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Review

Nuisance compounds in cellular assays

Jayme L. Dahlin,[1,15,*] Douglas S. Auld,[2] Ina Rothenaigner,[3] Steve Haney,[4] Jonathan Z. Sexton,[5] J. Willem M. Nissink,[6] Jarrod Walsh,[7] Jonathan A. Lee,[8] John M. Strelow,[9] Francis S. Willard,[9] Lori Ferrins,[10] Jonathan B. Baell,[11,12] Michael A. Walters,[13] Bruce K. Hua,[2,14] Kamyar Hadian,[3] and Bridget K. Wagner[2,14]

[1]National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, MD 20850, USA
[2]Novartis Institutes for Biomedical Research, Cambridge, MA 02139, USA
[3]Assay Development and Screening Platform, Helmholtz Zentrum Muenchen, 85764 Neuherberg, Germany
[4]Indiana Biosciences Research Institute, Indianapolis, IN 46202, USA
[5]Department of Internal Medicine, Gastroenterology, Michigan Medicine at the University of Michigan, Ann Arbor, MI 48109, USA
[6]Oncology R&D, AstraZeneca, Cambridge CB4 0WG, UK
[7]Hit Discovery, Discovery Sciences, R&D, AstraZeneca, Alderley Park SK10 4TG, UK
[8]PDD4Patients LLC, Indianapolis, IN 46205, USA
[9]Eli Lilly and Company, Indianapolis, IN 46285, USA
[10]Department of Chemistry and Chemical Biology, Northeastern University, Boston, MA 02115, USA
[11]Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia
[12]School of Pharmaceutical Sciences, Nanjing Tech University, Nanjing 211816, People's Republic of China
[13]Institute for Therapeutics Discovery and Development, University of Minnesota, Minneapolis, MN 55414, USA
[14]Chemical Biology and Therapeutics Science Program, Broad Institute, Cambridge, MA 02140, USA
[15]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02140, USA
*Correspondence: dahlinjl@mail.nih.gov
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SUMMARY

Compounds that exhibit assay interference or undesirable mechanisms of bioactivity ("nuisance compounds") are routinely encountered in cellular assays, including phenotypic and high-content screening assays. Much is known regarding compound-dependent assay interferences in cell-free assays. However, despite the essential role of cellular assays in chemical biology and drug discovery, there is considerably less known about nuisance compounds in more complex cell-based assays. In our view, a major obstacle to realizing the full potential of chemical biology will not just be difficult-to-drug targets or even the sheer number of targets, but rather nuisance compounds, due to their ability to waste significant resources and erode scientific trust. In this review, we summarize our collective academic, government, and industry experiences regarding cellular nuisance compounds. We describe assay design strategies to mitigate the impact of nuisance compounds and suggest best practices to efficiently address these compounds in complex biological settings.

INTRODUCTION

Bioactive but low-quality compounds that interfere with cell-based readouts are an inevitable problem in high-throughput screening (HTS) including phenotypic and high-content assays. These are in contrast to high-quality chemical probes, which are potent, selective, and act by a defined mode of action (Arrowsmith et al., 2015). If not recognized early, low-quality compounds are published as "real" actives in reputable scientific outlets, included in literature reviews, and used by other researchers to propagate low-quality studies (Dahlin et al., 2015b, 2017). This consumes precious scientific resources across academia, industry, and government. For example, the prototypical nuisance compound curcumin has consumed greater than US$150 million of NIH funding, yet is still not recommended as a probe or drug (Nelson et al., 2016).

Nuisance compounds are now a recognized problem among the probe and drug-discovery communities, as well as scientific publishers (Aldrich et al., 2017). Robust tools to identify nuisance compounds in cell-free assays have been developed. However, there is still a significant gap in how best to address nuisance compounds in cellular assays, along with many misconceptions (Figure 1). Fortunately, the reoccurring nature of nuisance compound mechanisms of action (MOAs) can enable specific strategies in assay design and triage.

NUISANCE NOMENCLATURE AND MECHANISMS

Just as there is controversy about what constitutes ideal molecular structures (e.g., fraction of sp^3 carbons [Fsp^3], Lipinski's Rule of Five [Ro5], ligand efficiency, quantitative estimate of drug-likeness), there is no universally agreed-upon definition of what constitutes a "nuisance compound" (Bickerton et al., 2012). We propose some standardized nomenclature (Figure 1). A key distinction should be made between artifacts (i.e., compounds that do not modulate bioactivity but interfere with the assay readout; "technology-related interferences") and compounds that genuinely modulate bioactivity but through

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Misconceptions

FDA-approved drugs cannot act by nuisance mechanism of actions (MOAs)	Nuisance compounds are always toxic to cells	Nuisance compound phenotypes/cellular readouts can be reliably predicted a priori
Reproducible and/or orthogonal cellular activity always rules out nuisance activity	SAR is always attributable to MOA of interest	Concentration-dependent activity is indicative of a desirable MOA
Compounds that interfere with assay technologies cannot be viable leads	Obtaining a desired cellular readout rules out nuisance activity	Nuisance compounds only operate by a clear, single MOA
Virtual screening, fragment, and DEL hits cannot be cellular nuisance compounds	Biophysical confirmation can reliably remove nuisance compounds	Gain-of-signal readouts are impervious to nuisance activity

Key nomenclature

artifacts – compounds that modulate an assay readout by interfering with assay technology and produce apparent activities irrelevant to the biology under investigation ("technical false-positives")

assay interferences – compound behaviors that confound the interpretation of an assay readout or the identification of true-positive compounds

false positives – (1) compounds that give rise to a positive assay readout by means other than the anticipated modulation of bioactivity; in the strict context of engaging a specific cellular target, a pathway modulator could be considered a false positive ("off-target" activity); (2) compounds whose bioactivity cannot be replicated upon re-retesting ("statistical false-positives") due to technical errors in executing the protocol (e.g., liquid handling errors, plate edge effects, unstable reagents, etc.)

nuisance compounds – undesirable compounds whose apparent bioactivities are due to assay technology-related and non-technology-related interferences

pan assay interference compounds (PAINS) – compounds harboring chemical substructures that were associated with promiscuous bioactivity (Baell and Holloway, 2010); often informally (but incorrectly) used as a catch-all term for nuisance compounds

phenotypic assay – cell- or organism-based biological assays, including high-content assays, that measure a phenotype; can discover unanticipated mechanisms of action (MOA) by virtue of being target-agnostic

promiscuous compounds – compounds with greater than expected frequencies of significant activity across a variety of biological assays; this classification can be influenced by assay technology, choice of targets, institutional practices, as well as other confounding factors

screening actives – the initial set of chemical matter identified as active from the screen, containing all types of true and false mechanisms

true positives – compounds giving rise to the expected cellular readout in the assay (in target-based assays, engaging with the desired target or pathway)

Figure 1. Misconceptions and nomenclature regarding cellular nuisance compounds

undesirable MOAs (Figure 2). Therefore, a nuisance compound can still be a true positive in a given assay if it gives rise to a desired biological readout, albeit through an unsought MOA.

In cell-free assays, nonspecific electrophilicity, colloidal aggregation, redox cycling, and chelation are well-known nuisance behaviors. Nonspecific reactivity, redox activity, and chelation lead to biological targets (mainly proteins) being modulated by covalent and ionic interactions. Conversely, aggregators and surfactants perturb proteins by reversible contacts via partial or full secondary/tertiary structure denaturation (Coan et al., 2009).

