A GUIDE TO DRUG DISCOVERY — OPINION

Reducing safety-related drug attrition: the use of *in vitro* pharmacological profiling

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Abstract | *In vitro* pharmacological profiling is increasingly being used earlier in the drug discovery process to identify undesirable off-target activity profiles that could hinder or halt the development of candidate drugs or even lead to market withdrawal if discovered after a drug is approved. Here, for the first time, the rationale, strategies and methodologies for *in vitro* pharmacological profiling at four major pharmaceutical companies (AstraZeneca, GlaxoSmithKline, Novartis and Pfizer) are presented and illustrated with examples of their impact on the drug discovery process. We hope that this will enable other companies and academic institutions to benefit from this knowledge and consider joining us in our collaborative knowledge sharing.

Decreasing the high attrition rate in the drug discovery and development process is a primary goal of the pharmaceutical industry. One of the main challenges in achieving this goal is striking an appropriate balance between drug efficacy and potential adverse effects[1] as early as possible in order to reduce safety-related attrition, particularly in the more expensive late stages of clinical development. Gaining a better understanding of the safety profile of drug candidates early in the process is also crucial for reducing the likelihood of safety issues limiting the use of approved drugs, or even leading to their market withdrawal, bearing in mind the growing societal and regulatory emphasis on drug safety.

It has been estimated that about 75% of all adverse drug reactions (ADRs) are dose-dependent and can be predicted on the basis of the pharmacology profiles of the candidate compound (known as type A ADRs)[2,3]. The pharmacology profile of a preclinical compound can be classified into primary or secondary effects. Primary effects relate to the action of the compound at its intended target (or targets), whereas secondary effects are due to interactions with targets other than the primary target (or targets) (that is, off-target interactions). Off-target interactions are often the cause of ADRs in animal models or clinical studies, and so careful characterization and identification of secondary pharmacology profiles of drug candidates early in the drug discovery process might help to reduce the incidence of type A ADRs.

In vitro pharmacological profiling involves the screening of compounds against a broad range of targets (receptors, ion channels, enzymes and transporters) that are distinct from the intended therapeutic target (or targets) in order to identify specific molecular interactions that may cause ADRs in humans. New drugs pass through non-clinical safety pharmacology and preclinical toxicology assessments according to guidelines produced by the International Conference on Harmonisation (ICH): for example, ICH S7A[4], ICH S7B[5] and ICH M3(R2)[6]. These guidelines describe the (mostly *in vivo*) safety testing of drug candidates and are designed to prevent serious ADRs from occurring in clinical studies.

The only *in vitro* pharmacology assay that is absolutely required by regulatory authorities is one that measures the effects of new chemical entities on the ionic current of native (I_{Kr}) or heterologously expressed human voltage-gated potassium channel subfamily H member 2 (KCNH2; also known as hERG)[5]. The mechanism by which blockade of hERG can elicit potentially fatal cardiac arrhythmias (torsades de pointes) following a prolongation of the QT interval is well characterized[7,8], and the seriousness of this ADR is one reason why this assay is a mandatory regulatory requirement. Receptor binding studies are also recommended as the first-tier approach for the assessment of the dependence potential of novel chemical entities[9].

However, current regulatory guidance does not describe which targets should constitute an *in vitro* pharmacological profiling panel and does not indicate the stage of the discovery process at which *in vitro* pharmacological profiling should occur. Nevertheless, the general trend for most pharmaceutical companies is to perform this testing early in drug discovery to reduce attrition and to facilitate better prediction of ADRs in the later stages of drug discovery and development.

Here, for the first time, four major pharmaceutical companies (AstraZeneca, GlaxoSmithKline, Novartis and Pfizer) share their knowledge and experiences of the innovative application of existing screening technologies to detect off-target interactions of compounds. The objective of this article is to describe the rationale and main advantages for the use of *in vitro* pharmacological profiling, to discuss best practices and to share more widely the minimum panel of targets that, based on our collective experience, should be considered. We hope this will enable smaller companies and academic institutions to benefit from this knowledge and consider joining us in our collaborative knowledge sharing. The impact of generating such data during the different phases of the drug discovery process is illustrated through case studies.

Advantages of *in vitro* profiling

Early profiling of compounds against targets that are known to underlie ADRs (using the screening technologies devised for drug discovery and in particular for high-throughput screening) can help medicinal chemists to identify chemical series that lack this activity and are a potential liability, long before the final compound is selected (FIG. 1). Classical examples of well-characterized targets include hERG[7,8] (which is linked to cardiac arrhythmias, as noted above) and the 5-hydroxytryptamine (serotonin) receptor 2B (5-HT_{2B}) (which is linked to cardiac valvulopathy)[10,11]. If inherently 'clean' chemical series (that is, those with no off-target activities of safety concern) are not available at the lead selection stage, an understanding of the structure–activity relationship (SAR) can help to reduce or eliminate off-target activity while retaining or increasing activity at the primary target.

There is a growing awareness that *in vitro* pharmacological profiling[12,13], together with traditional safety pharmacology, can have a positive impact on the success rates of late-stage clinical development[14,15]; BOX 1 summarizes the main advantages of *in vitro* pharmacological profiling. Although the impact of *in vitro* pharmacological profiling data differs according to the stage at which the data are utilized in the drug discovery process, it can be maximized when generated throughout the discovery process (FIG. 1). Impact can be categorized into five aspects: first, early hazard identification and decision-making in the lead generation and selection phase; second, hazard elimination in the lead optimization phase; third, candidate selection; fourth, integrated risk assessment in early development; and fifth, risk management and mitigation in preclinical and clinical development.

First, once a chemical series has been identified and activity at the primary therapeutic target has been confirmed, secondary profiling can be performed to assess its promiscuity (that is, the percentage of targets hit at a specific concentration compared to the total number of targets tested). The importance of assessing promiscuity at this stage is discussed below. In addition, individual off-target activities at molecular targets with established linkage to ADRs can be identified at this stage.

Second, these data can then be used to develop a lead optimization plan, which can include analysis of the SAR at off-targets in order to influence chemical design. An advantage of *in vitro* profiling at this stage means that testing only requires a relatively short turnaround time (days) compared to *in vivo* studies, which may take weeks to read out. The compound amounts required are also substantially lower for *in vitro* studies compared to *in vivo* studies.

Third, at the end of lead optimization, the data can be used to select the candidate drug from a shortlist. The data can also be used to trigger and influence the design of *in vivo* discovery safety studies as part of an early integrated risk assessment. Most compounds are tested during the lead selection and lead optimization phases, as this is when the full benefits of chemical optimization in reducing potential liabilities can be achieved.

Fourth, during the later phases of lead optimization, the value of the data is enhanced when interpreted in the context of the predicted therapeutic free plasma concentration, which is often available by this stage. Based on the drug's affinity at the off-target and the predicted exposure levels, drug occupancy at off-targets can be estimated and safety margins can be calculated (this is described in more detail in the case study examples below).

Fifth, in the preclinical and clinical development stages, when regulatory safety studies are performed, a broad understanding of the pharmacological profile of the candidate drug is helpful for understanding the mechanistic underpinnings of the effects observed *in vivo*. In addition, the data can be used to build a patient risk management plan for Phase I trials if necessary. In some cases, additional end points or biomarkers may be included, which is a main advantage of profiling human targets from the beginning of the process.

