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30 Years of Kinase Inhibitors: A Historical Overview and some Brazilian Contributions

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The past 30 years have been transformative in the landscape of drug discovery, and certainly one of the most ground-breaking areas was kinase research. Since its start in the mid-1990s, kinase studies have reached notable hallmarks, ranging from the development of the first inhibitor, to innovative treatment options for a range of diseases. Today, kinases remain a pivotal protein class in drug discovery. Contemporarily, the Brazilian medicinal chemistry scene has also evolved, and followed up to such trends in the global landscape of research. Notably, the creation of the Summer School of Pharmaceutical and Medicinal Chemistry (EVQFM, in portuguese) in 1995, and its subsequent 30 consecutive editions, has raised the interest and knowledge of multiple generations in Brazil. This review aims to briefly outline the history of kinase inhibitor research, some of its landmarks, and highlight some of the contributions of Brazilian researchers to this important field of medicinal chemistry.

Keywords: protein kinase inhibitors, drug discovery

1. Introduction

Protein kinase inhibitors (PKIs) have made a profound impact on the prospect of treatment for various diseases, and remains a dynamically evolving area of research. The significance of protein kinases (PKs) as viable drug targets was first recognized in the early 1980s with the identification of kinase activity in the Src oncogene.[1] This pivotal discovery prompted extensive investigations into the potential involvement of these proteins in pathological developments and strategies for therapeutically modulating their activity.

Approximately 30 years ago, the field witnessed its initial breakthrough when the Rho-associated coiled-coil-containing kinase (ROCK1 and 2) inhibitor, fasudil, gained approval in Japan for treating cerebral vasospasm.[2] This milestone was succeeded by the approval of the mTOR inhibitor sirolimus (natural product rapamycin) by the Food and Drug Administration (FDA),[3] for organ rejection treatment.

The subsequent advancements in kinase inhibitor research have had many highlights, paralleling with progress in genomics, immunology, computational biology, crystallography, and synthetic chemistry. Currently, kinase inhibitors are one of the most relevant therapeutic classes in medicinal chemistry, and the potential for further exploration remains substantial.[4,5] In this review, we briefly trace the historical trajectory of PKIs, provide insights into the current landscape, and outline potential future directions. Additionally, we highlight some of the noteworthy contributions of Brazilian researchers, particularly those initiated during the 30-year journey of the Summer School of Pharmaceutical and Medicinal Chemistry (EVQFM in portuguese).

1.1. Functionality and structure

The superfamily of protein kinases is constituted by more than 500 members. They are responsible for catalyzing the transfer of the γ-phosphoryl group from adenosine triphosphate (ATP) to a free hydroxyl group of an amino acid side chain. They can be classified by their catalytic

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activity into serine/threonine kinases, tyrosine kinases, and dual-specificity Ser/Thr/Tyr kinases. Based on their primary sequence and structural features, PK are divided into eight groups: AGC (protein kinase A, G and C), CaMK (calcium/calmodium-dependent kinases), CMGC (cyclic-dependet kinases), MAP kinases, glycogen synthase kinases and casein kinases, TK (tyrosine kinases), STE (homologues of yeast sterile 7), CK1 (casein kinase), TKL (tyrosine kinase-like) and RCG (receptor guanylate cyclases). Additionally, there are "atypical kinases", which is the group that does not share relevant sequence homology with the others and lack even the canonical sequence motifs of the kinase catalytic binding site. And finally, the "pseudokinases", which have a typical kinase domain, but lack at least one conserved structural motif important for catalytic activity.[6]

The phosphoryl transfer is a signaling mechanism that regulates all essential physiological processes, such as cell growth, differentiation, and cell death. Therefore, irregularities in these signaling mechanisms are linked to various diseases. Hence, modulation of PK's function by inhibition is a significant therapeutic strategy, but relatively challenging. The catalytic site, occupied by ATP, is very conserved within the kinome and so are many of the binding features required for interaction. This also means that selectivity is very challenging when developing PKI, and much of ligand design involves exploring features that can differentiate between family members.[7-9]

PK are constituted by two lobes (C-terminal and N-terminal) which are connected by a flexible loop (hinge, Figure 1). Between these two lobes is the catalytic site, where ATP binds. The N-terminal lobe is constituted by five β-sheets (β1-β5) and one α-helix, known as the C-helix, or αC. Between β1 and β2 is a glycine rich flexible loop, known as P-loop, which participates in the coordination of ATP. The C-lobe has mostly α-helixes, but β6 and β7 of this lobe form the catalytic loop, that contains an Asp residue required for phosphate transfer. On the opposite of the hinge region is the activation-loop, or A-loop. In the A-loop, which is especially flexible, is the DFG (Asp-Phe-Gly), an important triad in the activation/disactivation of the protein.[4,10]

Upon activation, the conserved Asp residue of the DFG triad moves towards the Mg^{2+} ion, into the pocket, coordinating to the ATP-phosphates.[6] This orientation is named DFG-in and is often used to identify the protein in its active state. Conversely, the DFG-out relates to the inactive state, when the Asp residue has not been oriented for interaction. An additional describer of a kinase's active state is the mechanism between the regulatory spine (R) and the catalytic spine (C). When in its active state, the C-spine is formed by amino acids of the protein and the adenine ring of the ATP, and the R-spine is formed by four amino acids, including the Phe residue of the DFG. These two spines are connected by one amino acid of the N-lobe. Such structural features can only be achieved with ATP and the correct orientation of the Phe, and therefore are also characteristics of the protein's activation.[10]

Lastly, the catalytic site is separated into different regions: ATP-occupied regions, i.e., adenine binding region, sugar pocket and phosphate binding region; and non-ATP occupied regions, namely hydrophobic regions I and II, and deep pockets. These non-ATP occupied pockets offer more diverse interactions for ligands, and are often used in PKI to improve selectivity.[11]

There are generally three types of inhibitors.[10,12,13] The so called type-I inhibitors target the activated protein at the DFG-in conformation and are direct competitors of binding with ATP. The type-I inhibitors tend to be smaller ATP-mimetic ligands, and less selective. However, many type-I inhibitors explore the pocket that is located behind the so-called "gatekeeper", an amino acid close to the hinge that is flexible enough to open a space unexplored by ATP.

