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Role of Fyn Kinase Inhibitors in Switching Neuroinflammatory Pathways

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Abstract: Fyn kinase is a member of the Src non-receptor tyrosine kinase family. Fyn is involved in multiple signaling pathways extending from cell proliferation and differentiation to cell adhesion and cell motility, and it has been found to be overexpressed in various types of cancers. In the central nervous system, Fyn exerts several different functions such as axon–glial signal transduction, oligodendrocyte maturation and myelination, and it is implicated in neuroinflammatory processes. Based on these premises, Fyn emerges as an attractive target in cancer and neurodegenerative disease therapy, particularly Alzheimer disease (AD), based on its activation by Aβ via cellular prion protein and its interaction with tau protein. However, Fyn is also a challenging target since the Fyn inhibitors discovered so far, due to the relevant homology of Fyn with other kinases, suffer from off-target effects. This review covers the efforts performed in the last decade to identify and optimize small molecules that effectively inhibit Fyn, both in enzymatic and in cell assays, including drug repositioning practices, as an opportunity of therapeutic intervention in neurodegeneration.

Keywords: Fyn kinase, neurodegeneration, dasatinib, tyrosine kinase, Fyn inhibitors, pyrrolopyrimidine

Fyn is a non-receptor tyrosine kinase belonging to the Src family kinases (SFKs). SFKs are characterized by a highly shared domain structure based on three Src homology domains (SH1 to SH3), one variable *N*-terminus region and SH4 domain which hangs the kinase to the cytosolic plasma membrane.[1] Structural variations regarding these two latter regions seem to be responsible for the different functional roles among the SFKs.[2]

Since its identification and characterization in 1988, three isoforms of Fyn have been identified, but only two of them seem to play a prominent role at cellular level. FynT is mostly expressed in hematopoietic cells, while FynB is ubiquitously expressed, albeit at higher levels in the brain.[3] Particularly, Fyn activation occurs accordingly by ligands binding to SH2 and/or SH3 domains with the related phosphorylation/dephosphorylation of two critical tyrosine residues. Phosphorylation at Tyr417 in FynT (or Tyr420 in FynB) induces an open conformation of catalytic site which fosters ligand binding and enzymatic activity. Conversely, Tyr528 phosphorylation in FynT (or Tyr531 in FynB) represents a negative modulation by inducing structural rearrangement in a close conformation where the catalytic site is not affordable and thus inhibiting its kinase function.[4]

At cellular level Fyn plays crucial roles among a plethora of signaling processes such as growth factors, cytokines trafficking, T-/B-cell receptors, ion channel functions and cell differentiation. Because of its ubiquitous involvement, Fyn impairment is strictly related to several pathologies such as cancer and neurodegenerative diseases.[5] In fact, Fyn is known as proto-oncogene and its upregulation is observed in several malignancies like melanoma, squamous cell carcinoma, breast and prostate cancer, thus proposing in some cases Fyn expression as potential biomarker for some type of cancer.[6, 7] The oncogenic properties rely on the dysfunction of cell proliferation and adhesion paired to morphogenic transformations accordingly to Fyn overexpression or genetic alterations.[7] More recently, Fyn has gained increasing interest as potential target to tackle neuroinflammatory processes which orchestrate brain disorders. In the central nervous system (CNS), Fyn coordinates different transduction pathways involving synaptic plasticity, myelination and glia formation that once disrupted could lead to brain pathologies such as Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS). In this context, the hypothesis of inhibiting Fyn to counteract neurodegeneration was further corroborated by the clinical trials carried out with the Fyn blockers saracatinib, masitinib and dasatinib as potential AD modifying drugs.[8]

In the brain Fyn is mainly placed in limbic regions, cerebellum and striatum, with a significant extensive distribution detected during embryonic brain development which persists also in adult brain.[9-11] In this regard, in CNS Fyn's machinery represents a key factor directing brain development and its physiological functions.[4] For example, Fyn plays a fundamental role in CNS myelination by triggering signaling pathways important both at the initial stages of myelination during brain development and at myelin regeneration in chronic demyelinating diseases.[12, 13] Interestingly, its involvement in myelinating processes seems to be unique among other SFKs.[12] Furthermore, Fyn is strictly involved in synaptic transmission and plasticity, mainly through the modulation of N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) trafficking at excitatory synapses and thus regulating cellular mechanisms underpinning learning and memory such as long-term potentiation (LTP) and long-

term depression (LTD).[14] This is mostly due to the strategic localization of Fyn at postsynaptic density (PSD) fraction of neurons, thus emerging as one of the crucial gears functionally orchestrating synaptic signaling.

Particularly, activated Fyn can phosphorylate both NR2A and NR2B subunits of NMDAR, but it seems to exert a tighter influencing connection with NR2B, mainly through its Tyr1472 phosphorylation. This turns out in reduced NMDAR endocytosis and higher membrane stabilization with the resulting enhancement of synaptic transmission.[15] Lastly, neurite outgrowth and oligodendrocyte differentiation are other two essential Fyn functions in CNS, albeit its precise role and the underpinning cellular mechanisms in this context are still to be elucidated.[5, 16]

Several experimental evidence depicted a tight connection between Fyn dysregulation and neurodegenerative disorders since long time. Firstly, in 1993 AD brain sections revealed strong Fyn immunoreactivity, paired with abnormal tau phosphorylation, when compared to the healthy one, paving the way for decades of research aimed at defining Fyn role and possible Fyn-targeting approaches in brain diseases.[17] Moreover, Fyn-tau liaison seemed to play essential role in creating the characteristic neurotoxic environment. Tau is functionally required to localize Fyn at PSD, which in turn can phosphorylate tau residues (e.g., Tyr18 is one of the most common) and thus, if abnormally activated or stimulated in pathological conditions, it triggers the deposition of neurofibrillary tangles (NFT), hallmark of several tauopathies such as AD and other forms of dementia (Figure 1).[18] In confirmation of Fyn driving role in tau pathology, in a Fyn knockout coupled with tau overexpression mutation mouse model a strong reduction of NFT emerged as well as reduced synaptic tau accumulation.[19] Interestingly, the other histopathological feature of AD, i.e., amyloid-β peptide (Aβ), has proved to be one of the most important upstream inducer of impaired Fyn cellular activity.[20] In AD, the aberrant stepwise cleavage of amyloid precursor protein (APP) results in Aβ formation, which is prone to aggregation leading to the formation of neurotoxic oligomers and fibrils (Figure 1).[21] These latter are responsible for the deposition of characteristic extracellular Aβ plaques. Remarkably, Aβ soluble oligomers (Aβos) emerged as powerful mediator of synaptotoxicity more than the final aggregated fibrils.[22] In 1998 it was first reported that Fyn acts as essential mediator of Aβos-induced toxicity and inhibitor of synaptic plasticity.[23] Further biological investigations in mouse model demonstrated that Fyn ablation decreased Aβ-induced mortality, whereas Fyn overexpression exacerbated it.[24] More recently, in neurons of AD patients Fyn showed also to trigger the amyloidogenic cleavage of APP by phosphorylating it at Tyr682 whereas the kinase inhibition led to a reduction of Aβ42 release.[25] To close this neurotoxic circle, tau emerged as essential for expressing Aβ toxicity by relocating Fyn to PSD where plausibly it allows the breakthrough of neurotoxic events inside the neuron.[18] From these findings turns out the driving role of toxic triad Fyn-tau-Aβ in the physiopathology of AD.[26] Briefly, extracellular Aβos binds cellular prion protein (PrPC) at cell surface activating an intracellular signaling cascade, which involves metabotropic glutamate receptor 5 (mGluR5), and leading to intracellular Fyn activation.[27] Besides tau phosphorylation and aggregation, impairment of LTP, onset of excitotoxic condition due to overexcitation of NMDAR and synaptic loss are other Aβos-induced and Fyn-mediated cellular features (Figure 1).[8]

