Chapter 2

On the Organization of a Drug Discovery Platform

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Additional information is available at the end of the chapter

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Abstract

Some of the most exciting parts of work in the pharmaceutical industry are the steps leading up to drug discovery. This process can be oversimplified by describing it as a screening campaign involving the systematic testing of many compounds in a test relevant to a given pathology. This naïve description takes place without taking into consideration the numerous key steps that led up to the screening or the steps that might follow. The present chapter describes this whole process as it was conducted in our company during our early drug discovery activities. First, the purpose of the procedures is described and rationalized. Next follows a series of mostly published examples from our own work illustrating the various steps of the process from cloning to biophysics, including expression systems and membrane-bound protein purifications. We believe that what is described here presents an example of how pharmaceutical industry research can organize its platform(s) when the goal is to find and qualify a new preclinical drug candidate using cutting-edge technologies and a lot of hard work.

Keywords: drug discovery, validation, cloning & expression, biophysics, structural biology, organization

1. Introduction

Drug discovery involves a suite of processes as part of a program aimed at finding drug therapies for diseases. These programs encompass many different scientific steps from validation of the target (or attempts to do so) and characterization of the hits until the selection of candidates for medicinal chemistry programs. We felt that an accurate description of those steps has not previously been available and that such a description could be interesting.

In PubMed®, several thousand publications exist with the term 'drug discovery' in their titles. Most frequently, these reports share with readers how important the authors' particular area of

expertise is in this domain, covering almost everything from crystallography to screening and high-content screening, origin of the screened molecules, compound libraries, sample conservation, biochemical approaches, microarrays, proteomics, and aptamers. These publications are a useful source of inspiration for each new program. Clearly, the first step in drug discovery is inspired by the state of the art, and the literature will help drive research towards the 'bench-to-bed' goal of finding a new molecule that will eradicate some disease that affects our society.

We feel, however, that the complete picture of such processes is not very often characterized. Thus, here we exemplify how we built our drug discovery (DD) platform on basic science with the goal of following, in a unique location (a set of specialized laboratories), the logical successive steps of drug discovery from ideas to first hits. An oversimplified picture of the logical progression from start to end of these processes can be seen in **Figure 1**.

This configuration did not preclude failures but probably helped forward our successes. In the pharmaceutical industry, the attrition rate is around 9 of 10 projects, meaning that only 1 project in 10 will have a slight chance of ending up in clinical development, and a further 2–3 out of 5 of these will have a chance to reach patients. Therefore, in preclinical research, scientists will try to deliver on time and with maximal rigor the tools necessary for the pathological problem to be at least partially solved. In 9 cases out of 10, the efforts will be vain, as the programs will be terminated, either for lack of results or for a change in strategy. The reasons for attrition are many, and a key to decreasing number is certainly a better characterization of molecules entering the medicinal chemistry program that will drive candidates from hit to

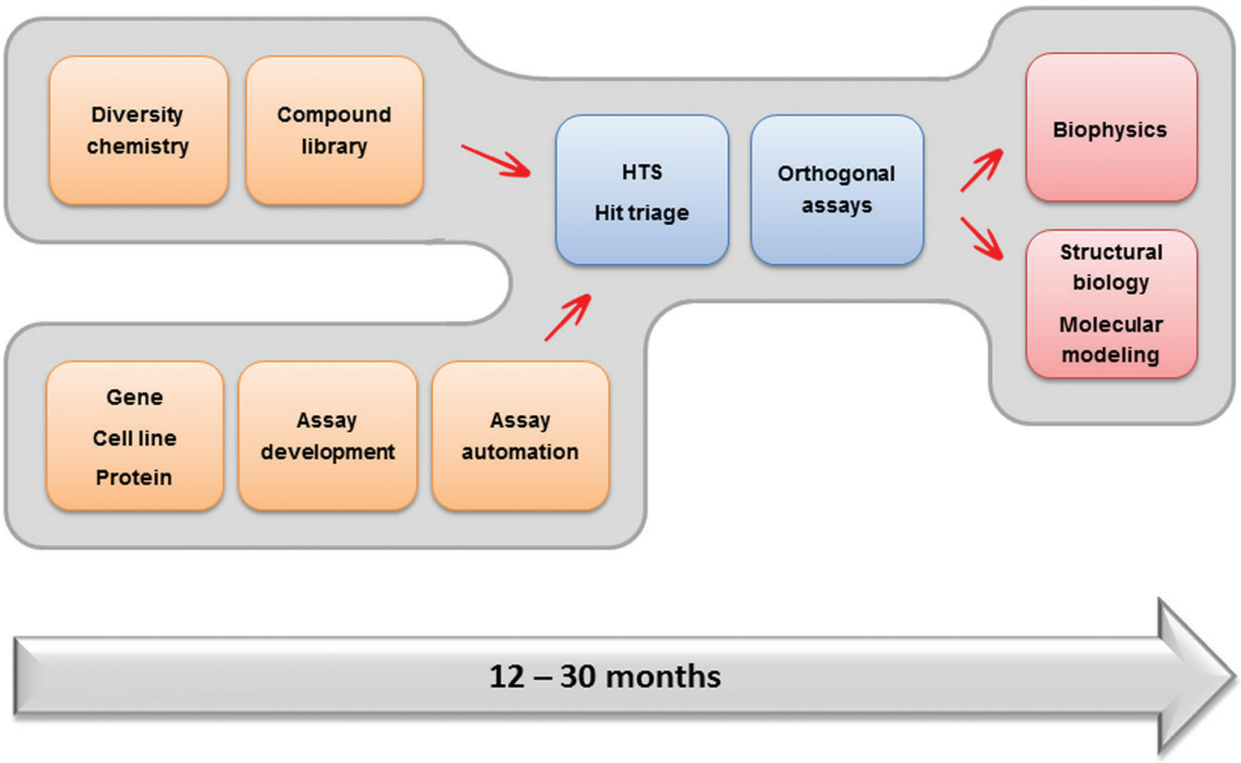

Figure 1. Schematic representation of a drug discovery process. Note that the exploratory/validation of the target process is not part of the schedule. Rather, these two steps might take several years to complete upstream.

lead. Among the characterizations that are essential is the specificity of the molecule within its class of targets (e.g., one given kinase among the 800 in the kinome) or its specificity towards the rest of the genome-encoded proteins. Another key is a better understanding of the molecule-target relationship at the atom level.

In addition to these scientific aspects, daily nonscientific aspects of business also require attention. Examples include practical questions, such as 'Can we hire new specialists to explore and assess a new field for a particular problem?'; economic questions, such as 'Can we spend the money necessary to acquire a particular technology'; and time-related questions, such as 'Can we spend the 12 to 20 months to deliver the results after the necessary assessment for a new technology/technique?'

Below, we offer our view through our experiences with all of these aspects, reflecting the work of a laboratory ultimately consisting of about 70 people, with some impressive achievements.

2. Basic sciences, organization?

Our group has a "simple" core job: the discovery of drug candidates. Programs are based on two types of knowledge: the pharmacological background linked to the various areas of therapeutics (e.g. cancer, cardiology, or neurosciences) and the techniques needed to explore the molecular side of the program (e.g. molecular biology, cellular biology, biochemistry, structural biology), in other words 'basic sciences for discovery'. That basis was a mixture of molecular and cellular pharmacology techniques that allowed starting from a protein target and finishing with a compound (or several) that interfered with this target in a reasonably specific manner. To this were added chemistry (for the compound collection) and molecular modeling (for dynamics and structural biology data interpretation). One of the major challenges was the identification of a given target in the pathological process, its role in the pathology, and its manipulation. These necessities meant that we also needed upstream access to experimental techniques such as siRNA, KO mice, alternative laboratory animals (such as yeast, flies, and zebrafish [1], and *Caenorhabditis elegans* [2]) often at a time when these techniques were barely validated. It also meant that we frequently had to make the choice of collaboration rather than pursuing these techniques internally.

2.1. Exploring pathology

"Exploring" means several things in this context, from exploring the disease to exploring the tools and the literature to validate a target. The literature is rather scarce in this context, but one resource is the essay by Scudamore, who tries to make us understand how difficult and diverse this area could be [3].

The exploration of pathology is the main objective of academia, but industry faces a challenging problem. Should it run brand-new programs using its internal research capacities or should it collaborate with academia to help them run such program? Considering that part of the work in industry is to choose relevant targets accessible to chemistry (even if chemistry has broadened its borders for the last decades) and that are truly and undoubtedly linked to

a pathological situation, it cannot start several million-euro projects without a validation that convinces everyone that the end discovery will help the patient.

Thus, industry must equilibrate its efforts among internal, confidential, and secured experiments that would identify and elucidate the role of a target in a pathological process while scouting externally in academia and biotech for new ideas, concepts, targets, and even sometimes candidates. In these last cases, however, internal reproduction of key data should not be underestimated in terms of time, money, and results (see Section 2.2).

We were involved intensively in several such programs. For example, N-myristoyltransferase is a protein-modifying enzyme with a key role in addressing substrate to the membrane [4], including oncogenes or viral proteins [5, 6]. Our discovery that one isoform was associated with rough endoplasmic reticulum led us to believe that this particular isoform potentially had a role in pro-virion maturation [7]. On another front, we discovered/characterized a new activity for the enzyme autotaxin, a lysophospholipase D, the activity of which explained why that protein was previously believed to be a migration factor [8, 9]. This enzyme could be deeply involved in fat accumulation processes [10] and in metastasis [11]. We pursued this program up to the discovery and characterization of a hit compound that failed for stability reasons [12]. Another example is N-arylalkylamine acetyltransferase (AANAT), an enzyme involved in the synthesis of melatonin, of which we characterized the properties of the human isoform [13], while its importance in regulating melatonin synthesis had been demonstrated [14]. Our approach to the uncoupling proteins was similar to this. We thought that UCP3 might be a good target for diabesity, if we could find an activator of this enzyme. The challenging idea was that by accelerating proton exchange, one could reasonably enhance the heat dispersed by muscles. We thus started to study this particular enzyme in various contexts [15, 16], up to an *in vivo* model for which elegant techniques were developed [17, 18].

Opportunities are numerous for scientists in the core of a network. As noted, the challenge is always to be original enough but not too much involved in fundamental research because the time frame is simply not suitable to the business of industry, at least for the most part.

2.2. Validating targets (as actor in a pathology)

The validation of the implied involvement of a given molecular target in a pathological process requires many different approaches and technical applications. A special issue of *Current Topics in Medicinal Chemistry* was published in 2017 on this subject (see the editorial by Henderson and Gibert in [19]). Furthermore, Kaelin published an interesting paper [20] on the pitfalls in preclinical cancer target validation. Indeed, one of the main aspects of the Kaelin essay is that many experiments reported even in major journals are hard to reproduce independently, if at all, and thus validation is a difficult task. Obviously, this has been an endless debate in the scientific community, and commenting on it further here will not add to the discussion. The *primum movens* is not the same between academia and industry. Basically, it is fair to say that the protocols are not regularly described in enough detail to be easily reproducible in a different laboratory context.

Without a strong, scientific knowledge about the pathology, cures will not be found except by chance. Thus, being able to present our program in a way that is reproducible is important.

One has to be able to ensure (or be reasonably sure) that the observations made are linked to the 'pathological' situation and not to experiment-generated artifacts. Of course, achieving this goal requires a mixture of pure luck, hard work, prepared minds, curiosity, and willingness to move forward.

Most of the time, however, only a portion of these experiments is feasible, either for timescale reasons or for cost. Therefore, the choice is often made to go to primary approaches such as knockout cells or knockout animals. We reported such an approach with cells [21], knocking out the product of a gene by measuring the catalytic activity of the corresponding enzyme, spontaneously expressed in a cell line, HT22, after siRNA and shRNA treatments. It took a double transfection with a shRNA encoding a siRNA specific of QR2 to knock down only 80% of the catalytic activity of the enzyme. This result suggests that knocking out a spontaneously expressed protein in a cell line—especially in a cancer cell line, which is often pluri-nuclear—is difficult, if not impossible beyond knocking it down somehow. It is interesting to compare this type of data, including those we obtained in similar conditions but that remained unpublished, with claims that cocktails of siRNA transfected directly into (cancer) cells would kill the activity of an enzyme within hours (if not minutes). A recent review on the subject [22] emphasized the fact that RNAi studies have been used and abused, suggesting that the key role of those RNAs should be kept to a tightly verified level to gain from this outstanding technology and fix once and for all the problem of *in cellulo* identification and engagement of a target in a given pathology. Technologies involving nucleases and CRISPR have changed the technological landscape of cellular biology because these techniques lead to complete knockout and are almost universal—until exceptions to their effectiveness or accuracy start to emerge.