In the type-II inhibition, DFG is in the "out" conformation, and this opens a new cleft in the binding pocket. This deep-pocket is explored by type-II inhibitors

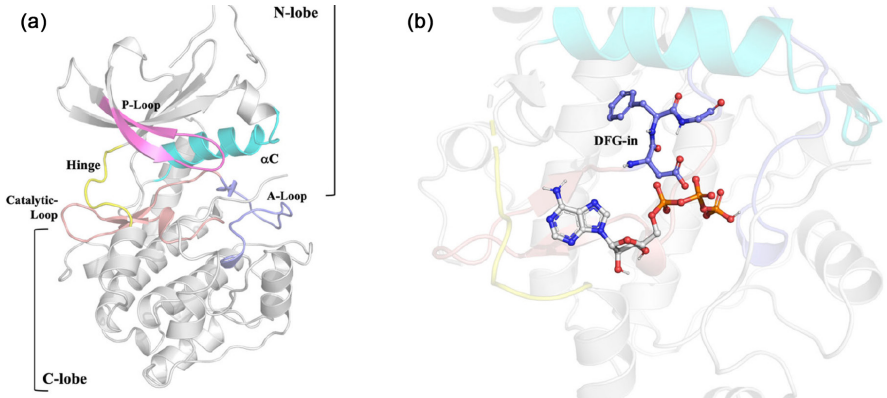

Figure 1. (a) Structure of the c-AMP-dependent protein kinase (PDB: 1ATP). Hinge is represented in yellow, P-loop in pink, αC in blue, A-loop in purple and catalytic loop in light pink. (b) In the cleft between lobes (same protein structure), is ATP and the DFG triad with the activated orientation.

to obtain better selectivity. This means that these inhibitors are usually longer molecules, with hydrophilic linkers to reach into the pocket.[13]

Lastly, the type-III inhibitors (or allosteric) comprehend a vast and diverse group of molecules that bind to allosteric sites of adjacent and non-adjacent pockets of the binding site. Type-III are usually non-ATP competitive inhibitors, and do not bind to the hinge. The identification of such pockets is very challenging, therefore, rational design of type-III inhibitors is often difficult.[12,13]

Other categorizations are possible, depending on different binding features. These include, for example, the intermediate type-I½ inhibitors,[12] that bind to the overall inactive state of the protein, but are able to interact with the Phe residue of the DFG, inducing a DFG-in conformation (often attribute of the active state). However, most of the inhibitors are generally divided into such three categories.

2. Historical Overview of Protein Kinase Inhibitors

2.1. Development of imatinib

Although the first officially approved protein kinase inhibitors were fasudil and sirolimus, a true breakthrough was made in 2001, when imatinib (**5**, Figure 2) was approved for the treatment of chronic myelogenous leukaemia (CML) a rare disease. Imatinib was the first rationally designed small molecule to target a protein kinase, namely Bcr-Abl.

The development of imatinib started with High-Throughput Screening (HTS) of a series of inhibitors of protein kinase C (PKC). The 2-phenylaminopyrimidine (**1**) scaffold was identified with good "lead-like" properties. Initial modifications were made by adding the 3'-pyridyl group at the 3-position of the pyrimidine ring that increased the PKC activity. Further structural modifications to an amide attached to the aniline ring, identified the BCR-ABL kinase as a second target.[14] The relevance of this kinase was evident. CML occurs as consequence of a shortened version of chromosome 22, known as the Philadelphia chromosome, that creates the BCR-ABL gene, which encodes a protein with elevated kinase activity. This was a drug target identified with a clearly differentiated activity between normal cells.

Analysis of structure-activity relationship (SAR) showed that substitution at position 6, the "flag-methyl" of the aniline ring completely abolished PKC activity. Finally, attachment of the *N*-methylpiperazine side chain provided improved pharmacokinetics profile, namely solubility and bioavailability. Compound STI571, or Gleevec and imatinib (**5**), was shown to bind to the Bcr-Abl kinase in its inactive form (type II), promoting antiproliferative activity in leukaemia cells from patients with Philadelphia-chromosome positive CML.[15]

Imatinib then entered clinical trials for CML and provided haematological and cytogenetic response that improved survival and progression-free survival of patients. Its record-breaking fast approval in 2001 was an important breakthrough for many reasons, including the fact that it changed CML from a rapidly fatal disease to a manageable condition. Also, imatinib was one of the world's most commercially successful drugs, reaching a peak of over US$ 4 billion in sales in 2012.[16] This attracted great focus on the field of kinase inhibitors and precision medicine, that has ultimately changed the prognosis of many different cancers and other diseases.

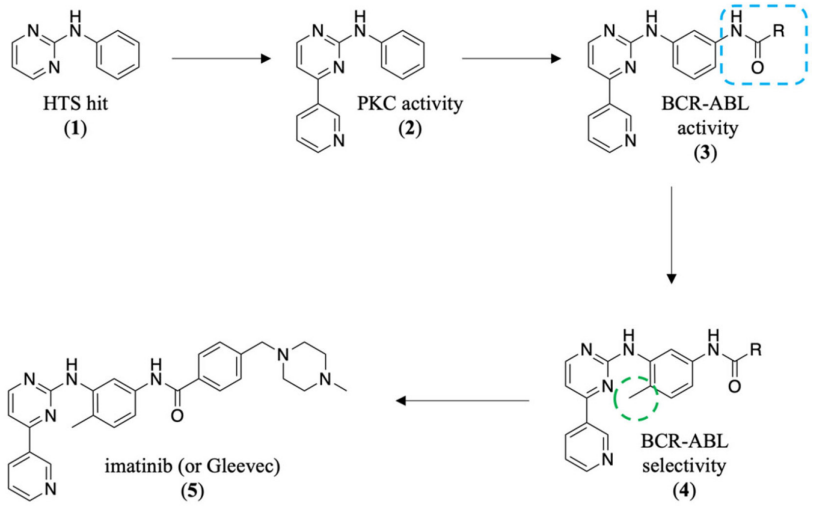

Figure 2. Schematic representation of the development of imatinib (**5**), from the identification of the aminopyrimidine core scaffold (**1**), attachment of the pyridine ring in (**2**), the amide group highlighted in **3**, which increased BCR-ABL activity; the flag methyl in **4**, highlighted in green, that abolished PKC activity, and finally, identification of the optimized structure of imatinib (**5**).

