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HETERO-BIFUNCTIONAL MOLECULES AS POSSIBLE THERAPEUTICS FOR
THE TREATMENT OF ALZHEIMER'S DISEASE

Presentata da: Alan Santini

Coordinatore Dottorato

Carmela Fimognari

Supervisore

Andrea Milelli

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Preface

This PhD thesis has been carried out at the Department of Life Quality Studies, Alma Mater Studiorum-University of Bologna (Italy), under the supervision of Prof. Andrea Milelli. The whole PhD thesis is devoted to the development of new agents for the treatments of neurodegenerative diseases, in particular Alzheimer's Disease. This thesis is divided in two main chapters: in the first chapter the development of new dual glycogen synthase kinase 3 β (GSK-3 β) / histone deacetylase (HDAC) inhibitors is described. Here two different projects are reported: the first one reporting compounds characterized by an ATP-competitive mechanism of GSK-3 β inhibition while the second focused on a series of compounds characterized by a non-ATP competitive mechanism for GSK-3 β inhibition. Design, synthesis, and preliminary biological evaluation are reported for both the projects. In the second chapter, the design and synthesis of a novel PROTAC directed towards GSK-3 β is reported, along with results and discussion of the biologic tests.

This entire work would have not been possible without the valuable collaboration of research groups coordinated by Prof. Vincenza Andrisano (UNIBO), Prof. Elisabetta Ciani (UNIBO), Prof. Andrea Tarozzi (UNIBO), Prof. Angela De Simone (UNITO) and Prof. Francesca Spyraakis (UNITO).

Abstract

Alzheimer's disease (AD) is the most common form of dementia, with a huge social impact for what concerns incidence, prevalence and cost of care. AD is currently affecting more than 50 million people worldwide and due to its debilitating nature, it causes enormous financial and emotional stress on patients and caregivers. For these reasons, in recent years attention towards this disease has risen in search for discovery and development of a drug that can stop it. Indeed, approved therapies for moderate to severe AD provide only temporary and incomplete symptomatic relief and only represent a palliative tool to slow down the clinical course of the disease. The cause for the incredible high attrition rate for AD drug discovery has been attributed to several factors, including the fact that the AD pathogenesis is not yet fully understood. Nevertheless, what is increasingly recognized is that AD is a multifactorial syndrome, characterized by massive deposits of amyloid- β ($A\beta$) peptide, neurofibrillary tangles (NTF) of the hyper-phosphorylated tau protein (P-tau), inflammatory mediators and reactive oxygen species (ROS), which may lead to neuronal death.

Given the complexity and multifactoriality of the disease, it is widely accepted that a chemical entity able to modulate more than one target would bring benefit to the therapy of AD. Based on this approach, the research group in which I spent my PhD period in 2019 reported the first dual inhibitor targeting two of the main enzymes involved in the onset of AD, glycogen synthase kinase 3 β (GSK-3 β) and histone deacetylase (HDAC). In the first chapter of this thesis, there are reported two projects regarding the design and synthesis of two new series of GSK-3 β /HDAC dual inhibitors. The first group of compounds were designed based on AR-A014418 as GSK-3 β inhibitor and Vorinostat and Entinostat as HDAC inhibitors. Therefore, two different series of compound were synthesized, characterized by two different zinc binding groups (ZBGs), the portion of the molecule able to bind HDAC in its active site. In particular, a series is characterized by the presence of an hydroxamic acid, characteristic of Vorinostat, and the other one by a benzamidic group, characteristic of Entinostat. Regarding the portion binding GSK-3 β , the ureic group of AR was taken as a common scaffold for both the series. All the compounds synthesized have been evaluated for their inhibitory activity towards the target enzymes. The best compounds of the series were selected for further biologic investigation to evaluate their properties. The second project focused on the design of non ATP-competitive GSK-3 β inhibitors combined with HDAC inhibition properties. In this case as GSK-3 β binding moiety the structure of thiadiazolidindione, characterizing Tideglusib, was selected. This compound showed high selectivity towards GSK-3 β and it demonstrated positive results in different animal models, where it showed improvement in cognitive performance, reduced tau phosphorylation and decreased $A\beta$ production. The heterocyclic ring of Tideglusib was combined with the

hydroxamate group of Vorinostat to generate a series of compounds that have been evaluated for their inhibitory ability toward GSK-3 β and HDAC and also in this case, the best compounds of the series were selected for biologic investigation to further evaluate their properties.

In chapter 2, the design and synthesis of a GSK-3 β -directed Proteolysis Targeting Chimeras (PROTAC) is reported. PROTACs are a new technology in drug discovery that gained enormous attention from the scientific community for their promising properties. Indeed, they act with a new paradigm that modulate target activity through degradation rather than inhibition. This mechanism involves the binding of a protein of interest (POI) and, at the same time, the recruitment of the E3 ubiquitin ligase, leading to ubiquitination and degradation of the target. One of the major advantages of PROTACs is their catalytic activity, thus avoiding high concentration side effects. The design and synthesis of a small series of GSK-3 β -directed PROTACs was achieved employing two different GSK-3 β ligands and the pomalidomide as E3 ligase recruiter element. *In vitro* assays were performed to evaluate the GSK-3 β -degradation ability, the effective involvement of E3 ubiquitine ligase in the process and their neuroprotective abilities. In conclusion, all these studies may represent a promising starting point for the development of new interesting molecules useful for the treatment of AD.

1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common form of dementia, being responsible for the 60-80% of the cases, and affects over 55 million people worldwide. These numbers are increasing every year, with an estimation of 150 million of patients by 2050.¹ AD is a neurodegenerative disease, so it worsens over time. The symptoms span from loss of memory to altered mood and behavior, which can interfere with the normal daily routine. In the final stages of the disease, the patient can hardly recognize family members and start being suspicious about them; speaking and walking can also become very difficult, as well as remembering anything occurred short time before.²

The onset of the disease is not completely understood, with several processes and metabolic pathways involved.³ It is believed that changes in the brain cells related to age are the main key, along with genetical and lifestyle factors. However, this is not always a valid scheme. Based on the age where the first symptoms happen, AD can be divided into early onset AD (EOAD), which develops under the age of 65, and late onset AD (LOAD), which is the most frequent, in case the patient is over the age of 65.⁴ Moreover, a further classification can be made according to genetic components:

- autosomal-dominant AD afflicts more than three members of the same family of different generations, with a tendency of developing in early ages. In most of the cases, genetical mutations of presenilin 1 and 2 (PSEN1 and 2) and Amyloid Precursor Protein (APP);⁵
- familiar AD occurs in more than one family member, within three degrees of relationships;
- sporadic AD, which is characterized by a single case in a family or separate cases with more than three degrees of relationship. In most of the cases, it happens in later ages.

The disease was firstly described by Alois Alzheimer, after whom it was named, in 1907, when he reported the case of a 55-year-old woman, Auguste Deter, in a conference held in Tübingen.⁶ He described neuropathological lesions and neurofibrillary tangles (NFTs), which led to gradual memory loss, psychosocial disability and even hallucinations. The surprising fact was the age of the patient, because most of the cases of dementia were usually noticed around the age of 70. After the death of Mrs. Deter, further examinations were performed on the brain cells, using the silver staining technique. They highlighted the presence of several abnormal structures, in the form of fibrils and miliary foci, which were subsequently called NFTs⁷ and β -amyloid ($A\beta$)⁸ plaques. Those structures are considered the main markers of AD (Figure 1), making it recognizable from normal dementia, and strictly connected to cognitive decline.

$A\beta$ plaques are structures which can be found outside the cells of hippocampus, amygdala, upper and lower temporal lobes. The central part is made of aggregated fibrils of $A\beta$ peptide, surrounded by

dystrophic neurites with abnormal axons. This kind of plaques is present even in brain of well-being old people, but in lower numbers⁹. NFTs are intracellular structures with a double helix scaffold made of phosphorylated tau protein, a cytoskeletal protein associated with the microtubules. They can be found in neurons of hippocampus, amygdala, basal prosencephalon and entorhinal cortex.⁷

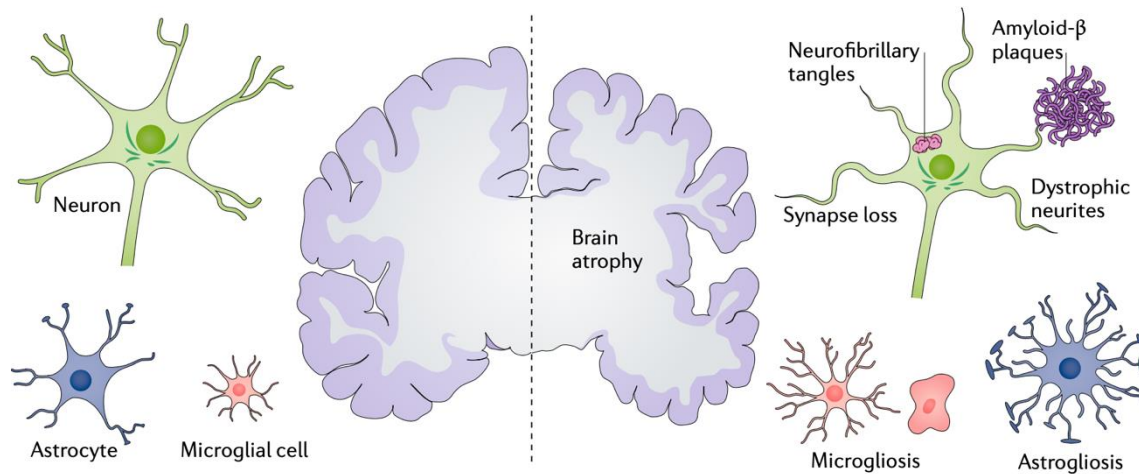


Figure 1. Histologic alteration of AD brain.¹⁰

In addition to NFTs and A β plaques, other alteration contributes to AD, such as neuronal loss deriving from oxidative stress¹¹ and inflammation.¹² This is frequently observed in areas like hippocampus, frontal, temporal and lower parietal cortex and Meynert basal nucleus, which is the main region responsible for acetylcholine (ACh) decreased levels. Then there is cerebral amyloid angiopathy, in which blood vessels in central nervous system (CNS) are partially blocked by A β deposits. Third, excitatory synaptic dysfunction, considered one of the first symptoms before neuronal loss. Finally, inflammation and reactive gliosis: tissue observation showed activated glial cells near A β deposits, mainly near A β plaques.

1.1 Etiopathogenesis

AD is considered a multifactorial pathology whose etiopathogenesis is not clear yet. Several hypotheses were formulated, considering genetical, biological and environmental components.

1) Cholinergic hypothesis

This theory was formulated for the first time in 1976 by Peter Davies and David Bowen, after they noticed that ACh is lacking in brain of AD patients.¹³ ACh is a neurotransmitter involved in attention, learning and mnemonic processes.¹⁴ ACh is synthesized in neurons by choline acetyl transferase (ChAT) from choline and acetyl-CoA and degraded by acetylcholinesterase (AChE).¹⁵ This enzyme is present in synapses and is responsible for the signal interruption. ACh can be degraded also by other enzymes, such as butyrylcholinesterase (BChE), even if with lower affinity. In AD patients, a loss up to 60-90% in ACh is registered in cortex and hippocampus neurons, similar to the one in Meynert basal nuclei (30-90%).¹⁶ Studies on rats showed that cholinergic antagonists, in particular antimuscarinic agents, induced an impairment in memory. Further studies on advanced age patients with AD highlighted damages on pathways associated with cognitive decline and general low level of ACh. From this evidence, the cholinergic hypothesis was developed, stating that a loss of cholinergic activity, in addition to advanced age, results in cognitive decline.¹⁷ In addition to cholinergic system, the ongoing of the disease is associated to alteration of the glutamatergic system. Indeed, neurotoxicity in AD is correlated also to overactivation of N-methyl-D-aspartate (NMDA) glutamate receptors.¹⁸ Those are the most sensible to calcium ion among the glutamatergic channel and the high concentration of Ca^{2+} produces damages and cell death. Excitotoxicity NMDA related, in combination with lower ACh activity, can lead to excitatory neurons loss in pyramidal cortex, which contributes to the neuropsychological deficit in AD patients.¹⁹ This evidence originates a glutamatergic theory that strengthen the cholinergic one.

2) A β hypothesis

In 1991, John Hardy and David Allsop stated that the main event leading to AD was the presence in brain tissue of plaques and aggregates made up primarily by A β peptides. This protein is generated by APP through the activity of enzymes known as secretases (α , β and γ -secretase).²⁰ According to how APP is cleaved, two different pathways can be observed (Figure 2). Non-amyloidogenic pathway originates with the cleavage of APP at residue 687 by α -secretase, which releases a N-terminal soluble fragment and leaves a C-terminal fragment made up of 83 amino acids into cell membrane. Amyloidogenic pathway, on the other hand, involves in the first phase β -secretase, which cleaves APP in a soluble fragment, thus leaving a C-terminal fragment of 99 residues with A β sequence at its N-terminal region. Then, γ -secretase cuts this fragment, generating A β peptide, which can be 40 or 42 residues long.

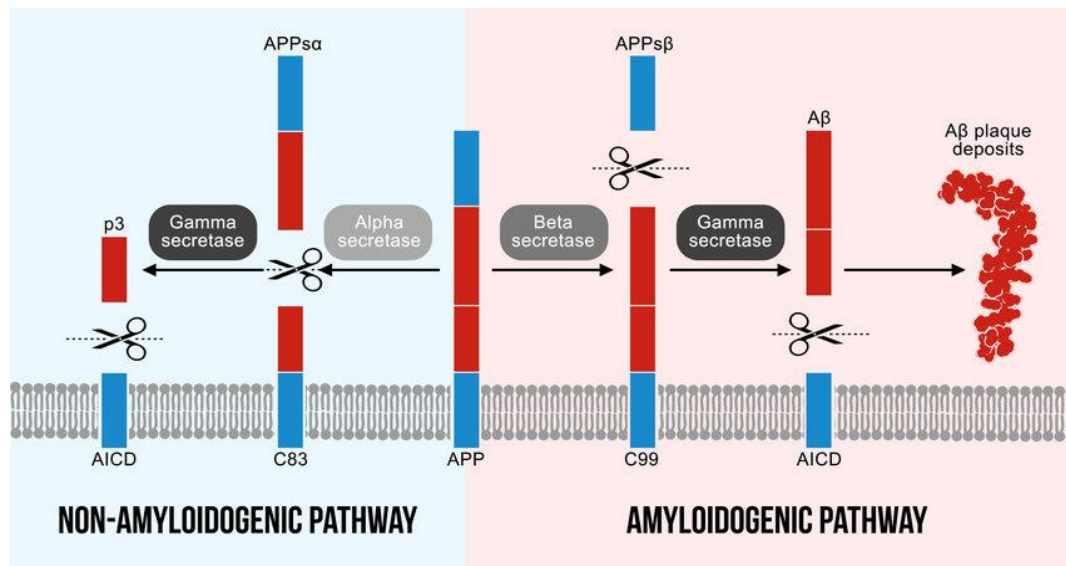


Figure 2. Non-amyloidogenic and amyloidogenic pathways representation.²¹

The more toxic fragment is the longer one, because it misfolds into a sheets structure that originates oligomers, proto-fibrils, fibrils and last insoluble plaques.²² Among these structures, oligomers are considered the more toxic for their interference in synaptic signaling, which leads to neuronal death. An example is the interaction with NMDA receptors and α -amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA) receptors, with loss in synaptic plasticity and memory formation.²³ Nevertheless, other A β structures damage neurons by compromising cellular membrane²⁴ or activating microglia and astrocytes, which product toxic and inflammatory intermediates, like nitric oxide, cytokines and reactive oxygen species (ROS) that cause neuronal death through apoptosis or necrosis.^{25, 26} A β seems also to be able to release Ca^{2+} from endoplasmic reticulum, increasing calcium concentration in mitochondria and damaging them. In this way, ROS and proapoptotic factors are released. Moreover, high cytosolic concentrations of Ca^{2+} are showed to induce A β aggregation and amyloidogenic pathway.²⁷ From a genetic point of view, mutations of genes for PSEN1 and PSEN2, as well as APP (50% of cases of autosomal-dominant AD), produce more-efficiently processable A β protein and increased activation of β - and γ -secretase.

The A β theory is the most studied and accepted, for its tight correlation with symptoms and multiple ways that lead to the onset of AD.

3) Tau protein hypothesis

This hypothesis indicates that excessive or abnormal phosphorylation of tau protein turns normal tau into coupled helicoidal filament (PHF-tau) and NFT.²⁸ Tau belongs to microtubules-associated

proteins MAP family proteins that bind tubulin subunits and stabilize microtubules. MAP is a large family of proteins, divided in two classes. Into class 1 there can be found MAP1a and MAP1b, while class 2 owns MAP2, MAP4 and tau protein. MAP2 and tau are the most studied and are located in dendrites and in axons, respectively. The C-terminal region of these proteins binds microtubules, while the N-terminal region is extended outside to interact with other proteins.²⁹ This bond is regulated by phosphorylation. However, excessive phosphorylation disrupts microtubules, so phosphorylated tau protein (ptau) can aggregate and form NFTs. This process is normally highly regulated, but in pathological conditions phosphorylation become irreversible and the mechanism collapses through activation of several kinases, such as glycogen synthase kinase 3 β (GSK-3 β) and cyclin-dependent kinase 5 (CDK-5).³⁰ On one side, NFTs compromise neurons and synaptic function, while on the other side ptau recruit normal tau, leading to microtubules disruption and alteration in axonal transport and neuronal plasticity. This mechanism can be also influenced by calcium concentration.³¹

Tau is a fundamental protein for the correct development of neurons, being involved in the formation of cytoskeleton, which gives the shape to the cell and guarantees axonal transport. Its action is regulated by microtubules affinity-regulating kinase 4 (MARK4), a serine/threonine kinase which phosphorylates MAP, making them separate from microtubules. A genetic modification of MARK4 can induce a tau modification generating a pathologic form which aggregates in an easier way and become insoluble.³² Moreover, tau released after neuronal death results toxic for near cells. Indeed, tau has an affinity ten times greater than ACh for M1 and M3 receptors, also managing to avoid their desensitization. Activation of muscarinic receptors increases intracellular levels of calcium, damaging neurons and circuits involved.³³

It is clear how tau plays a fundamental role in AD onset. However, it is not sufficient to explain all symptoms of AD. It is theorized that tau and A β are strictly connected and together contributes to the disease (Figure 3): at first, it seemed that they fortify each other, but a recent study showed that is A β to trigger tau pathogenesis.³⁴ Nevertheless, tau aggregates can be observed even in early stages of AD, making them valuable biomarkers for early diagnosis.³⁵

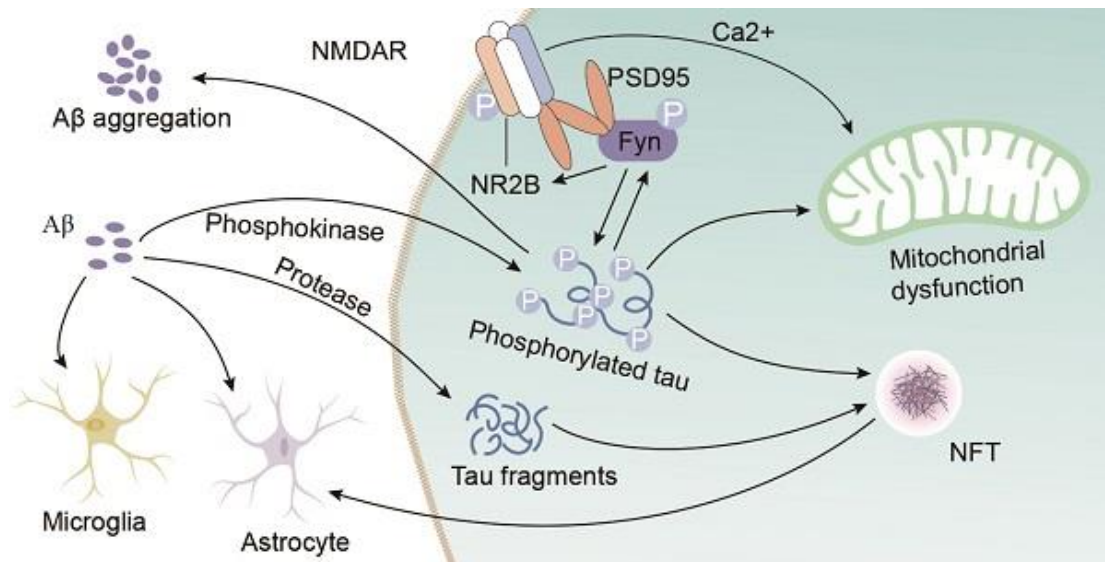


Figure 3. Schematic representation of the interaction between A β and tau.³⁶

4) Inflammatory hypothesis

Inflammation is one of the most important hypotheses to describe AD pathogenesis. It is based on the principle that progressive inflammation leads to neurodegeneration.³⁷ At this point there is no specific inflammatory factor associated with AD, but it is suggested that inflammation can be triggered by AD markers (NFT and A β) or fragments of degenerated neurons. It is thought that these factors excite glial cells (microglia and astrocytes) to release pro-inflammatory cytokines, like tumoral necrosis factor α (TNF- α), interleukin-1 β , interleukin 16 (IL-1 β and IL-16) and C-reactive protein (CRP). Increased levels of these factors can produce more ptau, A β and pro-inflammatory molecules.³⁸ Some evidences of this theory are the correlation between A β plaques and active microglia, reactive astrocytes and inflammatory factors mentioned before, the ability of TNF- α , IL-6 and IL-16 can trigger the production of A β 42 and tau phosphorylation, which stimulates the production of the same factors (Figure 4).³⁹ Inflammatory intermediates are neuroprotective at low levels, but can cause neurodegeneration at higher levels: this implies that every process which can cause inflammation contributes to onset and progression of AD.⁴⁰ To support this theory, there are studies in which AD patients were treated with anti-inflammatory drugs. Although these studies are still at the beginning, encouraging results showed that the contrast of inflammation manages to slow AD pathology.⁴¹

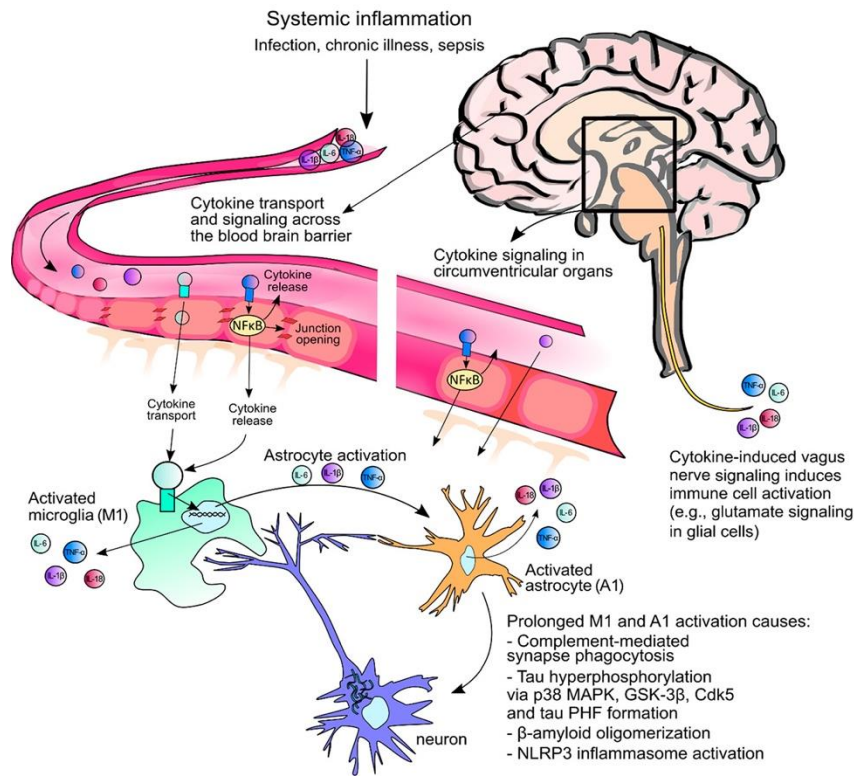


Figure 4. Impact of inflammation on AD onset.⁴²

5) Oxidative stress hypothesis

Oxidative stress is the perturbation of normal balance of oxidant species, usually radicals based on oxygen (ROS), and antioxidant defense mechanism that neutralize them. It can be the cause of early aging, as well as some chronic and degenerative diseases. In detail, CNS is one of the principal targets of ROS and oxidative stress, which is consequently considered a cofactor in neurodegenerative diseases. Oxidative stress in AD is correlated with mitochondrial dysfunction, which lead to excessive ROS production and damages to synaptic neurons (Figure 5).⁴³ In AD patients it is observed that progressive increase in oxidants is paired with loss of antioxidant elements. It was demonstrated that Aβ accumulation can trigger oxidative stress.⁴⁴ This leads to oxidation and nitration of proteins. The use of natural antioxidants found in food and medicinal plants, like resveratrol, curcumin, epigallocatechin gallate (EGCG) and others, showed significant impairment in AD patients, preventing ROS excessive generation and relative oxidative stress.⁴⁵

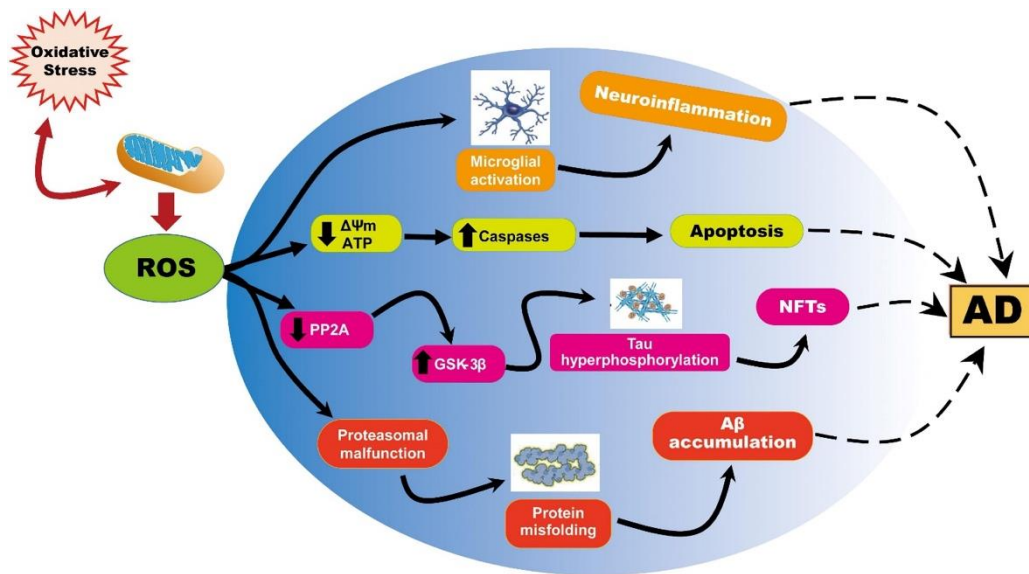


Figure 5. Role on ROS in AD pathology.⁴⁶

6) Metal hypothesis

Metal ions, in particular iron, zinc and copper (Fe, Zn and Cu), are present in trace amount in the brain, where they contribute to maintenance of its function. If the metal balance is impaired, neurodegenerative events can occur.⁴⁷ There are evidences which relates metal homeostasis to AD. Bush and Tanzi showed that A β oligomerization depends on Zn and Cu (Figure 6).⁴⁸ Cu binds single monomer, creating crossed species that are very difficult to eliminate for the cell, leading to neuronal death. Moreover, Cu can generate free radicals that cause oxidative damages. Zn is responsible for precipitation of A β . A β plaques seize Zn and Cu, blocking these metals from doing their normal action, although there is no evidence of negative effects of reduced level, as observed in mice model.⁴⁹ Targeting interaction between A β and metals seems to be a therapeutic approach capable to normalize metal homeostasis and reduce A β toxic oligomers in brain. The most reasonable approach was the design of molecule able to chelate those metal involved, but this was not successful, maybe due to multifactorial nature of AD.⁵⁰

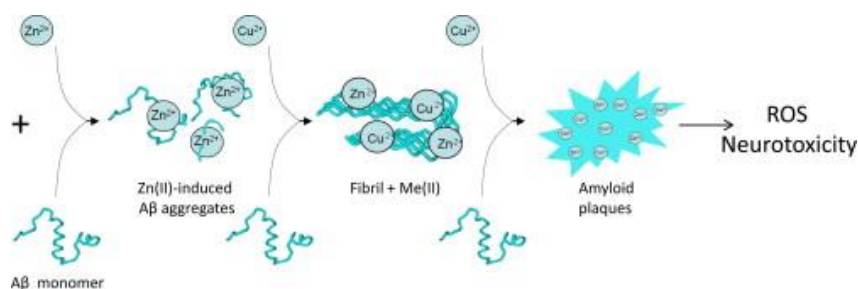


Figure 6. The metal hypothesis of AD.⁵¹

7) Autophagy

Autophagy is a biological process fundamental for cells survival, through the elimination of mutated proteins, damaged mitochondria and other waste materials. In this way, those substances can be degraded and recycled to synthesize new components. Recent studies indicate that autophagy can be involved in tissue degeneration linked to AD.⁵² When this mechanism abnormally works, waste materials start to accumulate inside neurons, resulting in oxidative stress and neuronal death.⁵³ The regulation of autophagic processes can become a winning strategy to protect cells and find new therapeutic approaches for AD.

1.2 Therapeutic approaches to AD

The complexity of AD has encouraged the research of ligands for almost every pathological mechanism involved in the disease. Over the years, the appearance of several hypothesis about the onset of the disease allowed scientists to design different therapeutic strategies. To date, only five small molecules were approved by US Food and Drug Administration (FDA) for the treatment of AD, in addition to two recent monoclonal antibodies, Aducanumab and Lecanemab.^{54, 55} The first class of drugs successfully used for AD were AChE inhibitors (AChEIs), with tacrine being approved in 1993, even though later it has been withdrawn due to hepatotoxicity. Other AChEIs available are donepezil, rivastigmine and galantamine. The last drug approved by FDA was memantine, a NMDA antagonist, approved in 2003 (Figure 7).

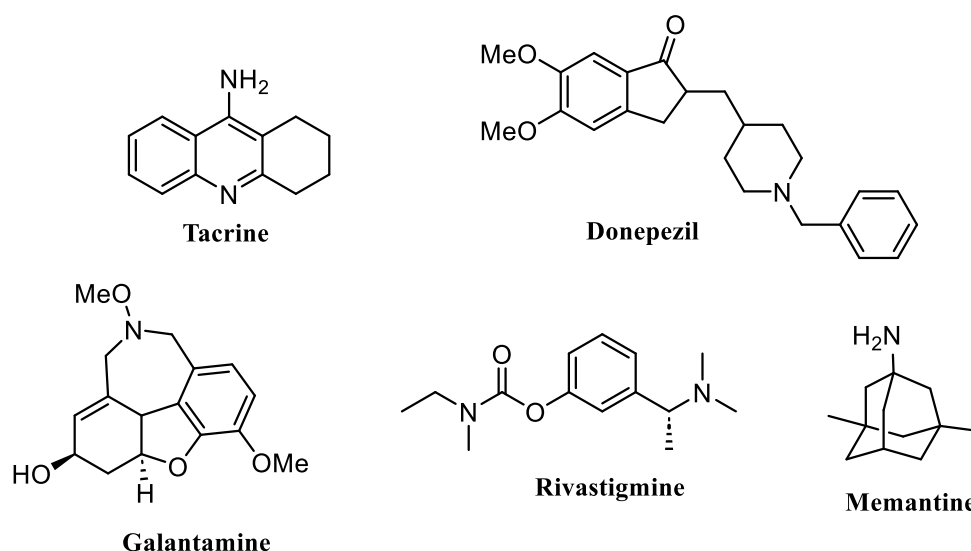


Figure 7. Structures of approved drugs by FDA.

- **Cholinergic therapy:** AChEIs were the first class of drugs approved for AD therapy. Tacrine (or tetrahydroaminoacridine) was the very first drug for AD treatment approved in 1993,⁵⁶ under the name of Cognex®. It is also a competitive ligand for muscarinic receptors,⁵⁷ and inhibits MAO A and MAO B,⁵⁸ with high lipid solubility. However, it suffers from several disadvantages, such as low oral bioavailability and side effects. In particular, it is metabolized to quinone derivative in hepatic cells and its hepatotoxicity is the main cause for its market retirement. Donepezil (Aricept®) is a reversible AChEI approved in 1997 able to improve cognitive functions, daily life activities, behavioural and neuropsychiatric symptoms in patients with mild or severe AD. Rivastigmine (Exelon®) is another selective, reversible AChEI, active also on BChE. It was approved in 2000 as a transdermal patch, which efficiently improves cognitive and global functions. Galantamine (Reminyl®) was approved in 2001 as a selective and reversible inhibitor of AChE able to modulate also nicotinic receptors (nAChRs).^{59, 60} It improves cognition in patients with AD by increasing ACh levels and, therefore, cholinergic transmission.

Recently, the focus was on M1 receptors agonists, since M1 modulation can mediate several cognition promoting effects.⁶¹ However, the lack of selectivity produces several side effects, which critically limits their use in AD. M2 receptors are also studied, since they are involved in learning, memory and neuronal plasticity.⁶² Unfortunately, developed M2 antagonist (SCH57790 and BIBN-99), although their ability to raise extracellular levels of ACh, showed poor pharmaceutical profile. Lastly, some nAChR agonists (SIB1553A and ABT418) were

developed to increase nicotinic activity, which is reduced in AD.⁶³ The use of these agonists is limited by the fast desensitization of nAChRs.

In general, use of AChEIs produce a temporary stabilization of cognitive decline and delay in neurodegeneration, as well as interference with A β accumulation and precipitation. The ability to contrast two important factors in AD onset, which are A β plaques and ACh breakdown, leads to believe that AChIs will continue to be constantly present in anti-AD drug discovery.