The overall benefits of performing *in vitro* pharmacological profiling throughout the drug discovery and development process are clear. However, so far, there is little consensus regarding the minimal panel

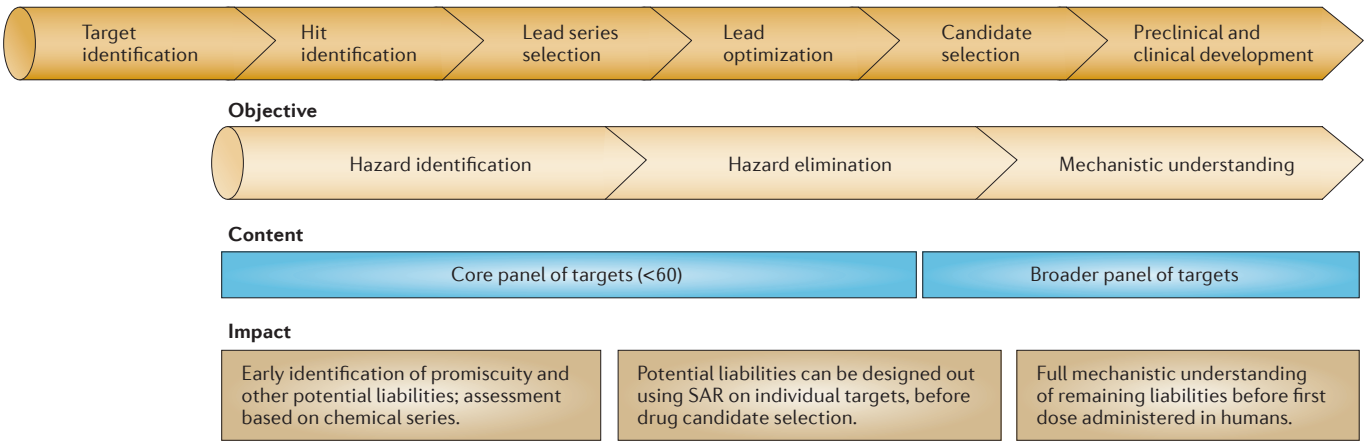


Figure 1 | **Alignment of *in vitro* pharmacology profiling to the drug discovery and development process.** In the early phases of the drug discovery process, the main objective is to identify hazards and understand the potential for promiscuity (that is, having affinity at a broad range of targets) in the initial lead series. The application of a standard panel of targets (fewer than 60), annotated for linkage to safety liability, provides data that can be used for early decision-making regarding which lead series to select for further development. Data from this initial hazard identification profiling can be used in the lead optimization phase to eliminate the liability by building structure–activity relationship (SAR) models and using the data to drive chemical design. The profiling panel can also be used to select key candidates to progress into development as well as to trigger and influence the design of investigative *in vivo* studies. In the later phases of drug discovery, data can be generated in a broader panel of targets and used for mechanistic understanding of effects *in vivo*. Importantly, it is desirable to obtain a comprehensive understanding of the broad pharmacological profile of the drug candidate before carrying out first-in-human trials.

of targets that should be used in these profiling campaigns. Below, we describe the pooling of information from four pharmaceutical companies, along with some of the lessons that have been learnt.

'Cross-pharma' knowledge

In vitro pharmacological profiling evolved in similar ways within different pharmaceutical companies. In the past, as part of the general pharmacology studies that were routinely carried out, a few advanced leads were tested against small panels of targets from protein classes closely related to the therapeutic target. With the development of screening technologies that enabled increased throughput at reduced costs, targets were added from members of major target classes that are involved in the regulation of vital systems such as the cardiovascular, respiratory and nervous systems.

The panels designed by these four pharmaceutical companies aim to cover a broad range of targets (receptors, ion channels, enzymes and transporters), and the inclusion of these targets is carefully considered by the weight of evidence for each target based on multiple parameters. These include knowledge of the following concepts: the expression and fundamental role of the target in physiology (including knockout mouse models and genetic evidence from humans); whether pharmacological modulation translates into a biological effect; and, more importantly, whether there is evidence from clinical (especially post-marketing) safety data that links an ADR to a specific target.

Key to whether clinical experience links an ADR to a specific target is the testing of marketed drugs through *in vitro* assays. As a result of an improved understanding of the pharmacological significance of each target, but also owing to budgetary constraints (see below for more details), most companies have reduced the number of targets that are included in their *in vitro* profiling panels. Now, only those targets that have a strong relevance to safety are routinely profiled. However, it should be noted that the application of larger, more diverse profiling panels on key drug candidates is also useful to facilitate a comprehensive understanding of their pharmacological profile before conducting first-in-human studies.

One of the main factors affecting each company's decision to include or exclude a particular target is the cost–benefit calculation of the probability of a hit at the target compared to the magnitude of the impact of this hit. Targets with a high hit rate and a high impact (for example, hERG and muscarinic acetylcholine receptor M_1) are readily prioritized for inclusion, whereas targets with a low hit rate and a low impact are readily prioritized for exclusion.

More judgement is required for potentially high-impact targets that have a low hit rate. For example, the endothelin A receptor is included in the panel despite having a low hit rate (<1%), as the cardiovascular and potential teratogenic effects of ligands at this receptor are profound and may not be identified until long-term *in vivo* studies are performed[16]. Conversely, melatonin receptor MT_3 has an extremely high hit rate (>30%) but as this has no definitive linkage to an adverse outcome, this target is not included in the minimum panel. Targets that have significant potential impact but very low hit rates (for example, transient receptor potential cation channel subfamily V member 4 (TRPV4), which has hit rates of <0.5%) are often not included based on this cost–benefit assessment. Each company has applied these considerations independently to derive its target panel. Hit rates are assessed at 10 μM with a broad range of chemically diverse compounds (more than 1,000 compounds) from each company's compound library and are continuously monitored over periods of routine screening. In general, there was a good correlation among the four companies regarding the hit rates at each target.

The minimal panel of targets

Here, by comparing the lists of targets from each of the four companies, we have defined a minimum panel of targets that are tested in at least three out of the four companies. Despite large variations in the size of the panels, as well as the tactics and technologies used among the companies, there is substantial overlap among the screened targets. Nineteen molecular targets were identical in all four companies and another 25 targets were tested in three out of the four companies.

The evolution of the target panels was based on decades of drug discovery experience with the targets and their validation in the clinic, so it is understandable that most targets are from the G protein-coupled receptor (GPCR) superfamily, with 24 representatives from 12 subfamilies. There are seven ion channel targets, six intracellular enzymes, three neurotransmitter transporters, two nuclear hormone receptors and one kinase. At present, we recommend that these 44 targets are considered as a minimal panel that should provide a broad early assessment

Box 1 | Major advantages of *in vitro* pharmacological profiling

The list below summarizes the main advantages of *in vitro* pharmacological profiling.

- Off-target interactions can be identified at an early stage — for example, at lead selection — and can be followed up in structure–activity relationship (SAR) studies to mitigate the activity
- Clinical side effects can be predicted that may be missed during *in vivo* safety pharmacology studies, toxicology studies or clinical trials: for example, valvulopathy with 5-hydroxytryptamine (serotonin) receptor 2B (5-HT_{2B}) agonists
- Large numbers of compounds, including metabolites, can be tested cost-effectively *in vitro* on human targets associated with clinical adverse drug reactions (ADRs)
- Representative chemical series can be tested in early stages of drug discovery for best leads and candidate selection, for all programmes in a portfolio
- Data from human targets correlate better with clinical side effects in humans (but perhaps not with those observed in animal models owing to species differences)
- Results and potential explanations of off-target effects can be obtained in a shorter time than with *in vivo* toxicology studies
- Data from SAR studies can help to optimize predictive *in silico* models
- Mechanism-based linkage of targets to established toxicities can be achieved
- Pharmacological promiscuity, which is usually indicative of significant ADRs, can be addressed at early stages
- Data from safety pharmacology profiling, first pharmacokinetic experiments, ADME (absorption, distribution, metabolism and excretion) and efficacy models can all be integrated to form an early safety risk assessment, giving greater confidence in the potential success of a development candidate
- Compounds entering development with minimal or no off-target activities require fewer investigative *in vivo* safety studies and therefore result in fewer delays in development, fewer animal models used and lower costs
- Data can be used to benchmark against key competitor compounds in order to develop a best-in-class strategy

of the potential hazard of a compound or chemical series. The targets, annotated with physiological relevance, are listed in TABLE 1.

GPCRs. The GPCR targets include representatives from the major neurotransmitter classes (adenosine, adrenergic, cannabinoid, dopamine, histamine, muscarinic, opioid and serotonin receptors) as well as from peptidergic GPCRs (cholecystokinin, endothelin and vasopressin receptors).

There was a good consensus on which GPCRs to include in the minimal panel; however, the decision on which muscarinic receptors to include in the panel provides an example of the impact of the individual experiences in each company. Muscarinic receptors have a fundamental role in physiology and their unintended inhibition or activation should therefore be avoided[17,18]. All four companies test against the M_2 receptor subtype (owing to the well-known cardiovascular effects of M_2 receptor agonists and antagonists), and two test against M_1 and M_3 receptors in addition to M_2 receptors. The other two companies test either M_1 or M_3 receptors (in particular for the cognitive and gastric effects of M_1 receptor antagonists and the involvement of M_1 and M_3 receptors in constipation, disturbed vision and dry mouth). The rationale for only testing either M_1 or M_3 receptors (and not both) is that despite the undesirability of affecting either of these receptor subtypes, there is likely to be redundancy; that is, a compound that is not designed to have activity at a specific muscarinic receptor is likely to be non-selective across multiple members of the muscarinic class of receptors.

