Preclinical safety pharmacology is designed to flag adverse clinical effects at a low cost and can prevent high attrition rates during clinical trials

Keynote review:

In vitro safety pharmacology profiling: an essential tool for successful drug development

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Broad-scale *in vitro* pharmacology profiling of new chemical entities during early phases of drug discovery has recently become an essential tool to predict clinical adverse effects. Modern, relatively inexpensive assay technologies and rapidly expanding knowledge about G-protein coupled receptors, nuclear receptors, ion channels and enzymes have made it possible to implement a large number of assays addressing possible clinical liabilities. Together with other *in vitro* assays focusing on toxicology and bioavailability, they provide a powerful tool to aid drug development. In this article, we review the development of this tool for drug discovery, its appropriate use and predictive value.

The development of new drugs involves the assessment of three major elements in drug design. First, the molecule needs to be active at the selected target. Second, once the desired route of administration has been established, it should be bioavailable and reach the target in the organism (e.g. optimized for absorption, protein binding, membrane permeability and favorable metabolic clearance). Finally, the drug should be tolerated by the organism. Although traditional toxicology can eliminate the major 'zero tolerance' actions of the molecules, there could be many other actions that produce minor or even major 'tolerable' side-effects. The extent of tolerance depends, for example, on the indication, patient population (e.g. age and gender), length of treatment and seriousness of the illness. Traditionally, screening for drug safety starts at a relatively late phase of lead optimization. Any liability discovered at this phase of drug discovery can easily clog development pipelines and cause high, late attrition rates associated with escalating costs. However, recent advances in automation and information technologies have provided the pharmaceutical industry with platforms to translate clinical liabilities into simple, fast and cost-effective *in vitro* screening assays, applicable to the early phases of drug

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discovery. These assays can flag and potentially avoid late, high attrition rates, making drug development more cost effective.

The success rate of compounds from first-in-human to registration was a meager 11% for the pharmaceutical industry during the decade 1991–2000 [1]. Whereas lack of efficacy was a leading cause of termination during the 1990s, toxicity and safety issues contributed together to ~30% of failures. Intriguingly, pharmacokinetic- and formulation-related losses decreased dramatically, largely because of changes in drug discovery practices [e.g. introduction of early predictive absorption, distribution, metabolism, elimination (ADME) assays and more-predictive pharmacokinetic (PK) models]. Recently, tools for early evaluation of mechanism-based toxicity have been introduced but very little has been done to screen for pharmacological promiscuity, which also can seriously affect success rate and influence side-effect profiles. (*In vivo* experiments addressing this matter before clinical application are common practice; however, correlations between animal and human data are questionable.) The importance of this component of drug discovery was recognized a long time ago but was applied to few compounds only, just before clinical trials. This had a serious effect on the pipelines, because at this late stage of the drug discovery process only little or no chemistry capacity was available for corrections. In many cases, projects were abandoned without establishing whether the undesirable side-effect was associated with a particular pharmacophore specific for the scaffold or just an 'accidental' effect of the individual molecule.

It is important to emphasize that regulatory authorities require 'safety pharmacology' investigations that integrate pharmacology, physiology and toxicology of the new chemical entities (NCEs) to test their safety for human use (methods are outlined in ICH S7A and S7B guidance documents). Preclinical safety pharmacology integrates *in vitro* [e.g. determination of human ether-a-go-go related gene protein (hERG) inhibition] and *in vivo* pharmacological data to assess potential undesirable pharmacodynamic (PD) effects in humans [2–4]. Although conventional safety pharmacology assays are an essential element of drug discovery, they are not suitable for testing large numbers of compounds because of the complexity and cost of these assays. Reliable, fast and cost-effective alternatives are needed to address potential liabilities at lead selection and lead optimization. We propose to call the pharmacology-related element of this effort *in vitro* 'safety pharmacology profiling' (SPP). Whereas safety pharmacology is concerned largely with risk assessment of a single (or several) molecule(s) and prevents NCEs with liabilities to enter the clinics, SPP concentrates on early hazard identification to guide drug discovery projects minimizing or abolishing these effects by SAR considerations. Early compound profiling by *in vitro* SPP can flag for receptor-, enzyme-, transporter- and channel-related liabilities of compounds and interprets these data in conjunction with ADME and toxicity (ADME–Tox) characteristics, determined either *in vitro* or *in vivo*.

Designing assay panels for preclinical safety pharmacology

Since the early 1990s, compounds ready to enter clinical trials have been screened against pharmacological targets within a multi-parallel assay scenario. Several contract research organizations (CROs), such as Panlabs (now part of MDS Pharma), Cerep SA and Novascreen, provided screening services that were largely based on radioligand-binding (RLB) filtration assays. These assays are based on a collection of known pharmacological targets and are often limited to the availability of membrane preparations from rodent tissues, with little relevance to functional aspects (e.g. agonist, antagonist or modulatory properties). However, as technology advanced and more targets were discovered, these assays became more sophisticated by using membranes from engineered cell lines, thus providing human targets. This aspect is important because of possible differences in species specificity of several targets. The use of human targets gives *in vitro* SPP the power to create a window into clinical performance at or before the time of *in vivo* animal tests. The *in vitro* SPP assay panels expanded, which inevitably led to prioritization and selection of subclasses, defined by the importance of the targets. One of the major aspects of these efforts was to define a relatively short list of assays that would predict for major clinical liabilities. Accumulating data from preclinical (including knockout animals) and clinical research provided ammunition for these efforts. Databases revealed the most common side-effects associated with drugs during clinical use and links were established between pharmacological targets and these adverse effects [5–7]. Based on the expected contribution of molecular targets to serious clinical liabilities, panels of assays were designed to flag for the most commonly occurring side-effects of NCEs (e.g. spectrum screen by MDS Pharma). We consider these efforts to be the core of what we call *in vitro* SPP. Although the assay panels that are offered by CROs are all slightly different, there is a common effort to satisfy some key requirements: prediction for the most commonly expected side-effects; speed of service; economical use of compounds; cost effectiveness. Certainly, every factor mentioned above creates a major challenge.

