Small molecules and their roles in effective pre-clinical target validation

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As demonstrated in multiple historical analyses, there are two main causes of clinical attrition; firstly drugs are not efficacious, and secondly they cause unacceptable toxicity, both of which can be the result of poor pre-clinical target validation. Target validation, one of the early stages of a drug discovery program, is the process of (in)validating a drug target to ensure it is significant to the intended disease, and unlikely to drive undesired toxicity. Target validation is vital in preventing late stage failures in the clinic and, if done effectively, can save pharmaceutical companies a great deal of time and money. As such, target validation is treated extremely seriously, as demonstrated by the formation of public-private partnerships, such as *Open Targets*, aimed to provide evidence of biological validity and the possible likelihood of pharmacological intervention. Central to the variety of molecular tools available for use in target validation are high quality small molecules called chemical probes.

A chemical probe is a tool molecule that selectively binds to a target protein, used to answer questions about the targets biological function and potential role in disease[1]. The strength in target validation is directly related to the quality of the chemical probe used. Consequently the misuse of chemical probes, the use of under characterized probes, and the sheer lack of high quality chemical probes (compared to the number of potential biological targets) can lead to inappropriate conclusions, for example the use of curcumin as a tool for target validation, highlighted recently by Nelson *et al.*[2].

Unfortunately, curcumin is just one of many small molecules compromising attempts at target validation today. Recently however, the area of chemical probes has evolved dramatically, with experts from medicinal chemistry, pharmacology, biology, industry and academia coming together to address these issues, and help learn from past mistakes. In particular, work carried out by Bunnage *et al*. has been instrumental in defining a new set of requirements. By adapting the three pillars of survival, Bunnage and coworkers created the following four guidelines for chemical probes: 1) The probe must be able to reach the site of action at pharmacologically relevant concentrations; 2) display *in vitro* evidence of target engagement and selectivity; 3) provide sufficient data to assign phenotypic results to an original structure or a well characterized derivative; and 4) provide cellular activity data to answer a hypothesis on the role of the target[3].

In addition to these guidelines, it has become evidently important for chemical probes to be accompanied by a structurally similar negative control. In the often paraphrased words of R. B. Woodward “the only model system worth using is the enantiomer” and chemical probes are no exception to this, although it should be noted that enantiomeric controls are not always possible. A

final consideration when designing chemical probes is the importance of structural diversity. With a selection of different chemotypes comes a greater confidence in the experimental outcome, a reduced probability of finding common off targets, greater conviction that observed phenotypes are due to target engagement, and compensation for the many known unknowns.

Although these guidelines have helped establish a new higher standard for chemical probes used within research, the transition of replacing the previously used under characterised chemical probes with new higher quality probes, is largely thanks to a collection of public-private consortiums such as the Structural Genomics Consortium (SGC)[4] and chemicalprobes.org[5]. Partnerships such as these have sought to raise awareness of the 'promise and perils' of chemical probes, whilst providing access to all the information available on existing probes, even offering guidance on the optimal probe for a particular target and appropriate concentrations for its use.

The combination of higher quality probes and an improved awareness has already led to a number of breakthroughs in scientific research, arguably none bigger than for the BET bromodomain family, which will now be used as a case study to demonstrate the power of small molecule chemical probes and their evolving use in target validation.

In 2010 two separate groups identified **(+)-JQ-1** and **I-BET762** as potent and highly selective inhibitors of the BET bromodomains. Whilst being high quality, well characterized chemical probes, they were both also accompanied by a negative control in the form of their inactive enantiomer, allowing for phenotypic screening studies[6]. Instead of keeping this information to themselves, the structures and properties of both molecules were made available for the scientific community. Several other groups followed suit with other BET probes, such as **I-BET151**, **PFI-1** and **OXF BD 02**, which together created a diverse portfolio of chemotypes, suitable to thoroughly investigate the BET bromodomains target validity. In doing so, the biological function of this family of bromodomains, and the role they play in a number of diseases, was vigorously interrogated, ultimately accelerating bromodomain drug discovery[7]. As of 2017 there are currently 16 BET bromodomain clinical candidates spanning 14 different companies, underpinning the value of **(+)-JQ-1** and **I-BET762** as extremely successful and effective chemical probes[1,8,9].