This type of knowledge can be used to inform a risk–benefit analysis for the inclusion of only one of the receptor subtypes in the *in vitro* pharmacological panel rather than multiple members of a family. However, there is much flexibility with regard to deciding which receptor subtypes to include in a panel at which stage. It may be preferable to screen against most members of a particular family upfront, or it may be preferable to pick individual members as representatives and only screen other members if there is activity in the initial screen.

Ion channels. The feasibility of developing or running an assay also has an influence on the original selection of targets. Owing to their prominent physiological roles in cellular excitation, drug interactions with ion channels have long been associated with undesired effects. It can be challenging to develop pharmacologically relevant assays for voltage-gated ion channels that are capable of screening large numbers of compounds. These assays require high-throughput electrophysiological techniques and must capture various modes of action and different channel states. The availability of instruments to perform high-throughput electrophysiology[19] screening has enabled some of these targets to be included in the safety pharmacology profiling repertoire. Some examples include cardiac voltage-gated sodium channel subunit α (Nav1.5), voltage-gated calcium channel subunit α (Cav1.2) as well as potassium voltage-gated channel KQT-like member 1 (KCNQ1) co-expressed with minimal potassium channel (MinK; also known as KCNE1). However, these assays are often run in separate dedicated laboratories and at a later stage of the drug development process. Assessments of hERG channel activity are sometimes run as a separate assay owing to the exceptionally high hit rate at this target and the consequently higher number of compounds that need profiling. Ligand-gated ion channels such as 5-HT_3, GABA (γ-aminobutyric acid), NMDA (*N*-methyl-D-aspartate) and nicotinic acetylcholine receptors are often amenable to the development of binding assays and are included in the minimal panel.

Enzymes. Following the withdrawal of rofecoxib from the market and the consequent concerns about the increased risk of heart attack and stroke associated with long-term use of cyclooxygenase 2 (COX2)-specific inhibitors, all four companies screen against this enzyme, and some also screen against COX1.

Monoamine oxidase (MAO) inhibitors were extensively used for treating psychiatric and neurodegenerative disorders, but their use was severely limited by the occurrence of centrally mediated side effects. In particular, MAO inhibitors induce hypertensive crisis when combined with pressor amines — for example, tyramine, which is present in cheese (leading to the term 'cheese effect' being coined for this interaction). Moreover, combining MAO inhibitors with other serotonin agonists or selective serotonin reuptake inhibitors also induces a severe risk of a life-threatening serotonin syndrome. Consequently, MAO isoforms are also included by all four companies in their profiling panels.

Most of the phosphodiesterase 4 (PDE4) inhibitors developed so far, in particular for the treatment of asthma and chronic obstructive pulmonary disease, have failed to reach the market owing to on-target adverse effects such as emesis; therefore, PDE4 is also included in the profiling panels. PDE3 is another phosphodiesterase that is included in the profiling panel. PDE3 inhibitors such as milrinone have been used as cardiotonic agents for patients who have suffered from heart failure; these drugs have shown beneficial effects on relieving symptoms, but their long-term use (longer than 48 hours) in patients with severe congestive heart failure is associated with pro-arrhythmic activities and increased mortality. The other enzyme included in the panel is acetylcholinesterase (AChE). AChE inhibition causes side effects of varying severity, partly depending on the reversibility of inhibition, and secondary mechanism-of-action studies to determine reversibility may be performed on compounds that have high activity against this target.

As with cardiac ion channels, kinase screening is an example of how the four different companies similarly developed a procedure whereby kinase screening panels operate semi-independently from the *in vitro* pharmacology panels. Some companies have selected a handful of kinases (between four and ten) to act as 'sentinel' representatives of the family. Hits in these sentinel kinases trigger screening in wider kinase panels. There was little overlap in the sentinel kinases chosen by the four companies; indeed, there was only a single common kinase in three out of the four companies: lymphocyte-specific protein tyrosine kinase (LCK).

The lack of overlap among the four companies probably reflects the relative lack of understanding regarding the implications of modulating specific kinase activity. For instance, there is evidence of cardiotoxicity associated with the clinical use of some poorly selective kinase inhibitors, but information on the individual kinases responsible is often based on knockout mice or transgenic models, which could be misleading[20,21]. However, for some kinases there is evidence of potential safety liabilities due to known human genetic mutations, and in particular there are now a few highly specific kinase-directed antibodies on the market (some with black box warnings), which provide more direct evidence for specific kinases that are responsible for serious ADRs. We recommend that LCK should not be the only kinase in the panel; rather, a small selection of kinases should be included. However, the challenge is to identify suitable *in vitro* kinase profiling assays that will be predictive for clinical adverse effects[22]. These kinases should then be added into the profile.

Table 1 | **Recommended targets to provide an early assessment of the potential hazard of a compound or chemical series**

Targets (gene)	Hit rate*		Main organ class or system	Effects		Refs§
	Binding	Functional or enzymatic		Agonism or activation	Antagonism or inhibition	
G protein-coupled receptors						
Adenosine receptor A_{2A} (*ADORA2A*)	High	Low (agonist)	CVS, CNS	Coronary vasodilation; ↓ in BP and reflex; ↑ in HR; ↓ in platelet aggregation and leukocyte activation; ↓ in locomotor activity; sleep induction	Potential for stimulation of platelet aggregation; ↑ in BP; nervousness (tremors, agitation); arousal; insomnia	57
α_{1A}-adrenergic receptor (*ADRA1A*)	High	Low (agonist); high (antagonist)	CVS, GI, CNS	Smooth muscle contraction; ↑ in BP; cardiac positive ionotropy; potential for arrhythmia; mydriasis; ↓ in insulin release	↓ in smooth muscle tone; orthostatic hypotension and ↑ in HR; dizziness; impact on various aspects of sexual function	58
α_{2A}-adrenergic receptor (*ADRA2A*)	High	Low (agonist); medium (antagonist)	CVS, CNS	↓ in noradrenaline release and sympathetic neurotransmission; ↓ in BP; ↓ in HR; mydriasis; sedation	↑ in GI motility; ↑ in insulin secretion	59
β_1-adrenergic receptor (*ADRB1*)	Medium	NA	CVS, GI	↑ in HR; ↑ in cardiac contractility; electrolyte disturbances; ↑ in renin release; relaxation of colon and oesophagus; lipolysis	↓ in BP; ↓ in HR; ↓ in CO	60
β_2-adrenergic receptor (*ADRB2*)‡	High	Medium (agonist); medium (antagonist)	Pulmonary, CVS	↑ in HR; bronchodilation; peripheral vasodilation and skeletal muscle tremor; ↑ in glycogenolysis and glucagon release	↓ in BP	61
Cannabinoid receptor CB_1 (*CNR1*)	Medium/ high	Medium (antagonist)	CNS	Euphoria and dysphoria; anxiety; memory impairment and poor concentration; analgesia; hypothermia	↑ in weight loss; emesis; depression	62
Cannabinoid receptor CB_2 (*CNR2*)	Medium	Medium (agonist)	Immune	Insufficient information	↑ in inflammation; ↓ in bone mass	63
Cholecystokinin A receptor (*CCKAR*)	Low/ medium	NA	GI	↓ in food intake; gallbladder contraction; pancreatic enzyme secretion; ↑ in GI motility; activation of dopamine-mediated behaviour	↑ in development of gallstones	64
Dopamine receptor D_1 (*DRD1*)‡	Medium/ high	Medium (antagonist)	CVS, CNS	Vascular relaxation; ↓ in BP; headaches; dizziness; nausea; natriuresis; abuse potential	Dyskinesia; parkinsonian symptoms (tremors); anti-emetic effects; depression; anxiety; suicidal intent	65
Dopamine receptor D_2 (*DRD2*)‡	Medium/ high	Medium/high (agonist); medium (antagonist)	CVS, CNS, endocrine	↓ in HR; syncope; hallucinations; confusion; drowsiness; ↑ in sodium excretion; emesis; ↓ in pituitary hormone secretions	Orthostatic hypotension; drowsiness; ↑ in GI motility	66
Endothelin receptor A (*EDNRA*)	Low	NA	CVS, development	↑ in BP; aldosterone secretion; osteoblast proliferation	Teratogenicity	67
Histamine H_1 receptor (*HRH1*)‡	High	Very high (antagonist)	CVS, immune	↓ in BP; allergic responses of flare, flush and wheal; bronchoconstriction	Sedation; ↓ in allergic responses; ↑ in body weight	68
Histamine H_2 receptor (*HRH2*)	High	Low (agonist)	GI, CVS	↑ in gastric acid secretion; emesis; positive inotropy	↓ in gastric acid secretion	69
δ-type opioid receptor (*OPRD1*)	Medium/ high	NA	CNS, CVS	Analgesia; dysphoria; psychomimetic effects; cardiovascular effects; convulsion	↑ in BP; ↑ in cardiac contractility	70
κ-type opioid receptor (*OPRK1*)‡	High	Medium (agonist and antagonist)	GI, CNS, CVS	↓ in GI motility; ↑ in urinary output; sedation and dysphoria; confusion; dizziness; ↓ in locomotion; tachycardia	Insufficient information	71
μ-type opioid receptor (*OPRM1*)‡	High	Medium (agonist and antagonist)	CNS, GI, CVS	Sedation; ↓ in GI motility; pupil constriction; abuse liability; respiratory depression; miosis; hypothermia	↑ in GI motility; dyspepsia; flatulence	72