- **Anti-amyloid strategies:** the modulation of A β production can be achieved by targeting the various enzymes that participates in amyloidogenic pathway.⁶⁴ The strategy is to block the activity of β - and γ -secretase, responsible for A β production, and/or to activate α -secretase, which cleaves APP to start the non-amyloidogenic pathway, to block the aggregation of A β fibrils and to catalyse its degradation. β -secretase (BACE-1) is the aspartic protease that cleaves APP at the β -secretase site and has emerged as a promising target, due to evidence that mice lacking in BACE-1 do not produce A β .⁶⁵ OM99-2, a peptidomimetic which represent the first generation of BACE-1 inhibitors, has an IC₅₀ of 2 nM, but has a poor oral bioavailability and, like other peptidomimetics, hardly cross BBB. For these reasons it was modified to reduce its low molecular weight, obtaining two compounds characterized by a IC₅₀ of 5 and 20 nM, but better pharmacokinetic profile.⁶⁶

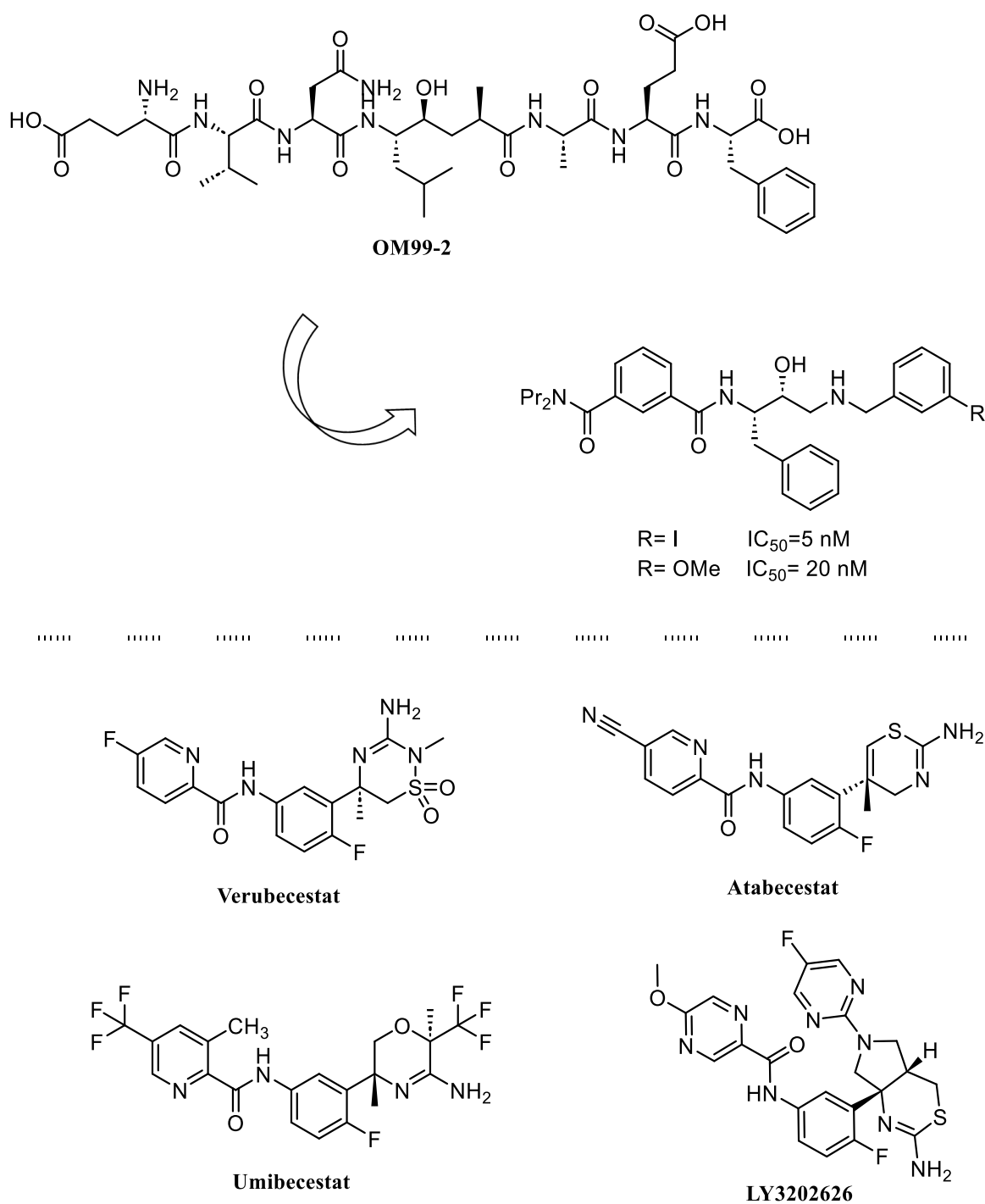


Figure 8. Structure of peptidomimetics OM99-2 and derivatives; structures of non-peptidic-BACE-1 inhibitors.

Verubecestat, Atabecestat, Umibecestat and LY3202626 are among the most recent non peptidic-BACE-1 inhibitors working their way through the trial pipeline (Figure 8).⁶⁵

γ -secretase is the other enzyme responsible for APP cleavage and, like BACE-1, is a good target to prevent A β formation. Its inhibitors may be useful in AD therapy, despite interfering with Notch signalling. One of these inhibitors, DAPT, has reached late-phase clinical trials, inhibiting the enzyme with an IC₅₀ of 115 nM.⁶⁷ LY-411575 was the most potent γ -secretase inhibitor (IC₅₀ of 119 pM), but its interference in Notch signalling compromises the maturation of B- and T-lymphocytes (Figure 9).⁶⁸

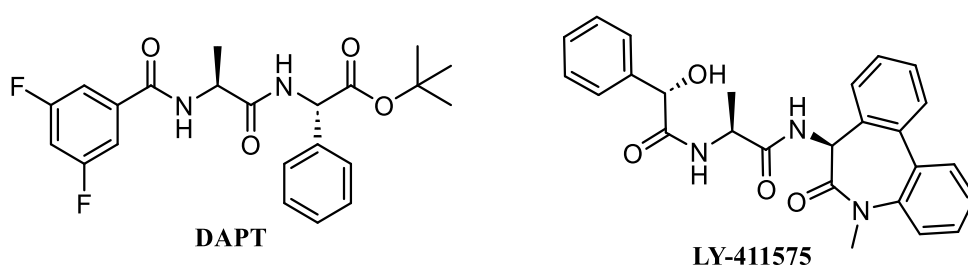


Figure 9. Structure of DAPT and LY-411575.

Non Steroidal Anti Inflammatory Drugs (NSAIDs), like ibuprofen and indomethacin, in addition to reducing the inflammatory processes, manage to interfere with the release of A β peptides, producing shorter and less amyloidogenic forms of the peptide.⁶⁹ This effect is achieved by direct interaction with γ -secretase complex. R-flurbiprofen (Tarenflurbil®) reached phase 3 of clinical trial, before being stopped for poor results in slowing the onset of the disease.⁷⁰

β -sheet breakers are a category of compound which interferes with A β formation by complexing to it through hydrophobic interactions. In this way, it forces changes in the β -sheet structure. The limitation to the use of these compounds, like iA β 5⁷¹ (Figure 10), is due to their pharmacokinetic profile, which makes them poorly drug-like.

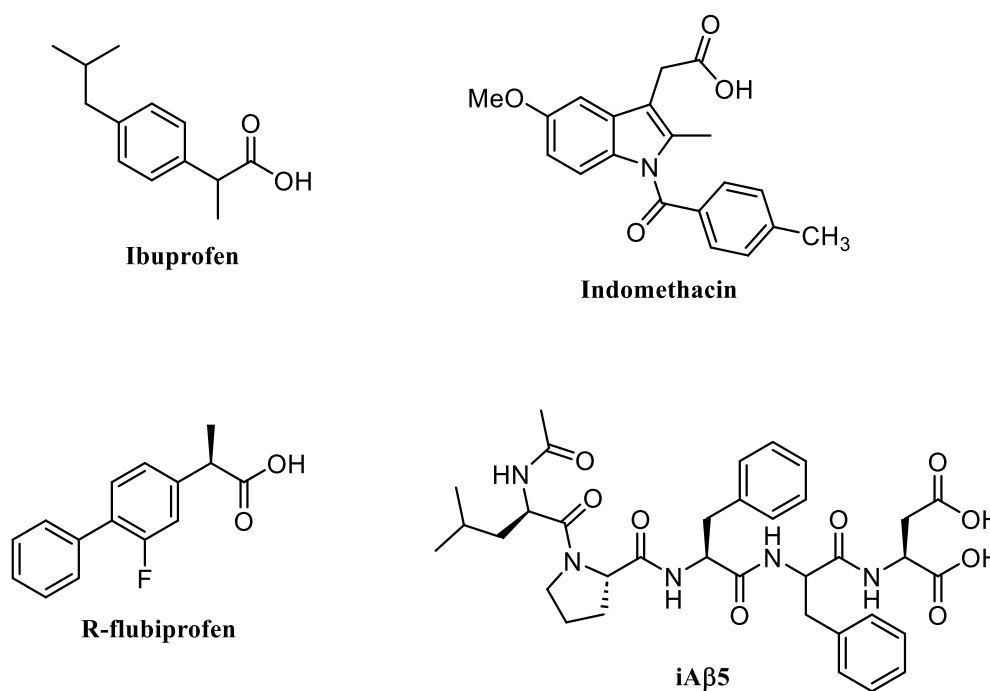


Figure 10. Structure of some NSAIDs and of β -sheet breaker iA β 5.

Finally, a very popular strategy nowadays is the A β immune therapy. Currently, anti-A β immunotherapies are active (vaccines) and passive (exogenous antibodies) immunotherapy. Active immunization stimulates the immune system by administering A β , thereby triggering an immune response to produce endogenous antibodies against A β .⁷² Passive immunotherapy uses humanized monoclonal antibodies that targets A β structures with high selectivity, promoting their disruption. At the moment, only two drugs are approved by FDA, Aducanumab and Lecanemab, with some controversial about their release, especially for Aducanumab.⁵⁴ Aducanumab is a human IgG1 mAb that targets amino acids 3–7 of the A β peptide and targets A β aggregates, including soluble oligomers and insoluble fibrils.⁷³

- **Tau hyperphosphorylation inhibition strategies:** NFTs are the other marker of AD, along with A β . The main strategy to avoid their formation is kinases inhibition, thus reducing tau hyperphosphorylation. More detailed information will be given following in the paragraph.
- **Antioxidant therapy:** this strategy aims to contrast oxidative stress. Antioxidants, many of which can be found in natural products, protects cells from free radicals trough their ability to catch radicals and free electrons.⁷⁴ Another advantage of these molecules is the absence of any kind of toxicity at the tested concentrations. Vitamins are a well-known class of compounds with antioxidant properties. Vitamin A (retinoids) is not directly involved in antioxidant processes in brain, but it can interact with A β , thus showing neuroprotective effects. Vitamin E (α -tocopherol) is a fundamental antioxidant factor in brain, as it can cross

the blood brain barrier (BBB) and prevent lipid oxidation by ROS. Its effects are powered up by vitamin C (ascorbic acid), which regenerates vitamin E back to its reduced form. Vitamin C is also the co-factor of ascorbate peroxidase, an enzyme that reduce H_2O_2 to H_2O , and it participates to several neuronal development processes. Phenolic compounds are a macro-class of antioxidant molecules widely present in plants. Their activity is strictly related to their structures, which presents several hydroxylic and substituted aromatic rings. These functional groups can scavenge free radicals and are able to give hydrogen atoms and electrons, in addition to chelate metal ions.⁷⁵ Phenolic compounds can be divided into flavonoids or non-flavonoids, based on their structures. Carotenoids are compounds characterized by a long polyenic chain, which interacts with free radicals. Some of them also carry out neuroprotective properties, like crocin, which is used in treatment of AD and PD (Figure 11).⁷⁵

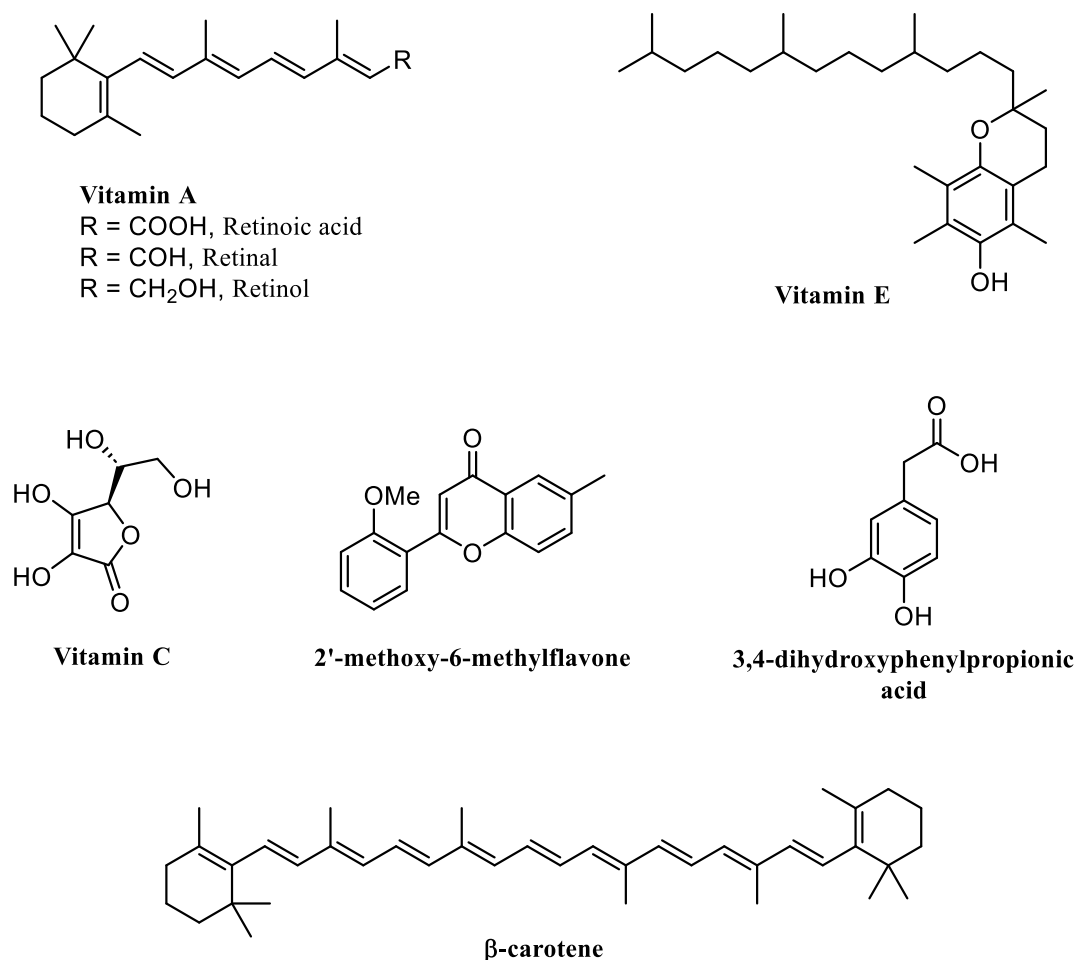


Figure 11. Structure of some antioxidant compounds. In order, Vitamin A, E and C; 2'-methoxy-6-methylflavone, a flavonoid antioxidant; 3,4-dihydroxyphenylpropionic acid, a non-flavonoid compound; β -carotene.

- **NMDAR mediated toxicity:** Among the several biochemical pathways leading to neuronal death in AD, NMDARs plays a key role. Its excessive activation leads to toxic levels of Ca^{2+} in the cells, therefore the use of NMDAR antagonist could be an interesting approach. However, many NMDA receptor antagonists also produce several undesirable side effects at doses within their putative therapeutic range. As a consequence, the only drug approved for AD treatment that does not interfere with the cholinergic system is Memantine. It is a non-competitive NMDAR antagonist derived from amantadine, an antiflea agent, and it was approved in 2003 for the treatment of moderate to severe case of AD. Memantine exerts its effect on NMDAR activity by binding at or near the Mg^{2+} site within the ion receptor channel.⁷⁶
- **Metal-chelating approach:** according to the metal hypothesis, high concentration of transition metals, such as Cu^{2+} , Fe^{2+} and Zn^{2+} , contributes to $\text{A}\beta$ plaques formation and precipitation. Metal chelating agents, like diethylenetriaminepentaacetic acid (DTPA), triethylenetetramine (TETA) and deferoxamine (DFO) (Figure 12), seize those metals from $\text{A}\beta$, reducing its formation and making it soluble. Those agents are also able to reduce oxidative stress deriving from amyloid plaques.⁷⁷

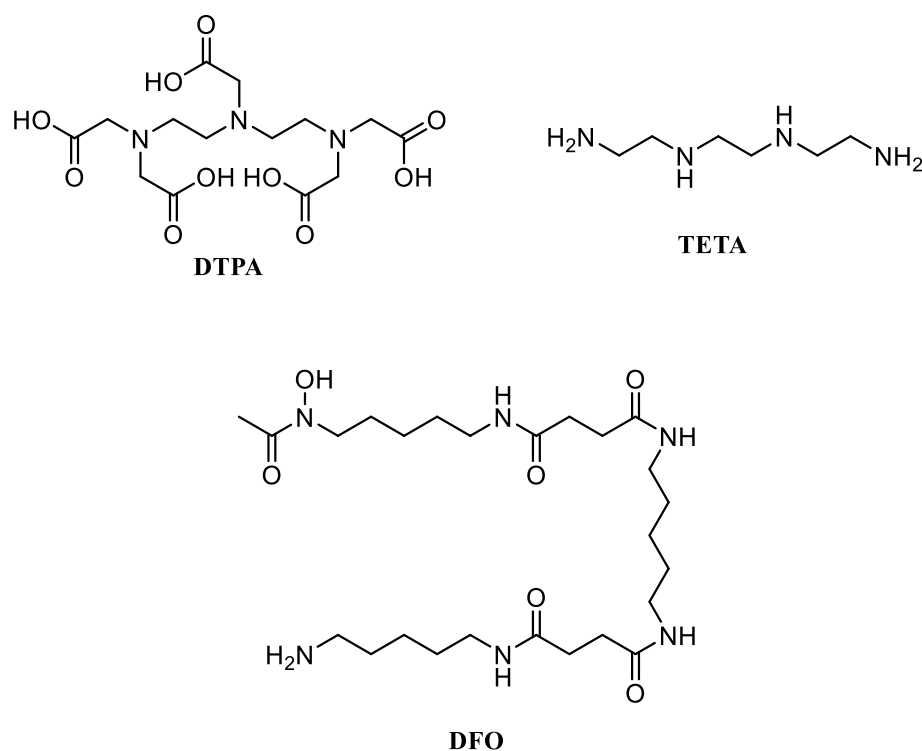


Figure 12. Structures of DTPA, TETA and DFO.

1.3 Glycogen synthase kinase 3 β

Glycogen synthase kinase 3 β (GSK-3 β) is an ubiquitous serine/threonine kinase involved in several cellular processes, from glycogen metabolism to microtubules' stability.^{78, 79} It exists in two isoform, GSK-3 α and GSK-3 β , generated from two separated genes. In mice, deletion of the gene for GSK-3 β is lethal, while deletion of the one for GSK-3 α results in lack of coordination, locomotion and aggressivity.⁸⁰ In brain cells, the β isoform is the most abundant and, in patients affected with AD, where it has a pivotal role, is overexpressed.⁸¹ Given its importance in this disease, GSK-3 β is under the spotlight of the scientific community since the 80s.

1.3.1 Structure and functions

The structure GSK-3 β was first described at the beginning of the century by Bax and his research group.⁸² The main regions are a N-terminal domain (residue from 25 to 134) and a C-terminal domain (from 135 to 380). In those macroregions can be found the ATP-binding site (in the N-terminal domain) and the activation site, in the C-terminal domain.

The activity of the enzyme can be regulated at different level, as highlighted from the crystal structures that gives important information about the activation or inhibition of the GSK-3 β .⁸³ The main mechanism of regulation is the post translational phosphorylation of a residue of serine in position 9, which acts as a substrate to the Substrate Binding Domain, preventing the binding of enzymatic targets (Figure 13). This phosphorylation is promoted by different protein kinases, such as protein kinase A (PKA), protein kinase B (PKB or Akt) and protein kinase C.

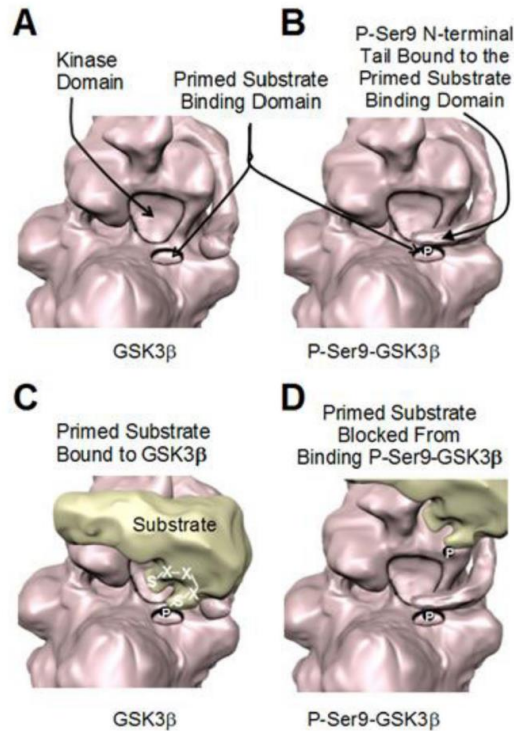


Figure 13. Representation of the active sites of GSK-3 β , with the kinase domain and the near site for the substrate (A). Substrate bound to its site on GSK (C). Conformation of the inactivated enzyme: phosphorylated Ser9 tail covers the site for the substrate (B), blocking the binding of the substrate (D).⁸⁴

A second site for the regulatory phosphorylation of GSK-3 β is the residue Tyr216, whose phosphorylation state can promote the binding of the primed substrate. Indeed, it is reported that the inhibition of this phosphorylation prevents the bond of the primed substrate, which is essential for the enzyme activity.⁸³ A secondary regulation mechanism is the protein-protein interaction. GSK-3 β can form complex with other protein taking part in several pathways, like the canonical Wnt signaling pathway. Other examples are the complexes of phosphorylated β -catenin, Atk and GSK-3 β in erythropoietin-induced glioma proliferation.⁸⁵ Last, it seems that the intracellular distribution of the enzyme can condition its activity. Although it is a cytoplasmatic protein, it can be found even in nucleus and mitochondria, where it is more active.⁸⁶

As previously stated, GSK-3 β is an ubiquitous enzyme involved in several signaling pathways, in which his role is essential. Over 100 suitable substrates have been recognized as targetable by GSK-3 β , some of them already been discussed.

The first important GSK-3 β substrate discovered was the glycogen synthase (which gives the name to the enzyme). GSK-3 β phosphorylates three residues of serine⁸⁷, thus decreasing its activity and, consequently, the synthesis of glycogen. In this way, glucose stays in solution and increases glycemia. The activity of GSK-3 β is inhibited by insulin through the activation of PKB, that phosphorylates Ser9 of GSK-3 β .⁸⁸ This evidence led to a promising path to GSK-3 β inhibitors as possible lead in the treatment of type-II diabetes.⁸⁹

A second major contribution is in the Wnt/ β -catenin signaling pathway. This system of 20 proteins regulates embryonic development, cells proliferation, cell fate determination and cell migration, in addition to carcinogenesis.⁹⁰ GSK-3 β phosphorylates primed β -catenin, starting the ubiquitin/proteasome degradation mechanism. Wnt can redirect GSK-3 β activity to LRP5/6, suppressing β -catenin phosphorylation.⁹¹

Lastly, GSK-3 β is responsible for the phosphorylation of tau protein, one of the main markers in AD. Normally, tau is a protein associated with microtubules, which are stabilized. Hyperphosphorylated tau protein can aggregate in structures called NFTs, with the loss of the stabilizing activity and the consequent dissociation of the microtubules. This results in a compromise in the cytoskeletal structure and in the neurotransmission mechanism (Figure 14).⁹²

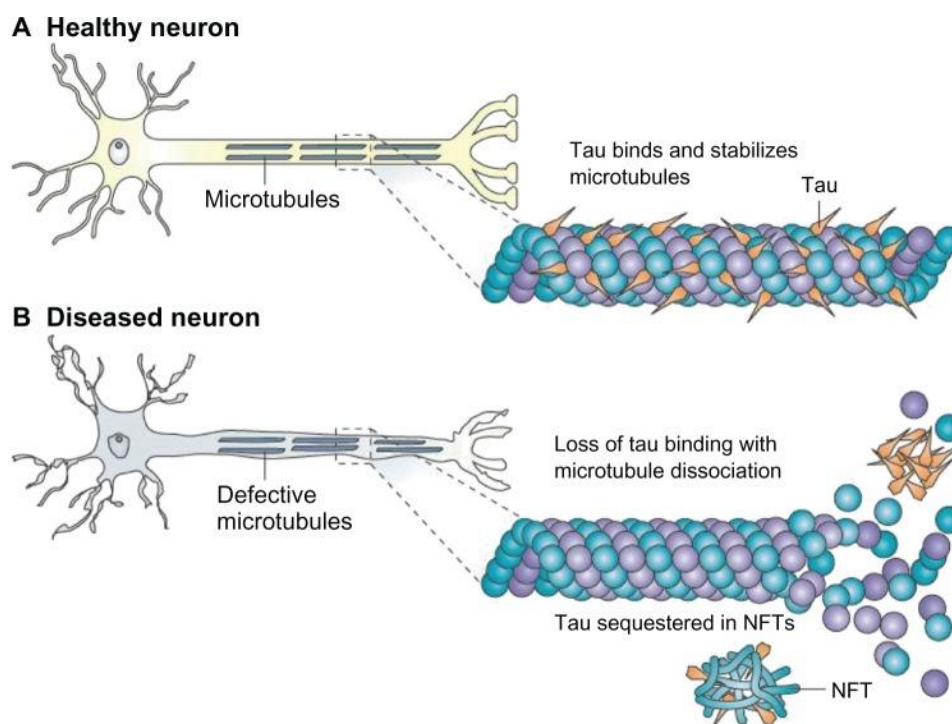


Figure 14. Tau protein in association with microtubules in a healthy neuron (A) and in a taupatic neuron (B). P-tau aggregates and makes microtubule less stable, leading to its dissociation.⁹³

1.3.2 GSK-3 β involvement in various diseases

Given the great number of biological targets, it is not surprising the involvement of GSK-3 β in many diseases and physiological disorders (Figure 15).

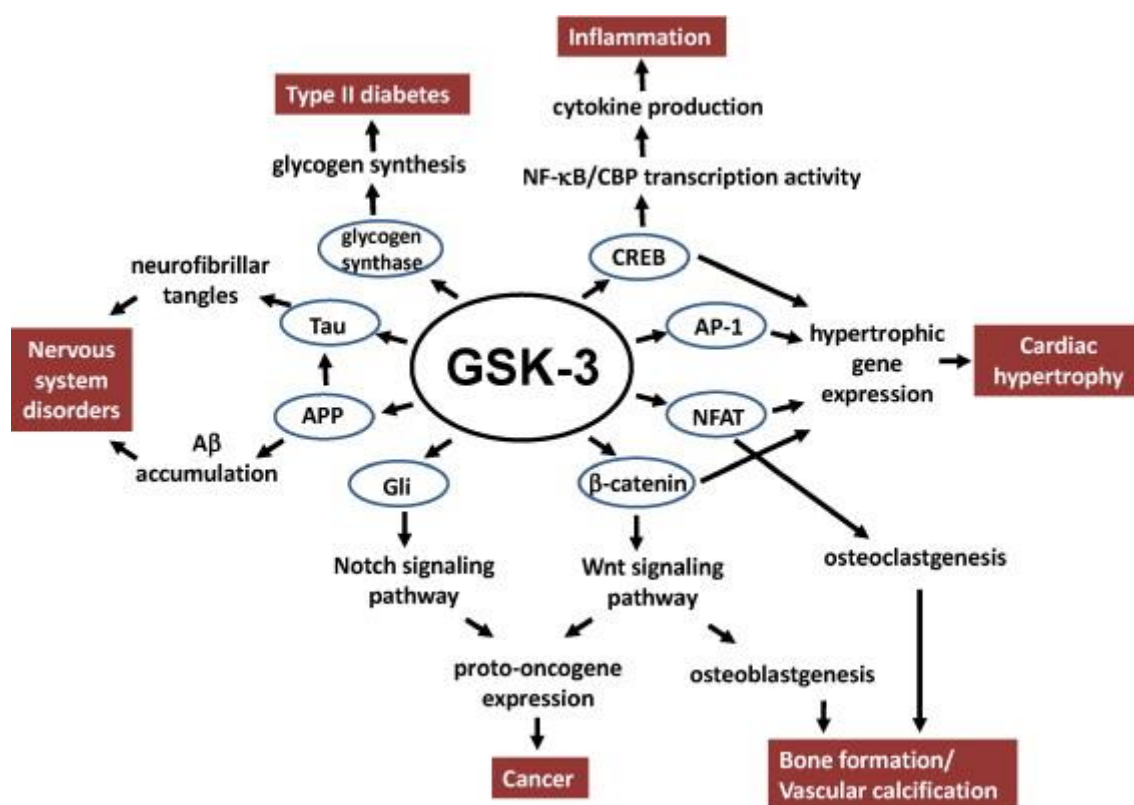


Figure 15. Involvement of GSK-3 β in some diseases through regulation of several targets.⁹⁴

- **Inflammation:** GSK-3 β can stimulate the inflammatory response through regulation of phosphorylated CREB, which raises the levels of interleukins, like IL-6, IL-1 β and IL-12, and tumor necrosis factor (TNF).⁹⁵ These factors activate several Toll-like receptors in monocytes and blood mononuclear cells. At the same time, production of anti-inflammatory IL-10 is reduced. Administration of GSK-3 β inhibitors can reduce the production of pro-inflammatory factors and increase IL-10 levels.⁹⁶ In addition, some studies showed that GSK-3 β inhibitors, in mice model, can protect against endotoxin shock;⁹⁷
- **Cancer:** the first evidence about GSK-3 β participation in cancerogenesis highlighted a tumor suppressing role. As previously reported, one of the targets of GSK-3 β is β -catenin,

a protein taking part in the Wnt signaling pathway. Abnormal regulation of β -catenin makes it accumulate and enter in the nucleus, where it promotes gene transcription. On the other hand, phosphorylated β -catenin is marked and directed to be degraded by ubiquitine/proteasome system, preventing its accumulation. GSK-3 β participates in the complex responsible for this phosphorylation, thus suppressing the tumoral generating cascade. Nevertheless, some studies suggests that GSK-3 β may be involved in cancer development. Higher levels of GSK-3 β were observed in tumors affecting liver, ovaries, pancreas, liver, prostate and colon.⁹⁸ In ovarian cancer, it is reported its positive contribution to cancer cells proliferation and survival, increasing cyclin D1 expression and cell cycle progression.⁹⁹ The same effect is observed in pancreatic cancer.¹⁰⁰ In addition, it seems that GSK-3 β inhibition can confer resistance to cis-platinum. This was observed in ovarian cancer cells with increased levels of pGSK-3 β (Ser9);¹⁰¹

- **Type 2 diabetes:** also known as insulin-resistant diabetes, type 2 diabetes is characterized by a reduced production of insulin or increased levels of insulin, paired with a poor capacity of the cells to recognize it. GSK-3 β participates in both this mechanism. It raises blood level of glucose by inhibiting glycogen synthase, thus inhibiting production of insulin.¹⁰² Insulin resistance is achieved by degradation of insulin receptor, induced by high level of glucose and phosphorylation by GSK-3 β , triggering the degradation via ubiquitine/proteasome;¹⁰³
- **Nervous system disorders:** GSK-3 β is highly expressed in the CNS, where it has a role in neuronal development, migration and differentiation. Being so diffuse in CNS, it is no surprising that a dysregulation of its activity can generate various nervous disorders. GSK-3 β is responsible for alteration in cytoskeletal disfunction, like microtubules alteration, and the production of toxic metabolites which can lead to neuronal death. Depending on the area in which the loss of neuronal functionality is located, some neurodegenerative diseases can occur, like AD (cholinergic system¹⁰⁴), Parkinson's disease (dopaminergic neurons in substantia nigra pars compacta¹⁰⁵), Huntington's disease (mainly neurons in dorsal striatum¹⁰⁶). If the neurons are concentrated in particular areas, the loss can result in an ischemic stroke.¹⁰⁷

For the purpose of this research, the involvement of GSK-3 β in the onset of AD will be deeper described.

1.3.3 GSK-3 β role in Alzheimer's disease

Through the years more and more evidence of the role of GSK-3 β in AD onset emerged, highlighting several contributes of its activity to the damaging of neurons.^{79, 81, 92} These damages are mainly related

to production of abnormal protein aggregates, such as hyperphosphorylated tau protein or A β protein aggregates; in addition, GSK-3 β takes part in the mitochondrial intrinsic apoptotic pathway,¹⁰⁸ increasing neuronal functionality loss (Figure 16 and 17).