Cellular validations are often not complete enough to give strong support to a pathophysiological hypothesis. It is thus necessary to move to another approach: the genetic deletion of the target in mice, or, more recently, in rats. The bias of this approach, besides its cost and often the length of the process, is linked to the potential capacity of animals to compensate for the absence of a particular gene (or the absence of a particular catalytic activity). This compensation might happen often, but proofs of such situations are rare. The two main difficulties we have encountered were either the lethality of the KO in mouse embryos [23] or the complete absence of an obvious phenotype due to the loss of this particular gene [24, 25], a surprisingly frequent feature. The former example offers a rare case. When we mutated a single amino acid in the catalytic site of lysophospholipase D, which renders the enzyme completely catalytically inactive, the mouse embryos died in the very early stages of their development [23]. This outcome was unexpected but proved at least that the enzyme activity was a key player in mouse embryogenesis [26, 27]. Knockout animals can also be fantastic tools to prove that the compound engages the target. For instance, our antagonist of the melanin-concentrating hormone (MCH) receptor, S38151, has no activity in MCHR1 KO animals, while it reduces feeding wild-type ones, demonstrating that the compound exerts its expected activity only through its binding to the receptor [28]. Other examples have been generated in our DD platform, such as with the deletion of trace amine 1 receptors, revealing the involvement of those receptors in the actions of the drug Ecstasy (MDMA) [29]. Unexpected properties of the modified animals are sometimes found long after the knockout has been performed, resulting in the resuscitation of the program. For example, the knockout of Ucp2, an uncoupling protein expressed in almost all mouse tissues, confers an unexpected resistance to *Toxoplasma gondii*

infection [30]. Finally, particularly difficult is the choice of the mouse strain usable for the KO line. Indeed, some examples of animals totally deprived of circulating melatonin led to slightly different observations about the impact of the knockout when compared to a strain presenting a "normal" level of circadian melatonin [31]; thus, not only are some strains partially blind but consequently their daily rhythm is profoundly affected.

Again, recent progress in cellular surgery, thanks to CRISPR techniques [32], might make the development of KO animals faster and even commonplace in the near future, but today the process remains a lengthy one.

Alternatively, in addition to these genetic manipulations *in vivo* or *in cellulo*, the pharmacological approach remains a possibility. Strategically, though, it is rare for a pharmacological set of tools for a "new" target to exist beforehand, and these molecules need to be specific and/or powerful enough to help validate the system. An upstream decision must be made about whether the high-throughput screening (HTS) approach that will probably deliver compounds can be used very early in the discovery process to bring tools rather than drug candidates to validate the target around which the program was/will be built. This theoretical situation can provide the community with very early tools to validate their activities (see reviews by [33, 34]).

Of interest, another approach has become possible in recent years, thanks to our collaborative work with the Shanghai Institute of Materia Medica (SIMM): a validation with molecules of poor specificity but engaging the target. Indeed, as soon as the first compounds are issued from this screening process, even if they are far from perfect, attempting high doses of such molecules on an animal model relevant to the pathology brings interesting and important information about the validation of the pathological hypothesis. Only a couple of examples have been published following these attempts, one with thioredoxin-interacting protein (i.e., TixNip) modulators [35] and the other with a gpr119 antagonist [36].

2.3. Cloning/expressing/purifying the targets

Obviously, targets come from independent experimental observations that have suggested their role in a given pathology in a given system through the measure of activity such as a catalytic activity or a potential binding site. Ultimately, purification should be performed and should lead to the unequivocal characterization of the protein the program targets. A recent trend is to consider that this given protein is a brick in the pathway(s) leading to the pathology. In this framework, the protein should retain its cellular context to allow for consideration of the potential role of its partners and cellular neighborhood. This situation is no doubt optimal. Nevertheless, finding compounds that will hit the target should begin with a simplification of the system. Without this simplification, one should consider the multiple interferences from the cell milieu (see also the HCS discussion below, Section 2.6.3).

This is an old debate between the pros and cons of protein purification. The pros consider that the candidate molecules should be aiming directly at the protein and that structural biology will deliver key information on the target-molecule relationship providing that the system is as simple as possible. The cons remind us that inside the cytosol, an enzyme interacts or is in

proximity to many other proteins, some of which can involve interference. Rather than being an assortment of simple linear pathways, the cytosol in reality is more like a bag of marbles—proteins—rolling around and bumping into each other.

As far as enzymes are concerned, is it acceptable to work on a partially purified extract or should we aim for the pure version? For screening purposes (the *nec plus ultra* of the work of such a DD platform), the need for membranes overexpressing a receptor is key to the success of the next processes, so are there alternatives?

Cloning remains a source of novelty. Obviously, using already published sequences for new targets involves a bias because these targets are already known in the literature, so that the results with them are less novel that would be desirable for the drug market. Thus, cloning efforts of new, not already described targets, are at best a challenge. Our first attempts were to control the expression of receptors or enzymes, together with an associated protein (whether naturally associated or needed for building an assay for this target). Particularly interesting was trying to find ways by which the expression of two proteins—or two peptides—could be stoichiometric. For such a goal, the best approach was to use and organize IRES promoters in different geometries [37]. This method led us to the successful expression of several multi-peptide proteins, such as the nicotinic receptors.

It is interesting to see now the progress in what was once called cellular surgery, first with nuclease approaches and now with CRISPR. In past years, we used nuclease-based techniques to produce cellular models (most of the time, unfortunately, based on cancer cells) that would express a single copy of the transgene, integrated into the cell genome at a single, neutral position [38]. This method was interesting because for comparisons of the molecular pharmacology of a receptor from different species, often from human and one or two rodent species, the comparison was more relevant because only the sequence of the receptors drove the differences between the pharmacological profiles. Indeed, it was integrated into the same cellular host—CHO or HEK cells—at an identical 'neutral' position, with a single copy of the gene, leading to a validity and accuracy in the data that was difficult to obtain whenever the transfection was less controlled.

When the enzymes have already been described/cloned, expression is easier in a host cell—often bacteria—but then the purification (of an active enzyme) often becomes the key. Typical examples of such purification strategies can be found for chymase [39], AANAT [13, 40], and indoleamine-2,3-dioxygenase [41].

Regarding other aspects of research the DD platform has conducted, we should mention work on already known targets that suddenly take on added interest because they turn out to be involved in an unexpected physiopathological situation. One example is the role of quinone reductase 2 beyond its presumed detoxifying role. The enzyme has been associated with cognitive processes on the one hand [42] and with melatoninergic systems on the other [43]. Aware of these observations, our attempt was to build a network around the notion that inhibitors of this enzyme could be interesting tools or drugs in understanding cognition and cognition deficits [24, 44], as well as better characterizing the MPTP Parkinson model in mice [45, 46]. We reported several examples in which we explored new pathways and

found evidence for new proteins or new activities. For example, the observation that lysoPLD activity was present and active in an adipocyte culture medium led us to hypothesize that this medium contained a protein capable of catalyzing the breakdown of lyso-phosphatidylcholine. We reported this discovery [8], after having purified, characterized, and discovered alternative splicing forms [9], built the KO mice [23], and started a search for active inhibitors [12]. It is interesting to look at it from a time-frame perspective. Indeed, the initial observations were made in 1999 while our initial report was published in 2003 [8] and the last report on the compound, S32826, in 2008 [12]. Thus, it took about 10 years before all tools were in place and the program had sufficient experimental evidence to allow for a process to lead to an actual drug discovery program *per se*. Only in 2011 evidence in the literature has shown the probable implication of this target in metastasis [47]. The work on autotaxin also concerned an already known motility factor [48] that turned out to be an enzyme generating this motility factor, with a potential role in metastasis [47, 49].

Broadening the observation that N-myristoyltransferase was key to the maturation and delivery of oncoproteins, such as src, to the membrane, we became interested in the other proteins that were myristoylated [6] and sought to differentiate their cytosolic activity from the poorly described membrane-associated activity. These studies led us to wonder if there was a target for inhibitors that would turn out to be antiviral compounds (myristoylation of the membrane viral protein gag is key in the virus life cycle). Very early on, we became interested also in kinases and some of their modifying enzymes (e.g., NMT, see above). Indeed, we started our program by choosing to explore the main tyrosine protein kinase expressed in the HL60 cancer cell line [50, 51] and purification was the only option at a time when cloning was rather rare.

We still believe that following the expression of the target (enzyme) in bacteria, for instance, activity must be purified to homogeneity. Indeed, the further process of testing the activity should lead to unambiguous attribution to the target protein and not to a similar activity catalyzed by a bacterial endogenous enzyme. Therefore, much effort was often put into the purification process, even though the current literature reflects a lack of enthusiasm about obtaining a pure enzyme. Furthermore, the need for biophysical as well as structural data necessitates a pure product, in any case. Therefore, what is done at that stage serves at least three purposes: obtaining uniformity of the enzyme in the preparations; gaining the possibility of measuring unambiguously the relationship between molecules and their targets; and finally, achieving crystallogenesis, which requires the purest possible preparation to start with.

Considering receptors, the situation might be slightly more favorable to a less simplified system. Indeed, the main property of receptor binding is certainly that the binder is specific to the receptor; thus, considering membranes as an acceptable receptacle for receptor studies seems to be an adequate compromise. For instance, in a program linked to our historical involvement in melatonin receptor pharmacology, we had to find a way to check for the activity of agonist candidates in an *in vivo* model of depression that was a non-rodent and diurnal animal (melatonin being heavily involved as a master switch of the circadian rhythm as well as the circannual one) [52, 53]. Such models were not that common, especially at a time when the exact nature of the chronobiology was not understood. We then turned to a sheep model with the difficulty that, at that time, it was believed that two animals were known to be natural

knockouts for one of the melatonin receptors (MT_2). Although this was clearly demonstrated for one strain of hamster [54], it was less clear for the sheep. Thus, we embarked on a challenge to finally clone, express, and characterize the MT_2 receptor from sheep, destroying this myth at the same time [55]. Such approaches became one of our interests in our multiple attempts to crystalize G-protein-coupled receptors (see also the structural biology section, below). In brief, a survey of the sequences of the receptors of a common family (e.g., melatonin) in various animals indicated variations of sequences with retention of specificity (towards melatonin). By measuring the thermal stability of the receptors, we can deduce the strongest structure that could be amenable to expression, solubilization, purification, and crystallogenesis attempts [56, 57], as anticipated a few years ago [58]. In other words, despite the apparent futility, cloning/expressing receptors from multiple sources might lead to findings of major importance. More recently, however, an attempt to clone the second melatonin receptor from the European hamster, previously reported as a natural knockout (as in the other hamster species), partially failed because of difficulty establishing the appropriate conditions to copy a particularly rich G-C region of the gene, as we had done for the sheep MT_2 receptor [55]. Finally, in our quest for stable versions of the melatonin receptors, we engaged in a series of cloning programs of these GPCRs from different animals, including bats, birds, snakes, and various mammals, most of which were not published (Guenin and Boutin, unpublished). Indeed, we ended up trying to characterize the melatonin receptors of the strangest mammal, the platypus. We did clone the ancestor of gpr50, a.k.a., Mel1c, and characterized its unique pharmacology; gpr50 has lost its melatonin-binding capacity in all mammals except for the platypus (Gautier et al., in press). Following the same line of work, we started a program aimed at validating the existence of a third melatonin-binding site [59], as reported earlier [60], to finally attempt and succeed in purifying it [43]. This effort led us to a series of studies demonstrating the key role of this enzyme in many different contexts [61, 62].

Of course, channels and receptors (as well as membrane-associated enzymes) require a system of expression in which the channel activity can be followed unambiguously because quite often, purification of these proteins is extremely difficult to achieve.

2.4. Testing the enzyme or the receptor (or else)

As noted, and as can be understood from the scheme in **Figure 1**, testing the enzyme or the receptor is the cornerstone of the whole process. Assaying a target involves several important requirements: the test should be robust, fast but reproducible, easy to handle and cheap and if possible should address only the activity that is targeted with minimal interference from the compounds in the assay ingredients. Several reviews have been published on this issue. For instance, the race for label-free solutions [63] or the use of instruments that measure directly the amount of product formed (or consumed) during the assay [64] are two perspectives on the never-ending moving/changing landscape that rises into view as soon as the screening process is involved. Books could be written on the way a screen must or can be done. Below are just a few examples of the choices we made.

Choosing the right test is a key decision that will influence the rest of the program. In the past, we spent some time trying to identify the best assay for our kinase program and evaluated the

whole literature for the best possible options [65] before finally attempting to run our own original version [66]. We frequently found that the assay described for our target class was not fully adaptable to our instruments or presented potential interference with the assay component(s). Several dozens of assays were adapted from the literature and/or from the material we had in hand, such as the systematic use of HPLC [9, 39, 41, 67–69] and more recently, mass spectrometry [70]. Worth noting here is the use of a global technique such as NMR. Indeed, a feature of this method is of great interest for us: it has poor sensitivity but a high robustness [71] and can be used not only for screening purpose but also for monitoring poor-affinity target/compound interactions, as in the first step of the search for receptor ligands. In addition to its obvious use as an analysis technique, we have adapted this method in various frames, including a way to better trace enzymatic activity [72], use a fluorine-labeled spy molecule as a ligand for an enzyme (that screened compounds can chase) [73], or verify changes in a component of a reaction [74]. These techniques are all automatable and thus can be used with less staff power as long as the automate is running by itself—day and night—and the validation of the assay guarantees stability of the components over time.

When turning to non-enzymatic assays, such as receptor binding or protein-protein interaction, the constructs necessary to reveal the activity of the candidate compounds might be complex. For the receptor assays, where binding is the known first step, the functionality is the key information: e.g., agonists as well as antagonists are ligands in a binding assay, and only the functional assay—often cellular [75–77], but with exceptions [78]—thus should be able to distinguish between both entity types.

