Chapter 9
Microglial Biology in Neuroinflammatory Disease: Pharmaco-industrial Approach to Target Validation

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Abstract Profound changes continue to shape scientific and business strategies in the pharmaceutical industry. Up until very recently, academic centers and corporations worked somewhat in isolation. However, over the last few years, two specific changes started to change this situation. On the one hand, academic organizations became more engaged in operations previously conducted mainly in the industry, such as actual drug design and high-throughput screening. Capabilities were enhanced, both human and technical, and consolidation of available "know-how" led to the establishment of several academic centers capable of influencing and making key contributions to early drug discovery research. Simultaneously, the pharmaceutical industry recognized the need to enhance their sources of innovation and engage in hitherto mainly unexplored areas of research, such as neuroinflammation. In the process, previously insular organizations became more open to collaborating, exchanging information and building knowledge with external partners. Challenges remain to maximize the productivity of these interactions, and to benefit the collaborating partners, and ultimately society, by boosting the success of drug discovery. Developing a common language to communicate respective views is a key step towards enabling the partners to learn from each other and work together. In recent years our company has established a variety of successful collaborations with external partners. This chapter summarizes at a high-level some of our current research processes, the learnings from our interactions with academic partners and our assessment of how to build strong academic-industry research partnerships.

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9.1 Introduction

Neuroinflammation has long been recognized as a pathological hallmark of a wide variety of neurological diseases. While activation of central inflammatory cells, particularly microglia, was initially interpreted as a reactive response to neuronal damage, growing evidence over the past decade suggests that neuroinflammation may contribute to disease progression (Weydt et al. 2002; Garden and Möller 2006; Hanisch and Kettenmann 2007; Möller 2010; Weinstein et al. 2010; Ellrichmann et al. 2013; Gandy and Heppner 2013; Nolan et al. 2013; Zhao et al. 2013). Consequently, efforts in the pharmaceutical industry have been mounting to target neuroinflammation as a therapeutic strategy to treat disorders of the central nervous system (CNS). This chapter aims to outline the high-level strategy by which companies approach drug discovery research, with a particular emphasis on novel microglial targets, and to address the critical criteria required to successfully bring new therapies to patients.

9.2 Target Identification and Validation

The successful development of a new drug is a herculean endeavor, with many potential pitfalls at each step of the way. On average it takes longer than a decade and more than a billion US$ to progress a drug discovery program from target identification to market (Bains 2004). Only a very small fraction of compounds make it through the whole drug discovery process and become *bona fide* drugs (Arrowsmith 2011). Therefore, it is critically important to minimize the risk of failure as early as possible, beginning with the selection of the best drug targets (Fig. 9.1).

One way to reduce this risk is to attempt to improve on existing drugs with well-established modes of action. However, to most significantly impact unmet medical need, new drug targets with novel mechanisms are required. New drug targets are discovered through a variety of means, from serendipitous discoveries in basic biology research to directed target identification screening efforts. Regardless of the source, it is critical to build a strong case that the target is relevant to the pathophysiology of the disease and that intervention would result in therapeutic benefit (Table 9.1). Furthermore, there must be a high likelihood that a successful drug can be developed with the appropriate pharmacological effect on the target. Often the first question is whether expression of the target is altered in the disease or associated with disease pathophysiology. Increased expression of a target in disease, for

Fig. 9.1 Stages in the drug discovery process. The drug discovery process often begins with the selection of a target using a variety of validation criteria. High-throughput screening (HTS) campaigns are then conducted as one of the ways to identify pharmacological modulators of the target. The output of high-throughput screens are relatively low potency, non-selective molecules called "hits". Hits are evaluated through a number of secondary assays and further optimized through chemical modifications to identify the most promising chemical matter, or "leads". Leads are further optimized to improve potency, increase specificity, and achieve the preferred physicochemical and metabolic properties

instance, not only increases confidence that the target contributes to the disease process, but also suggests that inhibition of the target may restore balance to a healthier state without resulting in significant adverse effects. Reduced expression of a target in the disease state, on the other hand, may point towards a need to augment functional activity. Unfortunately, it is particularly challenging to generate target expression data for CNS disorders compared to other disease areas, since human CNS tissue is obviously not as accessible as peripheral tissues where biopsies or blood draws are routine.

Alterations in expression are merely correlative and do not demonstrate any functional consequences. For this reason, demonstration that the target shows a reproducible genetic association with disease often provides more confidence of a mechanistic role. Mechanistic information can also be gleaned by pharmacologically or genetically assessing the function of the target in disease-relevant *in vitro* or *in vivo* disease models. As we discuss below, the latter criterion is fraught with many challenges, since multiple factors need to be met in order to ensure that the target function is indeed being accurately probed and that the models are indeed disease-relevant.

Table 9.1 Linking target to disease

Factors that support the role of a putative drug target in disease
Altered expression in disease
Component of disease pathophysiology
Mechanistic link to disease etiology
Genetic association with disease
Demonstration of beneficial effects in disease-relevant *in vitro* model
Knockout animals display beneficial phenotype
Pharmacological intervention using qualified probe compound(s) shows therapeutic benefit in animal model

Table 9.2 Criteria for selecting a new drug target

Target selection criteria	Examples
Link to disease	Genetic association, altered expression, preclinical or clinical validation
Chemically tractable	Precedents for target class; small molecule endogenous substrate/ligand
Likelihood for specificity	Some divergence from related family members
Safety	Restricted expression to target cell/organ; no overt knockout phenotype

There are other more pragmatic considerations for selecting a target, in addition to linking it to the disease (Table 9.2). Notably, the target must be considered chemically tractable, or "druggable". This is a somewhat amorphous term that is open to broad interpretation. Traditionally, a druggable target would be one in which a small molecule can bind with high affinity and specificity and mediate the desired biological effect. For instance, enzyme inhibition is considered a chemically tractable approach since a small molecule could be envisioned to occupy the active site and antagonize normal activity. Activation of a G-protein coupled receptor would also be considered feasible since a small molecule may be able to alter the conformation of the protein in the same way that the endogenous ligand would. By contrast, blocking a protein-protein interaction has traditionally been considered less druggable: since these are generally large surface interactions mediated by van der Waals forces as well as electrostatic interactions, they were thought to be difficult to disrupt with a small molecule. However, scientific advances are changing this view, and modulation of protein-protein interactions by small molecules is now an area of active growth (Higueruelo et al. 2013). Indeed, the scope of druggable targets continues to expand as new precedents are set, as drug screening technologies become more sophisticated, and as new drug modalities, such as monoclonal antibodies and antisense oligonucleotides, become available.