In a similar manner, Fyn showed to orchestrate and trigger several key pathological pathways even in PD pathogenesis.[28] Particularly, activated Fyn phosphorylates specifically α-synuclein at Tyr125 that, in turn is able to activate downstream neurotoxic cascade PrPC-mediated (in a similar way to what is reported in Figure 1 regarding Aβ). Furthermore, upon oxidative or inflammatory stimuli, Fyn can further trigger oxidative stress and inflammatory response from glial cells leading to the characteristic dopaminergic neuronal loss through the phosphorylation of protein kinase C δ (PKCδ) and other signaling pathways such as MAPK, NF-κB and Nrf2.[28] To note, under neurodegenerative conditions Fyn emerges as potential molecular plug linking the neurotoxic pathways involved in the onset and development of neuroinflammatory processes. Fyn is physiologically expressed in microglial and astrocytic cells while it is persistently upregulated in neuroinflammatory conditions where it seems to play a driving role.[29] In different disease in vitro and in vivo models Fyn proved to be essential for fostering the pro-inflammatory response (e.g., Aβ, α-synuclein, LPS, TNFα) made up by increased cytokines release and iNOS activation, whereas in Fyn knockout model a consistent reduction of key inflammatory markers was registered.[20, 29, 30] As a confirmation of this, in a recent work by Monteiro and colleagues Fyn showed to act as pivotal cellular decision-maker in controlling immunoreceptor status by managing inflammatory conditions.[31]

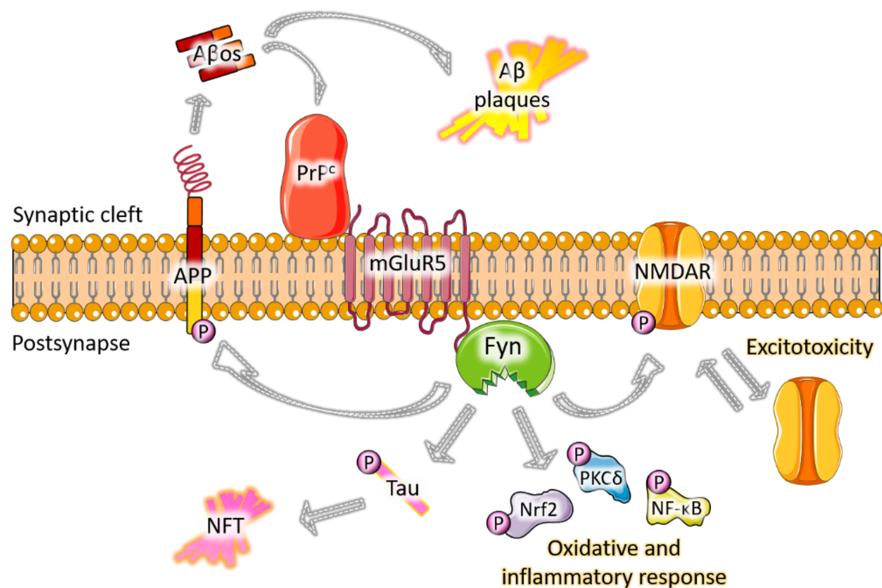


Figure 1. Overview of Fyn-mediated neurotoxic cascade at cellular level in AD. Aβos = β-amyloid soluble oligomers; APP = amyloid precursor protein; NFT = neurofibrillary tangles; PrP^c = cellular prion protein; mGluR5 = metabotrope glutamate receptor 5; Nrf2 = Nuclear factor-erythroid factor 2-related factor 2; PKCδ = Protein kinase C δ; NF-κB = nuclear factor κ-light-chain-enhancer of activated B cells; NMDAR = *N*-methyl-*D*-aspartate receptor.

Conversely to physiological condition, FynT isoform is selectively upregulated in AD neocortex neurons simultaneously with a FynB decrease, suggesting an induced pathological alternative splicing directly associated to the formation of NFT. Furthermore, in astrocytes an Aβ-induced increased FynT to FynB ratio was found, confirming astrocytic activation and astrocyte-mediated inflammatory response in AD.[32] A similar upregulation under prolonged proinflammatory stimuli (i.e., TNF) was found, which corroborates the isoform-specific role of FynT in fostering persistent neuroinflammatory conditions by coordinating other cellular inflammatory pathways (e.g., PKCδ and NF-κB).[33]

Based on this preclinical evidence, over the years Fyn has been explored as potential therapeutic target for neurodegenerative disorders. In animal models of brain diseases, Fyn inhibition showed promising behavioural improvement paired with reduced pathological markers (e.g., NFT, proinflammatory cytokines) and glial activation.[34, 35] These experimental findings set the stage for years of research regarding potential Fyn inhibitors with the aim to dissect the pathological network underpinning neuroinflammatory processes mediated and orchestrated by Fyn. A deepen comprehension on how Fyn is involved in those neurotoxic loops at molecular level could also help for the development of future Fyn-targeting effective treatments. Nevertheless, the dark side of the medal indicates Fyn as promising but unfortunately tricky target. Firstly, the high structural analogy among SFKs hampered the identification of potent and selective Fyn inhibitors, that otherwise can lead to side effects due to interaction with other SFKs. Furthermore, Fyn has to deal with essential and several physiological functions, as previously mentioned, which should be preserved by pharmacological treatment to avoid the onset of toxic effects. With the aim of paving the way for future Fyn-targeting therapeutic treatments of neurodegenerative disorders, herein we have reported the most promising Fyn inhibitors and their pharmacological characterization, highlighting most recent progresses in the development of selective pharmacological tools in the context of neuroinflammatory pathways.

2. FYN INHIBITORS

To date, no selective Fyn kinase inhibitors exist due to the tight structural homology among SFK members.[36] As reported above, SFKs have very similar sequences and only differ at the N-terminus and at the linker connecting the SH2 domain to the kinase one.[37] An important starting point in the design and synthesis of selective Fyn inhibitors has been represented by the crystal structure (PDB - 2DQ7) of Fyn complexed with the pan-kinase inhibitor staurosporine.[38] Its flat structure lays in the groove between the C- and the N-lobes stabilized by several hydrophobic interactions while establishing three driving H-bonds with Ser348 and the backbone of Glu342 and Met344.[38] From this emerged for the first time some crucial interactions in the ATP-binding site and structural requirements which can result in a competitive inhibition mechanism. Therefore, most of the Fyn inhibitors developed so far competitively bind to a conserved hinge region within the ATP-binding site,[39] thus blocking the triggering transfer of a phosphate group from ATP to Tyr416 residue.[40] Although bearing the traditional issue of selectivity, several potent Fyn inhibitors have been developed and entered in clinical trials for the treatment of neurodegenerative disorders. Most of them were originally developed to treat various types of cancers and later underwent drug-repurposing campaigns in order to find potential AD-modifying drugs (Figure 2).