The recent evolution in understanding of receptor bias ligands has considerably changed our view of receptology. Indeed, for GPCR studies, compounds can be specific antagonists of a given signaling pathway (see, for example, a recent survey on the melatonin receptors [79]) but partial agonists in another measured pathway. Even if the bottom line is that an agonist should lead to the internalization of the receptor—which the antagonists should not—there will be exceptions and changes in paradigm(s) ([80], for instance, and Legros and Boutin [unpublished]).

Of course, in some cases, we had to develop and assess a completely new assay, such as when we addressed the difficult question of PPARγ ligands and had to use a new method of binding onto this soluble protein [81].

Globally, it is fair to say that four molecular target types cover most of the druggable biochemistry of living organisms: the enzymes, receptors, channels, and protein-protein interactions. In fact, our view is that a full spectrum of possible techniques should be available to play with and adapt to the current project, while validating as much as possible the potential biases of such techniques. We decided very early to double-check the results of a screen (i.e., validate the hits) by using an orthogonal assay (see Section 2.9), that is, an assay that does not have the same technical approach as the initial one.

2.5. Automatizing the assay

Automation is the capacity for an industrial laboratory to run literally hundreds or thousands of assays a day and be able to extract from this formidable amount of data a promising

candidate that will fulfill at least some of the criteria for future development. It is thus important to have a decent number of automated stations without losing the capacity to understand and control the various parameters of such an assay.

We decided to go with small, independent stations that were not entirely automatized. One of the reasons for this choice was the fact that we could collaborate with the SIMM group that had all the capacities for running mammoth screen campaigns (over one million compounds). In addition, we believed—and still do—that some modularity is essential for the involvement of the technical personnel in these very repetitive tasks. Such modularity is more adaptive to numerous situations than a single, heavily integrated robot that will take weeks if not months to reconfigure for another assay, especially when the assays cover a very large spectrum of techniques. Indeed, we faced campaigns based on assays for widely different proteins and targets, including enzymes, receptors, protein-protein interactions, and channels, requiring being able to adapt the material at hand to the various approaches for screening these different target types.

Another aspect that is particularly important for us is repeatability of the screens. Indeed, we chose to run assays in independent duplicates at two different calendar dates and possibly with two different operators, leading to a higher level of robustness for the results, as long as both results were close to each other (see below).

2.6. Screening

Screening is the modern version of intelligently seeking the needle in the haystack. It concerns a test in which literally hundreds of thousands of compounds are evaluated, most of the time without any *a priori* information on their chemical structure or class (e.g., peptides, fragments, drug-like compounds, aptamers, toxins, natural extracts, natural compounds, etc.). Therefore, for cost constraint reasons, it may become important to screen in a smarter way [82]. This search has become a science in itself, a science that relies on chemical diversity. We view it as follows: the more diverse the compound library is, the more chemical space it covers, and the more successful we might be in finding one or several hits for a particular target.

In a given library of compounds, what are the chosen compounds that will lead to a new drug (i.e. active, specific, non-toxic, and patentable)? A perspective from some leading pharmaceutical companies was published in 2011 [83]. They gave their common view of the HTS programs, what they delivered, and what they failed to deliver. This particularly unusual publication (co-signed by the major pharmaceutical companies) exemplifies the reality of HTS expectations (too high) and of the quality/amounts of the results (the new drugs, too low). Nevertheless, as with the main progress in life science technologies in recent decades, the rise of HTS has led to plenty of new discoveries and approaches. We should not forget that all of these techniques accelerated discoveries, and even if those are not yet transformed into new drugs for the patient, they still add to the understanding of physiopathological processes. Mayr and Bojanic described the basic organization of an HTS laboratory—among other types—a few years back [84].

2.6.1. High-throughput screening

We embarked about 15 years ago on establishing a minimal HTS department in which a couple of screening robots together with an integrated analysis system permitted 'rapid' screening of several thousand compounds. The adaptability of the machine quickly became a problem: expanding our screening capacity—in terms of diversity of assays as well as number of compounds—would require several identical instruments running in parallel on which specialized personnel would apply their skills in an unvarying way. We chose something else for a time. We aimed at having small stations independent from one another to be able to treat or read between 40 and 50 384-well plates (~15,000 compounds). There are no general rules about how to arrange such a laboratory: it depends on the space, money, and personnel available. We felt that the whole process should be rigorously simple, with several key steps (go/no-go decisions) from the basic setup of the assay (and its validation with reference compounds) to the automation and test on a small scale of 10,000 compounds and finally both full-deck campaigns.

Figure 2 shows a series of typical examples of data obtained with this set of methods. In general, we screened about 10,000 compounds using the final conditions (set up previously) in two independent campaigns with the same compounds and different operators, if possible. The analysis of those data is what will permit moving forward to the full campaigns of 250,000 compounds, tested twice on the target. Without going too much into practical details, we found this methodology to be the most appropriate. Even if 2 or 3 out of 30 HTS campaigns turned out to be mute (no compound out of the HTS campaigns), the rest delivered several classes of compounds that could be pushed to the program downstream.

The figures show the repartition of the compounds according to their level of activity (left) while the graphs show the actual data where the results of the first test are plotted versus the second series of tests. The two tests are experimentally identical but run on a different day and if possible with a different operator. Because most of the compounds should be inactive, the Gaussian curves are centered to 0. The graphs should be aligned according to the diagonal of the rectangle because the data are theoretically identical, and should be massively located around 0. In **Figure 2A**, the first robotic setup led to a fat repartition of the compounds on the Gaussian curve, suggesting poor reproducibility, which is confirmed by the format of the correlation graph (or rather the lack thereof). In **Figure 2B**, with another setup, the data are more centered to the 0 and diagonal areas, respectively, in both figures, and in **Figure 2C**, the test was run first with a set of molecules, as in a feasibility attempt. We designated this process as a pre-HTS campaign. The data show a very good reproducibility of the tests, with compounds gathered along the diagonal of the graph.

2.6.2. HTS system: factory or small business?

It is the fashion to elaborate big systems that are very integrated and can run 'any' type of assays for any given number of compounds in a library. Unfortunately, despite beautiful examples of successful efforts with such machines, the issue of time frame in such situations is an elusive one. Indeed, moving on the same instrument from an enzymatic assay (e.g., with

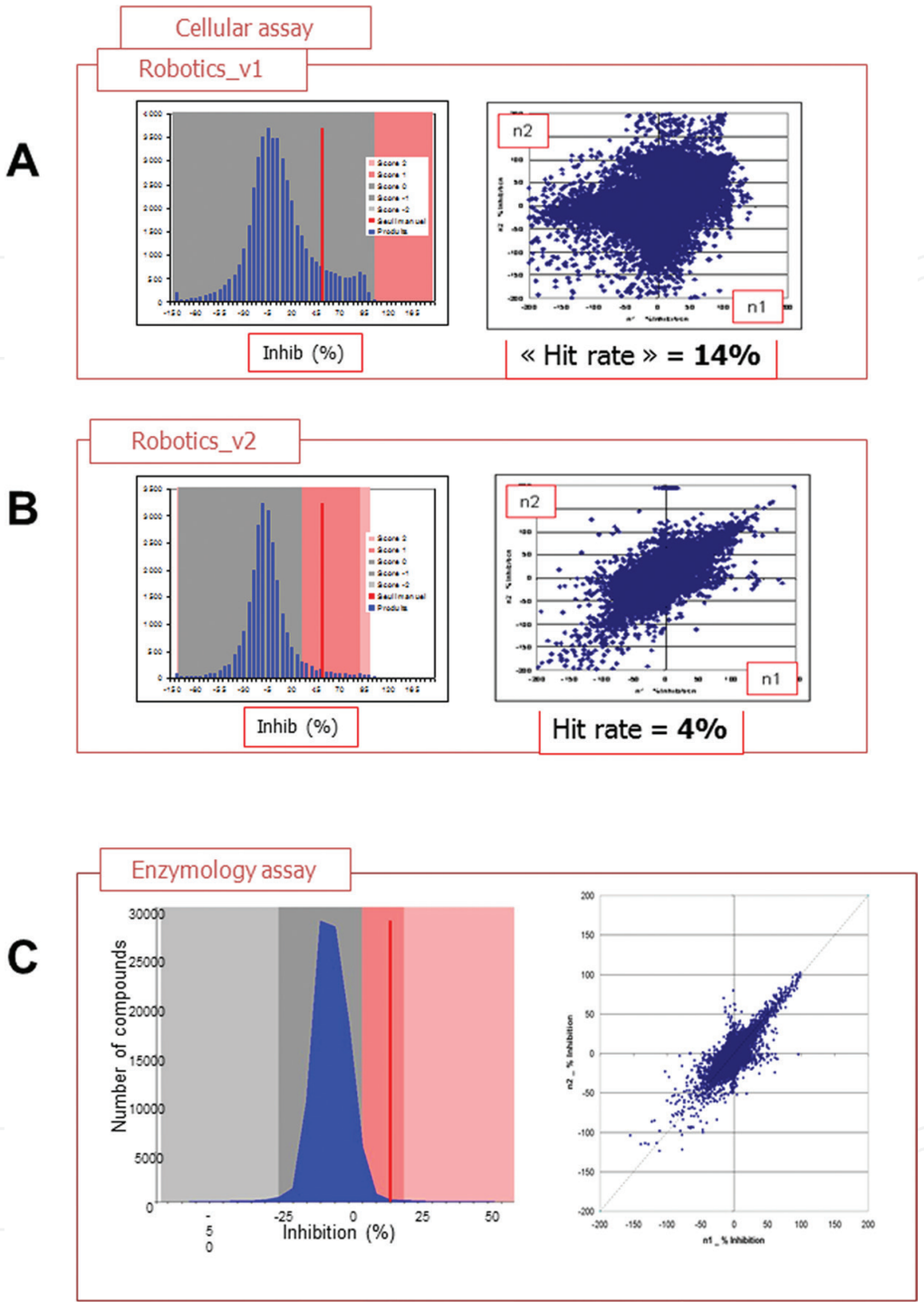

Figure 2. A typical and real example of the influence of the robotic setup on the quality of the screening results. The test was a cellular-based assay (*), comprising the detection of an intra-organelle color change potentially due to the tested compounds (see Jensen et al. for full description of the test [85]). A and B show cellular-based assay results; * note the use of differentiated primary cells; C shows an enzymatic assay. All results (whatever the biological sources) should resemble panel C. Poor results such as A and B, even if partially corrected by adapting robotics (between A and B), would lead to massive amounts of false positive hits.

fluorescent probes) to a cellular assay (e.g., with engineered cells) involves different instruments, setups, and prerequisites. One cannot honestly predict the length the preparation will require, and to a certain extent, the length of the global process (assessment + campaigns).

In the particular case of cellular assays, and as discussed elsewhere, the use of cancer cell lines brings difficulties, due to their genetic baggage, that is, to say the least, abnormal, leading to overexpression of many proteins. Alternatively, cells might be primary cells differentiated or not, the use of which leads to major variations from cells to cells, ultimately leading to massive false positive amounts (up to 14%, see **Figure 2**) and poor reproducibility from batches to batches. Finally one possible choice would be to work with differentiated stem cells, as their robustness is enhanced due to the current state of the art, with more and more publications describing works in this area. All these cells can be engineered with modern transfection techniques.

It is always possible for extreme cases to use different approaches. For instance, in a premodern time, we ran a HPLC assay for AANAT, ending with interesting hits despite the "low" number of compounds tested [67]. It is certainly an option whenever the available test involves extremely costly reagents or engineered fragile cells.

2.6.3. High-content screening (HCS) and its obligatory companion, functional genomic screening

High-content screening is a fast cellular-based approach that became possible in recent years, thanks to progress in imaging, computing, capturing, analyzing, and automating those processes. Moffatt et al. has summarized these approaches [86]. One also has to keep in mind that whatever the complexity of the engineered cell-based assay that is designed to identify compounds specific to given (cellular) pathways, it is probably less complex than the cellular metabolism. In other words, once the compound has been identified, one must check the *in situ* nature of its target. Treatment of siRNA libraries of the cellular systems and/or reverse pharmacology can be used to such an end. Saeidnia et al. [87] has reviewed the reverse pharmacology and possible solutions that are available.

The latest trend is to screen phenotypically engineered cells for the pathway in which the target is involved. If a compound is found, the validation process seems cumbersome to us. In fact, the deconvolution of the phenotype, to identify the actual target(s) of the hit compound(s), is a long process that is interesting, difficult, and expensive (in time and money). Cautions also have been published recently, warning scientists using such methodologies to be extremely careful in turning to these approaches because the number of biases is immense. We only very recently entered onto this path. In the meantime, we had serendipitously prepared the next steps by a very early attempt to industrialize our previous success in reverse pharmacology: the discovery of the third melatonin-binding site as quinone reductase 2 [43]. Nevertheless, this success has not been the rule. Several attempts done internally to find the target of given pharmacological agents have so far failed.