2.2. Epidermal growth factor receptor (EGFR) inhibitors, mutations and resistance

Directly after the approval of imatinib (**5**), two new inhibitors went into the market: gefitinib (**6**) and erlotinib (**7**), for the treatment of non-small cell lung cancer (NSCLC). Solid tumors were usually treated with cytotoxic therapy. Yet, this has limited efficacy, due to lack of specificity and considerable toxicity. The transmembrane PK epidermal growth factor receptor (EGFR) had been identified as an important partaker in the growth and progression of tumors, being expressed in almost 80% of NSCLCs.[16,17]

Both gefitinib (**6**) and erlotinib (**7**) are 4-aminoquinazoline derivatives and were determined and inhibit EGFR as type I. They entered trials and were approved in 2002, performing very well in the clinic, by significantly prolongating life expectancy for terminal patients.[18,19] Not long after their approval, acquired mutations began to be identified and response to treatment varied between groups of patients. Genetic sequencing identified a predominant early single point mutation, which substitutes arginine for a leucine at codon 858. The L858R mutation was classified as an activating mutation, as it led to a ligand-independent activation. This results in an increased net activity of EGFR, reduced affinity to ATP and therefore increased sensitivity to gefitinib and erlotinib treatment.[20] However, eventually response to treatment would decrease, which was linked to a number of compensatory events leading to resistance.[21]

The principal second point mutation correlated to acquired resistance was T790M (threonine to methionine), a gatekeeper amino acid. This was thought to be a selective pressure mutation, as it was not identified in patients that had not received treatment with EGFR inhibitors. At that point, these mutations decreased significantly the affinity not only to gefitinib (**6**) and erlotinib (**7**), which are first generation PKI, but also to second generation EGFR inhibitors (Figure 3), like afatinib (**8**),[21,22] which have been developed as covalent inhibitors to overcome resistance in first generation EGFR treatment[23] (more on covalent inhibitors in section 2.3).

A third generation was eventually developed to specifically target the gatekeeper mutant T790M without binding on wild-type EGFR. Osimertinib (**10**) was approved in 2015 and was used as alternative treatment for patients that had already stopped responding to PKI initial treatment. Still, through a series of both EGFR-dependent and EGFR-independent mechanisms, combined with the observed C578S point mutation of the key cysteine amino-acid to a serine, the third generation was also eventually bypassed.

Current strategies to circumvent resistance problems with EGFR include a fourth generation of inhibitors and

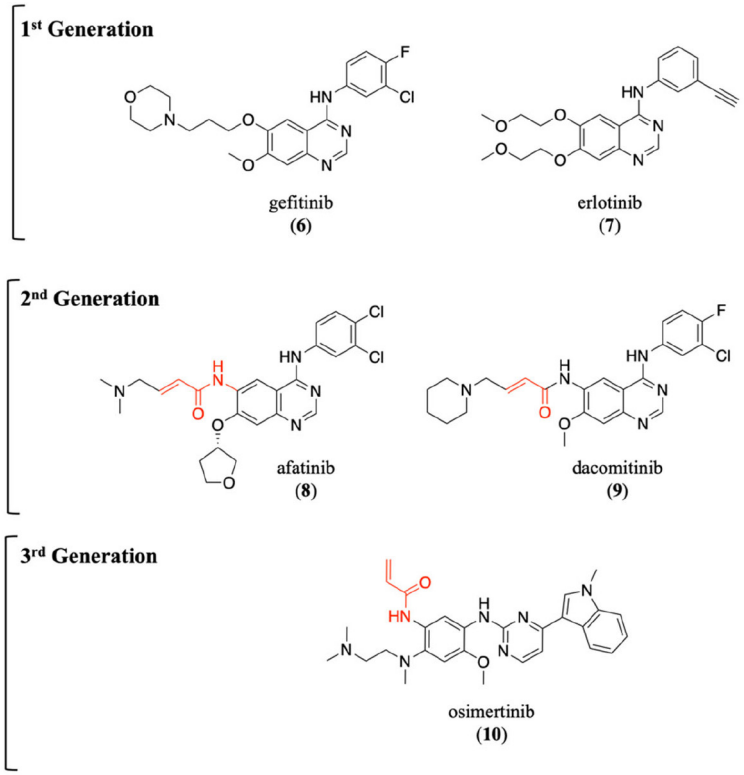

Figure 3. Chemical structures of first, second and third generation EGFR inhibitors. Second and third generation inhibitors have the electrophilic moiety for covalent binding highlighted in red.

combinatory treatment.[23,24] However, the high genomic instability of EGFR was an important lesson learned for PKI, that tumors are inherently adaptable and develop several mechanisms to evade therapeutic pressure. Currently, tumor treatment is understanded to require monitoring of oncogene addiction for a successful outcome.

2.3. Covalent inhibitors

Throughout the history of medicinal chemistry, covalent inhibitors were characterized as such after clinical use. For a long time, covalent drugs were thought to be toxic and/or cause problematic side effects. However, in the past few decades, the potential for this class of drugs has been recognized, and the rational design of such small-molecules gained attention. In fact, the use of covalent inhibitors is a great strategy to improve features like selectivity and duration of action.[25]

In covalent inhibitor design, ligands should form a covalent bond to the target protein after nucleophilic attack (usually by a cysteine) to an electrophilic moiety, or warhead. Such inhibitors are primarily designed to bind to the catalytic site uncovalently, and then form the covalent bond. For such, targeted covalent inhibitors (TCI) explore the strength of warheads, that are in many cases Michael Acceptors such as acrylamide,[26,27] to obtained the desired covalent bond. The fine tuning of the warheads, can be used in PKI to design irreversible covalent inhibitors or reversible covalent inhibitors, which is a dynamic way of modulating the kinases' inhibition.[28,29]

In the broad field of kinases, identification of nucleophilic amino acids in the binding site of a PK has enabled the development of TCIs.[30,31] The cysteinome, for example, which is the "targetable cysteine" component of the kinome, contains around 200 kinases, many of which have not yet been explored as drug targets.[31]

As mentioned before, afatinib (**8**) was a covalent inhibitor designed with an acrylamide moiety to react with Cys797 in the catalytic site of EGFR. This targeted covalent bond strategy was maintained in the third-generation EGFR inhibitors, such as osimertinib (**10**), with the exchange of the usual 4-aminoquinazoline scaffold to a pyrimidine, which offered selectivity for mutant T790M EGFR.

