytotoxicity, providing additional evidence to support evaluation of

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Supporting Information

Table S1: 65 compounds in 4 reporter gene assays related with development (SBE-*bla*, WNT-, RAR-, SHH-*luc*) and pSMAD assay, and cell viability assay on SBE-HEK 293T and NSCs; Table S2: chemicals in enriched clusters from primary screening of SBE-*bla* assay; Table S3: reference to assign curve classes and activity category as described in method section 2.7. Figure S1: inhibitory effects on SBE, WNT, RAR, and SHH pathways and cytotoxicity of 65 confirmed SBE antagonists.

these compounds for developmental toxicity. We profiled the inhibitory patterns of putative SBE antagonists toward other developmental signaling pathways, including wingless-related integration site (WNT), retinoic acid α receptor (RAR) and sonic hedgehog (SHH). The profiling results from SBE-*bla* assay identify chemicals that disrupt TGF β /SMAD signaling as part of an integrated qHTS approach for prioritizing putative developmental toxicants.

Graphical Abstract

Keywords

TGF β /SMAD; developmental and neurotoxicity; quantitative high throughput screening (qHTS)

1. Introduction

The transforming growth factor β (TGF β) superfamily of secreted signaling molecules and their cognate receptor systems are critical regulators of embryogenesis and tissue homeostasis.^{1, 2} Various ligands including TGF β s, nodal, activins, bone morphogenic proteins (BMPs), and growth/differentiation factor (GDF), regulate cell fate and behavior that underlie differential growth and patterning via complex biological networks.³ Despite the complexity of combinatorial ligand-receptor interaction in the TGF β superfamily, the downstream regulation of target gene expression has been shown to be mediated through SMAD (Small Mothers Against Decapentaplegic).⁴ Canonical SMAD-dependent signaling is transduced by two distinct SMAD complexes, SMAD2/3/4 or SMAD1/5/8, in response to different ligands.^{5, 6}

Transcriptional activity regulated by the SMAD2/3/4 complex plays an important role in neurogenesis, hematopoiesis, angiogenesis, osteogenesis and epithelial-mesenchymal transitions.^{7–10} Evidence exists to implicate SMAD-dependent TGF β signaling pathway during developmental toxicity. For example, a recent study on gestational exposure to the carbamate pesticide carbofuran in rat revealed increased mRNA level of TGF β , SMAD2 and 3 in hippocampus, and that adverse consequences of these compounds on neurogenic stem

cells could be ameliorated with a pharmacological inhibitor (SB431542) and Smad-3 siRNA.¹¹ In contrast, genomic analysis of zebrafish embryos exposed to the fungicides thiram and disulfiram implicated inhibition of the TGF β pathway, including an essential transcription factor for zebrafish craniofacial development, SRY-box-containing gene 9a (sox9a), which resulted in craniofacial abnormalities.¹² These findings, together with a recognition of the critical role for SMAD-dependent TGF β signaling in normal embryogenesis¹⁰ and tissue homeostasis¹³, motivated the broad screening of chemical compound libraries for potential disrupters of this pathway, as few environmental and industrial chemicals have been tested for such effects. This study focused on the antagonist modes because inhibition of TGF β /SMAD signaling leads to failure in mesoderm formation and during gastrulation in the mouse development.¹⁴

To identify potential TGF β /SMAD inhibitors, the current study used a cell-based reporter gene assay to determine whether a chemical has the potential to disrupt TGF β 1 signaling via the SMAD 2/3/4 complex.¹⁵ TGF β 1 binding to its cognate receptor results in the phosphorylation of SMAD2 and SMAD3, which then dissociate from the receptor and bind to SMAD4.¹⁶ Nuclear translocation of the SMAD2/3/4 complex results in a trans-activation of the beta-lactamase (*bla*) reporter via the SMAD Binding Element (SBE). Inhibition of SBE-*bla* activity¹⁷, independent of significant cytotoxic effect, indicates suppression of SMAD2/3/4-dependent transcriptional activity. Here, we performed a quantitative high-throughput screen (qHTS) against the Tox21 10K compound library (7872 unique chemicals defined by CASRNs) using the SBE-*bla* HEK 293T cell line to identify potential TGF β /SMAD pathway inhibitors (dataset available in PubChem AID: 1347032). Since we have in-house assays of other developmental related pathways (WNT, RAR and SHH), the selected compounds' effects in SBE-*bla*, WNT-, RAR-, and SHH-*luc* assays were evaluated and compared. WNT signaling is critical for neurodevelopmental processes, such as central nervous system (CNS) patterning and differentiation.^{18, 19} Retinoic acid (RA) is an essential morphogen in vertebrate development^{20, 21} through binding with homeobox genes regulated by nuclear retinoic acid receptors (RARs) for patterning the neuroaxis.^{21–23} SHH plays a crucial role in early patterning and organization of CNS as well.^{24–26} Therefore, perturbations in WNT, RAR, or SHH signaling may disrupt neurodevelopmental patterning and result in disorders related to the structure and function of the CNS. The experimental design and workflow are outlined in Figure 1. We identified several known TGF β /SMAD pathway inhibitors [e.g. flavopiridol hydrochloride (CASRN: 131740-09-5) and dasatinib (CASRN: 302962-49-8)], and several compounds including pharmaGSID_48519 (CASRN: 686756-87-6) and ziram (CASRN: 137-30-4) that were not previously known to interact with the TGF β /SMAD pathway and found that they inhibited SMAD3 phosphorylation. As such, a SBE-*bla* reporter gene assay coupled to SMAD3 phosphorylation conveys a robust and efficient means to identify potential disruptors of TGF β /SMAD signaling for mechanistic evaluation in other assays relevant to developmental signaling (eg, WNT, RAR, SHH).

2. Materials and Methods

2.1 Cell lines and culture conditions:

CellSensor® (SBE-*bla* HEK 293T) cell line, containing a beta-lactamase reporter gene under control of the SBE stably integrated into HEK 293T cells, was purchased from Life Technologies Corporation (Carlsbad, CA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% dialyzed fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 25 mM HEPES, 100 U/mL penicillin and 100 µg/mL streptomycin, and 5 µg/mL blasticidin (Life Technologies Corporation). HepG2 cells, purchased from American Type Culture Collection (ATCC, Manassas, VA), were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA), and 100 U/mL penicillin and 100 µg/mL streptomycin. Neural Stem Cells (NSC) derived from human embryonic stem cell (hESC-H9) were obtained from Life Technologies (Carlsbad, CA) and cultured in StemPro NSC serum free medium from Thermo Fisher (Grand Island, NY). The cells were subcultured by release with StemPro Accutase and seeded on Matrigel-coated flasks.

Human cortical glutamatergic neurons were ready-to-use iPSC-derived neurons purchased from BrainXell (Madison, WI) and were cultured in DMEM/F12 and neurobasal medium supplemented with B27 supplement, N2 supplement, GlutaMAX, Geltrex (Thermo Fisher, Grand Island, NY) and BDNF, GDNF and TGFβ1 (Peprotech, Rocky Hill, NJ). TCF/LEF-*luc* HEK293 cells were cultured in DMEM supplemented with 10% FBS, and 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies Corporation, Carlsbad, CA). C3RL4 cells stably transfected with RARE-*luc* were cultured in Eagle's Basal Medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 2 µg/mL puromycin and 100 U/mL penicillin and 100 µg/mL streptomycin. Gli1-*luc* NIH/3T3 cells were cultured in DMEM medium supplemented with 10% bovine calf serum, BCS (ATCC, Manassas, VA), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, and 2 µg/mL puromycin (Life Technologies Corporation, Carlsbad, CA). All the cells were maintained at 37°C under a humidified atmosphere and 5% CO₂.