2.7. Screening what?

At the start of the year 2000, the question was, "How many compounds do you have in your library?" Without reference to the diversity of the compounds, some big players in the game had more than a million compounds available. Indeed, diversity is a complex problem that

has been discussed at length by various authors (see Gillet et al., in particular [88, 89]). In the chemist community, it is often joked that there are as many definitions of diversity as individual chemists. Briefly, we chose to stay on the following path. Based on a definition of diversity that we found clear and attractive [90] and that gather all the basic concepts of diversity (and their measurement), we chose to base our own measure of diversity on the pharmacophore concept [91]. We felt that the more we wanted to pinpoint this diversity concept, the more we became lost in endless descriptors of the molecules [92]. Ultimately, we used the approach of Lepp [93] to establish a representative sub-library of compounds that can serve as a simplification of our complete library for pre-test purposes (see above) or to limit the number of compounds whenever the screening campaign uses a test that is either too expensive or cellular (i.e., "too" complex). Another way to define diversity is to measure the similarity between compounds, another approach that has also been discussed at length [94].

The diversity of the product collection that will be tested is indeed important and allows for anticipation of the discovery of new series on which the medicinal programs will be developed. Achieving diversity, however, will require the inclusion in the library of compounds with unusual or poorly documented structures.

A company like ours has a 60-year history of chemical synthesis of drug candidates. These molecules are still available in the compound collection and constitute an original source of diversity. To complete this panel while maintaining the largest diversity possible, we chose to integrate into the compound library natural pure products, extracts, peptides, and more generally 'drug-like' chemicals either synthesized internally or commercially available. We targeted having a total of about 250,000 compounds screened, systematically, for any targets of interest.

2.7.1. Natural compounds

A fundamental in this domain is certainly the belief that ethnopharmacology can identify interesting natural molecule(s) from traditional medicine (see Heinrich et al. for background in ethnopharmacology [95]). Ethnopharmacology focuses on a compendium of mixtures that medical practitioners in native populations prepared and gave to their patients, sometimes with outstanding success. The complexity of these approaches is immense. Indeed, besides the type of the plants used—not always easy to trace and not always easy to find in historical locations—there is the nature of the preparation, including the mixture of several different plants and associated products (of animal [including insects] or mineral origin). We tried to access the two main available sources of such compounds: pure compounds or extracts that need to be deconvoluted, usually by bio-directed strategies. We have reported our experiences with the latter in three publications [96–98] but rapidly abandoned these approaches for the following reasons: paucity of results; tediousness of the process; difficulty in repeating the exact same experiment, including trouble finding plants at the same location with the same properties; and last but not least, often difficulties with the structure identified, which turned out to be common or of a complexity beyond the reasonable feasibility of industrial production. It remains interesting to view those compounds as a mold of the target binding site and/or to use them as an inspiration for a future drug. At the other end of the spectrum,

commercially available, pure compounds are hard to find in a decent quantitative supply. Often, compounds are available from vendors, but they are either common (flavonoids, coumarins, peptides, terpenoids, and alkaloids) or provided in minute quantities, with barely any reload possible. We therefore balanced our library with some of those pure compounds, as an internal control to assay, because often flavonoids—while poorly soluble—have turned out to be enzyme inhibitors (see, for example, [40, 99]).

In the interest of completeness, we add that one other path is to provide some biotech companies with a target. They screen the target with their proprietary sources of natural compounds (often as microorganisms, plants, and similar extracts to start with).

2.7.2. Peptide libraries

We became interested in peptide combinatorial libraries at the very beginning of this approach and studied several aspects of those libraries: numbers [100], quality control [101], and activity [102, 103]. For solid-phase synthesis approaches, the actual number of peptides in a given library is limited by the availability of amino acids on the resin, and that number is limited by the number of individual peptide repeats in the initial mixture, which is obviously by far the most complex one. The trick turned out to be the way syntheses were conducted, by having the last amino acids as a constant in each vessel (see Houghten et al. [104]). Regardless, these approaches are often interesting to follow, particularly when soluble or secreted targets are concerned.

2.7.3. Compound libraries

Despite the large diversity of compounds available for screening, whether through scientific collaboration, subcontracts, or vendors, the nature of the compounds is one of the most sensitive secrets of the pharmaceutical companies. As stated above, we attempted to complement our more than 60 years of internal chemical synthesis with compounds bought or synthesized with the greatest care to try to achieve the widest diversity of compounds and to be able, at the end of the screening process, to validate original molecules with great potential. This strategy is based on our capacity to analyze and understand chemical diversity and thus be able to predict what should be synthesized to fill in the gaps in this diversity picture.

2.8. Choosing the hits for further programs

The question here is, "Choosing among which hits and why?" In the industry, the answer is simple: to heal and to make a drug. For other scientists, the answer might be slightly different: understanding a pathway (such as those at the core of a pathology) or finding tools that permit the study of the pathology, the receptor, or the enzyme. In any case, the path from hits to lead and then from lead to candidate is particularly long and painful. Thus, the structure of the various candidates (often from two or three different families of compounds) must be chosen extremely carefully to avoid the various obstacles: chemistry difficulties, including that the drug will be synthesized at the ton level at the end; specificity towards the other proteins of the body (not less); pharmacokinetics (often the compound should be given orally);

and patentability. It is interesting to note that exceptional universities, such as the University of Michigan, have published their reflections on this subject [105]. On the other hand, one should realize that the hit rate of an *ab initio* screening is somewhere between 0.01 and 0.1%. For 250,000 molecules, that translates to 25 and 250 compounds. It looks like plenty to choose from, although the hope is that among those compounds, some are similar, suggesting a structure-activity relationship that enforces the results of the HTS.

Sometimes, as noted, researchers will choose compounds for their availability or their known lack of toxicity, despite other problems such as a lack of specificity. The purpose then is different: finding tools to validate a pathway or the engagement of a target in a given pathology (see Section 2.2). Examples like melatonin [106], curcumin [107], or resveratrol [108] are also interesting to note. Indeed, these compounds are fantastic tools to better understand a network of pathways involved in various pathologies, but none of them shows any type of specificity. To the contrary, they seem active in many pathological situations because they interfere with many targets.

A more recent approach has also involved the systematic use of chemoinformatics analyses with its formidable capacity of calculus and prediction [109, 110]. Starting from there, it is possible to rationalize the choice of the best hits: among others, the previous history of the compound in the earlier campaigns on other targets, similarity with known compounds from the literature, patentability, and accessibility to the chemistry (not too complicated to allow 'easy' generation of a large series of analogues).

2.9. Confirming the hit activity

Once hits have been identified, the process continues in validating the molecules in their recorded interaction with the targets. Genick and Wright [111] addressed this subject, among others, in their recent paper. Essentially, it comprises three aspects: biochemical validation, biophysical measurement, and structural data.

Our first step is towards an orthogonal confirmation of the activity of the compound. Obviously, whenever an enzyme is concerned, it is 'quite easy' to invent or copy an alternative assay that engages different physical parameters, going from the old, global, radioactive phosphocellulose paper-based detection assay [112] to a more specific radioactivity-based assay [113] for a kinase, for instance; trying to go from a protein-based phosphorylation assay [114] to a more specific peptide-based assay [66] or lastly, going from an antibody-based assay to a more specific ubiquitinated peptide-based assay [115]. There are multiple examples of such orthogonal assays, such as the use of HPLC, fluorescence polarization, mass spectrometry, and fluorescence resonance energy transfer (FRET) technology. We always felt that part of the reproducibility problem(s) was (were) due to the result of the poorly understood interferences between components of the assay, even sometimes the totally aspecific actions of the compound onto the target, for instance, by precipitating the protein rather than inhibiting it, with a similar effect in the assay (lack of activity in the presence of the compound). Compounds at the concentration used in the screening process—often 10 μM—tend to precipitate. The addition of dimethylsulfoxide (DMSO) as a compatible organic solvent limits this precipitation to a certain extent, but the presence of DMSO might affect the enzyme or the reactivity medium

(not to mention the receptor, membrane-bound assay). This is also why we argue for work on purified targets to simplify, at least in this context, the environment of the protein.

For the receptor assay, it can be more complicated to find an alternative, often because of the uniqueness of the radioactive ligand used in the binding assay. We tried to circumvent this problem by developing options in this context, for example, in synthesizing other ligands, to have more than a single binder in our melatoninergic [116, 117] or MCH toolboxes [118]. If this goal is achievable for receptors that have been studied for decades (serotonin, adenosine, histamine, and melatonin), for other more recently studied receptors, it has been completely impossible, including the obvious case of orphan receptors for which ligands are not known and assays are built around receptor functionality. Thus, the study of such receptors has largely been done through efforts to de-orphanize them [119, 120] and to fully characterize them with all the tools of molecular pharmacology, as for gpr103 [121–124] or gpr50 [125, 126]. An easier alternative is to work with known ligands as peptides, as in our extensive work on melanin-concentrating hormone for which alterations in the natural sequence led to alternative ligands [118], agonists [127], or antagonists [28, 128]. Beyond the thrill of executing a *tour de force* design and the joy of manipulating peptides of short sequences, with their infinite possible variations, the work brought a panel of new tools that might ultimately contribute to understanding such integrated systems.

2.10. Structuring: what is the hit/target relationship at the atom level?

As noted, part of the validation is linked to the visualization of the molecular structure of the complex between the hit and the target. The key role of this approach has been reviewed numerous times (e.g., Scapin [129], Hu et al. [130], and Zheng et al. [131, 132]). It is now clear that the progression of the compounds from a hit to an elaborate drug will benefit immensely from structural biology data. We contributed also to a certain vision of how alternative methods will complete (and possibly compete with?) crystallography, particularly cryo-electronic microscopy [133].

We made the choice of an active and ambitious structural biology approach about 10 years ago. Creating efficient collaborations with some groups, our first attempts were to characterize the interactions between some of our compounds and a given target in neurogenerative diseases [134–136]. We then embarked on several kinase-related projects that ended with the discovery of powerful compounds [137–140]. However, we needed more freedom to operate and establish proof of concept of the importance of these approaches to complement our drug discovery programs. For this reason, we chose to install a dedicated laboratory inside a synchrotron (Soleil, St. Aubin, France) to gain full access not only to the beamlines but also to the vast scientific community behind this instrument. This choice led to several collaborative academic-oriented lines of research [136, 141] and to more internal research programs. In preparing the next steps of crystallization—the step involving the receptor and more generally membrane proteins—we assessed a series of methodologies for the solubilization and purification of functionally competent receptors [56, 57]. Furthermore, we also studied the impact of microfluidics on crystallization of proteins for structural biology studies [142, 143].

The companion science of structural biology is molecular modeling. It is key for discovery programs, either at the early stage (explaining and rationalizing the molecule-target relationship

and even quantifying it) or at a later stage when new molecules, derived from a hit in the screening campaign, will be synthesized and need to be paused inside the structure of the target by calculation or co-crystallization. We have reported some examples of the use of those techniques for glucokinase [144], gpr103 [124], and rev-erbα [145].

2.11. "Biophysing": understanding and measuring the forces responsible for the relation between the molecule and its target

Many reviews have nicely summarized recent community perspectives on this aspect of compound characterization [146–148]. No doubt, the rise of all the physical biology methods affords more and more tools that are essential to a modern drug discovery program, particularly when trying to understand and measure the relationship between the compound—that is, the drug candidate or its ancestor—and its protein target.

From the seminal work—for us—of Shuman et al. on HIV proteases [149], we started to wonder if the complicated concepts of thermodynamics could be applied to the process of moving from hit to lead compounds. What would be the help of measuring the actual parameters of a target-compound association, even if the target were not a receptor but an enzyme? We became adopters of surface plasmon resonance (SPR) [150], calorimetric instruments, fast-flow machines to measure events of enzymatic activity that occur faster than a second [151], and native mass spectrometry to gain information on the minute conformational changes of proteins [152].

It is now clear that those tools have become part of ongoing studies for helping medicinal chemists better evaluate and thus better understand the impact of the minute modifications they make to their compounds. For instance, the use of calorimetry to determine the actual affinity of an inhibitor for its enzyme has proved to be accurate [134], while standard methodologies such as binding turned out to be cumbersome if not wrong [153]. Furthermore, the determination of the number of compounds and their nature associated with a given protein was also a key step in our understanding of quinone reductase 2 enzymatic behavior [70].

These "new" tools are increasingly reliable. "Structuring" and "biophysing" help give access to precise details on the structure of the co-crystal together with actual thermodynamics measures and thus the relationships at the atom level between the target and its ligand (at least, and for the moment, for enzymes); however, crystallization of the membrane-bound proteins (such as GPCRs) is still a challenge, despite recent major progress [154, 155]. These newer tools permit visualization and quantification of these interactions. The medicinal chemist can use this information as the basis for a synthesis strategy for the evolution of hits towards a series of compounds with *ad hoc* properties that are easier to predict and rationalize.

3. What next?

Research has always been a question of strategic and/or opportunistic decisions at a given time to explore areas that look promising for a better future. These decisions, at the industry

level, are always difficult to make. Money, time, and energy go into anticipating what will be important—technology-wise—for the next decade of discovery.

Among the published reports that we attempted to explore were many approaches involving new, non-invasive techniques to better understand the role of a compound and its target inside a cell, the future of stem cells and of chemical proteins, and the modifications of proteins inside the cells.