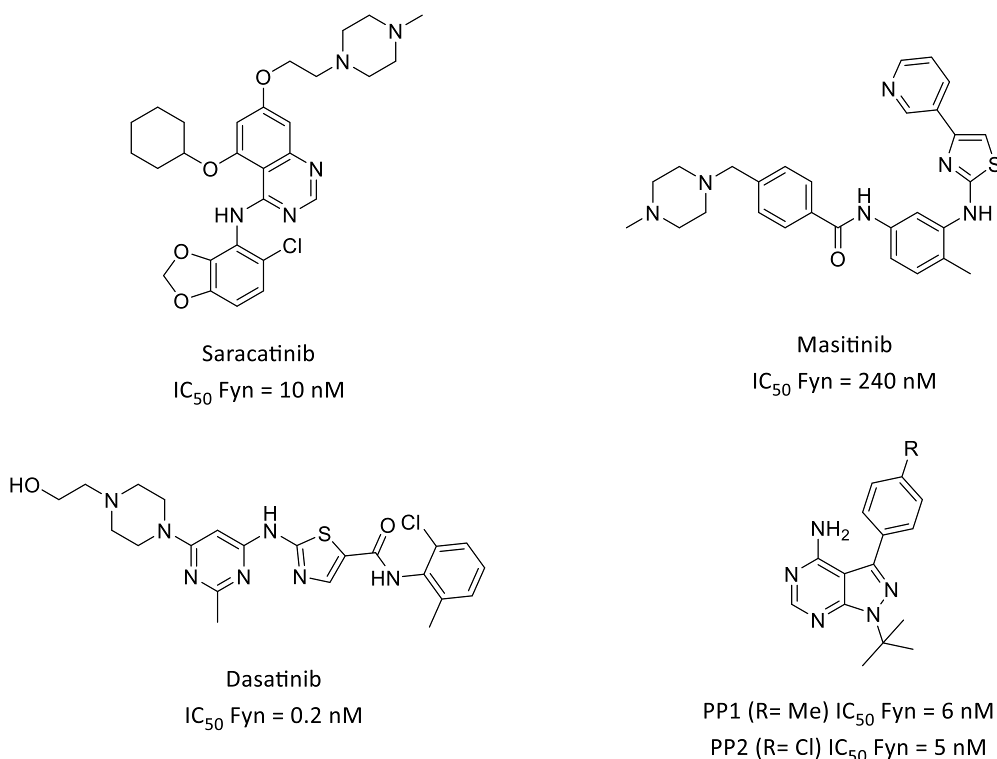


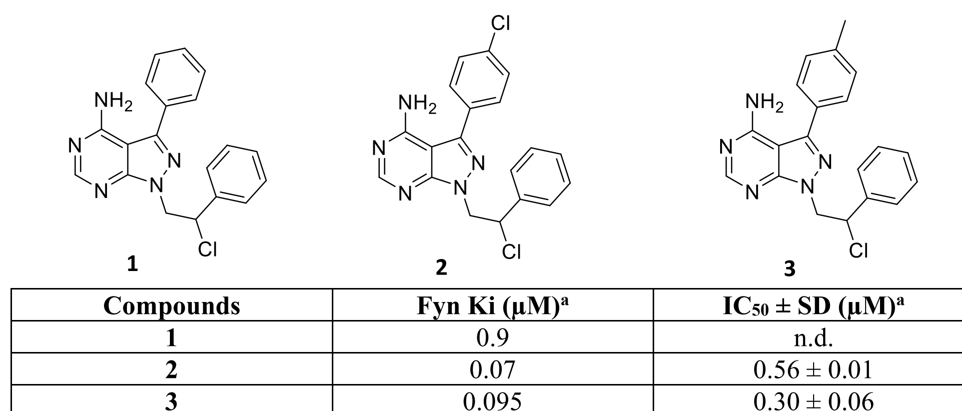
Figure 2. Fyn inhibitors in clinical trials for the treatment of neurodegenerative disorders.

Saracatinib (AZD0530, Figure 2) is a quinazoline-based inhibitor of SFKs with nanomolar potency, originally developed by AstraZeneca to treat solid tumors.[41] It was discontinued due to its limited efficacy as a single cancer-treating agent and it is currently investigated at Yale University as an AD-modifying drug, through the disruption of the Aβ-PrPC-Fyn cascade.[42] Here, studies carried out on AD mouse models showed that chronic Fyn inhibition reduces learning and memory impairments as well as restoring synapse density.[35] Then, a phase Ib clinical study conducted in people suffering from mild-to-moderate AD revealed that doses ranging from 50 to 125 mg daily - that is lower than the ones used for cancer treatment - are well tolerated, and this dosing regimen achieves CSF concentrations similar to those found in the brain of AD mice where memory impairment was restored.[43] A phase IIa study followed, that indicates no statistically significant reduction of AD progression upon treatment with saracatinib.[44]
Masitinib (AB1010, Figure 2) is an aminothiazole-based inhibitor of c-kit kinase designed by AB Science for the treatment of mast cell tumor in dogs.[45] Due to its affinity for Fyn and the other SFK family, it has been recently repurposed and studied as AD-treating agent.[46] In a phase II clinical trial masitinib was tested in an add-on therapy to the standard one over 24 weeks, showing a promising ability of slowing the rate of AD cognitive decline along with a good tolerance profile.[47] A phase III trial followed and was completed in December 2020 (NCT01872598) with no results reported so far. Furthermore, masitinib has been evaluated in ALS patients as add-on therapy to riluzole, showing significant benefit and slowing the functional decline, thus pushing it into phase III to corroborate these results.[48]
Dasatinib (BMS-354825, Figure 2) is an aminothiazole-derived Abl kinase inhibitor developed by Bristol-Myers Squibb for the treatment of chronic myelogenous leukemia.[49] Selectivity assays clearly demonstrate that dasatinib inhibits Fyn and the other SFK members with submicromolar potencies,[50] making it a potential candidate in the AD treatment. Indeed, studies conducted in murine AD models show that it promisingly reduces Aβ-dependent microgliosis.[51] Recently, treatment of AD mice with combination of dasatinib and quercetin proved to reduce neuroinflammation, decrease Aβ plaques and improve cognitive deficits leading to a Phase I and II clinical trials for AD and MCI in USA.[52]
Pyrazolopyrimidines PP1 and PP2 (Figure 2) are SFK inhibitors first discovered by Pfizer to understand the role of SFKs in T cell activation, both having IC50 values of 5 and 6 nM respectively for Fyn.[53] This class of compounds, along with structurally-similar molecules, have been investigated as kinase inhibitors for cancer treatment[54] and, particularly, PP1 and PP2 exhibited a promising activity in fighting breast cancer.[55] Although no studies are currently underway with PP1 and PP2 in human AD, they both might be employed to combat neuroinflammation due to their good activity towards Fyn.
Starting from these well-known Fyn inhibitors, several chemical entities bearing different scaffold were developed able to block Fyn kinase activity.[56] Herein we reported the most recent and promising small molecules developed as Fyn inhibitors which could be useful to investigate and disrupt Fyn role in the context of neuroinflammatory processes in neurodegenerative disorders. The compounds are sorted on the basis of their central scaffold ranging from ATP-like nitrogenated bicycles (e.g., pyrazolopyrimidine, pyrrolopyrimidine and oxindole), single heterocycles (e.g.,

aminothiazole and aminoimidazole) and polyphenolic derivatives. Furthermore, we also present some examples of computational-aided methods which were developed leading to the identification of new Fyn-inhibiting scaffolds that may represent an innovative, quick and cheap approach which could help towards the discovery of new selective and potent Fyn inhibitors.