2.2 SBE-*bla* assay and qHTS:

SBE-*bla* HEK 293T cells (passage 6) were seeded at 4000 cells/well/5 µL culture medium without blasticidin into 1536-well tissue culture treated black-wall/clear bottom tissue culture plates (Greiner Bio-One North America, NC) by using a Multidrop Combi (Thermo Fisher, Waltham, MA). Assay plates were incubated at 37°C overnight for cell attachment. The compound treatment of the Tox21 10K compound library was executed by a Wako Pintool station (Wako Automation, San Diego, CA) which transfer 23 nL of test compounds dissolved in DMSO into 5 µL culture medium, followed by addition of 1 µL of 0.25 ng/mL human TGFβ1 using BioRaptr FRD (Beckman Coulter, Brea, CA) to each well. The final compound concentrations in the wells were in the range of 0.98 nM to 77 µM, with assays run in 15-point concentration response in triplicates. After compound treatment, the assay plates were incubated for 5 h at 37°C. For SBE-*bla* readout, 1 µL of LiveBLAzer™ FRET B/G CCF4-AM substrate (Invitrogen, Carlsbad, CA) was added to each well and the

fluorescence intensity was measured after 2 h using an Envision plate reader (Perkin Elmer, Waltham, MA) with filter setting at Ex 320 nm, and Em 460 nm and 530 nm. SBE-*bla* activity was measured as ratio of reading at 460 nm/530 nm. As for SBE-*bla* assay data normalization, the readings of wells adding TGFβ1 only were used as 0% inhibition, and vehicle control DMSO wells were used as 100% inhibition since no TGFβ1 activity should be observed.

After measurement of SBE-*bla* activity, cell viability was measured by adding 5 μL CellTiter-Glo reagent (Promega, Madison, WI) to each well, and luminescence signal was measured through ViewLux plate reader (Perkin Elmer, Waltham, MA) after 30 min incubation at room temperature. Cell viability was measured by relative luminescence units. The positive control in the cell viability assay was tetra-N-octylammonium bromide (Sigma-Aldrich, St. Louis, MO). The readings of vehicle control DMSO wells were used as 0% activity, and positive control tetra-N-octylammonium bromide wells were used as –100% activity for cell viability data normalization.

2.3 Measurement of phosphor-SMAD3 (pSMAD3) level by homogeneous time resolved fluorescence (HTRF) technology:

HepG2 cells (passage 6) were seeded at 5000 cells/well/5 μL of culture medium into 1536-well PDL-coated tissue culture treated white plates using a BioRaptr FRD. The assay plates were incubated overnight at 37°C for cell attachment. The next day, 23 nL of test compound or positive control, a TGFβ1 receptor (ALK5) inhibitor, SB525334 (CASRN: 356559-20-1, Sigma-Aldrich), was added into assay plates, followed by addition of 1 μL of 1.5 ng/mL human TGFβ1 (R&D Systems, Minneapolis, MN) to stimulate SMAD3 phosphorylation. The final compound concentrations in the wells were in the range of 75 nM to 77 μM, with assays run in 11-point concentration response in triplicates. After the assay, plates were incubated at 37°C for 1 h and the supernatant from each well was removed with a light spin evacuation step using Bluewasher (GC biotech, Waddinxveen, The Netherlands). Then, 4 μL of 1X lysis buffer with blocking reagent (Cisbio, Bedford, MA) was added to each well. The assay plates were centrifuged at 1000 rpm for 20 s and incubated for 30 min at room temperature. Antibody mixture to detect pSMAD3 (Ser 423/425) was added after 30 min cell lysis, followed by 4 h incubation at room temperature. Fluorescence intensity was measured with filter setting at Ex 320 nm and Em 620 nm/ 665nm. The relative pSMAD3 level was expressed as the ratio of readings at 665nm/620nm. The readings of vehicle control DMSO wells were used as 0% activity, and positive control SB525334 (CASRN: 356559-20-1, Sigma-Aldrich) wells were used as –100% activity for pSMAD3 activity normalization.

2.4 Cell viability assays in human embryonic neural stem cells:

Human NSCs (passage 5) were seeded at 2000 cells/ well/5 μL culture medium supplemented with 1% Vitronectin (Thermo Fisher, Grand Island, NY)²⁷ into 1536-well white, solid bottom plates using a Multidrop Combi. The cells were incubated at 37°C for 16 h for attachment. Then, 23 nL of compound or positive control, tetra-N-octylammonium bromide, was transferred to the assay plates to reach 11 final concentrations from 0.8 nM to 92 μM. The cells were treated for 5 h or 24 h. Cell viability was measured with CellTiter-

Glo luminescent cell viability assay.²⁸ After compound treatment, 5 μ L CellTiter-Glo reagent was added to each well and luminescence signal was measured through ViewLux plate reader after 30 min incubation at room temperature. The readings of DMSO was used as a vehicle control (0% activity) and tetra-N-octylammonium bromide was the positive control (–100% activity) for data normalization.

2.5 Neurite outgrowth assay in iPSC-derived neurons:

iPSC-derived GFP-expressing cortical glutamatergic neurons (immediately usage after receiving from vendor) were thawed and directly seeded at the density of 800 cells per well in 5 μ L culture medium into low-base poly-D-lysine-coated 1536-well black-wall/clear-bottom assay plates (Aurora Microplates, Whitefish, MT) using BioRaptr FRD. The cells were incubated at 37°C in a humidified environment of 5% CO₂ in air for 42 h, followed by addition of 23 nL compounds to reach final concentrations from 90 nM to 92 μ M. The treated cells were cultured for 24 h.

GFP-expressing neurons allows imaging analysis of neurite outgrowth. Confocal 20 $\times$ water lens was used to capture 4-field images per well through Operetta CLS (Perkin Elmer, Shelton, CT). EGFP fluorescence intensities (460–490 nm excitation, 500–550 nm emission) were analyzed by Harmony 4.6 High-Content Imaging Analysis System. The neurite length was quantified based on the default image analysis function, maximum neurite length. Number of objects which indicated cell body number was used to normalize the number of cells in each well. The reduction of maximum neurite length comparing to untreated group was inhibition of neurite outgrowth. Normalized percentage of inhibition was according to the difference between maximum neurite length of vehicle control DMSO and positive control, rotenone (final concentration 11.5 μ M).

2.6 WNT-, RAR- and SHH-luc reporter gene assays:

2.6.1 WNT-luc assay: TCF/LEF-*luc* HEK293 cells (passage 9) were seeded at 2000 cells/well/4 μ L culture medium into 1536-well tissue culture treated white-solid plates using a BioRaptr FRD. The assay plates were cultured overnight at 37°C and 5% CO₂. Then 23 nL of compounds were transferred to the assay plates using Wako Pintool station, followed by the addition of 1 μ L of 200 ng/mL recombinant human WNT-3a (R & D Systems, Minneapolis, MN) using a BioRaptr FRD. The cells were treated for 24 h. The cell viability assay was multiplexed with reporter-gene readout by addition of 1 μ L of CellTiter-Fluor reagent into each well of the assay plates using a BioRaptr FRD and the assay plates were incubated for 1 h at 37°C before measuring luciferase reporter activity. Fluorescence intensity was measured using a ViewLux plate reader. For the luciferase reporter assay, 4 μ L ONE-Glo reagent (Promega) was added to each well and luminescence signal was measured using ViewLux plate reader after 30 min incubation at room temperature. Data were expressed as relative fluorescence units (cell viability assay) and relative luminescence units (luciferase reporter assay).

2.6.2 RAR-luc assay: The use of C3RL4 cells (passage 8) stably transfected with the retinoic acid response element (RARE) in qHTS screening was previously reported.²⁹ The cells were seeded at 1000 cells/well/4 μ L culture medium in 1536-well white-solid plates

using a Multidrop Combi. After assay plates were incubated at 37°C and 5% CO₂ overnight, 23 nL of compounds were transferred to the assay plates by Wako Pintool station, followed by addition of 1 µL of 1.0 µM retinol using a BioRaptr FRD. The assay plates were incubated for 6 h. To multiplex the cell viability assay with reporter-gene readout, 1 µL CellTiter-Fluor reagent was added into each well of the assay plates using a BioRaptr FRD and the assay plates were incubated for 1 h at 37°C before measuring luciferase reporter activity. Fluorescence intensity was measured using a ViewLux plate reader. For luciferase reporter assay, 4 µL of ONE-Glo reagent was added to each well, and luminescence signal was measured through a ViewLux plate reader after 30 min incubation at room temperature. Data were expressed as relative fluorescence units (cell viability assay) and relative luminescence units (luciferase reporter assay).