3.1. Stem cells as expressing systems

Stem cells have been the new frontier for some time and remain so. One debate has been whether to target therapy with stem cells, directly (in cell therapy) or indirectly (by using the secretosome of differentiated stem cells) or to use the cells as hosts for a target that would then be expressed in an optimal environment, as opposed to the classical use as hosts of cancer cell lines that are either very derived from the original cell lines or comprise numerous nuclei with profoundly abnormal karyotypes. We chose the isolation, differentiation, production, and characterization of such stem cell-derived cardiomyocytes [156]. Potentially, these techniques open new roads to the use of natural hosts that would be a better context for the expression of targets in a natural environment. Furthermore, as demonstrated in several instances, stem cells can be derived from patients, and some can recapitulate the disease at the cellular level, leading to a possibly better understanding of the molecular cause(s) of a given disease [157, 158].

It might be important in the coming years or decades to be able to cope with the therapeutic use of such stem cells, despite initial concerns [159] and the extraordinary characteristics [160] of these cell types.

3.2. Chemical proteins

A boom in the area of chemical proteins followed the discovery—among others—of chemical ligation [161]. It became possible to synthesize enzymes chemically and measure their catalytic activity. After the chemical synthesis (by solid-phase synthesis) and use of ubiquitin, described for several decades, we chose a small enzyme (120 aa), calstabin, that we completely synthesized with our partner. We crystallized this enzyme and measured its catalytic activity. In doing so, we learned two important things: (1) such an approach is feasible, even though it led to small quantities of material, at least to start with, and the material was indistinguishable from the recombinant version and (2) the apparent mono-peak analysis of the result of the synthesis revealed, after further analyses, that the protein could be separated in two distinct peaks, one a fully refolded protein with catalytic activity and the other a denatured protein with no catalytic activity and no refolding [141]. These findings led us to consider that large peptides/proteins that are chemically synthesized and often described as being used in the literature (e.g., EPO, growth factors) might be a mixture of active and inactive substances if not appropriately characterized.

Our aim was and still is the validation of a process encompassing both the recombinant expression of pieces of a large protein—such as an antibody—that contained only natural

amino acids and other pieces of the same protein, in which exotic amino acids were inserted. The fusion between those pieces would then be performed by chemical ligation(s). These approaches will open avenues to the incorporation of key functional groups in protein for derivatization with drugs (antibody-drug conjugates) or to incorporation into proteins of exotic chemical functions.

3.3. Ligases

Among the seminal studies of the *in cellulo* modifications of proteins are the numerous efforts of Alice Ting's group [162]. Using a modified ligase, it became possible to directly label target proteins in a cellular context. The label can be a fluorophore, radiolabeled compound, or NMR probe, for example, and the chemistry of the modification is the main limitation of this process. Coupling it with the power and resolution of magnifying instruments, including specialized beam lines in a synchrotron, can lead to the cartography of the protein inside a living cell. We began the process first by characterizing the enzymology beyond this ligase-catalyzed enzymatic reaction, using purified enzyme and reagents in an acellular context [163]. This technique will be adapted to intracellular processes with the goal of modifying a recombinant protein *in situ* and to allow labeling of a protein inside the cell to follow its fate.

4. Outside the box

Anticipating tomorrow's needs in modern research is a challenge that requires triangulating the many different areas necessary for drug discovery. It has thus been difficult to choose various avenues to make our efforts more successful, modern, and interesting and at the service of future trends and needs in drug discovery for therapies and cures. Choosing to enhance our capacities to incorporate new approaches is always a challenging but moderate risk, based on the way we perform these exploratory strategies. Indeed, one critical choice was to hire postdocs to scrutinize those ideas. In doing so, we not only identified and incorporated strong scientists but also could assign new technologies to categories such as 'dream', 'usable soon', 'needs more time', etc. Among the discoveries that we have published are the ligase use for *in cellulo* transformation of proteins [163], including antibodies; cellular imaging using large synchrotron instruments; synthetic proteins that can be as active as the recombinants [141]; and synthesis of proteins and peptides modified by ubiquitination the way it happens in the cell (as opposed to what was commercially available at that time) [115]. In addition, we started our first trials in thermodynamics, structural biology, new crystallization techniques, native mass spectrometry for structural biology approaches [152] and for target-molecule relationships, and the use of human stem cells as an alternative to rat cardiomyocytes in first culture, among other initiatives.

The engineering of cells and their use as therapeutic agents bring new challenges and new wonders almost daily. New challenges emerge because the necessary work to achieve therapies for tomorrow may not necessarily be what was done in the recent past. These challenges

often require new approaches, particularly in the use of treatments with stem cells from the patient, or with cells that have been engineered to fulfill a task that only science fiction writers dreamed about in the 1950s. Just one example is the seminal paper by Schukur et al. [164] on the possibility of engineering a cell to produce a factor (such as tumor necrosis factor) following a native regulatory pathway in the patient in whom these cells will be grafted. The number of new technologies required to reach that end is just immense. If we do not prepare for such wonders, the industry will have an extremely complicated time reorienting to tomorrow's health-related demands.

5. Conclusions

The body of techniques developed and applied in the DD platform throughout the years has contributed to the introduction of several molecules on the market. The contribution of this platform concerned the initial steps of compound discovery that proceeded towards final molecules. Or else, the platform was involved in providing data on the biophysics of the interaction, most of the time, all steps of drug discovery contributed to the final results. Some findings have been published and showed the completeness of the approach that laid the foundations for the discovery and early clinical development of drug candidates [138, 139, 165].

There is obviously confusion between the use of research that is the replication of data obtained elsewhere (the role of the industry) and the de novo research programs that are developed in the pharmaceutical industry. The confusion is that work consisting of new research should not be done in the industry, but in academic institutions, while repeating published data should be done in the industry. In that way, by limiting the novelty of the industrial work, the novelty of the cure(s) found in such context might be limited, thus leading to a situation where alternate paradigms must be found. For instance, a way to access to novelty might be to fund biotechnology companies to explore new research areas and new ideas.

To echo arguments already made above, most of the published research is difficult (if not impossible) to repeat. And as noted, there has been a long debate in the scientific literature on the repeatability of data [166, 167]. Therefore, part of any efforts at clarity and repeatability should be sorting the reality from the fantasy in what we are reading. Furthermore, the key to success is often to be the one company that delivers first on something for the patients' benefit. Anyone coming in after the first- or second-place entry will be less rewarded. As mentioned in the introduction, research attrition in the pharmaceutical industry is around 90%, meaning that 9 out of 10 projects fizzle before reaching the clinic. In the meantime, the (discovery) show must go on, and the company must sustain this endeavor with internal and investor money or turn to other sources of innovation.

Finally, research is a never-ending dynamic process. If development of new techniques and new concepts ceases, such a drug discovery platform can rapidly become obsolete.

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References

[1] Gao G, Chen L, Huang C. Anti-cancer drug discovery: Update and comparisons in yeast, Drosophila, and zebrafish. Current Molecular Pharmacology. 2014;**7**:44-51

[2] O'Reilly LP, Luke CJ, Perlmutter DH, Silverman GA, Pak SC. *C. elegans* in high-throughput drug discovery. Advanced Drug Delivery Reviews. 2014;**69-70**:247-253

[3] Scudamore CL. Integrating pathology into human disease modelling—How to eat the elephant. Disease Models & Mechanisms. 2014;**7**:495-497

[4] Kamps MP, Buss JE, Sefton BM. Mutation of NH2-terminal glycine of p60src prevents both myristoylation and morphological transformation. Proceedings of the National Academy of Sciences of the United States of America. 1985;**82**:4625-4628

[5] Boutin JA, Ferry G, Ernould AP, Maes P, Remond G, Vincent M. Myristoyl-CoA:protein N-myristoyltransferase activity in cancer cells. Purification and characterization of a cytosolic isoform from the murine leukemia cell line L1210. European Journal of Biochemistry. 1993;**214**:853-867

[6] Boutin JA. Myristoylation. Cellular Signalling. 1997;**9**:15-35

[7] Boutin JA, Clarenc JP, Ferry G, Ernould AP, Remond G, Vincent M, Atassi G. N-myristoyltransferase activity in cancer cells. Solubilization, specificity and enzymatic inhibition of a N-myristoyl transferase from L1210 microsomes. European Journal of Biochemistry. 1991;**201**:257-263

[8] Ferry G, Tellier E, Try A, Gres S, Naime I, Simon MF, Rodriguez M, Boucher J, Tack I, Gesta S, Chomarat P, Dieu M, Raes M, Galizzi JP, Valet P, Boutin JA, Saulnier-Blache JS. Autotaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation. Up-regulated expression with adipocyte differentiation and obesity. Journal of Biological Chemistry. 2003;**278**:18162-18169

[9] Giganti A, Rodriguez M, Fould B, Moulharat N, Coge F, Chomarat P, Galizzi JP, Valet P, Saulnier-Blache JS, Boutin JA, Ferry G. Murine and human autotaxin alpha, beta, and gamma isoforms: Gene organization, tissue distribution, and biochemical characterization. The Journal of Biological Chemistry. 2008;**283**:7776-7789

[10] Sun S, Wang R, Song J, Guan M, Li N, Zhang X, Zhao Z, Zhang J. Blocking gp130 signaling suppresses autotaxin expression in adipocytes and improves insulin sensitivity in diet-induced obesity. Journal of Lipid Research. 2017

[11] Boutin JA, Ferry G. Autotaxin. Cellular and Molecular Life Sciences. 2009;**66**:3009-3021

[12] Ferry G, Moulharat N, Pradere JP, Desos P, Try A, Genton A, Giganti A, Beucher-Gaudin M, Lonchampt M, Bertrand M, Saulnier-Blache JS, Tucker GC, Cordi A, Boutin JA. S32826, a nanomolar inhibitor of autotaxin: Discovery, synthesis and applications as a pharmacological tool. The Journal of Pharmacology and Experimental Therapeutics. 2008;**327**:809-819

[13] Ferry G, Loynel A, Kucharczyk N, Bertin S, Rodriguez M, Delagrange P, Galizzi JP, Jacoby E, Volland JP, Lesieur D, Renard P, Canet E, Fauchere JL, Boutin JA. Substrate specificity and inhibition studies of human serotonin N-acetyltransferase. The Journal of Biological Chemistry. 2000;**275**:8794-8805

[14] Ganguly S, Gastel JA, Weller JL, Schwartz C, Jaffe H, Namboodiri MA, Coon SL, Hickman AB, Rollag M, Obsil T, Beauverger P, Ferry G, Boutin JA, Klein DC. Role of a pineal cAMP-operated arylalkylamine N-acetyltransferase/14-3-3-binding switch in melatonin synthesis. Proceedings of the National Academy of Sciences of the United States of America. 2001;**98**:8083-8088

[15] Mozo J, Ferry G, Masscheleyn S, Miroux B, Boutin JA, Bouillaud F. Assessment of a high-throughput screening methodology for the measurement of purified UCP1 uncoupling activity. Analytical Biochemistry. 2006;**351**:201-206

[16] Mozo J, Ferry G, Studeny A, Pecqueur C, Rodriguez M, Boutin JA, Bouillaud F. Expression of UCP3 in CHO cells does not cause uncoupling, but controls mitochondrial activity in the presence of glucose. The Biochemical Journal. 2006;**393**:431-439

[17] Guigal N, Rodriguez M, Cooper RN, Dromaint S, Di Santo JP, Mouly V, Boutin JA, Galizzi JP. Uncoupling protein-3 (UCP3) mRNA expression in reconstituted human muscle after myoblast transplantation in RAG2−/−/gamma c/C5(−) immunodeficient mice. The Journal of Biological Chemistry. 2002;**277**:47407-47411

[18] Riquet FB, Rodriguez M, Guigal N, Dromaint S, Naime I, Boutin JA, Galizzi JP. In vivo characterisation of the human UCP3 gene minimal promoter in mice tibialis anterior muscles. Biochemical and Biophysical Research Communications. 2003;**311**:583-591

[19] Henderson LC, Gibert Y. Editorial: Validation techniques for therapeutic molecules in drug discovery. Current Topics in Medicinal Chemistry. 2017;**17**:2005

[20] Kaelin WG. Cell biology: Divining cancer cell weaknesses. Nature. 2006;**441**:32-34

[21] Chomarat P, Coge F, Guenin SP, Mailliet F, Vella F, Mallet C, Giraudet S, Nagel N, Leonce S, Ferry G, Delagrange P, Boutin JA. Cellular knock-down of quinone reductase 2: A laborious road to successful inhibition by RNA interference. Biochimie. 2007;**89**:1264-1275

[22] Kaelin WG. Molecular biology. Use and abuse of RNAi to study mammalian gene function. Science. 2012;**337**:421-422

[23] Ferry G, Giganti A, Coge F, Bertaux F, Thiam K, Boutin JA. Functional invalidation of the autotaxin gene by a single amino acid mutation in mouse is lethal. FEBS Letters. 2007;**581**:3572-3578

[24] Benoit CE, Bastianetto S, Brouillette J, Tse Y, Boutin JA, Delagrange P, Wong T, Sarret P, Quirion R. Loss of quinone reductase 2 function selectively facilitates learning behaviors. The Journal of Neuroscience. 2010;**30**:12690-12700

[25] Mailliet F, Ferry G, Vella F, Thiam K, Delagrange P, Boutin JA. Organs from mice deleted for NRH:quinone oxidoreductase 2 are deprived of the melatonin binding site MT3. FEBS Letters. 2004;**578**:116-120