However, it is not merely necessary to be able to generate a potent pharmacological agent against the target; it is also important that the molecule can specifically modulate the target. Understanding the gene family to which the target belongs is useful in predicting the likelihood for specificity. For instance, if the target is highly related to another protein that it is not desirable to inhibit, then it may be difficult to identify specific inhibitors, and the value of the target will be diminished.

The final, and arguably most important, criterion for target selection is safety. Many drugs fail in the clinic, not because of lack of efficacy, but due to adverse safety events. Therefore, it is important to select targets with the greatest likelihood of being safe from the outset, and to address any potential safety concerns experimentally as early as possible. The first question to ask is how broadly the target is expressed. A target expressed specifically in the target cell and tissue presents some advantages, as there is less likelihood for adverse effects unrelated to the desired mechanism of action. Unfortunately, in the case of microglial targets, there are very few genes expressed in microglia that are not expressed in peripheral cells of monocytic origin, so immunosuppression is a persistent concern. This is in contrast to neuronal or astrocytic pathways, where it is often possible to identify targets with expression restricted to the CNS.

The potential safety of a target can also be assessed to some extent if a knockout animal for the target is available. If the knockout animal does not have any observable phenotypes, this is a good sign, with the major caveat that other genes may compensate for the complete loss of function of the target throughout development. A conditional knockout model where the target can be globally ablated only in the adult animal would be more informative than a constitutive knockout. If there are safety concerns, whether due to a knockout phenotype, suspected mechanistic roles, or some other information, it is important to address these directly in an experimental system. For instance, if immunosuppression is a concern, it might be important to challenge animals with an infection after drug administration to determine whether they are able to resolve it. Potential safety issues may not necessarily lead to immediate termination of a program, since there may be a large gap between doses that result in therapeutic efficacy and those that result in toxicity, thereby providing a therapeutic window. However, it is not sufficient to merely hope for the best and determine whether there is a viable therapeutic window in the clinic. The therapeutic window must be diligently determined in preclinical models using all tools available.

9.3 Assay Development

Once a target is identified, the next step is to screen for modulators of the target. A common approach is to employ high-throughput screening (HTS) of small molecule libraries to identify agonists, antagonists or allosteric modulators. In the pharmaceutical industry, an HTS campaign traditionally prosecutes libraries containing 10^5–10^6 compounds, and therefore must be run in a high-density plate format, usually 1,536-well plates. More recently, screening has become more sophisticated, employing smaller diverse libraries, fragment libraries, or other emerging technologies, rather than brute force approaches (Manly et al. 2008; Langer et al. 2009; Mayr and Bojanic 2009). The screens themselves are generally quite rapid. Most of the time and effort goes into developing the assay for the screen, and it is the quality of the assay that often determines the success of the screen.

Table 9.3 Assay development quality control

Factor	Measure
Precision of liquid handling	Coefficient of variation of volume transfer
Reagent stability	Assay performance remains the same over course of screen
Plate effects	Coefficient of variation of endpoint across plate; discernible patterns
Assay performance	Z′
Day-to-day consistency	Coefficient of variation of Z′ over different assay runs
Assay connectivity	EC_{50} or IC_{50} values comparable across multiple assays

EC_{50}, the concentration of a drug that gives half-maximal response; IC_{50}, the concentration of an inhibitor where the response (or binding) is reduced by half

High-throughput screening assays require more quality control (QC) than assays run in research labs (Table 9.3). This is in part because the assays must be robust enough to perform consistently from day-to-day executed by different scientists, and in part because each compound is run as a single replicate for practical reasons. A common metric used in research for assay performance is the coefficient of variation (CV), the ratio of the standard deviation (σ) to the mean (μ). However, this is not a sufficient metric for an HTS assay, since it does not incorporate the variability at the extremes of the assay and the size of the effect. A better metric to QC an HTS is the Z′ (Z-prime, also known as Z-factor) (Zhang et al. 1999). The Z′ is a measure of the magnitude of the window between the positive (p) and negative (n) controls as well as their variability:

$$Z' = 1 - 3\left(\sigma_p + \sigma_n\right) / \left|\mu_p - \mu_n\right| \tag{9.1}$$

The factor of 3 is chosen to multiply the sums of the standard deviations because in a normal distribution, 99% of values occur within 3 standard deviations of the mean. An assay with no variability ($\sigma = 0$) will have a Z′ of 1, and therefore 1 is the highest Z′ possible. However, due to the factor of 3 in the numerator, Z′s are very sensitive to assay variability. Indeed, in an assay with three replicates, a Z′ can be negative and still show highly significant differences between positive and negative controls, whereas a cutoff of 0.5 is generally required for HTS. This highlights the increased rigor required for screening compared to assays run in a more traditional basic research setting.

Developing *in vitro* assays in microglial cultures poses additional unique challenges. Microglia are specifically adapted to respond dramatically to the slightest signal of damage or infection. Therefore, even tiny amounts of contaminants, such as endotoxin present on most labware, can have major consequences on assay performance, even though they would not cause any noticeable effects on most other cell types. Standard protocols must be adapted so that special care is taken to minimize the effects of environment on microglia (Witting and Möller 2011).

There are additional considerations when establishing a screening assay. If automation is used, which is usually the case, the precision of liquid handling needs to be assessed. This can be achieved by transferring a dye, such as tartrazine (Petersen

and Nguyen 2005), at the desired volume to several plates and measuring the optical density against a standard curve. The assay endpoint should also be assessed across several plates to determine whether there are any artifacts amongst wells of a plate or from plate-to-plate. Since automation often adds time delays and assays are run over several hours, the stability of the assay components and of the assay itself needs to be determined. Finally, the assay must perform robustly from day-to-day over the course of the entire screen. Each run should include the appropriate controls, such as a whole column of the positive control and a whole column of the negative control on every plate, to ensure that the assay continues to perform with the appropriate metrics.

9.4 Hit-to-Lead and Lead Optimization

The output of screening campaigns using compound libraries are generally poor pharmacological agents, called "hits" or "actives", that serve as mere starting points in the drug discovery process. Hits must go through a number of quality assurance steps, secondary assays and some chemical optimization before promising lead compounds are identified (Fig. 9.2).

The first step is to retest a relatively large number of actives to confirm the biological activity found in the N = 1 screen. At this stage, some compounds will replicate the finding, and some will not. The former are considered "confirmed actives". Often a freshly-prepared sample is then tested to further confirm the *in vitro* activity. A number of counterscreens are performed to eliminate the possibility of false positives, such as tests on a parental cell line not expressing the receptor being studied, as well as a number of orthogonal tests which are usually run under more physiologically-relevant conditions than the screening assay.