In the year 2012, when afatinib (**8**) was approved, another covalent inhibitor went into the market: ibrutinib (**11**, Figure 4), an inhibitor of Bruton's Tyrosine Kinase (BTK). BTK first became a relevant target because of its downstream signaling of B cell receptors, and consequently, B cell malignancies. Ibrutinib development began with the design of acrylamide-containing molecules to be used as a tool compound to fluorescently labeled BTK for the study of treatments for rheumatoid arthritis (RA). The cysteine residue targeted, Cys481 (Figure 4), is present on a small number of other kinases, making its inhibition fairly selective. The tool-compound that was identified had already suitable activity and drug-like properties and subsequently entered for clinical trials. Ibrutinib (**11**) has since been approved for the treatment of mantle-cell lymphoma, chronic lymphocytic leukaemia, Waldenstrom's macroglobulinaemia and chronic graft versus host disease (GVHD).[32] Other BTK inhibitors, like zanubrutinib, and acalabrutinib (which bears an alkynamide warhead) were subsequently approved for other haematological malignancies.[33]

The field of covalent inhibitors has still a lot to offer. Since 2013, eight covalent PKI have been approved, stablishing this class as a viable option to approach selectivity, clinical efficacy, overcome resistance and address new targets.[34,35] Recent advances have been made towards new warheads, and currently new amino acid targets beyond cysteine are being explored, for example, lysine or tyrosine.[25] Exploring covalent PKI will be an important tool to address the un-explored part of the kinome, and hopefully gain insight into new therapeutic possibilities.

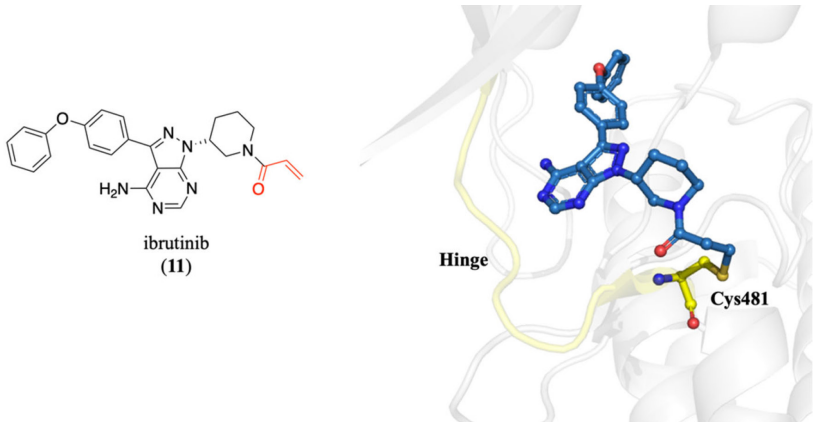

Figure 4. Chemical structure (on the left) and crystal structure (on the right) of ibrutinib (**11**) covalently bonded to the Cys481 of the BTK kinase (PDB: 5P9J).

2.4. JAK-inhibitors and kinases for autoimmune diseases

Inflammation response of the immune system is an essential process to heal and repair tissue and fight infections. However, unresolved or inappropriate inflammatory response is the primary cause of various diseases. Rheumatoid arthritis (RA), inflammatory bowel disease, Chron's disease and so on, are all consequences of chronic inflammation. The identification of targets related to production of inflammatory mediators, including protein kinases, is a strategy for treatment of such diseases. It poses, however, a great challenge, as cytokines have a natural primary role of fighting microbial pathogens, a mechanism that when not working properly, would lead to infection and sepsis. In this sense, the idea of targeting a protein kinase for a chronic inflammatory disease had the great challenge of being safe and non-toxic. Initially, protein kinase inhibitors that had been approved were targeting cancer, and in many cases, side effects of the treatment were circumstantially tolerable, something that would impose great loss of quality of life in a treatment for a chronic disease.[16,36]

The approval and success of anti-TNF antibody adalimumab and TNF-binding protein etanercept, both for chronic diseases, gave the pharmaceutical companies confidence that targeting the inflammatory cascade could be a safe approach for treatment. One of the most promising kinase targets at the time was the Janus Kinase (JAK) family.[16]

The JAK family includes three isoforms (JAK1, JAK2 and JAK3) and a non-receptor tyrosine kinase TYK2. They are intracellular kinases but are non-covalently associated with cytokine receptors. Upon cytokine activation, a conformational change bridges JAK isoforms, that upon phosphorylation, recruit members of the STAT (signal transducers and activators of transcription) family. After phosphorylation and dimerization, the STAT members translocate to the nucleus for gene regulation. This mechanism is essential for inflammatory response, and has been associated in various pathogenic pathways, such as RA.[36-38]

The history of PKI targeting inflammation began in the early 1990s at the laboratories of the NIH (US National Institute of Health), when the therapeutic potential of JAK inhibition was first described. The project was incorporated by Pfizer, initially focused on organ transplantation, but soon moved to a synthetic small-molecule.[39] Tofacitinib (**12**, Figure 5) was identified initially as having inhibitory activity in JAK3. It proceeded into clinical trials, and was approved for treatment of RA, by JAK 1/3 inhibition, in 2012 by the FDA.[39]

In the year before, ruxolitinib (**13**) had already been approved as a JAK1/2 inhibitor for the treatment of myelofibrosis, another inflammatory disease. However, its side effects and loss of efficacy eventually led to its discontinuation.[40] Instead, tofacitinib proved to be less toxic than its JAK inhibitor predecessor and is guarded as the first kinase inhibitor success history outside of the field of oncology. Indeed, it has been later determined that while