Table 1 (cont.) | **Recommended targets to provide an early assessment of the potential hazard of a compound or chemical series**

Targets (gene)	Hit rate*		Main organ class or system	Effects		Refs§
	Binding	Functional or enzymatic		Agonism or activation	Antagonism or inhibition	
G protein-coupled receptors (cont.)						
Muscarinic acetylcholine receptor M_1 (*CHRM1*)	High	Low (agonist); high (antagonist)	CNS, GI, CVS	Proconvulsant; ↑ in gastric acid secretion; hypertension; tachycardia; hyperthermia	↓ in cognitive function; ↓ in gastric acid secretion; blurred vision	73
Muscarinic acetylcholine receptor M_2 (*CHRM2*)‡	High	Low (agonist); medium (antagonist)	CVS	↓ in HR; reflex; ↑ in BP; negative chronotropy and inotropy; ↓ in cardiac conduction (PR interval); ↓ in cardiac action potential duration	Tachycardia; bronchoconstriction; tremors	74
Muscarinic acetylcholine receptor M_3 (*CHRM3*)	High	NA	GI, pulmonary	Bronchoconstriction; ↑ in salivation; GI and urinary smooth muscle constriction	Constipation; blurred vision; pupil dilation; dry mouth	75
5-HT_{1A} (*HTR1A*)	Medium/ high	Low (agonist); medium (antagonist)	CNS, endocrine	↓ in body temperature; reduced REM sleep; ↑ in ACTH; cortisol and growth hormone secretion	Potentially anxiogenic	76
5-HT_{1B} (*HTR1B*)	High	High (agonist); medium (antagonist)	CVS, CNS	Cerebral and coronary artery vasoconstriction; ↑ in BP	↑ in aggression	77
5-HT_{2A} (*HTR2A*)‡	Very high	Low/medium (agonist); medium/high (antagonist)	CVS, CNS	Smooth muscle contraction; platelet aggregation; potential memory impairments; hallucinations; schizophrenia; serotonin syndrome	Insufficient information	78
5-HT_{2B} (*HTR2B*)	High/ very high	Low (agonist); high (antagonist)	CVS, pulmonary, development	Potential cardiac valvulopathy; pulmonary hypertension	Possible cardiac effects, especially during embryonic development	79
Vasopressin V_{1A} receptor (*AVPR1A*)	Medium	High	Renal, CVS	Water retention in body; ↑ in BP; ↓ in HR; myocardial fibrosis; cardiac hypertrophy; hyponatraemia	Insufficient information	80
Ion channels						
Acetylcholine receptor subunit α1 or α4 (*CHRNA1* or *CHRNA4*)‡	Medium/ high	Low (opener); very high (blocker)	CNS, CVS, GI, pulmonary	Paralysis; analgesia; ↑ in HR; palpitations; nausea; abuse potential	Muscle relaxation; constipation; apnoea; ↓ in BP; ↓ in HR	81
Voltage-gated calcium channel subunit α Cav1.2 (*CACNA1C*)‡	NA	Medium/high (blocker)	CVS	Insufficient information	Vascular relaxation; ↓ in BP; ↓ in PR interval; possible shortening of QT interval of ECG	82
$GABA_A$ receptor α1 (rat cortex) BZD site (*GABRA1*)‡	Medium	NA	CNS	Anxiolysis; muscle relaxation; ataxia; anticonvulsant; abuse potential; sedation; dizziness; depression; anterograde amnesia	Seizure (when used as a BZD antidote)	83
Potassium voltage-gated channel subfamily H member 2; hERG (*KCNH2*)	High	High	CVS	Insufficient information	Prolongation of QT interval of ECG	84
Potassium voltage-gated channel KQT-like member 1 (*KCNQ1*) and minimal potassium channel MinK (*KCNE1*)	NA	Low	CVS	Atrial fibrillation	Long QT syndrome; potential hearing impairment, deafness and GI symptoms	85
NMDA receptor subunit NR1 (*GRIN1*)‡	Low/ medium	Medium (blocker)	CNS	Psychosis (schizophrenia-like); hallucinations; delirium and disoriented behaviour; seizures; neurotoxicity	Insufficient information	86
5-HT_3 (*HTR3A*)‡	Medium	Very high	GI, endocrine	Emesis; gastric emptying; hyperglycaemia; possible ↑ in HR	Constipation; dizziness	87
Voltage-gated sodium channel subunit α Nav1.5 (*SCN5A*)	NA	High	CVS	Insufficient information	Slowed cardiac conduction; prolonged QRS interval of ECG	88

Table 1 (cont.) | **Recommended targets to provide an early assessment of the potential hazard of a compound or chemical series**

Targets (gene)	Hit rate*		Main organ class or system	Effects		Refs§
	Binding	Functional or enzymatic		Agonism or activation	Antagonism or inhibition	
Enzymes						
Acetylcholinesterase (*ACHE*)	NA	High	CVS, GI, pulmonary	Insufficient information	↓ in BP; ↓ in HR; ↑ in GI motility (↓ at high doses); bronchoconstriction; ↑ in respiratory secretions	89
Cyclooxygenase 1; COX1 (*PTGS1*)	NA	Medium	GI, pulmonary, renal	Insufficient information	Gastric and pulmonary bleeding; dyspepsia; renal dysfunction	90
Cyclooxygenase 2; COX2 (*PTGS2*)‡	NA	Medium/high	Immune, CVS	Insufficient information	Anti-inflammatory activity; anti-mitogenic effects; myocardial infarction; ↑ in BP; ischaemic stroke; atherothrombosis	91
Monoamine oxidase A (*MAOA*)‡	NA	Medium	CVS, CNS	Insufficient information	↑ in BP when combined with amines such as tyramine; DDI potential; dizziness; sleep disturbances; nausea	92
Phosphodiesterase 3A (*PDE3A*)	NA	High	CVS	Insufficient information	↑ in cardiac contractility; ↑ in HR; ↓ in BP; thrombocytopaenia; ventricular arrhythmia	93,94
Phosphodiesterase 4D (*PDE4D*)‡	NA	Very high	CNS, immune	Insufficient information	Anti-inflammatory activities; antidepressant-like activities; emesis; vasculitis and arteritis; possible thymus atrophy	95,96
Lymphocyte-specific protein tyrosine kinase (*LCK*)	NA	Medium/high	Immune	T cell activation	T cell inhibition; SCID-like immunodeficiency	97
Transporters						
Dopamine transporter (*SLC6A3*)	High/very high	NA	CNS	Insufficient information	Addictive psychostimulation; depression; parkinsonism; seizures; dystonia; dyskinesia; acne	98
Noradrenaline transporter (*SLC6A2*)‡	High/very high	NA	CNS, CVS	Insufficient information	↑ in HR; ↑ in BP; ↑ in locomotor activity; constipation; abuse potential	99
Serotonin transporter (*SLC6A4*)‡	High	NA	CNS, CVS	Insufficient information	↑ in GI motility; ↓ in upper GI transit; ↓ in plasma renin; ↑ in other serotonin-mediated effects; insomnia; anxiety; nausea; sexual dysfunction	100
Nuclear receptors						
Androgen receptor (*AR*)	Medium	Medium	Endocrine	↑ in prostate carcinoma; oedema; androgenicity in females; ↑ in muscle mass; ↑ in hostility; sleep apnoea; liver complications	↓ in spermatogenesis; impotence; gynecomastia, mastodynia; ↑ in breast carcinoma	101,102
Glucocorticoid receptor (*NR3C1*)	Medium	Medium	Endocrine, immune	Immunosuppression; hyperglycaemia; insulin resistance; muscle wasting; ↑ in body weight; osteoporosis; glaucoma; ↑ in BP; ↓ in plasma potassium and arrhythmia	Hypoglycaemia	103