Selection of targets by clinical relevance: criteria for *in vitro* SPP

Over 90% of adverse clinical reactions are associated with frequently taken drugs, such as analgesics, anticoagulants, anticancer drugs, antimicrobials, antidiabetics, diuretics and steroids. Most cases relate to exaggerated effects at the primary target (i.e. mechanism-based toxicity), dosing difficulties, prolonged and/or permanent use, cytotoxicity, in particular hepatotoxicity and bone-marrow toxicity,

BOX 1

Major adverse effects associated with the clinical use of drugs

GI tract:
Hepatitis and/or hepatocellular damage
Constipation
Diarrhea
Nausea and/or vomiting
Ulceration
Pancreatitis
Dry mouth

Hematology:
Agranulocytosis
Hemolytic anemia
Pancytopenia
Thrombocytopenia
Megaloblastic anemia
Clotting and/or bleeding
Eosinophilia

Dermatology:
Erythemas
Hyperpigmentation
Photodermatitis
Eczema
Urticaria
Acne
Alopecia

Cardiovascular:
Arrhythmias
Hypotension
Hypertension
Congestive heart failure
Angina and/or chest pain
Pericarditis
Cardiomyopathy

Metabolic:
Hyperglycemia
Hypoglycemia
Hyperkalemia
Hypokalemia
Metabolic acidosis
Hyperuricemia
Hyponatremia

Respiratory:
Airway obstruction
Pulmonary infiltrates
Pulmonary edema
Respiratory depression
Nasal congestion

Musculosceletal:
Myalgia and/or myopathy
Rhabdomyolysis
Osteoporosis

Renal:
Nephritis
Nephrosis
Tubular necrosis
Renal dysfunction
Bladder dysfunction
Nephrolythiasis

Endocrine:
Thyroid dysfunction
Sexual disfunction
Gynecomastia
Addison syndrome
Galactorrhea

Neurological:
Seizures
Tremor
Sleep disorders
Peripheral neuropathy
Headache
Extrapyramidal effects

Psychiatric:
Delirium, confusion
Depression
Hallucination
Drowsiness
Schizophrenic and/or paranoid reactions
Sleep disturbances

Ophthalmic:
Disturbed color vision
Cataract
Optic neuritis
Retinopathy
Glaucoma
Corneal opacity

Otological:
Deafness
Vestibular disorders

by the compounds themselves and by their active metabolites. Individual alterations, polymorphism, differences in enzyme expression, non-linear kinetics, as it was described with phenytoin [8], steep dose–response curves, all can contribute to adverse effects. In addition, there is an extensive list of 'idiosyncratic' effects associated with specific molecular targets. It would be ideal to use preclinical safety pharmacology to predict for any possible clinical liability. However, considering logistics, cost and practicality, this tool has to be used in a hierarchical way. At early phases of drug discovery, the most prevalent liabilities should first be eliminated. These include those associated with selectivity to a particular receptor channel family and with some highly promiscuous proteins. It should be emphasized that the primary role of these assays is to raise flags. These panels are not designed for mechanistic studies, although they should trigger them and also initiate further *in vivo* tests. Before addressing questions of acceptability of side effects, the most common clinical adverse drug reactions (ADRs) need to be recognized. Once this has been established, ADRs can be linked to targets and the corresponding *in vitro* assays can then be set up.

Box 1 shows the most commonly occurring ADRs associated with the clinical use of drugs, sorted by therapeutic areas. It can be noticed that the seriousness of these common ADRs are different, ranging from potentially life-threatening (e.g. arrhythmia) to tolerable inconveniences (e.g. minor skin reactions). The permitted severity of the side-effect profile of any given drug depends on the indication. Although bepridil has serious effects at the hERG channel and could cause TdP (torsade de pointes), it has some use in angina, when diltiazem fails to work. However, several compounds were withdrawn from clinical use or stopped during development because of the hERG channel inhibition (see later).

Many of the side effects become a major problem only during chronic treatment, when they interfere with everyday life (e.g. skin reactions and nausea). Any ADR listed in Table 1 can prevent a drug from entering the clinic under some circumstances. Even minor side-effects could result in non-compliance and alternative drugs that do not have these side effects could be more successful.

Translating these effects into simple assays and flagging ADRs is becoming common practice in drug discovery. However, economical and logistic considerations require prioritization. Balance between the cost of early testing for elimination and/or optimization of ADRs and late termination of a project has to be considered. Early testing

TABLE 1

A selection of cardiovascular targets included in the *in vitro* SPP assay panel used by Novartis

Targets	Possible ADRs[a]
Adenosine, Ad_1	Bradycardia, atrioventricular block. Renal vasoconstriction.
Adenosine, Ad_{2a}	Hypotension, coronary vasodilation. Facilitation of platelet aggregation.
Adenosine, Ad_3	Enhanced mediator release could exacerbate asthma and allergic conditions.
Adrenergic alpha, Al_{1a}	Hypertension and positive inotropic effect. Orthostatic hypotension.
Adrenergic alpha, Al_{1b}	Orthostatic hypotension.
Adrenergic alpha, Al_{2a}	Might inhibit insulin secretion, resulting in hyperglycemia. Hypertension exacerbates heart failure.
Adrenergic alpha, Al_{2b}	Hypertension, cardiac ischemia (block), vasoconstriction of arteries. Peripherally exacerbates heart failure, centrally reduces blood pressure.
Adrenergic alpha, Al_{2c}	Hypertension, cardiac ischemia. Increased muscular, skeletal blood flow.
Adrenergic beta, $Beta_1$	Positive inotropic and chronotropic effects, ventricular fibrillation. Facilitation of bronchospasm, impairs cardiovascular performance.
Adrenergic beta, $Beta_2$	Facilitates cardiac arrest, bronchodilation. Increased bronchospasm, impairs exercise stress cardiovascular performance.
Angiotensin II, AT_1	Increases blood pressure, cell proliferation and migration, tubular Na^+ resorption.
Bradykinin, B_1	Enhances nociception, inflammation, vasodilation and cough.
Bradykinin, B_2	Enhances nociception, inflammation, vasodilatation and cough.
Calcitonin gene-related peptide, CGRP	Hypocalcaemia and hypophosphatemia.
Ca channel type L, benzothiazepine	Hypotension.
Ca channel type L, phenylalkylamie	Hypotension.
Dopamine, D_1	Treatment of Parkinson's disease; induces dyskinesia, extreme arousal, locomotor activation, vasodilatation and hypotension. Schizophrenia, neurodegeneration, coordination disorders.
Endothelin, ET_a	Might cause vasoconstriction, positive inotropy, cell proliferation (e.g. smooth muscle and mesangial cells) and aldosterone secretion.
Endothelin ET_b	Causes initial vasodepression, vasoconstriction, bronchoconstriction and cell proliferation. Vasodilatation, platelet aggregation.
Ghrelin, GHS	Energy homeostasis, GH release, effects on glucose homeostasis, cardiovascular effects.
Histamine, H_3	Impairs memory, causes sedation, vasodilatation, bronchodilation, negative chronotropy and reduces gastrointestinal motility.
Muscarinic, M_1(h)	Vagal effects, blood pressure changes, secretory functions. Decreases gastric acid secretion.
Muscarinic, M_2	Vagal effects, blood pressure changes. Tachycardia.
Muscarinic, M_3	Vagal effects, blood pressure changes, salivation. Reduces incontinence, bronchoconstriction and gastrointestinal motility. Interferes with ocular accommodation, dry mouth.
Muscarinic, M_4	Vagal effects, blood pressure changes. Facilitation of D1 CNS stimulation.
NE transporter	Inhibitor increases adrenergic hyperactivity and facilitate a1 adrenergic activation.
Nicotinic acetylcholine	Stimulates autonomic cardiovascular, gastrointestinal functions. Palpitation, orthostatic hypotonia, nausea, sweating, muscle tremor, bronchial secretion. Effects on muscular and vegetative ganglionic functions.
NPY, Y_1	Antidepressant, causes vasoconstriction (venous), inhibits gut motility, gastric emptying, acid secretion, pancreatic exocrine secretions. Anxiogenic, inhibits ischemic brain injury.
Potassium Ch (hERG)	QT interval (electrocardiogram) prolongation.
Potassium Ch [ATP]	Hypotension. Hypoglycemia.
Serotonin, 5-HT_{2b}	Cardiac valvulopathy.
Serotonin, 5-HT_4	Facilitates gastrointestinal transit, mechanical intestinal allodynia. Useful in treatment of irritable bowel syndrome, cardiac arrhythmias.
Sodium Ch (site 2)	Antagonist causes cardiac arrhythmia.
Thromboxane a2 receptor, TP	Facilitates vascular, uterine and bronchial constriction, gastrointestinal spasm, allergic inflammation and platelet aggregation. Useful in treatment of chronic productive cough, thrombosis, atherosclerosis.
Vasopressin V_{1a}	Vasopressor.
Vasopressin V_{1b}	Vasopressor, anxiogenic.