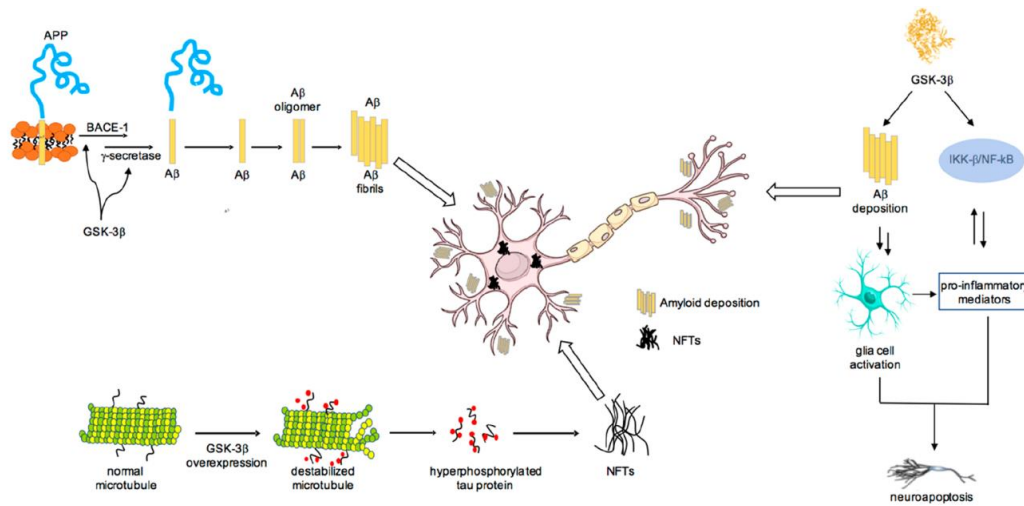


Figure 16. Contribution of GSK-3 β in AD onset.¹⁰⁹

- Tau hyperphosphorylation:** as previously seen, tau protein is normally associated with microtubules. In condition of overexpression of GSK-3 β , tau is hyperphosphorylated and tend to aggregate, breaking its bond to the microtubules, which are no longer stabilized. Great part of phosphorylated residues is close to the microtubule-binding region. Therefore, GSK-3 β inhibitors are reported in several works, in which they show interesting abilities to reduce hyperphosphorylation of tau protein, thus being a good candidate for treatment of AD.⁸¹ Highly selective inhibitor SB-216763, when administered in vitro before the cells were treated with A β , protected them against A β toxicity. Moreover, at the concentration of 40 nM, it produces a reduction of GSK-3 β activity of 39%, with 44% reduction on glycogen levels. This indicates that SB-216763 reduces the enzyme activity, without abolishing it.¹¹⁰
- Amyloid plaques:** amyloid plaques were considered for many years the key element for the onset of AD.¹¹¹ This protein originates from APP when cleaved by two secretase, BACE-1 and γ -secretase, in fragment of 37-49 amino acids. The main monomers are A β 40 and A β 42. These aggregate in oligomers, which are still soluble, and then in mature fibrils, which are insoluble and are considered the toxic form.¹¹² GSK-3 β is involved in A β production, as it activates BACE-1 and γ -secretase, promoting their cleavage of APP.^{113, 114} GSK-3 β is also activated by A β ,¹¹⁵ probably trough inflammation generated by protein extracellular

deposition (even though the full mechanism is still unknown)¹¹⁶ and by inhibition of Wnt regulation of GSK-3 β .¹¹⁷

- **Neuronal cells death:** GSK-3 β contributes to loss of neurons by targeting several intermediates which results in the activation of apoptotic processes. In addition to β -catenin, already mentioned, other important targets are p53, a tumor suppressor factor which induces apoptosis when activated, heat shock factor-1 (HSF1), a survival-promoting transcription factor inhibited by phosphorylation and NF- κ B, a nuclear factor which regulates apoptosis. Moreover, mitochondria are also affected by activity of GSK-3 β , which targets several proteins leading to the disruption of mitochondria and the consequent cellular death.¹⁰⁸

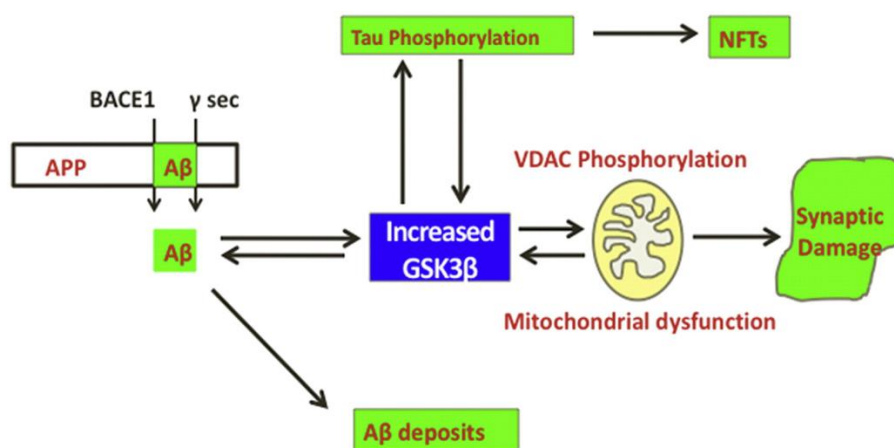


Figure 17. Contribution of GSK-3 β in AD pathology.¹¹⁸

1.3.4 GSK-3 β as a pharmacological target

Considering the contribution and the involvement of GSK-3 β in several pathologies and biological pathways, it is no surprising that hypothesis of GSK-3 β inhibition as potential therapeutic treatment for such diseases as AD and Parkinson's disease is popular.^{119, 120} With the progresses achieved in researching information on structure and potential lead compounds, nowadays tens of different compounds are available, characterized by good selectivity. Those can be distinguished for their origin, which can be natural, metallic cation or small synthetic molecules, and their mechanism of action, being ATP-competitive or non-ATP competitive.¹²¹

The first GSK-3 β inhibitor discovered was lithium, which was largely used as a salt in bipolar disorder as a mood stabilizer. It directly and indirectly inhibits GSK-3 β with two different mechanisms.¹²² Directly, it acts as an uncompetitive inhibitor for co-factor magnesium. On the other

hand, it promotes the activity of AKT, which phosphorylates GSK-3 β and reduces its activity. There are contrasting studies about its benefits when used in AD therapy,^{123, 124} which add to toxic side effects in elderly patient.¹²⁵ For these evidence, clinical studies were interrupted.

Regarding synthetic ATP-competitive inhibitors, the first major category reported were the aminopyrimidine, purine analogs including CHIR98014, CHIR98023 and CHIR99021, potent and selective compounds which inhibit GSK-3 β in nanomolar concentration range.¹²⁶ These compounds were able to reduce tau phosphorylation in neuronal systems. Another class of inhibitors is that of arylindolemalimides, where SB-216763 is the prototype characterized by activity in the range nanomolar concentration and ability to reduced tau phosphorylation.¹²⁷ SB-216763 also displayed neuroprotective effects against several damages leading to apoptosis.¹²⁸ AR-A014418, an amino thiazolic compound developed by AstraZeneca in 2003, showed high selectivity for GSK-3 β when tested with other kinases.¹²⁹ In addition, it resulted neuroprotective in apoptotic conditions, preventing also neurodegeneration induced by A β peptide.¹²⁹ Lastly, a class of tetracyclic compounds, called paullones, inhibits GSK-3 β in the nanomolar concentration range. Alsterpaullone and kenpaullone protect neuronal cells from death in response to several insults;¹³⁰ moreover, alsterpaullone reduces tau phosphorylation,¹³¹ while kenpaullone reduces A β production in cells overexpressing APP (Figure 18).¹²⁴

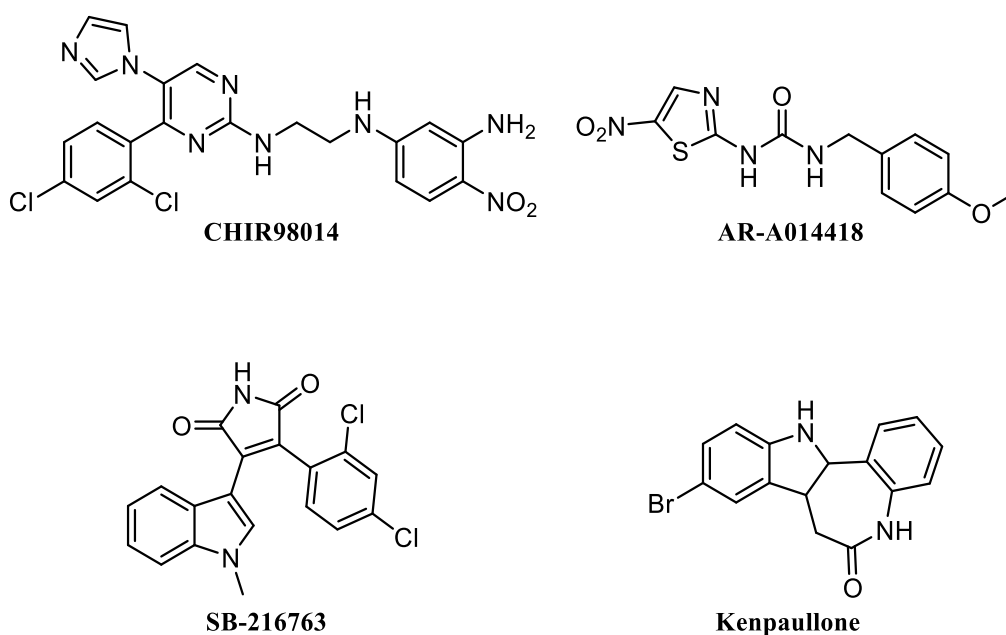


Figure 18. Structures of some synthetic GSK-3 β inhibitors.

Regarding non ATP-competitive compounds, they are more selective towards GSK-3 β due to interactions with unique regions on the enzyme, thus modulating its activity in a finer way than simply blocking ATP binding. Indeed, they can act as covalent inhibitor, allosteric inhibitor or substrate competitive inhibitor. Non ATP-competitive GSK-3 β inhibitors are promising drugs which can contrast AD efficiently in a safer way.

Besides natural inhibitors, like Manzanine A, an alkaloid with IC₅₀ towards GSK-3 β of 10 μ M, there are several synthetic non ATP-competitive inhibitors. The first family of this compounds were thiadiazolidinones (TDZD), reported in 2002 by Ana Martinez and her research group.¹³² These heterocyclic compounds interact with Cys199, a crucial residue located in active site, managing to reduce tau phosphorylation and showing neuroprotective ability.¹³³ The most important member of this class of inhibitors is Tideglusib (NP-12) (Figure 19), which reached phase 2 in clinical study against AD.¹³⁴ Tideglusib has high selectivity towards GSK-3 β over other kinases like PKA, PKC, casein-kinase 2 (CK2) and cyclin-dependent kinase 1 (CDK1). It demonstrated positive results after several studies in different animal models, where it showed improvement in cognitive performance, reduced tau phosphorylation and decreased A β production. Furthermore, it showed the ability to cross the BBB.¹³⁵

Other relevant compounds are halomethylketone (HMK) derivatives, which form a carbon-sulfur covalent bond with Cys199.¹³⁶ Those compounds are permeable in cells and can decrease tau phosphorylation (Figure 19).

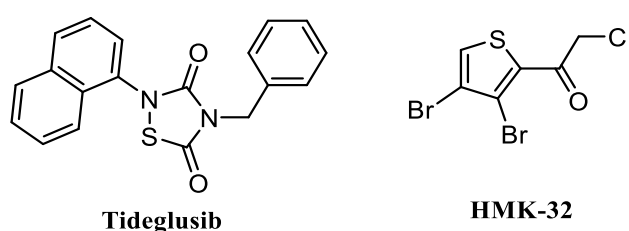


Figure 19. Structure of non ATP-competitive GSK-3 β inhibitors Tideglusib and HMK-32.

1.4 Histone deacetylase – HDAC

Although the knowledge about AD has greatly improved over the years, there are many aspects that are still not fully understood, especially regarding sporadic AD. The main contribution in this area is given from epigenetic, a branch of genetics which study the gene expression in an unchanged sequence of nucleotides. The term epigenetic refers to change in gene expression without alteration

in the DNA sequence. Epigenetic modifications reversibly affect long-term gene expressions through covalent alterations of DNA or proteins involved in the DNA package, such as hydroxymethylation, methylation and acetylation.¹³⁷ Histones are the main DNA-binding proteins, through interactions between their positively charged aminoacidic chains (mainly Lysine and Arginine) and DNA's phosphate groups, which have a negative charge. There are 5 different histone proteins (H1, H2A and B, H3, H4) which form the scaffold of nucleosome, the fundamental unit of chromatine. Those units have not just a structural function, but take part in gene expression by being modified, like previously said. The terminal N-region exits from nucleosome and its aminoacidic residues can be methylated on Arg and Lys, phosphorylated on Ser and Thr and, above all, acetylated on Lys residues.¹³⁸ Those changes increase or decrease availability of chromatine towards transcription (Figure 20).

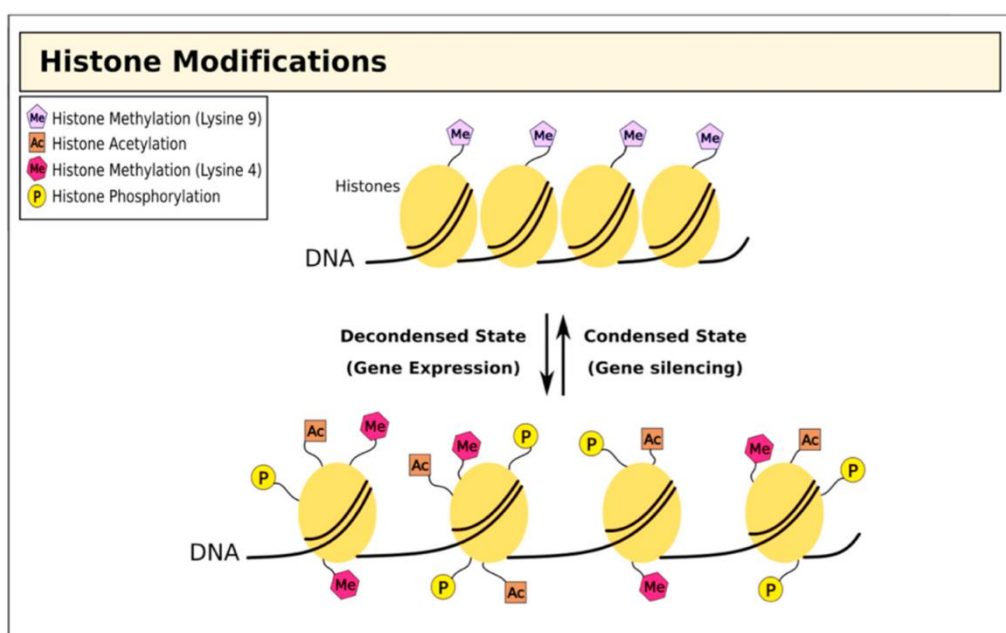


Figure 20. Histone modification and their effects on gene expression.¹³⁹

Acetylation or deacetylation of Lys residues are performed by histone acetyltransferase (HAT) and histone deacetylase (HDAC), respectively. Histone deacetylation removes acetyl groups from Lys, increasing the positive charge and making chromatin denser. This reduces DNA transcription. On the other hand, histone acetylation promotes transcription and is usually associated with memory and learning processes.¹⁴⁰

HDAC is a superfamily of 18 different isoforms, divided in 4 groups by differences in structure, nature of the co-factor and cellular distribution (Figure 21). These classes I, II and IV share the same dependence towards zinc as co-factor:

- class I includes HDAC1, HDAC2, HDAC3 and HDAC8, which are mainly nuclear (isoforms 3 and 8 can move into cytoplasm);
- class II includes HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 further divided into IIa (isoforms 4, 5, 7 and 9) and IIb (HDAC6 and HDAC10). Those two classes are different for the presence of two deacetylating domain in class IIb, while there is only one in class IIa. HDAC6 is mainly located in cytoplasm, where it targets proteins like α -tubulin, cortactin and HSP (heat-shock protein).¹⁴¹ Besides microtubule and actin cell mobility, it also plays a crucial role in clearance of misfolded protein. Activity of HDAC10 is still not clear.
- class IV only consists of HDAC11, a nuclear HDAC which is similar to class I and II and targets fatty acids.¹⁴²
- class III, whose HDAC are called sirtuins, is totally different. Instead of zinc, they are NAD^+ dependent enzymes located in mitochondria, cytoplasm and nucleus. Class I, II and IV are highly expressed in neuronal cells and oligodendrocytes, but not in astrocytes. Class I, especially HDAC1 and HDAC2, are highly expressed in brain areas that regulate memory and learning, like hippocampus and cortical areas.¹⁴³

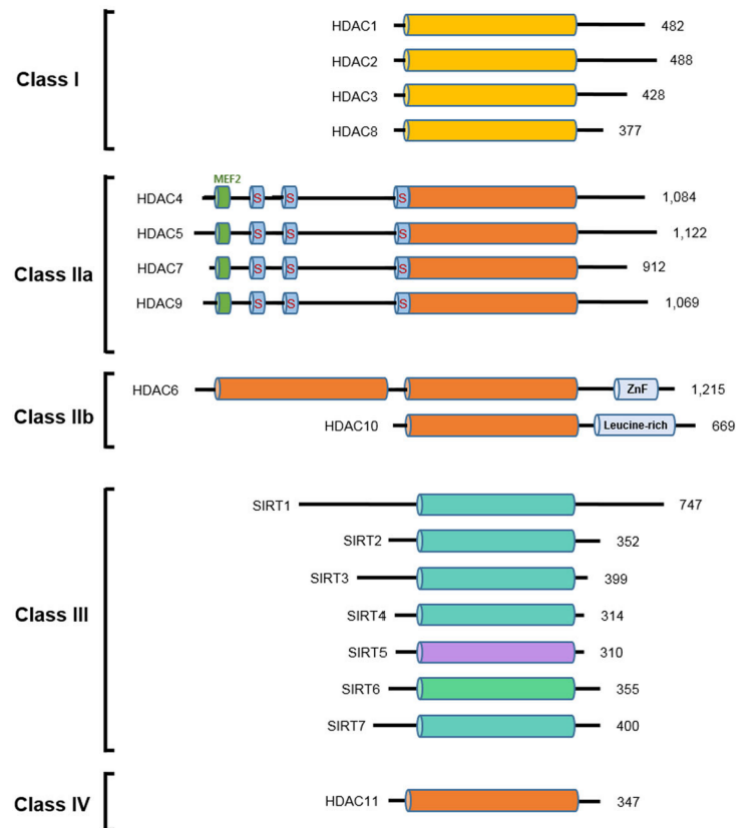


Figure 21. Classification and domain composition of HDACs.¹⁴⁴

1.4.1 HDAC and AD

All HDAC isoforms are implicated in several neurodegenerative diseases. In AD, the most involved HDAC isoforms are 2 and 6. HDAC2 belongs to class I and has 85% of structural similarity with HDAC1, which is found in the same protein complex with. However, it is HDAC2 to be involved in cognitive process. Overexpression of HDAC2 in neurons decreases the density of dendritic spine as well as synapses numbers and synaptic plasticity. These effects depend on interaction between HDAC2 and memory-promoting genes, such as brain-derived neurotrophic factor (BDNF) and early growth response protein 1 (Egr1). Suppression of these factors is mediated by CoREST, a co-repressor required for gene expression, which is deactivated by HDAC2. Consequently, neuronal gene expression is suppressed. On the other hand, deficiency of HDAC2 increases synapse numbers and plasticity. The same effect is gained with the chronic treatment with HDAC inhibitors (HDACIs).¹⁴⁵ Those results suggest that HDAC produces effects that negatively regulates memory and learning, thus promoting research of selective HDAC2 inhibitors to contrast memory affecting diseases. Nevertheless, carefulness is required to prevent problems of neurogenesis in adult brain, inquired to HDAC inhibition.¹⁴⁶ Regarding HDAC1, its role is less clear, as both neuroprotective and

neurotoxic effects are highlighted. In CK-25 mice model, p25 binds to HDAC1 and affects its activity, leading to augmented DNA breaks, abnormal cell-cycle activity and neuronal death. In addition, in cooperation with SIRT1, it promotes genomic stability.¹⁴⁷ On the other hand, it seems that neurotoxic effects are related to cellular distribution. Indeed, HDAC1 induces the block of axonal transport and axonal loss, even though only when it is taken from nucleus to cytoplasm, where it binds to α -tubulin and kinesins.¹⁴⁸ Finally, its presence catalyzes HDAC3 phosphorylation by GSK-3 β , which leads to neuronal death in cerebellar granule neurons.¹⁴⁹ The most important HDAC isoform involved in neurodegeneration is HDAC6, a class II HDAC located in cytoplasm, where regulates acetylation of α -tubulin. Through its activity with microtubules, HDAC6 is able to simplify the transport of protein aggregates, making the cell deal with abnormal amounts of misfolded proteins.^{150, 151} Its main contribution in AD is the interaction with tau protein: HDAC6 promotes tau aggregation and toxicity through deacetylation of Lys321 residues, making the protein more accessible to phosphorylation on Ser324, which promotes aggregation in filaments.¹⁵² The role of other HDAC isoforms in AD are not fully understood.

1.4.2 HDAC inhibitors

As already reported, epigenetic modifications, such as methylation or acetylation of DNA, are crucial in several diseases. HDAC modulates acetylation of histones, producing ipoacetylation, which is a typical situation in neurodegenerative disorders. On the other hand, iperacetylation obtained through HDACIs produces neuroprotective effects. Selectivity over the different isoforms is a fundamental aspect, being they so similar, even though there are not fully selective HDACIs for a specific isoform. However, it is reported that HDACIs can be used in the treatment of AD.¹⁵³

Although HDACIs can be divided in categories based on structure, they share the same pharmacophoric features (Figure 22) in which there can be found:

- a Zinc Binding Group (ZBGs), which bind the zinc atom through H-bonds and electrostatic interactions, blocking the activity of the enzyme;
- a linker domain capable of hydrophobic nature, aliphatic or aromatic;
- a cap group, able to interact with the external surface and usually having an aromatic structure.



Figure 22. Pharmacophoric features of HDACIs.¹⁵⁴

Based on structure, four main different groups can be distinguished:

- hydroxamic acids: this is the first class of HDACIs discovered. They are pan-inhibitors, with no specific selectivity for any isoform belonging to class I, II and IV. They are powerful inhibitors, but with a short half-life. The main compounds of this category are **Tricostatin A** (TSA), **Vorinostat** (SAHA), **Belinostat** (PXD101) and **Panobinostat** (LBH589). Progenitor of hydroxamic acids is TSA, a natural compound used as antibiotic whose structure inspired the second generation of inhibitors. It has highly toxic, depending on its low selectivity. For this reason, it is often used as a model for the synthesis of other HDACIs. SAHA is the most advanced inhibitor, it is well tolerated, quickly absorbed and metabolized in non-pharmacological compounds. Its structure is similar to TSA, which shares the mechanism of action with. They inhibit HDAC by imitating Lys side chain to enter in the active site, where they chelate the zinc moiety.¹⁵⁵ Treatment with SAHA in AD mice model showed improvement of spatial memory and reduction in the expression of genes related to inflammation.¹⁵⁶ Another important inhibitor is **Tubastatin A** (Figure 23), the first HDACIs hydroxamic acid-based without neurotoxic effects. In the original paper, it showed dose-manner neuroprotective effects against induced neuron model of oxidative stress.¹⁵⁷ Further studies displayed its ability to increase levels of acetylated α -tubulin in vivo and vitro.¹⁵⁸

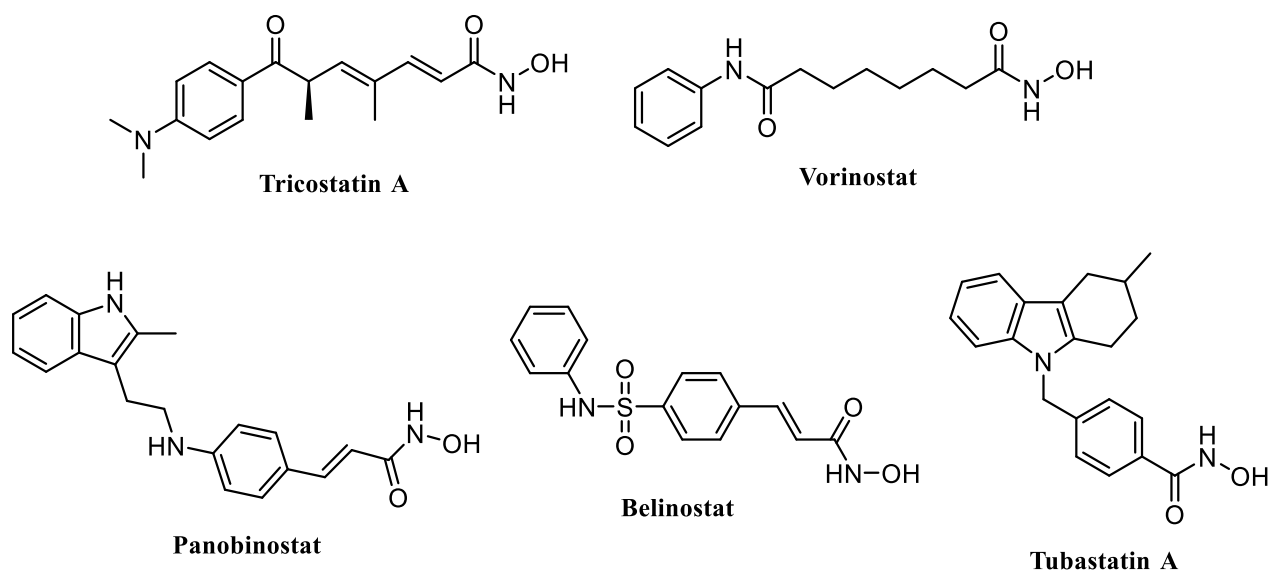


Figure 23. Main hydroxamic acid-based HDACIs.

- cyclopeptides: like hydroxamic acids, this class inhibits the enzyme through interaction with the zinc ion. However, given their chemical structure variability, the research and development of this class as a potential drug in AD is strenuous. Apicidin and Romidepsin (FK-228) belong to this category, but only the second one is used in therapy, inhibiting HDAC 1 and 2. This molecule (Figure 24) acts as a pro-drug: indeed, the S-S bond is reduced in cell to unleash the thiolic function, which interacts with the zinc ion.¹⁵⁹

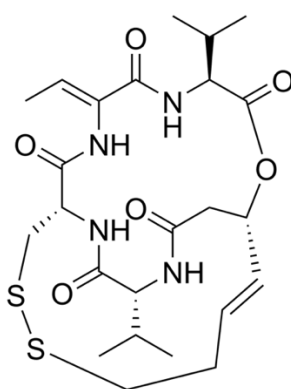


Figure 24. Structure of romidepsin/FK-228.

- benzamides: this class owns relatively selective inhibitors, in general, less active than hydroxamic acids. Its main inhibitors are Entinostat (MS-275) and Mocetinostat (Figure 25). MS-275 and its derivatives inhibit HDACs in vitro in micromolar concentration range. It is

hypothesized that the aniline group forms interaction with amino acids in the active site and with the zinc ion, along with the amide group. MS-275 is a synthetic HDACIs which targets mainly HDAC1, with no activity towards HDAC4, 6, 7 and 9.¹⁶⁰

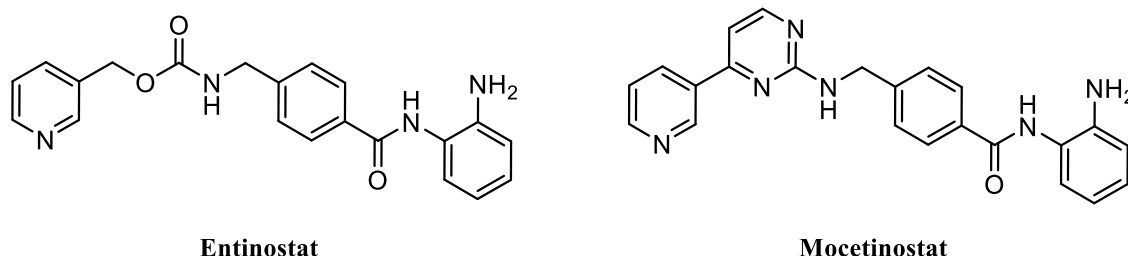


Figure 25. Structure of benzamidic HDACIs Entinostat and Mocetinostat.

- short chain fatty acids: these compounds are characterized by a short aliphatic chain, which poorly interacts with the enzymatic active site and results in low power (Figure 26). They have major affinity for HDAC class I and II.¹⁶¹ Studies showed that long treatment with these HDACIs improved memory in mice model, even in late AD, with high acetylation of histones in hippocampus and higher expression of genes associated with associative learning. This makes short chain fatty acids potential drugs for the treatment of AD, even in later stages.¹⁶²

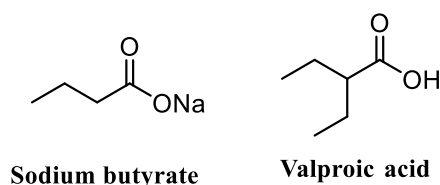


Figure 26. Structures of sodium butyrate and valproic acid.

1.4.3 HDACIs and AD

The benefits given by the use of HDACIs in treatment of AD and other similar neurodegenerative diseases is consolidated by several studies. Fischer et al. treated p25 mice with intraperitoneal administration of sodium butyrate (NaBu) for four weeks, reporting restored learning abilities and improved memory. The authors stated that this is probably due to effects on both histone and non-histone proteins.¹⁶³ In another study, they reported restoration of spatial memory and decreased inflammation-inducing genes by treatment with Vorinostat.¹⁵⁶ NaBu, in co-administration with

valproic acid (VA) and Vorinostat, was tested on familial AD mice model in a treatment of 2-3 weeks. It was reported that inhibition of class I HDACs completely restored memory.¹⁶⁴ The same mice model was used in a study of treatment with TSA, which increased H4 acetylation with improvement in memory formation.¹⁶⁵ Ricobaza et al reported two interesting studies on effects of five weeks treatment with sodium 4-phenylbutyrate in TG2576 AD mouse model. In the first, decreased levels of phosphorylated tau were observed, along with restored H4 acetylation and induced expression of synaptic plasticity markers.¹⁶⁶ In the second, reversion of mnemonic deficits was reported, due to augmented clearance of A β deposits and reduced endoplasmic reticulum stress.¹⁶⁷ VA mitigated memory deficits and decreased A β production in APP23 mice model, by reducing the cleavage of APP promoted by GSK-3 β induced γ -secretase.¹⁶⁸ Finally, some HDAC selective drug were used in studies on APP/PS1 mice model. Indeed, tubastatin A and ACY-1215, selective HDAC6 inhibitors, improved cognitive function, increased acetylation of α -tubulin and decreased A β production and tau hyperphosphorylation.¹⁶⁹

1.5 Multi-target-directed ligand drug discovery strategy

Neurodegenerative diseases like AD and PD develop from several factors, following their multifactorial nature. In case of AD, NFTs, A β low levels of ACh and oxidative stress are the major contributors to its onset. The complex nature of this disease highlighted the needs for ligands directed to multiple targets, to contrast more aspects of the disease at the same time. This led to a new approach which overcome the old paradigm “one target-one compound”, paving the way for the multi-target-directed ligands (MTDLs) approach.¹⁷⁰ In this way, some critical aspects of the combination of single targets drugs can be avoided, producing a safer activity profile. To achieve it, a possible ligand must be designed as the combination of two different drugs or as a “chimera”, which is a single entity capable of interacts with more biological targets. Indeed, MTDLs can be divided into hybrid drugs or chimera drugs. The first ones are designed trough the combination of different target drugs via linker, which can be stable or degradable in vivo. In the case of degradable linker, the two portions freed are then able to interact independently with their targets. In the other case, the liker itself takes part to the activity of the drugs. This means that the length of the linker plays a crucial role in interactions with both the targets. Chimera drugs, on the other hand, are designed by fusing different pharmacophores in a unique structure (Figure 27).^{171, 172}

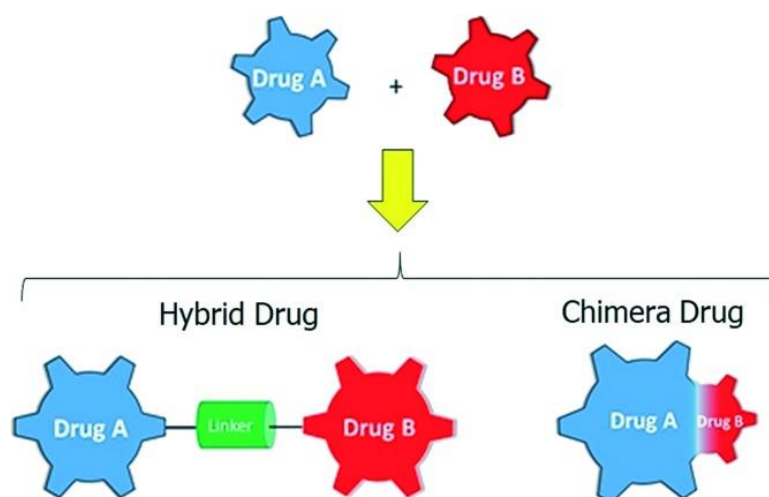


Figure 27. Differences in the design of hybrid drugs and chimera drugs.¹⁷³

In comparison to multi-drugs therapy, MTDLs are advantageous in many aspects, such as lower risk of drug-drug, greater bioavailability and a better pharmacokinetic profile. The affinity for a specific target is generally lower, but this is not always a disadvantage, because it reduces the risk of side effects.¹⁷⁴

In the design of new MTDLs for the treatment of AD, molecular hybridization is the most common strategy. AChE holds its popularity in the target choice, even in multi-target era, in combination with other significant actors in AD onset.¹⁷⁵ An example of MTDL targeting AChE is Lipocrine (Figure 28), a hybrid molecule obtained by the combination of Tacrine, the first AChEI approved for AD, and Lipoic acid, an anti-oxidant with neuroprotection ability against A β toxicity.¹⁷⁶ The resulting compound inhibits AChE in low nanomolar concentration range, reduce A β aggregation induced by the cholinesterase and protects cells from ROS toxicity.

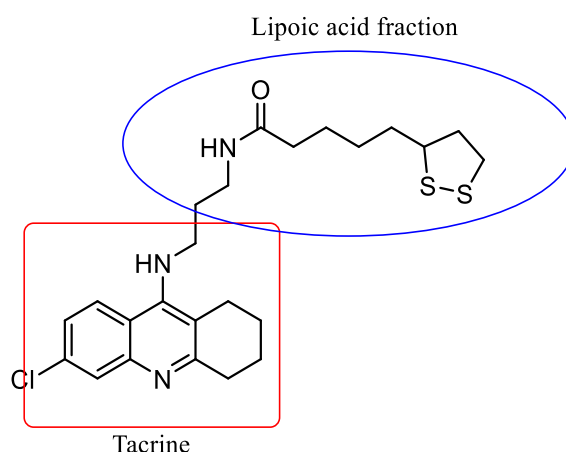


Figure 28. Structure of Lipocrine.

Regarding tacrine, Pang et al. developed a dimer of tacrine with the aim to improve its affinity towards AChE (Figure 29).¹⁷⁷ Bis(7)tacrine resulted 500 more potent than its monomer in inhibiting AChE. In addition, it showed antagonism activity against NMDAR and inhibiting capacity toward oxide synthase and BACE-1, thus reducing in vitro A β formation.

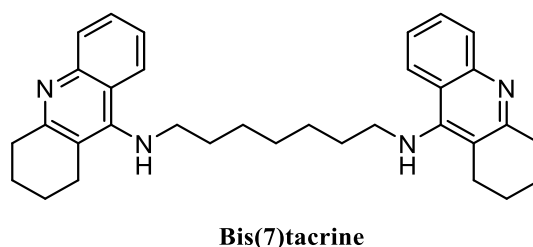


Figure 29. Structure of bis(7)tacrine.