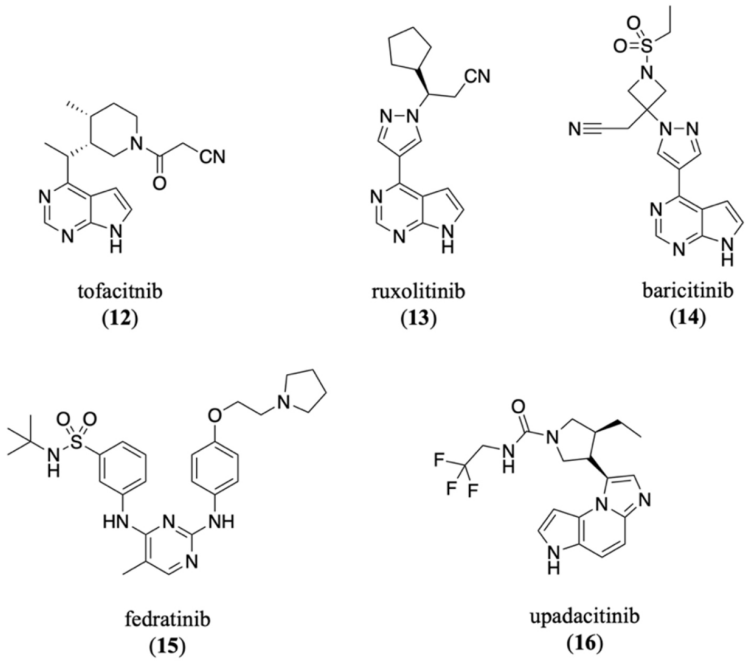

Figure 5. Chemical structure of some JAK inhibitors.

JAK1 and 2 have a broad spectrum of functions, JAK3 is more closely related to immune response, being considered the primary isoform to be targeted in such treatments.[41] Tofacitinib was the first orally available treatment for RA for more than 50 years, and it was subsequentially approved for psoriatic arthritis, ulcerative colitis and juvenile idiophatic arthritis.[16]

After tofacitinib (**12**) and ruxolitinib (**13**), approval of other JAK inhibitors followed, namely baricitinib (**14**, 2018), fedratinib (**15**, 2019) and upadacitinib (**16**, 2019). Obviously, safety and potential side effects for all drugs targeting inflammation is an issue that is always considered and re-evaluated, and different strategies are being tested. Currently evaluated options include targeting pseudokinase domains of JAK and combinational therapy. New targets, like IRAK1/4 and the SYK families, have had growing interest, and are currently being explored for the treatment of inflammatory diseases.[36]

2.5. Current challenges in PK research

2.5.1. Diseases at the central nervous system

As kinase-targeted investigation advanced, more targets were validated, new chemical entities were introduced to the clinic and PK research had stablished itself, naturally researchers would eventually look into central nervous system (CNS) disorders. Already validated targets, such as BCR-ABL, EGFR and VEGFR (vascular endothelial growth factor receptor) began to be studied for their participation in brain tumors and neurological inflammatory diseases, such as Alzheimer's. The initial generally accepted approach was to try to relocate existing drugs, and so imatinib (**5**) and gefitinib (**6**), for example, entered clinical trials for glioma. On the other hand, drugs like osimertinib (**10**) were being evaluated for their neurotoxicity.[16] But it was quickly realized that not only new targets should be identified, but also new chemical entities had to be developed, as the penetration of blood-brain barrier (BBB) was a major challenge.[42]

One of the first targets to draw attention in CNS disease treatment was the glycogen synthase kinase 3 (GSK3), a serine-threonine kinase family that seems to contribute both to the amyloid and tau pathologies that characterize Alzheimer's.[43-45] This target, however, did not yield a successful treatment yet, but it is still under intense investigation. In parallel, mitogen-activated protein kinase (MAPK) also seemed to be a good target, due to its participation in various inflammatory pathways, including Alzheirmer's, ALS (amyotrophic lateral sclerosis) and cerebral ischaemia.[46,47] The p38 MAPK pathway was deeply explored, though it also did not advance into clinics due to several safety issues.[42] Simultaneously, many of the validated and new targets in oncology still are under investigation for their potential in neurooncology, with the design of new inhibitors with more suitable physicochemical properties for brain penetration.[48] However, this is still an unmet need in the clinics.

Currently, the most promising target for CNS disorders has been the already mentioned BTK, for multiple sclerosis. Several inhibitors of the BTK inhibitor's class have proven to be suitable for BBB penetration. Evobrutinib, fenebrutinib, remibrutinib and tolebrutinib have reached phase III trials, and orelabrutinib is in phase II.[49]

2.5.2. PROTACs for kinases

The combination of PROTAC (proteolysis targeting chimera) strategy with kinase inhibition has also been a rapidly developing field. PROTAC ligands are designed by linking two structures, so to bind to a target protein and recruit the E3 ligase, which is responsible for degrading the protein via the ubiquitin-proteasome pathway. After the first report of a PROTAC in 2001,[50] the number of targets identified with PROTAC potential had only reached about 40 by 2019.[51] Since then, however, there has been a resurgence, and the number from 2020-2021 alone has exceed the previous 18 years. It is expected that around 54 kinases can be targeted by PROTACs, which encompasses 45% of total number of PROTAC targets currently. The fact that many kinases have known and effective inhibitors, presumes a straightforward adaptation to a PROTAC, by connecting linkers targeting the E3 ligase.[51,52]

PROTAC technology in kinase inhibitors have been tested in a diverse portfolio of targets and diseases. Many known ligands have been used in PROTAC design, including gefitinib, dasatinib, and even covalent inhibitors, such as ibrutinib.[52] One advantage of PROTAC design is that the protein of interest (POI) ligand can be nonselective, as the degrader can induce specific protein-protein interactions. This provides a great number of possibilities on how to combine existing kinase inhibitors, that may have not progressed to clinics, with the desired degrader.