[a]Possible ADRs or other physiological effects expected to occur when these targets are hit by compounds. Most of these targets are included in available *in vitro* assay sets provided by various CROs for early *in vitro* safety testing.

for the major ADRs saves time and cost by preventing the clinical toxicology pipeline from clogging with low-quality compounds. On the other hand, a non-selective, large-scale testing campaign for ADRs can slow down the lead optimization phase. Also, the quality and predictive value of the assays should be a concern.

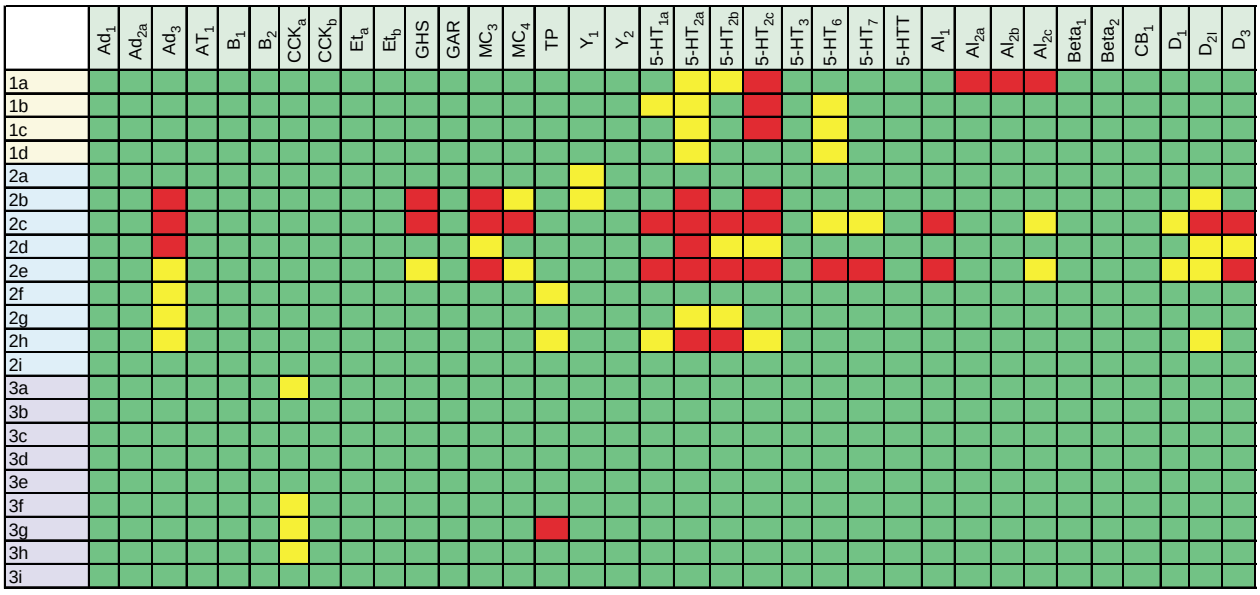

FIGURE 1

Random pattern of activity for a selection of compounds from three different projects. Numbers in the left-hand side of the map mark compounds which have the same scaffold. Percentage inhibition at 10 μM concentration of the compounds is color coded: <50% inhibition of binding is green; 50–75% is yellow and >75% is red. The map demonstrates two important elements: highlights the most active sites of the compounds and emphasizes the extent of promiscuity. Both elements are taken into consideration when compounds are ranked by their promiscuity. Abbreviations: AT_1, angiotensin II AT1 receptor; CCK_a and CCK_b, cholecystokinin a and b receptors; ET_a and ET_b, endothelin a and b receptors; GAR, gaba a receptor; TP, thromboxane a2 receptor; 5-HTT, serotonin transporter.

Drug discovery projects outline the expected product profile of the end product, at the beginning of the program. This includes an ADR profile, which is based on the knowledge of the target class, compound structures and existing clinical experience (if any). Thus, the chance of adverse effects should be defined and eliminated or reduced during the lead optimization phase. The question is when and how to do this. In a more-conservative drug discovery environment, this was done at the late phase of lead optimization by addressing organ-specific cytotoxicity and non-human *in vivo* toxicity (e.g. hepatotoxicity, cardiotoxicity and bone-marrow toxicity). *In vitro* or *in vivo* experiments addressing these toxic effects usually had a go or no-go end point. Little mechanistic insight was gained and projects that produced NCEs with toxic effects were abruptly terminated. However, mechanistic studies that revealed more molecular background of ADRs changed the way drug discovery is conducted today. In recent years, many of the molecular targets for cardiotoxicity, for example, have been discovered and translated into simple *in vitro* assays, which are inexpensive, fast and use a small amount of compound. In addition, statistical analyses help to define the frequency of contribution of these targets to an ADR. In the case of cardiotoxicity, the frequent involvement of the hERG channel made this protein a target for early toxicity screening. Whereas the more complex toxicological experiments are important in the process of drug discovery, early studies using simple assays for selected targets help eliminate the major causes of a given ADR.

Table 1 represents a set of assays based on the most commonly occurring cardiovascular ADRs associated with the clinical use of drugs. Binding to the listed human targets could induce (patho)physiological effects, which can severely disable the patient or lead to significant complications, such as lasting organ damage. Targets included in the *in vitro* SPP panels are not only representatives of 'culprits' causing side-effects, but also many of them are relevant target molecules for various therapies. However, their unintended modulation, irrelevant to the original therapeutic use, can introduce unwanted side-effects, for example lowering blood pressure while targeting allergic reactions or central nervous system (CNS) functions can cause complications such as orthostatic hypotension. The comparison of various available lists of *in vitro* SPP assays used by CROs and pharmaceutical companies shows a remarkable uniformity. This is defined by two elements: availability of the assay and common knowledge of the targets associated with ADRs.