Another example of MTDL is Memoquin, a molecule deriving from caproctamine obtained by inserting a fragment of coenzyme Q10 into its aliphatic chain (Figure 30).¹⁷⁸ Coenzyme Q10 is an antioxidant compound with ROS scavenging properties and A β deposition preventing abilities. Memoquine showed the same antioxidant ability of coenzyme Q10 and a nanomolar potency against AChE, thus avoiding AChE-induced A β aggregation. In addition, it resulted able to inhibit BACE-1 with an IC₅₀ of 108 nM. Finally, when tested on AD mice model, it managed to improve cognitive impairment and to reduce tau phosphorylation and A β deposition.

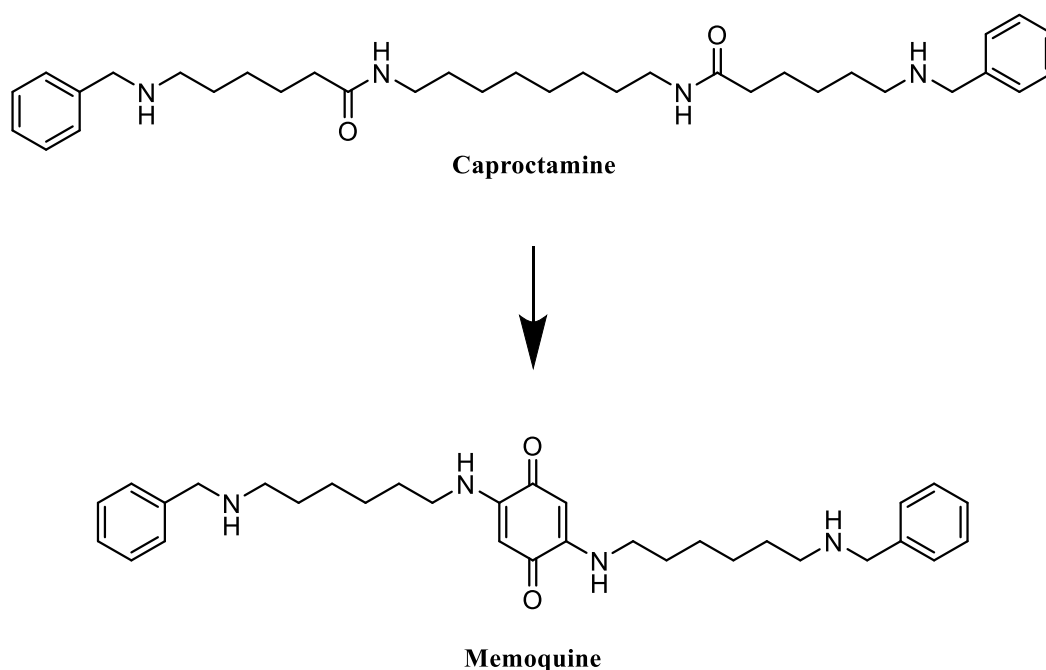


Figure 30. Design of Memoquin starting from caproctamine.

Ladostigil was designed as a ChEI with antioxidant properties. Its structure presents the rivastigmine carbamate moiety substituted into position 6 of rasagiline (Figure 31). It was reported to inhibit BuChE 100 times more efficiently than AChE. However, activity of AChE was reduced by 20-80% after oral administration, increasing Ach levels in dose-dependent manner. Moreover, it was able to inhibit both MAO isoforms in vivo but not in vitro, probably through transformation in active metabolite.¹⁷⁹

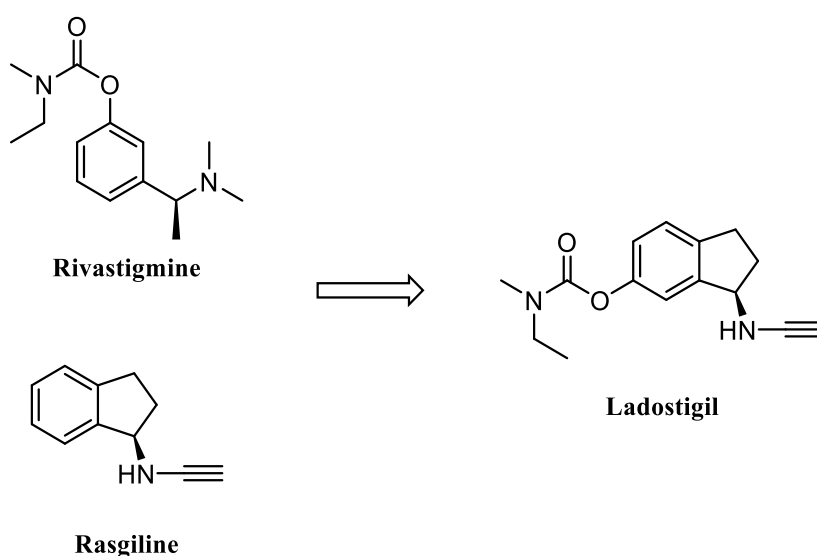


Figure 31. Structure and design of Ladostigil.

Other MTDLs used in AD are based on HDACI and GSK-3 β inhibitors. Regarding HDACI, it is very common to find MTDL presenting hydroxamic acids as ZBG, while the second pharmacophore acts as CAP group in addition to its first activity.¹⁸⁰ A first example of HDACI base MTDL are those designed in combination with phosphodiesterase 5 (PDE5). PDE5 in CNS is located in Purkinje cells, hippocampus, substantia nigra and in cerebellum, where it hydrolyses cGMP (cyclic guanosine monophosphate) and activates protein kinase g, which starts several signal pathways. Moreover, cGMP modulates ionic channels sensible to nucleotides, which plays an important role in neuronal physiology¹⁸¹. Some studies point out the possible implication of PDE5 in neuronal cells survival and, if alteration occurs, in the onset of neurodegenerative diseases like AD, PD, Huntington disease and more. In detail, PDE5 is more active in AD brain and its inhibition promotes the transcription of CREB genes involved in long term memory improvement.¹⁸² A combination of Vorinostat and Tadalafil, known PDE5 inhibitor, weakened AD symptoms in murine model of AD.¹⁸³ It was observed that synergic inhibition of HDAC and PDE5 modulates histone acetylation, overtaking strong inhibition of class I HDAC, which generates toxic effects. Other PDE5 inhibitors, as Sildenafil and Vardenafil, were used as CAP group, after a study in X-ray showed that a linker could be inserted without compromising activity towards the enzymes. Finally, the results of testing of several linkers led to compound CM-414 (Figure 32).¹⁸⁴ This molecule displayed high inhibitory activity against PDE5 and HDAC6 and a lower one towards class I HDAC. In mice model of AD, it showed the ability to reduce levels of A β 42, APP e phosphorylated tau, reversing the cognitive impairment.¹⁸⁰

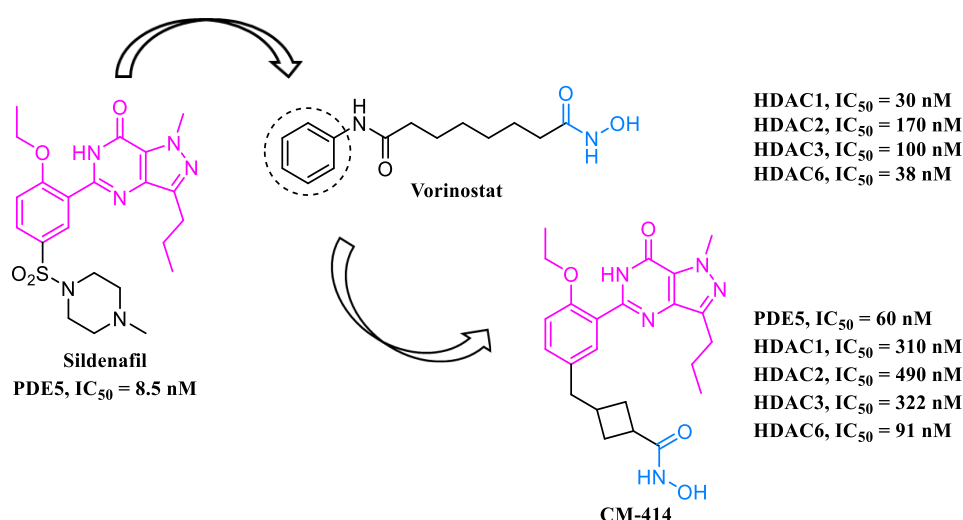


Figure 32. Design of dual inhibitor HDAC/PDE5 CM-414.¹⁰⁹

Another example of HDACI based MTDL is RBC-2008, a HDAC inhibitor with antioxidant properties obtained by the combination of Tubastatin A and Ebselen (Figure 33). It displayed good activity towards HDAC6 and good selectivity against class I HDACs. In antioxidant tests, it resulted more active than Ebselen in oxygen radicals' absorption (ORAC) and in preventing ROS accumulation. Furthermore, it was non-toxic up to 20 μ M in PC3 cell line, in which showed protective abilities against oxidative stress induce by hydrogen peroxide and 6-hydroxydopamine.¹⁸⁵

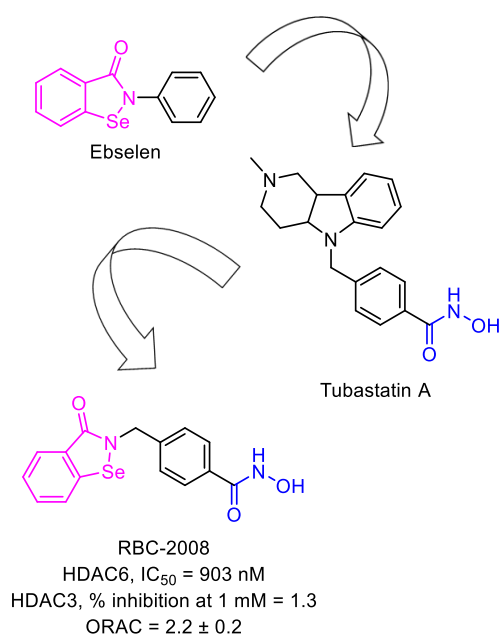


Figure 33. Design of RBC-2008.

Regarding GSK-3 β , in 2015, Cavalli, Bolognesi and co-workers reported one of the first examples of MTDLs towards GSK-3 β as neuroprotective agent. As second target they focused on BACE-1, whose inhibitors (Lanabecestat, Verubecestat and similar) can contrast the development of A β in nanomolar concentration range. In addition to the importance of tau and A β in AD onset, some connections between the two enzymes were reported. GSK-3 β increases BACE-1 activity, which produces more A β and thus activates GSK-3 β , increasing levels of NFT, inflammation and cognitive decline. The researcher studied several inhibitors, detecting the guanidine moiety of BACE-1 inhibitors and the cyclic amide of GSK-3 β inhibitors. Those fragments were combined to obtain different dual inhibitors with substituted triazinone scaffold^{186, 187}. Compound **I** was the most promising, inhibiting both enzymes in low micromolar concentration range (Figure 34).

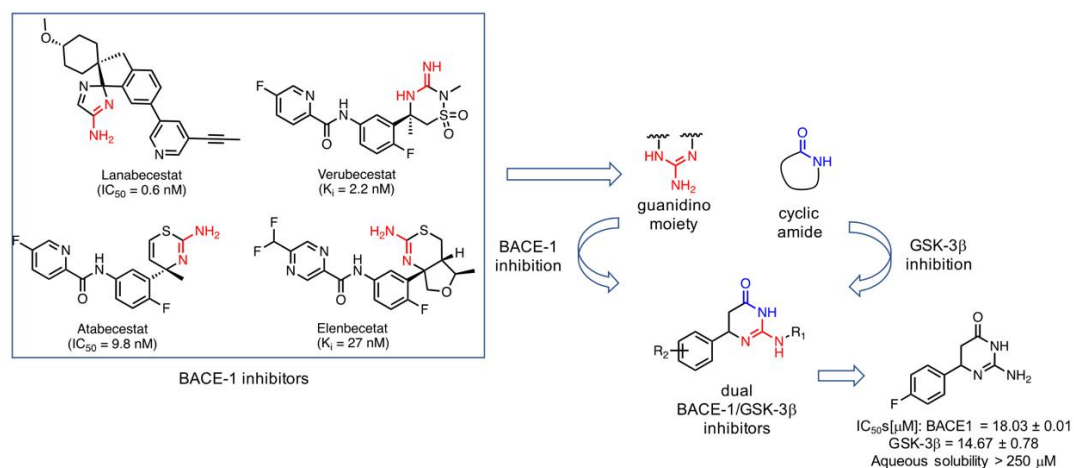


Figure 34. Design of dual inhibitor BACE-1/GSK-3 β I¹⁰⁹.

The same research group considered the structure of Tideglusib and tau-aggregation inhibitors, like hydantoin, thiohydantoin and rhodanine. The aim was to design a MTDL able to interfere with two crucial points in NFT formation: tau phosphosrylation and aggregation. Those molecules have a similar heterocyclic scaffold, which led to selected 2,4-thiazolidinedione fragment substituted in position 5 with an arilic moiety. In this way, several MTDL were obtained, in which compounds **II** and **III** resulted being the best for IC_{50} values, low toxicity and neuroprotection in SH-SY5Y cells (Figure 35). In addition, and most importantly, they were able to inhibit aggregation of tau fibrils at concentration of 50μ M.¹⁸⁸

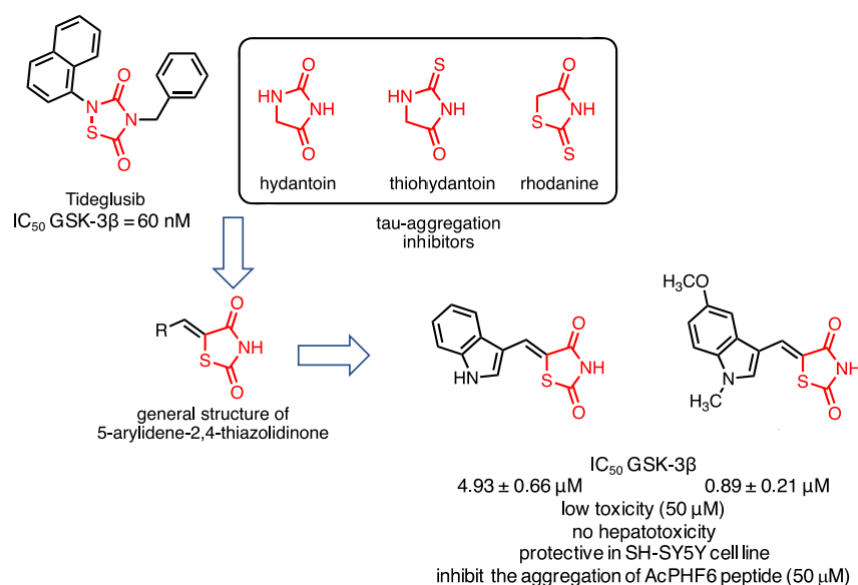


Figure 35. Design of compounds **II** and **III**, with summary of their principal properties.¹⁰⁹

In 2018, Sun et al. reported the first class of dual GSK-3 β /AChE inhibitors, moved by the hypothesis that this kind of dual ligand could bring great benefits in AD treatment. In particular, both tau phosphorylation by GSK-3 β and A β formation due to AChE activity could be inhibited. Compound **V** was designed starting from SAR studies on compound **IV**, which highlighted interaction of amidic carbonyl with Lys85, while methoxy and amide moiety are solvent exposed. This gave the opportunity to insert the linker in this portion, to link AChEI tacrine and obtain the desired product. **5** was able to inhibit both target in low nanomolar concentration range, preventing Ab aggregation at 20 μ M and tau phosphorylation at 10 μ M. Moreover, it showed improvement on cognitive disorders in vivo and, unlike tacrine, no hepatotoxicity was observed.¹⁸⁹ One year later, another example of dual GSK-3 β /AChE inhibitors was reported. In this case, tacrine was linked to valmerin, a potent GSK-3 β inhibitor, through a triazole. Among the synthesized compounds, **VI** was the most interesting for inhibition properties and low toxicity in SH-SY5Y cell line (Figure 36).¹⁹⁰

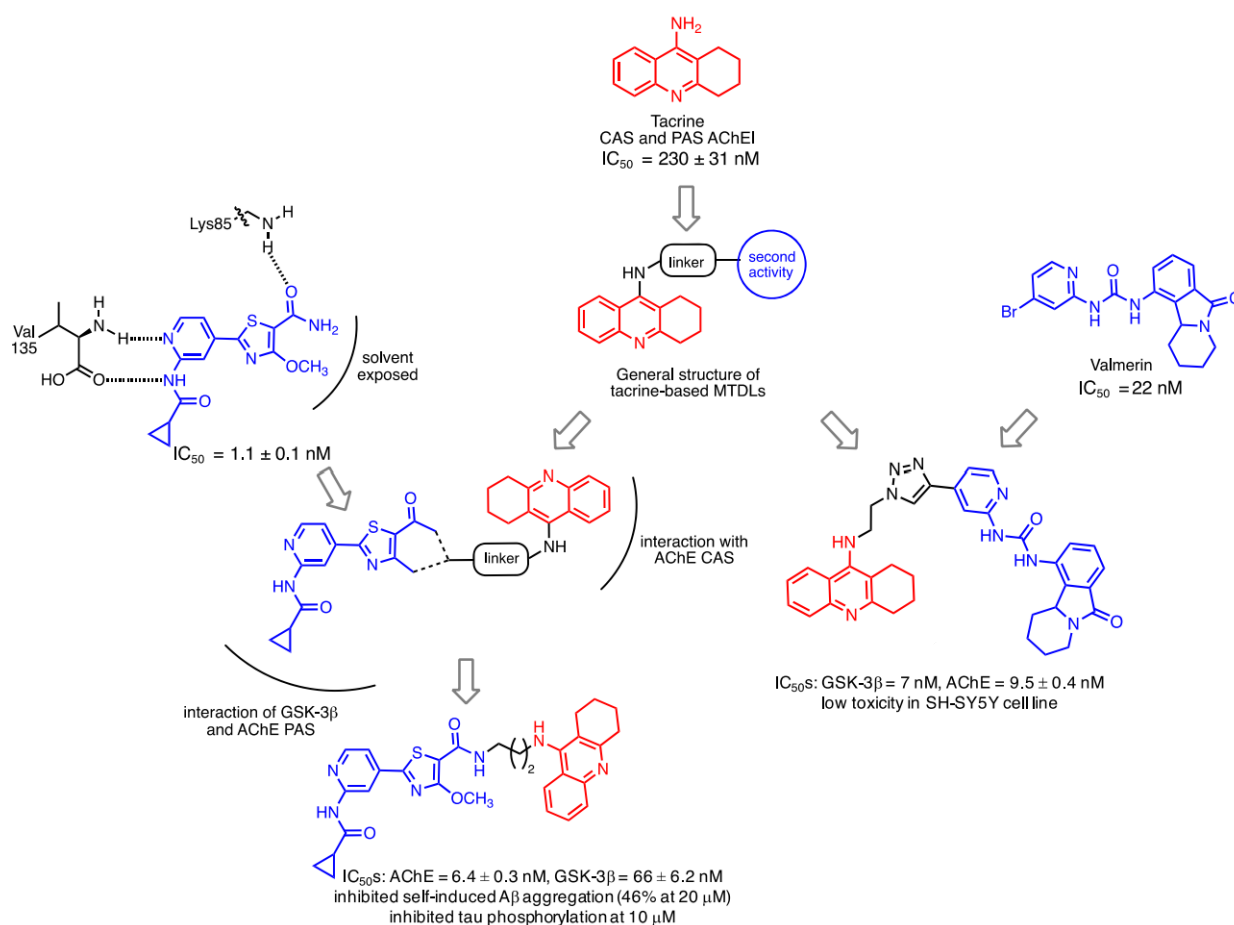


Figure 36. Design of compounds **V** and **VI**.

2 DUAL GSK-3 β / HDAC INHIBITORS

2.1 Aim of the project

Alzheimer's disease (AD) represents the most common cause of dementia, and it is a pathological condition for which a cure is not available. Unfortunately, it is also the disease with the highest attrition rates in drug discovery (99.6% of failure rate). Indeed, since Memantine's approval in 2003, only two monoclonal antibodies, aducanumab and lecanemab, has received approval from FDA, even though clinical data and therapeutic efficacy must still be proved. The failures of intense decades of research from both industry and academia may be due to the incomplete knowledge of the physiopathological events that lead to the onset of the pathology. Due to the complexity of AD, it is now widely accepted that it could be better contrasted by a chemical entity able to simultaneously modulate multiple targets involved in the onset of the disease. It is an accepted belief that a single compound able to fulfill such a task should more significantly affect the course of disease progression. However, the development of multitarget ligands is a huge challenge in terms of target selection, molecules design, and physical-chemical properties requirements of the designed molecules. Plenty of ligands have been developed so far hitting a single or multiple targets acting on different and not strictly related pathways believed important in AD pathogenesis, such as acetylcholinesterase, monoamino oxidases, oxidative stress, metal dyshomeostasis, etc. In addition to the aforementioned classical and deeply explored targets, one of the most attractive protein due to its pivotal role in this disorder is glycogen synthase kinase (GSK-3 β). It is a key signaling enzyme in AD, mainly known for its capacity to modulate tau hyperphosphorylation. Moreover, since the epigenetic modification is considered a new promising frontier for the study and treatment of AD, the interest in regulating histone acetylation through histone deacetylases (HDACs) is rising up. The emerging role of HDACs as AD target is also supported by the evidence of the role of histone acetylation in rescuing learning and memory impairment. Several connections between GSK-3 β and HDACs have been highlighted. D'Mello and co-workers have shown that the neurotoxic effects of HDAC1 depend on GSK-3 β activity and, in turn, the pharmacological blockage of such activity prevents HDAC1- induced neuronal death in cerebellar granule neurons (CGNs). In hippocampal neurons, GSK-3 β and HDAC6 were found in the same protein complex in which GSK-3 β phosphorylates HDAC6, enhancing its activity. It is worth noting that elevated HDAC6 activity increases tau phosphorylation and the HDAC6-selective inhibitor tubacin attenuates tau phosphorylation. Furthermore, both GSK-3 β and class I-II HDAC inhibitors are able to shift microglia from the neurotoxic activation (M1, proinflammatory) to the neuroprotective (M2, antiinflammatory) phenotype. Moreover, it is reported that combined inhibition of GSK-3 β and HDACs induces synergistic neuroprotective effects compared to the single drug with a potential improved therapeutic selectivity. Based on the above

considerations, the research group with which I spent my PhD recently reported about the first in class dual GSK3 β /HDAC inhibitors.¹⁹¹ To design such new class of dual inhibitors, the authors combined in a single chemical entity the pharmacophoric groups responsible for binding to GSK-3 β and HDACs (Figure 37). Analyzing the structures of known HDAC inhibitors (i.e., 1–3 in Figure 1), it emerges that the following pharmacophore features are present: the hydroxamic acid as ZBG to chelate the Zn²⁺ located in HDACs active site (in pink), an aromatic moiety, named cap group (in red), which occludes the entrance of the pocket, and a linker connecting the two moieties (in green). Particularly, polycyclic aromatic compounds are quite selective toward HDAC6 (see 2 and 3), due to its larger substrate binding pocket. As regards the GSK-3 β , many inhibitors, for instance 4–7, possess a phthalimide-like scaffold, like the aryl-maleimide or the aryl-pyrrolinone, which are acknowledged to competitively bind to the ATP binding site of the enzyme. Bearing in mind these observations, they thought to design a small series of molecules possessing a hydroxamic acid as ZBG and the phthalimide moiety as cap group. In fact, this rather large aromatic moiety might possibly fit in both HDAC1 and HDAC6 and bind the GSK-3 β enzyme. The hydroxamic acid has been connected to the phthalimide moiety through polymethylenic linkers of different length (**VIII–XI**).

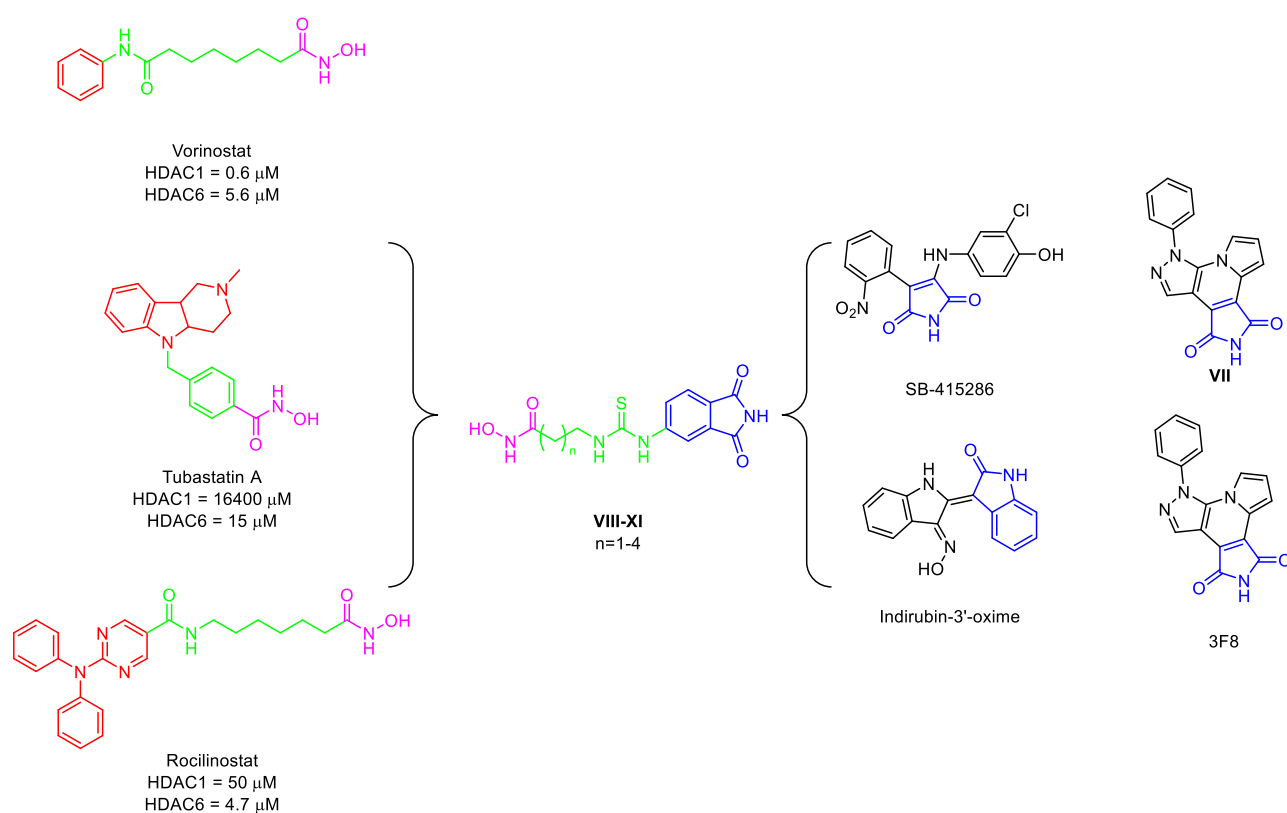


Figure 37. Design of compounds **VIII–XI**.

Among the different compound synthesized, compound **XI** (n=4) emerged as the most interesting of the series. In particular, it inhibits GSK-3 β , HDAC1 and HDAC6 with an IC₅₀ of 2.69 $\pm$ 0.01, 12.78 $\pm$ 0.11 and 3.19 $\pm$ 0.08 μ M, respectively. Interestingly, it shows balanced *in vitro* activities against the targets. Compound **XI** is able to induce enzyme inhibition also in cell-based assay being able to induce hyperacetylation of histone H3 at lysines 9/14 (H3K9K14ac) and tubulin in SH-SY5Y cell lines. In particular, this effect was already visible at 0.1 μ M and was concentration dependent. However, acetylation induced by compound **XI** was more marked in tubulin than histone H3. Since tubulin is a substrate of HDAC6 and acetylated tubulin levels function as a biochemical marker for HDAC6 cellular activity, the increase in the level of acetyl α -tubulin compared to H3K9K14ac could be ascribed to an intrinsic cellular selectivity of compound **XI** for HDAC6 isoform. Compound **XI** (10 μ M) completely counteracted copper mediated tau phosphorylation in SH-SY5Y cell line in a major extent compared to LiCl (10 mM), and Vorinostat (10 μ M) confirming the ability to block GSK-3 β activity in cell. Furthermore, it did not show any toxic effects in SH-SY5Y up to 100 μ M and it was able to counteract oxidative stress-induced neuronal death already at the concentration of 0.1 μ M. Interestingly, compound **XI** turned out to be more efficient than the combination of GSK3 β and HDAC inhibitors. Moreover, it promotes neurogenesis and displays immunomodulatory effects, shows no lethality in a wt-zebrafish model (<100 μ M) and high water solubility. Recently, it has been reported that compound **XI** improved survival of both human *CDKL5*-deficient cells and hippocampal neurons from *Cdkl5* KO mice suggesting that it may have a wider therapeutic benefit in *CDKL5* deficiency disorder patients.¹⁹¹

Aim of these two projects is the development of new series of dual GSK-3 β /HDAC inhibitors based on the structure of an ATP-competitive inhibitor AR-A014418 (project#1) and a non-ATP competitive GSK-3 β inhibitor Tideglusib (project#2)

2.2 Project#1. Design, synthesis, and biological evaluation of dual ATP-competitive GSK-3 β / HDAC inhibitors.

In this regard, we focused our attention on a GSK-3 β inhibitor with a heteroarylureidic structure, AR-A014418.¹²⁹ This compound, developed by Astra-Zeneca, inhibits GSK-3 β with an IC₅₀ of 104 $\pm$ 27 nM in an ATP-competitive manner. It demonstrates a good selectivity over CDK2 and CDK5, inhibits tau protein phosphorylation in cells and blocks the β -amyloid-induced neurodegenerative process in hippocampal neurons. From the analysis of the crystallographic structure (Figure 38), it has been observed that AR-A014418 binds in the hinge region forming three bonds with the protein: (i) H-bond between an NH ureidic and the carbonyl oxygen of proline 136, (ii) H-bond between a ureidic

NH and the carbonyl oxygen of valine 135, and (iii) H-bond between the N of the thiazole ring and the NH of valine 135. In addition, the nitro group occupies the central part of the of the ATP binding pocket. The methoxybenzyl group assumes an orientation of 108° relative to the inhibitor core, and the methoxy group points toward the region exposed to the solvent.

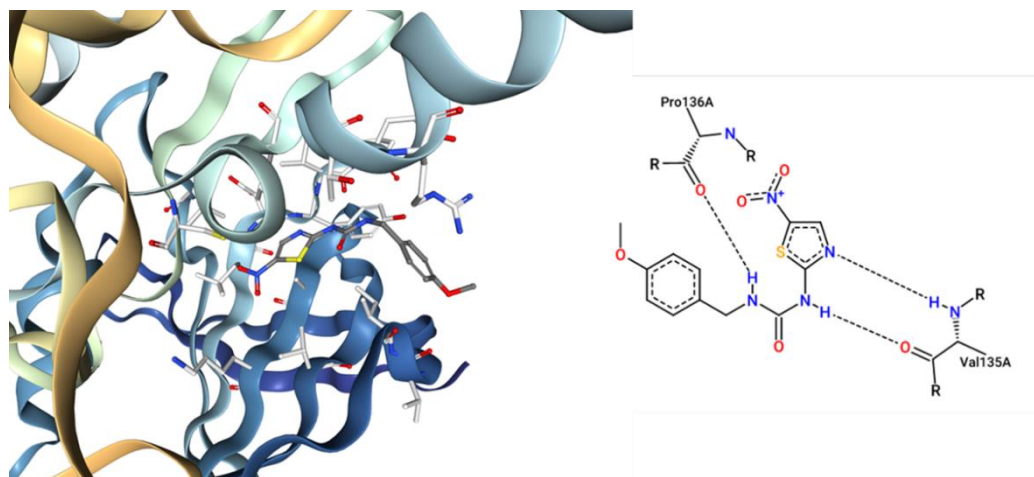


Figure 38. X-Ray analysis of AR-A014418 in the GSK-3 β ATP binding site (PDB:1Q5K).

Therefore, the methoxy group can be substituted without loss of activity. Considering these considerations, comparing the structure of AR-A014418 and known HDAC inhibitors, we designed a new class of dual inhibitors by replacing the methoxyl group of AR-A014418 with ZBGs, which can bind the Zn²⁺ ion located in the active site of HDAC. In particular, two series have been produced, one bearing as ZBG the hydroxamic acid, characteristic of Vorinostat and Nexturastat, and the other one bearing the benzamide, characteristic of Entinostat.

In order to obtain compounds with the best characteristics of enzyme inhibitions and physicochemical properties and in order to study Structure-Activity Relationships (SARs), we designed and synthesized a library of compounds in which aromatic or heteroaromatic rings differently substituted are introduced to ureidic group, obtaining **1-17** and **20-36**. Moreover, in order to confirm the necessity of a (hetero)-aromatic substituent at the ureidic group, we designed and synthesized the derivatives **18** and **37**, which have a cycloalkyl group. Finally, we synthesized derivatives **19** and **38**, that presents the nitrothiazole ring, which is characteristic of compound AR-A014418 (Figure 39).

