Reactive compounds and *in vitro* false positives in HTS

Gilbert M. Rishton

An important component of the successful high-throughput screening (HTS) strategy in drug discovery is the ability to assess HTS structure–activity data, and to distinguish between promising drug leads and the many useless false positives that can plague screening efforts. The author discusses simple chemistry guidelines for the evaluation of 'positives' in biochemical screens, with the aim of selecting stable, non-covalent binders (ligands) and eliminating protein-reactive compounds (reagents) from consideration as drug leads at an early stage.

The powerful tools of biotechnology have provided drug discovery groups with protein targets that are necessary for the examination of biological processes at the molecular level. Pharmacologists exploit such targets for the development of high-throughput screening (HTS) programs in which the testing of thousands of compounds per day in a biochemical screen is routine. The HTS paradigm promises to accelerate the identification of drug leads for new biological targets. An important component of the successful HTS strategy will be the ability of the drug discovery team to assess HTS structure–activity data, and to distinguish between the promising drug leads and the many useless false positives that often plague an HTS effort. This article is intended to provide simple chemistry guidelines for the evaluation of 'positives' in biochemical screens.

Feeding the screen

In the early stages of the drug discovery process, an HTS team must choose a strategy able to generate new chemical leads that exhibit activity in a biochemical screen. Combined strategies, including a rational design component and a complementary random screening component, are often employed in an attempt to adhere to a directed approach without precluding serendipitous drug discovery. Ready access to a large compound collection, in the form of a corporate sample bank, combinatorial libraries and vendor-acquired collections, is crucial to the HTS effort. The HTS team usually has little or no control over the classes of compounds in the collections being screened.

Evaluating the 'positives'

In the event of an *in vitro* 'positive', a medicinal chemist must determine if the chemical entity in question is suitable to serve as a lead compound for the next level of strategy, which involves optimization by iterative medicinal chemistry. The evaluation of the structure of the 'positive', and the decision to move forward with the chemical entity as a new drug lead, is probably the most important decision a discovery group can make. The decision to move forward involves the commitment of substantial resources and, ultimately, the commitment of years of time and millions of dollars.

A judicious selection of the good lead compounds, and an early and systematic removal of suspected 'false positives' is vital to the success of an HTS program. The most common false positives result from compounds that are chemically reactive towards protein. These are alkylating and acylating agents, which can easily be identified by a chemist's inspection of their chemical structures. They are generally prone to solvolysis or hydrolysis, and are characteristically reactive

Gilbert M. Rishton, AMGEN, 1840 DeHavilland Drive, Thousand Oaks, CA 91320, USA. tel: +1 805 447 1064, fax: +1 805 499 5714, e-mail grishton@amgen.com

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towards biological nucleophiles (Figure 1). Compounds that contain these functional groups should not be submitted for assay in a biochemical screen without the understanding that they are suspect false positives. This generalization intends to emphasize that reactive compounds that exert their effects in a biochemical screen via covalent bond-forming processes are false positives. The screening data associated with a reactive chemical entity are useless to a medicinal chemist, regardless of observed 'potency'. The false read-out is simply a consequence of chemical reactivity, not biological activity.

