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Metal Impurities Cause False Positives in High-Throughput Screening Campaigns

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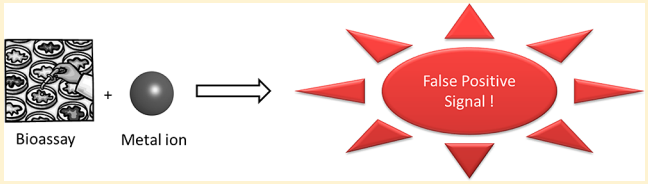
Supporting Information

ABSTRACT: Organic impurities in compound libraries are known to often cause false-positive signals in screening campaigns for new leads, but organic impurities do not fully account for all false-positive results. We discovered inorganic impurities in our screening library that can also cause positive signals for a variety of targets and/or readout systems, including biochemical and biosensor assays. We investigated in depth the example of zinc for a specific project and in retrospect in various HTS screens at Roche and propose a straightforward counter screen using the chelator TPEN to rule out inhibition caused by zinc.



KEYWORDS: *HTS, false positive, assay interference, Pad4, Jak3, Ras, zinc, promiscuous inhibitor, lead identification*

One of the dominant strategies for lead identification for small molecule drug discovery campaigns is high-throughput screening (HTS).[1−3] The main problems for medicinal chemistry teams when analyzing and following up hits derived from HTS are false negatives and false positives. Contrary to most false negatives that are missed from a screening effort entirely and may only be rescued by hit expansion methods, false positives that are pursued by project teams will always be discovered eventually. However, false positives can consume significant time and resources to be identified, characterized, and eliminated, thereby impeding progress toward lead identification and optimization.[4−7]

Many different mechanisms that cause false-positive signals have been discovered in recent years. Among them are nonspecific binding, interference with the assay detection method, organic impurities, compound aggregation, and redox reactions.[8−17] Here, we describe another source for false positives that we identified in recent HTS campaigns at Roche. These false-positive signals in HTS are caused by inorganic impurities. These inorganic impurities, the problems they may cause, and their removal at the developmental stage of drugs are known and widely discussed, but on- and off-target interferences from metals at the discovery stage of lead identification have not been reported.[18−21]

Most screening libraries are typically designed and built up over time. Compounds are assembled from various sources. At Roche, compounds of past projects are part of the HTS library as well as compounds specifically synthesized or purchased from various vendors over many years. As a consequence, the purity data on these compounds differ from being very elaborate for compounds derived from in-house project teams to very limited for commercial compounds that were purchased up to decades ago.

After screening a library, project teams usually select a number of compounds for hit verification and hit expansion studies. Initial identity and purity criteria include confirmation of NMR and mass spectra for the compound under investigation. As these methods are suitable to confirm the identity and purity of the organic component of solid compound material, they cannot reveal potential inorganic impurities such as transition metals that may be used in the synthesis of compounds.

During a recent lead identification campaign for the enzyme Pad4, the project team evaluated a number of HTS hit series.[22] Samples from four structurally different series appeared clean, and activity was retained throughout follow-up activities including an enzyme-linked immunosorbent (ELISA) enzyme assay and a standard enzyme assay. ForteBio and Biacore biosensor-based binding assays provided similar results and were consistent with these findings.[23] The measured IC_{50} and K_D values were in the low micromolar range, making these low molecular weight series attractive starting points for development into a lead series (compounds **1**.1, **2**.1, **3**.1, and **4**.1; Table 1).

The hits were resynthesized, and close analogues were prepared for early structure−activity relationship (SAR) exploration. All three series lacked conclusive SAR. Most exemplifying are the activities of different batches of the very

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Table 1. Activities and Binding Affinities of Various Batches

compd.batch)	IC_{50} (μM)	ligand efficieny	K_D (μM) ForteBio	zinc contamination (%)
1.1	11	0.29	23	7
1.2	59	0.25	45	2
1.3	>1000	<0.18	no binding	
2.1	4	0.39	10	20
2.2	>1000	<0.22	>500	
3.1	5	0.52	8	
3.2	>1000	<0.29	no binding	
4.1	14	0.25	10	
4.2	>1000	<0.15	no binding	

same compounds that exhibited very different activities from being low micromolar to inactive with IC_{50} values greater than one millimolar (Table 1). Additionally, the SAR of close analogues was either flat or very steep as indicated by compounds with minimal structural changes losing all activity (data not shown).

For these particular hits, we investigated these findings further. It was discovered that for one series, different routes of synthesis were used for the original preparation of the HTS library compound and its resynthesis. The historic synthesis made use of a zinc/titanium reduction step, whereas the new synthesis leading to inactive compounds did not. The schemes to prepare compounds of the other series also had steps involving zinc. Elemental analysis of the samples to determine the zinc content revealed that the active batches contained different amounts of zinc of up to 20% of total mass, whereas the inactive batches only had traces of zinc (Table 1). Hence, we hypothesized that if the assay readout was not affected by zinc, the enzyme that we were trying to target was sensitive to zinc ions.