Cellular assays tend to enrich for a variety of compounds that cause, in a broad sense, cellular injury. Examples we routinely encounter are genotoxins, lysosomotropic agents, membrane disruptors, and tubulin poisons. Depending on the context and goals of a particular screening effort, these mechanisms may be regarded as nuisance behaviors. Even high-quality chemical probes and drugs can be bioactive by nuisance MOAs when tested at sufficiently high concentrations. Cytotoxins are not necessarily prohibitive, but are more desirable when they display biological selectivity or mechanism-based cytotoxicity. Recent work by the NIH/EPA Tox21 program has illuminated the concept of "cytotoxicity burst," which occurs at relatively high compound concentrations when cellular activities are thought to result from the activation of multiple stress responses as opposed to originating from activation of a specific molecular target (Escher et al., 2020).

The finer details of nontechnology interferences are less well understood in cellular assays. Here, nuisance compounds will generally interfere by causing cellular injury. Due to their inherent lack of specificity, many nuisance MOAs are consistent across cell lines and assay types. Exceptions exist, such as metabolic toxins across cells with different energetic states (e.g., glycolytic versus oxidative phosphorylation). Colloidal aggregates do not appear to penetrate mammalian plasma membranes, but they can limit the activity of drugs in cell-based assays and perturb membrane targets such as G-protein-coupled receptors (GPCRs) (Owen et al., 2014; Sassano et al., 2013). Nonspecific electrophiles can disrupt specific proteins and also initiate adaptive responses in cells (e.g., NRF2 pathway).

Compounds can also interfere with cellular assay technologies. This often occurs through light-based interferences such as compound light absorption and autofluorescence. Even in microscopy assays, these compounds can produce diffuse and/or localized patterns that can mimic bioactive morphologies (Johnston et al., 2016). Compounds can also interfere with specific technologies (e.g., luciferase inhibitors, AlphaScreen $^1O_2$ quenchers, capture reagent disruptors) (Coussens et al., 2020).

BLACK BOXES: THE DIFFICULTY OF CELLULAR NUISANCE BEHAVIORS

The allure and curse of cellular assays is that compounds can usually elicit a particular response by known and novel MOAs. However, even active readouts in target-based cellular assays can occur by on- or off-target effects, and without detailed follow-up experiments the machinations behind a given readout are essentially a "black box" (Solinski et al., 2019). Compounds

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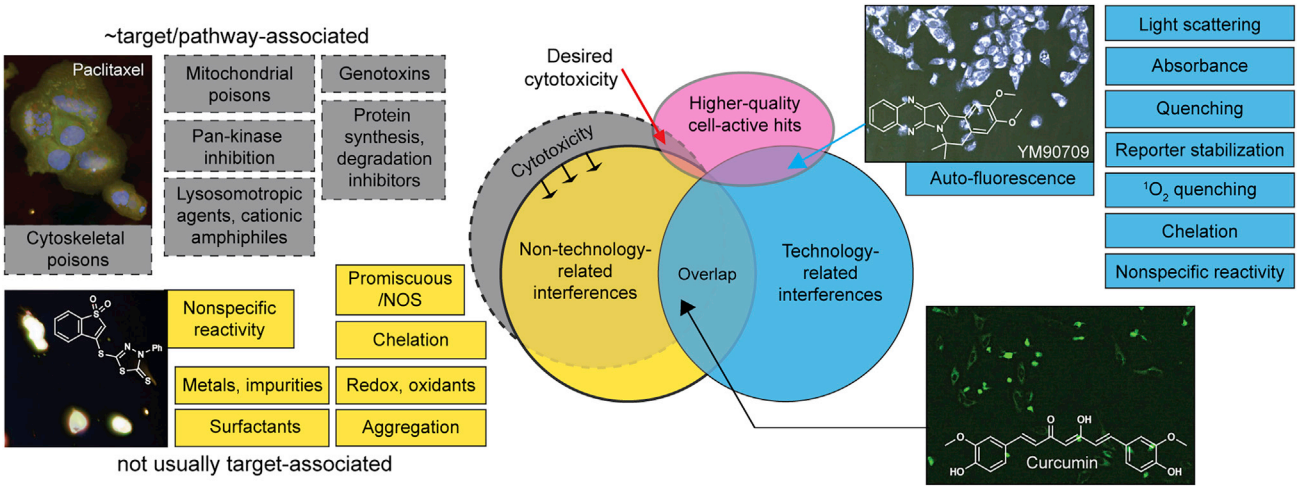

Figure 2. Cellular nuisance compound framework

Interferences can be broadly divided into technology-related ("artifacts") and non-technology-related categories (undesirable, nonspecific activity), and can overlap. Most non-technology-related interference mechanisms can lead to cytotoxicity. High-quality hits can still have interference such as autofluorescence or selective cytotoxicity.

may also interfere with the function of extracellular protein stimuli used in certain cellular assays (Blevitt et al., 2017). This lack of clear-cut MOAs makes compound prioritization difficult, as it is less obvious which factors should guide triage (Figure 3). A cascade of follow-up assays is essential for hit triage, perhaps even more so than traditional target-based approaches.

Care should be taken when correlating cell-free and cellular readouts. A common trap occurs when performing a biochemical validation assay, observing a desired response, and then equating the original cellular readout with this specific activity. The converse situation is also common: low-quality compounds derived from virtual or biochemical HTS can show apparent "confirmatory" cellular activity driven by a loss-of-signal or cytotoxic effect (Dahlin et al., 2017). Prototypical nuisance compounds can produce "positive" readouts in biophysical assays (Bista et al., 2012; Coussens et al., 2018). Biophysical assays will best support target-associated cellular readouts when compound series show sufficient combinations of potency (usually nanomolar or low micromolar), stoichiometry, and interpretable structure-activity relationship (SAR) (Lessene et al., 2013; Markwalder et al., 2017).

The case of the small molecule remodelin illustrates many of the above points (Figure 3). This hydrazone was identified by a phenotypic screen for compound-dependent nuclear remodeling in Lamin A/C-depleted cells. The remodelin-induced phenotype was attributed to NAT10 inhibition based on micromolar biochemical activity, pull-down of NAT10 (among >50 other proteins), and other experiments (Larrieu et al., 2014). NAT10 was specifically chosen for follow-up based on previous studies linking the parent phenotypic lead compound CPTH2 to KAT inhibition (Chimenti et al., 2009). Hydrazones such as CPTH2 are nonspecific electrophiles and promiscuous (Dahlin et al., 2017), making such a link tenuous. Follow-up studies demonstrate that remodelin does not actually modulate NAT10-dependent cytidine acetylation activity in cells (Shrimp et al., 2020). Nonspecific compounds such as remodelin may "phenocopy" knockdown phenotypes of crucial genes such as NAT10 purely by coincidence. This is particularly dangerous if a phenotype is far removed from the biochemical activity of the proposed target (e.g., nuclear circularity and RNA acetylation). Reviewers of such papers must assess the strength of evidence, and determine whether it constitutes rigorous demonstration or is merely "consistent with" a proposed mechanism and subject to confirmation bias.

POTENTIAL FOR NUISANCES IN DRUG REPURPOSING

The popularity of using phenotypic screening libraries of clinical compounds with the hope of a simple repurposing path runs counter to the significant hurdles seen when repurposing drugs (Edwards, 2020). However, drug repurposing is not immune to nuisance activities. Cationic amphiphilic drugs (CADs) frequently appear as bioactive in repurposing screens, and exhibit an array of nonspecific cellular perturbations such as lysosomal accumulation and phospholipidosis (Gunesch et al., 2020; Salata et al., 2017). Antimicrobial, anticancer, and other suspiciously broad cell-based activities are commonly reported (Costa Silva et al., 2017; Gunesch et al., 2020; Jang et al., 2019; Kaiser et al., 2015; Lagadinou et al., 2020; Li et al., 2016; Salata et al., 2017; Vazão et al., 2017; Weeks et al., 2018). In these offending cases, activities can be weakly potent (micromolar).