5-HT_{1A}, 5-hydroxytryptamine (serotonin) receptor 1A; ACTH, adrenocorticotropic hormone; BP, blood pressure; BZD, benzodiazepine; CNS: central nervous system; CO, cardiac output; CVS, cardiovascular system; DDI, drug–drug interaction; ECG, electrocardiogram; $GABA_A$, γ-aminobutyric acid type A; GI, gastrointestinal; HR, heart rate; NA, not applicable; NMDA, *N*-methyl-D-aspartate; REM, rapid eye movement; SCID, severe-combined immunodeficiency. *Hit rates were determined at 10 μM. 'Low' corresponds to <1% hit rate; 'medium' corresponds to 1–5% hit rate; 'high' corresponds to 5–20% hit rate; 'very high' corresponds to >20% hit rate. ‡Targets that were included in the panels of all four companies. §The references cited are key references giving details of some of the main adverse drug reactions (ADRs) for each target, but not all of the ADRs listed are mentioned in the cited publications.

Transporters. Neurotransmitter transporters are important drug targets; however, many are associated with safety liabilities such as increases in blood pressure and abuse liability, and thus three (dopamine, noradrenaline and serotonin transporters) are included in the minimal *in vitro* pharmacological profiling panel. Transporters that are involved in drug secretion or uptake (for example, liver-specific organic anion transporter 1 (LST1); also known as OATP1B1) or drug-metabolizing enzymes (for example, the cytochrome P450 enzymes) are often tested in separate departments (that is, those that assess the ADME (absorption, distribution, metabolism and excretion) properties of the candidate drugs). These departments have different workflows and so these types of transporters are not included in this pharmacological profiling panel.

Nuclear receptors. Nuclear receptors are represented by two targets: the androgen receptor and the glucocorticoid receptor. Neither androgenic nor anti-androgenic effects would be desirable properties in most drugs, whereas interactions with the glucocorticoid receptor could cause immunodeficiency, impaired glucose tolerance, muscle wasting and hypertension.

Profiling methods and testing strategies

In spite of the similar make-up of the target panels screened by the different pharmaceutical companies, different technical approaches have been adopted in establishing assays for these targets, particularly in the use of ligand binding and functional assays. Typically, binding assays utilize purified preparations of membrane or protein from recombinant or tissue sources expressing the target and a labelled high-affinity ligand that incorporates either a radioisotope or fluorescent probe. Compounds are tested for their ability to displace the binding of this labelled ligand. Functional assays measure activation, inhibition or modulation of the actual activity of a target, either in a purified preparation or expressed in a host cell. They encompass many different techniques, often with several approaches per target class.

GPCR assays generally rely on the measurement of second messenger (calcium or cyclic AMP) release or ^{35}S-labelled GTPγS binding, although numerous alternative approaches are possible[23]. For ion channels, technologies allowing automated electrophysiological measurements of channels expressed in a host cell (for example, using so-called 'population patch-clamping') are utilized[24,25]. Enzymes, including kinases, may be purified and assayed by incubation with substrates or products that have clear read-outs. Nuclear receptors are usually expressed in cells in combination with gene reporters, although biochemical assays using time-resolved fluorescence resonance energy transfer (TR-FRET) and fluorescence polarization can also be used. BOX 2 outlines some of the factors that influence the use of functional and binding assays in the context of *in vitro* pharmacological profiling, and summarizes the different information generated.

Ultimately, these approaches are complementary and all four companies rely on a combination of both ligand binding and functional assays to establish effective assay panels[26]. All assays included in early profiling panels, binding or functional, need to be robust (that is, they have to produce reliable and reproducible results over years) and they have to have a predictive value for safety. It can be challenging to achieve the level of robustness required when utilizing cell-based functional assays. Regardless of whether binding or functional assays are used as the first-pass test, care needs to be taken to confirm activity and specificity, as with any hit in a high-throughput assay. This is often achieved with a secondary assay using the complementary technology (that is, a binding assay followed by a functional assay, or a functional assay followed by a binding assay).

Ideally, initial testing should be performed at multiple test concentrations to allow a direct estimation of the AC_{50}. This provides a faster and more reliable result than if testing is performed initially at a single test concentration (typically 10 μM in duplicate) and only the hits are followed up by performing concentration–response curves. However, the former strategy can have a higher cost, especially for targets with a low hit rate, and this has to be calculated against the benefits.

With robust binding or enzymatic assays, it is possible to estimate the IC_{50} from single concentration data in which the percentage inhibition is between 20% and 80%, and this can provide the minimum information required for early hazard identification and elimination of off-target activity at a lower cost. However, this approach is not recommended when quantitative assessment of safety margins is required or if the percentage inhibition is greater than 85%. If the data are intended for regulatory submission, it is advisable to explore the concentration–response relationship and generate quantitative data (such as K_i, IC_{50} or EC_{50})

Box 2 | Factors influencing the assay mode chosen for *in vitro* profiling assays

The lists below outline some of the factors influencing the use of functional and binding assays in the context of *in vitro* pharmacological profiling, and the different information generated by these methods.

Binding assays

- Direct measure of affinity
- Single, defined site of binding only on the target
- Widely established technology that is applicable to different targets and target classes
- No differentiation of modes of action (that is, agonist or activator versus antagonist, blocker or inhibitor)
- Multiple assays for complex targets with multiple binding sites (for example, ion channels)
- Single assay to capture multiple modes of action; may need to deconvolute with downstream functional assay
- Usually require radioligand
- High-efficacy, low-affinity agonists may be missed

Functional assays

- Indirect or no measure of affinity
- Ability to determine end result of binding at any site on the target
- Different technologies for different targets and target classes; may require extensive technical expertise
- Ability to differentiate between modes of action
- Single assays for complex targets with multiple binding sites (for example, ion channels)
- May require separate assays for different modes of action or different second messenger pathways
- Usually non-radioactive
- Ability to directly measure agonist EC_{50} (effector concentration for half-maximum response)

to enable interpretation of the results in the context of a safety margin. Also, when a functional cell-based assay is used as the primary profiling assay, it is important to evaluate a full concentration–response effect, as partial activation of a target would otherwise be missed.

A common theme among the four companies is to screen compounds starting from the earliest hit and lead identification stages of drug discovery onwards. At these stages, *in vitro* pharmacological profiling data are used in the selection of lead series for further chemical optimization. When unexpected off-target activity is detected, chemically related compounds will typically be screened against the primary therapeutic and the undesirable target (or targets) to establish a divergent SAR. Additional targets for which the pharmacophore space is similar to the undesirable target may also be included. *In silico* and data mining tools based on near-neighbouring or other similarity methods may be deployed — for example, to identify previously tested and well-annotated compounds with similar profiles[27].

During lead optimization, off-target activities are considered in the context of the nature of the hazard to determine further courses of action; that is, whether an off-target activity is a high- or low-impact event. At this stage, drug discovery teams begin to predict therapeutic plasma concentrations. A key objective during this phase of discovery is to achieve a desired effect following dosing in an animal model of disease (that is, to achieve efficacy). Availability of *in vitro* profiling data enables the development of leads with the best safety margins, rather than simply those that have efficacy at the lowest plasma concentration. Prioritization of safety pharmacology or toxicology studies may also occur on the basis of the *in vitro* findings to investigate a potential liability at early stages. It should be noted that it is strongly recommended to build these profiling panels using human (as opposed to animal orthologue) targets, as ultimately the aim is to predict ADRs occurring in humans. For some molecular targets (for example, muscarinic acetylcholine receptors), there is a good correlation in affinity between human and rodent targets, but this is not the case for other targets (for example, chemokine receptors). This needs to be considered when using data from pharmacological assays to design or interpret experiments in preclinical species.