Most of the targets included in Table 1 are primarily associated with well-described cardiovascular (CV) side-effects. For example, the G protein-coupled receptors (GPCRs) for angiotensin, endothelin and α-adrenoreceptors responsible for blood pressure regulation and heart failure are all included [6,9,10]. GPCRs often dominate the *in vitro* SPP assay sets, but enzymes, transporters, nuclear

receptors and channels are also included. All of these assays can be performed in an automated RLB format [filtration or scintillation proximity assay (SPA)]. The assay panels can be easily used to aid lead optimization by prioritizing compounds for selectivity and can contribute to SARs-based design. Early screens for ADRs should primarily flag unusually high pharmacological promiscuity and high affinity at prohibiting targets, such as hERG [11], or serotonin 5-HT_{2b} [12]. Although the agonist–antagonist effects are not determined in these assays, the affinity to the receptor channel or the interaction with any protein at a defined site can be clearly demonstrated and alert to possible ADRs. In addition, cell-based and other functional assays could be implemented, although the relatively inexpensive RLB assays remain the first line of screening. Only when compounds show a reasonable safety profile in the *in vitro* SPP panel and activity at only one or a few unwanted targets 'survive' lead optimization and cause concern, it is necessary to address the physiology behind the interaction of the compound with these targets.

The use of first-line *in vitro* SPP approach is demonstrated in Figure 1. A set of 22 compounds synthesized for the same therapeutic target was tested in an *in vitro* SPP assay panel, similar to that described in Table 1. A heat map was generated, based on the affinity of the compounds to the various targets. Low affinity to these targets might not be a problem if the compound has a significantly higher affinity at the primary target. The compounds in this test set belonged to three different classes, as marked on the left-hand side of the color map. Group 3 is represented by nine compounds, with no major effect at the targets. Group 1 and 2 are to some extent similar in their pattern of pharmacological promiscuity and both compound classes hit the 5-HT family at high frequency but, intriguingly, no compound from this indication has an effect at the 5-HT_3 channel. Furthermore, group-2 compounds have activity at the adenosine-3 (Ad_3), ghrelin (GHS), melanocortic ($MC_{3,4}$) and dopamine receptors (D_1 and D_{2L}).

Side effects associated with the listed receptors have to be considered; for example, the high non-selective affinity to the α-adrenergic (Al) receptors and the 5-HT_{2b} receptor alone raises several flags for possible cardiovascular ADRs. In addition, the affinity to the dopamine D_{2L} receptor subtype could indicate potentially serious CNS side-effects, such as dyskinesia (antagonist) or erectile dysfunction and neurodegeneration.

This type of information can help chemists and biologists to rank and prioritize compounds according to pharmacological profile and to optimize their structures without losing the affinity to the primary target. Unfortunately, in some cases this is not an option and the project has to be abandoned. In the example shown in Figure 1, one compound in the second structural class was remarkably 'clean' and the *in vitro* SPP panel provided valuable information for further optimization.

Once compounds are optimized for an acceptable level of promiscuity (in addition to affinity to the primary target and drug-like ADME–Tox properties), teams can make final decisions to promote the 'fittest' for final risk assessment (e.g. safety pharmacology, toxicology studies), before clinical development. As an example, two compounds (at a late lead-optimization phase) have been selected for an anti-inflammatory indication to demonstrate the application of *in vitro* SPP (Table 2). Both have relatively low promiscuity: compound 1 shows affinity only to two (of 140) targets at a moderate level, whereas compound 2 remains considerably more promiscuous, although with moderate activities (with one exception, phosphodiesterase-3). The activities of these compounds at the *in vitro* SPP targets are at least 200 times less than at their primary target. This gives a comfortable margin, which would allow both compounds to proceed for safety pharmacology testing, with a priority given to compound 1.

Although the above evaluation permits relatively straightforward decision making in terms of identification of targets associated with ADRs, it remains open whether other properties of the compounds would influence the clinical performance. Thus, ADME properties can still raise serious flags for further development.

Pharmacological promiscuity of drugs

Statistical analysis of data collected from 70 assays demonstrates a high hit rate for 894 compounds submitted for profiling during lead selection and optimization phase (Figure 2). A compound is a 'hit' when IC_{50} <10 μM, determined by a concentration response curve in duplicates. These datasets are largely biased because projects that experience pharmacological promiscuity of their lead compounds tend to test higher numbers to reveal possible SAR for the unwanted effects. The promiscuity is emphasized by the distribution of hits (Figure 2). Almost half of the tested compounds had no affinity to any of the targets, whereas others were attracted to multiple targets. Known promiscuous targets, such as hERG (not included in this analysis) and 5-HT_{2b}, attract a large proportion of NCEs. By contrast, some structural classes are attracted by many similar binding sites on different proteins. Genetic variations of large receptor classes, such as the adrenoceptor family, associated with a broad spectrum of physiological functions, further complicate the picture [13].

Taken into consideration that endogenous ligands are usually promiscuous and large receptor families are excited by the same molecule (e.g. serotonin for the 5-HT receptor family [14]), it is not a surprise that man-made molecules act in the same way. In addition, HTS utilizes molecular libraries that are most likely promiscuous, at least within a particular target class.

However, targeting a particular member of a receptor class is often the goal of a therapeutic approach. Selectivity of the NCEs within the same receptor class becomes a must under these conditions. For example, the nicotinic

TABLE 2

Alert for possible serious side-effects

Target	Compound 1[a]		Compound 2[a]		Potential liabilities
	% inhibition at 10 μM	IC_{50} (μM)	% inhibition at 10 μM	IC_{50} (μM)	
Cardiovascular					
Adenosine3 receptor, hr Ad_3	70	6.8	95	2.5	Myocardial ischemia.
Norepinephrine transporter, hr NET	5	>10	79	1.6	Adrenergic hyperactivity; attention deficit–hyperactivity disorder; depression; cardiovascular effects.
Thromboxane a2 receptor, hr TP	44	>10	72	7.0	Vaso-, broncho-constriction; platelet aggregation; capillary permeability.
Vasopressin receptor, hr V_{1a}	35	9.7	31	>10	Vasopressor effects.
Phosphodiesterase3, hr PDE_3	32	10.2	98	0.3	Might induce positive cardiac inotropic effect.
CNS					
Dopamine transporter, hrDAT	18	>10	78	5.0	Dopaminergic hyperactivity; attention deficit–hyperactivity disorder; depression; Parkinsonism; psychotic disorders; seizure; dystopia; dyskinesia.
Opiate mu receptor, hr op-mu	−7	>10	54	9.6	Analgesia; sedation; physical dependence; bowel dysfunction; respiratory depression; modulation of cough reflex.
Inflammation					
Leukotriene, hr $CysLT_1$	21	>10	79	No data	

[a]The two compounds for the indication of rheumatoid arthritis are from the same chemical class, with different pharmacological profile. Whereas compound 1 does not carry a major liability, compound 2 could cause unwanted cardiac side-effects because of high affinity to PDE_3.