Complex cellular behavior by CADs may confound their identification as nuisance compounds at screening concentrations. A given CAD may influence cellular signaling without gross cytotoxicity, giving a superficial impression that the compound is selective via a specific mechanism. For example, chlorpromazine can increase membrane permeability through nonspecific interaction while amiodarone alters lipid dynamics by interacting with the membrane bilayer hydrophobic core (Salata et al., 2017). Some CADs may therefore give a readout while others will not, imparting an impression of SAR. Important caveats must be mentioned: a CAD may still be subject to successful target-based optimization when undertaken rationally, and CADs designed to specifically target physicochemical differences in pathogen membranes may still represent fruitful avenues (Moretti et al., 2019; Waldschmidt et al., 2017).

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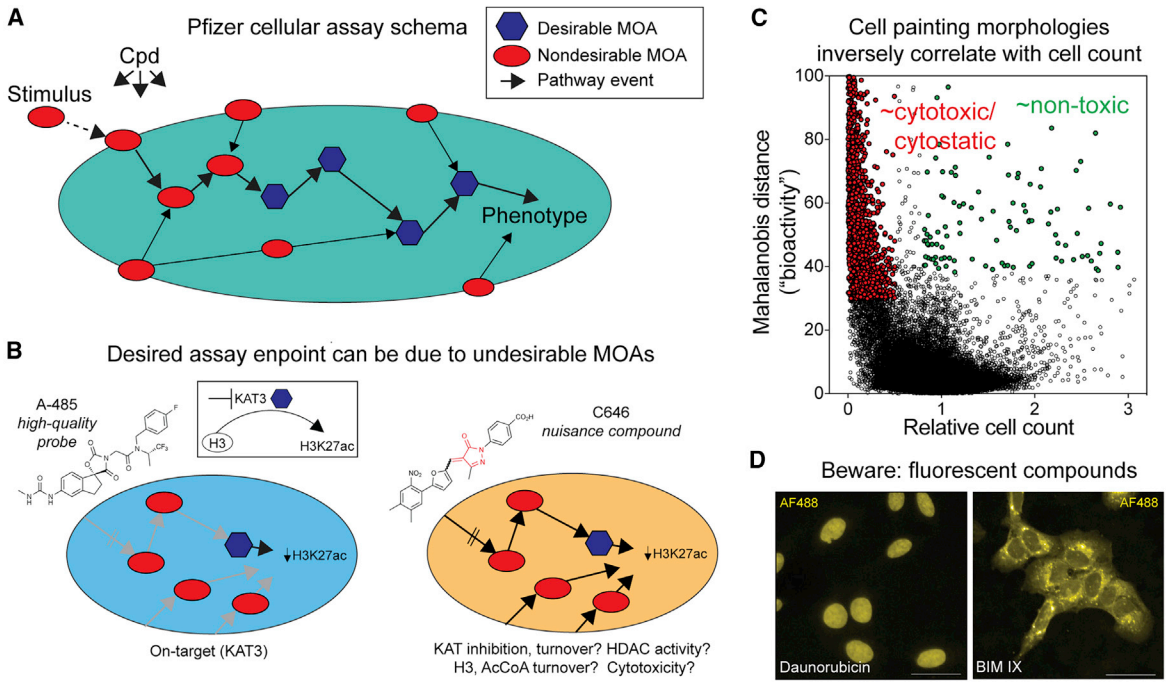

Figure 3. Common pitfalls of cellular nuisance compounds

(A) Pfizer model for conceptualizing cellular assay readouts (Vincent et al., 2015).

(B) KAT3 inhibitors A-485 and C646 both decrease cellular H3K27ac levels as expected, but through specific and nonspecific target engagement, respectively (Dahlin et al., 2017; Lasko et al., 2017; Shrimp et al., 2015).

(C) Active cell-painting morphologies are enriched in compounds that decrease final cell number (Bray et al., 2017).

(D) Compounds can fluoresce and interfere with the interpretation of readouts in microscopy-based cellular assays. Scale bars, 50 μm.

(E) Nuisance compounds from phenotypic assays can appear "active" in subsequent target-based assays and are at risk for confirmation bias.

NATURAL PRODUCTS

Natural products (NPs) represent a valuable source of chemical diversity, but they can still be nuisance compounds interfering by all the aforementioned MOAs including membrane perturbation (Ingólfsson et al., 2014). NP samples present unique challenges; extracts can have uncharacterized chemical contents, be enriched for endogenous substances (salts, fatty acids, lipids), and can include tannins (nonspecific-binding polyphenols). NPs themselves can contain notoriously problematic chemotypes such as quinones (Appleton et al., 2007; Baell, 2016). Potential solutions include testing NP samples at multiple concentrations and prefractionating NP extracts (Cheng et al., 2015; Thornburg et al., 2018). Difficulties with the reproducibility and scale-up of NP biosyntheses, chemical characterization, synthesis, and retesting present additional challenges. Compound extraction and chemical instability can introduce various chemical artifacts and unnatural structural rearrangements, and incorrect chemical characterizations such as stereochemistry are not uncommon (Capon, 2020; Maltese et al., 2009). The perspective that NPs at screening concentrations may signal nonspecifically in bioassays can be obscured by their association with drug discovery as privileged structures (Baell, 2016).

NUISANCE COMPOUNDS AND EMERGING TECHNIQUES

Newer cell-based screening techniques and nuisance compounds will inevitably intersect. There is growing interest in applying fragment-based and DNA-encoded library methods to cellular assays (Cai et al., 2019; Parker et al., 2017; Wu et al., 2015). Given the

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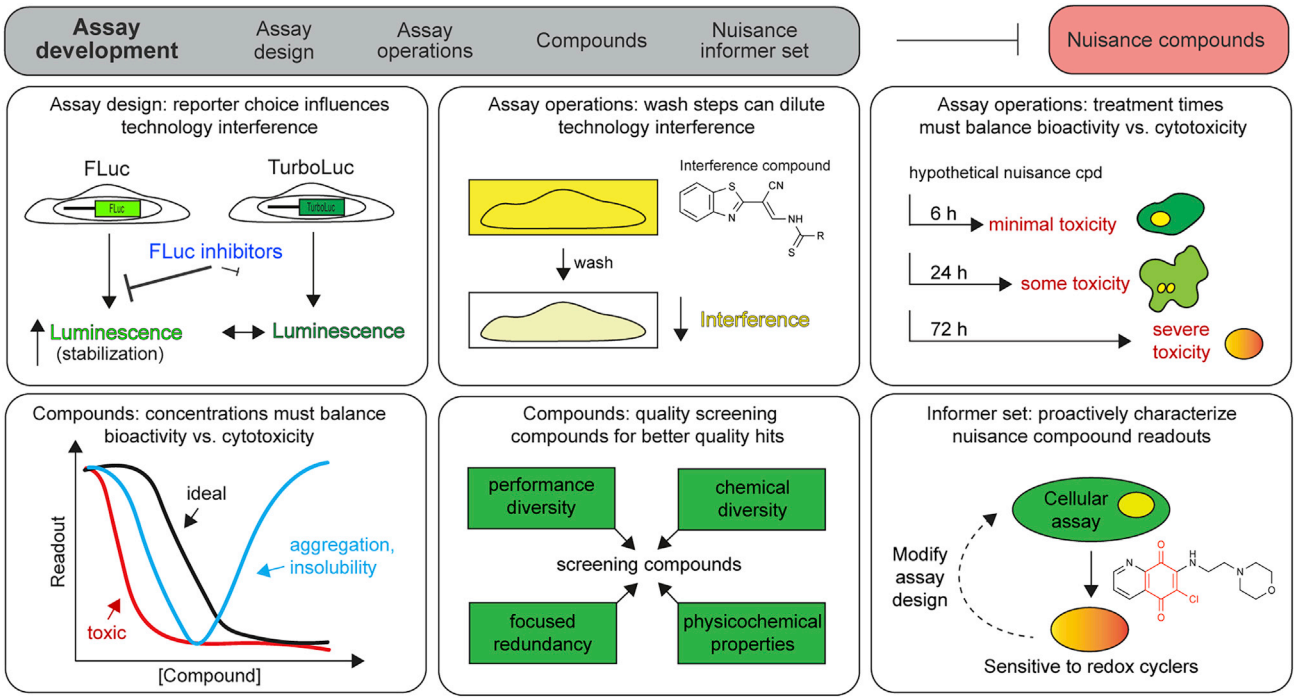