2.6.3 SHH-luc assay: 3T3 Gli1-*luc* cells (passage 7) were seeded at density of 2000 cells per well in 4 µL of assay medium into a 1536-well tissue culture treated white plates using a Multidrop Combi. The assay plates were incubated for 4 h at 37°C for cell attachment, and then followed by the transfer of 23 nL test compounds by a Wako Pintool station. To screen for SHH signaling inhibitors, 1 µL of conditioned medium was added using a BioRaptr, FRD and the assay plates were then incubated for 24 h. To multiplex the cell viability assay with reporter-gene readout, 1 µL of CellTiter-Fluor reagent was added into each well of the assay plates using BioRaptr FRD after 23 h of compound treatment. The assay plates were further incubated for additional 1 h at 37°C. Fluorescence intensity was measured using a ViewLux plate reader (Perkin Elmer, Waltham, MA). Then 4 µL of Amplite Luciferase reporter gene assay reagent (AAT Bioquest, Sunnyvale, CA) was added to each well of the assay plates. After 30 min incubation at room temperature, the luminescence intensity was measured using a ViewLux plate reader. Data were expressed as relative fluorescence units (cell viability assay) and relative luminescence units (luciferase reporter assay).

2.7 Data analysis:

qHTS data analysis.—Data normalization and concentration-response curve fitting for the data from the qHTS screening and follow up studies were performed as previously described.³⁰ Briefly, raw plate reads for each titration point were first normalized relative to the positive control compound and 32 DMSO-only wells (0.25 ng/µL human TGFβ-1 = 0%, DMSO = -100%) as follows: % Activity = $((V_{\text{compound}} - V_{\text{DMSO}})/(V_{\text{pos}} - V_{\text{DMSO}})) \times 100$, where V_{compound} denotes the compound well values, V_{pos} denotes the median value of the positive control wells, and V_{DMSO} denotes the median values of the DMSO-only wells (final concentrations ranging from 0.38 to 0.45%), and then corrected by applying a NCATS in-house pattern correction algorithm using compound-free control plates (i.e., DMSO-only plates) at the beginning and end of the compound plate stack.³¹ Concentration-response titration points for each compound were fitted to a four-parameter Hill equation yielding concentrations of half-maximal inhibitory activity (IC₅₀) and maximal response (efficacy) values.³²

Compounds were designated as Class 1–4 according to the type of concentration-response curve observed.^{33, 34} Each compound was assigned an activity outcome, inactive, active or

inconclusive, based on the type of concentration response curve and reproducibility (3 independent runs) as described previously³³ and summarized in Table S3. Each replicate curve is first assigned an activity class based on its curve class as follows: inactive (class 4), active antagonist (class -1.1, -2.1; class 5 due to super potency ($IC_{50} < \text{lowest test concentration}$), antagonist (class -1.2, -2.2), inconclusive antagonist (all other cases). Each activity class is then assigned a score: active antagonist (-3), antagonist (-2), inconclusive antagonist (-1), inactive (0). The pair-wise activity score differences for all replicate curves of each sample are then averaged and the % of inactive calls for the sample calculated to determine the reproducibility call: active match (average score difference < 1.1 , %inactive call $< 25\%$), inactive match (average score difference < 1.1 , %inactive call $> 50\%$), mismatch (average score difference > 2.5), inconclusive (all other cases). Compounds with active or inactive match calls are considered reproducible.

2.8 Chemical structure cluster analysis.

The Tox21 10K compound collection was first grouped into 1014 clusters based on structural similarity (9242-bit fingerprints, Leadscape®, Columbus, OH, USA) using the self-organizing map (SOM) algorithm.^{35, 36} Each cluster was evaluated for its enrichment of active antagonists and significance of enrichment was determined by the Fisher's exact test ($p < 0.01$).

2.9 Compound management.

The Tox21 10K compound collection was dissolved in DMSO and stored at -80°C . Three sets of the Tox21 10K compound collection was made as replicates. Each copy of a compound was located in the different plate location. The primary screening against three sets of the compounds were run independently. When screening started, the plates were kept in the room temperature for 4 months after thawing. Quality control testing assess the compound stability and reported in <https://tripod.nih.gov/tox21/samples>.

3. Results

3.1 SBE-bla assay optimization:

The SBE-*bla* assay was used to measure the activation of SMAD2/3/4 signaling. As shown in Figure 2A, all three native TGFR ligands (TGF β 1, TGF β 2, and TGF β 3) triggered activity in the SBE-*bla* assay with effects that were both concentration-dependent and similar potency ($EC_{50} = 0.11 \text{ ng/mL}$). Accordingly, we chose antagonism of TGF β 1-induced SBE-*bla* activity to indicate potential inhibition of TGF β signaling. To identify chemicals and drugs that inhibit TGF β /SMAD signaling, we screened the Tox21 10K compound library in an SBE-*bla* antagonist mode against stimulation by 0.25 ng/mL (EC_{85}) TGF β 1 multiplexed with a cell viability assay (CellTiter-Glo). Compounds that were reproducibly active in both the ratio of 460/530 nm and 460 nm readouts and not apparently cytotoxic (meaning the IC_{50} value is significantly more potent (at least 3-fold; $p < 0.05$) in the SBE-*bla* assay than the IC_{50} value in the viability assay) in the primary screen were selected for confirmation and follow-up studies.

3.2 qHTS performance and reproducibility:

The primary screening tested 7872 unique compounds at 15 concentrations ranging from 0.98 nM to 77 μ M in three independent runs (n=3). To evaluate qHTS performance, a set of parameters were calculated as described previously.³⁷ For SBE-*bla* assay screening, the signal to background (S/B) ratio was 2.89 ± 0.22 , the coefficient of variation (CV) of DMSO vehicle control was 4.66 ± 1.16 , and the average Z' factor from 408 plates of DMSO vehicle control and TGF β 1 across all the plates was 0.76 ± 0.03 . The Z' was calculated based on the standard deviation (SD) and mean of negative (vehicle control) and positive control (TGF β) wells in each plate. This indicated excellent performance for a large-scale screen. Cell viability assayed simultaneously yielded an S/B ratio of 166.80 ± 4.64 with CV of 7.18 ± 0.68 and Z' factor of 0.79 ± 0.03 , again consistent with excellent results. The qHTS reproducibility parameters in Table 1 are described in the data analysis section. The activity for each compound from three independent runs was defined as an active, inconclusive, or inactive based on curve classes. It showed that the three independent runs reproduced well; no mismatch was found.

3.3 Identification of SBE-*bla* antagonists:

The primary screen identified 68 compounds that potently inhibited TGF β 1-induced SBE-*bla* activity with efficacy > 50% and IC₅₀ < 10 μ M. Re-testing in SBE-*bla* HEK 293T cells using in-house compound stock solutions from the existing Tox21 10K library confirmed 65 of 68 (95%) actives which are listed in Table S1. Note that these compounds are mainly anti-cancer drugs and pesticides. Concentration response curves of several known TGF β /SMAD pathway inhibitors are shown in Figure 2B, such as the cyclin-dependent kinase inhibitor flavopiridol hydrochloride (IC₅₀ at 0.02 μ M) and the SRC/ABL inhibitor dasatinib (IC₅₀ at 0.26 μ M). We identified several potential novel TGF β /SMAD actives such as ziram and PharmaGSID_48519, with IC₅₀ values of 0.52 μ M and 0.57 μ M, respectively (Figure 2C).