A further powerful use of *in vitro* profiling is for influencing the design of preclinical *in vivo* studies and for understanding the molecular mechanisms of the adverse effects observed. Regulatory guidance (ICH S7B)[5] draws on the importance of integrated risk assessment to define the risk–benefit profile of a drug candidate. Integrated risk assessments incorporate *in vitro* pharmacology data (AC_{50}) and compare these data to the exposure (free C_{max}) observed in preclinical species or to the predicted therapeutic free plasma concentration in humans, alongside biomarkers for adverse events such as blood pressure, cardiac contractility or circulating liver enzymes[28,29]. This comparison offers a holistic representation of risk versus benefit to facilitate the decision to proceed to clinical studies.

Drug metabolites are usually identified and available in purified or synthesized forms only at later stages of development, but they should still be considered for *in vitro* pharmacological profiling to account for the potential ADRs that might limit patient safety. The high capacity and low relative cost of *in vitro* profiling means that any available metabolite can be profiled, including those generated in low proportion to the parent compound. The relative exposure to the metabolite compared with the parent compound can then be considered to ascertain the probability of activities translating into ADRs in humans. A classic example is the sodium channel blocker procainamide and its metabolite *N*-acetylprocainamide. The parent compound is used as an anti-arrhythmic drug but its metabolite exerts pro-arrhythmic effects via blockade of cardiac potassium channels[30]. The adverse effects are accentuated by the fact that the metabolite is equipotent at sodium and potassium channels and has a longer half-life than the parent molecule.

Glossary

AC_{50}
Concentration required to elicit a 50% response in an *in vitro* assay. IC_{50} refers to an inhibitory response (the half maximal inhibitory concentration) and EC_{50} refers to an effect (the effector concentration for half-maximum response), usually an activation or stimulation. AC_{50} is a collective term used for any activity.

Adverse drug reactions
(ADRs). Any noxious, unintended and undesired effects of a drug, occurring at doses used in humans for prophylaxis, diagnosis or therapy. These exclude therapeutic failures, intentional and accidental poisoning and drug abuse.

EC_{50}
The concentration of an agonist that is required to produce 50% of the maximum response of that agonist.

Free C_{max}
The fraction of the C_{max} (peak total plasma concentration of a drug at a certain dose) that is not bound to plasma proteins. The percentage of the bound drug is determined separately and the C_{max} is corrected accordingly.

IC_{50}
The half maximal inhibitory concentration, or the concentration of an inhibitor that is required for 50% inhibition of the maximum control response in a biochemical or cellular assay.

K_i
Inhibition constant; can be derived from the IC_{50} (half maximal inhibitory concentration) if the concentration of ligand or substrate and its dissociation or Michaelis constant is known. Should be used in preference to IC_{50} for binding assays.

Safety margins
Ratios of an AC_{50} (concentration required to elicit a 50% response in an *in vitro* assay) — or the inhibition constant K_i — of a drug at a target known to mediate specific adverse drug reactions (ADRs) and the therapeutic free plasma concentration. The latter can be directly determined in preclinical or clinical studies, or estimated from models. The AC_{50} is taken from the most relevant assay available for that target. Safety margins should be used as early as possible in the preclinical phase to continually assess the risk of an ADR occurring in the clinic.

Selectivity
The ratio of the AC_{50} (concentration required to elicit a 50% response in an *in vitro* assay) — or the inhibition constant K_i if available — of a drug at any target that is known or suspected to mediate an adverse drug reaction, and the primary (therapeutic) target.

Therapeutic free plasma concentration
The concentration of a compound in the plasma following a therapeutic dose. Often quoted as the maximum exposure.

Therapeutic index
In a drug development setting: the quantitative ratio of the exposure level at the chosen safety end point divided by the exposure level at the chosen efficacy end point, typically the ratio of the highest exposure to the drug that results in no toxicity over that which produces the desired efficacy. This term is often used incorrectly to describe the safety margin.

Impact in drug discovery — case studies

Application of the above-described strategies in early stages of drug discovery has many advantages, including early identification of safety signals, ability to influence chemical design, ability to provide a mechanistic elucidation of *in vivo* effects and, importantly, assistance in decision-making at crucial junctures in the development process.

The impact of the data generated from pharmacological profiling screens depends on the context of the individual project. This can include the therapeutic indication and patient population, route of

administration, duration of proposed treatment, anticipated nature and severity of the adverse effect linked to the target, central nervous system penetration and primary therapeutic target affinity. When available, predicted safety margins are important for interpreting the data and assessing the level of concern and action required. For example, for an oncology indication it may be appropriate to progress a compound with off-target activities that would not be tolerated by a juvenile asthmatic population.

Below, we outline some case studies in which the application of *in vitro* pharmacological profiling had a major impact on the project outcome.

Promiscuity analysis. The application of a consistent *in vitro* pharmacology profiling strategy over time enables the generation of a data matrix whereby novel compounds can be benchmarked against known compounds. Such data can be used to calculate a promiscuity index (which is generally the percentage of targets hit at a specific concentration compared to the total number of targets tested)[31,32], and the profile of a company's portfolio of development-stage candidate drugs can be compared with either successfully marketed or withdrawn drugs.

A promiscuity analysis of clinical and development-stage candidates from Novartis is shown in FIG. 2. The level of promiscuity of failed candidate drugs from Novartis was similar to that of withdrawn drugs, but owing to the implementation of early *in vitro* pharmacological profiling activity from late 2003 onwards the level of promiscuity of Novartis's development-stage candidates decreased significantly over time (FIG. 2).

A promiscuity index depends heavily on the composition of the panel and the number of targets included in the panel. A link between promiscuity and the propensity for toxicity has been shown[33,34]. As this link has been increasingly recognized, it has led to the use of smaller panels (10–25 targets), which are mostly made up of assays with a high hit rate that are used to estimate the level of promiscuity of a compound or series[35]. In these smaller screens, the aim is not to accurately define the consequences of a compound's interaction with an individual protein; rather, it involves estimating the promiscuity of the compound (or series) in line with the hypothesis that the more proteins a compound interacts with, the greater the likelihood of observing ADRs in toxicological or clinical studies. These pharmacological results are often combined with data on physicochemical properties to prioritize the chemical series[34,36].

Lead optimization. A project at Boehringer Ingelheim, developing novel ribosomal protein S6 kinase α3 (S6Kα3; also known as RSK2) inhibitors for the treatment of heart failure, identified BIX 02565 as an initial lead compound[37]. *In vitro* profiling of this lead compound in a panel of 68 targets revealed substantial off-target activities at multiple adrenergic receptors.

The translation of these *in vitro* off-target activities into *in vivo* cardiovascular effects was demonstrated in rat aortic ring studies, an anaesthetized rat cardiovascular screen and telemetry in conscious rats. Acute hypotension was shown to correlate with the affinity of this chemical series at the α_{1A}-adrenergic receptor but not with its activity at the primary target (RSK2). SAR studies allowed the separation of this off-target activity from RSK2 activity, as illustrated in FIG. 3.

These early *in vitro* pharmacology profiling data and subsequent SAR studies identified a new potent RSK2 inhibitor (compound 15) without substantial affinity for α_{1A}-adrenergic receptor and with no adverse cardiovascular effects *in vivo* at high plasma concentrations.

Interpretation and translation of profiling data. *In vitro* pharmacological profiling is an integral part of the drug discovery process, including predefined and *ad hoc* follow-up strategies. If a compound is designated a 'hit' in a binding assay then the next step should be to confirm functional activity. Designation of what is a hit of interest, and what is not, should take into account the affinity at the primary target and the degree of selectivity: if the ratio is sufficiently large, then the off-target hit can be de-prioritized.

However, assumptions on how well the AC_{50} at either the primary target or the off-target translates into a physiological effect can be highly misleading, so a similar level of effort to that applied for the primary target should be applied to the off-target to take into account how the activity translates from biochemical to cellular assays and from these into *in vivo* outcomes in both animal models and in humans.