We tested this hypothesis by determining the IC_{50} for $ZnCl_2$, which was found to be 1 μM in an ELISA functional enzyme assay. Also, in Biacore and ForteBio binding studies, we could confirm zinc binding with a K_D of 1 μM with Pad4 (Supporting Information).

We concluded that the observed strange SAR was caused by zinc contaminations in the compound samples. Zinc-free batches of the compounds showed no activities. Different routes of synthesis or varying methods of working up the compounds, which removed or only partially removed the zinc contaminations, caused inconsistent and false activities of the investigated compounds. We also checked other metals and found that many of them are active against Pad4, although with lower potency (Table 2).

To check all HTS hits for zinc contaminations, we rescreened the hits in the presence of a selective Zn chelator

Table 2. IC_{50} of Different Metals against Pad4

metal	IC_{50} (μM)
zinc (Zn^{2+})	1
iron (Fe^{3+})	192
palladium (Pd^{2+})	231
nickel (Ni^{2+})	242
copper (Cu^{2+})	279
barium (Ba^{2+})	>1000
calcium (Ca^{2+})	>1000
magnesium (Mg^{2+})	>1000

TPEN (*N*,*N*,*N*′,*N*′,-tetrakis(2-pyridylmethyl) ethylenediamine) that has been used frequently to remove zinc effects in various assays.[24−27] We identified 90 compounds in our hit set that showed greater than 7-fold potency shift in the presence of TPEN. We concluded that these compounds were very likely to be contaminated with zinc ions; possibly, the real number is larger, but we chose a 7-fold shift as a cutoff to be conservative and only include contaminated compounds in the following analysis.

We asked the question how often metals—or in our case zinc—cause problems in HTS screens. We used the identified 90 compounds as "probes" and analyzed 175 past HTS screens at Roche. Because the HTS campaigns were completed over a number of years during which the screening library evolved, not all compounds were screened in all assays. We analyzed only those HTS data sets with at least 10 compounds from the list of 90 tested (Table S1 in the Supporting Information).

Of the 175 HTS screens, 41 showed a hit rate of zinc-probing compounds of at least 25% as compared to a randomly expected hit rate of <0.01%. We consider this very high, as we do not expect for all compounds to be active in a zinc-sensitive screen, since we assume differing impurity levels of zinc in the compound samples as well as different sensitivities of the various assays or targets in addition to a false-negative rate expected for any HTS.

Most of the 41 suspicious HTS campaigns belonged to nonactive projects at Roche; however, four projects were still active at the time of this investigation. We assayed zinc in each of these assays and found each of them were susceptible to zinc inhibition at various concentrations (Table 3).

Table 3. Measured IC_{50} Values in Various Assays (For Details, See the Supporting Information)

assay	target class	$ZnCl_2$ IC_{50} (μM)
1	Jak3 (kinase)	14.9
2	protein−protein interaction	4.9
3	protein−protein interaction	2.7
4	Ras/Raf	<47[a]

[a]Assay conditions contain EDTA, and the complexation of zinc is expected to increase the measured IC_{50} value.

The Ras screen was one of the screens showing the highest hit rate for zinc contaminated compounds with all 36 compounds in the screen known to contain zinc giving a positive signal. This screen was an exception in that it was not a HTS but a fragment-based screen (FBS). This was the only FBS considered for this study and was run at a compound concentration of 250 μM. It is conceivable to assume that FBSs, which are typically run at much higher compound concentrations, should be more prone for false-positive signals from zinc and metal-contaminated compounds. The data for the Ras FBS support this.

We conclude that zinc and potentially other metal impurities in screening library compounds may affect a large number of targets or assays. The zinc impurity in false-positive compounds may cause false-positive signals in the low micromolar range, simulating potencies relevant for selection by project teams. We found zinc-contaminated compounds synthesized using different reactions involving zinc. Many reactions using metals including zinc are employed frequently by medicinal chemists and should be considered as a potential source of zinc contamination.[28,29] Whether or not zinc is transferred as an

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impurity depends not only on the workup procedure but also on the property of the organic compound. For example, we found organic compounds with zinc impurities that may have formed complexes with multivalent cations and survived workup procedures.

Because these impurities do not raise flags by simple purity checks of the organic material and often maintain activity throughout orthogonal assays including binding assays, they can lead a project team in the wrong direction, wasting valuable resources and time. In our case, the zinc inhibitory activity appeared to be on target. This is not novel since inhibitory or regulatory effects of metals either alone or in complex with a small molecule are well-known.[30−35] Other nonfunctional mechanisms of metal interference on readout systems may also be possible and are more likely to cause false-positive signals. However, we have not observed an enrichment of a particular assay type among the suspicious HTS assays (see the Supporting Information).

When analyzing HTS hits, inorganic impurities should always be considered and checked for before starting any lead optimization campaign. Assaying selected hits or the entire hit selection in the presence of a nonselective chelator such as EDTA or a more selective chelator such as TPEN can be a simple but very effective method to rule out false-positive hits. Metal contamination in compound samples from synthesis can also cause inconclusive SAR in later lead optimization stages.

■ ASSOCIATED CONTENT

Supporting Information

Statistics of activity of zinc-contaminated compounds in HTS assays and experimentals for Pad4, Jak3, and Ras/Raf assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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