The Tox21 10K compounds were grouped into clusters based on structural similarity using a self-organizing map (SOM). A heatmap was generated to show the enrichment of SBE-*bla* antagonists in each structural class (Figure 3A). Among the structural clusters containing active SBE-*bla* antagonists in the Tox21 10K compound collection, a cluster of dithiocarbamates (DTCs, cluster k35.18) is the most significantly enriched ($p < 10^{-20}$) with 10 selected active SBE-*bla* antagonists reported in Figure 3B. We sub-categorized the DTCs based on the type of metal ion, R- group and the number of sulfides in the molecule. As shown in Figure 3B, dialkyl dithiocarbamate metal that complexes with different metal ions (M), and tetraalkyl thiuram disulfides/polysulfides that have different R-groups both exhibited different IC₅₀ and efficacy values in the SBE-*bla* assay. Among the dialkyl dithiocarbamate metal complexes, the ones with heavy metals and higher valent metals were more potent in blocking TGF β 1-induced SBE-*bla* activity. For example, ferbam, ziram, copper dimethyl dithiocarbamates and sodium dimethyl dithiocarbamate share the same organic component, but different metal ions, i.e., Fe³⁺, Zn²⁺, Cu²⁺ and Na⁺, respectively. The rank order of potency (based on IC₅₀ value) is Fe³⁺ (0.05 μ M) > Zn²⁺ (0.66 μ M) > Cu²⁺ (1.62 μ M) > Na⁺ (9.33 μ M).

Tetraalkyl thiuram disulfide was the most potent inhibitor in DTCs. Thiram, disulfiram and dicyclopentamethylenethiuram blocked more than 50% of TGF β 1-induced SBE-*bla* activity at <1 μ M. The size of the R group seemed to have an effect on compound potency, such that compounds with the larger polysulfide group were less potent in inhibiting SBE-*bla* activity. For example, bis-1-piperidinylthioxomethyl hexasulfide (IC₅₀ = 7.2 μ M) was 12-fold less potent than its disulfide counterpart, dicyclopentamethylenethiuram (IC₅₀ = 0.58 μ M). To further investigate their structure-activity relationship, the SBE-*bla* antagonists in the DTC cluster were selected for follow-up targeted studies.

3.4 Inhibition of SMAD3 phosphorylation:

To investigate the effect of potential SBE-*bla* antagonists on inhibition of downstream SMAD3 phosphorylation, intracellular pSMAD3 (Ser 423/425) level was measured using HTRF technology. HepG2 cells were co-treated with 0.15 ng/mL TGF β 1 and test compound for 1 h. As shown in Figure 4A, pSMAD3 level was decreased upon treatment of dasatinib³⁸ or cantharidin³⁹, which are known inhibitors in TGF β 1-induced SMAD3 phosphorylation. The novel SBE antagonists identified here reduced TGF β 1-induced pSMAD3 level with potency similar to dasatinib. For example, at a concentration of 15 μ M, pharmaGSID_48519 eliminated TGF β 1-elevated pSMAD3 levels; and ziram inhibited SMAD3 phosphorylation with an IC₅₀ at 22 μ M. Interestingly, a group of organometallics decreased the pSMAD3 level (Figure 4B). The most potent example is phenylarsine oxide, with an IC₅₀ value of 5 μ M and complete abolishment of TGF β 's effect around 10 μ M. None of the compounds that reduced pSMAD3 levels had cytotoxicity at the measure time point.

3.5 Cytotoxic effects of potential SBE antagonists on human embryonic H9-derived neural stem cells (NSCs):

The primary and confirmatory screens identified 65 SBE antagonists independent of cytotoxic effect in HEK293T cells. A counter screen was performed on NSC cell viability. IC₅₀ values of viability in HEK293T cells (SBE viability) and NSCs were listed in Table S1. Among the 65 SBE-*bla* antagonists, 36 did not exhibit cytotoxicity in SBE-*bla* HEK 293T cells after 5 h compound treatment. However, 16 out of 35 showed cytotoxicity in NSCs after 5 h treatment, and 33 of them showed cytotoxicity after 24 h treatment (Table S1). Compared to iPSC-derived neurons, NSCs appeared to be more sensitive to cytotoxic effects due to their active proliferation state. For example, several kinase inhibitors (dasatinib, flavopiridol hydrochloride, pp242, PharmaGSID_48519) invoked NSC cytotoxicity at 24 h, but not at 5 h. Additionally, nine organometallics (dicyanoaurate, phenylmercuric acetate/lactate, phenylarsine oxide, triphenyltin fluoride, dibutyltin and zinc pyrithione) had cytotoxic effects in NSCs but were 10-fold less cytotoxic in SBE-*bla* HEK 293T cells. Among these organometallics, phenylarsine and phenylmercuric and dicyanoaurate inhibited TGF β 1-elevated SMAD3 phosphorylation (Figure 4). The cytotoxicity in NSCs motivated follow-up studies related to developmental or neurotoxicity.

3.6 Inhibitory effects of SBE-antagonists in WNT, SHH and RAR pathways:

Regarding NSC cytotoxicity of SBE antagonists in NSCs, their potential toxicity was evaluated through profiling developmental-related pathways using three different luciferase reporter gene assays.

For the DTCs, we found similar inhibition patterns across WNT, RAR and SHH pathways (blue box, Figure S1). DTCs are organosulfur compounds represented by a general structure [(R1R2)N-(C=S)-S]_n-M, where R can be substituted by an alkyl group, and M usually by a metal ion. Since differences in the R-group and metal ion affected SBE-*bla* activity (Figure 3B), we sub-grouped these DTCs by either metal ion (Table 2) or R group (Table 3). As shown in Table 2, dimethyldithiocarbmates complexed with higher oxidation state metal ions demonstrated more potent and efficacious inhibition of TGFβ, WNT, RAR, and SHH pathways. Among them, ferbam (Fe³⁺) was the most potent in inhibiting all pathways with IC₅₀ values of 0.05 μM, 0.57 μM, 0.83 μM and 0.05 μM in SBE-*bla*, WNT-*luc*, RAR-*luc*, and SHH-*luc* assays, respectively. We also compared outcomes in developmental pathways based on the size of R group (Table 3). Sodium dimethyldithiocarbamate inhibited all developmental pathways at 10 μM while sodium dibutyldithiocarbamate was inactive. The rank order of general inhibition effects in WNT, RAR and SHH pathways were in accordance with their potency in inhibiting SBE-*bla* activity (Table 3). In addition, most of the anti-cancer drugs inhibited SBE and RAR pathways, but not SHH or WNT pathways (purple box, Figure S1). These compounds inhibited SBE and RAR reporter gene activities at concentrations that did not compromise cell viability, as evidenced by the comparison of the IC₅₀ values of reporter gene assay and viability assay resulting in a ratio greater than 3.

3.7 Inhibition of neurite outgrowth by selected SBE antagonists:

To further explore potential neurotoxicity, selected SBE antagonists were tested in a neurite outgrowth assay. iPSC derived neurons were treated by 6 DTCs and 13 anti-cancer drugs for 24 h post-neurite extension. High-content imaging of neurite length and viable cell body relative to the control (DMSO) group are shown in Figure 5. Ziram and ferbam inhibited neurite outgrowth at 0.1 μM and were 18-fold more potent than sodium dimethyldithiocarbamate (Figure 5B); all three of these compounds share a similar structure in their organic moiety but differ in metal ion. A general pattern among organically-similar DTCs was decreasing IC₅₀ values (increase of potency) for neurite inhibition with increasing valency in the metal ions. Differential inhibition of neurite outgrowth was also observed for DTCs having different R groups. For example, disulfiram and thiram had IC₅₀ values of 0.18 and 0.1 μM, respectively, versus that for dicyclopentamethylenethiuram disulfide at 0.82 μM. The R group in the case of dicyclopentamethylenethiuram disulfide is dicyclohex versus thiram (R=methyl) and disulfiram (R=ethyl) that were 10-fold more potent. This pattern followed the structure-related activity of DTCs observed in the SBE-*bla* assay (Figure 3B), indicating that potency toward neurite outgrowth was dependent both on metal ion status and R group.

Among anti-cancer drugs, flavopiridol hydrochloride, canertinib, 5-Azacitidine, idarubicin hydrochloride, vinblastine sulfate, bortezomib, pharmaGSID_48505, and nemorubicin inhibited neurite outgrowth (Figure 5D). Surprisingly, dasatinib, pharmaGSID_48519 and pp242 did not inhibit neurite outgrowth in iPSC-derived neurons despite their activity on the SBE-*bla* assay.