2.1 Bicycles and polycycles

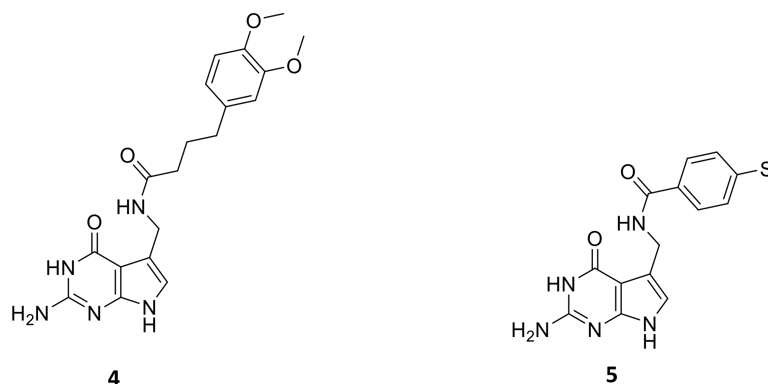
The highly shared structural homology of ligand binding pockets among kinases represents one of the biggest issues to face in designing selective competitive inhibitors. Therefore, a comprehensive exploration on spatial conformation and key residues involved in ligand interaction is crucial to achieve strong Fyn inhibitor. To this aim, a deepen investigation on ATP-binding site of Fyn was conducted, assisted by in vitro and in silico analyses, starting from the promising 4-aminopyrazolopyrimidine moiety characterizing previously reported PP1 and PP2.[57] A preliminary screening on different substitution patterns in position 1, 3, 4 and 6 afforded derivative 1 with low micromolar profile bearing a primary amine in C4, an unsubstituted phenyl in C3 and a 2-chloro-2-phenylethyl side chain in N1 (Figure 3). Consistently, in docking studies 1 proved to locate inside the binding site same as that previously identified for most potent PP2 by crystallographic experiment. Key interactions are represented by H-bonds of exocyclic amine with carbonyl of Glu343 and N5 with Met345, whereas the two phenyl rings stabilize the adopted conformation lying in hydrophobic regions. From these outputs, a small hit-to-lead optimization campaign was conducted to improve 1 potency toward Fyn by placing different functionalizations in positions C3, C6 and N1. The thiomethyl moiety inserted in C6 led to loss of affinity, because in docking studies it seemed to force away the pyrazolopyrimidine nucleus from interactions with key residues reported above. Hydrophobic para-substitutions in C3-attached phenyl ring generally increase binding affinity such as chlorine and methyl for 2 and 3 which emerge as the best Fyn inhibitors of the series. Furthermore, substitution with chlorine in N1 side chain led to the most potent compounds of the series. Starting from docking poses of most promising inhibitors, further insights with molecular dynamics simulations helped to elucidate pivotal ligand-target interactions. Stable hydrogen-bond between carbonyl backbone of Glu343 and exocyclic amine and a weaker one between Met345 and N5 stabilize residues facing the binding pocket with a different conformation recognized as the active conformation of Fyn which is commonly overexpressed in pathological conditions. For example, the inhibitor-induced loss of Thr342-Glu343 interactions and stabilized network among Lys299-Glu314-Asp408-Phe409 contribute to the stabilization of the complexes in active state. Furthermore, lower flexibility in complexes with respect to the free receptor were found with stronger inhibitors, especially in a glycine-rich loop, suggesting its possible involvement in determining potencies against Fyn. Interestingly, 2 exerts selectivity toward a panel of kinases, but still lacking it among SFK members as well as with kinases bearing high structural similarity with SFKs. Fyn inhibitory potencies of 2 and 3 were also confirmed in neuroblastoma cell line. Particularly, after Aβ-induced insult they proved to reduce Tyr18-Tau phosphorylation in a dose-dependent manner over 6h.



Compounds	Fyn Ki (μM)[a]	IC_{50} ± SD (μM)[a]
1	0.9	n.d.
2	0.07	0.56 ± 0.01
3	0.095	0.30 ± 0.06

Figure 3. Structures and Fyn inhibitory potencies of Pyrazolo[2,3d]pyrimidine derivatives. n.d. = not determined. [a]Fyn inhibition determined by an enzymatic assay using radiolabeled ATP.

Pyrrolo[2,3-d]pyrimidine nucleus among nitrogen heterocycles was widely exploited to inquire kinases' binding site, albeit they usually tend to exert slight selectivity over Fyn.[58] Particularly, Ölgen and colleagues exploited a dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-one core targeting SFKs to explore different aromatic substituents connected to position 5 with an amido linker (Figure 4).[59] All the pyrrolopyrimidine derivatives showed weak inhibitory activities in enzymatic fluorescence-based assays, especially toward Fyn, whose best inhibitor 5 at 100 μM exert a blockage of around 50%. Unfortunately, 5 inhibits with the same extent also Lyn, displaying selectivity only over c-Src (Figure 4). Conversely, compound 4 with 3,4-dimethoxyphenyl fragment emerged as the stronger non-selective inhibitor of the series. From in silico insights on the plausible binding mode inside the predicative binding site, only inhibitor 4 showed to make three hydrogen bonds with Fyn. All derivatives are placed in Fyn active site similar to PP2, but none of them is well overlapped with it. From these computational and in vitro analyses, pyrrolopyrimidine scaffold confirmed to behave as good starting point for designing SFK inhibitors, albeit long and flexible substitutions in position 5 exert an important influence in lowering the inhibitory potency and selectivity.



Compounds	% Fyn inhibition[a]	% c-Src inhibition[a]	% Lyn inhibition[a]
4	38 ± 4	54 ± 4 (IC_{50}= 21 µM)	32 ± 1
5	46 ± 3	14 ± 4	42 ± 4

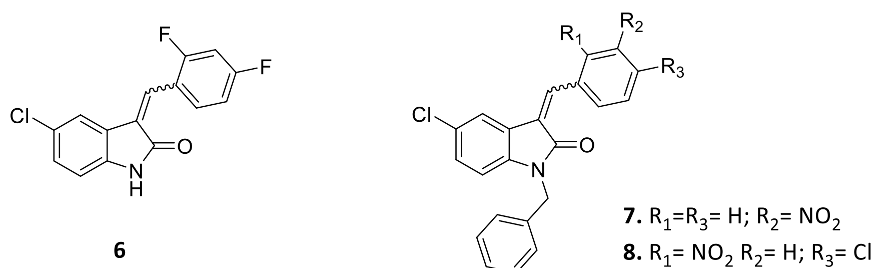
[a] % inhibitory effects of selected compounds at 100 µM. Lyn alongside c-Src belong to SFKs.

Figure 4. Structures and biological activities of Pyrrolo[2,3d]pyrimidine-based compounds.