The field of PROTAC itself and its combination with kinase inhibition still have many challenges to overcome, for example, the design of the linker between the POI and E3 ligase, pharmacological issues due to their high molecular weight, and specifically for kinases, the recurring mutation and drug resistance problem. Still, many advancements on PROTAC will be seen in the upcoming years, and without a doubt, kinase inhibitors will be among them.[51,52]

2.5.3. Viral infections

Another area that has received growing attention is the use of kinase inhibitors for antiviral treatment. Various protein kinases have been associated with the entry stage

of the virus into the host cell, by means of the use of its cell machinery for replication cycles. Although targeting host cellular kinases has the disadvantaged of undesired toxic effects, this approach has been generally used, as it provides a higher barrier to resistance and offer a broad virus spectrum range.[53]

Kinases that have been linked to antiviral treatment include c-Abl, EGFR, JAK and BTK. The main strategy used so far for identification of inhibitors has been repurposing of approved drugs or candidates, as it accelerates the development stages. On the same note, HTS has also been widely used to identify potential candidates for kinase antiviral treatment. More than 20 of the approved kinase inhibitors have been investigated on their antiviral activity, and more than half of those have already been introduced in clinical trials. Imatinib (**5**), for example, has reached phase III for treatment of coronavirus virus disease-19 (COVID-19).[53]

Naturally, with the emergence of the COVID-19 pandemic, investigation of PKI for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections has increased rapidly. The most promising has been the JAK/STAT pathway, specially the JAK2/JAK3 selective inhibitors. The main challenges encountered so far have been related to duration of treatment required for pharmacological effects to take place, which is not suitable for COVID-19 patients that require immediate response, and all the possible side effects associated with kinases of immune response. Nonetheless, many efforts are being made in this field, and combination therapy has been a promising path.[54]

3. Brazilian Contributions in PKI Development

The field of Pharmacological and Medicinal Chemistry has had, since a long time, great interest from Brazilian researchers, and many hallmarks throughout decades paved the way to the current landscape.[55-57] Examples of the significance of Medicinal Chemistry in Brazil are attested by the creation of Division on Structure and Activity Relationship in 1991 by the Brazilian Chemical Society (later renamed as Division of Medicinal Chemistry), the creation of the Summer School on Pharmacological and Medicinal Chemistry (EVQFM, in portuguese), and the Brazilian Symposium on Medicinal Chemistry (BrazMedChem). The EVQFM specifically, which takes place at the Federal University of Rio de Janeiro (UFRJ), has been of the utmost importance in the training of young researchers and the dissemination of the field throughout the country. During its past 30 editions, many prominent international researchers have also had the opportunity to contribute to the school, and consequently, form connections and collaborations with Brazilian researchers.[58]

Through interdisciplinary collaborations and innovative approaches, Brazilian scientists have thus made many contributions to the development of novel bioactive compounds. A notable mention is the discovery and description of bradykinin, which would later be pivotal for the development of captopril.[59,60] Following the world tendency, PKI research has also been a strong focus. The establishment of a branch of the Structure Genomics Consortium (SGC) at Universidade Estadual de Campinas (UNICAMP), for example, illustrates the relevance and potential of the field in Brazil.

In this following session, we will display some of the contributions of Brazilian researchers that were made in collaboration with the University of Tübingen, as a result of cooperations that were stablished during the EVQFM.

3.1. Dual EGFR/VEGFR-2 inhibitors

Protein kinase EGFR, as previously mentioned, is a mediator of several signals implicated in solid tumors. In parallel, EGFR pathways also stimulate the vascular endothelial growth factor (VEGF), which is associated with angiogenesis.

The VEGF promotes vascular permeability in solid tumors, by a signaling mechanism mediated by its receptor VEGFR, another receptor tyrosine kinase. The isoform VEGFR-2 and EGFR share common downstream signal transduction pathways, and play a vital role in promoting growth and vascularization of solid tumors. The inhibition of the VEGFR-2 pathway contributes to the antitumor effects of EGFR inhibitors, and on the other hand, when VEGF expression is activated, EGFR's resistance response mechanism is facilitated.

Both of these protein kinases had been clinically validated by the approval of gefitinib and erlotinib as EGFR inhibitors for NSCLC treatment, and sunitinib as a VEGFR-2 inhibitor for renal cell carcinoma. Therefore, the approach of a dual inhibition of these targets was an interesting strategy for the treatment of solid tumors.

In 2011, vandetanib (**17**, Figure 6) had been approved as a dual EGFR/VEGFR-2 inhibitor for the treatment of late-stage metastatic medullary thyroid cancer. Vandetanib (**17**) has the 4-aminoquinazoline core structure, which is shared among several EGFR inhibitors.[61] It was, however, not equipotent between targets.

On an effort to optimize potency for both EGFR and VEGFR-2, Barbosa *et al.*[62] designed a series of compounds based on the 6,7-dimethoxy-2-chloro-4-aminoquinazoline

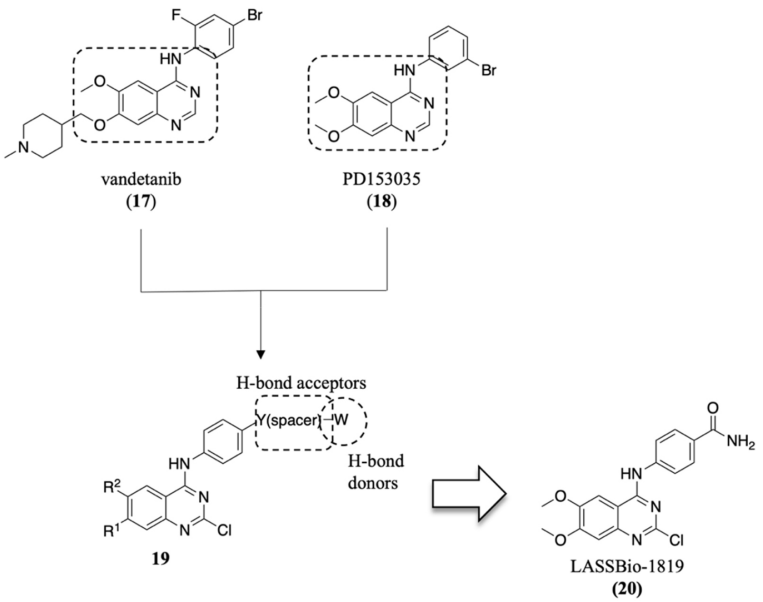

Figure 6. Design of dual inhibitors targeting EGFR and VEGFR-2. To the 2-aminoquinazoline scaffold (highlighted with dotted lines), various anilines were attached, with *para*-substituents to gain hydrogen interactions in both kinases.

derivative PD153035 (**18**), which was the most potent EGFR inhibitor known. Additionally, different *para*-substituents containing sulfonamides and amides were attached to the aniline ring, which was a strategy to gain hydrogen bonds with key amino acids Glu855 and Asp1046 of VEGFR-2 (Figure 6).