This exercise often delivers up to a few hundred compounds ready to move forward. Sometimes an early readout of possible structure-activity relationships can be established. This is extremely valuable at this early stage, as it supports chemical tractability of the biological activity, which should not be taken for granted. Compounds are then typically analyzed based on a number of *in silico* physicochemical descriptors, which are used as surrogates of the drug-like quality of the compounds being studied. The most common descriptors used include molecular weight (MW), lipophilicity (cLogP), polar surface area (PSA), rotatable bonds (RB), and hydrogen bond donors and acceptors (HBD and HBA, respectively). For the specific case of CNS drugs a drug likeness central nervous system multiparameter optimization (CNS MPO) algorithm may be used (Wager et al. 2010).

After all these efforts, generally a number of distinct chemical series, called leads, are prioritized and the lead optimization work starts, aiming at delivering highly-optimized compounds known as drug candidates. The optimization process navigates through a number of hurdles to maximize confidence that the compound will be efficacious and safe when taken to the expensive clinical trials. For most projects this implies assuring oral bioavailability, good absorption and pharmacokinetics,

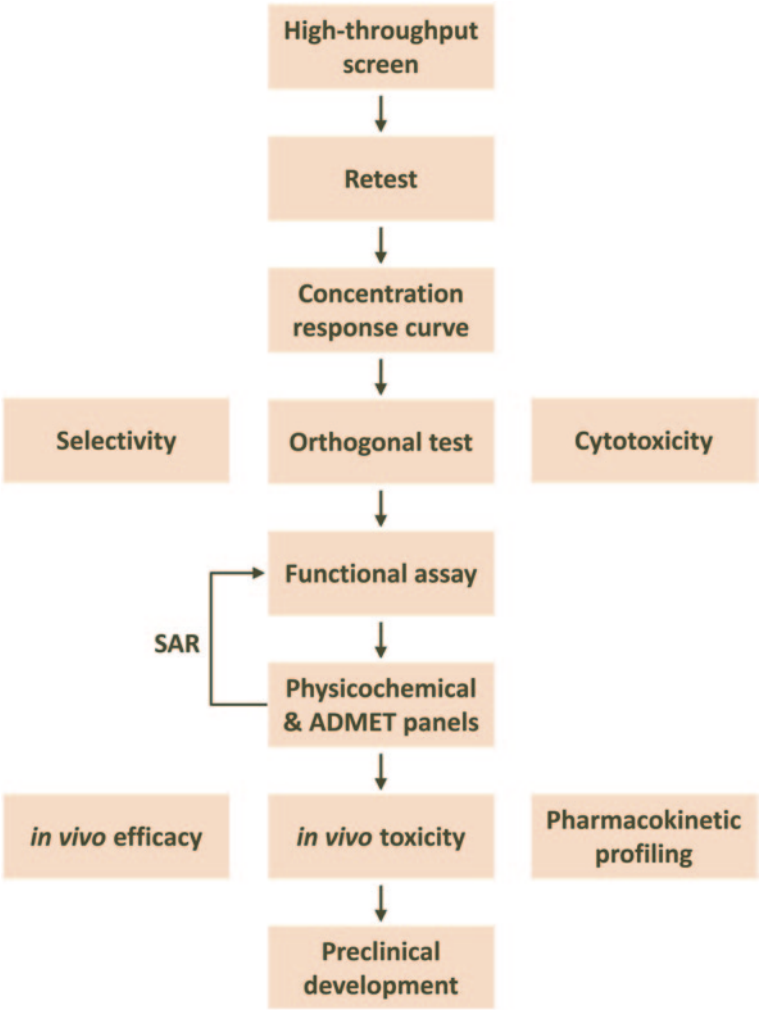

Fig. 9.2 Generic drug discovery screening cascade. After hits are identified from a high-throughput screen, they are first confirmed through retesting at the screening concentration, including parental cell line. Confirmed hits are then tested over a range of concentrations to generate concentration response curves and determine potency. Orthogonal tests confirm hits using technology completely independent of the screening assay to rule out potential screening artifacts. Orthogonal assays are usually more physiological than the HTS assay. At this point, compounds may also be evaluated for selectivity against proteins related to the target and, if they are going to be taken from a cell-free to cell-based assay, for general cytotoxicity. Secondary assays are then run to confirm the functional consequences of modulating the target. Compounds are then evaluated through a variety of *in vitro* physicochemical and ADMET (absorption, distribution, metabolism, excretion, toxicity) assay panels used to predict pharmacokinetic (PK) properties and potential safety liabilities. Structure-activity relationships (SAR) are determined via iterative medicinal chemistry to optimize PK, safety and activity, producing lead compounds. Lead compounds are tested *in vivo* to determine their PK, safety and efficacy profiles. The best compounds are progressed towards full preclinical development to identify candidates that will enter the clinic. It should be noted that this is a generic example. In keeping with the spirit and scope of this work, greater details are given for the early part of this process, and much less so for the later one. (For details on the later part of the preclinical drug development process, the reader is suggested to access a number of references (Wermuth 2008).)

efficacy at a reasonable daily dose, lack of drug-drug interactions derived from cytochrome P-450 (CYP) inhibition or induction, minimizing interactions with active transporters (*e.g.*, P-glycoprotein for CNS drugs), establishing an effect-exposure relationship *in vivo*, efficacy in phenotypic and mechanistic pre-clinical models (hopefully with good translational validity, *vide infra*), as well as a large battery of selectivity and safety tests *in vitro* and *in vivo*, to determine a therapeutic index and a maximum tolerable exposure during the clinical work (van de Waterbeemd 2009).

It is important to understand that, for the vast majority of new drug projects, the development candidate is not a compound that already exists as a member of a chemical library. On the contrary, medicinal chemistry efforts are often described as "threading the needle", to symbolize the highly sophisticated process that generally results in a very small number of molecular entities with acceptable attributes. Another point worth discussing is that *in vitro* potency should not be used in isolation as the key driver to rank-order compounds for further profiling. Indeed, it has been concluded that marketed oral drugs seldom possess single-digit nanomolar potency (50 nM is the average potency) (Gleeson et al. 2011). Therefore, a development candidate is often a compound with a number of balanced attributes rather than the molecule that performs significantly better in every possible test in the screening cascade.

9.5 Biological Validity Criteria: Predictive, Face and Construct Validities

A critical step in validating targets and evaluating lead molecules is to test them in preclinical animal models. A major pitfall has been the indiscriminate use of preclinical animal models without sufficient understanding of their validity. This paradigm worked for a while, when the exploited biological target space was a continuation of past successes. However, once these "low hanging fruit" of drug discovery were eventually depleted, and to continue to address unmet patient needs, industry had to venture into newer, more complex areas, and hence explore more sophisticated animal models (Meier et al. 2013).