Oxindoles (or indolin-2-ones) have been widely studied as scaffolds for the design of inhibitors directed to various tyrosine kinases.[60] Many efforts have been made in order to improve the selectivity of oxindole towards SFK members: for example, some oxindoles bearing a tetrahydroindole side chain have proved selective for SFK, in particular being active against Fyn in the low-micromolar range,[61] while oxindoles with an imine side chain were only active in the high-micromolar range. [62] Also indole derivatives have been used as scaffolds for the development of Src inhibitors, namely N-benzylated indole esters[63] and indole-3-imine and amine derivatives[64] being active in the medium-micromolar range.

In light of these findings, Kilic-Kurt et al. synthesized two series of oxindole derivatives, namely 5-chloro-3-(substituted-benzylidene)indolin-2-ones and 1-benzyl-5-chloro-3-(substituted-benzylidene)indolin-2-ones (Figure 5) and tested them as inhibitors of SFK.[65]

Final products came out as mixtures of E/Z isomers, with the majority of them being in the E configuration, probably due to the hydrogen bonding between the oxygen of the carbonyl group in position 2 of the indole scaffold and the hydrogen in position 10 (or 14) of the phenyl ring.



Compounds	% Fyn inhibition (0.01 mM)	% Fyn inhibition (0.001 mM)
7	5	0
8	15	5

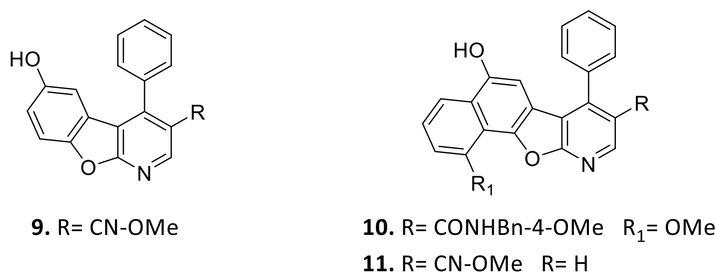
Figure 5. Structures and Fyn inhibitory potencies of oxindole derivatives.

In enzymatic fluorescence-based SFK assay protocol, compounds 7 and 8 were slightly active against Fyn, when tested at 0.01 mM and totally inactive against the other SFK members. Compound 6 was a very weak Fyn inhibitor, underlining the importance of nitro group and the N-benzyl moiety for maintaining a slight of inhibitory activity.

Docking studies were performed on the most active compound 8 to unveil its binding pose into Fyn catalytic site: it is proposed to bind into the ATP binding site by establishing a hydrogen bond between the oxygen of the nitro group and the hydrogen of the amino group of Asp148 residue. Conclusions can be drawn that the ability of forming H-bonds is important for the activity as well as the E/Z configuration: indeed, the least active compound 6 is mainly an E isomer, while the most active 7 and 8 have a majority of Z one.

However, docking studies run both on 8 and on the Fyn inhibitor PP2, taken as reference, confirmed that the latter has a better interaction with the ATP binding site. Probably the benzylidene core, with is more flexible than the p-chloro phenyl

ring of PP2, is responsible for the lower activity of 8. This new N-benzyl oxindole derivative, although owing a low activity against SFKs, could represent a starting point for further optimizations towards more potent Fyn inhibitors.
More recently, bulkier furopyridine-based compounds were developed and evaluated as multi-kinase inhibitors including also Fyn (Figure 6). In particular, two series with different grade of steric hindrance were tested for their ability to block pathological activities of a variety of kinase involved in the development of neuroinflammation in AD besides Fyn, such as cyclin-dependent kinase 1 (Cdk1), cyclin-dependent kinase 2 (Cdk2), c-Jun N-terminal kinase 3 (JNK3) and glycogen synthase kinase 3β (GSK-3β).[66]
In a first series, characterized by a three-fused-ring core, oxime-benzylether moiety attached in position 3 instead of benzylamide fragment seemed to be favoured for Fyn, pointing out compound 9 as the most active on Fyn, with a low micromolar affinity constant, although showing no selectivity among tested kinases. In the second series, based on a four-fused-ring core, both benzylamide-bearing compound 10 and oxime derivative 11 showed similar inhibition values for Fyn compared to 9, however they had a better selectivity among tested kinases, underlining the positive contribution of increased planarity and encumbrance to achieve Fyn selectivity. Particularly, derivative 10 resulted as the most potent and selective compound of both series, with a Fyn affinity constant of 1.71 μM.[66]



Compounds	% inhibition at 10 μM (affinity constants when determined)				
	Fyn[a]	Cdk1	Cdk2	JNK3	GSK-3β
9	56 (K= 2.25 μM)	76	73	50	27
10	54 (K= 1.71 μM)	0	16	16	20
11	64 (K= 1.81 μM)	24	22	14	9

Figure 6. Structures and Fyn inhibitory potencies of furopyridine derivatives compared to other tested kinases. [a]Fyn inhibition determined by an enzymatic assay using radiolabeled ATP.

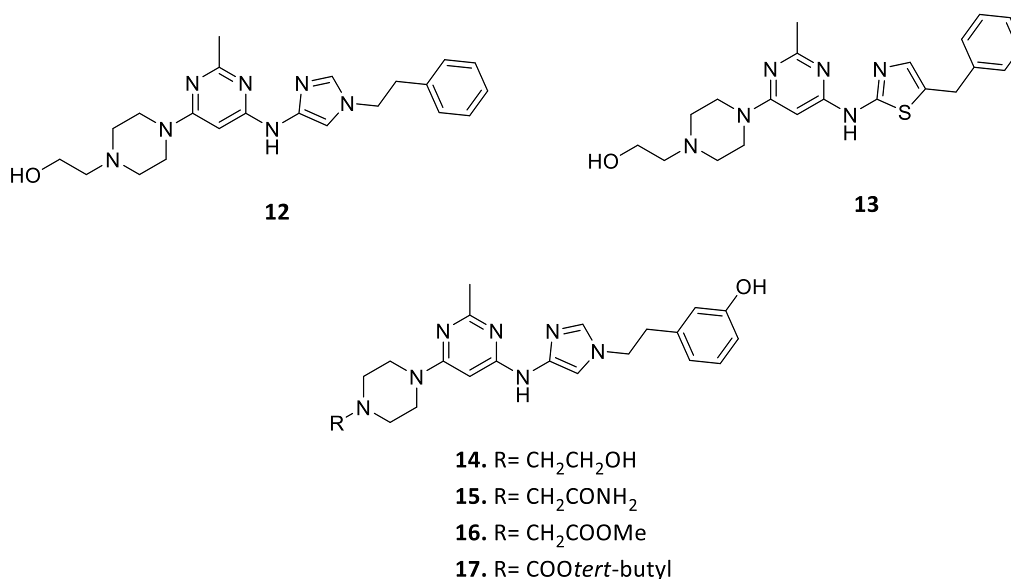
2.2 Aminothiazoles and aminoimidazoles
Through structural simplification and deconstruction of previously discussed bicyclic pharmacophoric scaffold, different potent Fyn inhibitors were developed characterized by nitrogenated monocyclic structures. In particular, starting from the dasatinib pharmacophore these inhibitors bear heteroaromatic moieties such as the pyrimidine scaffold attached to a 2-aminothiazole/imidazole moiety, that establishes hydrogen bonds with key residues within the hinge region.[67]
On this basis, Francini et al. designed compounds with a 2-methylpyrimidine scaffold bound to an aminoimidazole (12) or an aminothiazole (13) moiety (Figure 7), taking inspiration from the structure of dasatinib, which were further evaluated as SFK inhibitors. Then, structure of aminoimidazole derivatives were modified through the insertion of different substituents in aromatic region and piperazine nucleus (14-17, Figure 7), to figure out how these modifications affect the observed activities.[68, 69]
Both aminoimidazole- and aminothiazole-bearing compounds, tested in FRET-based enzymatic assays, were active towards SFKs in the nanomolar range, albeit generally more selective for in respect for Fyn, with compounds 12 and 13 emerging as the most interesting of the series.
Given that these compounds are more active towards c-Src, docking studies were executed on the ATP binding site of this enzyme: results showed that in the aminoimidazole series the nitrogen atom in position 3 of the scaffold undergoes H-bond, the phenyl ring makes van der Waals interactions in the hydrophobic region (HR) of the ATP site and the two-carbon alkyl chain seems to be the optimal distance allowing the aromatic ring to fit in the HR. In the aminothiazole series the phenyl ring establishes van der Waals interactions in the HR and the R substituent goes into the solvent-exposed area of the catalytic site. Moreover, aminothiazole derivatives undertake an alternative binding mode with the pyrimidine being rotated by 180°, the methyl group of the scaffold directed into the hinge region and the R substituent forming electrostatic interactions.
Lastly, these compounds were tested on neuroblastoma cell line (SH-SY5Y) and on leukemia cell line (K562): results were similar to those previously discussed, with compound 13 displaying the lowest IC50 values of 3.6 μM in SH-SY5Y cells and of 0.5 μM in K562 ones, regarding the antiproliferative profile.
The structure of aminoimidazoles was further modified with the aim of optimizing their activity on SFKs in in vitro assays (14-17 in Figure 7).[69] In particular, they investigated: i) the influence on the steric hindrance within the hinge region by inserting alkyl chains of different lengths between aminoimidazole and phenyl ring; ii) the electrostatic interactions

