Epigenetic chemical probes to identify therapeutic targets in oncology

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Background

The genetic and epigenetic abnormalities in cancer offer potential opportunities to identify tumor-specific vulnerabilities that can be targeted with small molecule therapeutics. Chemical probes are powerful tools for the discovery of such pharmacologic vulnerabilities and epigenetic targets are especially attractive for cancer therapy as they govern dynamic post-translational modifications of DNA and histones that lead to alterations in the chromatin structure and compaction, thus controlling tissue- (or tumor-) specific gene expression programs. Ablated epigenetic cellular states, such as blocking differentiation, can play a key role in the development and progression of cancer. Chemical probes are potent, selective and cell-active small molecules that inhibit the biochemical function of a specific protein or small set of closely related protein targets (1). Examples include selective inhibitors of catalytic activity of a histone methyltransferase or demethylase (2, 3), and antagonists of protein-protein interaction domains that "read" acetyl or methyl marks on histones (4).

Chemical probes are highly complementary to genetic methods of target validation with some distinct advantages. First, because most protein targets function as part of larger multifunctional multi-protein complexes, protein knockdown or knockout experiments can lead to disruption of entire complexes, which does not always phenocopy inhibition of a single enzymatic function. Secondly, unlike knockdowns, the degree and duration of inhibition with a chemical probe is readily controlled through variation of dose and incubation time and is applicable to many different cell types. Thirdly, chemical probes most closely match the eventual mode of therapeutic targeting and can provide a sense of "druggability" of the target for future drug development programs. For example, although many proteins can appear to be excellent targets based on knockout/down studies, many such proteins do not have the right structural or biophysical properties for inhibition by drug-like small molecules. Finally, chemical probes can readily be tested in combination with other agents – an emerging trend in oncology. Thus, the use of chemical probes enables more sophisticated and complex studies to be carried out in search of new therapeutic targeting strategies.

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How and When to Use Chemical Probes

Discussion

What makes a good chemical probe?

Chemical probes are well-characterized drug-like small molecules that potently and selectively inhibit the target of interest \textit{in vitro} with a defined mechanism of action. For initial target validation studies in cell lines or primary patient samples cultured \textit{in vitro}, the probe must be cell-permeable and stable in cells, but need not necessarily have all the properties of a drug, such as favourable \textit{in vivo} pharmacokinetics. Importantly, the probe should be able to bind to and inhibit the intended target in the cell at low μM concentrations or less. Before embarking on a study with a chemical probe(s), one should familiarize oneself with some of the key features of the molecule(s) being used.

- \textit{What is the mode of action and IC}_{50} of the probe for the target? This should have been demonstrated by measuring the probe’s effect \textit{in vitro} on a known biochemical activity of the target such as enzymatic activity or protein-protein interaction.

- \textit{Is the probe sufficiently selective for the target of interest}, especially with respect to closely related proteins? If selectivity has not been demonstrated, then one cannot draw conclusions about the role of the target in disease biology. For example the early kinase inhibitor literature was “polluted” with studies attributing anticancer effects of staurosporine to protein kinases C before it was widely recognized that this molecule inhibits most kinases (5).

- \textit{Is there a known cellular biomarker that can be used to monitor probe activity}? Changes in the level of an enzyme product, such as a histone post translational modification are often used for this purpose as a measure of primary response to a probe (Fig. 1).

- \textit{What is the maximum tolerated concentration} of the compound in several transformed and non-transformed cell lines before nonspecific toxicity is observed?

- \textit{Is there a closely related, but inactive compound that can be used as a negative control}? The use of vehicle alone does not control for potential off-target effects of a chemical probe.

Application of Chemical Probes in Oncology Target Discovery

In the quest for new cancer therapeutics, the field has traditionally searched for agents that selectively kill rapidly growing cancer cells such as cytotoxic chemotherapies, and more recently targeted therapies that selectively kill specific cancer genotypes. Epigenetic chemical probes may be thought of as potential targeted therapies that can take advantage of the unique genetic or epigenetic state of specific cancer genotypes. Importantly, they have the potential to selectively alter gene expression programs, thereby, reprogramming cellular states. Therefore, in addition to evaluating the classical parameter of cell death or viability, a host of additional, clinically relevant phenotypes are of interest, such as differentiation, self-renewal, epithelial-to-mesenchymal transition, radiosensitization, or reversal of drug resistance (chemosensitivity).