The risk of failure is especially high for compounds reaching late clinical trials, where hundreds of millions of dollars may be spent for just one clinical study. In order to manage the risk involved in clinical translation of efficacy from preclinical models, a number of concepts were developed under the umbrella of the validity. While the concept is not new (Willner 1984), it is now a subject of increased focus (Fineberg et al. 2011).

There are three primary domains of validity that are sought after in animal models of disease: predictive, face and construct validity (Willner 1984; Markou et al. 2008; Becker and Greig 2010; Dzirasa and Covington 2012). Predictive validity refers of the ability of the model to predict pharmacological efficacy in the clinic. For instance, the Forced Swim Test (Porsolt et al. 1977) in which rodents are placed in a beaker of water has some predictive validity for depression because

monaminergic-based antipressants reduce animal immobility in this assay (although the observed rapid onset of efficacy in this model does not reflect the clinical experience). The major limitation for models with predictive validity is that they may not respond to drugs with novel, and maybe more efficacious, mechanisms of action. Furthermore, if there are no effective drugs in the clinic for a given disease, it would not be possible to know whether the model possesses any predictive validity for that disease.

Models with face validity overtly resemble the symptoms of the disease. For example, models in which dopamine neurons are ablated using exogenous toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (William Langston et al. 1984), exhibit some of the same motor symptoms as Parkinson's disease. The caveat for preclinical models with face validity is that they may not reflect the etiology of the disease in humans, and therefore may not respond to therapeutic interventions that target disease pathogenesis. They may however respond to treatments aimed at the symptoms of the disease.

The third type of biological validity is construct validity. Construct validity means that the model captures the underlying pathophysiology of the disease. This could be at the molecular level, such as when a transgenic animal model is generated by overexpressing a human mutant protein that causes an inherited monogenic disorder; or at the systems levels, such as by altering neuronal circuitry in a way that mimics alterations found in disease. The phenotypic manifestation of a model with construct validity may not resemble the disease at all. For instance, *Drosophila* mutants with loss of the *parkin* gene are arguably a model for early-onset Parkinson's disease with construct validity, yet the phenotype manifests as difficulty emerging from their pupal cases and defects in flight due to degeneration of muscle cells (Greene et al. 2003; Whitworth et al. 2005).

Ideally, a disease model will have all three types of biological validity. This is rarely, if ever, the case. Nevertheless, models with only one or two types of biological validity can be very useful in the drug discovery process, so long as their limitations are clearly understood and they are used to answer the right questions.

9.6 Additional Validity Criteria: Chemistry, Quantitative Pharmacology and Bias-free

In addition to the well-described types of biological validity, we propose three additional types of validity to consider when conducting preclinical studies using pharmacological probes in animal models. These are chemistry, quantitative pharmacology and bias-free validity (Fig. 9.3).

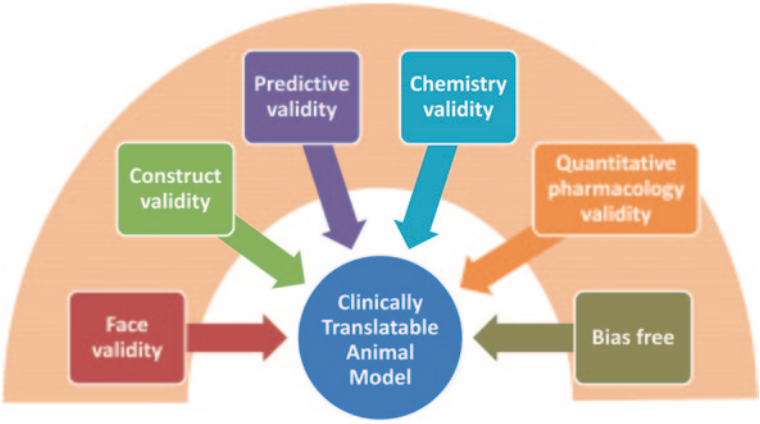

Fig. 9.3 Schematic proposal for enhancing biology-based validity criteria to include chemistry, quantitative pharmacology and decision-making principles useful in supporting scientific research aiming at validating biological targets for drug discovery projects

9.6.1 Chemistry Validity

The primary intent of a chemical probe is to establish the relationship between a molecular target and the broader biological consequences of modulating that target (Frye 2010). To this aim, a certain level of qualification of the compound being used across a number of areas is required. The goal of this exercise is to make sure the compound is actually doing what one thinks it must do, the way one expects it should do it, by ruling out other potential interferences of chemical origin.

Answering a number of key questions will go a long distance to support phenotypic observations and link these to a true pharmacological modulation of a biological target by a tool compound or drug candidate. For example,

- Is there a class effect? Do different compounds, with diverse chemical structures but similar target profiles, demonstrate similar pharmacology? Or is this a "one-off" effect, only seen with one compound and not seen with very close analogs?
- Does the tool compound have any druggability flaws derived from its chemical structure?

A number of properties related to the chemical structure of a certain tool compound may cloud the interpretation of phenotypic screens. Among these, the most common are shown in Table 9.4 (Davis and Erlanson 2013).

It is important to understand that these are not rigid rules, and are meant only to exemplify some of the weaknesses that may be encountered that bias data interpretation. For example, not all compounds containing a certain chemical moiety will be pan-assay interference compounds (PAINS) (Baell and Holloway 2010); it varies on a case-by-case basis and should be considered only as a risk management strategy.

Table 9.4 Some fundamental factors to be considered for available probe compounds depending on the nature of the study being planned

in silico	*in vitro*	*in vivo*, single-dose	*in vivo*, multiple-dose
Unequivocal structure	Functional activity and binding affinity at the target	Dosing routes: IV, IP, PO, SC, ICV	Dosing routes: IV, IP, PO, SC, minipump, ICV
Chemically synthesizable	Solubility in buffer used	Unbound drug concentration commensurate with *in vitro* activity	Understand exposure time profile and unbound drug concentration commensurate with *in vitro* activity at peak and trough
	Selectivity (binding and functional screens) against anti-targets and broad panel	Selectivity	Selectivity
		Possible active metabolites	Possible active metabolites CYP induction

IV intravenous, *IP* intraperitoneal, *PO* per os, i.e. oral administration, *SC* subcutaneous, *ICV* intracerebroventricular

9.6.2 What Type of Study Are You Planning?

Not all of a compound's properties are equivalent or critical in terms of qualifying it as an appropriate tool compound for the planned study. So, which molecular attributes are truly needed and how to define them? A useful way to address this issue is based on the type of pharmacological study to be conducted: *in silico*, *in vitro*, acute *in vivo* or multiple-dose (sub-chronic) *in vivo*.

For *in silico* work, one can of course design any virtual molecule "on paper" as long as its structure is unambiguously defined, but that does not mean the compound's structure can actually be put together, or that it will stay that way in the actual experiment. Care must be taken with chiral centers and other structural features that lead to an ambiguous definition of a molecule or an unstable arrangement of atoms (*e.g.*, tautomers, unstable atom arrangements, Fig. 9.4).