that may be established within the HR by introducing both hydrophilic and hydrophobic groups on the phenyl ring; iii) the influence of various heterocyclic R substituents in the solvent-exposed area.

In summary, inserting a hydroxyl group in meta position of the phenyl ring of 12 causes around ten-fold increase in Fyn inhibitory potency, as it is reported for derivative 14, showing an IC50 of 10 nM, which is similar to that of dasatinib (Figure 7); then, keeping the m-hydroxyl group different substituents were evaluated at the terminal nitrogen of piperazine ring, ranging from amide group (15) to small or branched ester functions (16 and 17 respectively). Interestingly, all tested substitutions at this position were well tolerated, maintaining IC50 in the low nanomolar range (Figure 7).

In cellular assays 16 and 17 showed the highest antiproliferative activities: in SH-SY5Y cells IC50 values were 8.6 and 7.8 μM respectively, that are similar or higher than dasatinib, while in K562 cells IC50 values were 11.7 and 18.9 μM, this time being higher than dasatinib.

These two reported studies investigated the activity of aminoimidazole and aminothiazole derivatives against SFKs on the heels of dasatinib potential: they show a prominent antiproliferative potential, however efforts can be made to obtain a higher selectivity towards Fyn. Importantly, they proved that aminothiazole can be replaced by aminoimidazole to act as hinge binder within the ATP pocket of SFK.



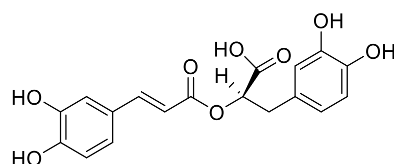
Compounds	IC_{50} ± SD (nM)			
	Fyn	c-Src	Yes	Lyn
12	167 ± 30	220 ± 30	689 ± 100	1300 ± 20
13	252 ± 30	93 ± 4	233 ± 20	318 ± 20
14	10 ± 0.4	93 ± 19	3 ± 1	20 ± 0.8
15	12 ± 2	40 ± 3	3 ± 0.7	10 ± 1.2
16	9 ± 1	40 ± 2	3 ± 1	18 ± 1
17	14 ± 1.5	50 ± 3	7 ± 0.35	26 ± 3
Dasatinib	<9	<4	<3	<0.9

Figure 7. Structures and biological activities of aminoimidazole (**12** and **14-17**) and aminothiazole (**13**) derivatives. Yes alongside Lyn and c-Src belong to SFKs.

2.3 Polyphenols

Natural or nature-based chemical probes have always represented a powerful arsenal to dissect complex cellular mechanisms. Particularly, polyphenols revealed a wealth of beneficial properties ranging from antioxidant to antiaggregating at in vitro and in vivo levels. Kinases' inhibition may account for beneficial effects of many polyphenolic derivatives, albeit categorization between specific binding or indirect interactions has to be done. Notably, some of these direct binders engage allosteric pockets, representing an interesting starting point for drug discovery campaign which could avoid aspecific effects of ATP-binding site competitors.[70] This is the case of rosmarinic acid, a caffeic acid's ester exhibiting strong antioxidant and antinflammatory properties in several animal models.[71, 72] Rosmarinic acid behaves as Fyn inhibitor with micromolar profile by interacting with a binding pocket on the opposite site of ATP-binding pocket (Figure 8). The new site engagement could sterically disrupt physiological ATP binding, thus justifying rosmarinic acid's linear-mixed inhibitory mechanism.[73]

More recently, the indirect inhibition of Fyn observed for rosmarinic acid contributed to define the multi-layered antioxidant profile of the compound, probing its therapeutic potential in the context of Aβ-induced oxidative damage.



Rosmarinic acid

IC_{50} Fyn = 1.3 µM

Figure 8. Structure and Fyn inhibitory potency of rosmarinic acid.

Indeed, rosmarinic acid has shown to reduce ROS production induced by Aβ25-35 insult in PC12 cell line, a cellular model with high sensitivity to oxidative injury used to study neurodegenerative disorders. In particular, in a stratified cellular machinery, rosmarinic acid indirectly leads to Nrf2 accumulation into the nucleus through AKT activation and GSK-3β inactivation, which in turn reduces the amount of phosphorylated Fyn. Therefore, Fyn, once phosphorylated, represents a negative regulator of Nrf2 by inducing its nuclear removal and subsequent degradation. Thus, when Fyn dephosphorylation occurs, phase-II enzymes (e.g., HO-1, NQO1) Nrf2/ARE-mediated increases counteracting oxidative damage, which partially accounts for rosmarinic acid neuroprotective efficacy.[74] Further studies in cancer cell lines demonstrated how rosmarinic acid could also inhibit Fyn expression by tackling the PI3K/Akt/NF-κB signaling pathway.[75]

Polyphenolic compounds exhibit neuromodulation effects through the activation of various signaling pathways involved in neuroprotection as well as scavenging ability against ROS species.[76, 77] In particular, O- and C-glucosylpolyphenols have proved to act as antidiabetic, antiamyloidogenic and antioxidant agents, with the sugar moiety conferring them an enhanced ability of disrupting the amyloid plaques.[78]

Based on these premises, De Matos et al. designed a series of glucosylpolyphenols to explore their therapeutic potential against AD and type 2 diabetes mellitus (T2DM),[79] starting from 8-β-D-glucosylgenistein (Figure 9), a natural compound that proved to be active against Aβ(1-42)-induced neurodegeneration and islet amyloid polypeptide (IAPP) aggregation in diabetes.[80] Through a molecular simplification approach, they first synthesized simplified C-gluycosyl polyphenols derived from acetophloroglucinol and hydroquinone (both showing antidiabetic potential[81, 82]), that later served as starting points to rationally design analogues of 8-β-D-glucosylgenistein.