[26] van Meeteren LA, Ruurs P, Stortelers C, Bouwman P, van Rooijen MA, Pradere JP, Pettit TR, Wakelam MJ, Saulnier-Blache JS, Mummery CL, Moolenaar WH, Jonkers J. Autotaxin, a secreted lysophospholipase D, is essential for blood vessel formation during development. Molecular and Cellular Biology. 2006;**26**:5015-5022

[27] Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, Noji S, Yatomi Y, Aoki J, Arai H. Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. The Journal of Biological Chemistry. 2006;**281**:25822-25830

[28] Della-Zuana O, Audinot V, Levenez V, Ktorza A, Presse F, Nahon JL, Boutin JA. Peripheral injections of melanin-concentrating hormone receptor 1 antagonist S38151 decrease food intake and body weight in rodent obesity models. Frontiers in Endocrinology. 2012;**3**:160

[29] Di CB, Maggio R, Aloisi G, Rivet JM, Lundius EG, Yoshitake T, Svenningsson P, Brocco M, Gobert A, De GL, Cistarelli L, Veiga S, De MC, Rodriguez M, Galizzi JP, Lockhart BP, Coge F, Boutin JA, Vayer P, Verdouw PM, Groenink L, Millan MJ. Genetic deletion of trace amine 1 receptors reveals their role in auto-inhibiting the actions of ecstasy (MDMA). The Journal of Neuroscience. 2011;**31**:16928-16940

[30] Arsenijevic D, Onuma H, Pecqueur C, Raimbault S, Manning BS, Miroux B, Couplan E, Alves-Guerra MC, Goubern M, Surwit R, Bouillaud F, Richard D, Collins S, Ricquier D. Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nature Genetics. 2000;**26**:435-439

[31] Tosini G, Owino S, Guillaume JL, Jockers R. Understanding melatonin receptor pharmacology: Latest insights from mouse models, and their relevance to human disease. BioEssays. 2014;**36**:778-787

[32] Charpentier E. CRISPR-Cas9: How research on a bacterial RNA-guided mechanism opened new perspectives in biotechnology and biomedicine. EMBO Molecular Medicine. 2015;**7**:363-365

[33] Jung D, Choi Y, Uesugi M. Small organic molecules that modulate gene transcription. Drug Discovery Today. 2006;**11**:452-457

[34] Uesugi M. Synthetic molecules that modulate transcription and differentiation: Hints for future drug discovery. Combinatorial Chemistry & High Throughput Screening. 2004;**7**:653-659

[35] Zhang M, Feng Y, Wang J, Zhao J, Li T, He M, Yang D, Nosjean O, Boutin J, Renard P, Wang MW. High-throughput screening for GPR119 modulators identifies a novel compound with anti-diabetic efficacy in db/db mice. PLoS One. 2013;**8**:e63861

[36] Li T, Lin GY, Zhong L, Zhou Y, Wang J, Zhu Y, Feng Y, Cai XQ, Liu Q, Nosjean O, Boutin JA, Renard P, Yang DH, Wang MW. W2476 ameliorates beta-cell dysfunction and exerts therapeutic effects in mouse models of diabetes via modulation of the thioredoxin-interacting protein signaling pathway. Acta Pharmacologica Sinica. 2017;**38**:1024-1037

[37] Allera-Moreau C, Chomarat P, Audinot V, Coge F, Gillard M, Martineau Y, Boutin JA, Prats AC. The use of IRES-based bicistronic vectors allows the stable expression of recombinant G-protein coupled receptors such as NPY5 and histamine 4. Biochimie. 2006;**88**:737-746

[38] Cabaniols JP, Ouvry C, Lamamy V, Fery I, Craplet ML, Moulharat N, Guenin SP, Bedut S, Nosjean O, Ferry G, Devavry S, Jacqmarcq C, Lebuhotel C, Mathis L, Delenda C, Boutin JA, Duchateau P, Coge F, Paques F. Meganuclease-driven targeted integration in CHO-K1 cells for the fast generation of HTS-compatible cell-based assays. Journal of Biomolecular Screening. 2010;**15**:956-967

[39] Ferry G, Gillet L, Bruneau V, Banales JM, Beauverger P, Coge F, Galizzi JP, Scalbert E, Okamoto T, Urata H, Boutin JA. Development of new assays and improved procedures for the purification of recombinant human chymase. European Journal of Biochemistry. 2001;**268**:5885-5893

[40] Ferry G, Ubeaud C, Dauly C, Mozo J, Guillard S, Berger S, Jimenez S, Scoul C, Leclerc G, Yous S, Delagrange P, Boutin JA. Purification of the recombinant human serotonin N-acetyltransferase (EC 2.3.1.87): Further characterization of and comparison with AANAT from other species. Protein Expression and Purification. 2004;**38**:84-98

[41] Ferry G, Ubeaud C, Lambert PH, Bertin S, Coge F, Chomarat P, Delagrange P, Serkiz B, Bouchet JP, Truscott RJ, Boutin JA. Molecular evidence that melatonin is enzymatically oxidized in a different manner than tryptophan: Investigations with both indoleamine 2,3-dioxygenase and myeloperoxidase. The Biochemical Journal. 2005;**388**:205-215

[42] Brouillette J, Quirion R. Transthyretin: A key gene involved in the maintenance of memory capacities during aging. Neurobiology of Aging. 2008;**29**:1721-1732

[43] Nosjean O, Ferro M, Coge F, Beauverger P, Henlin JM, Lefoulon F, Fauchere JL, Delagrange P, Canet E, Boutin JA. Identification of the melatonin-binding site MT3 as the quinone reductase 2. The Journal of Biological Chemistry. 2000;**275**:31311-31317

[44] Rappaport AN, Jacob E, Sharma V, Inberg S, Elkobi A, Ounallah-Saad H, Pasmanik-Chor M, Edry E, Rosenblum K. Expression of quinone reductase-2 in the cortex is a

muscarinic acetylcholine receptor-dependent memory consolidation constraint. The Journal of Neuroscience. 2015;**35**:15568-15581

[45] Janda E, Parafati M, Aprigliano S, Carresi C, Visalli V, Sacco I, Ventrice D, Mega T, Vadala N, Rinaldi S, Musolino V, Palma E, Gratteri S, Rotiroti D, Mollace V. The antidote effect of quinone oxidoreductase 2 inhibitor against paraquat-induced toxicity in vitro and in vivo. British Journal of Pharmacology. 2013;**168**:46-59

[46] Janda E, Isidoro C, Carresi C, Mollace V. Defective autophagy in Parkinson's disease: Role of oxidative stress. Molecular Neurobiology. 2012;**46**:639-661

[47] Samadi N, Bekele R, Capatos D, Venkatraman G, Sariahmetoglu M, Brindley DN. Regulation of lysophosphatidate signaling by autotaxin and lipid phosphate phosphatases with respect to tumor progression, angiogenesis, metastasis and chemo-resistance. Biochimie. 2011;**93**:61-70

[48] Stracke ML, Krutzsch HC, Unsworth EJ, Arestad A, Cioce V, Schiffmann E, Liotta LA. Identification, purification, and partial sequence analysis of autotaxin, a novel motility-stimulating protein. The Journal of Biological Chemistry. 1992;**267**:2524-2529

[49] Benesch MG, Tang X, Maeda T, Ohhata A, Zhao YY, Kok BP, Dewald J, Hitt M, Curtis JM, McMullen TP, Brindley DN. Inhibition of autotaxin delays breast tumor growth and lung metastasis in mice. The FASEB Journal. 2014;**28**:2655-2666

[50] Boutin JA, Ernould AP, Genton A, Cudennec CA. Partial purification and characterization of a new p36/40 tyrosine protein kinase from HL-60. Biochemical and Biophysical Research Communications. 1989;**160**:1203-1211

[51] Ernould AP, Ferry G, Barret JM, Genton A, Boutin JA. Substrate phosphorylation capacities of the major tyrosine protein kinase from the human promyelocytic cell line, HL-60. International Journal of Peptide and Protein Research. 1994;**43**:496-504

[52] Wood S, Loudon A. Clocks for all seasons: Unwinding the roles and mechanisms of circadian and interval timers in the hypothalamus and pituitary. The Journal of Endocrinology. 2016;**228**:X1

[53] Wood S, Loudon A. The pars tuberalis: The site of the circannual clock in mammals? General and Comparative Endocrinology. 2017;**S0016-6480**(17):30350-30357

[54] Weaver DR, Liu C, Reppert SM. Nature's knockout: The Mel1b receptor is not necessary for reproductive and circadian responses to melatonin in Siberian hamsters. Molecular Endocrinology. 1996;**10**:1478-1487

[55] Coge F, Guenin SP, Fery I, Migaud M, Devavry S, Slugocki C, Legros C, Ouvry C, Cohen W, Renault N, Nosjean O, Malpaux B, Delagrange P, Boutin JA. The end of a myth: Cloning and characterization of the ovine melatonin MT(2) receptor. British Journal of Pharmacology. 2009;**158**:1248-1262

[56] Logez C, Berger S, Legros C, Baneres JL, Cohen W, Delagrange P, Nosjean O, Boutin JA, Ferry G, Simonin F, Wagner R. Recombinant human melatonin receptor MT1 isolated in

mixed detergents shows pharmacology similar to that in mammalian cell membranes. PLoS One. 2014;**9**:e100616

[57] Logez C, Damian M, Legros C, Dupre C, Guery M, Mary S, Wagner R, M'Kadmi C, Nosjean O, Fould B, Marie J, Fehrentz JA, Martinez J, Ferry G, Boutin JA, Baneres JL. Detergent-free isolation of functional G protein-coupled receptors into nanometric lipid particles. Biochemistry. 2016;**55**:38-48

[58] Alkhalfioui F, Magnin T, Wagner R. From purified GPCRs to drug discovery: The promise of protein-based methodologies. Current Opinion in Pharmacology. 2009;**9**:629-635

[59] Paul P, Lahaye C, Delagrange P, Nicolas JP, Canet E, Boutin JA. Characterization of 2-[125I]iodomelatonin binding sites in Syrian hamster peripheral organs. The Journal of Pharmacology and Experimental Therapeutics. 1999;**290**:334-340

[60] Duncan MJ, Takahashi JS, Dubocovich ML. 2-[125I]iodomelatonin binding sites in hamster brain membranes: Pharmacological characteristics and regional distribution. Endocrinology. 1988;**122**:1825-1833

[61] Vella F, Ferry G, Delagrange P, Boutin JA. NRH:quinone reductase 2: An enzyme of surprises and mysteries. Biochemical Pharmacology. 2005;**71**:1-12

[62] Boutin JA. Quinone reductase 2 as a promising target of melatonin therapeutic actions. Expert Opinion on Therapeutic Targets. 2016;**20**:303-317

[63] Rocheville M, Jerman JC. 7TM pharmacology measured by label-free: A holistic approach to cell signalling. Current Opinion in Pharmacology. 2009;**9**:643-649

[64] Leveridge M, Buxton R, Argyrou A, Francis P, Leavens B, West A, Rees M, Hardwicke P, Bridges A, Ratcliffe S, Chung CW. Demonstrating enhanced throughput of RapidFire mass spectrometry through multiplexing using the JmjD2d demethylase as a model system. Journal of Biomolecular Screening. 2014;**19**:278-286

[65] Boutin JA. Tyrosine protein kinase assays. Journal of Chromatography. B, Biomedical Applications. 1996;**684**:179-199

[66] Boutin JA, Ernould AP, Ferry G, Genton A, Alpert AJ. Use of hydrophilic interaction chromatography for the study of tyrosine protein kinase specificity. Journal of Chromatography. 1992;**583**:137-143

[67] Ferry G, Boutin JA. High-capacity screening of arylalkylamine N-acetyltransferase inhibitors using a high-performance liquid chromatography system. Journal of Biomolecular Screening. 2000;**5**:361-368

[68] Thomas M, Sabatini M, Bensaude F, Mignard B, Ortuno JC, Caron I, Boutin JA, Ferry G. A microplate assay for the screening of ADAMTS-4 inhibitors. Matrix Biology. 2006;**25**:261-267

[69] Letellier MA, Guillard J, Caignard DH, Ferry G, Boutin JA, Viaud-Massuard MC. Synthesis of potential rho-kinase inhibitors based on the chemistry of an original heterocycle:

4,4-dimethyl-3,4-dihydro-1H-quinolin-2-one. European Journal of Medicinal Chemistry. 2008;**43**:1730-1736

[70] Antoine M, Marcheteau E, Delagrange P, Ferry G, Boutin JA. Characterization of cofactors, substrates and inhibitor binding to flavoenzyme quinone reductase 2 by automated supramolecular nano-electrospray ionization mass spectrometry. International Journal of Mass Spectrometry. 2012;**312**:87-96

[71] Shapiro MJ, Wareing JR. NMR methods in combinatorial chemistry. Current Opinion in Chemical Biology. 1998;**2**:372-375

[72] Ferry G, Ubeaud C, Mozo J, Pean C, Hennig P, Rodriguez M, Scoul C, Bonnaud A, Nosjean O, Galizzi JP, Delagrange P, Renard P, Volland JP, Yous S, Lesieur D, Boutin JA. New substrate analogues of human serotonin N-acetyltransferase produce in situ specific and potent inhibitors. European Journal of Biochemistry. 2004;**271**:418-428