4. Discussion

Normal biological development requires specific signaling pathways that regionalize and pattern the growth of tissue. Many pathways including TGF β , WNT, BMP, hedgehog, RAR and Notch-delta were identified as essential signaling in development and they were conserved from model organisms, laboratory animals to humans.^{40, 41} The current study used the SBE-*bla* assay as a first-tier screen to determine whether a chemical has the potential to inhibit TGF β 1-induced SMAD2/3/4 signaling. TGF β signaling plays an important role in early (before organogenesis and cytodifferentiation) and later development (tissue growth and renewal).^{42, 43} For example, disruption of TGF β signaling leads to craniofacial defects⁴⁴ or embryoletality.⁴⁵ TGF β is also essential for the survival of embryonic dopaminergic neurons and survival of embryonic dopaminergic neurons.^{46, 47} Our results showed the following main findings: (1) several kinase inhibitors, organometallic compounds, and DTCs were found across the Tox21 library to inhibit TGF β 1-induced SMAD signaling independent of cytotoxicity; (2) counter-screen of SBE antagonists demonstrated differential cytotoxicity on human NSCs providing evidence for potential developmental toxicity; (3) SBE antagonist profiles showed potential effects on other key developmental signaling pathways, including WNT, RAR, and SHH; and (4) some but not all compounds with inhibitory effects on the SBE assay also impaired neurite outgrowth. These results show that the SBE-*bla* assay is an effective qHTS approach for identifying chemicals that disrupt TGF β /SMAD signaling in a manner relevant to predicting a potential developmental hazard.

The qHTS SBE-*bla* platform identified both known and previously unreported inhibitors of SMAD-dependent TGF β 1 signaling. For example, a failed drug, pharmaGSID_48519, which was initially designed as a kinase inhibitor, inhibited SMAD3 phosphorylation and consequently directly blocked TGF β 1 signaling through its cognate receptor (ALK5). Flavopiridol hydrochloride (a pan-CDK/GSK3 inhibitor), pp242 (a mTOR inhibitor) and dasatinib (a BCR-ABL inhibitor) inhibited TGF β 1-induced SBE-*bla* activity at a similar concentration as reported in Mv1Lu cells¹⁶, normal fibroblast⁴⁸, panc-1 and colo-357 cells, respectively.⁴⁹ Dasatinib and flavopiridol hydrochloride's inhibition on TGF β 1's effect has been reported as a treatment or for prevention of pathophysiologic conditions caused by excessive TGF β activity, such as cancer³⁸, pulmonary fibrosis⁵⁰ or atherosclerosis.⁵¹ In the current toxicology study, dasatinib, pp242 and pharmaGSID_48519 were cytotoxic in NSCs after culturing for 24 h but didn't inhibit neurite outgrowth. These results point to a potential effect on developmental toxicity, but not via a mechanism involving impact on neurite outgrowth.

Some organometallic environmental chemicals were active in the SBE-*bla* and pSMAD3 assays with broad toxicity observed in NSCs as well. The mechanism of organometallic chemicals' inhibition in TGF β signaling remains unknown. It has been reported that phenylarsine oxide inhibited internalization of TGFR and limited the access of activated receptor to intracellular substrates, which may explain the inhibition in TGF β signaling.⁵² Organometallics has been used as anticancer agents because of their inhibition in protein kinases.⁵³ Therefore, they may inhibit SMAD phosphorylation which is required for SBE-*bla* activity. Transition metals are generally toxic due to oxidative stress.⁵⁴⁻⁴² Heavy metals

such as mercury, lead, and arsenic lead to an alteration of neurogenesis, resulting in motor and cognitive function deficits.^{55, 56} Perhaps adverse effects on TGF β 1 signaling may contribute to neuroinflammation⁵⁷ and neurological disorders.⁵⁸

DTCs are used as fungicides, accelerators in the rubber industry, animal repellants, and biocides in many household products. Previous studies have suggested that DTCs were neurotoxic at different stages of life and affected multiple organs.⁵⁹ In our study, the rank order of DTC-induced inhibition in WNT-, RAR and SHH-*luc* assays were associated with a reduction in SBE-*bla* activity. Thiram, disulfiram, ziram, and ferbam were the most potent actives in the SBE-*bla* assay, and showed a similar trend inhibiting SHH, WNT and RAR pathways. These results are consistent with existing evidence about their broad developmental toxicity, including embryo-fetal toxicity (e.g., thiram and disulfiram induced-skeletal malformations in rats, human, hamster and rabbits, and cleft palate in mice),^{60–62} neurodevelopmental defects (e.g., ferbam-induced hydrocephalus in rat development and thiram-induced anophthalmia and microphthalmia),⁶³ and peripheral neuropathy effects (e.g., ziram induced-spinal cord histopathology in rat, and thiram-induced degeneration of ventral horn and lower lumbar region of spinal cord).⁶⁴

With respect to redox status⁶⁵, the pattern observed was that less stable DTCs show a stronger inhibition of TGF β /SMAD signaling. This may be explained by metal ionization state and/or increasing R group size. For the former, DTCs complexed with transition metals, such as Zn²⁺ (ziram), and Fe³⁺ (ferbam), were more effective in the inhibition of SBE-*bla* activity than the Na⁺ salt. Under physiological conditions, most DTCs reoxidize to thiuram disulfide. Higher valent heavy metals, such as Zn²⁺ or Fe³⁺, are relatively unstable and can be easily more readily oxidized to thiuram disulfide. Thiuram disulfides can then react with other molecules to form thiol groups suppressing a 'cysteine knot' in the SMAD2/3/4 complex, resulting in dysregulation of TGF β downstream targets. As to the R-group pattern, inhibition of SBE-*bla* activity by the DTC series declined with longer alkyl chains. Steric hindrance from large alkyl groups against reoxidization may decrease their ability to generate isothiocyanates, which are believed to be responsible for the antifungal activity and general inhibition of developmental pathways. Subsequent studies showed that the mutagenicity of DTCs also followed this pattern.⁶⁶ Rank order potency of DTCs on WNT-, RAR-, and SHH-*luc* assays in the present study also followed this pattern. This can help explain their potential to cause developmental effects through the impact of complexed metals or alkyl groups. We speculate that the ability to form thiuram disulfides may be critical to the differential neuropathic effects of DTCs.

Current prenatal toxicity testing guidelines monitor the potential action of toxicants at many levels of normal development and assess disruption of overall processes of developmental, reproductive, and teratogenic effects of pharmaceuticals, food additives, and pesticides.^{67–69} Faster, inexpensive high throughput *in vitro* assays, such as the SBE-*bla* assay, could be used as a quantitative high-throughput (qHTS) screen to identify chemicals for developmental toxicity assessment. For example, 15 SBE-*bla* antagonists in the Tox21 SBE-*bla* assay have been reported to downregulate TGF β protein levels as determined by ELISA in a ToxCast Bioseek study (noted with * in Table S1).⁷⁰ The concordance of downregulation of TGF β expression and inhibition of TGF β /SMAD signaling showed associations in these *in vitro*