In vitro pharmacology is mostly conducted in buffered aqueous solutions, generally at pH values close to the physiologically germane value of 7.4 (unless a specific study is done to accommodate broader pH values that are found in certain tissues). If the test substance remains as a separate physical phase and cannot be brought in contact with the rest of the biological system under study (*i.e.*, it is insoluble) within the time frame of duration of the assay, the pharmacological experiment cannot be conducted. When this occurs, a number of solubilization techniques are routinely used to circumvent this major issue. However, one is frequently better off looking for a more water-soluble compound, as often times lack of solubility comes together

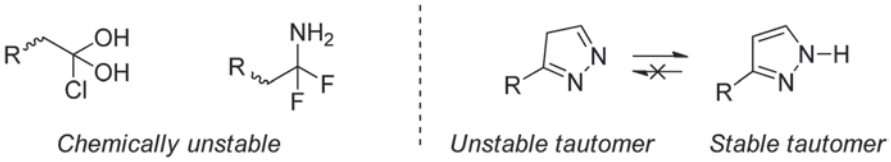

Fig. 9.4 Examples of chemical structures that may be drawn on "paper" for *in silico* studies but will not lead to synthesizable compounds due to chemical instability

with high non-specific binding (to plastic or glass surfaces as well as to the biological matrix, such as protein and lipids).

Importantly, selectivity of tool compounds against an anti-target (a biological receptor that must be avoided) or cell toxicity in toxicological screens is often assessed based on *in vitro* tests conducted at relatively high compound concentrations (in the 10–100 μM range). In this regards, solubility may hinder or confound the determination of these key enabling attributes.

If the compound can reach meaningful solubility in water and it interacts with the biological target being tested, the path forward is simpler. For overexpressed, recombinant systems used often for *in vitro* work, selectivity is not an issue. However, if then one turns to cell-based assays or *ex vivo* tissues for *in vitro* work, potential off-target interactions with other receptors that may now be expressed at similar levels as the target of interest must be kept in mind. Likewise, good cell membrane permeability is now an important requirement for the compound to reach certain types of targets (*e.g.*, intracellular enzymes).

For acute (single dose) *in vivo* studies, a different set of criteria comes to play to minimize the risk of misinterpreting phenotypic observations as related to the temporal concentration effects of the test compound. A number of routes of administration can be used, including intraperitoneal, intravenous, oral, subcutaneous, intracerebroventricular, etc. As long as the vehicle used is among those considered viable, any route may be fine. This is of course with the caveat that not all formulation vehicles are suitable to be administered in all routes. Capsules containing solid dosage forms for oral administration may be considered for some higher species like dog, with some distinct advantages.

The purpose of using an appropriate route of administration with relevant formulation is to deliver the drug to the desired site of action in large enough concentrations, as related to an *in vitro* measure of target affinity or functional potency, generally expressed as an IC_{50}, EC_{50}, etc. If the *free drug hypothesis* (*vide infra*) (Smith et al. 2010) is to hold within the target vicinity, then the unbound (free) drug concentration can be estimated. This estimation is calculated adjusting the total measured drug concentration by taking the unbound tissue fraction into consideration as shown in Eq. 9.2.

$$[Drug]_{unbound} = [Drug]_{total} \times [UB]_{tissue} \tag{9.2}$$

Unbound (UB) tissue fraction parameters (e.g., plasma protein or brain free fraction) are generally determined *in vitro* using relevant tissue homogenates. Often, for an antagonist (Kenakin 2009), receptor occupancy (RO) may be estimated based on Eq. 9.3.

$$\mathrm{RO\%} = 100/\left[1+\mathrm{IC}_{50}/[Drug]_{\mathrm{unbound}}\right] \tag{9.3}$$

So, when comparing similar compounds, a class effect may be established if a structurally diverse set of compounds provides a phenotypic effect at similar receptor occupancy values (Melhem 2013).

For multiple-dose *in vivo* work, in addition to the aforementioned criteria for single-dose studies, one has to consider the effects that prolonged drug exposure over time may have on the animal being studied. Thus, understanding the compound exposure-time profile is essential and achieving steady-state conditions may be of importance in these models. Potential changes range from those relatively simpler to explore, such as reduction of drug exposure due to CYP induction, or increases in drug exposure due to CYP inhibition or drug accumulation, to those more challenging to understand, such as changes in gene expression levels. In addition, the formation of circulating metabolites with their own pattern of biological activity, may contribute to the phenotypic readout otherwise attributed only to the parent drug. While this can certainly occur during single-dose studies, it becomes a much more significant risk during multiple-dose paradigm with *in vivo* work. If feasible, conducting a metabolite identification study with compounds of interest generally provides valuable information to mitigate this risk and design better multiple-dose studies.

Finally, we would like to express a word of caution about "repurposing" a compound used in human clinical work or even a marketed drug for use in preclinical studies. "Rats are not small humans." Their drug disposition mechanisms, as well as clearance pathways, may well not be the same, contributing to some of the caveats discussed earlier.

9.7 The Four Pillars of Target Validation

As discussed above, a full characterization of the chemical probes is essential to support the unbiased interpretation of biological experiments, which is a key element of rigorous preclinical target validation. It is to everyone's best interest that before making the commitments required to launch a drug discovery program, the biological target has been effectively validated using relevant assays. However, for a number of reasons this is not always the case, and several broadly used chemical probes exist which do not meet generally accepted potency and selection criteria, and therefore conclusions made from their use are suspect at best.

Pfizer scientists Mark E Bunnage, Eugene L Piatnitski Chekler & Lyn H Jones have put forward a framework for using chemical probes known as "the four pillars

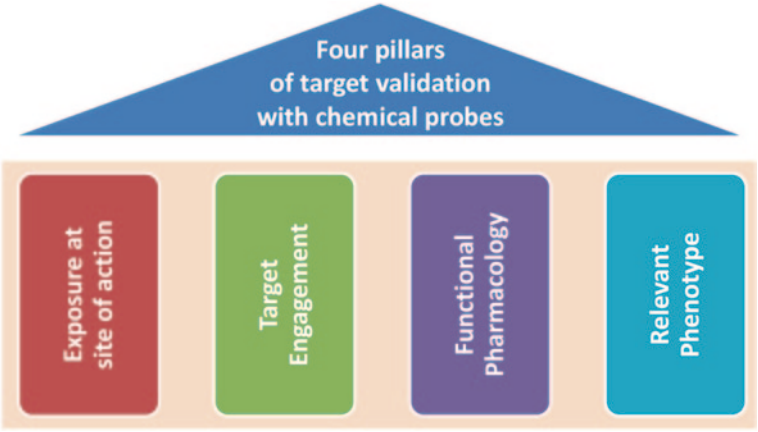

Fig. 9.5 The four pillars of target validation using chemical probes. Additional criteria may apply depending on the specifics of the target under investigation

of target validation" (Bunnage et al. 2013) (Fig. 9.5). While this is not meant to be a "one size fits all" solution to target validation, it is a good example of how strategic thinking can be applied during target validation using chemical probes. These key elements, which are not totally disconnected from those used in clinical drug studies (Morgan et al. 2012), are: establishing drug exposure at the relevant biophase, confirming target engagement, and confirming there is an actual functional pharmacology which is also relevant to the biological hypothesis under investigation.