Figure 4. Proactively addressing cellular nuisance compounds during assay development

emergence of covalent fragments (Keeley et al., 2020), one can imagine this method being increasingly applied to cells (Backus et al., 2016). The success of modern targeted covalent modulators (Singh et al., 2011) may also inspire more primary screening of compounds with covalent warheads. These newer methods are still expected to enrich for nuisance MOAs, given the high compound concentrations typically used in fragment-based screening. In cases of covalent-binding hits (fragments or otherwise), a premium should be placed on cellular health assays and characterizing proteome-wide selectivity (Lanning et al., 2014).

MITIGATION OF CELLULAR NUISANCE BEHAVIOR BY ASSAY DESIGN

Maximizing knowledge of the cellular assay in response to nuisance compounds prior to large-scale screening is highly advantageous. Choosing conditions to maximize robustness can prevent follow-up on irreproducible hits, while other conditions can render readouts less susceptible to nuisance MOAs (Figure 4).

Assay technologies and readouts

Assay technology and reporter choices may enrich for specific compound interferences. For example, PTC-124 was identified and likely optimized in a cellular luciferase expression assay for nonsense codon suppression (Welch et al., 2007). It stabilized the translated FLuc reporter used for its discovery in a manner that paralleled the desired read-through activity in the assay, creating debate regarding its MOA (Auld et al., 2010; McElroy et al., 2013). By understanding the fundamentals of luciferase interference, one can exploit the different small-molecule inhibition profiles of luciferase constructs by counter-screens of coincidence reporters to better select noninterfering and noncytotoxic compounds (Auld et al., 2018; Cheng and Inglese, 2012). Reporters can have a significant bearing on the prevalence of compound-dependent optical interferences, as reporters with longer excitation/emission wavelengths (e.g., far-red versus green fluorophores) and time-resolved fluorophores are less prone to interferences (Imbert et al., 2007; Vedvik et al., 2004).

Gain-of-signal/gain-of-function (GOS/GOF) and rescue experiments are generally more resistant to cytotoxic nuisance compounds. Disrupting a cellular process is comparatively easier than enhancing an already highly optimized process, such as cancer screening assays where hits usually lower assay outputs such as cell proliferation and viability (Kaelin, 2017). In line with this thinking, many cancer drugs are found to kill target cells by off-target toxic activity (Lin et al., 2019). Assays where hit compounds increase signal output or certain cellular functions can have fewer false positives. For example, differential GOF cellular assays can be constructed to rescue or inhibit basal cytotoxicity (Brito et al., 2020). However, not all biological systems are amenable to GOS/GOF design, and these technical manipulations may perturb the relevant biology. Care should still be taken when interpreting GOS readouts, as simple readouts such as increased cellular GFP expression can select for green autofluorescent compounds (Johnson et al., 2008). Higher-quality readouts might include functional elements that are usually resistant to simple interferences.

Assay operations

The order, timing, and technical settings of each assay step can have profound effects on nuisance activities. Autofluorescent compounds constitute a small overall fraction of a screening library, though more frequently in the blue and green fluorescent regions (Simeonov et al., 2008). These types of nuisance

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compounds are often diluted by repeated washing steps, or can be rendered unimportant by assay design (Henrich et al., 2006; Johnson et al., 2008). Washing is not a care-free step, as compounds that disrupt cellular adhesion can lead to decreased cell counts. Furthermore, washing may not effectively remove intracellular nuisance compounds to prevent compound-dependent interference, as shown in an assay for GFP reporter interference that identified all of the primary active hits from a 315K compound HTS as autofluorescent artifacts (Ibáñez et al., 2017). Treatment times are also critical determinants of certain nuisance MOAs. Many cellular nuisance compounds are nonspecific and ultimately cytotoxic, and often these irreversible actions will be time dependent (Hsieh et al., 2017). Grossly cytotoxic nuisance mechanisms usually require at least 6–24 h to induce apoptosis or other cell-death pathways (Galluzzi et al., 2018). Greater treatment times (48 h, 72 h) can also enrich for compounds that cause cellular injury or reduce cell adhesion.

Choice of compounds and concentrations

Most interference mechanisms are concentration dependent, and one can be falsely reassured by observing a concentration-dependent response in cellular assays. A compromise is usually struck between testing "low-enough" compound concentrations to minimize interferences/off-target effects and "high-enough" concentrations to identify bioactive compounds. A good strategy is to run a pilot screen at multiple compound concentrations and include known nuisance compounds to select an optimal screening concentration.

Both high-quality and nuisance compounds should be tested in concentration-response curves (CRCs), either in the primary screening (quantitative HTS [qHTS]) or the primary confirmation stage (Cheng et al., 2015). The complexities of cellular assays confounds CRC interpretation, although primary qHTS data can be quite pharmacologically revealing (Kinder et al., 2020; Sotoca et al., 2010). Membrane permeability, xenobiotic metabolism, serum protein binding, and compound efflux are some confounding factors. Certain curve features can be suggestive of interference MOAs. For example, steep and bell-shaped CRCs with lower asymptotes above 10 μM are consistent with but not definitive proof of aggregation and/or poor solubility (Owen et al., 2014).

Precedent often shapes compound screening collections, but it is worthwhile to curate collections for cell screening (Figure 4) (Spear and Brown, 2017). Screening collections for cell-based assays should contain sufficient chemical and performance diversity, ideally with built-in chemical and bioactivity redundancy to help establish preliminary SAR and hypotheses (Dahlin and Walters, 2014; Wawer et al., 2014). Libraries can also be culled for highly problematic chemotypes, with a caveat that many substructure filters were derived from cell-free HTS data as well as high concentrations less applicable to cell screening. For capacity reasons, libraries can also be designed for physicochemical properties that favor cellular activity such as permeability. However, a caveat is that impermeable compounds may give rise to extracellularly-mediated effects by affecting transmembrane proteins (e.g., GPCRs, ion channels, solute carriers). Prediction of passive and active permeability is not straightforward, but it is possible to remove known cell-impermeable compounds and those with a high likelihood of impermeability based on their physicochemical properties (e.g., polar surface area, ionization, Ro5).

Informer sets

Screening is an enrichment exercise. Purposefully testing nuisance compounds during assay validation can inform screeners about an assay's sensitivity to unwanted MOAs and the associated readouts. This information can help to design more effective cellular assays and screening cascades for hit triage (Figure 4). Screening focused collections of high-quality reference compounds is a standard industry practice when piloting cellular assays. Several industrial screening operations also take an opposite approach, employing informer sets composed of nuisance chemical matter (Hansson et al., 2018). Challenging assays with endogenous and exogenous interferants is routine in clinical assay validation (Kroll and Elin, 1994). Some reference compounds will show similar phenotypes in high-content assays across different cell lines (Hughes et al., 2020; Warchal et al., 2019; Willis et al., 2020). However, compound phenotypes may not transfer across cell lines or altered experimental conditions, suggesting value in routinely using informer sets. Confidence in nuisance-related readouts can be enhanced by including inactive analogs (e.g., weaker leaving groups or warheads for electrophiles) (Dahlin et al., 2015b).