The new proposed series of quinazolines showed a good equipotency of EGFR and VEGFR-2. The results exhibited the relevance of hydrogen bond donating substituents at the *para*-position of the aniline for interaction of both EGFR and VEGFR-2. The standout compound LASSBio-1819 (**19**) had a half-maximal inhibitory concentration (IC_{50}) = 0.9 μM for EGFR and 1.17 μM for VEGFR-2, which were less potent than vandetanib (**17**), but had good equipotency. Compared to its prototype PD153035 (**18**), potencies in cells were 7-fold and 11-fold higher, respectively.

3.2. Novel scaffolds for mutant EGFR inhibitors

On an ongoing effort to identify new scaffolds to overcome EGFR resistance, do Amaral *et al.*[63] proposed a bioisosteric substitution of the quinazoline ring, present in many EGFR inhibitors (Figure 7). The goal was to obtain ligands that would inhibit the clinically relevant mutant EGFR.

Molecular design was based on the quinoxaline ring, which maintains the sp^2 nitrogen atom of the quinazoline for relevant hydrogen bond interactions in the hinge region. Additionally, the aniline moiety, commonly attached to the quinazoline core, was substituted by an urea linker to

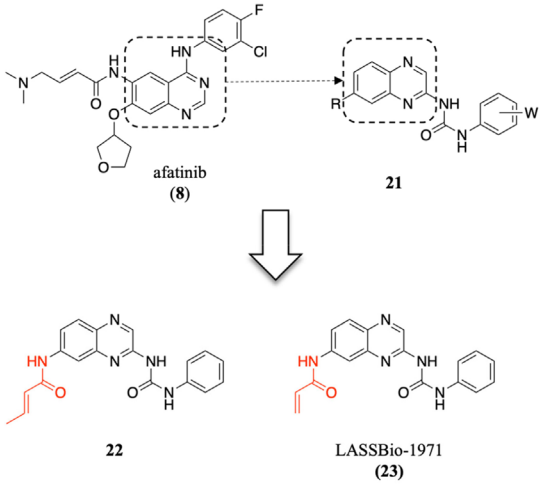

Figure 7. Molecular design of new inhibitors of mutant EGFR. The quinazoline ring in afatinib (**8**) was bioisosterically switched to the quinoxaline ring in **21**.

a phenyl ring. And lastly, to target the covalent interaction with Cys797, various electrophilic subunits were used.

Biological activity of the derivatives in three types of EGFR (wt, L858R and L858R/T790M) primarily showed that the bioisosteric substitution to the quinoxaline subunit was successful, and the compounds were generally active in the protein. SAR analysis demonstrated that substitution in the phenylurea subunit was not easily tolerable, and was overall deleterious to EGFR inhibition. The non-substituted derivatives **22** and **23**, bearing the acrylamide group as warhead, displayed inhibitory activity at the low nanomolar range. Compound **23** (LASSBio-1971) also showed cytotoxicity in various tumor cell lines, and selectivity for specific lines with EGFR-inhibitor resistance.

3.3. Bioisosters for p38 MAPK inhibitors and prolonged target-residence time (TRT)

The relevance of targeting MAPK pathway is due to its participation in cellular stress, and its regulation of the biosynthesis of various proinflammatory cytokines. Of the four isoforms of p38 (α, β, δ and σ,), p38α is the only to be ubiquitously expressed in all tissues, and is associated with tumor development upon deregulation of expression levels. Skepinone-L (**24**, Figure 8) had been previously identified as a highly selective p38α inhibitor, displaying an IC_{50} of 5 nM.[64] The crystal structure of Skepinone-L (**24**) in complex with p38α revealed a glycine flip at the hinge region due to its dibenzosuberone interaction, which provides great selectivity within the kinome. Further optimization identified compound **25**, with an amide linker to an aromatic subunit.[65] This compound also displayed exceptional potency in enzymatic assays (IC_{50} < 3 nM), and great selectivity. Crystal analysis then revealed an edge-to-face interaction between the aromatic residues of the eastern amide and Phe169 of the DFG motif, which assembles the regulatory spine (R-spine), in an active-like conformation.[66] These compounds were named type I ½ inhibitors.

Interaction with the R-spine greatly influences the inhibitor's target residence time (TRT). Skepinione-L (**24**) has a relatively short TRT of 88 s whereas **25** displays an increase to 746 s. Optimization of the TRT is an important tool in medicinal chemistry as it improves pharmacokinetics.[67] Pedreira *et al.*[68] proposed a molecular design based on **25** to improve TRT by the bioisosteric substitution of thiophene to selenophene, to intensify the aromatic interaction with Phe169, and therefore R-spine stabilization. Additionally, the *N*-acylhydrazone (NAH) moiety was used instead of the amide linker to improve the metabolic profile of the compounds (Figure 8).

The proposed derivatives displayed good inhibitory potency at the nanomolar range, comparable to their prototype **25**. Also, the longer NAH moiety did not seem to negatively influence interaction. Cell activity mostly correlated to the enzymatic inhibition values, and TNF-α quantification in plasma showed good inhibitory activity for compound **27**. The TRT of the selenophenic derivative **29** was substantially longer compared to its thienyl analog **28**, corroborating the hypothesis of R-spine stabilization by aromatic interaction effects. Representation of spine interaction is displayed for compound **27** in Figure 9. Compound **27**, which has the best overall profile, was evaluated in a metabolic stability study, and displayed better metabolic stability compared to the amide-derivative.

4. Conclusion and Perspectives

After nearly 30 years of kinase inhibitor development, and currently (as of May, 2024), 80 PKI approved by the FDA[69] (Figure 10 and Table S1, Supplementary Information section), much has been achieved and learned about kinase targeting. In the field of oncology, lessons about mutation and acquired resistance through genetic instability have changed the way PKI mediated cancer treatment is approached, and this is still one of the great challenges when entering a PKI into clinic. Ideally, tracking specific biomarkers of tumors should be done prior to entering treatment.