Pillar 1: Exposure at the Site of Action This refers to the fact that regardless of the mechanism by which a tool compound exerts its effects, it must be able to reach the receptor localized at an appropriate bio-phase at pharmacologically relevant concentrations. For example, compounds exerting CNS effects *via* centrally-located receptors must be assessed for brain penetration. Importantly, the lack of exposure at the site of action does not rule out that the pharmacology observed may be real; it just negates the hypothetical mechanism of action. In particular, for intracellularly located biological targets, the ability of the chemical probe to penetrate the cell membrane (permeability) must be demonstrated. This is particularly important when assessing "false negatives" due to the probe's inability to enter a cell and reach its target rather than the lack of efficacy of a biological mechanism. Effective medicinal chemistry strategies exist to deal with this issue (Kerns and Di 2008).

Actual probe compound concentrations are often assumed to be "nominal concentrations" calculated based on those in a stock solution added to the test system. However, the presence of active uptake, transporter-mediated efflux or just slow permeability may hinder the probe compound from reaching the target. Hence, an experimental determination of the drug concentration *at the site of action* should be conducted. Exposures must also be commensurate with respect to the desired efficacious concentration of probe compound, as if these are in great excess over the on-target *in vitro* activity there may imply an erosion of the off-target selectivity, leading to misinterpretations.

Pillar 2: Target Engagement Receptor occupancy is a key element to link relevant drug concentrations to observed pharmacological effects. A number of different experimental techniques are routinely used to accomplish this task. Ideally, preclinical positron emission tomography studies provide valuable information in an *in vivo* setting (Linnman et al. 2013). A number of *ex vivo* techniques are often used. However, the interpretation of the data is not always straightforward (Grimwood and Hartig 2009). A possible approach to estimate preliminarily the levels of receptor engagement is to calculate the theoretical receptor occupancy (RO) using Eq. 9.4. This requires the determination of the free drug concentration in the appropriate biophase [A], and a measure of target affinity generally based in an *in vitro* assay (e.g., K_i).

$$\mathrm{RO\%}=100/\left[1+\mathrm{K_i}/[\mathrm{A}]\right] \tag{9.4}$$

During target validation, it is critical to minimize the risk that the activity measured in a given assay does not originate from an interfering biological target. In this regard, selectivity criteria for tool compounds are more stringent than for marketed drugs, where activity at a secondary target may be tolerable or even desirable.

Chemical probes acting *via* covalent binding to their biological targets deserve a special paragraph. While discouraged in the past due to potential for idiosyncratic toxicity, current views may be changing as can be inferred from an increasing number of drug candidates with a covalent mechanism of action progressing through clinical trials. It must be noted that technical challenges to fully characterize irreversible chemical probes are significant; a number of new strategies and tools have emerged and are expected to be expanded in the future (Mah et al. 2013)

Pillar 3: Expression of Functional Pharmacology Experimental means may be established to add support for the pharmacology derived from introducing a chemical probe into a biological system. Examples are the observation of changes of concentrations of an enzyme substrate or product (*e.g.*, the phosphorylated reaction product of a kinase) or measure changes in brain electrical circuitry using electrophysiology (*e.g.*, inhibition of post-synaptic excitatory currents).

Pillar 4: Proof of Phenotype Perturbation Preclinical-to-clinical translational challenges are well-known, especially for novel biological mechanisms of action. In part, this reflects a currentlyincomplete understanding of human and preclinical species disease and healthy-state physiology. Still, for a good number of indications, phenotypic tests exists that correlate with demonstrated efficacy in the clinic. These tests generally capture the most relevant biological changes in the context of the human disease. In such cases, probe compounds may be used to gain support for biological target validation.

Probe compounds may help achieve target validation even when providing negative results in a phenotypic screen. One such case, for example, is ruling out that a certain endpoint is derived from cell death instead of inhibition of a particular target (false positive). Another often used example is taking advantage of a chiral center in the chemical probe and when the biological activity resides mostly in one of the enantiomers. In such case, the biologically “inactive” enantiomer may be used

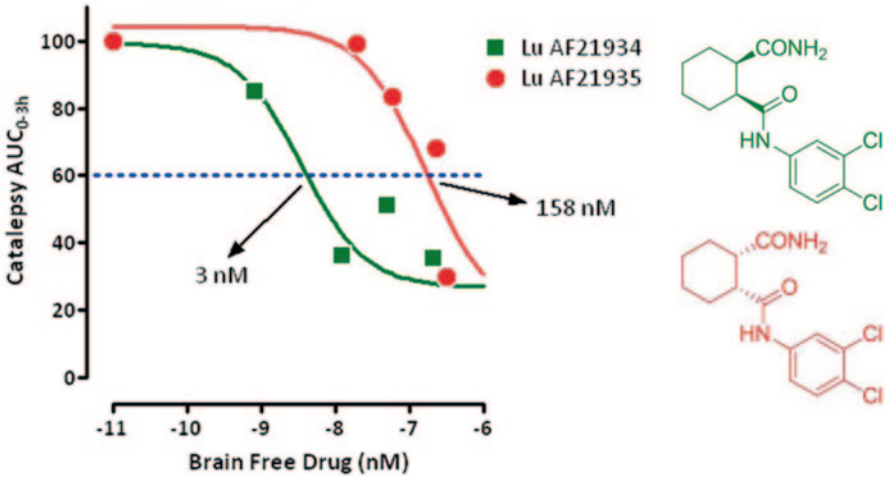

Fig. 9.6 Graph showing the a shift to the right for the relationship between the reversal of haloperidol-induced cataleptic response (phenotypic screen) and the unbound brain drug concentration of Lu AF21934 (in *green*) and Lu AF21935 (in *red*), the "active" and "inactive" enantiomers probe compounds, respectively, used in the validation of metabotropic glutamate receptor 4 as a Parkinson's disease target. (Bennouar et al. 2013)

as a negative control, as long as cross-reactivity and relevant tissue exposures are appropriately validated. These tests involve using probe compound doses beyond the minimum efficacy dose (MED), and often times a positive phenotypic response is obtained at a high dose or concentration, which originates as a result of a small percentage of the active enantiomer present as a contaminant, or due to potential cross-reactivity at probe high exposures. Practically, the extent of the "shift to the right" in the concentration- or dose-response graph must then be determined for the two enantiomers (Fig. 9.6). The observation of biological activity in a phenotypic screen *at high exposures* of the inactive enantiomer does not necessarily rule out a certain mechanism of action.