endpoints, which suggests TGF β perturbation by chemicals such as ziram, thiram, disulfiram may be predictable through cheminformatics analysis. We recognize the identification of chemical-induced TGF β 1 blockage effects on reporter gene assay will not necessarily predict specific developmental or neurotoxicity. Since TGF β subtypes are indistinguishable in most biological assays with cell lines cultured *in vitro*, it is expected the SBE-*bla* assay responds to TGF β 's -1, -2, -3 non-differentially. Therefore, it is important to conduct follow-up tests that measure structurally similar chemicals in functional assays to maximize the probability of detecting neurotoxicity. Further experiments can be done in more biologically complex functional assays involving neurobehavioral, neurochemical, and neuropathologic measurement. For developmental toxicants in general, the cell viability results in hESC-derived NSC offers a potential counter-screen. Because some but not all SBE inhibitors impaired neurite outgrowth in human neurogenic cells derived from H9 embryonic stem cells, the modeling the impact of TGF β /SMAD, WNT, RAR, and SHH pathways provides a pragmatic means to address developmental toxicity (and potential neurotoxicity) at a systems level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Wu MY; Hill CS, Tgf-beta superfamily signaling in embryonic development and homeostasis. *Dev Cell* 2009, 16 (3), 329–43. [PubMed: 19289080]
2. Linask KK; D'Angelo M; Gehris AL; Greene RM, Transforming growth factor-beta receptor profiles of human and murine embryonic palate mesenchymal cells. *Exp Cell Res* 1991, 192 (1), 1–9. [PubMed: 1845792]
3. Massagué J, TGF β signalling in context. *Nature reviews Molecular cell biology* 2012, 13 (10), 616. [PubMed: 22992590]
4. Feng X-H; Derynck R, Specificity and versatility in TGF- β signaling through Smads. *Annu. Rev. Cell Dev. Biol* 2005, 21, 659–693. [PubMed: 16212511]
5. Lucarelli P; Schilling M; Kreutz C; Vlasov A; Boehm ME; Iwamoto N; Steiert B; Lattermann S; Wasch M; Stepath M; Matter MS; Heikenwalder M; Hoffmann K; Deharde D; Damm G; Seehofer D; Muciek M; Gretz N; Lehmann WD; Timmer J; Klingmuller U, Resolving the Combinatorial Complexity of Smad Protein Complex Formation and Its Link to Gene Expression. *Cell Syst* 2018, 6 (1), 75–89 e11. [PubMed: 29248373]
6. Shi Y; Massague J, Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003, 113 (6), 685–700. [PubMed: 12809600]
7. Araujo AP; Diniz LP; Eller CM; de Matos BG; Martinez R; Gomes FC, Effects of Transforming Growth Factor Beta 1 in Cerebellar Development: Role in Synapse Formation. *Front Cell Neurosci* 2016, 10, 104. [PubMed: 27199658]
8. van Praag H; Schinder AF; Christie BR; Toni N; Palmer TD; Gage FH, Functional neurogenesis in the adult hippocampus. *Nature* 2002, 415 (6875), 1030–4. [PubMed: 11875571]

9. Millan FA; Denhez F; Kondaiah P; Akhurst RJ, Embryonic gene expression patterns of TGF beta 1, beta 2 and beta 3 suggest different developmental functions in vivo. *Development* 1991, 111 (1), 131–43. [PubMed: 1707784]
10. Gehris AL; Greene RM, Regulation of murine embryonic epithelial cell differentiation by transforming growth factors beta. *Differentiation* 1992, 49 (3), 167–73. [PubMed: 1618373]
11. Seth B; Yadav A; Agarwal S; Tiwari SK; Chaturvedi RK, Inhibition of the transforming growth factor- β /SMAD cascade mitigates the anti-neurogenic effects of the carbamate pesticide carbofuran. *Journal of Biological Chemistry* 2017, jbc. M117. 798074.
12. van Boxtel AL; Pieterse B; Cenijn P; Kamstra JH; Brouwer A; van Wieringen W; de Boer J; Legler J, Dithiocarbamates induce craniofacial abnormalities and downregulate sox9a during zebrafish development. *Toxicol Sci* 2010, 117 (1), 209–17. [PubMed: 20530235]
13. Mercado-Pimentel ME; Runyan RB, Multiple transforming growth factor-beta isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. *Cells Tissues Organs* 2007, 185 (1–3), 146–56. [PubMed: 17587820]
14. Dunn NR; Vincent SD; Oxburgh L; Robertson EJ; Bikoff EK, Combinatorial activities of Smad2 and Smad3 regulate mesoderm formation and patterning in the mouse embryo. *Development* 2004, 131 (8), 1717–1728. [PubMed: 15084457]
15. Wilkinson P Functional Adenosine Receptor 2B Drives Profibrotic and Proinflammatory Signaling in Renal Fibroblasts Through Modulation of TGF β Receptor Signaling. University of the Sciences in Philadelphia, 2017.
16. Wang G; Matsuura I; He D; Liu F, Transforming growth factor- β -inducible phosphorylation of Smad3. *J Biol Chem* 2009, 284 (15), 9663–73. [PubMed: 19218245]
17. Chin J; Adams AD; Bouffard A; Green A; Lacson RG; Smith T; Fischer PA; Menke JG; Sparrow CP; Mitnaul LJ, Miniaturization of cell-based beta-lactamase-dependent FRET assays to ultra-high throughput formats to identify agonists of human liver X receptors. *Assay Drug Dev Technol* 2003, 1 (6), 777–87. [PubMed: 15090224]
18. Rosso SB; Inestrosa NC, WNT signaling in neuronal maturation and synaptogenesis. *Frontiers in cellular neuroscience* 2013, 7, 103. [PubMed: 23847469]
19. Bielen H; Houart C, The Wnt cries many: Wnt regulation of neurogenesis through tissue patterning, proliferation, and asymmetric cell division. *Developmental neurobiology* 2014, 74 (8), 772–780. [PubMed: 24488703]
20. Niederreither K; Dollé P, Retinoic acid in development: towards an integrated view. *Nature Reviews Genetics* 2008, 9 (7), 541.
21. Duester G, Retinoic acid synthesis and signaling during early organogenesis. *Cell* 2008, 134 (6), 921–931. [PubMed: 18805086]
22. Das BC; Thapa P; Karki R; Das S; Mahapatra S; Liu T-C; Torregroza I; Wallace DP; Kambhampati S; Van Veldhuizen P, Retinoic acid signaling pathways in development and diseases. *Bioorganic & medicinal chemistry* 2014, 22 (2), 673–683. [PubMed: 24393720]
23. Zieger E; Schubert M, New insights into the roles of retinoic acid signaling in nervous system development and the establishment of neurotransmitter systems In *International review of cell and molecular biology*, Elsevier: 2017; Vol. 330, pp 1–84. [PubMed: 28215529]
24. Choy SW; Cheng SH, Hedgehog signaling In *Vitamins & Hormones*, Elsevier: 2012; Vol. 88, pp 1–23. [PubMed: 22391297]
25. Álvarez-Buylla A; Ihrie RA In *Sonic hedgehog signaling in the postnatal brain*, *Seminars in cell & developmental biology*, Elsevier: 2014; pp 105–111.
26. Kirschen GW; Xiong Q, Primary cilia as a novel horizon between neuron and environment. *Neural regeneration research* 2017, 12 (8), 1225. [PubMed: 28966631]
27. Dai S; Li R; Long Y; Titus S; Zhao J; Huang R; Xia M; Zheng W, One-Step Seeding of Neural Stem Cells with Vitronectin-Supplemented Medium for High-Throughput Screening Assays. *J Biomol Screen* 2016, 21 (10), 1112–1124. [PubMed: 27647668]
28. Xia M; Huang R; Shi Q; Boyd WA; Zhao J; Sun N; Rice JR; Dunlap PE; Hackstadt AJ; Bridge MF, Comprehensive Analyses and Prioritization of Tox21 10K Chemicals Affecting Mitochondrial Function by in-Depth Mechanistic Studies. *Environmental health perspectives* 2018, 126 (7), 077010. [PubMed: 30059008]