IDENTIFYING BADLY ACTING COMPOUNDS AFTER SCREENING

Guided by the relevant disease biology and desired safety profiles, we recommend multiple experimental- and knowledge-based approaches to investigate cell-active compounds for nuisance behaviors (Figures 5 and 6).

Cell-free counter-screens

Fortunately, many nontechnology interferences (thiol reactivity, aggregation) and technology interferences (luciferase inhibitors, optical interference) already have robust biochemical counter-screens developed. Since their readouts are more straightforward to interpret, we recommend testing cell-active compounds for a comprehensive panel of compound interferences. However, cell-free conditions may not model the behavior of compounds in cells, and these assays are themselves susceptible to compound interferences. Compounds may show "clean" behaviors in cell-free reactivity counter-screens but have "masked electrophiles" or metabolites that are reactive in cells (Eaton et al., 2019). A lack of cell-free selectivity versus a panel of related and unrelated molecular targets (e.g., kinases, GPCRs) can hint at nuisance MOAs in cells (Dahlin et al., 2017).

Secondary cellular assays

For cell-based hits, we recommend a combination of (1) technology counter-screens to rule out artifacts, (2) counter-screens to rule out undesirable MOAs, and (3) orthogonal cellular assays to confirm desired bioactivity. The timing and arrangement can vary due to logistics, the number and nature of hit compounds, individual preference, and pilot experiments with informer compound collections (Dahlin and Walters, 2014).

Characterizing technology interferences by counter-screens determines the level of confidence in a particular readout. A straightforward counter-screen for compound autofluorescence is parallel image acquisition of compound-treated cells with and without reporters (Ibáñez et al., 2017) (Figure 5). A related counter-screen for readout attenuation interference (e.g.,

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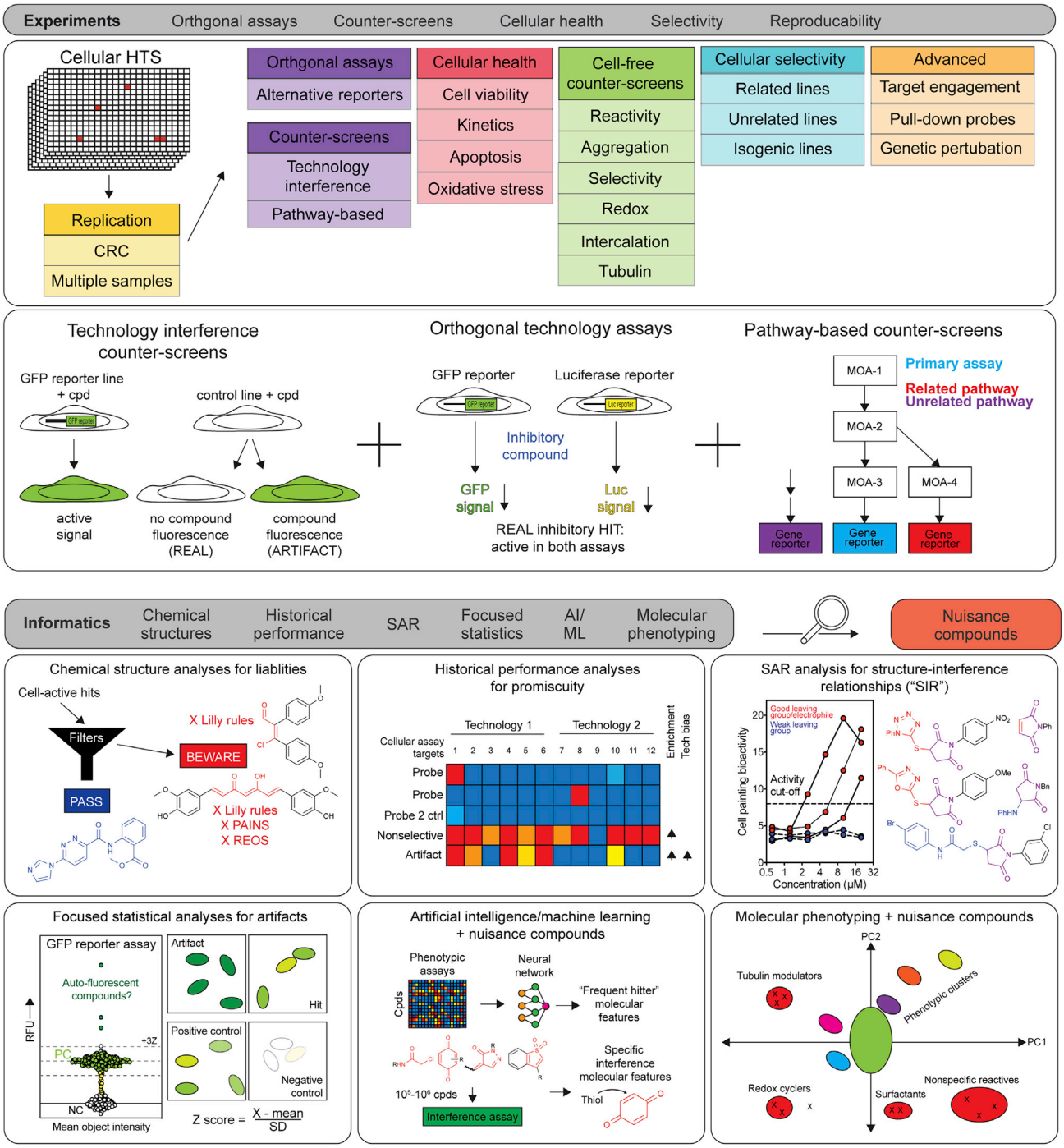

Figure 5. Approaches to identify cellular nuisance compounds

quenchers) is to spike compound into a constitutively expressed or prestained control.

It is central dogma in screening to confirm bioactivity by orthogonal methods. In cell-free assays, this is traditionally done with independent assay technologies, while cellular orthogonal assays may instead incorporate different cell lines, pathway components, or readouts. Reliance on a single readout or technology is unwise, as it can drive optimization toward an artifactual bioactivity (Auld et al., 2009). The choice of primary and secondary assay design, and what biological (and technological) spaces they cover, will determine the types of active compounds (Vincent et al., 2020). For instance, follow-up assays on a pathway process upstream of the primary assay may fail to confirm compounds acting more downstream in the pathway of interest. Similarly, orthogonal assays that are susceptible to the same interferences as the primary assay may instill false