[73] Assemat O, Antoine M, Fourquez JM, Wierzbicki M, Charton Y, Hennig P, Perron-Sierra F, Ferry G, Boutin JA, Delsuc MA. (1)(9)F nuclear magnetic resonance screening of glucokinase activators. Analytical Biochemistry. 2015;**477**:62-68

[74] Boutin JA, Marcheteau E, Hennig P, Moulharat N, Berger S, Delagrange P, Bouchet JP, Ferry G. MT3/QR2 melatonin binding site does not use melatonin as a substrate or a cosubstrate. Journal of Pineal Research. 2008;**45**:524-531

[75] Luttrell LM, Maudsley S, Gesty-Palmer D. Translating in vitro ligand bias into in vivo efficacy. Cellular Signalling. 2017

[76] Luttrell LM, Maudsley S, Bohn LM. Fulfilling the promise of "biased" G protein-coupled receptor agonism. Molecular Pharmacology. 2015;**88**:579-588

[77] Maudsley S. G protein-coupled receptor biased agonism: Development towards future selective therapeutics. Mini Reviews in Medicinal Chemistry. 2012;**12**:803

[78] Audinot V, Fabry N, Nicolas JP, Beauverger P, Newman-Tancredi A, Millan MJ, Try A, Bornancin F, Canet E, Boutin JA. Ligand modulation of [35S]GTPgammaS binding at human alpha(2A), alpha(2B) and alpha(2C) adrenoceptors. Cellular Signalling. 2002;**14**:829-837

[79] Cecon E, Oishi A, Jockers R. Melatonin receptors: Molecular pharmacology and signalling in the context of system bias. British Journal of Pharmacology. 2017. DOI: 10.1111/bph.13950

[80] Boutin JA, Bonnaud A, Brasseur C, Bruno O, Lepretre N, Oosting P, Coumailleau S, Delagrange P, Nosjean O, Legros C. New MT(2) melatonin receptor-selective ligands: Agonists and partial agonists. International Journal of Molecular Sciences. 2017;**18**, pii: E1347

[81] Ferry G, Bruneau V, Beauverger P, Goussard M, Rodriguez M, Lamamy V, Dromaint S, Canet E, Galizzi JP, Boutin JA. Binding of prostaglandins to human PPARgamma: Tool assessment and new natural ligands. European Journal of Pharmacology. 2001;**417**:77-89

[82] Langer T, Hoffmann R, Bryant S, Lesur B. Hit finding: Towards 'smarter' approaches. Current Opinion in Pharmacology. 2009;**9**:589-593

[83] Macarron R, Banks MN, Bojanic D, Burns DJ, Cirovic DA, Garyantes T, Green DV, Hertzberg RP, Janzen WP, Paslay JW, Schopfer U, Sittampalam GS. Impact of high-throughput screening in biomedical research. Nature Reviews. Drug Discovery. 2011;**10**: 188-195

[84] Mayr LM, Bojanic D. Novel trends in high-throughput screening. Current Opinion in Pharmacology. 2009;**9**:580-588

[85] Jensen VK, Nosjean O, Dziegiel MH, Boutin JA, Sorensen MG, Karsdal MA, Henriksen K. A quantitative assay for lysosomal acidification rates in human osteoclasts. Assay and Drug Development Technologies. 2011;**9**:157-164

[86] Moffat JG, Vincent F, Lee JA, Eder J, Prunotto M. Opportunities and challenges in phenotypic drug discovery: An industry perspective. Nature Reviews. Drug Discovery. 2017;**16**:531-543

[87] Saeidnia S, Gohari AR, Manayi A. Reverse pharmacognosy and reverse pharmacology; two closely related approaches for drug discovery development. Current Pharmaceutical Biotechnology. 2016;**17**:1016-1022

[88] Gillet VJ. New directions in library design and analysis. Current Opinion in Chemical Biology. 2008;**12**:372-378

[89] Gillet VJ. Diversity selection algorithms. WIREs Computational Molecular Science. 2011;**1**:580-589

[90] Mount J, Ruppert J, Welch W, Jain AN. IcePick: A flexible surface-based system for molecular diversity. Journal of Medicinal Chemistry. 1999;**42**:60-66

[91] Makara GM. Measuring molecular similarity and diversity: Total pharmacophore diversity. Journal of Medicinal Chemistry. 2001;**44**:3563-3571

[92] Maldonado AG, Doucet JP, Petitjean M, Fan BT. Molecular similarity and diversity in chemoinformatics: From theory to applications. Molecular Diversity. 2006;**10**:39-79

[93] Lepp Z, Huang C, Okada T. Finding key members in compound libraries by analyzing networks of molecules assembled by structural similarity. Journal of Chemical Information and Modeling. 2009;**49**:2429-2443

[94] Jenkins JL, Glick M, Davies JW. A 3D similarity method for scaffold hopping from known drugs or natural ligands to new chemotypes. Journal of Medicinal Chemistry. 2004;**47**:6144-6159

[95] Heinrich M, Lardos A, Leonti M, Weckerle C, Willcox M. Best practice in research: Consensus statement on ethnopharmacological field studies—ConSEFS. Journal of Ethnopharmacology. 2018;**211**:329-339

[96] Bousserouel H, Litaudon M, Morleo B, Martin MT, Thoison O, Nosjean O, Boutin JA, Renard P, Sevenet T. New biologically active linear triterpenes from the bark of three new-caledonian Cupaniopsis species. Tetrahedron. 2005;**61**:845-851

[97] Litaudon M, Jolly C, Le CC, Cuong DD, Retailleau P, Nosjean O, Nguyen VH, Pfeiffer B, Boutin JA, Gueritte F. Cytotoxic pentacyclic triterpenoids from *Combretum sundaicum* and *Lantana camara* as inhibitors of Bcl-xL/BakBH3 domain peptide interaction. Journal of Natural Products. 2009;**72**:1314-1320

[98] Litaudon M, Bousserouel H, Awang K, Nosjean O, Martin MT, Dau ME, Hadi HA, Boutin JA, Sevenet T, Gueritte F. A dimeric sesquiterpenoid from a Malaysian Meiogyne as a new inhibitor of Bcl-xL/BakBH3 domain peptide interaction. Journal of Natural Products. 2009;**72**:480-483

[99] Boutin JA, Chatelain-Egger F, Vella F, Delagrange P, Ferry G. Quinone reductase 2 substrate specificity and inhibition pharmacology. Chemico-Biological Interactions. 2005;**151**:213-228

[100] Boutin JA, Fauchere AL. Combinatorial peptide synthesis: Statistical evaluation of peptide distribution. Trends in Pharmacological Sciences. 1996;**17**:8-12

[101] Boutin JA, Hennig P, Lambert PH, Bertin S, Petit L, Mahieu JP, Serkiz B, Volland JP, Fauchere JL. Combinatorial peptide libraries: Robotic synthesis and analysis by nuclear magnetic resonance, mass spectrometry, tandem mass spectrometry, and high-performance capillary electrophoresis techniques. Analytical Biochemistry. 1996;**234**:126-141

[102] Ferry G, Boutin JA, Atassi G, Fauchere JL, Tucker GC. Selection of a histidine-containing inhibitor of gelatinases through deconvolution of combinatorial tetrapeptide libraries. Molecular Diversity. 1997;**2**:135-146

[103] Boutin JA, Marande W, Petit L, Loynel A, Desmet C, Canet E, Fauchere JL. Investigation of S-farnesyl transferase substrate specificity with combinatorial tetrapeptide libraries. Cellular Signalling. 1999;**11**:59-69

[104] Houghten RA, Pinilla C, Blondelle SE, Appel JR, Dooley CT, Cuervo JH. Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. Nature. 1991;**354**:84-86

[105] Dahlin JL, Walters MA. The essential roles of chemistry in high-throughput screening triage. Future Medicinal Chemistry. 2014;**6**:1265-1290

[106] Tordjman S, Chokron S, Delorme R, Charrier A, Bellissant E, Jaafari N, Fougerou C. Melatonin: Pharmacology, functions and therapeutic benefits. Current Neuropharmacology. 2017;**15**:434-443

[107] Shehzad A, Qureshi M, Anwar MN, Lee YS. Multifunctional curcumin mediate multitherapeutic effects. Journal of Food Science. 2017;**82**:2006-2015

[108] Tsai HY, Ho CT, Chen YK. Biological actions and molecular effects of resveratrol, pterostilbene, and 3′-hydroxypterostilbene. Journal of Food and Drug Analysis. 2017;**25**:134-147

[109] Xu J, Hagler A. Chemoinformatics and drug discovery. Molecules. 2002;**7**:566-600

[110] Humbeck L, Koch O. What can we learn from bioactivity data? Chemoinformatics tools and applications in chemical biology research. ACS Chemical Biology. 2017;**12**:23-35

[111] Genick CC, Wright SK. Biophysics: For HTS hit validation, chemical lead optimization, and beyond. Expert Opinion on Drug Discovery. 2017;**12**:897-907

[112] Radziejewski C, Miller WT, Mobashery S, Goldberg AR, Kaiser ET. Purification of recombinant pp60v-src protein tyrosine kinase and phosphorylation of peptides with different secondary structure preference. Biochemistry. 1989;**28**:9047-9052

[113] Barret JM, Ernould AP, Ferry G, Genton A, Boutin JA. Integrated system for the screening of the specificity of protein kinase inhibitors. Biochemical Pharmacology. 1993;**46**:439-448

[114] Cooper JA, Esch FS, Taylor SS, Hunter T. Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases in vivo and in vitro. The Journal of Biological Chemistry. 1984;**259**:7835-7841

[115] Bacchi M, Fould B, Jullian M, Kreiter A, Maurras A, Nosjean O, Coursindel T, Puget K, Ferry G, Boutin JA. Screening ubiquitin specific protease activities using chemically synthesized ubiquitin and ubiquitinated peptides. Analytical Biochemistry. 2017;**519**:57-70

[116] Legros C, Matthey U, Grelak T, Pedragona-Moreau S, Hassler W, Yous S, Thomas E, Suzenet F, Folleas B, Lefoulon F, Berthelot P, Caignard DH, Guillaumet G, Delagrange P, Brayer JL, Nosjean O, Boutin JA. New radioligands for describing the molecular pharmacology of MT1 and MT2 melatonin receptors. International Journal of Molecular Sciences. 2013;**14**:8948-8962

[117] Legros C, Brasseur C, Delagrange P, Ducrot P, Nosjean O, Boutin JA. Alternative radioligands for investigating the molecular pharmacology of melatonin receptors. The Journal of Pharmacology and Experimental Therapeutics. 2016;**356**:681-692

[118] Audinot V, Lahaye C, Suply T, Beauverger P, Rodriguez M, Galizzi JP, Fauchere JL, Boutin JA. [125I]-S36057: A new and highly potent radioligand for the melanin-concentrating hormone receptor. British Journal of Pharmacology. 2001;**133**:371-378

[119] Rodriguez M, Beauverger P, Naime I, Rique H, Ouvry C, Souchaud S, Dromaint S, Nagel N, Suply T, Audinot V, Boutin JA, Galizzi JP. Cloning and molecular characterization of the novel human melanin-concentrating hormone receptor MCH2. Molecular Pharmacology. 2001;**60**:632-639

[120] Chartrel N, Alvear-Perez R, Leprince J, Iturrioz X, Reaux-Le GA, Audinot V, Chomarat P, Coge F, Nosjean O, Rodriguez M, Galizzi JP, Boutin JA, Vaudry H, Llorens-Cortes C. Comment on "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". Science. 2007;**315**:766

[121] Le MO, Neveu C, Lefranc B, Dubessy C, Boutin JA, Do-Rego JC, Costentin J, Tonon MC, Tena-Sempere M, Vaudry H, Leprince J. Structure-activity relationships of a series of analogues of the RFamide-related peptide 26RFa. Journal of Medicinal Chemistry. 2011;**54**:4806-4814

[122] Chartrel N, Alonzeau J, Alexandre D, Jeandel L, Alvear-Perez R, Leprince J, Boutin J, Vaudry H, Anouar Y, Llorens-Cortes C. The RFamide neuropeptide 26RFa and its role in the control of neuroendocrine functions. Frontiers in Neuroendocrinology. 2011;**32**:387-397

[123] Neveu C, Lefranc B, Tasseau O, Do-Rego JC, Bourmaud A, Chan P, Bauchat P, Le MO, Chuquet J, Guilhaudis L, Boutin JA, Segalas-Milazzo I, Costentin J, Vaudry H, Baudy-Floc'h M, Vaudry D, Leprince J. Rational design of a low molecular weight, stable, potent, and long-lasting GPR103 aza-beta3-pseudopeptide agonist. Journal of Medicinal Chemistry. 2012;**55**:7516-7524

[124] Neveu C, Dulin F, Lefranc B, Galas L, Calbrix C, Bureau R, Rault S, Chuquet J, Boutin JA, Guilhaudis L, Segalas-Milazzo I, Vaudry D, Vaudry H, Santos JS, Leprince J. Molecular basis of agonist docking in a human GPR103 homology model by site-directed mutagenesis and structure-activity relationship studies. British Journal of Pharmacology. 2014;**171**:4425-4439

[125] Ould-Hamouda H, Chen P, Levoye A, Sozer-Topcular N, Daulat AM, Guillaume JL, Ravid R, Savaskan E, Ferry G, Boutin JA, Delagrange P, Jockers R, Maurice P. Detection of the human GPR50 orphan seven transmembrane protein by polyclonal antibodies mapping different epitopes. Journal of Pineal Research. 2007;**43**:10-15