29. Chen Y; Sakamuru S; Huang R; Reese DH; Xia M, Identification of compounds that modulate retinol signaling using a cell-based qHTS assay. *Toxicology in Vitro* 2016, 32, 287–296. [PubMed: 26820057]
30. Huang R, A Quantitative High-Throughput Screening Data Analysis Pipeline for Activity Profiling. *Methods Mol Biol* 2016, 1473, 111–22. [PubMed: 27518629]
31. Wang Y; Huang R, Correction of Microplate Data from High Throughput Screening In High-Throughput Screening Assays in Toxicology, 1 ed.; Zhu H; Xia M, Eds. Humana Press: 2016; Vol. 1473.
32. Wang Y; Jadhav A; Southal N; Huang R; Nguyen DT, A grid algorithm for high throughput fitting of dose-response curve data. *Curr Chem Genomics* 2010, 4, 57–66. [PubMed: 21331310]
33. Huang R, A Quantitative High-Throughput Screening Data Analysis Pipeline for Activity Profiling In High-Throughput Screening Assays in Toxicology, 1 ed.; Zhu H; Xia M, Eds. Humana Press: 2016; Vol. 1473.
34. Inglese J; Auld DS; Jadhav A; Johnson RL; Simeonov A; Yasgar A; Zheng W; Austin CP, Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci U S A* 2006, 103 (31), 11473–8. [PubMed: 16864780]
35. Kohonen T, Self-organizing neural projections. *Neural Netw* 2006, 19 (6–7), 723–33. [PubMed: 16774731]
36. Attene-Ramos MS; Miller N; Huang R; Michael S; Itkin M; Kavlock RJ; Austin CP; Shinn P; Simeonov A; Tice RR, The Tox21 robotic platform for the assessment of environmental chemicals—from vision to reality. *Drug discovery today* 2013, 18 (15–16), 716–723. [PubMed: 23732176]
37. Zhang J-H; Chung TD; Oldenburg KR, A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *Journal of biomolecular screening* 1999, 4 (2), 67–73. [PubMed: 10838414]
38. Bartscht T; Rosien B; Rades D; Kaufmann R; Biersack H; Lehnert H; Gieseler F; Ungefroren H, Dasatinib blocks transcriptional and promigratory responses to transforming growth factor-beta in pancreatic adenocarcinoma cells through inhibition of Smad signalling: implications for in vivo mode of action. *Mol Cancer* 2015, 14, 199. [PubMed: 26588899]
39. Li K; Shi C; Huang J; Tang K, Cisplatin plus norcantharidin alter the expression of TGF- β 1/Smads signaling pathway in hepatocellular carcinoma. *Bratislavske lekarske listy* 2017, 118 (2), 85–88. [PubMed: 28814088]
40. Grandjean P; Landrigan PJ, Developmental neurotoxicity of industrial chemicals. *The Lancet* 2006, 368 (9553), 2167–2178.
41. National Research Council (U.S.). Committee on Developmental Toxicology.; National Research Council (U.S.). Commission on Life Sciences., Scientific frontiers in developmental toxicology and risk assessment. National Academy Press: Washington, DC, 2000; p xviii, 327 p.
42. McCartney-Francis NL; Mizel DE; Frazier-Jessen M; Kulkarni AB; McCarthy JB; Wahl SM, Lacrimal gland inflammation is responsible for ocular pathology in TGF-beta 1 null mice. *The American journal of pathology* 1997, 151 (5), 1281. [PubMed: 9358754]
43. Sanford LP; Ormsby I; Gittenberger-de Groot AC; Sariola H; Friedman R; Boivin GP; Cardell EL; Doetschman T, TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997, 124 (13), 2659–2670. [PubMed: 9217007]
44. Greene RM; Pisano MM, Perspectives on growth factors and orofacial development. *Curr Pharm Des* 2004, 10 (22), 2701–17. [PubMed: 15320737]
45. Dickson MC; Martin JS; Cousins FM; Kulkarni AB; Karlsson S; Akhurst RJ, Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 1995, 121 (6), 1845–54. [PubMed: 7600998]
46. Roussa E; und Halback O. v. B.; Krieglstein K, TGF- β in Dopamine Neuron Development, Maintenance and Neuroprotection In Development and Engineering of Dopamine Neurons, Springer: 2009; pp 81–90.

47. Hegarty SV; Sullivan AM; O'Keeffe GW, Roles for the TGF β superfamily in the development and survival of midbrain dopaminergic neurons. *Molecular neurobiology* 2014, 50 (2), 559–573. [PubMed: 24504901]
48. Chang W; Wei K; Rosen G, Suppression Of Lung Fibrosis By Dual MTOR Inhibitors. In B28. SCARRING IN THE LUNG: PRINCIPLES AND PERSPECTIVES, American Thoracic Society: 2011; pp A2718–A2718.
49. Huang R; Xia M; Cho MH; Sakamuru S; Shinn P; Houck KA; Dix DJ; Judson RS; Witt KL; Kavlock RJ; Tice RR; Austin CP, Chemical genomics profiling of environmental chemical modulation of human nuclear receptors. *Environ Health Perspect* 2011, 119 (8), 1142–8. [PubMed: 21543282]
50. Kanemaru R; Takahashi F; Kato M; Mitsuishi Y; Tajima K; Ihara H; Hidayat M; Wirawan A; Koinuma Y; Hayakawa D, Dasatinib Suppresses TGF β -Mediated Epithelial-Mesenchymal Transition in Alveolar Epithelial Cells and Inhibits Pulmonary Fibrosis. *Lung* 2018, 1–11.
51. Rostam MA; Shajimoon A; Kamato D; Mitra P; Piva TJ; Getachew R; Cao Y; Zheng W; Osman N; Little PJ, Flavopiridol Inhibits TGF- β -Stimulated Biglycan Synthesis by Blocking Linker Region Phosphorylation and Nuclear Translocation of Smad2. *Journal of Pharmacology and Experimental Therapeutics* 2018, 365 (1), 156–164. [PubMed: 29438988]
52. Zwaagstra JC; El-Alfy M; O'Connor-McCourt MD, Transforming Growth Factor (TGF)- β 1 Internalization MODULATION BY LIGAND INTERACTION WITH TGF- β RECEPTORS TYPES I AND II AND A MECHANISM THAT IS DISTINCT FROM CLATHRIN-MEDIATED ENDOCYTOSIS. *Journal of Biological Chemistry* 2001, 276 (29), 27237–27245. [PubMed: 11356827]
53. Zaki M; Hairat S; Aazam ES, Scope of organometallic compounds based on transition metal-arene systems as anticancer agents: starting from the classical paradigm to targeting multiple strategies. *RSC advances* 2019, 9 (6), 3239–3278.
54. Hogarth G, Transition metal dithiocarbamates: 1978–2003. *Progress in inorganic chemistry* 2005, 53, 71–561.
55. Jaako-Movits K; Zharkovsky T; Romantchik O; Jurgenson M; Merisalu E; Heidmets LT; Zharkovsky A, Developmental lead exposure impairs contextual fear conditioning and reduces adult hippocampal neurogenesis in the rat brain. *Int J Dev Neurosci* 2005, 23 (7), 627–35. [PubMed: 16150564]
56. Sigel A; Sigel H; Sigel RKO, Neurodegenerative diseases and metal ions. Wiley: Chichester, West Sussex, England ; Hoboken, NJ, 2006; p xxiii, 463 p.
57. Shinoda Y; Ehara S; Tatsumi S; Yoshida E; Takahashi T; Eto K; Kaji T; Fujiwara Y, Methylmercury-induced neural degeneration in rat dorsal root ganglion is associated with the accumulation of microglia/macrophages and the proliferation of Schwann cells. *The Journal of toxicological sciences* 2019, 44 (3), 191–199. [PubMed: 30842371]
58. Salehi F; Normandin L; Krewski D; Kennedy G; Philippe S; Zayed J, Neuropathology, tremor and electromyogram in rats exposed to manganese phosphate/sulfate mixture. *Journal of Applied Toxicology: An International Journal* 2006, 26 (5), 419–426.
59. Kanchi S; Singh P; Bisetty K, Dithiocarbamates as hazardous remediation agent: A critical review on progress in environmental chemistry for inorganic species studies of 20th century. *Arabian Journal of Chemistry* 2014, 7 (1), 11–25.
60. Miller DB, Neurotoxicity of the pesticidal carbamates. *Neurobehav Toxicol Teratol* 1982, 4 (6), 779–87. [PubMed: 6763154]
61. Lee CC; Peters PJ, Neurotoxicity and behavioral effects of thiram in rats. *Environ Health Perspect* 1976, 17, 35–43. [PubMed: 1026416]
62. Frisoni GB; MONDA VD, Disulfiram neuropathy: a review (1971–1988) and report of a case. *Alcohol and Alcoholism* 1989, 24 (5), 429–437. [PubMed: 2554935]
63. Hodge HC; Maynard EA; Downs WL; Coye RD Jr.; Steadman LT, Chronic oral toxicity of ferric dimethyldithiocarbamate (ferbam) and zinc dimethyldithiocarbamate (ziram). *J Pharmacol Exp Ther* 1956, 118 (2), 174–81. [PubMed: 13368055]