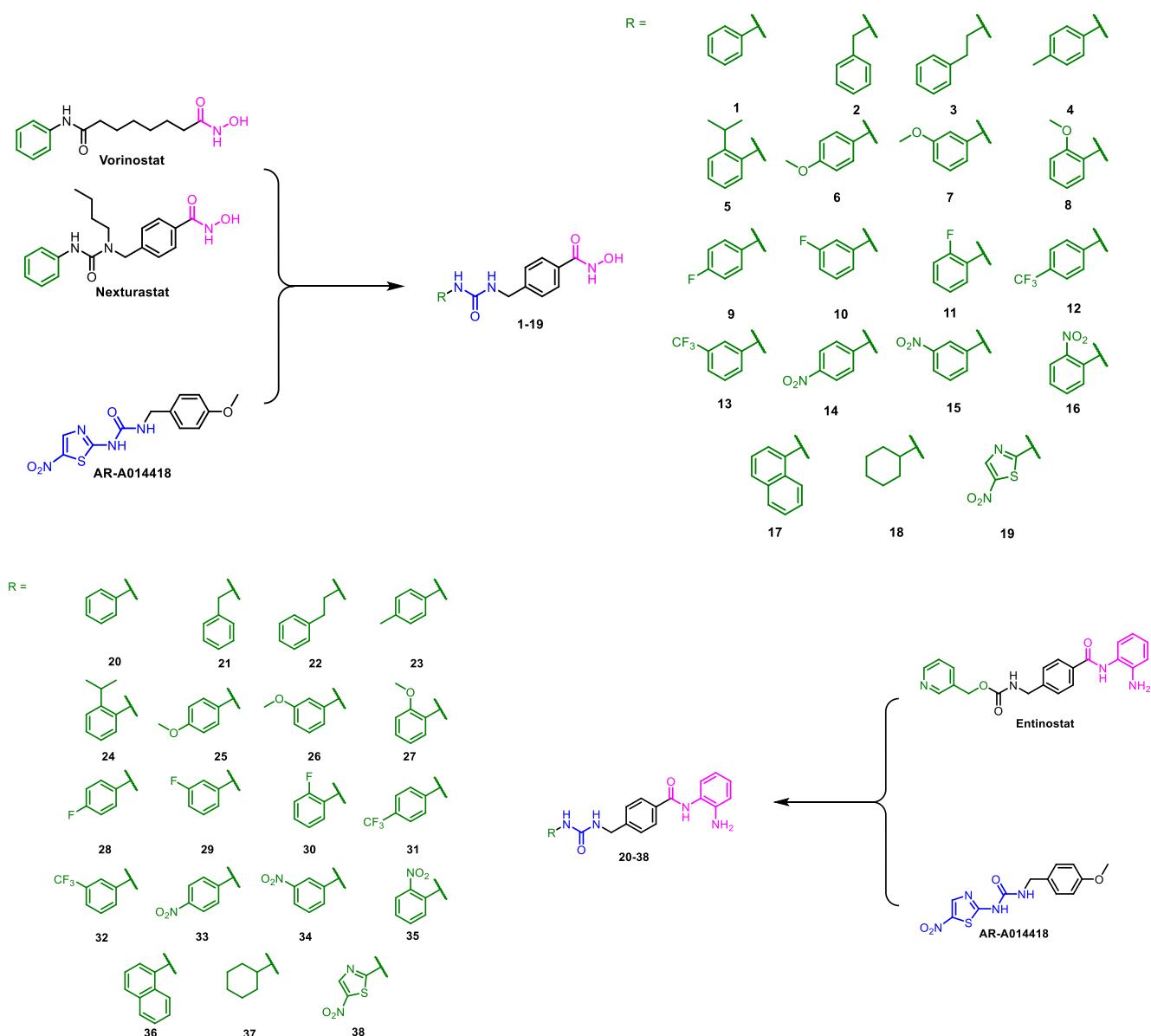
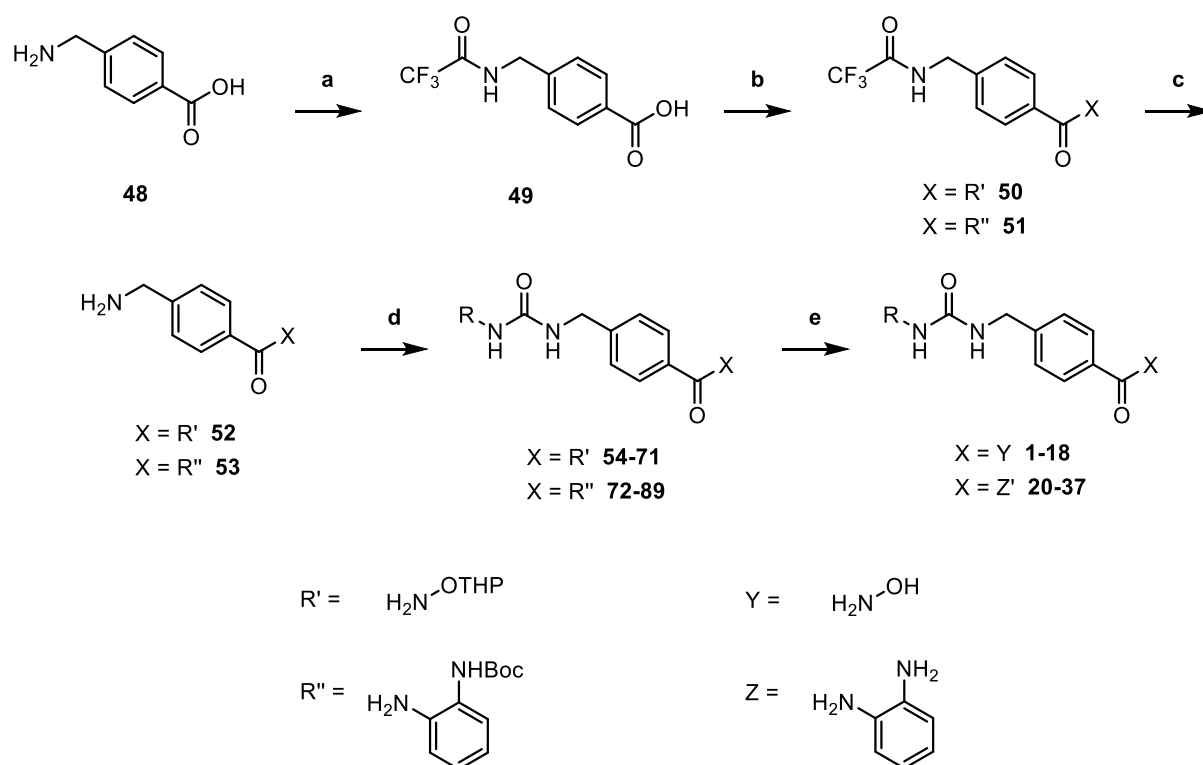


Figure 39. Drug design leading to dual compounds 1-38.

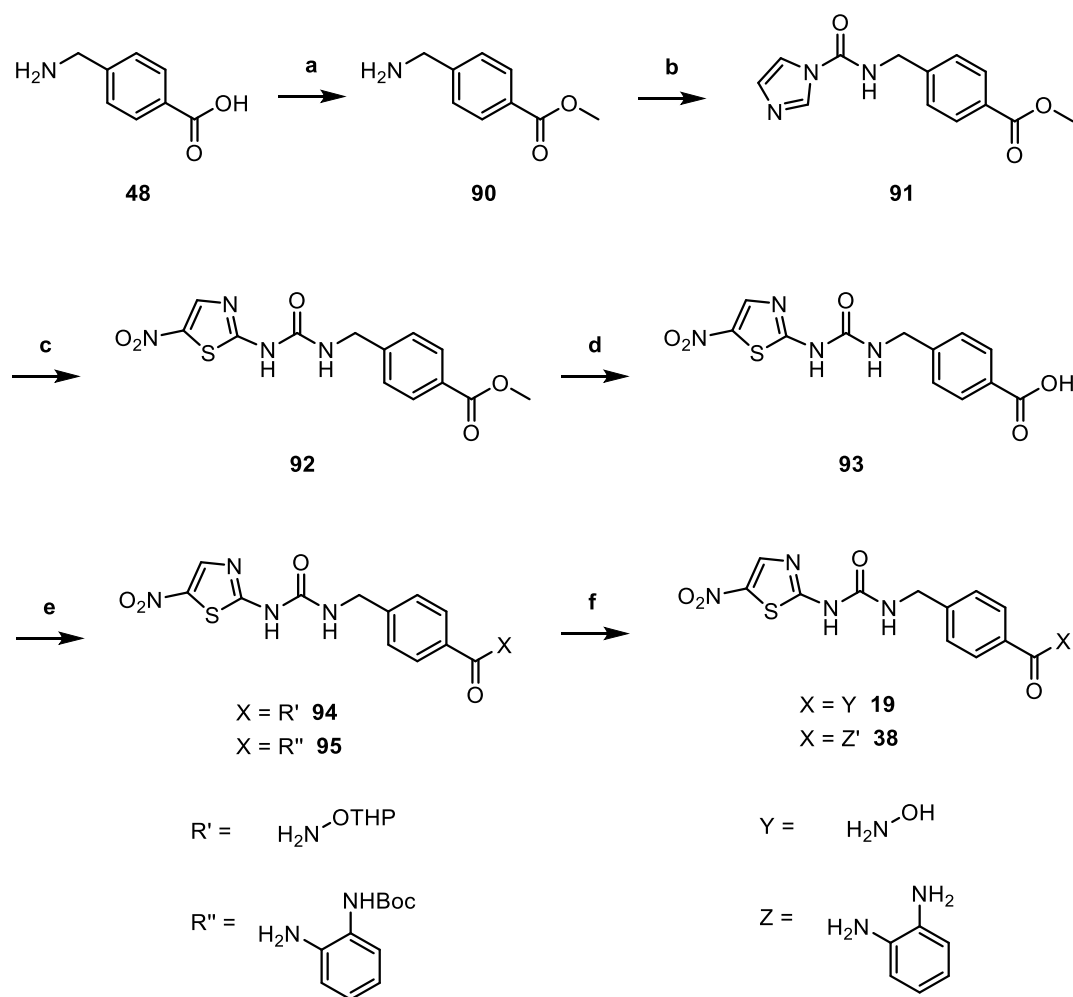
2.2.1 Chemistry section

The synthesis of the target compounds is reported in Scheme 1. Commercially available amino acid **48** was protected on the amino group using trifluoroacetic anhydride to obtain compound **49**. Amydation reaction was performed using tetrahydropyran-protected hydroxylamine and Boc-protected o-phenyldiamine to obtain **50** and **51**, respectively. These two compounds were reacted with different isocyanates to give two series of ureic derivatives, **54-71** and **72-89**. Subsequent deprotection led to final products **1-38**.



SCHEME 1. Synthesis of compounds **1-18** and **20-37**. Reagents and conditions. A) $(\text{CF}_3\text{CO})_2\text{O}$, rt, 2h; b) R'/R'' , EDCI, DMAP, THF, rt, 12h, N_2 ; c) K_2CO_3 , $\text{H}_2\text{O}/\text{MeOH}$, rt, 12h; d) $\text{R}-\text{NCO}$, DCM, rt, 2-12h; e) TFA, DCM, rt, 12h.

Regarding the synthesis of compounds **19** and **38**, since 2-isocyanato-5-nitrothiazole is not commercially available, an alternative procedure has been developed (Scheme 2). Amino acid **48** was esterified with MeOH in presence of sulfuric acid to obtain ester **90**. Amidation reaction with carbonyldiimidazole was performed to give mixed urea **91**, which was converted in compound **92** by reaction with 5-nitro-2-aminothiazole activated by NaH. Subsequent hydrolysis of ester group to carboxylic acid gave **93**, which was reacted with the two protected ZBGs to obtain intermediates **94** and **95**. Final deprotection with TFA led to products **19** and **38**.



SCHEME 2. Synthesis of compounds **19** and **38**. Reagents and conditions. a) H_2SO_4 , MeOH, rt, 5h; b) CDI, THF/DCM, rt, 12h; c) 5-nitro-2-aminothiazole, NaH, DMF, rt, 12h, N_2 ; d) Na_2CO_3 , MeOH/ H_2O , rt, 12h; e) R'/R'' , EDCI, DMAP, THF, rt, 12h, N_2 ; f) TFA, DCM, rt, 12h.

2.2.2 Results and discussion

To determine the potential interest of compounds **1-38**, their inhibitory potency on GSK-3 β , HDAC2, and HDAC6 in comparison to their parent compounds AR-A014418, Nexturastat and Vorinostat will be determined. Compounds have been first evaluated for their inhibitory activity at the concentration of 15 μM and IC_{50} values have been determined for compounds that showed an inhibitory activity higher than 50 % at that concentration (Table 1). Up to now, all the compounds, with exception of compounds **15**, **22**, **34** and **35**, have been evaluated for their inhibitory activity on GSK-3 β at 15 μM . By considering these data, it turned out that some of the hydroxamic acid-based compounds **1-18** are good inhibitors. Compounds **1**, **4**, **6**, **7**, **17** and **19** show inhibitory activity at higher than 50% and their IC_{50} s values have been determined. In particular, compound **19** characterized by the nitroaminothiazole substituent, as AR-A014418, is the best inhibitor of the series with an IC_{50} of 0.04 ± 0.01 μM . It is important to point out that compound **19** is even more active than the parent pure

GSK-3 β inhibitor AR-A014418 characterized by an IC₅₀ of 0.10 $\pm$ 0.05 μ M. Regarding compounds characterized by benzamide as ZBGs (**20-38**) are very weak GSK-3 β inhibitors and none of them is able to inhibit more the 50% at the concentration of 15 μ M. The only exception is represented by compound **38**, characterized by the nitroaminothiazole substituent, with an IC₅₀ value of 0.15 $\pm$ 0.06 μ M, slightly higher than the inhibitory activity of its parent compound AR-A014418.

Table 1. Effects of Compounds **1-38**, AR-A014418 and Nexturastat against GSK-3 β .

Compound	GSK-3 β		Compound	GSK-3 β	
	% inhibition @15 μ M ^a	IC ₅₀ (μ M) ^{a,b}		% inhibition @ 15 μ M ^a	IC ₅₀ (μ M) ^{a,b}
1	73.48 $\pm$ 7.74	10.75 $\pm$ 0.82	21	43.82 $\pm$ 5.47	n.d. ^e
2	39.72 $\pm$ 7.04	n.d. ^e	22	16.46 $\pm$ 3.06	n.d. ^e
3	22.94 $\pm$ 0.08	n.d. ^e	23	25.74 $\pm$ 3.72	n.d. ^e
4	51.49 $\pm$ 5.32	26.48 $\pm$ 3.80	24	10.59 $\pm$ 3.63	n.d. ^e
5	39.59 $\pm$ 4.57	n.d. ^e	25	15.59 $\pm$ 1.34	n.d. ^e
6	49.27 $\pm$ 7.62	29.16 $\pm$ 1.77	26	22.44 $\pm$ 3.70	n.d. ^e
7	59.34 $\pm$ 2.24	21.59 $\pm$ 1.60	27	23.00 $\pm$ 3.50	n.d. ^e
8	39.35 $\pm$ 3.6	n.d. ^e	28	23.70 $\pm$ 1.47	n.d. ^e
9	2.74 $\pm$ 2.83	n.d. ^e	29	42.14 $\pm$ 0.97	n.d. ^e
10	50.85 $\pm$ 3.83	n.d. ^e	30	7.53 $\pm$ 1.60	n.d. ^e
11	40.72 $\pm$ 1.65	n.d. ^e	31	29.30 $\pm$ 5.09	n.d. ^e
12	39.27 $\pm$ 3.55	n.d. ^e	32	n.i. ^d	n.d. ^e
13	11.57 $\pm$ 2.76	n.d. ^e	33	17.35 $\pm$ 1.27	n.d. ^e
14	50.76 $\pm$ 4.40	n.d. ^e	34	n.d. ^e	n.d. ^e
15	n.d. ^e	n.d. ^e	35	n.d. ^e	n.d. ^e
16	7.70 $\pm$ 0.16	n.d. ^e	36	34.43 $\pm$ 1.70	n.d. ^e
17	53.23 $\pm$ 0.24	26.07 $\pm$ 2.98	37	19.45 $\pm$ 2.06	n.d. ^e
18	17.74 $\pm$ 2.51	n.d. ^e	38	t.i. ^c	0.15 $\pm$ 0.06
19	t.i. ^c	0.04 $\pm$ 0.01	AR-A014418	n.d. ^e	0.10 $\pm$ 0.05
20	38.20 $\pm$ 2.84	n.d. ^e	Nexturastat	37.60	n.d. ^e

^a values are reported as a mean value of three or more determinations; ^b IC₅₀ values are defined as the drug concentration that reduces by 50% the target activity and are reported as a mean value of three or more determinations; ^c t.i.= total inhibition; ^d n.i. = not inhibition; ^e n.d. = not determined.

Regarding HDACs isoform inhibitory activity, up to now only few data are available (Table 2). Compounds have been preliminarily evaluated against HDAC2 and HDAC6 isoforms at 15 μ M concentration. As it is reported in Table 2, most of the compounds show total enzyme HDAC2 inhibition at the tested concentration and a slightly lower activity against HDAC6. This selectivity seems to be more pronounced in the benzamide series (**20-38**).

Table 2. Effects of selected Compounds **1**, **16**, **19**, **33**, **38** against HDAC2 and HDAC6.

Compound	% inhibition @ 15 μ M			% inhibition @ 15 μ M	
	HDAC2	HDAC6		HDAC2	HDAC6
1	90.91 $\pm$ 0.44	95.81 $\pm$ 4.19	20	48.58 $\pm$ 4.71	43.39 $\pm$ 0.15
2	n.d. ^c	n.d. ^c	21	t.i. ^b	54.46 $\pm$ 2.43
3	t.i. ^b	87.64	22	t.i. ^b	61.87 $\pm$ 3.12
4	t.i. ^b	t.i. ^b	23	t.i. ^b	65.77
5	t.i. ^b	t.i. ^b	24	32.61 $\pm$ 5.64	22.67 $\pm$ 0.89
6	t.i. ^b	t.i. ^b	25	t.i. ^b	60.73 $\pm$ 0.52
7	t.i. ^b	t.i. ^b	26	54.03 $\pm$ 5.40	34.24 $\pm$ 2.26
8	n.d. ^c	n.d. ^c	27	n.d. ^c	n.d. ^c
9	n.d. ^c	n.d. ^c	28	t.i. ^b	64.16
10	t.i. ^b	t.i. ^b	29	t.i. ^b	67.39 $\pm$ 0.49
11	n.d.	n.d. ^c	30	t.i. ^b	64.74 $\pm$ 0.53
12	t.i. ^b	t.i. ^b	31	t.i. ^b	53.76 $\pm$ 4.35
13	t.i. ^b	t.i. ^b	32	t.i. ^b	64.13 $\pm$ 2.31
14	n.d. ^c	n.d. ^c	33	88.73 $\pm$ 1.07	81.76 $\pm$ 0.46
15	n.d. ^c	n.d. ^c	34	n.d. ^c	n.d. ^c
16	84.80 $\pm$ 0.95	76.53 $\pm$ 2.14	35	t.i. ^b	64.49 $\pm$ 0.62
17	t.i. ^b	t.i. ^b	36	29.64 $\pm$ 3.47	24.42 $\pm$ 5.03
18	t.i. ^b	n.d. ^c	37	t.i. ^b	58.24 $\pm$ 1.73
19	t.i. ^b	t.i. ^b	38	82.69 $\pm$ 0.08	65.86 $\pm$ 3.68

^a values are reported as a mean value of three or more determinations. ^b t.i.= total inhibition; ^c n.d. = not determined.

In order to evaluate whether the *in vitro* inhibitory activities translate into intracellular inhibition of HDACs, further in-cell-based assays for compounds **1** and **19** were carried out. Western blotting analysis was performed using human neuroblastoma SH-SY5Y cell line to determine the effects of selected compounds on the acetylation levels of tubulin and histone H3 at lysines 9/14 (H3K9K14ac) (Figure 40). Cells were treated with selected compounds **1** and **19** and their parent compounds AR-A014418 and Nexturastat for 30 h at three different concentrations (0.1, 1.0, 10 μ M). Compound **19** turned out to be a better in-cell inhibitor compared to **1**. Indeed, compound **1** does not seem to induce any increase in acetylated histone H3 at none of the tested concentrations. **1** induce a slight increase in acetylated tubulin only at 10 μ M. At the opposite, **19** induces a significant increase in both acetylated histone H3 and tubulin already at 1 μ M and regarding acetylated tubulin in a dose-dependent manner. It is clear that **19** induces a higher acetylation of tubulin respect to histone H3. Since tubulin is a substrate of HDAC6 and acetylated tubulin levels function as a biochemical marker for HDAC6 cellular activity, the increase in the level of acetyl tubulin compared to H3K9K14ac could be ascribed to an intrinsic cellular selectivity of compound **19** for HDAC6 isoform. As expected,

relevant effects on tubulin and histone H3 were not observed when cells were treated with GSK-3 β inhibitor AR-A014418, while Nexturastat confirmed its HDAC inhibitory properties.

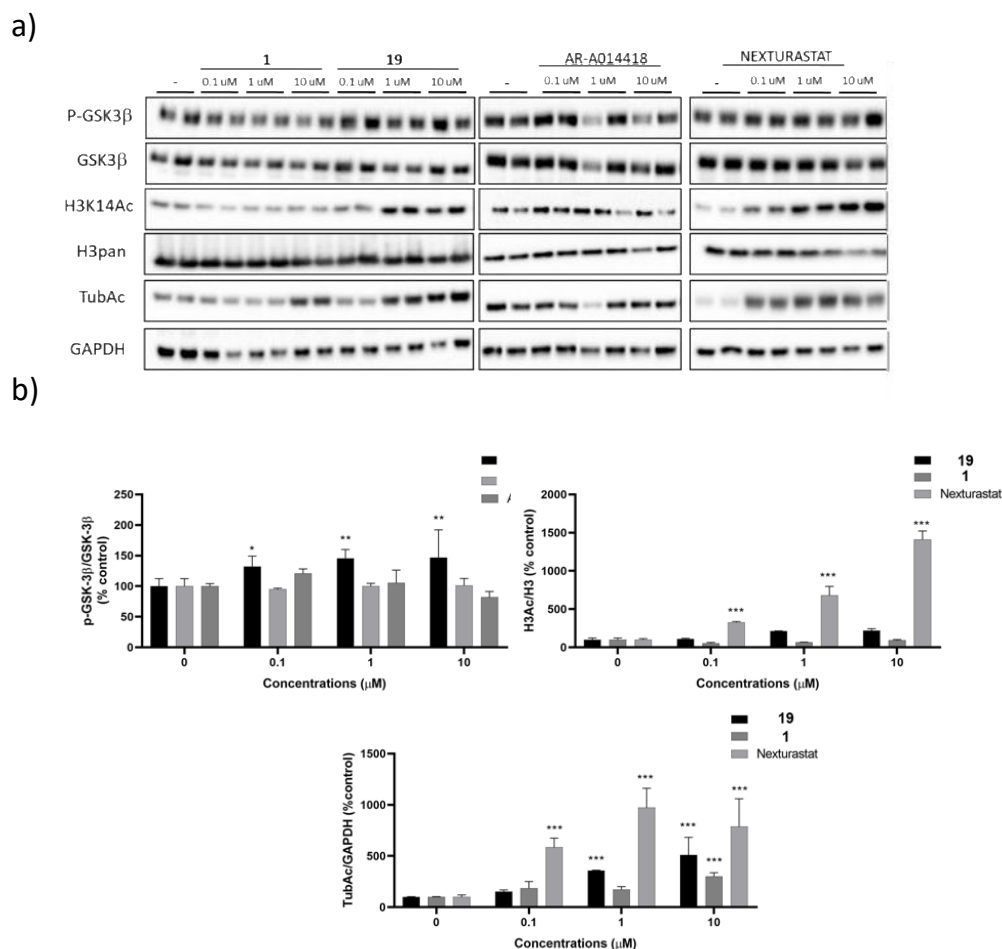


Figure 40. a) Western blot probing for phosphorylated GSK-3 β , acetylated histone H3 and α -tubulin in SH-SY5Y cells after 24h treatment. b) Densitometry analysis regarding western blotting results of **1** and **19** on GSK-3 β phosphorylation in comparison to AR-A014418, histone H3 acetylation and tubulin acetylation in correlation with Nexturastat. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to untreated cells.

Then, to assess the effect of selected compounds **1** and **19** on tau hyperphosphorylation, the protein's phosphorylation was induced in differentiated SH-SY5Y cells upon incubation with copper. Copper induced a time-dependent tau phosphorylation in differentiated SH-SY5Y cells, with a peak after 16 h of incubation. Based on these data, 400 μ M copper for 16 h was chosen as experimental condition. Then, the ability of the compounds to counteract copper-induced tau phosphorylation was examined. AR-A014418 was used as reference control (Figure 41). As reported, compound **19** (10 μ M) counteracted copper mediated tau phosphorylation in a major extent compared to AR-A014418 (10

μM), and **1** ($10\ \mu\text{M}$). These data suggest that a dual-target molecule may offer advantages in terms of cellular localization that enable an enhancement of its efficacy.

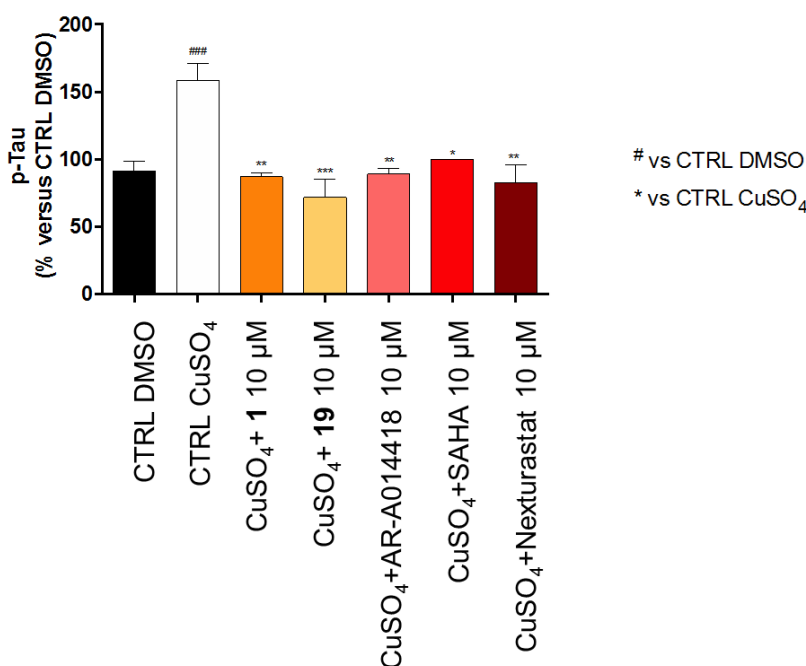


Figure 41. Effects of the compounds on tau phosphorylation. The cells were pretreated with **1**, **19**, AR-A014418, Vorinostat, Nexturastat ($10\ \mu\text{M}$) for 1 h and then with copper ($400\ \mu\text{M}$) for 16 h. Following incubation time, cells were harvest by scraping, and the pellet was obtained. Subsequently, the extent of tau phosphorylation was determined by immunoenzymatic assay. Absorbance was measured at 450 nm. Values represent the mean $\pm$ SEM; * $p < 0.05$, *** $p < 0.001$ vs control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs cells treated with copper.

Since HDAC inhibitors are used in therapy as anticancer drugs, selected compounds **1** and **19** were evaluated for their cytotoxic potential in SH-SY5Y cell line in comparison to AR-A014418, Vorinostat and Nexturastat at six different concentrations (0.1 , 1 , 10 , 25 , 50 , $100\ \mu\text{M}$) using MTT assay (Figure 42). Compound **19** does not show any cytotoxic effect up to $100\ \mu\text{M}$, while **1** shows cytotoxicity only at the highest concentration ($100\ \mu\text{M}$). Among HDACIs, Vorinostat shows significant toxicity already at $10\ \mu\text{M}$ while Nexturastat shows toxicity only at $50\ \mu\text{M}$. AR-A014418 shows slight cytotoxicity at $25\ \mu\text{M}$.

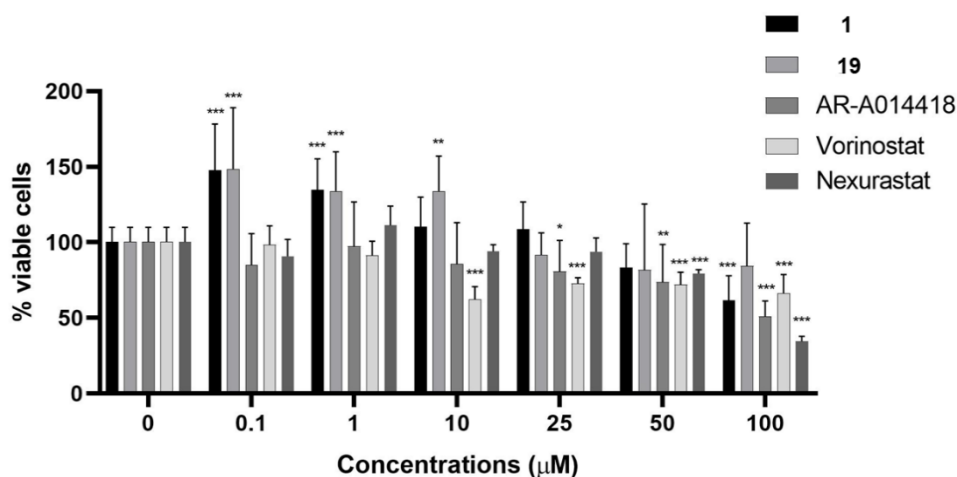
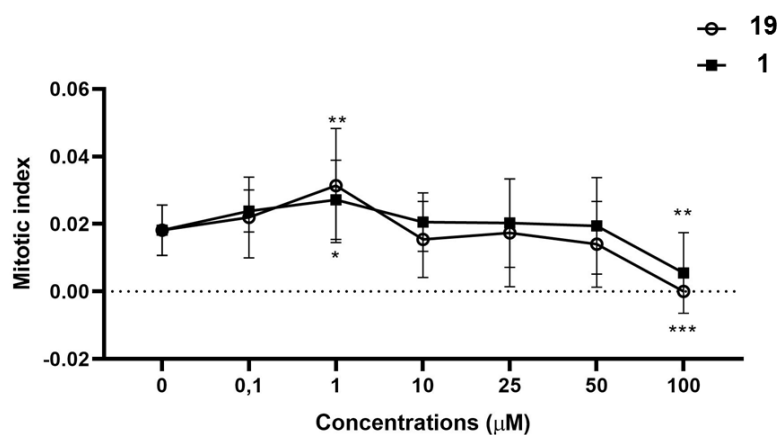


Figure 42. Cytotoxicity of compounds **1**, **19**, AR-A014418, Vorinostat and Nexturastat in SH-SY5Y cells. Cells were incubated for 24h with different concentration of the studied compounds (0.1 – 100 μM). At the end of incubation, the cell viability was measured by MTT assay. Data are reported as mean ± SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to untreated cells.

Since compounds **1** and **19** at the concentration of 0.1 and 1 μM induced an increase in the percentage of viable cells, mitotic and pycnotic index have been determined to clarify cells behavior. In particular, mitotic index is a measure of the proliferation status of a cell population (i.e. the proportion of dividing cells) and it is elevated during cellular division. From Figure 43a, it is clear that both compounds are characterized by an increased mitotic index at the concentration of 1 μM confirming the results obtained with the MTT assay. On the other hand, pyknotic index is a measure of cells undergoing necrosis or apoptosis. In this context, in Figure 43b, it can be observed that up to 50 μM compounds **1** and **19** do not increase the number of dying cells, in line with the results of MTT (Figure 42) and mitotic index (Figure 43a) assays.

a)



b)

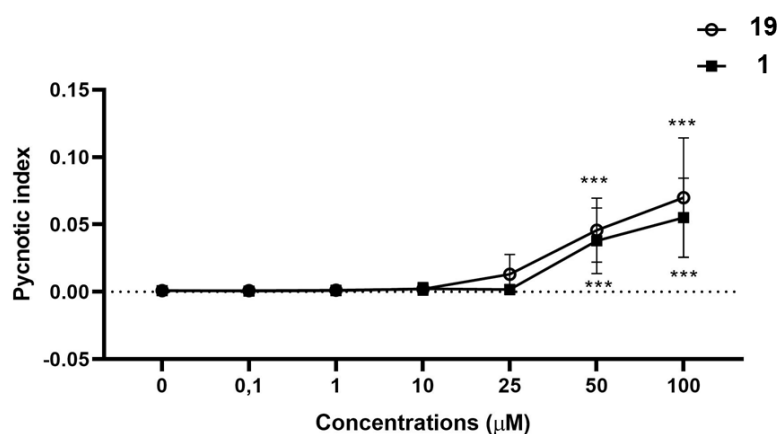


Figure 43. a) Mitotic and b) pyknotic index of compounds **1** and **19** in SH-SY5Y after 24h compounds exposure. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to untreated cells.

One of the main challenge in designing multiple ligands is related to the physical–chemical properties of the designed molecules. In silico prediction of some pharmacokinetic and ADME properties of compounds **1** and **19** were obtained from SwissADME,^{192, 193} an online web tool, and displayed in Table 3. As it can be observed, both compounds fulfill the requirements for the Lipinsky rule of Five and are predicted to not be a P-gp substrate. Furthermore, very importantly, both compounds are not identified as PAINS.

Table 3. In silico pharmacokinetic data for **1** and **19**.^a

	1	19		1	19
MW	285.30	338.32	MR	77.35	80.26
Rotable Bonds	7	8	GI absorption	high	low
H-bond A	3	6	P-gp substrate	no	no
H-bond D	4	5	Leadlikeness	yes	yes
TPSA	90.46	181.25	Lipinsky	yes	yes
Log $P_{o/w}$	1.24	-4.15	PAINS	no	no

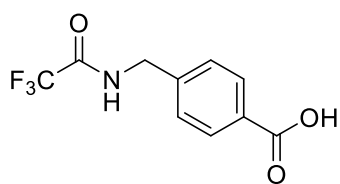
^a Obtained from <http://www.swissadme.ch/>, access date January13, 2023

2.2.3 Conclusions

In conclusion, two new series of dual GSK-3 β /HDAC inhibitors, based on urea moiety of AR-A014418 and ZBGs of Vorinostat and Entinostat, were developed. The obtained compound **1-38** were tested on their activity against target enzymes at the concentration of 15 μ M, and compound **1**, characterized by phenyl substituent, and compound **19**, substituted with 5-nitro-2-aminothiazole, resulted being the best and were chosen for further evaluation in SH-SY5Y cell line. Western blotting assay was performed to compare activity of **1** and **19** with parent inhibitors: compound **19** resulted being more selective in increasing levels of acetylated tubulin instead of histone H3 compared to Nexturastat, hinting at a selectivity against HDAC6 rather than HDAC2. Moreover, both compounds **1** and **19** showed low toxicity in SH-SY5Y cell lines. Compound **19** was also able to counteract Copper-mediated tau phosphorylation, reducing its level under control values. In general, compound **19** proved to be an interesting dual inhibitor against GSK-3 β and HDAC, having good inhibiting ability and cytoprotecting properties when tested in SH-SY5Y cell line.