[126] Sidibe A, Mullier A, Chen P, Baroncini M, Boutin JA, Delagrange P, Prevot V, Jockers R. Expression of the orphan GPR50 protein in rodent and human dorsomedial hypothalamus, tanycytes and median eminence. Journal of Pineal Research. 2010;**48**:263-269

[127] Audinot V, Beauverger P, Lahaye C, Suply T, Rodriguez M, Ouvry C, Lamamy V, Imbert J, Rique H, Nahon JL, Galizzi JP, Canet E, Levens N, Fauchere JL, Boutin JA. Structure-activity relationship studies of melanin-concentrating hormone (MCH)-related peptide ligands at SLC-1, the human MCH receptor. The Journal of Biological Chemistry. 2001;**276**:13554-13562

[128] Audinot V, Zuana OD, Fabry N, Ouvry C, Nosjean O, Henlin JM, Fauchere JL, Boutin JA. S38151 [p-guanidinobenzoyl-[des-Gly(10)]-MCH(7-17)] is a potent and selective antagonist at the MCH(1) receptor and has anti-feeding properties in vivo. Peptides. 2009;**30**:1997-2007

[129] Scapin G. Structural biology and drug discovery. Current Pharmaceutical Design. 2006;**12**:2087-2097

[130] Hu T, Sprague ER, Fodor M, Stams T, Clark KL, Cowan-Jacob SW. The impact of structural biology in medicine illustrated with four case studies. Journal of Molecular Medicine. 2018;**96**:9-19

[131] Zheng H, Handing KB, Zimmerman MD, Shabalin IG, Almo SC, Minor W. X-ray crystallography over the past decade for novel drug discovery—Where are we heading next? Expert Opinion on Drug Discovery. 2015;**10**:975-989

[132] Zheng H, Hou J, Zimmerman MD, Wlodawer A, Minor W. The future of crystallography in drug discovery. Expert Opinion on Drug Discovery. 2014;**9**:125-137

[133] Venien-Bryan C, Li Z, Vuillard L, Boutin JA. Cryo-electron microscopy and X-ray crystallography: Complementary approaches to structural biology and drug discovery. Acta Crystallographica Section F. 2017;**73**:174-183

[134] Calamini B, Santarsiero BD, Boutin JA, Mesecar AD. Kinetic, thermodynamic and X-ray structural insights into the interaction of melatonin and analogues with quinone reductase 2. The Biochemical Journal. 2008;**413**:81-91

[135] Pegan SD, Sturdy M, Ferry G, Delagrange P, Boutin JA, Mesecar AD. X-ray structural studies of quinone reductase 2 nanomolar range inhibitors. Protein Science. 2011;**20**:1182-1195

[136] Cassagnes LE, Rakotoarivelo N, Sirigu S, Perio P, Najahi E, Chavas LM, Thompson A, Gayon R, Ferry G, Boutin JA, Valentin A, Reybier K, Nepveu F. Role of quinone reductase 2 in the antimalarial properties of indolone-type derivatives. Molecules. 2017;**22**, pii: E210

[137] Sancier F, Dumont A, Sirvent A, Paquay de PL, Edmonds T, David G, Jan M, De MC, Coge F, Leonce S, Burbridge M, Bruno A, Boutin JA, Lockhart B, Roche S, Cruzalegui F. Specific oncogenic activity of the Src-family tyrosine kinase c-Yes in colon carcinoma cells. PLoS One. 2011;**6**:e17237

[138] Burbridge MF, Bossard CJ, Saunier C, Fejes I, Bruno A, Leonce S, Ferry G, Da VG, Bouzom F, Cattan V, Jacquet-Bescond A, Comoglio PM, Lockhart BP, Boutin JA, Cordi A, Ortuno JC, Pierre A, Hickman JA, Cruzalegui FH, Depil S. S49076 is a novel kinase inhibitor of MET, AXL, and FGFR with strong preclinical activity alone and in association with bevacizumab. Molecular Cancer Therapeutics. 2013;**12**:1749-1762

[139] Ferry G, Studeny A, Bossard C, Kubara PM, Zeyer D, Renaud JP, Casara P, de NG, Wierzbicki M, Pfeiffer B, Prudhomme M, Leonce S, Pierre A, Boutin JA, Golsteyn RM. Characterization of novel checkpoint kinase 1 inhibitors by in vitro assays and in human cancer cells treated with topoisomerase inhibitors. Life Sciences. 2011;**89**:259-268

[140] Petit P, Antoine M, Ferry G, Boutin JA, Lagarde A, Gluais L, Vincentelli R, Vuillard L. The active conformation of human glucokinase is not altered by allosteric activators. Acta Crystallographica. Section D, Biological Crystallography. 2011;**67**:929-935

[141] Bacchi M, Jullian M, Sirigu S, Fould B, Huet T, Bruyand L, Antoine M, Vuillard L, Ronga L, Chavas LM, Nosjean O, Ferry G, Puget K, Boutin JA. Total chemical synthesis, refolding, and crystallographic structure of fully active immunophilin calstabin 2 (FKBP12.6). Protein Science. 2016;**25**:2225-2242

[142] Gerard CJJ, Ferry G, Vuillard L, Boutin JA, Chavas LM, Huet T, Ferte N, Grossier R, Candoni N, Veesler S. Crystallisation using tubing microfluidics permits both in situ and ex situ X-ray diffraction. Acta Crystallographica. Section F, Structural Biology and Crystallization Communications. 2017;**73**:574-578

[143] Zhang S, Gerard CJJ, Ikni A, Ferry G, Vuillard L, Boutin JA, Ferte N, Grossier R, Candoni N, Veesler S. Microfluidic platform for optimization of crystallization conditions. Journal of Crystal Growth. 2017;**472**:18-28

[144] Mollica L, Theret I, Antoine M, Perron-Sierra F, Charton Y, Fourquez JM, Wierzbicki M, Boutin JA, Ferry G, Decherchi S, Bottegoni G, Ducrot P, Cavalli A. Molecular dynamics simulations and kinetic measurements to estimate and predict protein-ligand residence times. Journal of Medicinal Chemistry. 2016;**59**:7167-7176

[145] Westermaier Y, Ruiz-Carmona S, Theret I, Perron-Sierra F, Poissonnet G, Dacquet C, Boutin JA, Ducrot P, Barril X. Binding mode prediction and MD/MMPBSA-based free energy ranking for agonists of REV-ERBalpha/NCoR. Journal of Computer-Aided Molecular Design. 2017;**31**:755-775

[146] Folmer RH. Integrating biophysics with HTS-driven drug discovery projects. Drug Discovery Today. 2016;**21**:491-498

[147] Renaud JP, Chung CW, Danielson UH, Egner U, Hennig M, Hubbard RE, Nar H. Biophysics in drug discovery: Impact, challenges and opportunities. Nature Reviews. Drug Discovery. 2016;**15**:679-698

[148] Renaud JP, Delsuc MA. Biophysical techniques for ligand screening and drug design. Current Opinion in Pharmacology. 2009;**9**:622-628

[149] Shuman CF, Hamalainen MD, Danielson UH. Kinetic and thermodynamic characterization of HIV-1 protease inhibitors. Journal of Molecular Recognition. 2004;**17**:106-119

[150] Vaissiere A, Berger S, Harrus D, Dacquet C, Le MA, Boutin JA, Ferry G, Royer CA. Molecular mechanisms of transcriptional control by Rev-erbalpha: An energetic foundation for reconciling structure and binding with biological function. Protein Science. 2015;**24**:1129-1146

[151] Antoine M, Boutin JA, Ferry G. Binding kinetics of glucose and allosteric activators to human glucokinase reveal multiple conformational states. Biochemistry. 2009;**48**:5466-5482

[152] Stojko J, Fieulaine S, Petiot-Becard S, Van DA, Meinnel T, Giglione C, Cianferani S. Ion mobility coupled to native mass spectrometry as a relevant tool to investigate extremely small ligand-induced conformational changes. The Analyst. 2015;**140**:7234-7245

[153] Mailliet F, Ferry G, Vella F, Berger S, Coge F, Chomarat P, Mallet C, Guenin SP, Guillaumet G, Viaud-Massuard MC, Yous S, Delagrange P, Boutin JA. Characterization of the melatoninergic MT3 binding site on the NRH:quinone oxidoreductase 2 enzyme. Biochemical Pharmacology. 2005;**71**:74-88

[154] Liu X, Ahn S, Kahsai AW, Meng KC, Latorraca NR, Pani B, Venkatakrishnan AJ, Masoudi A, Weis WI, Dror RO, Chen X, Lefkowitz RJ, Kobilka BK. Mechanism of intracellular allosteric beta2AR antagonist revealed by X-ray crystal structure. Nature. 2017;**548**:480-484

[155] Zhang H, Qiao A, Yang D, Yang L, Dai A, de GC, Reedtz-Runge S, Dharmarajan V, Zhang H, Han GW, Grant TD, Sierra RG, Weierstall U, Nelson G, Liu W, Wu Y, Ma L, Cai X, Lin G, Wu X, Geng Z, Dong Y, Song G, Griffin PR, Lau J, Cherezov V, Yang H,

Hanson MA, Stevens RC, Zhao Q, Jiang H, Wang MW, Wu B. Structure of the full-length glucagon class B G-protein-coupled receptor. Nature. 2017;**546**:259-264

[156] Bedut S, Seminatore-Nole C, Lamamy V, Caignard S, Boutin JA, Nosjean O, Stephan JP, Coge F. High-throughput drug profiling with voltage- and calcium-sensitive fluorescent probes in human iPSC-derived cardiomyocytes. American Journal of Physiology. Heart and Circulatory Physiology. 2016;**311**:H44-H53

[157] Kujala K, Paavola J, Lahti A, Larsson K, Pekkanen-Mattila M, Viitasalo M, Lahtinen AM, Toivonen L, Kontula K, Swan H, Laine M, Silvennoinen O, Aalto-Setala K. Cell model of catecholaminergic polymorphic ventricular tachycardia reveals early and delayed afterdepolarizations. PLoS One. 2012;**7**:e44660

[158] Novak A, Barad L, Lorber A, Gherghiceanu M, Reiter I, Eisen B, Eldor L, Itskovitz-Eldor J, Eldar M, Arad M, Binah O. Functional abnormalities in iPSC-derived cardiomyocytes generated from CPVT1 and CPVT2 patients carrying ryanodine or calsequestrin mutations. Journal of Cellular and Molecular Medicine. 2015;**19**:2006-2018

[159] Cohen J. Surviving the cure. Science. 2017;**357**:122-125

[160] Gornalusse GG, Hirata RK, Funk SE, Riolobos L, Lopes VS, Manske G, Prunkard D, Colunga AG, Hanafi LA, Clegg DO, Turtle C, Russell DW. HLA-E-expressing pluripotent stem cells escape allogeneic responses and lysis by NK cells. Nature Biotechnology. 2017;**35**:765-772

[161] Kent S. Chemical protein synthesis: Inventing synthetic methods to decipher how proteins work. Bioorganic & Medicinal Chemistry. 2017;**25**:4926-4937

[162] Fernandez-Suarez M, Ting AY. Fluorescent probes for super-resolution imaging in living cells. Nature Reviews. Molecular Cell Biology. 2008;**9**:929-943

[163] Rouch A, Fould B, Jeantet E, Marcheteau E, Antoine M, Nosjean O, Ferry G, Boutin JA. On the acellular use of lipoic acid ligase for labeling proteins. Advances in Biochemistry and Biotechnology. 2017;**110**

[164] Schukur L, Geering B, Charpin-El HG, Fussenegger M. Implantable synthetic cytokine converter cells with AND-gate logic treat experimental psoriasis. Science Translational Medicine. 2015;**7**:318ra201

[165] Kotschy A, Szlavik Z, Murray J, Davidson J, Maragno AL, Le Toumelin-Braizat G, Chanrion M, Kelly GL, Gong JN, Moujalled DM, Bruno A, Csekei M, Paczal A, Szabo ZB, Sipos S, Radics G, Proszenyak A, Balint B, Ondi L, Blasko G, Robertson A, Surgenor A, Dokurno P, Chen I, Matassova N, Smith J, Pedder C, Graham C, Studeny A, Lysiak-Auvity G, Girard AM, Grave F, Segal D, Riffkin CD, Pomilio G, Galbraith LC, Aubrey BJ, Brennan MS, Herold MJ, Chang C, Guasconi G, Cauquil N, Melchiore F, Guigal-Stephan N, Lockhart B, Colland F, Hickman JA, Roberts AW, Huang DC, Wei AH, Strasser A, Lessene G, Geneste O. The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. Nature. 2016;**538**:477-482

[166] Rosenblatt M. An incentive-based approach for improving data reproducibility. Science Translational Medicine. 2016;**8**:336ed5

[167] Jasny BR, Wigginton N, McNutt M, Bubela T, Buck S, Cook-Deegan R, Gardner T, Hanson B, Hustad C, Kiermer V, Lazer D, Lupia A, Manrai A, McConnell L, Noonan K, Phimister E, Simon B, Strandburg K, Summers Z, Watts D. Fostering reproducibility in industry-academia research. Science. 2017;**357**:759-761

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