64. Mulkey M The grouping of a series of dithiocarbamate pesticides based on a common mechanism of toxicity US Environmental Protection Agency, Office of Pesticide Programs, Office of Prevention, Pesticides and Toxic Substances, Washington, DC; SAP Report: 2001.
65. Mathieu C; Duval R; Xu X; Rodrigues-Lima F; Dupret J-M, Effects of pesticide chemicals on the activity of metabolic enzymes: focus on thiocarbamates. *Expert opinion on drug metabolism & toxicology* 2015, 11 (1), 81–94. [PubMed: 25391334]
66. Hedenstedt A; Rannug U; Ramel C; Wachtmeister CA, Mutagenicity and metabolism studies on 12 thiuram and dithiocarbamate compounds used as accelerators in the Swedish rubber industry. *Mutation Research/Genetic Toxicology* 1979, 68 (4), 313–325.
67. Guideline, I. H. T., Detection of toxicity to reproduction for medicinal products & toxicity to male fertility S5 (R2). Citeseer: 2005.
68. Cooper RL, Current developments in reproductive toxicity testing of pesticides. *Reproductive Toxicology* 2009, 28 (2), 180–187. [PubMed: 19409482]
69. Kimmel CA; Makris SL, Recent developments in regulatory requirements for developmental toxicology. *Toxicology letters* 2001, 120 (1–3), 73–82. [PubMed: 11323164]
70. Kleinstreuer NC; Yang J; Berg EL; Knudsen TB; Richard AM; Martin MT; Reif DM; Judson RS; Polokoff M; Dix DJ; Kavlock RJ; Houck KA, Phenotypic screening of the ToxCast chemical library to classify toxic and therapeutic mechanisms. *Nat Biotechnol* 2014, 32 (6), 583–91. [PubMed: 24837663]

Figure 1.

A flowchart of identification of potential TGF β /SMAD inhibitors. The primary screening of Tox21 10K library in SBE-*bla* assay generates a dataset with 5.6% active SBE-*bla* antagonists. From the 68 potent antagonists, 65 inhibitory compounds were confirmed in the confirmation screening of SBE-*bla* assay. These 65 compounds were further tested in a group of follow up studies including pSMAD3 assay, cell viability in NSCs, WNT-, RAR-, SHH-*luc* assays. Selected compounds were further tested in the neurite outgrowth assay.

Figure 2.

Effects of agonist and antagonist of SBE-*bla* assay in primary screening. SBE-*bla* activity of natural ligand TGFβ1 (A), reported TGFβ/SMAD inhibitors (B), and potential TGFβ/SMAD inhibitors (C), were plotted in concentration-response curves. Data were expressed as mean ± SD from three independent experiments.

Figure 3.

(A) Compound structural clustering of SBE-*bla* antagonist activity. Each cluster was screened in qHTS SBE-*bla* assay. Clusters were colored according to the enrichment of active compounds which is calculated by $-\log p$ -value. Some clusters were highlighted to represent the compounds of interest in follow up study. (B) Differential inhibitory effects of DTCs in SBE-*bla* activity. Ten SBE-*bla* antagonists in cluster k35.18 were sub-categorized into (1) dialkyl dithiocarbamate complexed with metal ion and (2) tetraalkyl thiuram disulfide/polysulfide have different R-groups. Their IC₅₀ and efficacy values in SBE-*bla* assay were colored based on potencies and efficacies.

Figure 4.

Inhibitory effect of SBE-*bla* antagonists on SMAD3 phosphorylation in HepG2 cells.

HepG2 cells were lysed after 1 h co-treatment of TGF β 1 and test compound. The phosphorylated SMAD3 level was measured by using HTRF technology. Inhibition of TGF β 1-induced SMAD3 phosphorylation were concentration dependent upon the treatment of known TGF β /SMAD inhibitors (A) and metal-organics (B). Data are expressed as mean $\pm$ SD from three independent experiments.

Figure 5.

Inhibitory effects of DTCs and anti-cancer drugs on neurite outgrowth. Neurite length is measured by maximum length method. The number of cells are counted as object number according to the shape of cell body. Neurons treated with DTCs and anti-cancer drugs for 24 h showed inhibition of neurite outgrowth. (A) Representative images of ferbam and thiram induced-neurite outgrowth inhibition. (B) Differential inhibitory effects of DTCs in neurite outgrowth and object number. Dialkyl dithiocarbamates with different metal ions, and tetra-alkyl thiuram disulfide with different R-group were compared separately. (C) Representative images of anti-cancer drugs-induced neurite outgrowth inhibition. (D) Differential inhibitory effects of anti-cancer drugs in neurite outgrowth assay and object number. Data from (B) and (D) are expressed as mean ± SD from three experiments.

qHTS reproducibility parameters. See Methods Section 2.7 for description of detailed criteria for assigning compounds to each reproducibility category.

Table 1.

Assay names	Reproducibility		
	Active match	Inactive match	Mismatch
qHTS SBE- <i>bla</i> assay	0.57%	98.82%	0.61%
qHTS viability assay	2.75%	94.53%	2.72%

During the primary screening of the Tox21 10K compound library, each compound was tested with SBE-*bla* assay multiplexed with cell viability assay in HEK293T cells. Data reproducibility was measured by the percentage of active match, inactive, mismatch and inconclusive compounds. As every compound in the Tox21 10K collection was tested three or more times with various activity outcomes, we assessed the reproducibility of these activity calls. “Active match” or “inactive match” means that most of the calls were active or inactive. “Mismatch” means that most of the calls were contradictory, e.g., half of the calls were active and half inactive. For cases where the discrepancies among the calls were not large enough to meet the “mismatch” criteria, these were grouped into the “inconclusive” category.

Table 2
Outcomes of dimethyldithiocarbamates complexed with different metals in SBE-*bla*, WNT-, RAR-, SHH- *luc* assays.

Sample Name	CASRN	Structure	SBE Antagonist		WNT Antagonist		RAR Antagonist		SHH Antagonist	
			IC ₅₀ (μM)	Efficacy (%)	IC ₅₀ (μM)	Efficacy (%)	IC ₅₀ (μM)	Efficacy (%)	IC ₅₀ (μM)	Efficacy (%)
Ferbam	14484-64-1		0.05	-80.99	0.57	-66.42	0.83	-93.29	0.05	-87.88
Ziram	137-30-4		0.52	-93.60	0.85	-81.79	1.39	-106.31	0.42	-92.50
Sodium dimethyldithiocarbamate	128-04-1		9.14	-68.19	Inactive	Inactive	17.37	-93.31	6.73	-87.30

Potency (IC₅₀) and efficacy (activity %) in inhibiting TGFβ WNT, RAR, and SHH pathways by ferbam, ziram, and sodium dimethyldithiocarbamates were compared in the table.

Table 3

Outcomes of Na-DTCs with different size of alkyl group in SBE-*bla*, WNT-, RAR-, SHH-*luc* assays.

Sample Name	CASRN	Structure	SBE Antagonist		WNT Antagonist		RAR Antagonist		SHH Antagonist	
			IC ₅₀ (μM)	Efficacy (%)	IC ₅₀ (μM)	Efficacy (%)	IC ₅₀ (μM)	Efficacy (%)	IC ₅₀ (μM)	Efficacy (%)
Sodium dimethylldithiocarbamate	128-04-1		9.14	-68.19	Inactive	Inactive	17.37	-93.31	6.73	-87.30
Sodium diethyldithiocarbamate	148-18-5		11.49	-58.53	3.69	-74.76	15.31	-113.16	8.92	-96.63
Sodium dibutylcarbarnodithioate	136-30-1		Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive

Potency (IC₅₀) and efficacy (activity %) in inhibiting TGFβ WNT, RAR, and SHH pathways by sodium dimethyl-, diethyl-, and dibutyl- dithiocarbarnates were compared in the table.