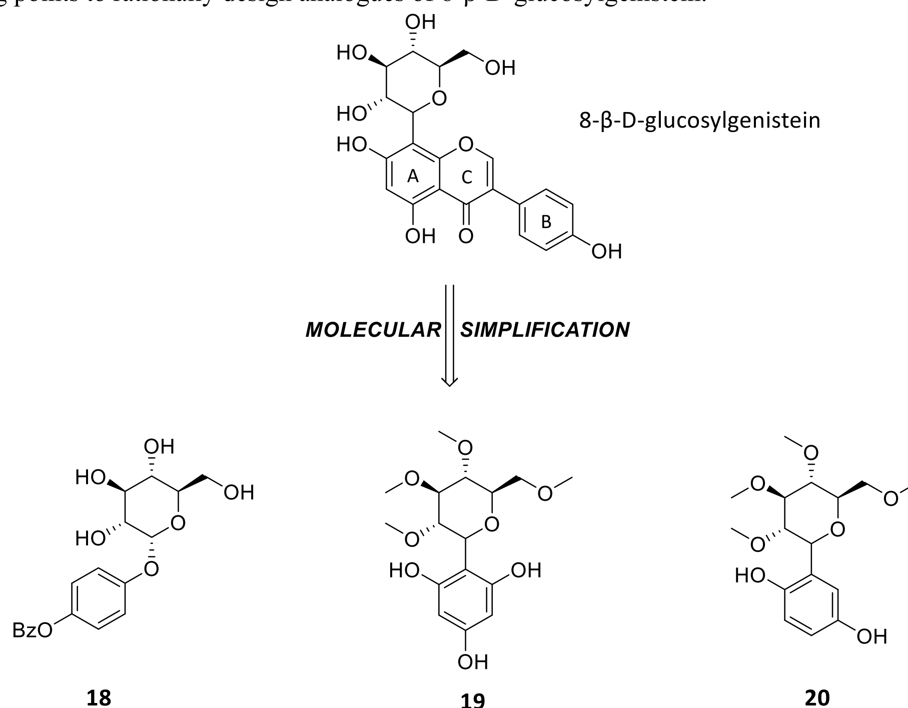
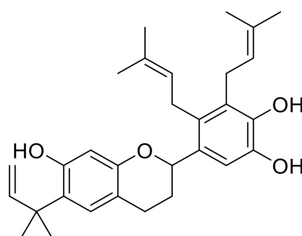


Figure 9. Structures of glucosylpolyphenols.

These compounds bear different hydroxylation patterns and, compared to the original 8-β-D-glucosylgenistein, with the exception of 18 maintain the β-C bond. They were tested for their ability to interact to multiple targets involved in the neurotoxic cascade, i.e., PrPc-Aβos binding interference, inhibition of Aβ-induced Fyn activation and inhibition of cholinesterase.

Compounds tested at 10 µM on HEK 293 cells, previously treated with Aβ oligomers, were able to decrease the Aβos-PrPc binding by 8-38%, with compound 18 being the most effective one.

Assays performed in human induced pluripotent stem cells (hiPSC) showed that per-O-methylglucosylphenol 19 remarkably lowered Aβ-induced Fyn activation.
In a luminescence ADP-Glo kinase assay, the activity of Fyn kinase was assessed in the presence of the synthesized compounds: interestingly 19 and 20 significantly reduced Fyn activity, while adding an acetyl group in the phenyl ring of 19 disrupts the activity.
The study showed that glucosylpolyphenols act as multitarget compounds against various pathological pathways involved in AD and T2DM; in particular, two of these scaffolds are able to directly bind to Fyn kinase, thus paving the way for a new possible therapeutic strategy in tackling these disorders.
Furthermore, a series of prenylated polyphenols demonstrated to reduce mitochondria dysfunction and oxidative stress in a Fyn-mediated manner.[83] Prenylation of polyphenols represents a natural-occurring modification, which occurs in several bioactive flavonoids endowed of antinflammatory activities.[84] Particularly, the antioxidant and cytoprotective properties of a series of kazinols, isolated from plants, seemed to operate through Fyn inhibition, albeit a direct activity was not proved. Interestingly, increasing Fyn inhibition and related antioxidant effects were directly correlated to the number of prenylic substitution and predicted cell permeability, underlining that the more-prenylated kazinols may be more effective through improved cell permeability. Three-substituted Kazinol E (Figure 10) emerged as the best antioxidant of the series with in vitro Fyn selective inhibition around 40% at 10 μM, whereas it was inactive on Src at the same experimental conditions. Moreover, it attenuated Fyn phosphorylation at Tyr417, abolished PDGF (platelet derived growth factor)-induced Fyn activation, like the Src family inhibitor SU6656 did in HepG2 cells, thus corroborating the hypothesis that it may directly inhibits Fyn. As a confirmation of Kazinol E inhibitory activity, two Fyn's negatively modulated downstream pathways, such as LKB1-AMPKα and Nrf2, were activated. The same Fyn-mediated cytoprotective effects were also confirmed after a cellular oxidative insult.[83]



Kazinol E

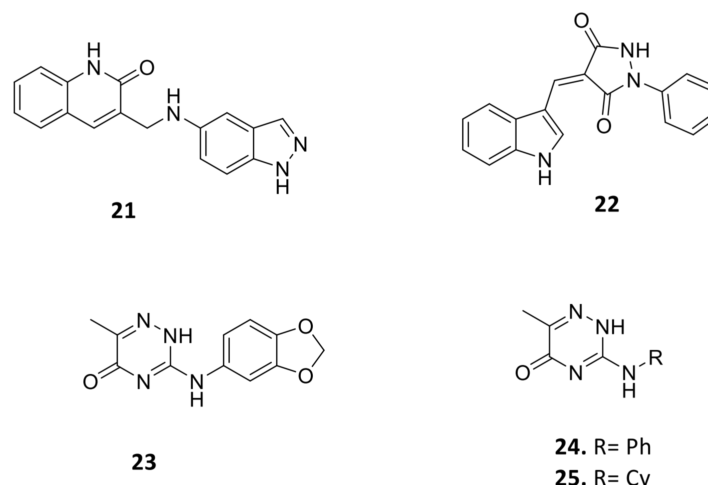
Figure 10. Most potent Fyn inhibitor among kazinol derivatives.