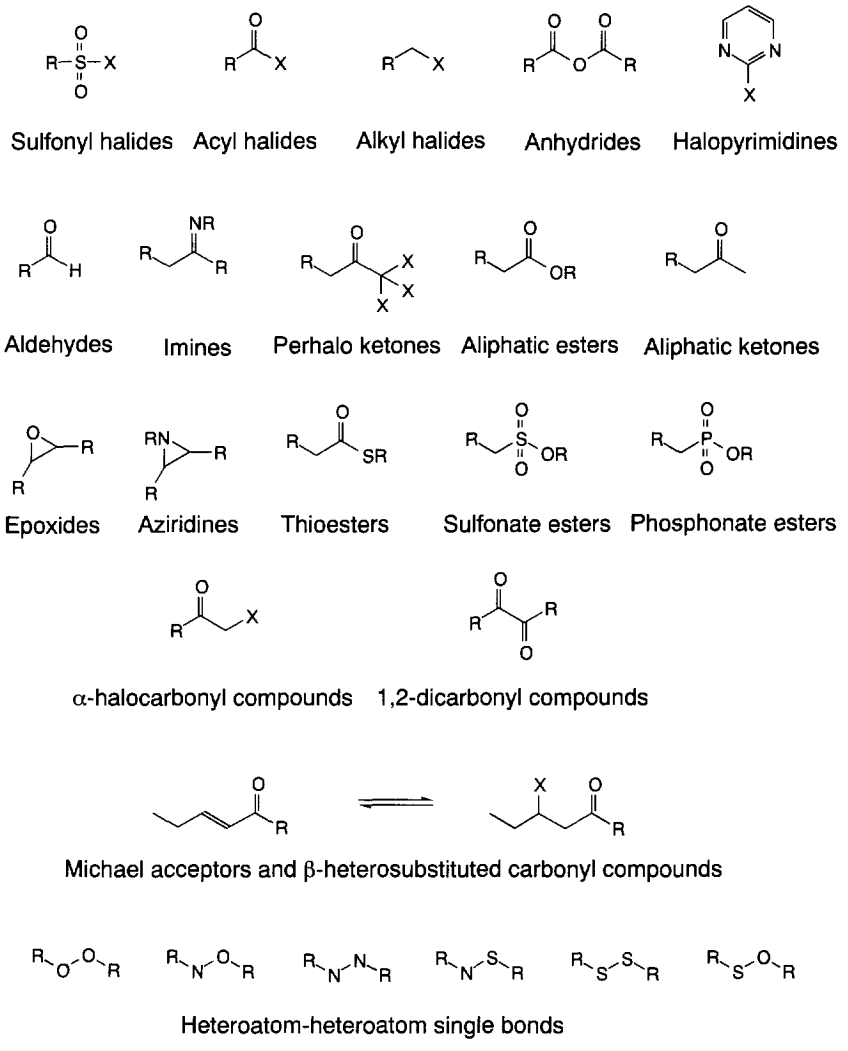

Figure 1. *Reactive functional groups responsible for* in vitro *false positives (X= F, Cl, Br, I, tosyl, mesyl, etc.; R = alkyl, aryl, heteroalkyl, heteroaryl, etc.). These reactive functional groups are generally prone to decomposition under hydrolytic conditions (i.e. aqueous Na_2CO_3/methanol). They are reactive towards protein and biological nucleophiles (e.g. glutathione, dithiothreitol), and they exhibit poor stability in serum.*

'Reactives' are everywhere

Ideally, only collections of compounds that are free of reactives should be screened. However, chemists have traditionally submitted reactive synthetic intermediates such as aldehydes, epoxides, alkyl halides and others to their corporate compound collections. In addition, chemists in the pharmaceutical industry have prepared reactive agents as antineoplastic and anti-infective drugs for decades. Many of these are also found in corporate compound collections.

These compounds are known to be reactive towards protein, and the success of these classes of agents in their respective therapeutic areas does not justify their screening in a target-driven HTS program. Such reactive compounds are notorious for causing false positives in receptor-based, enzyme-based and whole-cell assays. While removal of all suspect reactives from a compound collection may be impractical or impossible, it is vital that the HTS team recognizes a reactive compound as a suspect false positive and removes it from consideration as early in the process of lead selection as possible.

Beware the tools

Reactive compounds such as α-chloroketones, trifluoromethyl ketones, aldehydes and various other alkylating and acylating agents are studied under carefully controlled conditions *in vitro* as pharmacological probes to serve as 'protease poisons' and so-called 'suicide inhibitors'. Often, these reactive probes are used as tools to characterize an enzyme during assay development. These *in vitro* pharmacological studies, and the compounds associated with them, should not be confused with lead compounds for medicinal chemistry. While several hypotheses exist that rationalize medicinal chemistry approaches to these compound classes, their binding to protein is covalent and not truly reversible.

Reactive compounds such as these, which operate by alkylating and acylating mechanisms, are inappropriate for most assays, including enzyme assays. Structural analogues of these compounds will uncover structure–reactivity relationships (SRR), not structure–activity relationships (SAR). A typical SRR is provided as an example (Figure 2). This profile is certainly indicative of a false positive.