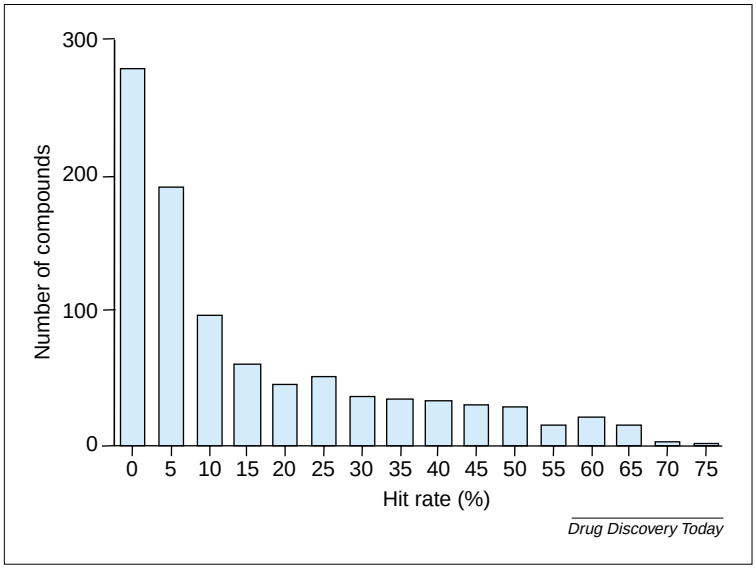

FIGURE 2

Frequency distribution of compounds from lead optimization for *in vitro* SPP. Frequency distribution shows the level of promiscuity of NCEs tested in an in-house panel of RLB assays (n=894; 70 assays). The average hit rate was 16.5%. The set of compounds were submitted for testing by drug discovery project teams (lead selection and lead optimization phase) from all Novartis sites. The assay panel includes a selection of GPCR, transporter and channel targets, identified by their potential to contribute to clinical liabilities if affected by NCEs.

α7 receptor is considered an adequate psychiatric target for schizophrenia. Additionally, compounds which affect the muscular and ganglionic nicotinic Ach receptors have to be avoided [15–17]. Whereas promiscuity within the same receptor family is usually addressed within the scope of a given drug discovery project, effects outside the family are considered by *in vitro* SPP assays. It is of great importance to find and include these promiscuous molecules in the *in vitro* SPP assay set. For example, binding of norfenfluramine, ergot drugs, non-selective 5-HT ligands to 5-HT_{2b} receptors in the heart could cause cardiac valvulopathy [12]. In the Novartis assay panel, this is the most promiscuous receptor. Although hit rates in assay panels depend to a large extent on the diversity of compounds tested, the panel used for this study was exposed to a random selection of compounds from different structures, synthesized in more than 70 projects. Certainly, those projects that encounter a problem with 5-HT_{2b} receptors would test their compounds with a higher frequency, thus biasing the interpretation. Interestingly, 5-HT receptors, in general, attract a very high hit rate: 5-HT_{2b} 49%, 5-HT_{2a} 39%, 5-HT_{2c} 31%, 5-HT_{1a} and 5-HT_{5a} 30%, 5-HT_7 and 5-HT_6 13%. The analysis of the high hit rate at 5-HT receptors might highlight some common SAR, which can be avoided during lead optimization. Also, testing the most commonly hit representative of the 5-HT receptor family could be enough to predict for the rest of the class.

Liabilities associated with promiscuous proteins

Analysis of a large amount of data from clinical trials suggests that, in addition to the primary targets, drugs interact with several non-target proteins with stunning frequency and cause ADRs. Among these targets, the hERG has a prominent position [18]. Inhibition of the hERG potassium channel can produce arrhythmias and ultimately TdP and death. For this reason, several drugs were withdrawn from the market (Figure 3).

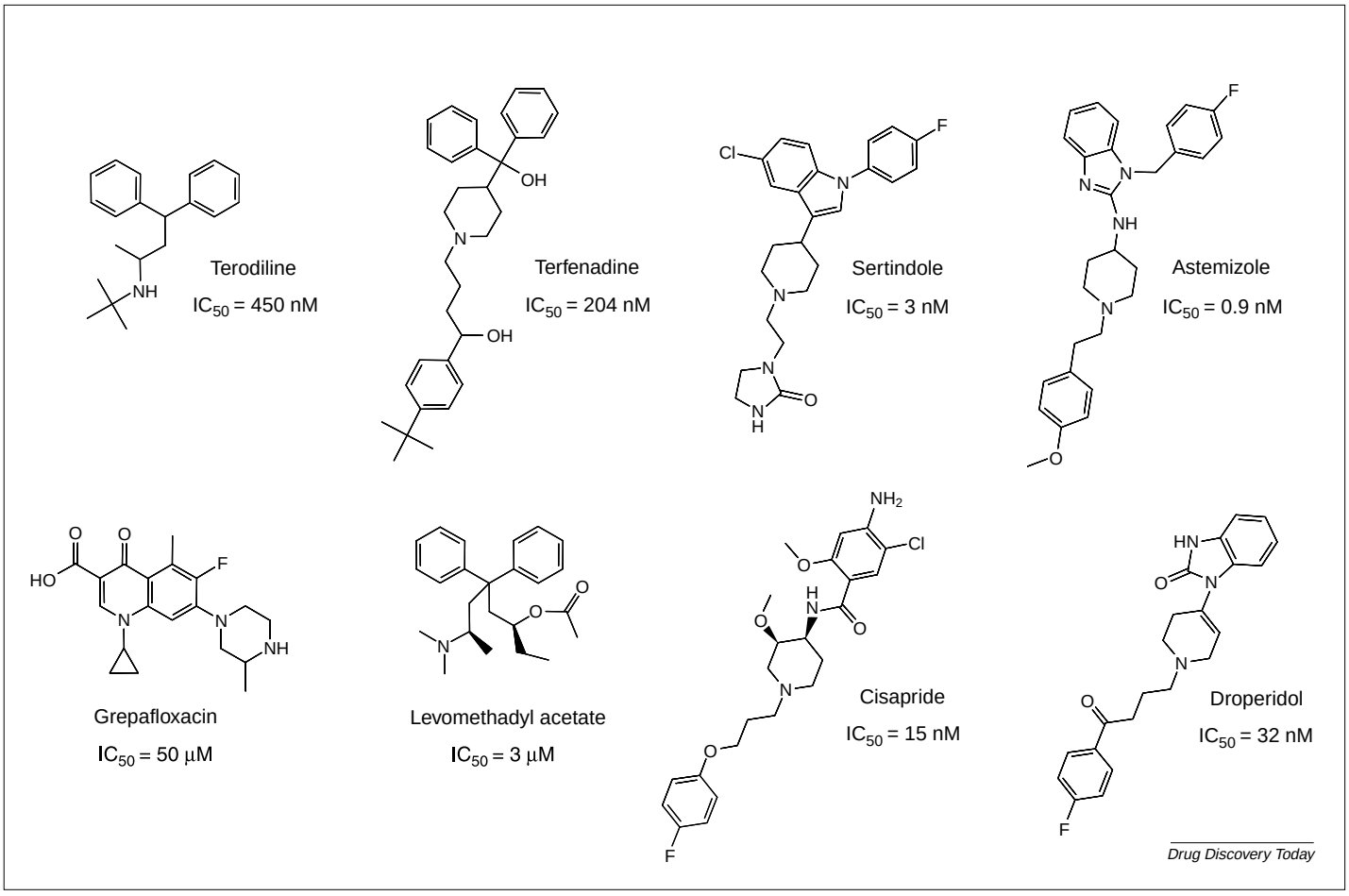

FIGURE 3

Drugs withdrawn from the market because of QT prolongation, TdP and fatalities.