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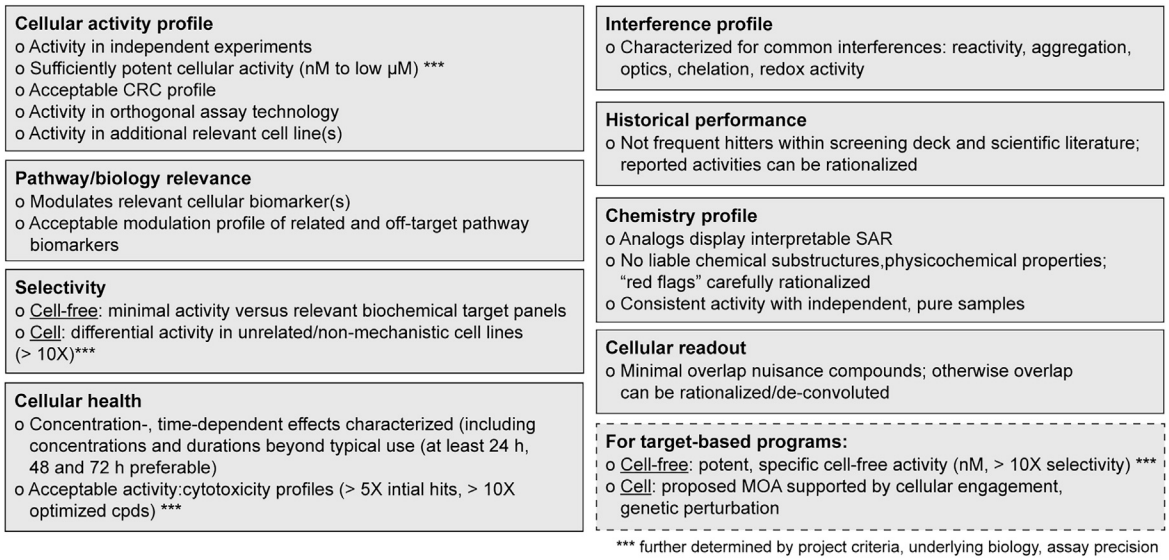

Figure 6. Decision aid for nuisance compound triage

confidence about a particular compound MOA. It is prudent to choose distinct readout technologies to understand the compound effect on a particular pathway/phenotypic signal output (Figure 5). Cellular orthogonal approaches can also utilize different readout classes such as biomarkers or cell function. For instance, in the identification of SMN2 splicing modifiers, primary luciferase gene reporter hits were confirmed with quantitative PCR for the full-length *SMN2mg* and Δ7 transcripts (Naryshkin et al., 2014).

Leveraging knowledge of disease- or mechanism-related biology and translational safety concerns can be advantageous, including measuring proximal or distal biomarkers (Vincent et al., 2015, 2020). For example, the discovery of the STING agonist SR-717 by phenotypic screening benefited from pathway-based counter-screens. The primary screen identified inducers of the downstream STING pathway component IRF, followed by counter-screens with STING and cGAS knockout cell lines (Chin et al., 2020). In another example, three unrelated transcripts (cyclic AMP response element, nuclear factor κB, transforming growth factor β) were profiled in parallel to exclude off-target activities and technology interference during a phenotypic screen for inhibitors of β-catenin-mediated transcription (Huang et al., 2009).

Cell-line dependence can be leveraged for counter-screening purposes. Similar activity in multiple related cell lines (or multiple donors in primary cells) can enhance confidence (Johannessen et al., 2017; Orellana et al., 2018). Cells without the relevant biological stimuli, or isogenic cells that lack the target of interest, are useful for assessing specificity (Torrance et al., 2001). Depending on the biological context, control experiments could be mutant or knockdown cell lines, nondiseased control tissues, or unrelated tissue types (including other species). Examples include comparing the cytotoxicity of NP extracts to mutant and wild-type c-KIT cell lines, and using mutant and species-specific proteosomes to confirm the target of the broad-spectrum antiprotozoal drug GNF6702 (Henrich et al., 2007; Khare et al., 2016). However, apparent selectivity of compounds between cell lines does not necessarily rule out nuisance MOAs, as other factors such as cell-line proliferation rates, culture conditions, and constructs may introduce bias (Bancos et al., 2013). Meaningful selectivity depends on the biology and assay precision (minimum significant ratio), but tenfold or greater differences are considered to be more believable (Eastwood et al., 2006).

Cellular health

Cellular health assays are high-yielding experiments for assessing cellular nuisance compounds, and should be run in parallel or immediately after the primary assay (Kinder et al., 2020). This is especially true in assays where grossly toxic compounds can reduce the reporter signal by killing cells. A distinction should be made between compound-dependent cellular injury at the screening and later preclinical stages (''toxicity''). A nonsurgical use of cytotoxicity criteria for triage can occasionally backfire. Concerns should especially be raised when the desired compound-dependent biology should not involve cytotoxicity (e.g., based on genetic perturbations or chemical inhibition of relevant pathways). The discovery of compounds that reduce viable cell counts may be desirable, for example in anti-neoplastic screens.

Whenever possible, a surrogate or direct cytotoxicity readout should be incorporated into a primary cellular screen, such as nuclear counts or confluence in high-content assays. If technically difficult, facile cellular viability assays such as CellTiter-Glo are useful options. We recommend image-based confirmation of cell counts, as cytotoxic compounds can interfere with metabolism-based assays (Chan et al., 2013). Follow-up cellular health assays should examine for evidence of cytotoxicity (loss of membrane integrity, intracellular dye reduction) and induction of apoptosis (caspase-3/7 activation, extracellular annexin V). More specialized assays should be performed as needed.

Cellular health assays should span a potency range to define the maximal noncytotoxic compound concentrations, and include

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time-course measurements that extend past primary time points for nonacutely toxic compounds. This information can be efficiently determined by live-cell imaging, multiplexed reagents, and/or aforementioned high-content-based strategies (Didiot et al., 2011). In our experience, nuisance compounds induce the active cellular readout within a few-fold concentrations of cytotoxicity, and in such cases should be cautiously pursued. Compounds can also be profiled for differential cytotoxicity across multiple cancer cell lines, such as the NCI60 or PRISM panels, to provide clues about MOA (Figure 5) (Shoemaker, 2006; Yu et al., 2016). Profiling hundreds of cancer cell lines helped to identify GPX-4 and ferroptosis as the primary molecular target and biological pathway of the electrophiles ML-162 and ML-210 (Viswanathan et al., 2017). Metrics such as GR_{50} may better account for inter-cell-line variations in division rate (Hafner et al., 2017).

False positives and compound integrity

Most nuisance compounds are actually highly reproducible. However, retested compounds that fail to reproduce with independent samples are often due to compound instability or contaminants from preparation procedures. Mechanisms of degradation include nonenzymatic oxidation, hydrolysis, thermal and photocatalyzed decomposition, and retro reactions (Baell et al., 2013; Olson et al., 2015; Stefaniak et al., 2018). Bioactive impurities can be synthetic contaminants such as metals (Zn, Hg), catalysts (Pd), and reaction side-products (Hermann et al., 2013; Morreale et al., 2017; Tarzia et al., 2007). The presence of trifluoroacetic acid and other agents can contribute to apparently selective and "noisy" activities, with some collections showing noisy activity in up to 80% of the set (Chakravorty et al., 2018; Stewart et al., 2014). High-priority compounds are best tested with freshly solubilized samples from solid stock, repurified samples, and even resynthesized samples (ideally by orthogonal synthetic routes) (Dahlin and Walters, 2014).

Compound stability is heterogeneous and multifactorial. However, initial purity is the main determinant of compound stability (Popa-Burke et al., 2014). Studies vary, but estimates ranging from 15% to 40% or more of screening compounds will show measurable degradation (Blaxill et al., 2009; Bowes et al., 2006; Shou et al., 2020). Ideally, all prioritized hits are assessed for purity using liquid chromatography-mass spectrometry approaches as a first indication whether a screening data point can be trusted. The increased availability of integrated higher-throughput compound characterization and reporting methods early in the hit evaluation cascade can increase the efficiency of this process (Shou et al., 2020).

Chemoinformatics and structural analyses

Certain discovery programs are rethinking structure-based triage in phenotypic screening (Vincent et al., 2020). Well-reasoned arguments include the potential for overzealous and subjective triage, and the inherent lack of knowledge regarding compound MOA to guide triage. As bioactivity is fundamentally determined by chemical structure, and because libraries may still be composed of highly dubious chemotypes, we still recommend analyzing cell-active chemical structures. If resources allow, we recommend secondary testing of all primary hits to allow for data-driven prioritization. Outright triage of cell-active NPs and atypical compounds may not be warranted, given the known progression of less-drug-like entities in the clinic (DeGoey et al., 2018). However, a hidden danger to retesting compounds with structural liabilities is that they can produce tantalizing bioactivity that needlessly prolongs their retainment.