9.8 Quantitative Pharmacology

Not so long ago, *in vivo* pharmacological tests would be interpreted solely based on the phenotypic readouts. Due in part to advances in ADMET (absorption, distribution, metabolism, excretion, toxicity) science, and in part due to the extent of clinical failures, a view emerged postulating the need to take more integrative approaches, by linking the pharmacodynamic (PD) actions of a chemical probe to its pharmacokinetics (PK). This approach, known as 'quantitative pharmacology' or pharmacokinetics/pharmacodynamics (PK/PD), maximizes the information content and clarifies the temporal interdependence of the pharmacological properties and relevant tissue exposure characteristics (Gabrielsson et al. 2010).

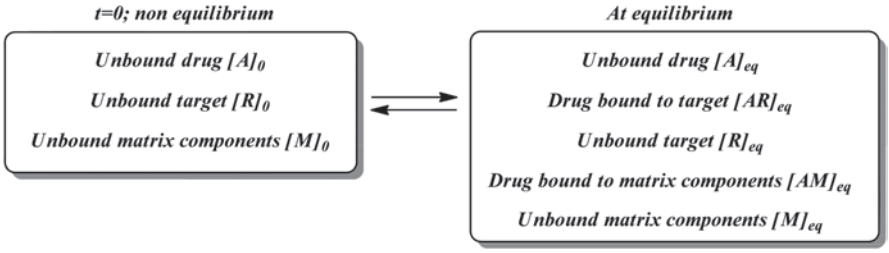

Fig. 9.7 Diagram showing changes in drug available to interact with the biological target. Non equilibrium concentrations correspond to nominal values. Equilibrium values take into account the non-specific binding that may occur to a variety of matrix components

An in-depth discussion of quantitative pharmacology is outside the scope of this work. Excellent discussions on practical PK/PD considerations for optimizing *in vivo* pharmacology studies have been published, and are highly recommended reading (Gabrielsson et al. 2010). However, two key points will be discussed in somewhat more detail here.

First, while human nature and probably our scientific training leads every one of us to simplify complex problems so that they become experimentally tractable and prone to be analyzed using mathematical models, we must not forget that Nature does not necessarily follow these principles. A large number of factors influence the way biological targets and their ligands interact in biological systems. Some of these are well-understood, but some are not. For example, for studies conducted *in vivo*, route of drug administration, vehicles used, rate and extent of drug absorption, dosing regimens, temporal differences and target turnover, receptor occupancy kinetics, differences in drug distribution, and non-specific tissue binding characteristics of drugs, all impact target engagement and therefore, are manifested in the observed pharmacology.

Second, in target validation studies, the *total* amount of drug at a given timepoint, in the relevant tissue is generally quantified using validated bioanalytical methods. However, as previously eluded to, the total concentration measured is often not what is available in the bio-phase to interact with the biological target. What is available is known as the *free (or unbound)* drug concentration. This difference is attributed to the vast majority of drug interacting with matrix components (*e.g.*, lipids and proteins *in vivo*, plastic or glass walls of labware used for *in vitro* tests) and as a consequence a new "equilibrium" state is generated (Fig. 9.7).

Thus, the free drug hypothesis is of particular importance when trying to establish the *relevant bio-phase* drug concentrations where the receptors are expressed (pillar 1, *vide supra*). Simply stated (Smith et al. 2010), the hypothesis is that:

a. at steady state, the free drug concentration is the same on both sides of any biomembrane
b. the free drug concentration at the site of action, the therapeutic target bio-phase, is the species that exerts pharmacological activity

The key implication is that measuring the total amount of drug in a certain tissue must be accompanied by a different measure of how strongly that drug interacts with matrix components. Exceptions are known to the free drug hypothesis, and include drugs with low passive permeability, or substrates of efflux or influx transporters present in the tissue expressing the therapeutic target.

9.9 Unpleasant Truths—Bias, Collaborations & (ir)Reproducibility

Sooner or later, data will be used to make decisions about a project. Given our imperfect understanding of disease biology, these decisions almost always imply incomplete knowledge. This is an area where better individual and team decision-making could enhance research performance. Indeed, it is well known that reproducible biases affecting human decision-making exist. Often known as cognitive biases, these jeopardize objectivity, and introduce a risk factor. These biases have been classified in overconfidence bias, calibration bias, availability bias, and excessive focus on certainty (Chadwick and Segall 2010). In a setting where projects compete against each other for (industry internal) resources, these biases pose a significant risk. Project leaders tend to start thinking of their projects as "their babies" and have a tendency to defend their projects beyond what would be considered reasonable. The *per se* laudable quality to "believe" in a project can turn into an obsession which keeps projects running against scientific evidence. In academia, where there is a constant need to publish, unsuccessful submission of manuscripts can have a limiting effect on work on "unpublishable" projects. In industry, where publications are usually a secondary aim and only happen with a delay during which intellectual property rights are secured, this external validation process is not in place. It is usually replaced by an internal review process which has to pay close attention not only to the scientific progress, but also needs to be aware of the "oh-so-human" biases. This internal review process is usually on an annual or biannual cycle. The outcome of such a project review determines the resources available to a project—they could be steady, increased or decreased. Due to the nature of pharmaceutical research, most projects will eventually close. The closure of projects leads to redistribution of resources and usually also affects academic collaborations.

Many investigators in academia have been in situations where they had industry contacts or even what they thought to be a successful collaboration with industry, and suddenly the industry partner walks away. Some academics may perceive this as "they were picking my brains and now they are secretly pursuing my idea". While we cannot speak for all in the pharmaceutical industry, we have not come across any case like this. Not only would we consider this unethical, this would deny our projects access to a valuable resource, the academic partner, who most likely is one of the leaders in their field. It is much more likely that a project which was working on a specific target got closed for the reasons discussed in earlier sections of this chapter.