Rule of thumb

If a 'positive' exhibits reactivity under the conditions and time course of the biochemical screen of interest, then the compound should be considered a false positive. As such reactivity/stability studies are not a routine component of an HTS approach, the HTS team should rely on the chemist's judgment to eliminate a false positive based on simple inspection of its chemical structure. If a more rigorous analysis is required, chemical stability studies can be performed (see Figure 1). Additional pharmacology, particularly multiple-point dose–response studies, will usually expose a reactive false positive as well.

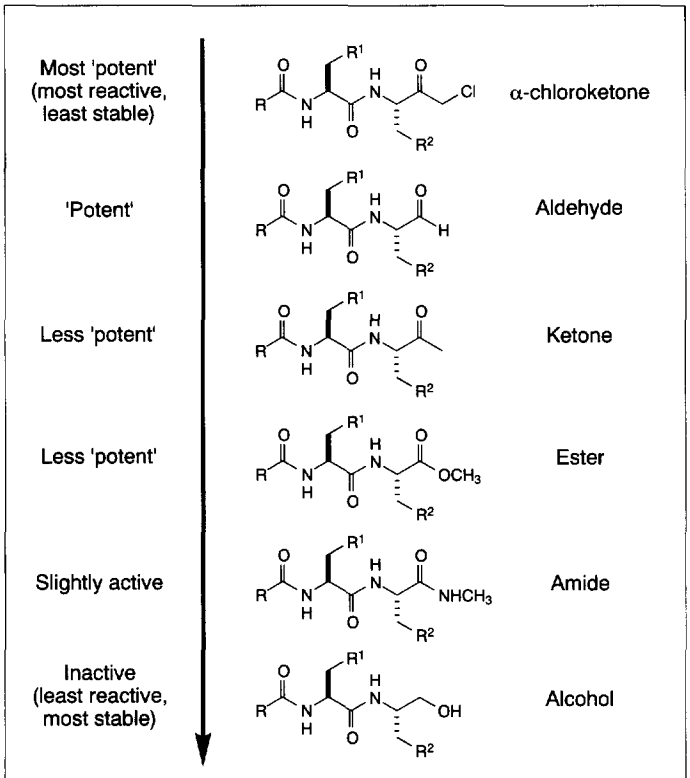

Figure 2. *Typical structure–reactivity relationship. In this example, analogues of the 'potent' compound vary only in the reactivity of the offending functional group. A graded structure–reactivity relationship (SRR) is revealed. This is a reliable indication of a false positive, as the 'activity' observed has no basis in a structure–activity relationship (SAR).*

Biochemical screens are designed to measure the competitive, reversible binding of a high-affinity ligand, not the covalent binding that is exhibited by reagents such as reactive molecular probes and suicide inhibitors. Only stable, reversibly binding ligands should be considered as truly active drug leads for the purposes of a target-driven biochemical screen.

Ramifications

The most insidious characteristic of a chemically reactive false positive is that it lacks intrinsic biological activity. It is the reactivity of the compound that is the basis of the 'positive' observed. This puts the medicinal chemist in an impossible situation of trying to 'fine tune' stability. Any amount of stabilization of the offending functional group of a reactive false positive is pointless because it will lead to a corresponding loss of the observed 'activity'. Commitment of further resources will ultimately prove unfruitful.

Future trends

In the future, the effective HTS team will manage the evaluation of 'positives' in the context of both potency and chemical stability, and will understand that hypotheses of drug action that involve covalent bond-forming processes are inappropriate for most biochemical screens. Drug discovery groups should become aware of the occurrence of reactive compounds in their corporate collections, in natural products extracts, and as impurities in libraries of compounds synthesized by automated methods. Managers of chemical compound collections should attempt to exclude reactive intermediates, and reactive antineoplastic and anti-infective agents, from the screening process. As a result of effective compound collection management, the drug discovery community will work towards the concept of the 'immaculate collection', including only compounds appropriate for a given biochemical screen.

The early identification and systematic elimination of reactive compounds as suspect false positives from any drug discovery process is vital. This is especially true in the HTS arena where hundreds of structurally dissimilar positives may be observed in a given screen. The selection of a stable, high-affinity, reversibly binding ligand will provide a good lead compound for optimization and drug development. In this way we can tap into the power and promise of HTS technologies.