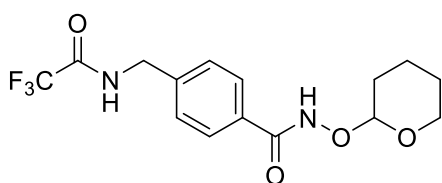
2.2.4 Experimental section

Chemistry



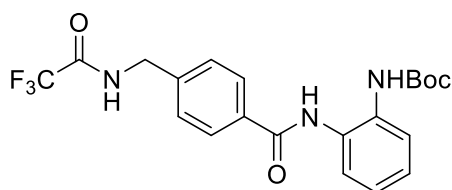
4-((2,2,2-trifluoroacetamido)methyl)benzoic acid (49). Amino acid **48** (4 g, 27 mmol) was solved in 10 ml of trifluoroacetic anhydride and stirred at rt for 2 h. Water was added and the precipitate filtered under vacuo. The filtrate was dried to obtain 6.6 g of white powder (quantitative yield).

General procedure for the synthesis of 50 and 51. To a solution of **49** in THF, protected hydroxylamine or o-phenylenediamine, EDCI and HOBt were added at 0°C under stream of nitrogen. Then the mixture was allowed to warm to room temperature (rt) and stirred for 12 h. Ethyl acetate and water were added, and the organic phase was washed with brine, dried over Na₂SO₄ and concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of petroleum ether / ethyl acetate to give the corresponding **50** and **51** as white solid.



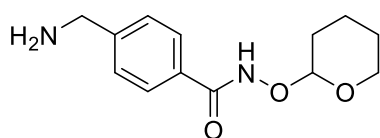
N-(((tetrahydro-2H-pyran-2-yl)oxy)-4-((2,2,2-trifluoroacetamido)methyl)benzamide (50). Synthesized from **49** (0.880 g, 3.55 mmol), O-(tetrahydro-2H-pyran-2-yl)hydroxylamine (0.500 g, 4.27 mmol), EDCI (1.36 g, 7.1 mmol) and HOBt (0.480 g, 3.55 mmol). Obtained 0.790g of a white solid (64% yield).

¹H NMR (400 MHz, DMSO) δ 11.61 (s, 1H), 10.05 (t, J = 6.0 Hz, 1H), 7.79 – 7.71 (m, 2H), 7.40 – 7.33 (m, 2H), 4.99 (d, J = 3.1 Hz, 1H), 4.44 (d, J = 6.0 Hz, 2H), 4.10 – 3.99 (m, 1H), 3.55 – 3.46 (m, 1H), 1.80 – 1.62 (m, 4H), 1.55 (dq, J = 8.4, 5.0 Hz, 3H). ¹³C NMR (100 MHz, DMSO) δ 174.00, 164.48, 157.08, 156.72, 156.00, 141.52, 131.87, 127.94, 127.71, 101.44, 61.83, 49.03, 42.76, 28.64, 25.16, 18.74.



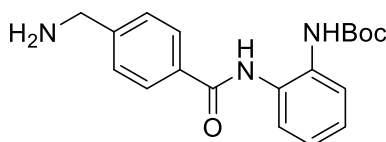
Tert-butyl (2-(4-((2,2,2-trifluoroacetamido)methyl)benzamido)phenyl)carbamate (51). Synthesized from **49** (1.200 g, 4.8 mmol), tert-butyl (2-aminophenyl)carbamate (1.000 g, 4.8 mmol), EDCI (1.840 g, 9.6 mmol) and HOBt (0.648 g, 4.8 mmol). Obtained 0.785 g of a pale yellow solid (38% yield).

General procedure for the synthesis of 52 and 53. To a solution of **50** or **51** in a mixture of methanol and water, potassium carbonate was added and the mixture was stirred at rt for 12 h. The solvent was concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of DCM / methanol with addition of ammonia to give the corresponding **52** or **53** as colorless oil.



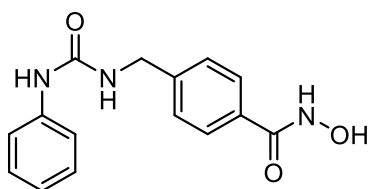
4-(aminomethyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (52). Synthesized from **50** (0.790 g, 2.29 mmol) and K₂CO₃ (0.956 g, 6.8 mmol). Obtained 0.512 g of a pale yellow solid (89% yield).

¹H NMR (400 MHz, DMSO) δ 7.80 – 7.66 (m, 2H), 7.44 (d, J = 8.3 Hz, 2H), 4.99 (d, J = 3.2 Hz, 1H), 4.12 – 3.98 (m, 1H), 3.83 (s, 2H), 3.52 (d, J = 11.3 Hz, 1H), 1.72 (s, 3H), 1.62 – 1.46 (m, 3H). ¹³C NMR (100 MHz, DMSO) δ 164.49, 146.02, 131.12, 127.72, 127.58, 101.42, 61.83, 49.03, 44.94, 28.37, 25.18, 18.77.



Tert-butyl (2-(4-(aminomethyl)benzamido)phenyl)carbamate (53). Synthesized from **51** (0.785 g, 2.30 mmol) and K₂CO₃ (0.953 g, 6.8 mmol). Obtained 0.500 g of a white solid (64% yield).

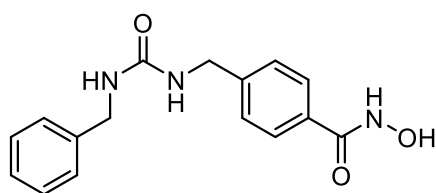
General procedure for the synthesis of 1-18 and 20-37. To a solution of **52** or **53** in DCM, corresponding isocyanates were added and the mixture was stirred at rt for 2-12 h and monitored via TLC. When the starting reagents were consumed, TFA was added and the mixture was stirred for 1-12 h. The solvent was concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of DCM / methanol to give the corresponding **1-18** and **20-37** as yellow/red solids.



N-hydroxy-4-((3-phenylureido)methyl)benzamide (1).

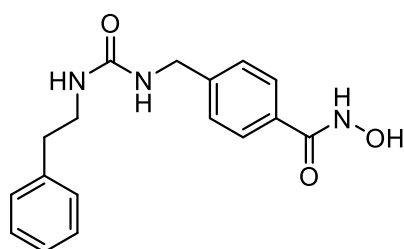
Synthesized from **52** (0.300 g, 1.2 mmol), isocyanatobenzene (0.157 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.062 g of light red solid (18% yield). ¹H NMR (400 MHz,

DMSO) δ 11.20 (s, 1H), 8.99 (s, 1H), 8.94 (s, 1H), 7.71 (d, J = 7.9 Hz, 2H), 7.41 (d, J = 7.9 Hz, 2H), 7.36 (d, J = 7.9 Hz, 2H), 7.21 (t, J = 7.7 Hz, 2H), 6.95 (s, 1H), 6.88 (t, J = 7.3 Hz, 1H), 4.33 (d, J = 5.1 Hz, 2H). ¹³C NMR (100 MHz, DMSO) δ 164.52, 155.82, 140.97, 131.63, 129.04, 127.30 (d, J = 4.5 Hz), 121.44, 118.04, 42.82.



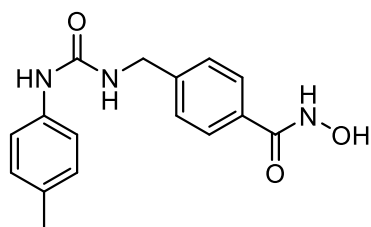
4-((3-benzylureido)methyl)-N-hydroxybenzamide (2).

Synthesized from **52** (0.300 g, 1.2 mmol), (isocyanatomethyl)benzene (0.175 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.030 g of light red solid (8% yield). ^1H NMR (400 MHz, DMSO) δ 11.17 (s, 1H), 8.98 (s, 1H), 7.69 (d, J = 7.8 Hz, 2H), 7.30 (d, J = 7.5 Hz, 4H), 7.28 – 7.16 (m, 4H), 6.57 (s, 2H), 4.26 (d, J = 5.3 Hz, 2H), 4.23 (d, J = 5.4 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 164.58, 158.56, 144.77, 141.30, 131.48, 128.63, 127.40, 127.23 (d, J = 7.4 Hz), 126.98, 43.39, 43.11.



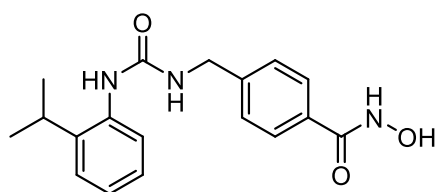
N-hydroxy-4-((3-phenethylureido)methyl)benzamide (3).

Synthesized from **52** (0.300 g, 1.2 mmol), (2-isocyanatoethyl)benzene (0.194 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.150 g of light red solid (40% yield). ^1H NMR (400 MHz, DMSO) δ 7.69 (d, J = 8.1 Hz, 2H), 7.33 – 7.24 (m, 4H), 7.20 (d, J = 6.8 Hz, 3H), 4.23 (s, 2H), 3.24 (t, J = 7.2 Hz, 2H), 2.69 (t, J = 7.2 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 164.52, 158.45, 144.86, 140.16, 131.43, 129.14, 128.74, 127.22 (d, J = 8.1 Hz), 126.43, 42.99, 41.44, 36.59.



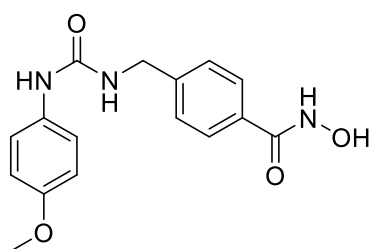
N-hydroxy-4-((3-(p-tolyl)ureido)methyl)benzamide (4).

Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-4-methylbenzene (0.175 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.087 g of light red solid (24% yield). ^1H NMR (400 MHz, DMSO) δ 11.17 (s, 1H), 8.98 (s, 1H), 8.66 (s, 1H), 7.71 (d, J = 8.2 Hz, 2H), 7.35 (d, J = 8.2 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 7.02 (d, J = 8.2 Hz, 2H), 6.76 (s, 1H), 4.32 (d, J = 5.7 Hz, 2H), 2.21 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 164.59, 155.90, 144.36, 138.42, 131.58, 130.13, 129.46, 127.33 (d, J = 6.0 Hz), 118.12, 42.82, 20.75.



N-hydroxy-4-((3-(2-isopropylphenyl)ureido)methyl)benzamide (5).

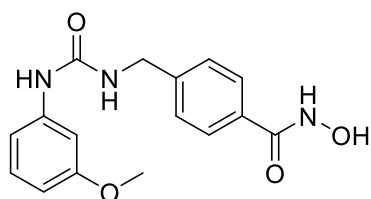
Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-2-isopropylbenzene (0.212 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.050 g of light red solid (13% yield). ^1H NMR (400 MHz, DMSO) δ 9.01 (s, 1H), 7.84 (s, 1H), 7.73 (d, J = 8.2 Hz, 2H), 7.63 (dd, J = 8.0, 1.1 Hz, 1H), 7.37 (d, J = 8.2 Hz, 2H), 7.23 (dd, J = 7.7, 1.4 Hz, 1H), 7.09 (td, J = 7.7, 1.5 Hz, 1H), 7.05 – 6.98 (m, 2H), 4.34 (d, J = 5.8 Hz, 2H), 3.19 – 3.07 (m, 1H), 1.16 (d, J = 6.8 Hz, 6H).



N-hydroxy-4-((3-(4-methoxyphenyl)ureido)methyl)benzamide

(6). Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-4-methoxybenzene (0.197 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.057 g of light red solid (15% yield).

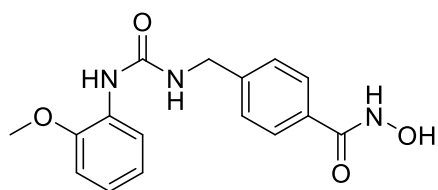
^1H NMR (400 MHz, DMSO) δ 11.24 (s, 1H), 9.13 (s, 1H), 9.01 (s, 1H), 7.71 (d, J = 7.6 Hz, 2H), 7.33 (dd, J = 13.5, 8.0 Hz, 4H), 6.79 (d, J = 8.3 Hz, 2H), 4.31 (s, 2H), 3.68 (s, 3H).



N-hydroxy-4-((3-(3-methoxyphenyl)ureido)methyl)benzamide

(7). Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-3-methoxybenzene (0.197 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.120 g of light red solid (32% yield).

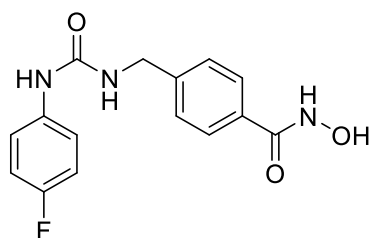
^1H NMR (400 MHz, DMSO) δ 10.17 (s, 1H), 7.97 (t, J = 5.4 Hz, 1H), 7.71 (d, J = 8.3 Hz, 1H), 7.35 (d, J = 8.3 Hz, 2H), 7.25 – 7.11 (m, 3H), 6.99 (ddd, J = 8.0, 2.0, 0.9 Hz, 1H), 6.93 – 6.86 (m, 1H), 6.63 (ddd, J = 8.3, 2.6, 0.9 Hz, 1H), 4.33 (d, J = 5.4 Hz, 2H), 3.73 (s, 3H).



N-hydroxy-4-((3-(2-methoxyphenyl)ureido)methyl)benzamide (8).

Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-2-methoxybenzene (0.197 g, 1.32 mmol) and TFA (2 ml) as result of two-steps

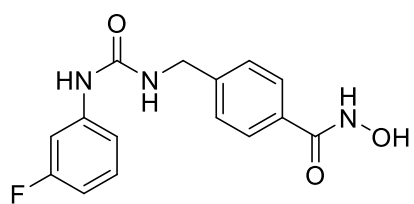
synthesis. Obtained 0.067 g of light red solid (19% yield). ^1H NMR (400 MHz, DMSO) δ 11.19 (s, 1H), 8.08 (dd, J = 7.7, 1.9 Hz, 1H), 8.05 (s, 1H), 7.72 (d, J = 8.3 Hz, 2H), 7.39 (t, J = 5.9 Hz, 1H), 7.35 (d, J = 8.3 Hz, 2H), 6.96 (dd, J = 7.9, 1.6 Hz, 1H), 6.85 (dtd, J = 17.8, 7.5, 1.7 Hz, 2H), 4.33 (d, J = 5.8 Hz, 2H), 3.83 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 155.64, 147.75, 144.03, 131.69, 129.76, 127.39 (d, J = 3.0 Hz), 121.56, 120.90, 118.40, 111.00, 56.09, 42.85.



4-((3-(4-fluorophenyl)ureido)methyl)-N-hydroxybenzamide (9).

Synthesized from **52** (0.300 g, 1.2 mmol), 1-fluoro-4-isocyanatobenzene (0.180 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.030 g of light red solid (8% yield).

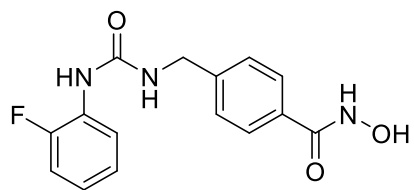
^1H NMR (400 MHz, DMSO) δ 10.81 (s, 1H), 10.21 (s, 1H), 8.73 (s, 1H), 7.60 (d, J = 8.2 Hz, 2H), 7.35 (d, J = 7.9 Hz, 2H), 7.16 (d, J = 8.9 Hz, 2H), 7.05 (s, 2H), 6.76 (t, J = 6.2 Hz, 1H), 4.35 (d, J = 6.0 Hz, 2H).



4-((3-(3-fluorophenyl)ureido)methyl)-N-hydroxybenzamide

(10). Synthesized from **52** (0.300 g, 1.2 mmol), 1-fluoro-3-isocyanatobenzene (0.180 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.043 g of light red solid (12% yield).

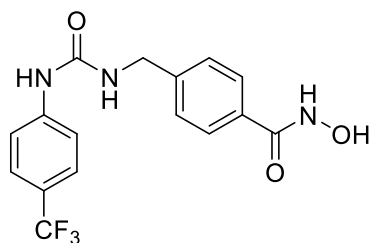
^1H NMR (400 MHz, DMSO) δ 11.19 (s, 1H), 9.16 (s, 1H), 7.71 (d, J = 8.2 Hz, 2H), 7.46 (dt, J = 12.3, 2.2 Hz, 2H), 7.35 (d, J = 8.1 Hz, 2H), 7.25 – 7.19 (m, 1H), 7.08 – 7.03 (m, 1H), 7.00 (t, J = 6.1 Hz, 1H), 6.69 (td, J = 8.4, 2.7 Hz, 1H), 4.33 (d, J = 5.9 Hz, 2H).



4-((3-(2-fluorophenyl)ureido)methyl)-N-hydroxybenzamide

(11). Synthesized from **52** (0.300 g, 1.2 mmol), 1-fluoro-2-isocyanatobenzene (0.180 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.048 g of light red solid (13% yield).

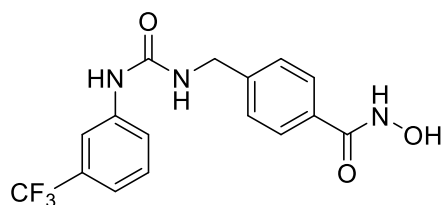
^1H NMR (400 MHz, DMSO) δ 9.01 (s, 1H), 8.47 (s, 1H), 8.11 (t, J = 7.8 Hz, 1H), 7.72 (d, J = 8.1 Hz, 2H), 7.36 (d, J = 8.1 Hz, 2H), 7.24 – 7.13 (m, 2H), 7.07 (t, J = 7.6 Hz, 1H), 6.93 (q, J = 6.5 Hz, 1H), 4.35 (d, J = 5.6 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 155.43, 153.33, 150.94, 143.85, 131.73, 128.69, 128.59, 127.39 (d, J = 8.0 Hz), 124.81 (d, J = 3.4 Hz), 122.15 (d, J = 7.3 Hz), 120.73, 115.37, 115.18, 42.88.



N-hydroxy-4-((3-(4-(trifluoromethyl)phenyl)ureido)methyl)benzamide

(12). Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-4-(trifluoromethyl)benzene (0.247 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.045 g of light red solid (11% yield).

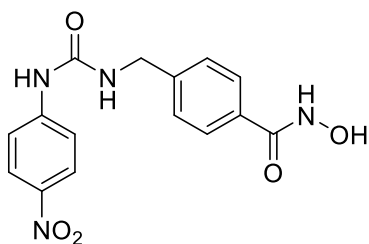
^1H NMR (400 MHz, DMSO) δ 11.20 (s, 1H), 9.76 (s, 1H), 9.00 (s, 1H), 7.71 (d, J = 7.7 Hz, 2H), 7.62 (d, J = 8.5 Hz, 3H), 7.55 (d, J = 8.3 Hz, 2H), 7.36 (d, J = 8.0 Hz, 2H), 7.23 – 7.11 (m, 1H), 4.35 (d, J = 5.4 Hz, 2H).



N-hydroxy-4-((3-(3-(trifluoromethyl)phenyl)ureido)methyl)benzamide

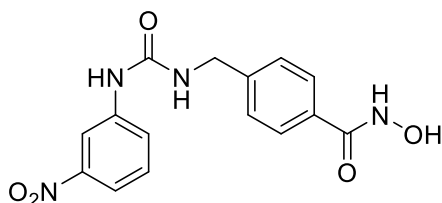
(13). Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-3-(trifluoromethyl)benzene (0.247 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.167 g of light red solid (39% yield).

^1H NMR (400 MHz, DMSO) δ 11.21 (s, 1H), 9.33 (s, 1H), 9.02 (s, 1H), 7.99 (d, J = 2.0 Hz, 1H), 7.72 (d, J = 8.0 Hz, 2H), 7.58 – 7.50 (m, 1H), 7.44 (t, J = 7.9 Hz, 1H), 7.36 (d, J = 8.0 Hz, 2H), 7.22 (d, J = 7.5 Hz, 1H), 7.06 (t, J = 6.1 Hz, 1H), 4.34 (d, J = 5.6 Hz, 2H).



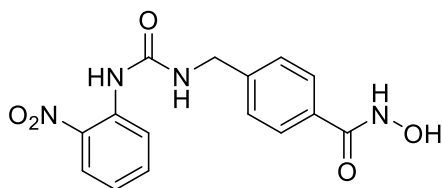
N-hydroxy-4-((3-(4-nitrophenyl)ureido)methyl)benzamide (14).

Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-4-nitrobenzene (0.216 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.089 g of light red solid (23% yield). ^1H NMR (400 MHz, DMSO) δ 11.19 (s, 1H), 9.97 (s, 1H), 8.99 (s, 1H), 8.14 (d, J = 8.4 Hz, 2H), 7.72 (d, J = 7.7 Hz, 2H), 7.65 (d, J = 8.3 Hz, 2H), 7.37 (d, J = 7.7 Hz, 3H), 4.37 (s, 2H).



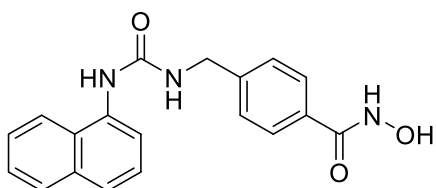
N-hydroxy-4-((3-(3-nitrophenyl)ureido)methyl)benzamide (15).

Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-3-nitrobenzene (0.216 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.109 g of light red solid (28% yield). ^1H NMR (400 MHz, DMSO) δ 11.21 (s, 1H), 8.56 (s, 1H), 7.71 (dt, J = 15.1, 7.9 Hz, 4H), 7.50 (t, J = 8.1 Hz, 1H), 7.36 (d, J = 8.1 Hz, 3H), 4.36 (d, J = 5.1 Hz, 2H).



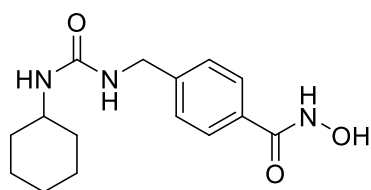
N-hydroxy-4-((3-(2-nitrophenyl)ureido)methyl)benzamide (16).

Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanato-2-nitrobenzene (0.216 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.159 g of light red solid (40% yield). ^1H NMR (400 MHz, DMSO) δ 11.20 (s, 1H), 9.49 (s, 1H), 9.01 (s, 1H), 8.28 (d, J = 8.4 Hz, 1H), 8.04 (d, J = 8.2 Hz, 2H), 7.73 (d, J = 8.0 Hz, 2H), 7.64 (t, J = 7.6 Hz, 1H), 7.38 (d, J = 8.0 Hz, 2H), 7.27 (t, J = 6.9 Hz, 1H), 7.13 (t, J = 7.6 Hz, 1H), 4.36 (d, J = 5.5 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 154.83, 143.40, 137.64, 136.05, 135.36, 129.08 (d, J = 2.8 Hz), 127.47 (d, J = 8.9 Hz), 127.20, 125.75, 122.55, 122.06, 43.11.



N-hydroxy-4-((3-(naphthalen-1-yl)ureido)methyl)benzamide (17).

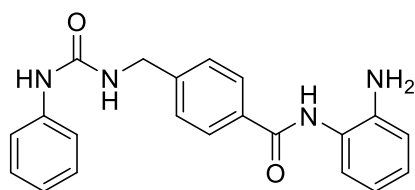
Synthesized from **52** (0.300 g, 1.2 mmol), 1-isocyanatonaphthalene (0.223 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.017 g of light red solid (4% yield). ^1H NMR (400 MHz, DMSO) δ 10.10 (s, 1H), 9.58 (s, 1H), 8.40 (dd, J = 8.5, 1.2 Hz, 1H), 8.06 (ddd, J = 17.3, 7.6, 1.1 Hz, 2H), 8.00 – 7.92 (m, 2H), 7.73 – 7.68 (m, 1H), 7.63 (dd, J = 9.7, 8.5 Hz, 2H), 7.60 – 7.53 (m, 2H), 7.49 (td, J = 7.9, 3.0 Hz, 2H), 7.42 (dt, J = 8.0, 3.8 Hz, 1H), 3.91 (t, J = 7.3 Hz, 2H).



4-((3-cyclohexylureido)methyl)-N-hydroxybenzamide (18).

Synthesized from **52** (0.300 g, 1.2 mmol), isocyanatocyclohexane (0.165 g, 1.32 mmol) and TFA (2 ml) as result of two-steps synthesis.

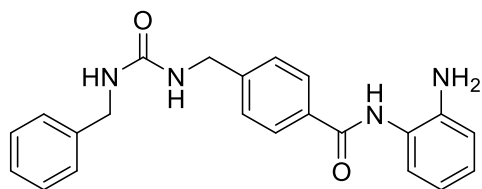
Obtained 0.050 g of light red solid (14% yield). ^1H NMR (400 MHz, DMSO) δ 11.16 (s, 1H), 8.97 (s, 1H), 7.75 – 7.60 (m, 2H), 7.28 (d, J = 8.0 Hz, 2H), 6.29 (s, 1H), 5.91 (d, J = 7.8 Hz, 1H), 4.22 (d, J = 6.0 Hz, 2H), 1.75 (dd, J = 12.7, 4.1 Hz, 2H), 1.63 (dt, J = 13.0, 3.9 Hz, 2H), 1.51 (t, J = 7.3 Hz, 1H), 1.33 – 1.20 (m, 3H), 1.19 – 1.02 (m, 3H). ^{13}C NMR (100 MHz, DMSO) δ 164.57, 157.98, 144.93, 131.38, 127.29, 127.16, 49.00, 48.20, 42.90, 33.70, 25.77, 24.88.



N-(2-aminophenyl)-4-((3-phenylureido)methyl)benzamide (20).

Synthesized from **53** (0.300 g, 0.88 mmol), isocyanatobenzene (0.115 g, 0.97 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.065 g of light yellow solid (20% yield).

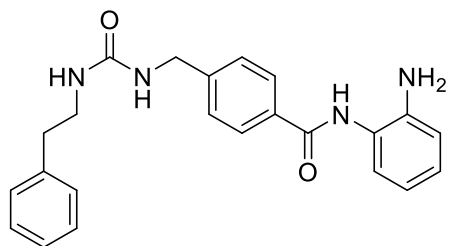
^1H NMR (400 MHz, DMSO) δ 10.77 (s, 1H), 10.07 (s, 1H), 8.65 (s, 1H), 7.93 (d, J = 8.0 Hz, 2H), 7.63 (dd, J = 8.0, 1.6 Hz, 1H), 7.50 (dd, J = 7.9, 1.7 Hz, 1H), 7.42 (dt, J = 14.9, 7.5 Hz, 4H), 7.33 (dtd, J = 15.1, 7.6, 1.7 Hz, 2H), 7.22 (t, J = 7.9 Hz, 2H), 6.90 (t, J = 7.3 Hz, 1H), 6.73 (t, J = 6.0 Hz, 1H), 4.38 (d, J = 5.9 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 165.98, 155.73, 145.04, 140.87, 132.78, 129.10, 128.30, 127.63, 127.34, 126.90, 126.66, 126.18, 121.60, 118.19, 42.94.



N-(2-aminophenyl)-4-((3-benzylureido)methyl)benzamide (21).

Synthesized from **53** (0.300 g, 0.88 mmol), (isocyanatomethyl)benzene (0.129 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis.

Obtained 0.300 g of light yellow solid (91% yield). ^1H NMR (400 MHz, DMSO) δ 9.69 (s, 1H), 7.94 (d, J = 8.1 Hz, 2H), 7.41 – 7.15 (m, 7H), 7.05 – 6.94 (m, 1H), 6.88 – 6.79 (m, 1H), 6.66 (t, J = 7.4 Hz, 1H), 6.59 (dt, J = 11.6, 6.0 Hz, 2H), 4.32 (d, J = 5.9 Hz, 2H), 4.25 (d, J = 5.9 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 158.58, 145.15, 141.34, 133.28, 128.67, 128.23, 127.44, 127.13 (d, J = 2.7 Hz), 127.01, 126.89, 124.35, 117.45, 117.08, 43.44, 43.18.

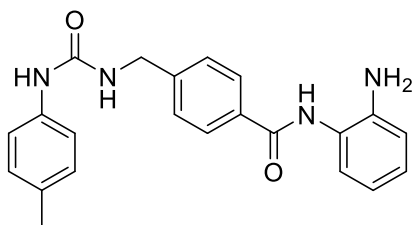


N-(2-aminophenyl)-4-((3-phenethylureido)methyl)benzamide (22).

Synthesized from **53** (0.300 g, 0.88 mmol), (2-isocyanatoethyl)benzene (0.143 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis.

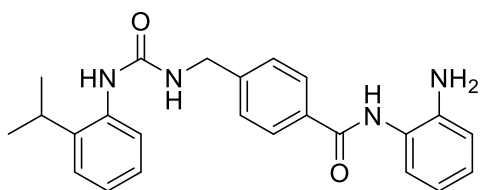
Obtained 0.120 g of light yellow solid (35% yield). ^1H NMR (400 MHz, DMSO) δ 9.80 (s, 1H), 7.94 (d, J = 8.1 Hz, 2H), 7.38 – 7.27 (m, 4H), 7.21 (q, J = 7.9 Hz,

4H), 7.05 (t, $J = 7.6$ Hz, 1H), 6.91 (d, $J = 7.8$ Hz, 1H), 6.76 (t, $J = 7.4$ Hz, 1H), 6.54 (s, 1H), 6.08 (s, 1H), 4.28 (d, $J = 5.0$ Hz, 2H), 3.26 (s, 2H), 2.70 (t, $J = 7.2$ Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 158.45, 145.32, 140.18, 133.11, 129.13, 128.74, 128.22, 127.14 (d, $J = 7.1$ Hz), 126.94, 126.43, 43.05, 41.46, 36.59.



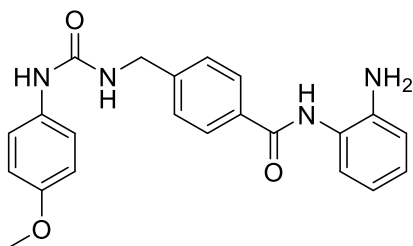
N-(2-aminophenyl)-4-((3-(p-tolyl)ureido)methyl)benzamide (23). Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-4-methylbenzene (0.129 g, 0.97 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.043 g of light yellow solid (13% yield). ^1H NMR (400 MHz, DMSO) δ 9.74 (s, 1H), 8.62 (s, 1H),

7.96 (d, $J = 8.2$ Hz, 2H), 7.43 (d, $J = 8.2$ Hz, 2H), 7.30 (d, $J = 8.4$ Hz, 2H), 7.22 (d, $J = 7.2$ Hz, 1H), 7.05 – 6.98 (m, 3H), 6.87 (dd, $J = 8.0, 1.2$ Hz, 1H), 6.76 (t, $J = 6.0$ Hz, 1H), 6.74 – 6.67 (m, 1H), 4.37 (d, $J = 5.9$ Hz, 2H), 2.22 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 165.69, 155.83, 144.73, 138.36, 133.32, 130.39, 130.26, 129.48, 128.31, 127.20 (d, $J = 8.6$ Hz), 126.93, 124.90, 118.27, 117.62, 42.94, 20.75.



N-(2-aminophenyl)-4-((3-(2-isopropylphenyl)ureido)methyl)benzamide (24).

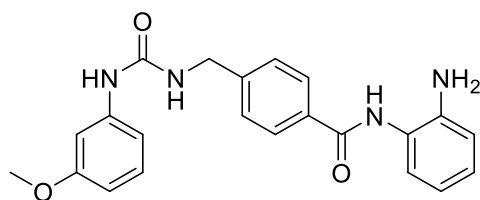
Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-2-isopropylbenzene (0.156 g, 0.97 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.140 g of light yellow solid (40% yield). ^1H NMR (400 MHz, DMSO) δ 10.78 (s, 1H), 10.08 (s, 1H), 7.95 (d, $J = 8.2$ Hz, 2H), 7.85 (s, 1H), 7.67 – 7.61 (m, 2H), 7.51 (dd, $J = 7.8, 1.4$ Hz, 1H), 7.46 (d, $J = 8.2$ Hz, 2H), 7.37 (td, $J = 7.7, 1.6$ Hz, 1H), 7.34 – 7.28 (m, 2H), 7.24 (dd, $J = 7.7, 1.4$ Hz, 1H), 7.19 – 7.07 (m, 3H), 7.02 (dd, $J = 11.0, 3.7$ Hz, 2H), 4.39 (d, $J = 5.5$ Hz, 2H), 3.14 (dt, $J = 13.6, 6.1$ Hz, 1H), 1.18 (d, $J = 2.0$ Hz, 6H). ^{13}C NMR (100 MHz, DMSO) δ 165.97, 156.46, 145.12, 139.59, 136.68, 133.02, 132.78, 129.16, 128.30, 127.63, 127.35, 127.09, 126.84 (d, $J = 10.4$ Hz), 126.67, 126.20, 126.10, 125.56, 123.87 (d, $J = 3.5$ Hz), 43.10, 27.16, 27.06, 23.50 (d, $J = 1.6$ Hz).



N-(2-aminophenyl)-4-((3-(4-methoxyphenyl)ureido)methyl)benzamide (25). Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-4-methoxybenzene (0.144 g, 0.97 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.200 g of light yellow solid (58% yield). ^1H

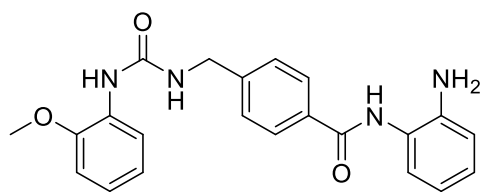
NMR (400 MHz, DMSO) δ 9.67 (s, 1H), 8.53 (s, 1H), 7.96 (d, $J = 8.1$ Hz, 2H), 7.42 (d, $J = 8.1$ Hz, 2H), 7.32 (d, $J = 9.0$ Hz, 2H), 7.19 (d, $J = 7.5$ Hz, 1H), 7.03 – 6.93 (m, 1H), 6.88 – 6.77 (m, 3H), 6.71

(t, $J = 6.0$ Hz, 1H), 6.64 (t, $J = 7.5$ Hz, 1H), 4.37 (d, $J = 5.9$ Hz, 2H), 3.69 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 155.99, 154.43, 144.73, 142.88, 134.04, 133.41, 128.28, 127.18 (d, $J = 9.8$ Hz), 126.90, 124.17, 119.94, 117.22, 116.92, 115.26, 114.33, 55.59, 42.97.



