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Screening in a spirit haunted world

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High-throughput screening (HTS) campaigns can be dominated by hits that ultimately turn out to be non-drug-like. These 'nuisance' compounds often behave strangely, with steep dose–response curves, absence of clear structure–activity relationships, and high sensitivity to assay conditions. Several mechanisms contribute to these artifacts, including chemically reactive molecules, those that absorb light in assays and those that affect redox conditions. One of the most common mechanisms behind artifactual inhibition is discussed in this review: at micromolar concentrations organic molecules can aggregate to form particles in aqueous buffers, and these aggregates can sequester and thereby inhibit protein targets. Aggregation-based inhibition is baffling from a chemical perspective, but viewed biophysically such behavior is expected. The range of molecules that behave this way, their rapid detection in a screening environment and their possible biological implications will be considered here.

If high-throughput screening (HTS) has changed drug discovery, it has also introduced into it a bestiary of peculiar molecules. Some of these have turned out to be interesting and important; others have proven to be 'nuisance' compounds with strange properties. Steep dose–response curves, flat structure–activity relationships and high sensitivity to assay conditions are unusual with classic, well-behaved drugs and reagents, but are common among nuisance hits. These are rarely suited for development, but much time and passion can be wasted chasing them before they are abandoned. Their prevalence has contributed to the evolution of screening practices towards high-quality compound libraries, the maintenance of dry stocks of pure compounds and ever-lower concentrations of compound in initial screens.

'Nonsense is always nonsense, but the study of nonsense can be scholarship', said Saul Lieberman of the Kabbalist Gershom Scholem. Much scholarly ink has been spilled on compounds in screening decks that are prone to artifactual inhibition. Lipinski's now famous rules [1] focused on the physical properties of drugs, reacting to an early tendency in HTS libraries toward large and hydrophobic molecules that were unlikely to be orally bioavailable. Subsequent studies, typically using retrospective analysis of hit lists, have focused on chemical reactivity [2], assay interference [3], high flexibility [4], oxidation potential [5], formal molecular charge [6], or liability to degradation and precipitation [7]. Indeed, these characteristics have been incorporated at most pharmaceutical companies using computational filters that flag likely nuisance compounds in screening collections, so that they can be scrutinized when reviewing screening hit lists. Whereas these filters have been implemented since the late 1990s, identifying pathological hits unambiguously using these criteria has proven difficult. As one class of nuisance inhibitor is identified, another emerges Hydra-like. This is partly a problem of the apparent specificity of nuisance compounds for particular assay conditions – a promiscuous hit in one assay can behave demurely in another, conferring on it a cruel imitation of fidelity. But there were also screening hits that did not obviously manifest the nuisance properties identified in the initial studies. These molecules did not appear to be chemically reactive, were not obviously interfering spectrally, passed internal filters and Lipinski rules and had little in common other than their similar behavior in assays. This assay behavior was unusual: many compounds had steep dose–response curves [3] and many series, on investigation, led to flat structure–activity relationships (i.e. when analogs were made around the initial hit, only small changes in affinity were observed). Such compounds were widely known among screeners but the mechanism and properties that related them were obscure. They were not publicly discussed.

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This review describes a single mechanism that explains the behavior of apparently unrelated nuisance hits and is consistent with their sensitivities to assay conditions and perverse variability. At micromolar and sometimes submicromolar concentrations, many drug-like organic molecules aggregate into colloid-like particles in aqueous media. These aggregates can sequester protein targets, thereby inhibiting them. Aggregating inhibitors are often unrelated chemically, although they typically share certain physical properties. Like colloids and vesicles, they are sensitive to assay conditions and target concentration. This contributes to their haunted, skittish behavior. From a chemical perspective this can be baffling, but from a biophysical point of view, such behavior is expected. Indeed, based on these features, aggregation-based 'promiscuous' inhibitors can be rapidly detected and controlled for. In this article, I summarize the range of molecules now known to behave this way, their mechanism of action, their rapid detection in a screening environment and their possible effects in biological environments.

Phenomenology of aggregation

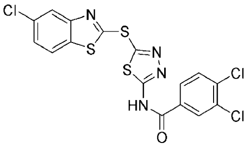
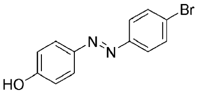
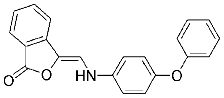
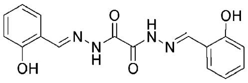
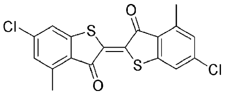
We encountered nuisance compounds by accident, while looking for inhibitors of the enzyme β-lactamase. We had tested tens of compounds predicted by virtual screening, finding many apparent inhibitors (Table 1). All had strange properties: they were noncompetitive, time-dependent and inhibited not only β-lactamase, but also dihydrofolate reductase (DHFR), chymotrypsin, β-galactosidase, and malate dehydrogenase (MDH) [8]. They also had unusually steep dose–response curves. Although there are reasons that can explain such curves, such as a high enzyme to K_i ratio [9], such dose–response curves are unusual. We initially thought that these compounds might be covalent inhibitors, but inhibition was reversible by dilution, inconsistent with such a mechanism of action. We then wondered if these inhibitors, so dissimilar structurally, were acting as denaturants. If this was true, we might have expected inhibition to be increased by guanidinium, urea or temperature. Instead, when we tested this, the opposite was true, inhibition was attenuated. Intriguingly, the potency of these compounds was strangely sensitive to protein concentration, diminishing considerably on addition of large amounts of bovine serum albumin or even increased amounts of the target enzyme [8]. Thus, increasing the concentration of β-lactamase in the assay ten-fold diminished potency dramatically. Correspondingly, increasing inhibitor concentration by a similar amount would return efficacy. This was difficult to reconcile with any classical mechanism of enzyme inhibition of which we were then aware. Rather, it seemed to point to a stoichiometric mechanism of inhibition, except that stoichiometries would not be 1:1 or even 10:1, but more like thousands of inhibitor molecules to one enzyme molecule. The only mechanism that we could think of with such high molar ratios was one where the inhibitors acted as colloid-like aggregates that somehow sequestered and inhibited enzyme targets without specificity.

A virtue of this hypothesis was that it was directly testable. Both dynamic light scattering (DLS) and transmission electron microscopy unambiguously detected particles when aqueous mixtures of these inhibitors were examined (Figure 1a and 1b). Both techniques agreed that these particles were huge (≥200 nm in diameter) – up to two orders of magnitude larger than the enzymes that they were inhibiting. Consistent with these particles being colloid-like aggregates of organic small molecules, they were sensitive to ionic strength. On moving to lower ionic strength, particle size decreased but the number of particles appeared to increase and inhibition improved. At high ionic strength, particle size increased and the apparent number of particles diminished, as did inhibition [8].

By 2001, it was clear that there was a chemically disparate class of nuisance compounds that inhibited enzymes not through a classic, single-molecule mechanism, but rather through sequestration. Because these compounds inhibited multiple enzymes, we began to call them 'promiscuous' inhibitors, a term probably first coined by Mic Lajines at Pharmacia. What remained unclear was

TABLE 1

Early inhibitors found to act through aggregation[a]

	IC_{50} (μM)				
Structure	**Original target(s)**	**β-Lactamase**	**Chymotrypsin**	**cDHFR**	**β-Gal**
	0.5 β-Lactamase[b]	0.5	2.5	5	15
	5 β-Lactamase[b]	5	25	35	90
	5 β-Lactamase[b]	5	15	N.D.	N.D.
	8 Malarial protease	10	55	70	180
	7 pDHFR	10	50	60	300