The recent approval of larotrectinib (2018) has been a good example of the potential of personalized medicine. This inhibitor was designed specifically to target tumors bearing the neurotrophic tyrosine receptor (NTRK) gene.[70,71] The (NTRK) gene fusion is a biomarker found in over 25 types of cancer. Larotrectinib therefore is a "tumor-agnostic" treatment, as it targets specific molecular characteristics

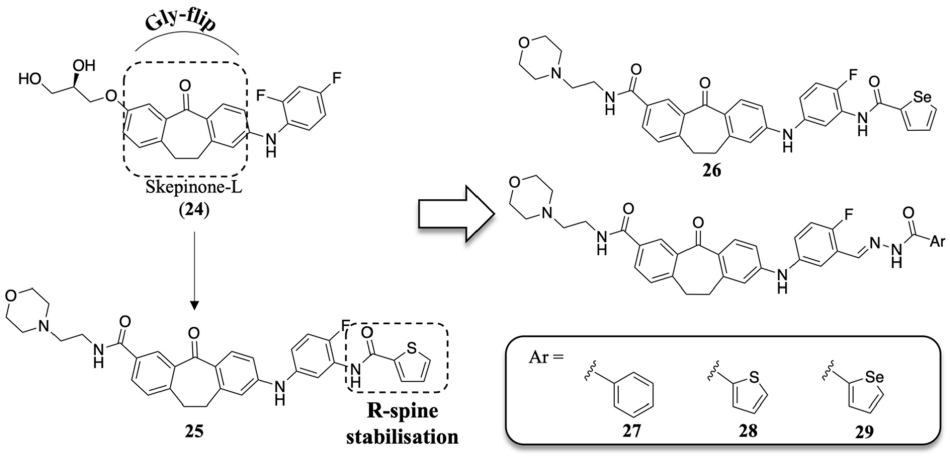

Figure 8. Chemical structure of Skepinone-L (**24**), and its derivative **25**, that displayed longer TRT. Structural modifications of the amide substituents identified compounds **26**, **27**, **28** and **29**.

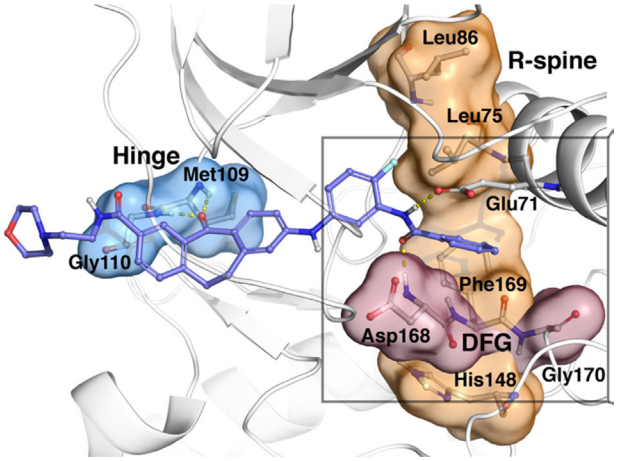

Figure 9. Representative shot of MD simulation of the NAH derivative **27** in the catalytic cleft of p38 MAPK (PDB: 5TCO), interacting with Phe169 of the DFG triad (purple) and therefore assembling the R-spine (orange).[68]

rather than the protein of a single tumor tissue.[72]

Selectivity of PKI will also always be a point of issue. However, the growing number of allosteric inhibitors offer a promising getaway from this issue. The approval of trametinib (2013), an allosteric MEK inhibitor for the treatment of melanoma, paved the way for allosteric PKI that reached the clinic. Cobimetinib (2015), binimetinib (2018) and selumetinib (2020) followed, the latter being approved for myelofibrosis.[73] In 2021, asciminib was approved for the treatment of CML by targeting BCR-ABL, binding in a remote site from the orthosteric binding pocket. Additionally, asciminib binding has been shown not to be affected by the T315I mutation, which blocks most ATP-competitive BCR-ABL inhibitors.[74] Rational development of allosteric inhibitors still pose many difficulties.

Finally, the repurposing of clinically approved kinase inhibitors, opens up an array of possibilities for treatment of untackled diseases. In fact, only 50 of the more than 500 kinase-encoded genes have been targeted for cancer, and approximately 70% of the kinome have not yet been explored.[4,75]

The progress of covalent inhibitors, recent growth of substrate specific ligands (probes), and the continuous effort into exploring the "dark kinome" will certainly impact PKI development. The ever-dynamic field of PK still have a lot to offer, and many new diseases to potentially tackle. As always, innovative developments should be expected of PKI in the next coming 30 years.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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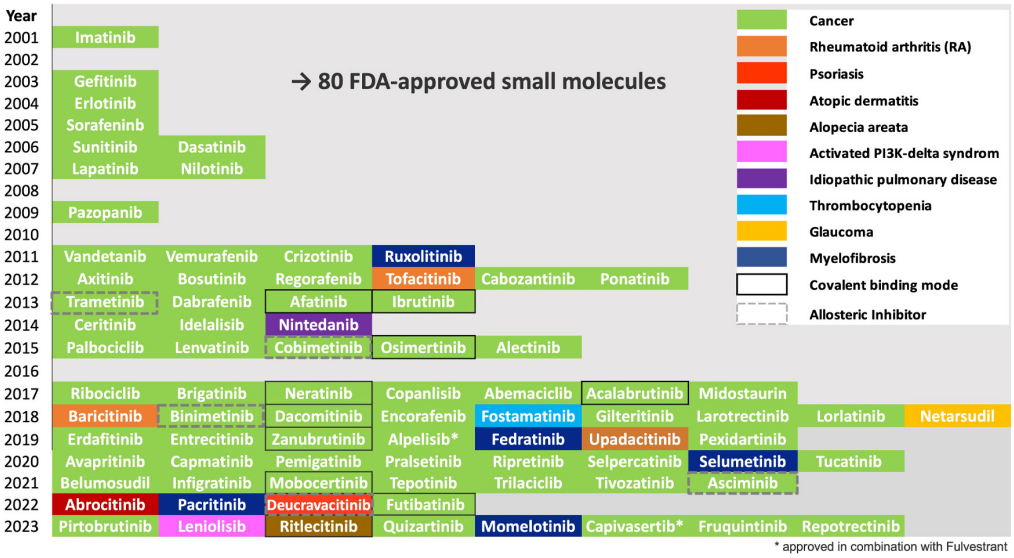

Figure 10. FDA-registered PKIs as of October 2023.

Tübingen Center for Academic Drug Discovery and Development, a member of the Brazilian Academy of Sciences since 2014, member of the scientific council of INCT-INOFAR (Instituto Nacional de Ciências e Tecnologia de Fármacos e Medicamentos) and visiting guest professor at the Federal University of Rio de Janeiro.

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