RLB assays are commonly used as primary screens for hERG inhibition, whereas patch clamp analysis remains the gold standard. Regardless of the assay format, this target attracts a large proportion of compounds, screened randomly during lead optimization. In our experience, the hit rate can reach 50–60%, when considering IC_{50} values <30 μM. Published literature includes high hit rates associated with drugs developed for different indications, including antihistamines and antipsychotics [19,20]. Several attempts have been made to establish predictive *in silico* models, based on physicochemical properties (electrostatic and hydrophobic interactions) and on related structural considerations, such as aromatic functions and the presence of a central nitrogen [21–23]. However, the diversity of the structures that have affinity to the hERG channel and the configuration of the channel itself make it difficult to produce reliable models [11]. Thus, *in silico* tools, based on published parameters, recognize the most potent binders, although the sensitivity is usually too high and cannot distinguish between weak binders and non-binders.

In our experience, of the 1832 compounds from lead optimization tested in our laboratory in a binding assay, 980 had an IC_{50} <30 μM for hERG. Again, this set of compounds is highly compromised, as projects that identify compounds with high affinity to the hERG channel use the assay with a much higher frequency than others, without this problem. Although the structure of the hERG channel is particularly amenable to promiscuity, this is not the only channel which attracts large number of NCEs. Many highly hydrophobic antagonists designed to inhibit GPCRs, such as neurokinin NK1 receptors, showed high affinity to Ca^{2+} channels [24].

The pregnane X-receptor (PXR) is another protein that attracts drug molecules with high frequency. PXR is a nuclear receptor, member of the ligand-activated transcriptional factor family, associated with the regulation of cytochrome P450 enzyme (CYP450) and transporter molecule induction [25,26]. PXR attracts a broad range of ligands, such as biliary acids, channel modulators, antiviral agents, herbal ingredients and, in general, a large number of xenobiotics and endobiotics. Because of its highly promiscuous nature and its involvement in the induction of important drug-metabolizing enzymes and transport molecules, induction of PXR has an enormous effect on metabolism, drug–drug interactions, multi-drug resistance and transport mechanisms.

Although several metabolic enzymes (e.g. CYP450, UDP-glycosyltransferases and sulphotransferases) and

membrane proteins, such as p-glycoprotein, are also notoriously promiscuous [11], their contribution is more prominent to ADME aspects of drug discovery, therefore we do not include them in the present review.

Effect of promiscuity on the clinical profile of drugs

The most common diseases are multifactorial, involving pathways with various elements. For the treatment of a disease, physicians can usually choose from a selection of drugs acting at different targets. This is particularly true when symptomatic treatments are used, such as pain medications (e.g. different classes of analgesics). As an example, we consider allergic reactions, where treatments with antihistamines or corticosteroids are available.

The first-line treatment of allergic rhinitis, atopic dermatitis, urticaria and asthma with H_1 receptor-antagonist antihistamines [27–29] is broadly used and considered safe. However, recent meta-analysis of the clinical use of these drugs raised alarms about some adverse effects associated with their promiscuity. Although the safety profile of these H_1 receptor antagonists is diverse, their use is not recommended during the first three months of pregnancy [30]. Major concerns include sedation, when compounds cross the blood brain barrier (BBB) and reach H_1 receptors in the brain, even if second-generation antagonists penetrate the BBB to a lesser extent. More importantly, some serious cardiotoxic effects associated with hERG inhibition were discovered with terfenadine (Seldane®) and astemizole [29,31,32]. In addition to their potent inhibitory effects, these compounds are extensively metabolized by 3A4 subfamily of CYP450 and thus are vulnerable to drug–drug interactions. For example, terfenadine has high affinity to the hERG channel but is metabolized rapidly and its active metabolite, fexofenadine, has only a weak inhibitory effect at the hERG channel. The risk arose when patients took drugs that blocked the metabolism of terfenadine, thus increasing its plasma concentration to dangerous levels, which could produce arrhythmia and consequently TdP. However, some H_1 antagonists, such as cetirizine, do not have inhibitory effects at the hERG channel [33].

Most of the first-generation H_1 antagonists had anticholinergic effects, which is relatively rare in the second-generation group [34,35]. Some H_1 antagonists, such as ketotifen and oxatomide, can also interfere with the serotonergic system [34]. These examples demonstrate the common ADR effects within the antihistamine, H_1 receptor-antagonist group. However, it also emphasizes that the development of structural diversity has produced diverse clinical profiles and optimization of first-generation compounds has greatly improved clinical performance by increasing safety margins.

A good example to highlight the importance of broad-scale pharmacological profiling for revealing mechanisms behind pharmacological effects, is the case of CGP 71683A, a potent and highly selective neuropeptide Y (NPY) Y_5 receptor antagonist [36]. This compound was shown to reduce food intake and lower body weight in animal experiments [37]. CGP 71683A showed very little activity at $Y_{1,2,4}$ receptors and reduced NPY-induced feeding. CGP 71683A was quickly gaining the status of a gold-standard reference compound to study the effects of a selective Y_5 receptor inhibition on food intake. However, during chronic treatment CGP 71683A lost its activity and food intake returned to normal. This phenomenon was explained as the effect of the activation of counter regulatory systems. Although this seemed to be plausible, it remained unclear why the body weight remained low. Nevertheless, data satisfied the scientific community, until recently published results by Zuana and colleagues [38] shed new light on the unusual profile of CGP 71683A. Although this study confirmed the original findings and reaffirmed that the compound was highly selective within the NPY receptor family, in a broad binding assay panel it also showed high affinity to the muscarinic receptor (K_i = 2.7 nM), the serotonin uptake recognition site (K_i = 6.2 nM) and to a lesser extent to α_2-adrenergic receptors. In addition, morphological observations suggested that CGP 71683A induced brain inflammation, which could have contributed to the prolonged weight loss in animals.

An independent study by Kanatani *et al.* [39] showed that a pure Y_5 receptor blockade is not enough for reducing food intake and that NPY knockout mice have normal food intake. It is very likely that Y_1 receptors also contribute to the maintenance of normal appetite.