Whilst **(+)-JQ-1** and **I-BET762** are classical examples of high quality chemical probes, there are multiple alternative approaches utilising small molecule probes for target validation. For example, the aforementioned BET chemical probes, whilst being highly pan-selective for BET bromodomains over other bromodomains, all display poor selectivity within the BET subfamily, preventing the direct assignment of BET inhibitor pharmacology to a particular bromodomain target. The "bump-and-hole approach", previously used in selective kinase inhibition, has attempted to address this issue, utilising the existing probe **I-BET762**. Mutation of a conserved leucine residue [L94 in Brd4(1)] to a sterically smaller alanine, and the introduction of an ethyl group to an **I-BET762** derivative, produced the aforementioned "hole" and "bump" respectively, from which BET subfamily selectivity was achieved. Displaying up to 540 fold selectivity for mutated bromodomains over regular, this ethyl derivative of **I-BET762** was used to dissect the individual role of Brd4's bromodomains in chromatin binding, thus demonstrating the potential for the bump-and-hole technique in the generation of new selective chemical probes, and their value in the interrogation of drug targets[10].

Utilising the structural information typically obtained during the development of chemical probes facilitates the development of bifunctional chemical biology tools. One such important research area

at the interface of drug discovery, chemical biology and target validation is proteolysis targeting chimeras (PROTACs). Fundamentally, PROTACs work by combining two protein binding regions, one region which binds to the target protein and another which binds to E3 ligase. Together they create a bifunctional molecule capable of facilitating ubiquitination of the target protein and thus ultimately proteolysis. A recent example of this is for the treatment of castration resistant prostate cancer (CRPC) where **(+)-JQ-1** was utilised to exploit its high selectivity and potency for BET bromodomains [11]. The result of which was **ARV-771**, a highly potent (IC_{50} <5 nm) BET PROTAC which has been shown to selectively degrade BET proteins and induce CRPC cell apoptosis *in vitro*. Furthermore, **ARV-771** has also been shown to reduce the levels of two key androgen receptors (full length androgen receptor and androgen receptor variant 7) present in prostate cancer cells, both of which are strongly linked to CRPC[12]. **(+)-JQ-1** based PROTACs have also been used to selectively degrade BRD4, again providing more information on selective BET subfamily suppression[13].

Also taking advantage of structural information around **(+)-JQ-1**, biotin was attached *via* a polyethylene glycol (PEG) linker to create **bio-JQ1**, a bifunctional small molecule capable of facile ligand-affinity capture. Used alongside Chem-seq, a parallel DNA sequencing technique, it was possible to map the direct interactions between small molecules and bromodomain containing proteins BRD2, BRD3 and BRD4 within the genome, providing further insight into the mechanisms by which BET inhibitors perturb gene expression[14].

Finally, photoaffinity labelling probes are an important chemoproteomic tool due to their ability to covalently label their biological targets. Seeking to replace the acetyl lysine mimetic of the CREBBP probe **SGC-CBP30**, Jones and co-workers identified a novel photoreactive tropolone warhead, and hypothesized that photoaffinity labels could be developed for bromodomains. Shown to indeed be possible, a clickable derivative was accessed which was used to understand the direct biological targets of the probe and assess BRD4 target engagement by **(+)-JQ-1** [15,16]. Clickable photoaffinity labelling probes have also been used to map thousands of site specific small molecule-protein interactions within human cells, from which more potent and selective small molecules can be optimised, presenting this technique as a versatile approach to small molecule discovery as we advance into the future[17].

As demonstrated with bromodomain probes, the area of chemical probes is continuously evolving, with more valuable and sophisticated approaches to small molecule enabled target validation being developed constantly. This is encapsulated by recent work investigating the epigenetic reader YEATS domain containing protein ENL, in the treatment of acute myeloid leukaemia. Using CRISPR-Cas9 gene editing and a targeted PROTAC approach, ENL was identified as an oncogenic transcription regulator involved in acute myeloid leukaemia. ENL displacement from chromatin was shown, with *in vitro* and *in vivo* evidence, to reduce oncogenic gene expression whilst simultaneously sensitizing leukaemia cells to BET inhibitors, presenting a promising stand alone epigenetic therapy for acute myeloid leukaemia, or as a combination therapy with BET inhibitors[18,19]. When considering the role of small molecules in target validation, it is of note that CRISPR often uses small molecule enhancers, such as **RS-1** and **L755507,** to improve efficiency and precision *via* various mechanisms[20].

In conclusion, high quality chemical probes and their derivatives are invaluable to the pre-clinical target validation tool set, acting as powerful research tools, resource savers, and a potential source for new medicines. This coupled with a greater understanding and appreciation of high quality

chemical tools will hopefully lead to an increase in successful target validation in the future, and a reduction in clinical attrition.

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