Chemical structure and physicochemical property filters (e.g., PAINS, REOS, APT) are an efficient approach to rapidly flag cell-active compounds for problematic chemotypes and properties (Figure 5) (Baell and Holloway, 2010; Cox et al., 2012; Walters and Namchuk, 2003). While these filters have limitations such as technology and historical biases, they are still helpful tools when used alongside the other components of a comprehensive screening cascade. In many of our practices, cell-active hits flagged by these filters are scrutinized and usually deprioritized unless additional experiments can be performed to verify optimizable bioactivity.

Since nuisance compounds often act by nonspecific MOAs, they are usually enriched in bioactivity across a variety of targets, cell types, and assay technologies. Mining historical screening data works best when the screening data are well curated, with a caveat being the potential for historical or institutional biases (e.g., repeated testing with similar targets, assay technologies). Bioassay promiscuity can be quantified by a variety of "frequent-hitting" indices such as pBSF or percent enrichment (Baell and Holloway, 2010; Chakravorty et al., 2018; Nissink and Blackburn, 2014). Large, high-quality annotated chemical activity databases allow for more quantitative approaches to grading repeatedly assayed compounds, such as a "Tool Score" algorithm that uses heterogeneous historical screening data to classify compounds as "tool-like" or "non-tool-like" (Wang et al., 2016). Adaptation and validation of such quantitative metrics should reduce subjective judgments and enhance objective compound classifications. While less quantitative and more laborious, it is also worthwhile to investigate the published scientific and patent literature related to cell-active compounds (Dahlin and Walters, 2015). Repeatedly published compounds across seemingly unrelated biological systems should raise caution.

Structure-activity relationships

SAR is one of the most important yet least appreciated arbiters of nuisance activity. This usually requires assembly of a focused SAR set using commercially available analogs and in-house chemistry (Yi et al., 2020). Assuming a cellular readout is governed by a dominant and specific target engagement, rational SAR with cellular and cell-free potencies spanning multiple logs of concentration should be forthcoming (Lee et al., 2012). Nuisance compounds will characteristically fail this test, often furnishing large tracts of flat SAR, indicating the compound series is not amenable to medicinal chemistry optimization (Baell, 2010, 2015). To add more complexity, nuisance compounds can also appear to display SAR when the compound set is insufficiently large. Different permeability and media-binding properties can add layers of variables to cellular SAR interpretation. A compound series can also show apparent SAR that can be completely explained by interferences ("structure-interference relationships," Figure 5) (Auld et al., 2009; Dahlin et al., 2015b).

Focused statistical analyses

Artifacts can be identified by straightforward *Z*-score calculations for significant deviations in key parameters relevant to

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particular interference MOAs (Figure 5). In microscopy assays, strongly autofluorescent and quenching compounds can characteristically exhibit high and low object-intensity *Z* scores in various imaging channels, respectively.

Artificial intelligence/machine learning

Advances in artificial intelligence/machine learning (AI/ML) have the potential to address cellular nuisance compounds. This could take two main approaches: model training based on chemical structures linked to undesirable nuisance behaviors, or model training on cellular assay readouts caused by prototypical nuisance compounds (Figure 5). The former case would benefit from library-wide profiling specifically for assay interferences including cytotoxicity (Lee et al., 2020; Schorpp et al., 2014; Simeonov et al., 2008). Already, this approach has been applied to aggregators and luciferase inhibitors (Alves et al., 2020; Ghosh et al., 2018). Advances in computation and profiling may also enable cell-level analyses of nuisance activities for cases where population ("well")-level readouts mask nuisance MOAs in cellular subpopulations (Gough et al., 2014). One foreseeable danger of AI/ML is models based on uncharacterized nuisance activities whereby users unknowingly optimize toward interferences.

ROLES OF ADVANCED EXPERIMENTS

The most egregious nuisance compound behaviors can be assigned using the aforementioned approaches. While seldom performed, suspected nuisance compounds can occasionally benefit from more complex experimental approaches to assess their compound-target engagement, selectivity, and overall MOA in accordance with high-quality probe criteria. Chemoproteomic probes labeled with capture or detection reagents by "click chemistry" can provide evidence of proteome-wide promiscuity, as in the cases of the reportedly selective KAT3 and MDMX inhibitors C646 and SJ-172550, respectively (Shrimp et al., 2015; Stefaniak et al., 2018). Target engagement assays such as CETSA, DART, and SPROX show potential utility for assessing specific compound-target interactions (Jafari et al., 2014; Lomenick et al., 2009; West et al., 2008). Continued advances in proteomics such as activity-based protein profiling can now quantify compound-protein binding across the proteome (Backus et al., 2016; Jessani and Cravatt, 2004). While these high-powered technologies hold considerable utility, in practice they are not yet routinely used for interrogating early leads, perhaps due to the resources and expertise required as well as the need for sufficient compound-target binding (often lacking early on in discovery campaigns) and chemistry support for probe design and synthesis. Higher throughputs and reduced costs may address these barriers. Similar arguments should apply to newer genetic-based target deconvolution strategies such as arrayed CRISPR libraries and pooled optical screening (Feldman et al., 2019; Jost and Weissman, 2018; Metzakopian et al., 2017).

Molecular phenotyping is another approach with the potential to evaluate for nuisance activity in cellular assays (Drawnel et al., 2017). Here, compound-treated cells are annotated by multiple parameters such as morphology, transcriptomics, proteomics, or metabolomics to create phenotypic "signatures." Compounds with readouts similar to nuisance compounds are "guilty by association." Hence, testing a collection of nuisance informer compounds can be highly valuable from the onset. Several classes of frequent-hitting cell-active compounds such as tubulin modulators and mitochondrial poisons can also be readily identified through commercial profiling services such as BioMAP (Berg et al., 2006). Compounds that cluster near or correlate with known nuisance compounds can then be more thoroughly examined for potential nuisance behaviors (Figure 5). However, most profiling studies understandably avoid testing nuisance compounds, so the potential for such advanced approaches would benefit from the purposeful inclusion of nuisance compounds.

For morphological assays, orthogonal profiling could include higher-throughput gene expression methods such L1000 and DRUG-seq (Subramanian et al., 2018; Ye et al., 2018). Compared with morphology, gene expression profiling is more amenable to shorter compound treatment times, and changes in gene expression can more directly lead to mechanistic hypotheses due to specific knowledge of transcript function. Cellular morphology and transcriptomic profiles can be nonredundant, and, notably, promiscuous and cytotoxic compounds have some of the most robust signatures in both techniques (Wawer et al., 2014). The role of molecular phenotyping in addressing nuisance compounds will likely benefit by the growth of open-source, reference perturbation datasets such as the Broad Connectivity Map (CMap), the Image Dataset Resource, and others (Tsherniak et al., 2017; Williams et al., 2017).

CONCLUSIONS

Nuisance compounds still represent a major yet surmountable burden in cell-based assays. Much work remains to fulfill the scientific promises of chemical biology, as illustrated by ambitious goals such as Target 2035 (Carter et al., 2019). In our view, major obstacles to realizing the full potential of chemical biology are not just difficult-to-drug targets or the sheer number of targets, but also nuisance compounds. This is due to the ability of nuisance compounds to waste significant resources and erode scientific trust. Successfully addressing cellular nuisance compounds involves understanding nuisance compound MOAs and exploiting relevant biology to design robust disease-relevant screening cascades. We propose a series of criteria to aid in nuisance compound triage (Figure 6).