In light of these data, the role of Y_5 receptors in food intake was strongly challenged. Serotonin can inhibit the synthesis and release of NPY, resulting in decreased food intake [40]. Interpretation of data with CGP 71683A are further complicated with a possible addition of a α_2-adrenergic (Al_2) receptor effect on food intake [41]. However, this latter aspect remains debatable in the absence of functional data. The affinity of CGP 71683A to the muscarinic receptors has less significance, as this receptor family has no direct effect on food intake. However, influences on locomotor activity and cognitive processes might contribute to the overall effect. It has been shown that muscarinic and NPY-containing neurons are associated in the limbic system and there is evidence that the cholinergic system has an effect on feeding behavior [42]. Thus, all the targets listed above have strong or moderate effect on food intake. The affinity of CGP 71683A to any of them can contribute to the decrease of food intake, even without considering an effect at the Y_5 receptor. Second-generation NPY Y_5 antagonists with clean *in vitro* SPP profiles were unable to induce a decrease in food intake [36].

The study by Zuana *et al.* [38] highlights the importance of determining the full pharmacological profile of a compound, particularly when complex mechanisms contribute to the development of a phenomenon (e.g. weight loss and decrease in food intake), when experimental data are interpreted with some difficulties and when speculation is substituted for sound scientific evidence.

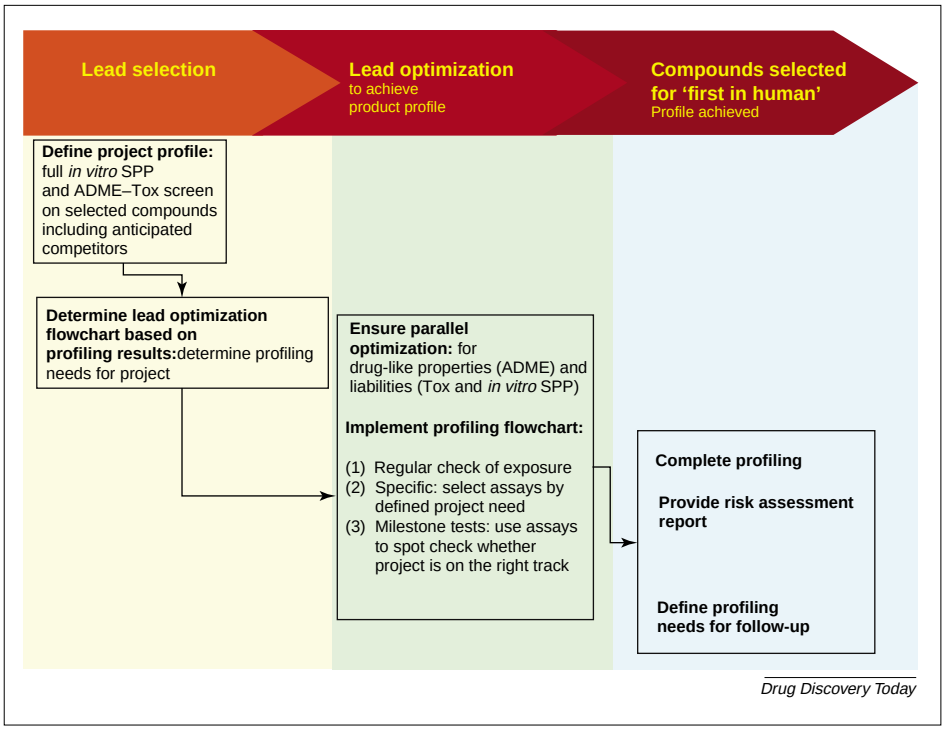

FIGURE 4

Recommended flowchart for profiling requirements and logistics at different stages of drug discovery.

Technical and logistic considerations

If *in vitro* SPP is so important and useful, why has not been used more systematically in the past? Certainly, since the mid- to late- 1990s *in vitro* SPP has been performed on a small scale (low number of assays and few compounds) by a few drug discovery companies, but many of the assays used non-human targets and the interpretation of the results was often based on animal pharmacology. Also, it often took several weeks to complete the panel.

The advent of HTS, efficient compound management and multi-well plate screening opened up opportunities for fast screens, using small amounts of compounds without compromising quality. Profiling, although thoroughly different in terms of logistics and process, utilizes many elements of HTS. SPA methods are broadly used in 384-well formats, and robotic liquid handling systems can enable parallel screening of hundreds of compounds in as many as 100 assays. However, a high level of automation of the assays is required to fulfill all the above criteria.

Another challenge of running so many assays in parallel is the management of the reagents. The logistics of automating sets of assays where the number of different reagents can be almost as high as the number of compounds being tested, locates *in vitro* SPP apart from HTS. These reagents must be available on a continuous basis, otherwise a fast turnaround of the compounds cannot be achieved. One limitation is associated with compound purity, which might be a problem in the early discovery phase. Also, racemates are regularly submitted to the *in vitro* SPP assays, before salt forms are provided. This could lead to differences in results obtained with different forms, and it outlines why data interpretation is such an important element of the process.

As *in vitro* SPP panels are relatively new, it is difficult to determine which targets should be included and what would be the optimal size of the assay set. The consensus is that first-line screening should address only major ADRs and the less important ones (e.g. lower frequency of appearance and less serious outcome) should remain for later tests, before clinical trials. However, safety should not be compromised, as coincidence of affinity at different targets might amplify mild side-effects and create a major crisis, such as immunodeficiency or anemia. It is recommended that compounds should be tested for *in vitro* SPP at the lead selection phase and major liabilities should be flagged. The remaining targets can be tested at a later stage, before testing in humans, with compounds that survived or rather evolved during lead optimization. A generalized flowchart or logistics for profiling is outlined in Figure 4.

Limitations

From the regulatory point of view, the limitations of the *in vitro* assay-based predictive tools are severe. No drug can enter the clinics based only on *in vitro* data from *in vitro* SPP assays. A series of *in vivo* examinations in animal species are essential for clearance, before entering clinical use. However, in some cases, when there are considerable genetic differences between human and experimental species, receptor binding and follow-up functional assays performed on human targets might be highly relevant and more predictive than results obtained from animal studies. For example, this is the case with B_1 and B_2 bradykinin receptors, which are significantly different in humans and rodent species [43,44] and require different molecules for inhibition.

Nevertheless, general pharmacological, toxicological and ADME evaluations *in vivo* are prerequisites for any clinical trial. *In vitro* studies cannot predict cumulative effects during chronic treatment and dose-related pharmacokinetic–pharmacodynamic (PK–PD) relationship [7], neither can these assays address the complexity of the *in vivo* performance of the compounds at the promiscuous targets.

Taking all of this together, one has to keep in mind that potency is not equivalent to clinical efficacy [45].

Consideration of therapeutic milieu

In vitro SPP can fail when used without considering ADME–Tox properties of the drug. Other reviews [5,11,46]

have highlighted this important aspect of profiling. Interpretation of *in vitro* SPP data is greatly enhanced by considering bioavailability, metabolism and drug–drug interactions. Differences in blood–brain barrier penetration can have significant impact on the performance of drugs within the same class, as for example in the case of H_1 antagonists. Pathological conditions, such as hepatic cirrhosis, renal or cardiac insufficiency, can significantly alter the PK conditions.

Interaction with housekeeping enzymes and receptors, such as Cox-1 and bradykinin B_1 receptors, could also cause serious side-effects. Furthermore, special conditions have to be considered, such as pregnancy and breast feeding, when the drug could pass to the fetus or infant.