Ideally, one would like to prioritize compounds from a lead series based on the initial result of the *in vitro* pharmacological panel. To do this, a high level of confidence is needed to predict not just whether a safety concern (that is, an ADR) may occur in

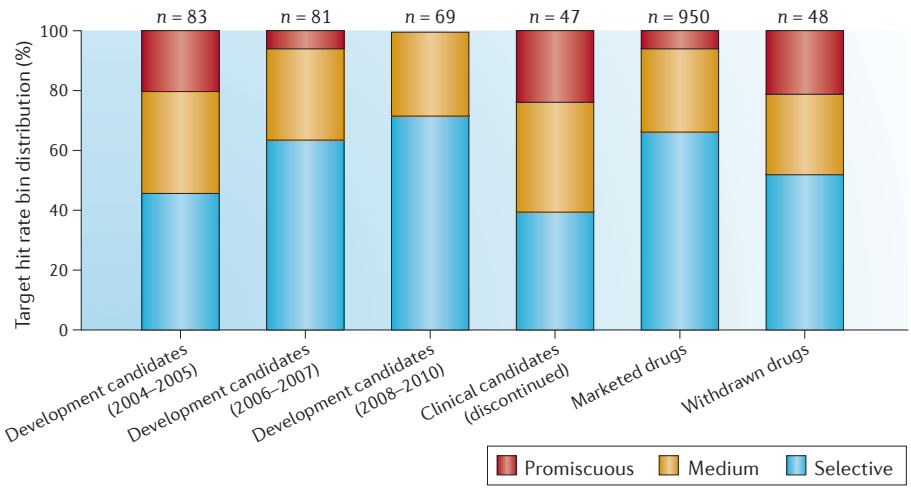


Figure 2 | **Levels of promiscuity among marketed drugs and Novartis's compounds.** A compound with a promiscuity index (the target hit rate, which is defined as the percentage of at least 50 targets giving greater than 50% inhibition at 10 μM) of greater than 20% is considered to be 'promiscuous', 5–20% considered to be 'medium promiscuous' and 0–5% considered to be 'selective'. Out of a set of 950 marketed drugs available in Novartis's internal compound archive (excluding antipsychotics), 66% were selective, whereas only 5% were promiscuous. Promiscuity increased to 22% in a set of 48 drugs that have been withdrawn or discontinued. The level of promiscuity among Novartis's clinical candidates that were discontinued was similar to the level of promiscuity of the withdrawn set of drugs (24%). The promiscuity of Novartis's development candidates, however, dropped from 21% to 0% between the 2004–2005 period and the 2008–2010 period. It remains to be seen whether this low level of promiscuity will translate into a lower attrition rate of the new clinical candidates. In this example, the levels of promiscuity (percentage of target hit rate bin distribution) are specific to this panel of more than 50 targets, and partially tested compounds have been excluded.

humans, but at what exposure the safety-related effect will be elicited to enable a reliable estimation of the safety margin.

Analysis of available translational data suggests that this type of early decision-making may be possible with *in vitro* hERG[8,38] and sodium channel Nav1.5 assays[39]. BOX 3 provides an example of how free plasma concentrations that are substantially below the K_i for Nav1.5 can result in adverse events in animals and in humans.

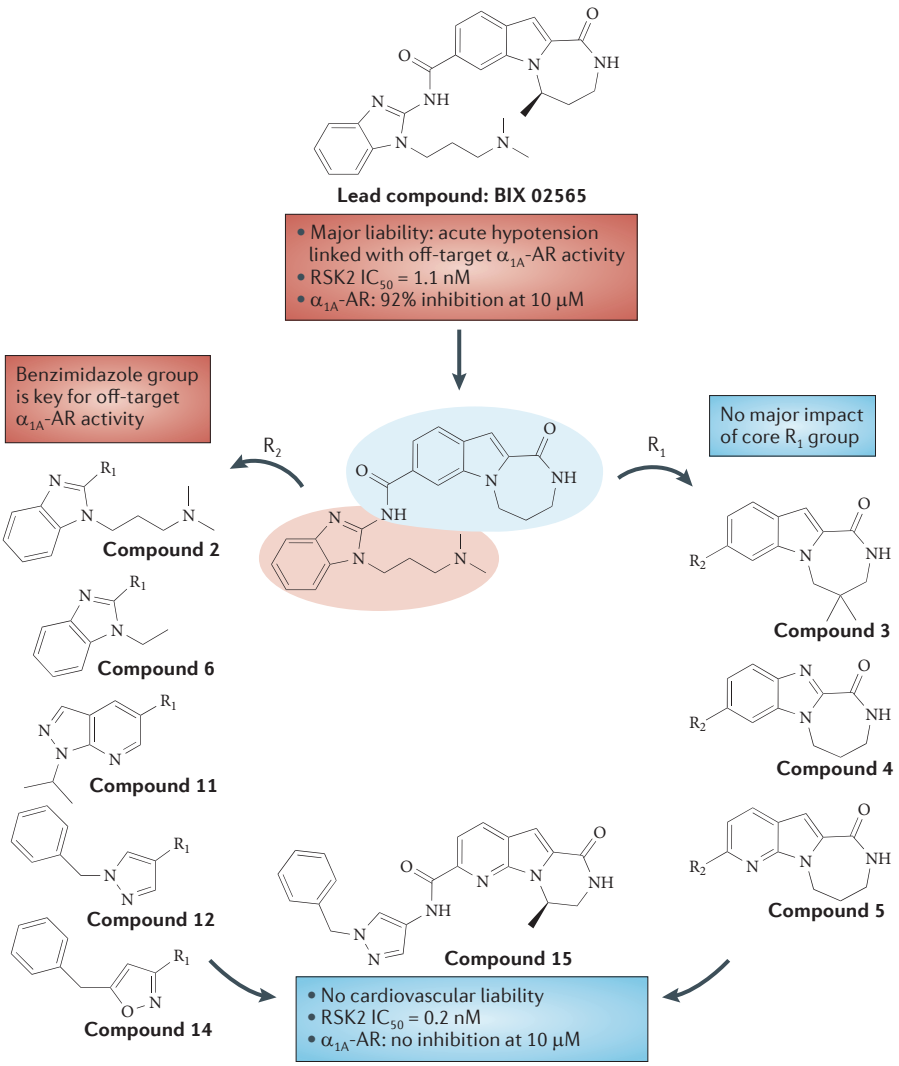
Assays for cannabinoid receptor 1 (CB_1) agonism provide another example for which data may have high translational predictive value. Compounds demonstrating CB_1 agonism are abused within the human population and there is emerging evidence that behavioural effects in rodents correlate with abuse potential in humans[40]. Unpublished data (Pfizer) show that the minimal effective concentration of compounds possessing CB_1 agonist activity in their pharmacological profile in rodent drug discrimination studies can correlate well with spontaneous reports of CB_1-like physiological effects in Phase I studies. Consistently, the minimal effective concentrations in rodents are below the EC_{50} of *in vitro* functional CB_1 agonist assays, which in turn are more potent than the binding K_i of the compound at CB_1. In reality, very few assays have this level of translational predictive value, and a stepwise approach through *ex vivo* and *in vivo* experimentation is necessary to confirm whether the compound possesses a safety liability as well as to refine the safety margin.

Future challenges and opportunities

The routine use of high-capacity *in vitro* pharmacology panels is one aspect of a broader trend in focusing on drug safety much earlier in the drug discovery process, with the aim of reducing the high rate of attrition. Selecting the minimal number of targets, and deciding which targets to include, in an *in vitro* pharmacological profiling assay is an exercise in judgement and experience, and also depends on budgetary and technical constraints.

It is estimated that the human genome contains more than 20,000 genes, and the number of proteins associated with undesirable pharmacological or toxicological effects is likely to be far in excess of the proposed 324 druggable proteins[41]. Even the largest of the *in vitro* profiling panels only covers ~1.5% of the total genome. Other targets will be added when confirmed links to ADRs are established.

Voltage-gated ion channels are proportionally under-represented in the current panels. This apparent under-representation



Compound	Primary target: RSK2 IC_{50} (nM)	Off-target: α_{1A}-AR (% inhibition at 10 μM)
R_2: benzimidazole group		
2	1.1	89
6	1.1	22
11	53	–5
12	4	0
14	30	2
R_1: core group		
3	21	92
4	91	69
5	240	68

Figure 3 | ***In vitro* profiling during lead optimization.** The initial lead inhibitor of ribosomal protein S6 kinase α3 (S6Kα3; also known as RSK2) — BIX 02565 — was shown to induce acute hypotension, which was linked with off-target activities on α-adrenergic receptors (α-ARs)[37]. To mitigate these off-target effects, a simple α_{1A}-AR binding assay was integrated into the flowchart of the project, and the structure–activity relationship (SAR) was established to decrease α_{1A}-AR activity while retaining potency at the primary target (RSK2). Some of these compounds were also tested in anaesthetized rats to confirm *in vitro* to *in vivo* translation between α_{1A}-AR binding activity and effects on cardiovascular function. Modification of the benzimidazole R_2 group, but not the core R_1 group, resulted in mitigation of the off-target activity and the discovery of a new lead compound (compound 15) displaying high activity on RSK2, no significant α_{1A}-AR-binding properties and no cardiovascular effects *in vivo*. IC_{50}, half maximal inhibitory concentration, or the concentration of an inhibitor that is required for 50% inhibition.