Everyone loses when nuisance activities are overlooked

Compound prioritization is both an art and a science (Dahlin and Walters, 2014). While egregious bad actors can be swiftly triaged, the most frustrating cellular nuisance compounds usually exhibit the following behaviors: weak-to-moderate activities, partial selectivity, imperfect SAR, and equivocal counter-screens. These compounds with "just enough" activity present a dilemma for project teams when there is a dearth of promising compounds to pursue. In academia, the need for publications and a cut-throat funding environment can incentivize the pursuit of lower-quality chemical matter in comparison with a more "industry-like" mentality that rewards efficient "go/no-go" decisions (Dahlin et al., 2015a).

A major limitation is the lack of quality datasets explicitly addressing cellular nuisance compounds. Decisions in screening centers are derived not only from the data at hand but also experience, expert opinion, and subjective biases. Fortunately, the widespread appreciation for compound interferences in cell-free assays has

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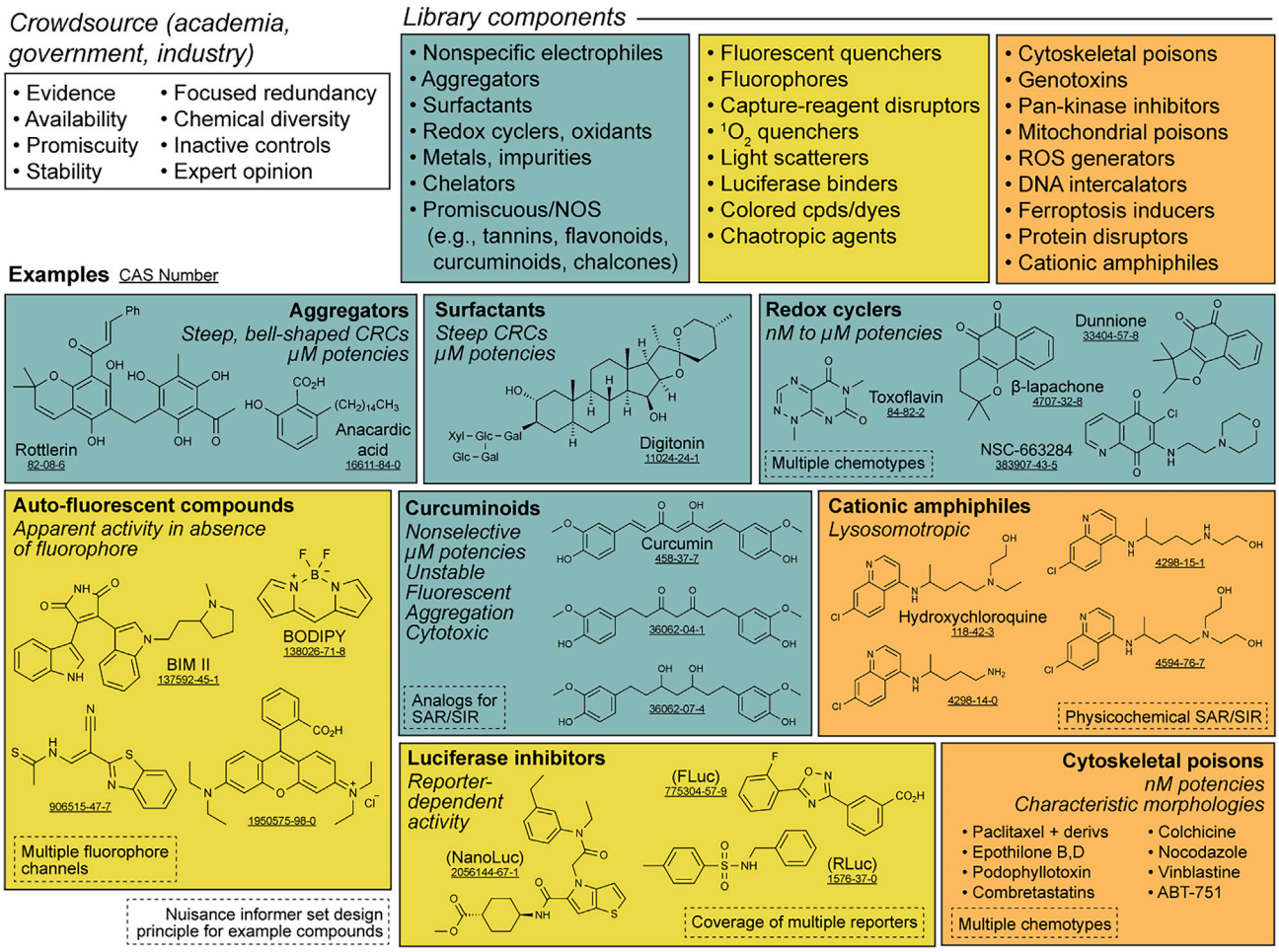

Figure 7. Framework for proposed nuisance compound informer set
See also Data S1.

enabled the creation of datasets and best practices that provide a roadmap for systematically tackling cellular nuisance compounds. A major barrier will be a lack of funding; while nuisance compounds are widely lamented, most public and private funding is directed for specific targets and diseases rather than general tools.

Other concerns are the behavior of nuisance compounds in *in vivo* assays (e.g., fungi, *Danio rerio*), three-dimensional tumor models, and tissue organoids. The effects of nuisance compounds in these highly complex systems are not well characterized, probably because of ethical considerations and resource limitations. However, nuisance compounds can indeed be identified by whole-organism screens (Hao et al., 2013; Larrieu et al., 2014), perhaps due to nonspecific xenobiotic stresses at subacute toxic concentrations.

Toward high-quality, open-source tools for tackling nuisance compounds

The drug- and probe-discovery communities would benefit from an open-source, evidence-based nuisance compound informer set. Toward this goal, we and interested collaborators are developing such a collection containing nuisance compounds nominated from academic, industry, and government screening experts. This set will be subject to prospective validation and published characterization experiments, and is expected to contain a diverse and comprehensive collection of non-technology-related and technology interference compounds, cytotoxins, inactive controls, and analogs (Figure 7 and Data S1).

SIGNIFICANCE

A major obstacle to realizing the full potential of chemical biology will be nuisance compounds, due to their ability to waste significant resources and erode scientific trust. Nuisance compounds are less characterized in cellular assays compared with cell-free assays. Cellular nuisance compounds are uniquely challenging because desirable and undesirable compound mechanisms of action (MOAs) can produce readouts that cannot be reliably predicted *a priori*. Confidently ascribing cellular activity to a nuisance MOA is challenging because of the vast number of molecular targets in cells. Cellular nuisance compounds include technology-related artifacts that mimic real bioactivity (e.g., autofluorescent compounds) and bioactive compounds that either perturb specific yet undesirable molecular targets/

pathways (e.g., microtubule poisons) or nonspecifically perturb molecular targets (e.g., nonspecific electrophiles). Nuisance compounds are best addressed by assay design principles and comprehensive post-screening cascades that leverage knowledge of both nuisance MOAs and the relevant disease biology. High-yield strategies include the use of nuisance control compounds to characterize nuisance readouts, orthogonal assays to confirm activity, counter-screens to triage undesirable MOA and interferences, and cellular health assays.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chembiol.2021.01.021.

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AUTHOR CONTRIBUTIONS

Writing – Original Draft, J.L.D., I.R., S.H., J.W.M.N., J.B.B., J.A.L., M.A.W, and K.H.; Writing – Review & Editing, all authors; Supervision, J.L.D.

DECLARATION OF INTERESTS

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