Remarkably, kazinols proved to inhibit Fyn in vitro, exerting promising antioxidant activities. Prenyl-substituted derivatives exhibit stronger inhibitory activity, probably due to the increased cellular permeability more than a greater enzyme affinity. Indeed, three-prenylated Kazinol E showed, respectively, seven, five and more than forty times higher EC50 values for cell viability than double-, mono- and non-prenylated analogues, whereas in vitro kinase inhibition for all prenylated polyphenols remained around the same amount.[83]

2.4 Computational studies

Pharmacophore is a concept that describes the steric and electronic features of a molecule to modulate a biological response[85] and is mainly used in virtual screening (VS), lead optimization and de novo design.[86] Over the years this concept has been used along with the one of "fingerprint", which describes a molecule as a unique data string and can be employed to understand similarities between molecules as well as to analyze those features triggering a biological response.[87]
Although pharmacophore-based methods have proved useful, over times new technologies have been tuned, generating complementary pharmacophores of protein binding sites together with ligand-based pharmacophores: this is the case of Fingerprint for Ligands and Proteins (FLAP), that identifies complementary pharmacophoric features of a given ligand for a protein binding site.[88] In particular, chemical probes are used to scan the enzyme cavity in order to detect favorable and unfavorable interactions and then calculations through the use of energy-based and space-coverage function lead to the generation of pharmacophore property-based points.[89]
In this context, Poli et al. identified new potential Fyn kinase inhibitors by using a FLAP-based approach and then enzyme and cellular-based assays were carried out to prove the inhibitory activity.[90] The FLAP-based analyses by using a ligand-based VS were carried out on Asinex database, a screening library providing lead-like molecules, macrocycles and fragments for drug discovery (http://www.asinex.com).
In an early filtering process, aimed at reducing the number of compounds to be analyzed, around 7500 molecules resulted as potential inhibitors, that were subsequently subjected to a docking study. Here around 250 molecules were predicted to be active and advanced to the next filtering step, namely their ability to bind to the hinge region within the ATP binding site of Fyn kinase. Indeed, this pocket is important, since many kinase inhibitors interact with the ATP binding site[36] by forming H-bonds. Therefore, only compounds being able to establish at least two H-bonds with the nitrogen of Met345 residue and with the oxygen of Glu343 and of Met345 ones in the hinge region of the ATP binding pocket were taken into account: as a result, only 69 molecules met these criteria, so they underwent molecular dynamics (MD) simulations

in order to assess the ligand-protein interactions. There, those capable of maintaining H-bonds with Glu343 and Met345 residues for at least the 90% of the entire MD process went forward. As a result, thirty-six compounds advanced and they were grouped according to their central scaffold, thus having 8 representative molecules.



Compounds	IC_{50} Fyn (μM)	IC_{50} MDA-MB-231 (μM)	IC_{50} A549 (μM)
21	15	136.4 ± 17.5	117.7 ± 13.9
22	11.5	62.7 ± 13.1	100.1 ± 17.5
23	4.8	145.0 ± 13.3	198.2 ± 9.1
24	>100	/	/
25	0.76	34.8 ± 4.6	101 ± 10.8
PP2	0.063	12.0 ± 1.4	14.3 ± 2.0

Figure 11. Structures and biological activities of compounds discovered through FLAP-based method.

To verify the reliability of this protocol, these eight molecules were tested in biological assays: in a luminescence kinase assay 21, 22 and 23 showed the highest activities with IC50 values of 15, 11.5 and 4.8 μM for Fyn, respectively (Figure 11). A test carried out on human breast MDA-MB-231 and human nonsmall-cell lung A549 cancer cells, were Fyn plays a key role in tumor progression,[91, 92] showed that 21, 22 and 23 improved the cell viability with IC50 values ranging from 62.7 to 198.2 μM.
Later, the compound with the best inhibitor activity, namely 23, was subjected to consensus docking, MD simulation and relative binding free energy assessment in order to shed light into its binding mode within Fyn catalytic site; structural optimization were also made with the aim of developing more potent derivatives.
The MD study showed that the triazinone scaffold of 23 forms two H-bonds with the nitrogen of Met345 and the oxygen of Glu343 in the hinge region of the enzyme, while the methyl group in position 6 of the scaffold is projected into the solvent-exposed area of the kinase. The benzodioxole moiety interacts through lypophilic bonds as well as through an H-bond with Lys299 residue and the amino group linking the scaffold to the benzodioxole establishes a H-bond with an hydroxyl group, although this interaction is not much stable. A possible explanation is that the amine group is highly conjugated with the benzodioxole and the triazinone scaffold and this does not allow the torsion of nitrogen, leading to a planar geometry of the whole molecule which impedes an efficient interaction between the amine moiety with the oxygen of Thr342.
The benzodioxole ring was then replaced by a phenyl ring and a cyclohexyl one, in order to get a deeper insight onto the role of H-bonds formed with Thr342 and Lys299 residues[93]. Compared to 23, compound 24, bearing a phenyl moiety, had a less stable interaction with the oxygen of Thr342, while compound 25, having a cyclohexyl ring, had a more stable H-bond with Thr342, due to the higher degree of torsional freedom of the amine group (Figure 11). In general, compound 25 has a higher affinity to Fyn kinase than 23, due to the more stable interaction with Thr342 that compensates the lack of the H-bond established with Lys299 residue.
Given these outcomes, the activities of 24 and 25 were tested in a luminescence kinase assay: as expected from MD simulations, compound 25 had an IC50 in the submicromolar range, thus resulting in the most active, with a six-fold increase in inhibition potency with respect to 23, while compound 24 had an IC50 higher than 100 μM.
Derivative 25 was also tested in human breast MDA-MB-231 and in human nonsmall-cell lung A549 cancer cells and compared to PP2. They showed IC50 values of 34.8 and 101 μM respectively, that were higher than the ones of PP2 (12 and 14 μM respectively) but lower than the ones of 23.
The two studies successfully applied an extensive computational study based on a FLAP approach to identify new Fyn inhibitors interacting with the hinge region of the ATP binding pocket. The following biological assays confirmed the results obtained from the FLAP analysis, with a cyclohexylaminotriazinone derivative that acted as a Fyn inhibitor in the submicromolar range.

CONCLUSION

The failure of the current therapies against most of the neurodegenerative diseases, including AD, boosted the research to explore novel molecular mechanism at the basis of their pathogenesis.

Several lines of evidence point out a role for Fyn kinase, belonging to the SFKs, in neuroinflammatory processes and in the pathogenesis of AD. The close interplay with both Aβ and Tau makes Fyn kinase as a noteworthy therapeutic target to contrast the major pathologic hallmarks of AD. In this context, in the last decades, the search for selective Fyn inhibitors to counteract neurodegeneration gained increasing attention for therapeutic drug development.

Herein, we have reported the most promising Fyn inhibitors and their pharmacological characterization, both in enzymatic and in cell assays, highlighting most recent progresses in the development of selective pharmacological tools in the context of neuroinflammatory pathways. Non-selective Fyn blockers such as saracatinib and dasatinib, in addition to being the subject of clinical trials for their potential repositioning in AD therapy, embodied the starting point for the selection of key scaffolds for the design of new small molecules that effectively inhibit Fyn. Also in this context, natural products have proved to be a valuable source of leads for drug discovery. Polyphenolic compounds such as rosmarinic acid and kazinol demonstrated to act as Fyn inhibitors through both direct binding to Fyn and indirect mechanisms involving Nrf2, opening new insight in the binding mode of Fyn inhibitors on kinase conformation.

However, from literature analysis it emerges that designing selective inhibitors represents a significant challenge due to the highly conserved ATP-binding sites of kinases. In this regard, FLAP-based approach here described, together with enzyme and cellular-based assays, gave a significant contribute to identify new potential Fyn kinase inhibitors.

Fyn controls many different processes in cells but its specific role in neurodegeneration needs further elucidating studies, in particular to distinct Fyn involvement respect to other kinases. Recently, it was developed a new biosensor to monitor the active form of Fyn in mammalian cells and, in the future, this could allow a more efficient screening of drug-like molecules to treat cancer and neurodegenerative diseases linked to Fyn activity.[94]

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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