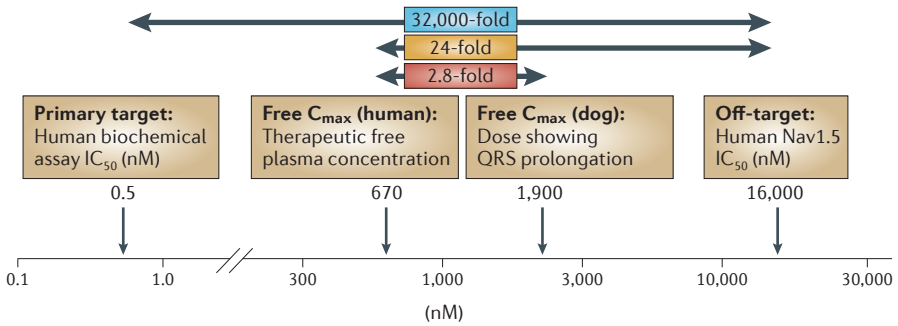
Box 3 | **Sodium channel Nav1.5 and cardiovascular toxicity**

Blockade of the cardiac voltage-gated sodium channel subunit α (Nav1.5) by a drug can lead to a prolongation of the QRS interval of an electrocardiogram (ECG), resulting in a block in ventricular conductance and potentially serious arrhythmias[54]. An association has also been reported between PR prolongation and the gene encoding the Nav1.5 channel (*SCN5A*) in humans[55].

A drug candidate being developed for a cardiovascular programme had a 32,000-fold difference (blue box) in the IC_{50} (half-maximal inhibitory concentration) values between the primary target and the off-target cardiac sodium channel Nav1.5. ECG measurements showed significant PR and QRS prolongation in a dog model at a free C_{max} (peak total plasma concentration of a drug) of 1,900 nM. Despite the very low IC_{50} value at the primary target, the therapeutic free plasma concentration (TFPC) in humans was only 2.8-fold lower (red box), resulting in an unacceptably low safety window.

The TFPC in the clinical study gave an estimated safety margin of 24-fold (orange box) against the Nav1.5 IC_{50}, which is within the 30-fold window suggested by Harmer *et al.*[39] as being a safety risk for QRS prolongation. A PR prolongation was observed in healthy volunteers at a free C_{max} of 1,200 nM. The resulting therapeutic index[104] of 1.8 was considered to be too low and so further development of the compound was terminated. As PR prolongation was seen to precede the QRS prolongation in dogs, the study was stopped at this point before a QRS prolongation could be seen. No suitable back-up compounds could be found and the programme was terminated. This case solidified our understanding of the safety margins required for Nav1.5 and will help future programmes to avoid the potential risks of hitting this target. For more information on this case study see REF. 56.

At early stages of drug discovery, only the *in vitro* biochemical and/or cellular activities are known, both on the primary target (or targets) and off-target (or off-targets). The magnitude of difference between these values can be calculated but — as demonstrated in this example — this may not translate well into the safety margin calculated between the off-target IC_{50} and the free C_{max} in the efficacy model, which is usually only obtained much later in the drug discovery process.



may be due to the lack of selective ligands available for validating any link to the safety liabilities associated with individual channels. Targets from new fields such as epigenetics are currently not included in the *in vitro* profiling panels as there is little understanding of the consequences of interfering with such targets. Similarly, kinases represent a relatively new target class; few kinase-directed compounds have reached the market and, with the exception of monoclonal antibodies, these compounds generally lack selectivity for individual kinases. Thus, although these compounds have fairly well-defined safety profiles, there is a substantial lack of strong evidence linking individual kinases to ADRs. The kinase field is, however, rapidly advancing with the development of translatable cell-based kinase assays, and so our understanding of the safety relevance of individual kinase targets will hopefully also advance accordingly[22].

Although there is potential to add more targets to the current panels, there also remains more to be learnt from — and about — the current panels. The companies surveyed in this article have been screening compounds for 5–10 years and have generated data that are ripe for structure–activity mining to develop *in silico* predictive tools similar to those that were previously generated for more familiar liabilities such as hERG[42] and those that were used in the prediction of genotoxic potential or metabolism (for example, Derek and Meteor software)[43]. The databases are now becoming large enough to proactively predict affinities across a range of chemotypes before carrying out compound synthesis[44,45], and computational tools for a more integrated approach to drug safety assessment are now also being developed[46,47]. However, owing to the requirement that data are acquired in a consistent manner (that is, using the same assay and technology), *in silico* models of pharmacological activity are currently restricted to individual company efforts.

With regard to the assessment of the secondary pharmacology of biologics (as opposed to small molecules, which are the focus of the efforts discussed here), it is generally accepted that profiling is not needed for monoclonal antibodies owing to their exquisite target selectivity. This argument has been made for not testing such therapeutics for activity at the hERG channel[48]. However, this may not hold true for all biologics. For example, changes to the amino acid sequence of small peptides can alter selectivity profiles, so possible off-target assessments could be restricted to close protein family members rather than a wider profile. If non-cytotoxic antibody–drug conjugates were to be developed, we would argue that these moieties would carry the same liability for off-target pharmacology as conventional small molecules because the pharmacological activity would originate from the small molecule and not the antibody, and so this is something to take into consideration for future profiling campaigns.

Although the ideal situation in drug development is elimination of the hazard, the biggest challenge is to interpret data from *in vitro* pharmacological profiling and provide advice on programme progression, as a development candidate is rarely absolutely 'clean'. *In vitro* pharmacological profiling of data must be performed in conjunction with a measure of efficacy (for example, primary target potency and therapeutic free plasma concentration) for comparison as well as to assess margins for safety effects. Interpretation of *in vitro* profiling data requires an understanding of the clinical impact of these off-target activities, which may be difficult to apply for novel targets. The translation of *in vitro* pharmacological panel data directly into knowledge that can reliably and accurately be used in safety-focused decision-making is at an early stage. As previously mentioned, several follow-up experiments of increasing complexity and cost are often needed to confirm that the safety risk is real (as exemplified in BOX 3). However, these follow-up assays suffer from the same well-documented limitations of many failures in Phase II efficacy studies: lack of predictivity from preclinical studies into clinical trials.

Expansion of the translational knowledge of safety end points could be achieved by closer cooperation among companies, academic institutions and governmental agencies that have data to share. An example of such data-sharing endeavours is the Predictive Safety Testing Consortium[49]. Other initiatives (for example, ToxCast, BindingDB, ChEMBL and the eTOX

Project) are attempting to generate and catalogue pharmacological data with the aim of predicting safety end points[50,51]. An Innovative Medicines Initiative (IMI)-like process to collaboratively validate *in vitro* pharmacological profiling assays could have a substantial positive impact. Mirams *et al.*[52] recently described how integration of data from multiple secondary *in vitro* pharmacology targets (in this case cardiac ion channels) provided improved *in silico* risk prediction, and similar approaches may be effective in other areas, for example, in emesis, convulsions or abuse potential. However, such a collaborative effort would need to take into account not only the differences in source technologies that generate the data (differing technologies will generate different *in vitro* affinities) but also the degree of risk tolerance cultures of the participants. The recent proposal to define the minimum information about a bioactive entity to be captured in public databases describing the activities of molecules is an attempt to standardize data annotation to include such important methodological information[53].

In summary, *in vitro* pharmacological profiling is a valuable tool that can allow the early identification of off-target pharmacological interactions that could cause safety liabilities in the clinic, and this early identification of safety liabilities could improve decision-making by discovery project teams. The use of the minimal panel of targets recommended in this article might help to reduce safety-related attrition of drug molecules during drug discovery and development. Further precompetitive knowledge management of this data could lead to the development of *in silico* tools that more accurately predict pharmacological activity, and integration of these data with robust *in vivo* models could enable efficient and cost-effective early decision-making based on accurate predictions of the exposures at which a safety liability may be expected in the human population. We hope that this article is a first step towards establishing a broad initiative to work closely on improving drug safety from early stages of drug discovery through to clinical development and at the post-marketing stage.

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