Patient population is another important determinant in the process. This can be correctly identified before registration, and the drug discovery process has to be adjusted accordingly. Elderly patients are more likely to have hepatic and renal dysfunctions, where β-adrenergic (Beta) receptors are less sensitive. Reproductive age also determines the safety margins for several drugs. High prevalence of diseases at advanced age will determine the possible coadministration of various drugs and highlight the importance of drug–drug interactions. Treatment of children, particularly at a younger age, represents challenges for PK estimation (distribution) because of ongoing development of organs and different metabolic rates. In addition, gender and genetic predisposition are further elements of the whole picture.

In vitro SPP is part of a complex preclinical profiling approach, which Novartis considers as an important element of drug discovery. It should clear out compounds before they proceed to preclinical development, including safety pharmacology. Ideally, compounds reaching this stage have a clean *in vitro* SPP profile, requiring no follow-up *in vivo* safety pharmacology investigation.

Interpretation of data for clinical predictive power

Despite the mentioned limitations of the *in vitro* assays, the power of the combination of these assays with *in vivo* and clinical ADME–Tox data is largely acknowledged. A significant element of *in vitro* SPP is the provision of an extensive, largely unbiased database, which contains annotation of individual compounds of great structural variety in the same assays. This database could enhance the predictive power of *in vitro* SPP, as it can refer to clinical performance of reference compounds. Several attempts have been made to do this, particularly for ADME properties [47,48]. Important efforts were introduced by Cerep with BioPrint™ [5], Iconix Pharmaceuticals with DrugMatrix™ (www.iconixpharm.com) and Novascreen's Receptor Selectivity Mapping database (RSMDB®; www.novascreen.com). These computational models are based on assays used to assess compounds during early stages of drug discovery. The strength of these and similarly built datasets is that they can determine QSAR by using modeling [49–52]. The size and diversity of the training sets largely define the predicting power of the model [5]. However, BioPrint and RSMDB® are more than simple collections of *in vitro* pharmacological data. They contain data from further *in vitro* assays predicting drug-like behavior, such as Caco-2 and PAMPA data (for absorption), CYP450 inhibition and induction (for drug–drug interaction) and microsomal stability (for metabolic stability) and, in case of DrugMatrix™, it incorporates elements of gene-expression datasets. Furthermore, they contain a large amount of clinical data from compounds that are in clinical use, withdrawn from the market or that failed registration for various safety reasons. These data are linked together with the *in vitro* dataset and provide strong predictive power for the clinical behavior of newly designed compounds. These types of models can predict clinical behavior of NCEs, based on their *in vitro* performance. However, the predictive power depends on the quality of the *in vitro* assays, on the chemical diversity of the training set and on the availability and relevance of clinical data (e.g. differences in protocols, quality control and data interpretation). Although the size of the datasets is crucial for the predictive value of the BioPrint model, it can also introduce significant variance, which makes it less reliable [5,53]. Also, the relevance of data obtained from *in vitro* ADME assays is of high significance for the predictive power of BioPrint. Although it is easy to predict ADRs that are associated with known targets, the clinical relevance will be ultimately determined by the physiological effect (agonist, antagonist or modulator) and the concentration of the compound at the target, which is highly dependent on its PK profile (e.g. D_1 receptor ligands have motor effects only if they penetrate the BBB).

These models cannot predict ADRs associated with little or unknown mechanisms (e.g. cardiovascular effects of Vioxx were difficult to predict before starting clinical trials [54–57]). Also, effects at different targets might balance each other, for example in the case of citalopram (Celera), where hERG inhibition and L-type Ca^{2+} channel inhibition act against each other [58] and as a result the drug has a good cardiosafety profile [59]. Therefore, careful screening of the clinical literature for metadata is very important for updating these types of databases.

Although it is not the topic of this review, the backbone tools implemented in BioPrint are briefly addressed here. In general terms, linear QSAR and predictive neighborhood methods are implemented in parallel to predict molecular properties. This arrangement produces a synergistic model, which enhances the chances for correct predictions [5]. The BioPrint environment basically assists in the clinical interpretation of *in vitro in vitro* SPP profiles of NCEs. Clusters can represent specific ADR profiles of drugs that are used for identical indications. Drug classes, such as antidepressants, seem to be highly promiscuous and interact with similar off-target molecules. These types of associations are broadly used to make more predictive

receptor-binding sets. The model puts all the new compounds in context, comparing their behavior with that of successful or failed drugs. Considering that major ADRs can be predicted by this method at a very early phase of drug discovery, the expert use of BioPrint type tools can have a significant effect on the success rate of these programs.

When is promiscuity an advantage?

The achievement of exquisite pharmacological selectivity is the Holy Grail of the pharmaceutical industry. However, when the disease seems to be of polygenic origin, some degree of promiscuity might be advantageous. For example, treatments for pain (e.g. Tramadol) and several anti-psychotic drugs (Risperidone, Clozapine [60]) act on more than one target and have advantages over compounds designed for a single target. So-called 'dirty drugs' are broadly used in psychiatry, when diseases seem to be polygenic, with the complication of changes in receptor densities and modified morphology of neuronal networks. Broad-based pharmacological screening is a way to address the therapy of these diseases [60]. Thus, *in vitro* SPP can give guidance by identifying compounds with similar pharmacological characteristics to those highly effective in psychiatric disorders, therefore assisting the lead optimization phase as well as identifying NCEs with predefined characteristics. In addition, promiscuity can be related to 'privileged substructures'. Because some classes of receptors share common structural configurations, it is not surprising to see close chemical derivatives displaying very different activities. A compound with a privileged substructure for a class of receptors will often reveal many different activities in this class. This property could be used, for example, for the design of ligands for orphan GPCRs [61]. *In vitro* SPP will help also in finding new privileged scaffolds, which could be used for the design of focused libraries.

Outlook and recommendations

Although not new [62], the *in vitro* SPP concept is increasingly being used by drug developers for the discovery and elimination of ADRs at an early phase. With the development of large, reliable databases, automation tools and greater computational power, the predictive value of BioPrint type approaches has improved significantly.

The development of new technologies using miniaturization and novel analytical techniques will ensure that this approach is more affordable and cost effective. It is likely that a selection of simple functional (e.g. introduction of screens using cryo-preserved cells) and phenotypic assays will soon be part of *in vitro* SPP screening, and associated *in silico* tools will become more predictive and will form a more integral part of drug design during lead selection and optimization. Whereas all the above is applicable to NCEs, new technologies should be developed to provide similar evaluations of biological drugs.

Taken all these together, *in vitro* SPP is becoming a common tool to test compounds before entering more-refined and costly assays at the next phase of drug discovery. Eventually, results obtained in *in vitro* SPP should enable these assays to be conducted earlier, thus speeding up drug discovery. However, interpretation of *in vitro* SPP data has always to be associated with ADME information, as PK–PD will ultimately define clinical efficacy.

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