The ultimate goal for the pharmaceutical industry is to develop safe and efficacious medication for patients with a high, unmet need. New projects are usually initiated with a survey of the scientific landscape around a disease or proposed disease biology mechanism. Many papers are read, discussed, prioritized, compared to our own expertise and in-house data and soon a picture emerges. Confidence builds around a target and its role in particular disease biology. However, one frequently lamented (Mullard 2011; Prinz et al. 2011; Begley and Ellis 2012; Nature-Editorial 2013b), but hitherto unresolved, issue might raise its ugly head: irreproducibility. This is certainly a challenge for industry where usually larger teams (and not only a sole graduate student) are used to pursue a project, and research costs quickly escalate. Yet, this issue affects the biomedical research community at large. The awareness around the topic has substantially increased in the recent years and the Nature publishing group has started a campaign in 2013 which is aptly called "Reducing our irreproducibility" and "Raising standards" (Nature-Editorial 2013a; Nature Neuroscience-Editorial 2013). Furthermore there is a web special on the issue (Nature-Editorial 2013b). In 2012 Nature had an editorial headlined "Must try harder" which bemoaned the perceived sloppiness of some research (Nature-Editorial 2012). Due to the touchiness of the topic, hard data is difficult to come by. However two recent non-peer reviewed correspondence pieces in Nature have tried to shed some light on the topic. In the first, titled "Believe it or not: how much can we rely on published data on potential drug targets?", a team from Target Discovery at Bayer Healthcare reports that out of 67 target validation studies, 43 (i.e. 64 %) were not reproducible (Prinz et al. 2011). In the second publication, results from a team at Amgen were reported. This group tried to validate 53 of what they considered landmark studies. Only six (i.e. 11 %) were successfully replicated (Begley and Ellis 2012). Based on these reports and the response they triggered from academia and industry alike, C. Glenn Begley wrote another comment which he termed the "Six red flags for suspect work" (Begley 2013). Based on his experience in trying to replicate published research, he raised the following six questions which might help identify "irreproducible" results. (1) Were experiments performed blinded? (2) Were basic experiments repeated? (3) Were all the results presented? (not just those that fit the story). (4) Were there positive and negative controls? (5) Were reagents validated? (6) Were statistical tests appropriate? These questions should resonate with any diligent reviewer for a scientific journal. However, it should come at nobody's surprise that many publications, including ones in high profile journals, do not hold up to these standards. Table 9.5 summarizes some of the key criteria we consider constitute a well-designed preclinical target validation study using pharmacological tools.

As detailed in the cited publications the reasons for irreproducibility can be multiple and our comments on this topic should not be perceived as finger-pointing. We simply want to raise awareness on the topic. In some instances, preclinical papers, which ultimately could not be reproduced, spawned an entire field. Sometimes, hundreds of follow-up publications expanded on elements of the original publication, without validating or falsifying its fundamental basis. More troubling though, some of the research led to the initiation of clinical studies implying that some

Table 9.5 Check-list for a well-designed preclinical pharmacological CNS study for target validation

Criteria	Importance
Drug exposure measured in brain at relevant time-points	Essential
Free fraction in target organ corresponds to compound potency	Essential
Pharmacodynamics endpoint	Essential
Researcher blinded to compound identity	Essential
Study size appropriately powered	Essential
Study repeated on independent cohorts of animals	Essential
Animals and groups randomized	Essential
Side-effect profile that may confound efficacy readout	Essential
Compounds tested in dose-response	Important if feasible
Evidence for target engagement (e.g., receptor occupancy)	Important if available
Multiple compounds of different chemotypes tested	Greatly improves confidence
Compounds tested in multiple disease models	Greatly improves confidence

patients might have been subjected to a trial of a drug or regimen that in all likelihood would not work. There is substantial difference if the end product of an effort is a publication or a drug which is given to people.

Conclusions

The beginnings of the twenty-first century are witnessing a strong wave of downsizing among corporations conducting new drug discovery. This is especially so in the area of central nervous system diseases. The lack of detailed knowledge about the biology of devastating diseases such as Alzheimer's disease, depression, or Parkinson's disease is a key determinant of extremely costly failures in clinical trals of drug candidates. Simultaneously, neuroinflammation biology is increasingly being recognized as playing a key role in these diseases, potentially enabling key progress in this area.

While several targets can be interrogated repositioning previously developed drugs for peripheral inflammation processes, a significant amount of new neuroinflammation targets require target validation and the discovery of new chemical probes to interrogate the relevant biologies. We hope the information provided in this chapter will aid organizations which have taken a leadership role and are making strides in the search for new drugs.

Scientists working in drug research projects end up making multitude of decisions—hopefully data-driven, good decisions. Given the time and efforts that are invested in the endeavor, it is not unexpected that personal biases will play a role and increase the chances of making a poor decision. Strategies to reduce sources of bias when assessing evidence exist (PLoS-Medicine-Editorial 2005). Understanding these "yellow flags" and becoming aware of them should also improve the probability of success. This may require special training and new approaches to analyzing data, data mining and visualization techniques. Drug discovery is a

task filled with uncertainty, and researchers must reach a level of comfort dealing with this issue. Differentiating preliminary from confirmatory studies or refutations, negative results, understanding biases and study design limitations, and potential confounding factors in the system under study should help managing the multiple inherent risks existing in target validation exercises.

Lastly, we would like to use our own experience pool (two of us are career pharmaceutical industry scientists, one is a recent transplant from academia) to suggest ways to optimize academia-industry interactions. Sometimes there seems to be too much focus on each other's weaknesses and very little is known about each other's strength. This can lead to misunderstandings, misconceptions and frustrations on either side. We believe there is tremendous potential for these collaborations to positively impact the future of human healthcare. To fulfill this potential, our interactions suggest that both parties should see each other as equal partners, and share as much knowledge and information as possible. To facilitate this aim, clear research collaboration agreements need to be in place, enabling the open exchange of data and ideas in both directions. Private parties, who must have a laser-focused objective to generate practical applications for human health, should clearly decide what deliverables they must own in order to justify their investment. Academic parties, typically more concerned about discovering new knowledge and making it available to the broader scientific community, need to clearly communicate their priorities and have a robust legal frame work to engage in frank discussions without the fear of being treated unfairly by the industrial partner. The key is to leverage each other's strength and compensate for potential weaknesses and generate mutually beneficial outcomes.

Nobody can discover drugs in isolation. It is our sincere hope that this book chapter highlights some of the needs and approaches of one of the partners and helps improve mutual understanding.

Acknowledgments We would like to sincerely thank Vlad and Alex to have given us the opportunity to write this chapter. We are grateful to all our colleagues in the Neuroinflammation Disease Biology Unit of Lundbeck Research USA for the enthusiasm with which they take every step in the exciting journey of uncovering the secrets of the intriguing and complex science required to improve our understanding of Neuroinflammation, and hopefully deliver new and effective treatments for underserved patients. In particular, we recognize Drs. Robb Brodbeck, Bob Nelson and Gamini Chandrasena for their encouragement. We acknowledge Drs. Stevin H. Zorn and Klaus Bæk Simonsen for their support.

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