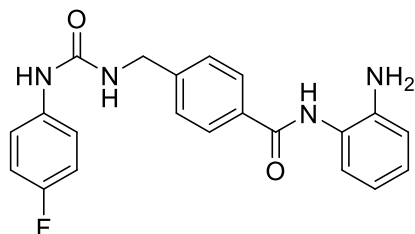
N-(2-aminophenyl)-4-((3-(3-methoxyphenyl)ureido)methyl)benzamide (26).

Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-3-methoxybenzene (0.145 g, 0.97 mmol) and TFA (2 ml) as result of two-steps synthesis. Obtained 0.180 g of light yellow solid (53% yield). ^1H NMR (400 MHz, DMSO) δ 10.76 (s, 1H), 10.06 (s, 1H), 8.69 (s, 1H), 7.93 (d, $J = 8.3$ Hz, 2H), 7.63 (dd, $J = 7.9, 1.5$ Hz, 1H), 7.50 (dd, $J = 7.8, 1.6$ Hz, 1H), 7.44 (d, $J = 8.4$ Hz, 2H), 7.36 (td, $J = 7.7, 1.7$ Hz, 1H), 7.31 (td, $J = 7.6, 1.6$ Hz, 1H), 7.17 – 7.09 (m, 4H), 6.89 (ddd, $J = 8.1, 1.9, 0.8$ Hz, 1H), 6.76 (t, $J = 6.0$ Hz, 1H), 6.48 (ddd, $J = 8.2, 2.5, 0.8$ Hz, 1H), 4.38 (d, $J = 5.8$ Hz, 2H), 3.70 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 160.57, 155.65, 130.62, 129.82, 128.29, 127.32, 55.43, 55.29.



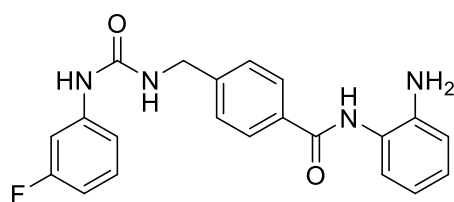
N-(2-aminophenyl)-4-((3-(2-methoxyphenyl)ureido)methyl)benzamide (27).

Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-2-methoxybenzene (0.145 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis. Obtained 0.140 g of light yellow solid (41% yield). ^1H NMR (400 MHz, DMSO) δ 8.19 (d, $J = 8.4$ Hz, 2H), 8.12 – 8.08 (m, 2H), 7.74 (dd, $J = 6.1, 3.1$ Hz, 2H), 7.56 (d, $J = 8.4$ Hz, 2H), 7.49 (t, $J = 5.9$ Hz, 1H), 7.41 (dd, $J = 6.1, 3.2$ Hz, 2H), 6.97 (dd, $J = 7.9, 1.6$ Hz, 1H), 6.86 (dtd, $J = 18.4, 7.5, 1.7$ Hz, 2H), 4.41 (d, $J = 5.8$ Hz, 2H), 3.84 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 158.37, 155.72, 150.39, 147.81, 129.74, 128.31, 127.81, 124.66, 121.63, 120.91, 118.45, 114.92, 111.02, 56.09.



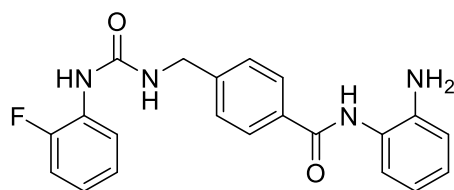
N-(2-aminophenyl)-4-((3-(4-fluorophenyl)ureido)methyl)benzamide (28).

Synthesized from **53** (0.300 g, 0.88 mmol), 1-fluoro-4-isocyanatobenzene (0.133 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis. Obtained 0.91 g of light yellow solid (27% yield). ^1H NMR (400 MHz, DMSO) δ 9.90 (s, 1H), 8.88 (s, 1H), 7.97 (d, $J = 8.1$ Hz, 2H), 7.44 (dq, $J = 9.0, 4.0$ Hz, 5H), 7.27 (d, $J = 7.6$ Hz, 1H), 7.06 (td, $J = 8.8, 1.8$ Hz, 3H), 6.98 (d, $J = 7.5$ Hz, 1H), 6.87 (dt, $J = 18.9, 6.6$ Hz, 2H), 4.38 (d, $J = 5.8$ Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 165.81, 158.52, 156.17, 155.84, 144.79, 137.29, 133.10, 128.35, 127.23, 127.00, 119.77, 119.70, 118.99, 115.63, 115.41, 42.93.



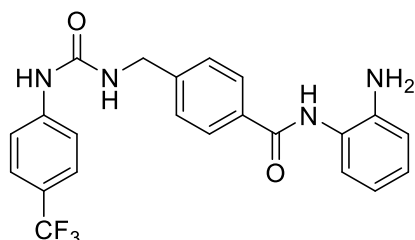
N-(2-aminophenyl)-4-((3-(3-fluorophenyl)ureido)methyl)benzamide (29). Synthesized from **53** (0.300 g, 0.88 mmol), 1-fluoro-3-isocyanatobenzene (0.133 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps

synthesis. Obtained 0.070 g of light yellow solid (21% yield). ^1H NMR (400 MHz, DMSO) δ 9.69 (s, 1H), 9.15 (s, 1H), 7.95 (d, J = 8.2 Hz, 2H), 7.48 (dt, J = 12.3, 2.3 Hz, 1H), 7.42 (d, J = 8.2 Hz, 2H), 7.28 – 7.16 (m, 2H), 7.15 – 7.04 (m, 2H), 7.03 – 6.95 (m, 1H), 6.83 (dd, J = 8.0, 1.3 Hz, 1H), 6.74 – 6.61 (m, 2H), 4.38 (d, J = 5.9 Hz, 2H).



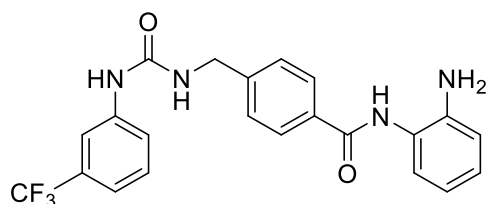
N-(2-aminophenyl)-4-((3-(2-fluorophenyl)ureido)methyl)benzamide (30). Synthesized from **53** (0.300 g, 0.88 mmol), 1-fluoro-2-isocyanatobenzene (0.133 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps

synthesis. Obtained 0.130 g of light yellow solid (39% yield). ^1H NMR (400 MHz, DMSO) δ 10.21 (s, 1H), 8.59 (s, 1H), 8.11 (t, J = 7.9 Hz, 1H), 8.05 (d, J = 7.5 Hz, 2H), 7.49 – 7.38 (m, 4H), 7.18 (dd, J = 19.5, 11.5 Hz, 3H), 7.13 – 7.02 (m, 2H), 6.99 – 6.86 (m, 1H), 4.41 (d, J = 4.5 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 165.94, 155.61, 155.56, 153.51, 151.11, 149.57, 146.03, 144.58, 133.43, 132.95, 128.49, 128.36 (d, J = 7.2 Hz), 127.38, 127.28, 126.35, 125.77, 124.79 (d, J = 3.2 Hz), 123.06, 122.34, 121.02, 115.41, 115.22, 114.92, 114.58, 42.95.



N-(2-aminophenyl)-4-((3-(4-(trifluoromethyl)phenyl)ureido)methyl)benzamide (31).

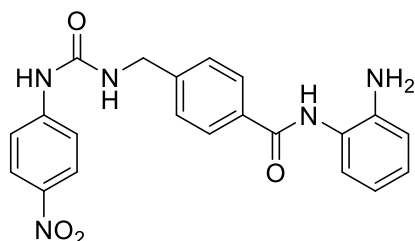
Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-4-(trifluoromethyl)benzene (0.182 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis. Obtained 0.172 g of light yellow solid (46% yield). ^1H NMR (400 MHz, DMSO) δ 9.95 (s, 1H), 9.30 (s, 1H), 7.98 (d, J = 8.2 Hz, 2H), 7.64 (d, J = 8.7 Hz, 2H), 7.57 (d, J = 8.8 Hz, 2H), 7.45 (d, J = 8.2 Hz, 2H), 7.29 (d, J = 7.7 Hz, 1H), 7.12 (t, J = 7.0 Hz, 2H), 7.03 (d, J = 7.8 Hz, 1H), 6.91 (t, J = 7.4 Hz, 1H), 4.41 (d, J = 5.8 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 165.87, 155.47, 144.70, 144.58, 133.11, 128.40, 127.26 (d, J = 5.7 Hz), 127.06, 126.39 (q, J = 3.4 Hz), 123.75, 121.64, 121.33, 119.48, 117.75, 42.95.



N-(2-aminophenyl)-4-((3-(3-(trifluoromethyl)phenyl)ureido)methyl)benzamide (32).

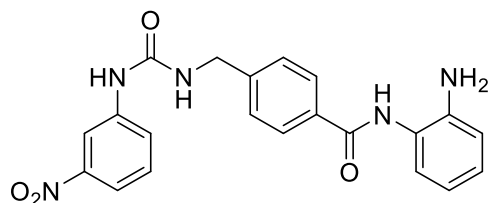
Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-3-(trifluoromethyl)benzene (0.182 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis. Obtained 0.135 g of light yellow solid (36% yield). ^1H NMR

(400 MHz, DMSO) δ 9.98 (s, 1H), 9.28 (s, 1H), 7.99 (d, J = 8.4 Hz, 3H), 7.55 (d, J = 8.2 Hz, 1H), 7.46 (t, J = 6.5 Hz, 3H), 7.31 (d, J = 7.8 Hz, 1H), 7.23 (d, J = 7.6 Hz, 1H), 7.10 (dt, J = 28.2, 7.6 Hz, 3H), 6.93 (t, J = 7.4 Hz, 1H), 4.40 (d, J = 5.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 165.87, 155.67, 144.67, 141.83, 133.06, 130.18, 130.02, 129.71, 128.41, 127.27, 127.04, 121.63, 117.74, 114.01 (d, J = 4.5 Hz), 42.95.



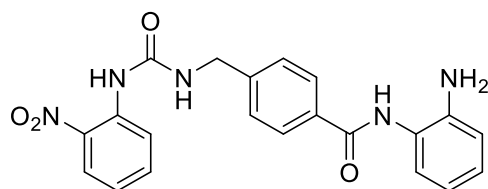
N-(2-aminophenyl)-4-((3-(4-nitrophenyl)ureido)methyl)benzamide (33). Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-4-nitrobenzene (0.159 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis. Obtained 0.103 g of light yellow solid (29% yield). ^1H NMR (400

MHz, DMSO) δ 9.80 (s, 1H), 9.64 (s, 1H), 8.15 (d, J = 9.0 Hz, 2H), 7.97 (d, J = 7.9 Hz, 2H), 7.67 (d, J = 9.0 Hz, 2H), 7.45 (d, J = 7.9 Hz, 2H), 7.23 (d, J = 7.8 Hz, 2H), 7.04 (t, J = 7.6 Hz, 1H), 6.90 (d, J = 7.9 Hz, 1H), 6.75 (t, J = 7.5 Hz, 1H), 4.42 (d, J = 5.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 165.73, 155.07, 147.64, 144.15, 140.91, 133.37, 128.37, 127.24 (d, J = 10.9 Hz), 127.01, 125.60, 125.21, 118.84, 117.98, 117.38, 43.01.



N-(2-aminophenyl)-4-((3-(3-nitrophenyl)ureido)methyl)benzamide (34). Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-3-nitrobenzene (0.159 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis. Obtained 0.079 g of light yellow solid (22% yield). ^1H NMR (400 MHz, DMSO) δ 9.65 (s,

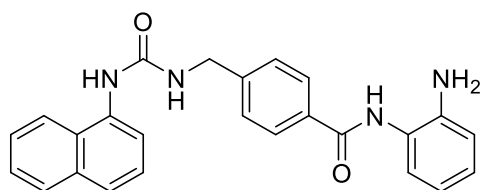
1H), 8.56 (s, 1H), 7.99 (d, J = 8.1 Hz, 1H), 7.95 (d, J = 8.0 Hz, 2H), 7.75 (dd, J = 8.1, 1.8 Hz, 1H), 7.69 (d, J = 8.1 Hz, 1H), 7.51 (t, J = 8.2 Hz, 2H), 7.43 (d, J = 8.1 Hz, 2H), 7.33 (s, 1H), 7.17 (d, J = 7.7 Hz, 1H), 6.96 (t, J = 7.4 Hz, 1H), 6.77 (d, J = 7.9 Hz, 1H), 6.59 (t, J = 7.3 Hz, 1H), 4.40 (d, J = 5.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 155.61, 148.56, 142.39, 130.35, 128.29, 127.23, 124.14, 115.94, 111.97.



N-(2-aminophenyl)-4-((3-(2-nitrophenyl)ureido)methyl)benzamide (35). Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanato-2-nitrobenzene (0.159 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis. Obtained 0.127 g of light yellow solid (36% yield). ^1H NMR (400 MHz, DMSO) δ 9.98 (s,

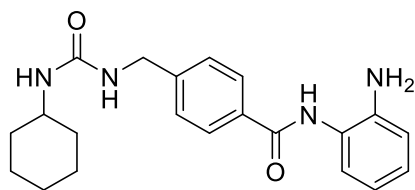
1H), 9.52 (s, 1H), 8.28 (d, J = 8.5 Hz, 1H), 8.12 (t, J = 5.8 Hz, 1H), 8.05 (dd, J = 8.4, 1.4 Hz, 1H), 7.99 (d, J = 8.2 Hz, 2H), 7.70 – 7.61 (m, 1H), 7.46 (d, J = 8.2 Hz, 2H), 7.31 (d, J = 7.2 Hz, 1H), 7.18 – 7.10 (m, 2H), 7.05 (d, J = 7.2 Hz, 1H), 7.02 – 6.85 (m, 3H), 4.41 (d, J = 5.8 Hz, 2H). ^{13}C NMR (101

MHz, DMSO) δ 165.86, 154.89, 144.09, 137.76, 136.02, 135.34, 133.19, 128.44, 127.41, 127.26, 127.08, 125.75, 122.61, 122.09, 119.70, 49.04, 43.13.



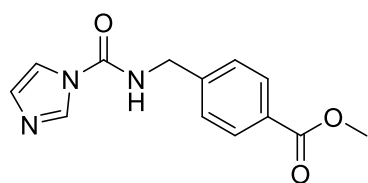
N-(2-aminophenyl)-4-((3-(naphthalen-1-yl)ureido)methyl)benzamide (36). Synthesized from **53** (0.300 g, 0.88 mmol), 1-isocyanatonaphthalene (0.164 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis.

Obtained 0.024 g of light yellow solid (6% yield). ^1H NMR (400 MHz, DMSO) δ 10.79 (s, 1H), 10.10 (s, 1H), 8.74 (s, 1H), 8.16 – 8.11 (m, 1H), 7.95 (d, J = 1.9 Hz, 3H), 7.92 – 7.83 (m, 3H), 7.64 (dd, J = 8.0, 1.6 Hz, 1H), 7.58 – 7.55 (m, 2H), 7.51 (d, J = 3.1 Hz, 2H), 7.43 (t, J = 7.9 Hz, 2H), 7.31 (td, J = 7.6, 1.7 Hz, 1H), 7.10 – 7.05 (m, 1H), 4.46 (d, J = 5.4 Hz, 2H).



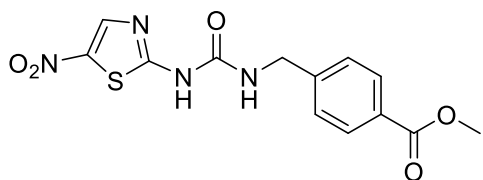
N-(2-aminophenyl)-4-((3-cyclohexylureido)methyl)benzamide (37). Synthesized from **53** (0.300 g, 0.88 mmol), isocyanatocyclohexane (0.121 g, 0.97 mmol) and TFA (2 ml) as a result of two-steps synthesis. Obtained 0.057 g of light yellow

solid (20% yield). ^1H NMR (401 MHz, DMSO) δ 9.67 (s, 1H), 7.93 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 8.1 Hz, 2H), 7.18 (d, J = 7.6 Hz, 1H), 6.98 (t, J = 7.1 Hz, 1H), 6.81 (d, J = 7.5 Hz, 1H), 6.63 (t, J = 7.3 Hz, 1H), 6.35 (t, J = 5.6 Hz, 1H), 5.96 (d, J = 7.7 Hz, 1H), 4.27 (d, J = 5.6 Hz, 2H), 1.82 – 1.69 (m, 2H), 1.69 – 1.59 (m, 2H), 1.59 – 1.47 (m, 1H), 1.35 – 1.02 (m, 5H). ^{13}C NMR (101 MHz, DMSO) δ 165.65, 157.85, 145.21, 133.23, 128.19, 127.11, 126.87, 124.18, 117.23, 116.93, 48.33, 43.00, 33.71, 25.73, 24.93.



Methyl 4-((1H-imidazole-1-carboxamido)methyl)benzoate (91).

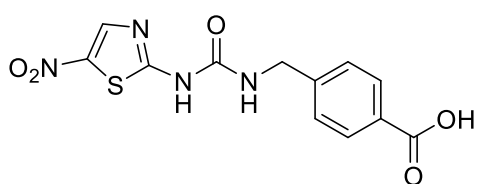
Compound **49** (0.330 g, 2 mmol) was solved in a mixture of THF and DCM and cooled to 0°C, then carbonyldimidazole (0.486 g, 3 mmol) was added. The reaction was allowed to stir at rt for 12 h, then the solvent was dried under vacuo and the crude purified via flash chromatography using DCM as mobile phase. Obtained 0.220 g of a white powder (35% yield). ^1H NMR (400 MHz, DMSO) δ 9.16 (s, 1H), 8.28 (dd, J = 1.3, 0.9 Hz, 1H), 7.98 – 7.92 (m, 2H), 7.71 (t, J = 1.5 Hz, 1H), 7.51 – 7.46 (m, 2H), 7.05 (dd, J = 1.6, 0.9 Hz, 1H), 4.55 (d, J = 3.1 Hz, 2H), 3.84 (s, 3H). ^{13}C NMR (100 MHz, DMSO) δ 166.49, 149.53, 144.54, 136.48, 130.15, 129.82, 128.93, 127.89, 117.07, 52.55, 43.69.



Methyl 4-((3-(5-nitrothiazol-2-yl)ureido)methyl)benzoate

(92). 5-nitro-2-aminothiazole (0.840 g, 5.78 mmol) was solved in DMF under stream of nitrogen. The mixture was cooled to 0°C, then NaH (0.230 g, 5.78 mmol) was added,

followed, after 30 minutes, by **91** (1.5 g, 5.78 mmol). The reaction was stirred for 12 h, then ethyl acetate was added and the mixture extracted with water. The organic phase was dried over Na₂SO₄ and concentrated under vacuo. The crude was purified via flash chromatography using DCM as mobile phase to give 0.620 g of a red oil (32% yield). ¹H NMR (400 MHz, DMSO) δ 11.84 (s, 1H), 8.51 (s, 1H), 7.94 (d, J = 8.4 Hz, 2H), 7.49 – 7.41 (m, 3H), 4.46 (d, J = 6.0 Hz, 2H), 3.84 (s, 3H).

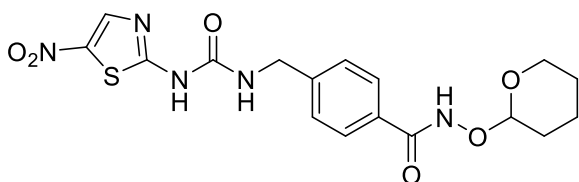


4-((3-(5-nitrothiazol-2-yl)ureido)methyl)benzoic acid

(93). Compound **92** (0.620 g, 1.85 mmol) was solved in a mixture of methanol and water, then Na₂CO₃ (0.588 g, 5.55 mmol) was added. The reaction was heated to 80°C and

stirred for 12 h, then methanol was evaporated under reduced pressure. Water was extracted with ethyl acetate, then acidified with HCl 2 N to pH 1 and extracted with fresh ethyl acetate. The organic phase was dried over Na₂SO₄ and concentrated under vacuo to give 0.32 g of red solid (54% yield). ¹H NMR (400 MHz, DMSO) δ 10.29 (s, 1H), 8.52 (s, 1H), 7.90 (d, J = 3.9 Hz, 2H), 7.78 (t, J = 5.8 Hz, 1H), 7.41 (d, J = 8.2 Hz, 3H), 4.45 (d, J = 6.0 Hz, 2H).

General procedure for the synthesis of 94 and 95. To a solution of **93** in DMF, protected hydroxylamine or o-phenyldiamine, EDCI and HOBt were added under stream of nitrogen and the mixture was stirred for 12 h. Ethyl acetate and water were added, and the organic phase was washed with brine, dried over Na₂SO₄ and concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of DCM / methanol to give the corresponding **94** and **95** as red solid.

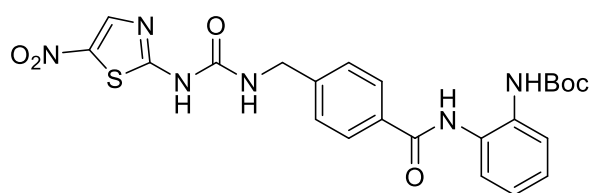


4-((3-(5-nitrothiazol-2-yl)ureido)methyl)-N-

((tetrahydro-2H-pyran-2-yl)oxy)benzamide (94).

Synthesized from **93** (0.229 g, 0.71 mmol), O-(tetrahydro-2H-pyran-2-yl)hydroxylamine (0.084 g,

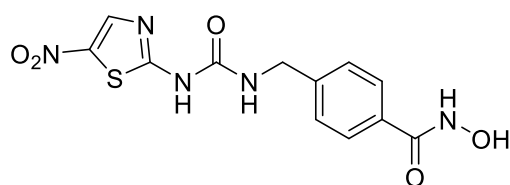
0.71 mmol), EDCI (0.272 g, 1.42 mmol) and HOBt (0.095 g, 0.71 mmol). Obtained 0.135 g of a red solid (45% yield).



Tert-butyl (2-(4-((3-(5-nitrothiazol-2-yl)ureido)methyl)benzamido)phenyl)carbamate (95).

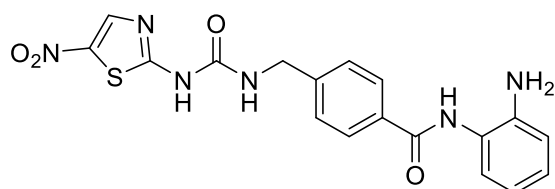
Synthesized from **93** (0.590 g, 1.83 mmol), O-(tetrahydro-2H-pyran-2-yl)hydroxylamine (0.380 g, 1.83 mmol), EDCI (0.702 g, 3.66 mmol) and HOBT (0.247 g, 1.83 mmol). Obtained 0.250 g of a red solid (27% yield).

General procedure for the synthesis of 19 and 38. To a solution of **94** or **95** in DCM, TFA was added and the mixture was stirred for 12 h. The solvent was concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of DCM / methanol to give the corresponding **19** and **38** as yellow solid.



N-hydroxy-4-((3-(5-nitrothiazol-2-yl)ureido)methyl)benzamide (19).

Synthesized from **94** (0.135 g, 0.32 mmol) and TFA (2 ml). Obtained 0.036 g of yellow solid (34% yield). ^1H NMR (400 MHz, DMSO) δ 12.00 (s, 1H), 11.19 (s, 1H), 9.00 (s, 1H), 8.50 (s, 1H), 7.72 (d, J = 8.1 Hz, 3H), 7.36 (d, J = 8.1 Hz, 2H), 4.42 (d, J = 5.4 Hz, 2H). ^{13}C NMR (100 MHz, DMSO) δ 164.83, 164.47, 154.27, 143.91, 142.68, 141.17, 131.97, 127.44 (d, J = 3.5 Hz), 43.22.



N-(2-aminophenyl)-4-((3-(5-nitrothiazol-2-yl)ureido)methyl)benzamide (38).

Synthesized from **95** (0.250 g, 0.49 mmol) and TFA (2 ml). Obtained 0.039 g of yellow solid (19% yield). ^1H NMR (400 MHz, dmso) δ 9.67 (s, 1H), 8.52 (s, 1H), 7.96 (d, J = 8.1 Hz, 2H), 7.55 (s, 1H), 7.49 – 7.38 (m, 2H), 7.18 (dd, J = 7.9, 1.6 Hz, 1H), 6.99 (ddd, J = 8.0, 7.2, 1.6 Hz, 1H), 6.81 (dd, J = 8.0, 1.5 Hz, 1H), 6.64 (td, J = 7.5, 1.5 Hz, 1H), 4.46 (d, J = 6.0 Hz, 2H).

2.3 Project#2. Design, synthesis and biological evaluation of dual non ATP-competitive GSK-3 β / HDAC inhibitors.

For the design of the first in class series of dual non ATP-competitive GSK-3 β / HDAC inhibitors we focused our attention on Tideglusib. It belongs to the series of Thiadiazolidinediones (TDZDs) developed in 2002 by Martinez and coworkers.¹³² It has shown neuroprotective, anti-inflammatory, and neurogenic properties in different neurodegenerative models.^{133, 194} Furthermore, good safety, tolerability, and efficacy have been reported in clinical trials for different neurological diseases. In particular, favorable cognitive evolution accompanied by a significant reduction in disease-specific biomarkers in cerebrospinal fluid has been shown in mild AD patients.¹³⁴ From a chemical point of view, Tideglusib presents a thiadiazolidindione core bearing two substituents on the nitrogen atoms: a naphthyl on the nitrogen in position 2 and a benzyl group on the nitrogen in position 4. Analyzing the SARs regarding Tideglusib, it turned out that as regards the benzyl ring in position 4, substituents in para position are well tolerated.¹³² Therefore, we designed a new series of dual GSK-3 β / HDAC inhibitors based on the Tideglusib structure by introducing the hydroxamic acid, as ZBGs, in para position of the benzyl ring.

In order to obtain compounds with the best characteristics of enzyme inhibitions and physicochemical properties and in order to study SARs, we designed and synthesized a library of compounds in which substituted aromatic rings are introduced in position 2 (compounds **39-47**) (Figure 44). Indeed, analyzing the SARs it turned out that either alkyl or aromatic substituents may be introduced on N in position 2 without comprising the GSK-3 β inhibitory activity.

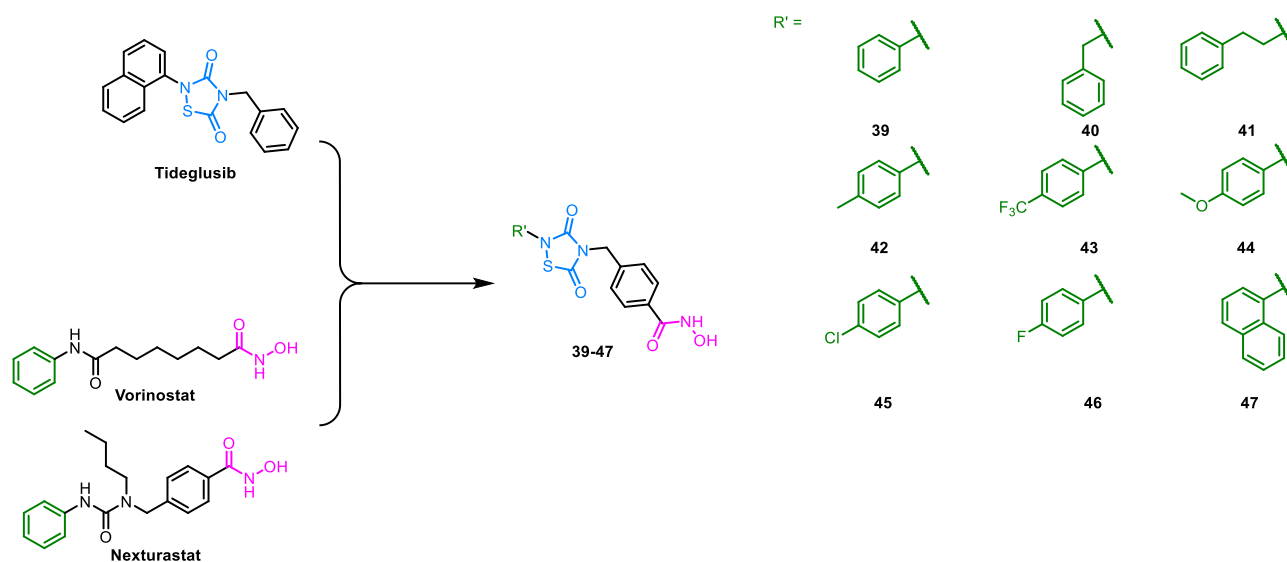
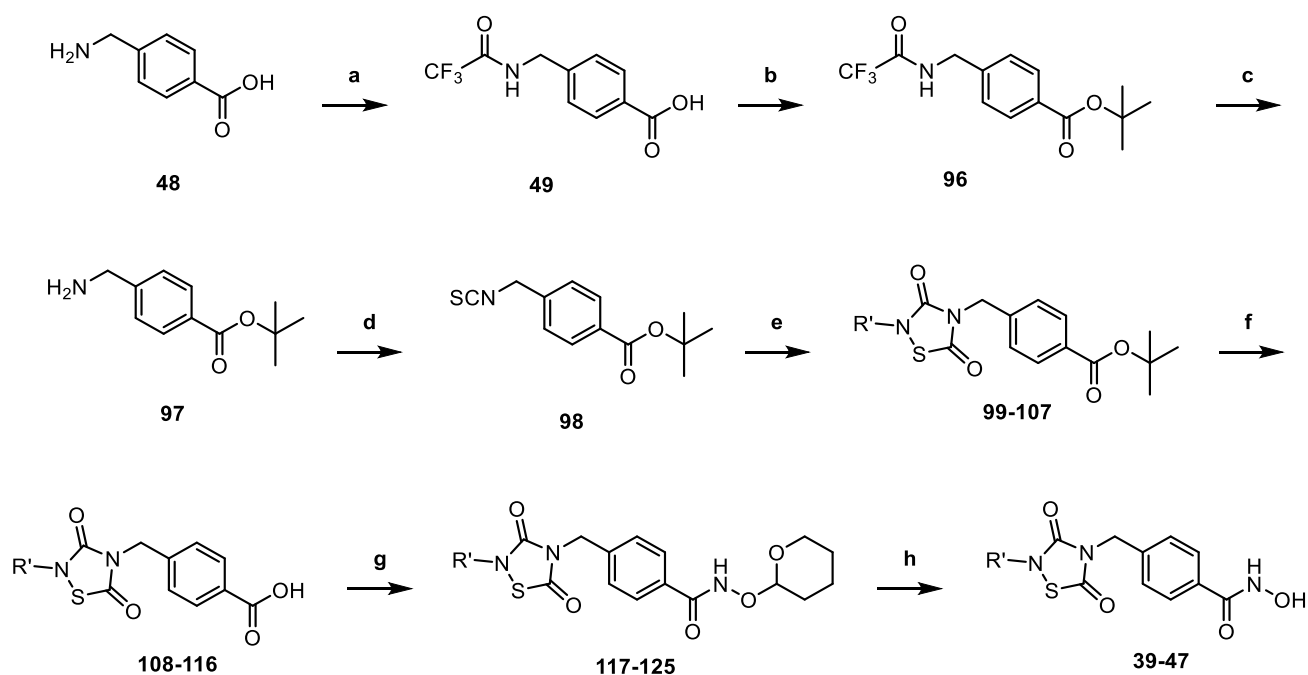


Figure 44. Drug design leading to dual compounds 39-47.

2.3.1 Chemistry section

Target compounds have been synthesized following procedure reported in Scheme 3. Commercially available amino acid **48** was protected on the amino group using trifluoroacetic anhydride to obtain compound **49**, whose carboxylic acid was protected with tert-butanol to give compound **96**. Amine group was deprotected using TFA and converted in isothiocyanate **97** by reaction with 1,1'-thiocarbonyldi-2(1H)-pyridone to obtain **98**. **98** underwent cyclization reaction with different isocyanates in presence of sulfur dichloride and subsequent oxidation in air to give compounds **99-107**. Then, tert-butyl protecting group was removed with TFA to obtain free carboxylic acid **108-116**, which was reacted with tetrahydropyran-protected hydroxylamine to give **117-125**. Final deprotection of hydroxamic acid gave products **39-47**.



SCHEME 3. Synthesis of compounds **39-47**. Reagents and conditions. A) $(\text{CF}_3\text{CO})_2\text{O}$, rt, 2h; b) $t\text{BuOH}$, EDCI, DMAP, THF, rt, 12h, N_2 ; c) K_2CO_3 , $\text{H}_2\text{O}/\text{MeOH}$, rt, 12h; d) 1,1'-thiocarbonyldi-2(1H)-pyridone, DCM, rt, 12h; e) $\text{R}'\text{-NCO}$, SO_2Cl_2 , THF, rt, 12h, then air, THF, rt, 30 min; f) TFA, DCM, rt, 12h; g) NH_2OTHP , EDCI, HOBt, DMF, rt, 12h, N_2 ; h) TFA, DCM, rt, 12h.

2.3.2 Results and discussion

Inhibitory effects of the compounds **1** and **19** together with the activity of parent compounds Tideglusib, Nexturastat and Vorinostat have been determined against human GSK-3 β , HDAC2, and HDAC6. Data are reported in Table 4. Compounds have been first evaluated for their inhibitory activity at the concentration of 1 μM and IC_{50} values have been determined for compounds that showed an inhibitory activity higher than 50 % at that concentration. Based on this, for compounds **39** with the phenyl substituent, **40** with the benzyl substituent and **43** with a p-trifluoromethyl phenyl substituent the IC_{50} values were not determined. Compounds **41-42** and **44-47** show IC_{50} values spanning from the submicromolar to the nanomolar range of concentrations against all three-target enzymes. In particular, regarding GSK-3 β inhibitory activity, all the tested compounds turned out to be more potent than the parent compound Tideglusib, confirming that introducing a hydroxamic acid in the para position of the N4 benzyl group does not affect the inhibitory activity. In the series, the most active inhibitor is compound **46**, characterized by a p-fluorophenyl substituent on N2 ($\text{IC}_{50} = 81.91 \pm 13.75$ nM) followed by compound **44**, characterized by a p-methoxyphenyl substituent on N2 ($\text{IC}_{50} = 83.74 \pm 19.79$ nM). Introduction of bulky group as naphthyl as in compound **47** is well tolerated ($\text{IC}_{50} = 142.07 \pm 5.55$ nM). Regarding HDAC6, all the compounds show satisfactory inhibitory

activity. Compound **47** with a naphthyl group is the most potent inhibitor with an IC_{50} of 45.10 ± 0.50 Nm, followed by compound **46** with a p-fluorophenyl substituent ($IC_{50} = 62.34 \pm 2.63$ Nm) and compound **45** with a p-chlorophenyl substituent ($IC_{50} = 73.55 \pm 1.08$ Nm). Regarding HDAC2, it can be observed as general trend that the compounds are more active against HDAC2 than HDAC6. In particular, the most active compound of the series is the p-methoxyphenyl derivative **44** ($IC_{50} = 2.0 \pm 0.19$ Nm) followed by p-methylphenyl derivative **42** ($IC_{50} = 10.86 \pm 1.75$ Nm), phenetyl derivative **41** ($IC_{50} = 23.05 \pm 2.58$ Nm), p-fluorophenyl **46** ($IC_{50} = 27.41 \pm 5.97$ Nm) and naphthyl derivative **47** ($IC_{50} = 29.72 \pm 4.65$ Nm).