However, it is first essential to demonstrate that the phenotypic effects being measured are dependent on specific target inhibition. For this purpose it is useful to classify cellular
responses into primary and secondary responses (Fig. 1). Primary responses reflect the biochemical activity of the target such as catalytic activity of a specific enzyme, while secondary responses are functional consequences of altered target activity such as effects on gene transcription, chromatin condensation, cell cycle, differentiation or apoptosis. Monitoring a target-specific response is critical for the successful application of chemical probes. The primary response can often be monitored by a suitable biomarker while the secondary functional readouts are often dependent on many other factors including those that are activated in response to a general toxic response. A general toxic response can be elicited by many chemotypes and may not necessarily be related to direct inhibition of the target. Thus, careful evaluation of the primary response in a time and dose dependent manner and comparison to the downstream functional and phenotypic events also measured in a dose dependent manner should be used to establish the causal relationship between the target and the phenotype.

Several possible scenarios for cellular responses to chemical probes are outlined in Fig. 2. We use here the example of cell viability as it is one of the most common assays in oncology. If, for example, an enzyme is 90 percent inhibited at a particular concentration of the probe (as measured by a confirmed biomarker) and cell viability starts to decrease at this concentration (Fig. 2A), then this is likely to be a target-specific cell death. Such a conclusion can be further supported by genetic knockdown of the target. In another scenario, if the cell death occurs at probe concentrations lower than that required for target inhibition (again as measured by a biomarker), then the response is most-likely not target related (Fig. 2B), and may be due to
unanticipated off-target or general cytotoxic effects of the probe. In such cases, the use of chemically similar, but target-inactive “negative control” compounds is very helpful. The parameters of the cell viability curve can also reveal off-target effects, as excessively steep or shallow slopes have been associated polypharmacology, population response heterogeneity, or non-specific toxicity (6). The third scenario is one in which the apparent functional effect occurs at much higher concentrations than target inhibition (Fig. 2C) and most often this kind of response is caused by non-specific compound toxicity at high concentrations. Again the slope of the toxicity curve can be revealing and negative control compounds are important. An additional helpful strategy is to use (if available) two or more chemically unrelated probes for the same target because two very different compounds having the same off-target activity is highly unlikely.

Another important consideration is the timeframe of the assay – especially for epigenetic targets whose inhibition may trigger a reprogramming event. The appropriate timeframe is highly dependent on the target. For example, inhibitors of kinases result in changes in substrate phosphorylation within minutes to hours while inhibition of histone methyltransferases such as DOT1L, G9a, and EZH2 require several days for a primary response (change in level of the histone methyl mark) and a week or more for a phenotypic response (2, 7–9). Selecting an appropriate time range for monitoring primary and secondary responses to target inhibition will ensure optimal interpretation of assay data.

**Fig. 2.** Dose response scenarios for the response biomarker and secondary events. A. Correlated and likely causal relationship or biomarker and viability responses. B. Viability changes occurring at lower concentrations than the target inhibition as measured by the biomarker indicates no-causal relationship. C. Dose response relationship where full biomarker inhibition does not elicit changes in cell viability likely indicates non-causal relationship.
Future Directions

Development of good quality chemical probes is an expensive endeavour relying on collaboration between biochemists, medicinal chemists and cell biologists in a multidisciplinary environment. As such, the majority of this activity has taken place within the drug discovery industry in the past. However, there is growing recognition of the importance of “industry quality” chemical probes as described above, and increasingly academia and industry are working together to develop and share such reagents (10–15).

The availability of a wide range of good quality chemical probes will enable the exploration of more complex biological systems such as collections of primary patient-derived cells that are more reflective of the disease than common cell lines (14). With such tools in hand one can envision an empirical chemical biology approach to target discovery by profiling selective chemical probes against collections of specific, genetically defined cancers at the cellular level. The results of these studies would justify the subsequent but expensive optimization of compounds (if required) for in vivo activity, paving the way to target validation studies in animal models and man.

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