Table 4. Effects of Compounds **39-47**, Tideglusib, Vorinostat on GSK-3 β , HDAC2, and HDAC6 activity.

Compound	% inhibition @1 μ M ^a			IC_{50} (nM) ^b		
	GSK-3 β	HDAC 2	HDAC 6	GSK-3 β	HDAC 2	HDAC6
39	44.78 $\pm$ 22.43	36.24 $\pm$ 13.40	36.99 $\pm$ 5.59	nd ^c	nd ^c	nd ^c
40	71.79	21.95 $\pm$ 2.43	37.23 $\pm$ 2.51	nd ^c	nd ^c	nd ^c
41	54.64 $\pm$ 6.07	55.93 $\pm$ 5.02	78.43 $\pm$ 0.74	176.67 $\pm$ 18.55	23.05 $\pm$ 2.58	95.75 $\pm$ 4.71
42	87.81 $\pm$ 2.90	86.65 $\pm$ 2.8 48.05@0.1 μ M	87.68 $\pm$ 0.23	83.74 $\pm$ 19.79	10.86 $\pm$ 1.75	222.30 $\pm$ 12.19
43	89.21 $\pm$ 3.14	63.07 $\pm$ 2.16	44.84 $\pm$ 3.08	nd ^c	nd ^c	nd ^c
44	91.93 $\pm$ 3.02	85.70 $\pm$ 3.87 43.38@0.1 μ M	88.36 $\pm$ 0.29	127.47 $\pm$ 7.52	2.0 $\pm$ 0.19	254.60 $\pm$ 7.41
45	94.45 $\pm$ 0.73	88.47 $\pm$ 1.25 37.78@0.1 μ M	86.8 $\pm$ 0.4	107.74 $\pm$ 20.64	31.32 $\pm$ 3.70	73.55 $\pm$ 1.08
46	90.31 $\pm$ 5.41	87.91 $\pm$ 2.45 46.07@0.1 μ M	86.99 $\pm$ 0.00	81.91 $\pm$ 13.75	27.41 $\pm$ 5.97	62.34 $\pm$ 2.63
47	95.33 $\pm$ 0.61	88.43 $\pm$ 0.49 52.65@0.1 μ M	89.39 $\pm$ 0.82	142.07 $\pm$ 5.55	29.72 $\pm$ 4.65	45.10 $\pm$ 0.50
Tideglusib				200 $\pm$ 15	nd ^c	nd ^c
Vorinostat				na ^{d,e}	119.63 $\pm$ 6.96	188.06

^a values are reported as a mean value of three or more determinations; ^b IC_{50} values are defined as the drug concentration that reduces by 50% the target activity and are reported as a mean value of three or more determinations; ^c nd = not determined; ^d na = not active up to 50 μ M; ^e data from ref.¹⁹¹

Based on these results, compound **47** characterized by a more balanced inhibitory profile has been selected for in deep biochemical analysis. To investigate the in cell HDAC inhibitory activity of compound **47**, the global levels of histone H3 (H3K9K14ac) and tubulin lysine acetylation were determined by western blotting in SH-SY5Y cells treated for 24 h at three different concentrations (0.1, 1 and 10 μ M) (Figure 45). The effect of compound **47** on acetylation was compared to that one of Nexturastat and Tideglusib, which is defective of the hydroxamic acid moiety. Compound **47** is able to induce a clear increase in the acetylation levels of both histone H3 and tubulin already at the

concentration of 0.1 μM and in a dose-dependent manner. In particular, the increased in the acetylation is more marked in tubulin with respect to histone H3 suggesting a clear selectivity towards HDAC6. Furthermore, compound **47** is more active than reference compound Nexturastat in inducing tubulin acetylation. As expected, Tideglusib, a pure GSK-3 β inhibitor, does not have any effect on histone H3 and tubulin acetylation.

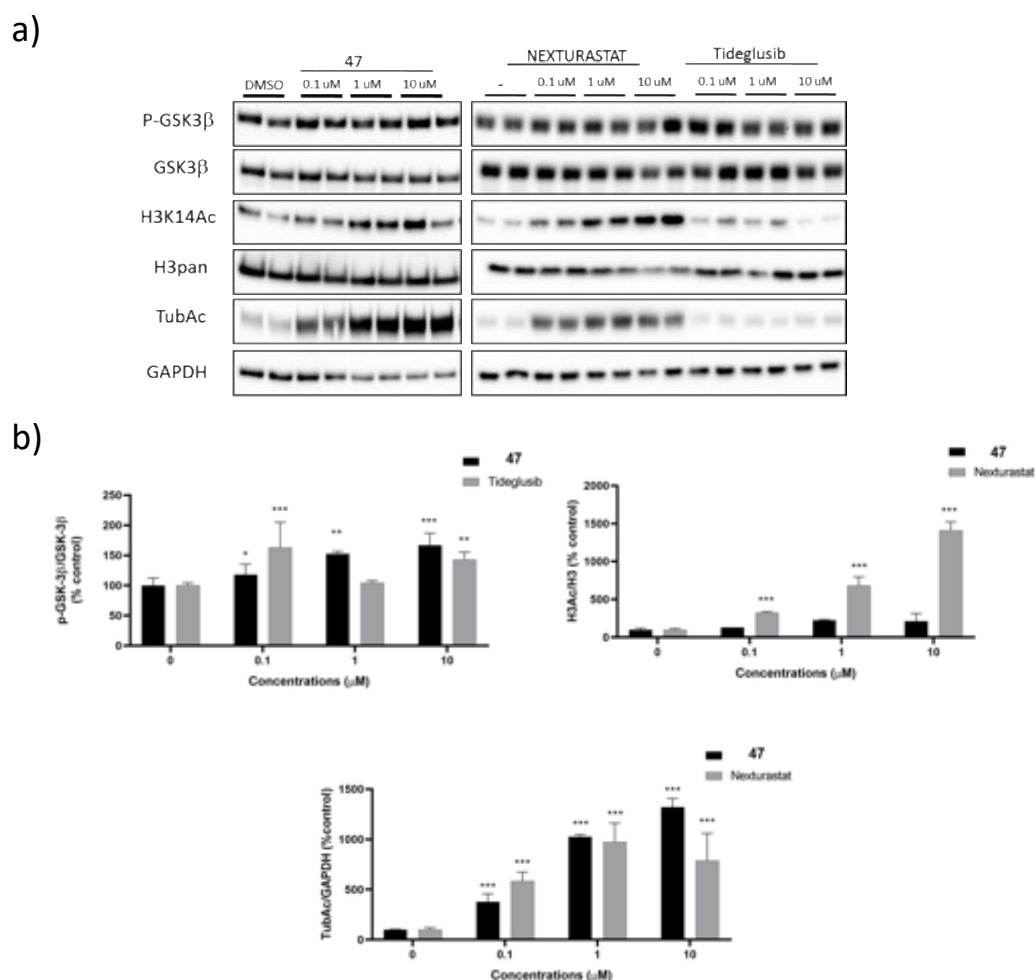


Figure 45. a) Western blot probing for phosphorylated GSK-3 β , acetylated histone H3 and α -tubulin in SH-SY5Y cells after 24h treatment. b) Densitometry analysis regarding western blotting results of **47** on GSK-3 β phosphorylation in comparison to Tideglusib, histone H3 acetylation and tubulin acetylation in correlation with Nexturastat. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to untreated cells.

Then to evaluate the ability of compound **47** to inhibit GSK-3 β in cell, its effect on counteracting copper-induced tau hyperphosphorylation has been determined in SH-SY5Y cell line. In particular, the protein's phosphorylation was induced in differentiated SH-SY5Y cells upon incubation with copper. Compound **47** at the concentration of 10 μM completely counteracted the phosphorylation of

tau protein. The observed effect is in line with the effect obtained with the same concentration of Tideglusib.

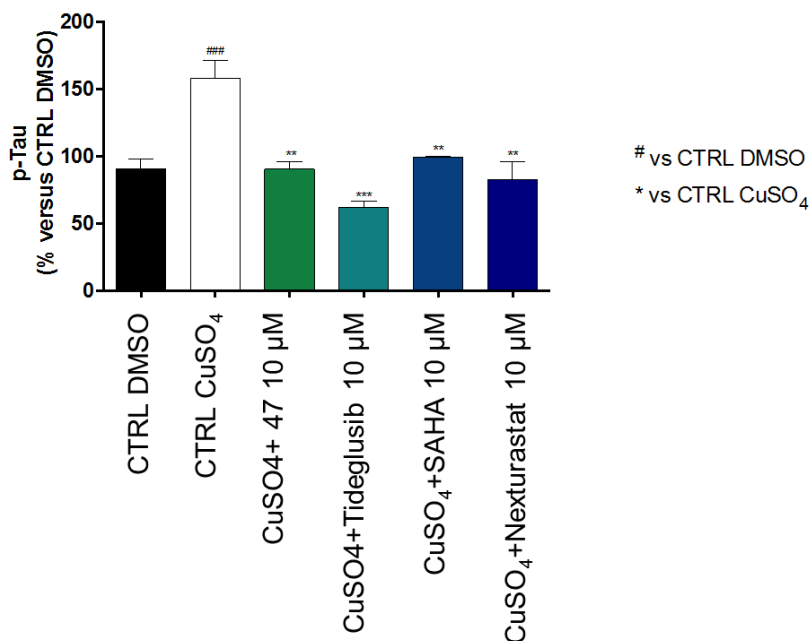


Figure 46. Effects of the compounds on tau phosphorylation. The cells were pretreated with **47**, Tideglusib, Vorinostat, Nexturastat (10 μM) for 1 h and then with copper (400 μM) for 16 h. Following incubation time, cells were harvest by scraping, and the pellet was obtained. Subsequently, the extent of tau phosphorylation was determined by immunoenzymatic assay. Absorbance was measured at 450 nm. Values represent the mean ± SEM; *p < 0.05, ***p < 0.001 vs control; # p < 0.05, ##p < 0.01, ###p < 0.001 vs cells treated with copper.

Since HDAC inhibitors are used in therapy as anticancer drugs, compound **47** was evaluated for its potential cytotoxicity in SH-SY5Y cells in comparison with Tideglusib, Vorinostat and Nexturastat (Figure 47). Compound **47** is not toxic up to 10 μM, being slightly more cytotoxic than Tideglusib and Nexturastat that show cytotoxic effects starting from the concentration 50 μM.

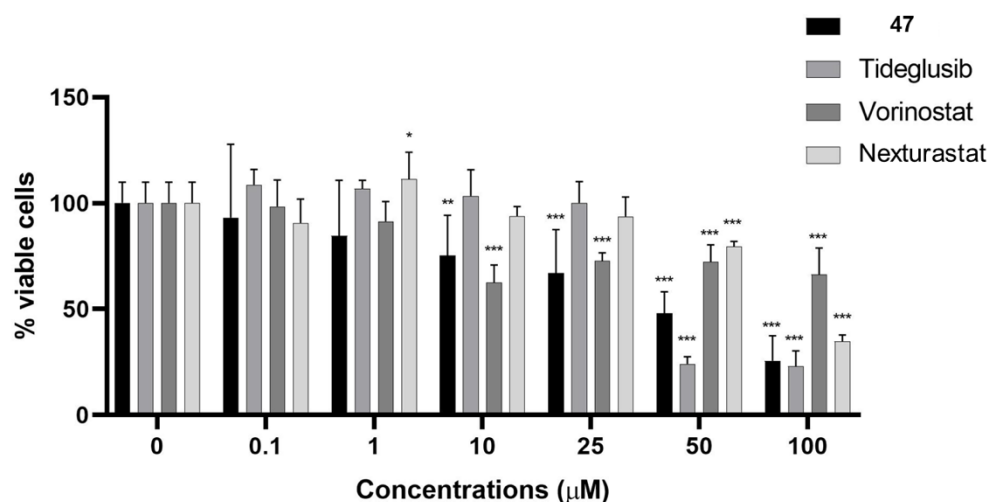


Figure 47. Citotoxicity of compounds **47**, Tideglusib, Vorinostat and Nexturastat in SH-SY5Y cells. Cells were incubated for 24h with different concentrations of the tested compounds (0.1 – 100 μ M). At the end of incubation, the cell viability was measured by MTT assay. Data are reported as mean $\pm$ SEM of three independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to untreated cells.

In silico prediction of some pharmacokinetic and ADME properties of compound **47** were obtained from SwissADME,^{192, 193} an online web tool, and displayed in Table 5. As it can be observed, compound **47** fulfills the requirements for the Lipinsky rule of Five and are predicted to not be a P-gp substrate. Furthermore, very importantly, it is not identified as PAINS.

Table 5. In silico pharmacokinetic data for compound **47** and Tideglusib.^a

	47	Tideglusib		47	Tideglusib
MW	393.42	334.39	MR	106.35	97.43
Rotable Bonds	5	3	GI absorption	high	high
H-bond A	4	2	P-gp substrate	no	no
H-bond D	2	0	Leadlikeness	no	no
TPSA	121.57	72.24	Lipinsky	yes	yes
Log $P_{o/w}$	2.67	3.02	PAINS	no	no

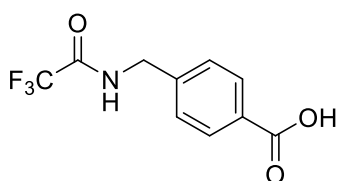
^a Obtained from <http://www.swissadme.ch/>, access date January13, 2023

2.3.3 Conclusions

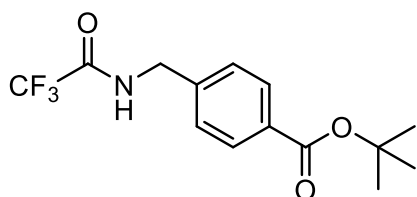
Concluding, a new series of dual GSK-3 β /HDAC inhibitors characterized by non ATP-competition was developed, based on Tideglusib and Vorinostat. Synthesized compound **39-47** were tested on their activity against target enzymes, showing activity in high to low nanomolar concentration range. Compound **47**, being the most balanced regarding activity towards the three enzymes on which was tested, was chosen for further evaluation in SH-SY5Y cell line. Western blotting assay confirmed its inhibiting properties, suggesting an increased selectivity against HDAC6 rather than HDAC2. Moreover, it resulted non cytotoxic up to 10 μ M. Summarizing, compound **47** resulted being an interesting inhibitor able to combine the characteristics of its parent compounds without reducing activity towards the targets, managing to be more active.

2.3.4 Experimental section

Chemistry

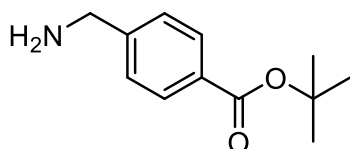


4-((2,2,2-trifluoroacetamido)methyl)benzoic acid (49). Amino acid **48** (4 g, 27 mmol) was solved in 10 ml of trifluoroacetic anhydride and stirred at rt for 2 h. Water was added and the precipitate filtered under vacuo. The filtrate was dried to obtain 6.6 g of white powder (quantitative yield).



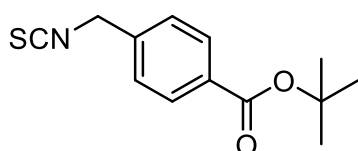
tert-butyl 4-((2,2,2-trifluoroacetamido)methyl)benzoate (96).

A solution of **49** (1.000 g, 4.0 mmol), tert-butanol (10 ml) and DMAP (0.490 g, 4 mmol) in anhydrous THF was cooled to 0°C under a stream of nitrogen. EDCI (2.31 g, 12 mmol) was added and the mixture was allowed to stir at rt for 12 h. The solvent was removed under reduced pressure and the mixture extracted with DCM and HCl 0.1 N. The organic phase was dried over Na₂SO₄, filtered and concentrated to obtain 0.96 g of a colorless oil (79% yield).



tert-butyl 4-(aminomethyl)benzoate (97). To a solution of **96** (0.885 g, 2.9 mmol) in a mixture of methanol / water (30 ml, 17:1 ratio), potassium carbonate (1.61 g, 12 mmol) was added at 0 °C and the reaction was allowed to reach rt and stirred overnight. Methanol was

removed in vacuo and the aqueous phase was extract with DCM. Organic phases were collected, dried over Na₂SO₄ and concentrated to give **10** (0.480 g, 79% yield) as yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 7.88 (d, 2H, J = 8.4 Hz), 7.33 (d, 2H, J = 8.4 Hz), 3.95 (s, 2H), 1.58 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 165.4, 131.4, 129.8, 127.6, 127.0, 81.1, 43.6, 28.1; ESI-MS = 208 (M+H)⁺.

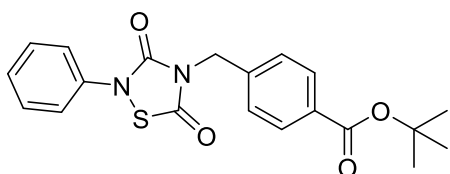


tert-butyl 4-(isothiocyanatomethyl)benzoate (98). To a solution of **97** (0.480 g, 2.32 mmol) in DCM under stream of nitrogen, 1,1'-Thiocarbonyldi-2(1H)-pyridone (0.533 g, 2.32 mmol) in DCM was added dropwise and the resulting mixture was stirred overnight at rt.

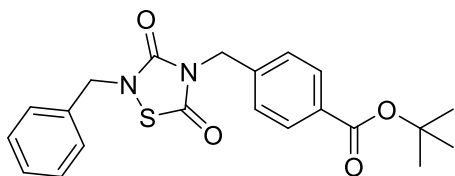
The solvent was removed in vacuo and the resulting residue was purified via flash chromatography using as mobile phase a mixture of petroleum ether / ethyl acetate (9.8:0.2) to give **11** (0.320, 55% yield) as yellow oil. ¹H NMR (400 MHz, CDCl₃) δ 8.01 (d, 2H, J = 8.0 Hz), 7.36 (d, 2H, J = 8.0 Hz),

4.77 (s, 2H), 1.59 (s, 9H); ^{13}C NMR (100 MHz, CDCl_3) δ 165.1, 138.5, 132.1, 130.1, 126.5, 81.3, 48.4, 28.1; ESI-MS = 250 ($\text{M}+\text{H}$) $^+$.

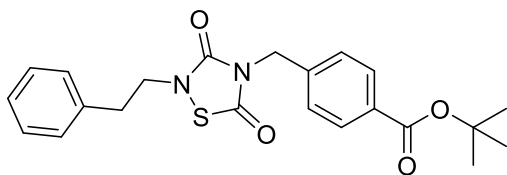
General procedure for the synthesis of 99-107. To a solution of **98** in anhydrous THF, corresponding isocyanates and sulfuryl chloride 1M solution in DCM (2 equivalents) were added and the mixture was stirred at rt for 12 h under stream of nitrogen. Then the mixture was allowed to stir in air for 30 minutes. Water was added and the phase extracted with DCM. The organic phase was collected and dried over Na_2SO_4 . After filtration, the solvent was concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of petroleum ether/ethyl acetate to give the corresponding **99-107** as white or yellow oils.



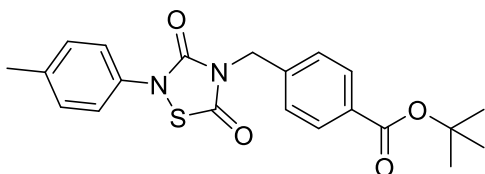
Tert-butyl 4-((3,5-dioxo-2-phenyl-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (99). Synthesized from **98** (0.330 g, 1.2 mmol), isocyanatobenzene (0.143 g, 1.2 mmol) and SO_2Cl_2 (2.4 mmol). Obtained 0.165 g of a white solid (36% yield).



Tert-butyl 4-((2-benzyl-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (100). Synthesized from **98** (0.950 g, 3.81 mmol), (isocyanatomethyl)benzene (0.508 g, 3.81 mmol) and SO_2Cl_2 (7.62 mmol). Obtained 0.396 g of a white solid (26% yield).

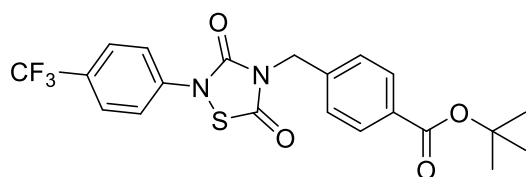


Tert-butyl 4-((3,5-dioxo-2-phenethyl-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (101). Synthesized from **98** (0.340 g, 1.365 mmol), (2-isocyanatoethyl)benzene (0.300 g, 1.365 mmol) and SO_2Cl_2 (2.73 mmol). Obtained 0.280 g of a white solid (49% yield).



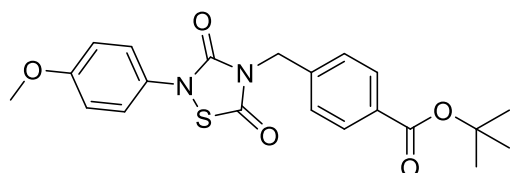
Tert-butyl 4-((3,5-dioxo-2-(p-tolyl)-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (102). Synthesized from **98** (0.500 g, 2 mmol), 1-isocyanato-4-methylbenzene (0.266 g, 2 mmol) and SO_2Cl_2 (4 mmol). Obtained 0.240 g of a white solid

(30% yield). ^1H NMR (400 MHz, CDCl_3) δ 7.99 – 7.95 (m, 2H), 7.54 – 7.50 (m, 2H), 7.35 (d, J = 8.5 Hz, 2H), 7.20 (dt, J = 8.0, 0.7 Hz, 2H), 4.94 (s, 2H), 2.35 (d, J = 0.8 Hz, 3H), 1.58 (s, 9H). ^{13}C NMR (100 MHz, CDCl_3) δ 165.30, 165.16, 150.94, 139.28, 137.43, 132.93, 132.07, 130.08, 129.90, 128.75, 123.74, 81.16, 45.71, 28.15, 20.99.



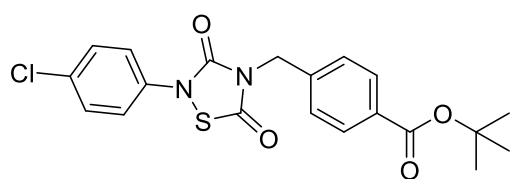
Tert-butyl 4-((3,5-dioxo-2-(4-(trifluoromethyl)phenyl)-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (103). Synthesized from **98** (0.430 g, 1.73 mmol), 1-isocyanato-4-(trifluoromethyl)benzene

(0.323 g, 1.73 mmol) and SO_2Cl_2 (3.46 mmol). Obtained 0.690 g of a white solid (88% yield).



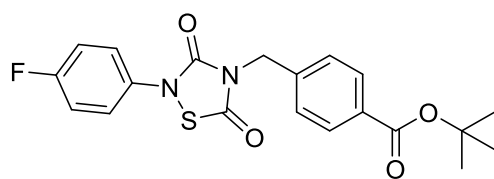
Tert-butyl 4-((2-(4-methoxyphenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (104). Synthesized from **98** (0.860 g, 3.45 mmol), 1-isocyanato-4-methoxybenzene (0.514 g, 3.45 mmol) and SO_2Cl_2 (6.9

mmol). Obtained 0.630 g of a white solid (44% yield). ^1H NMR (400 MHz, CDCl_3) δ 7.97 (d, J = 8.4 Hz, 2H), 7.53 (d, J = 8.3 Hz, 2H), 7.36 (d, J = 9.0 Hz, 2H), 6.92 (d, J = 9.0 Hz, 2H), 4.93 (s, 2H), 3.81 (s, 2H), 1.58 (s, 9H).



Tert-butyl 4-((2-(4-chlorophenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (105). Synthesized from **98** (0.330 g, 1.325 mmol), 1-chloro-4-isocyanatobenzene (0.203 g, 1.325 mmol) and SO_2Cl_2

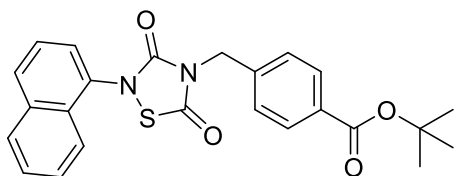
(2.65 mmol). Obtained 0.140 g of a white solid (33% yield).



Tert-butyl 4-((2-(4-fluorophenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (106). Synthesized from **98** (0.420 g, 1.68 mmol), 1-fluoro-4-isocyanatobenzene (0.230 g, 1.68 mmol) and SO_2Cl_2 (3.37

mmol). Obtained 0.097 g of a white solid (14% yield). ^1H NMR (400 MHz, CDCl_3) δ 7.98 (d, J = 8.2

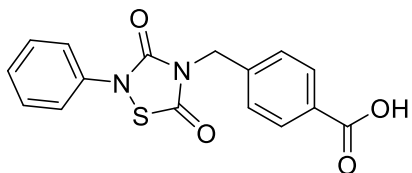
Hz, 2H), 7.52 (d, J = 8.3 Hz, 2H), 7.11 (dd, J = 9.1, 8.0 Hz, 2H), 7.01 (dd, J = 9.1, 8.2 Hz, 2H), 4.94 (s, 2H), 1.60 (s, 9H).



Tert-butyl 4-((2-(naphthalen-1-yl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (107). Synthesized from **98** (0.500 g, 2 mmol), 1-isocyanatonaphthalene (0.372 g, 2 mmol) and SO₂Cl₂ (4 mmol). Obtained 0.500 g of a white solid

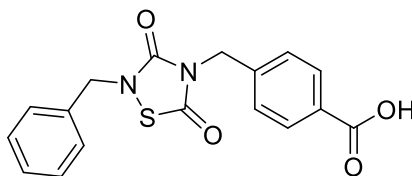
(58% yield). ¹H NMR (400 MHz, DMSO) δ 8.09 (t, J = 8.8 Hz, 2H), 7.97 – 7.90 (m, 3H), 7.80 (dd, J = 7.4, 1.2 Hz, 1H), 7.69 – 7.61 (m, 3H), 7.53 (d, J = 8.4 Hz, 2H), 4.97 (s, 2H), 1.55 (s, 9H).

General procedure for the synthesis of 108-116. To a solution of **99-107** in DCM, TFA was added and the mixture was stirred for 12 h. The solvent was concentrated under vacuo, then water was added and the phase extracted with DCM. The organic phase was collected and dried over Na₂SO₄. After filtration, the solvent was concentrated to give a residue that was eventually purified via flash chromatography using as mobile phase a mixture of petroleum ether/ethyl acetate to give the corresponding **108-116** as white or yellow solids.

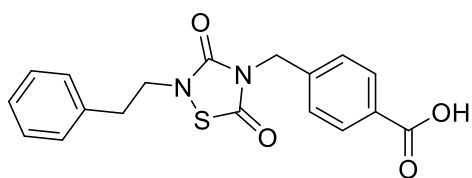


4-((3,5-dioxo-2-phenyl-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (108). Synthesized from **99** (0.165 g, 0.43 mmol) and TFA (2 ml). Obtained 0.059 g of a white solid (42% yield). ¹H NMR (400 MHz, DMSO) δ 7.94 (d, J = 8.3 Hz, 2H),

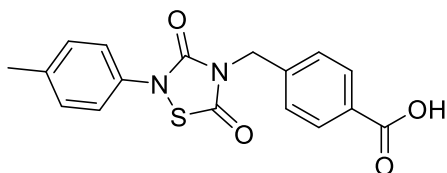
7.61 – 7.54 (m, 2H), 7.54 – 7.43 (m, 4H), 7.36 – 7.30 (m, 1H), 4.91 (s, 2H).



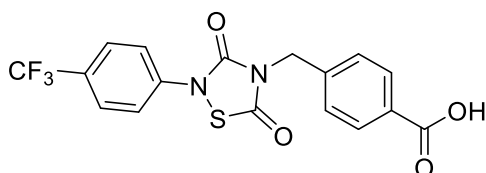
4-((2-benzyl-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (109). Synthesized from **100** (0.300 g, 0.75 mmol) and TFA (2 ml). Obtained 0.255 g of a white solid (98% yield).



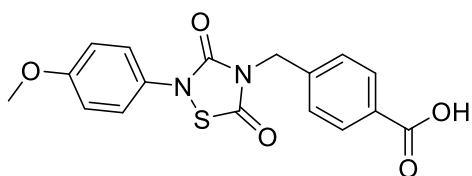
4-((3,5-dioxo-2-phenethyl-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (110). Synthesized from **101** (0.280 g, 0.68 mmol) and TFA (2 ml). Obtained 0.167 g of a white solid (69% yield).



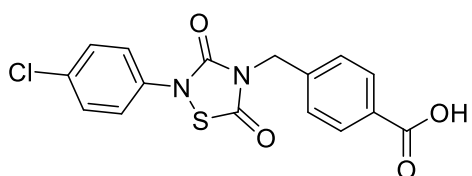
4-((3,5-dioxo-2-(p-tolyl)-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (111). Synthesized from **102** (0.240 g, 0.60 mmol) and TFA (2 ml). Obtained 0.125 g of a white solid (61% yield).



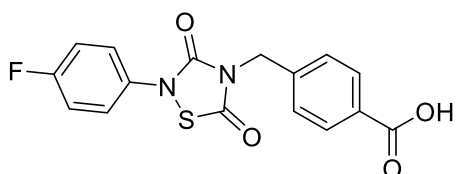
4-((3,5-dioxo-2-(4-(trifluoromethyl)phenyl)-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (112). Synthesized from **103** (0.500 g, 1.11 mmol) and TFA (2 ml). Obtained 0.370 g of a white solid (84% yield).



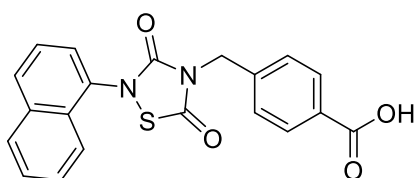
4-((2-(4-methoxyphenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (113). Synthesized from **104** (0.630 g, 1.51 mmol) and TFA (2 ml). Obtained 0.240 g of a white solid (44% yield).



4-((2-(4-chlorophenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (114). Synthesized from **105** (0.140 g, 0.33 mmol) and TFA (2 ml). Obtained 0.105 g of a white solid (88% yield).



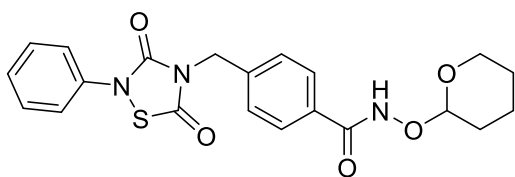
4-((2-(4-fluorophenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (115). Synthesized from **106** (0.097 g, 0.24 mmol) and TFA (2 ml). Obtained 0.055 g of a white solid (66% yield).



4-((2-(naphthalen-1-yl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (116). Synthesized from **107** (0.500 g, 1.15 mmol) and TFA (2 ml). Obtained 0.213 g of a white solid (49% yield). ^1H NMR (400 MHz, DMSO) δ 8.13 – 8.05 (m, 2H),

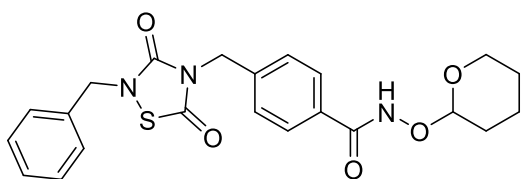
8.00 – 7.96 (m, 2H), 7.95 – 7.91 (m, 1H), 7.85 – 7.79 (m, 1H), 7.69 – 7.59 (m, 3H), 7.56 – 7.51 (m, 2H), 4.97 (s, 2H).

General procedure for the synthesis of 117-125. To a solution of **108-116** in DMF, protected hydroxylamine, EDCI and HOBt were added under stream of nitrogen and the mixture was stirred for 12 h. Ethyl acetate and water were added, and the organic phase was washed with brine, dried over Na_2SO_4 and concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of DCM / methanol to give the corresponding **117-125** as red solid.



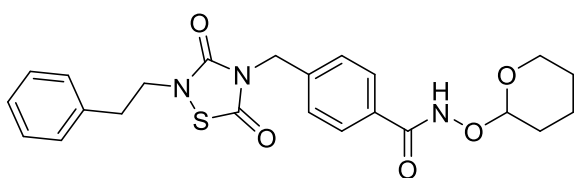
4-((3,5-dioxo-2-phenyl-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (117). Synthesized from **108** (0.254 g, 0.78 mmol), O-(tetrahydro-2H-pyran-2-yl)hydroxylamine

(0.215 g, 0.78 mmol), EDCI (0.299 g, 1.56 mmol) and HOBt (0.105 g, 0.78 mmol). Obtained 0.120 g of a red solid (36% yield).



4-((2-benzyl-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (118). Synthesized from **109** (0.255 g, 0.74 mmol), O-(tetrahydro-2H-pyran-2-yl)hydroxylamine

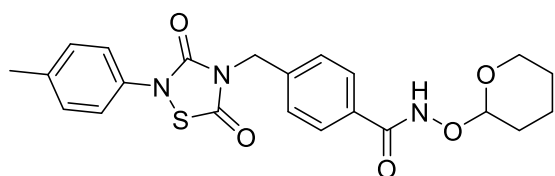
(0.087 g, 0.74 mmol), EDCI (0.283 g, 1.48 mmol) and HOBt (0.100 g, 0.74 mmol). Obtained 0.300 g of a red solid (92% yield).



4-((3,5-dioxo-2-phenethyl-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (119). Synthesized from **110** (0.167 g, 0.47 mmol), O-(tetrahydro-2H-pyran-2-yl)hydroxylamine

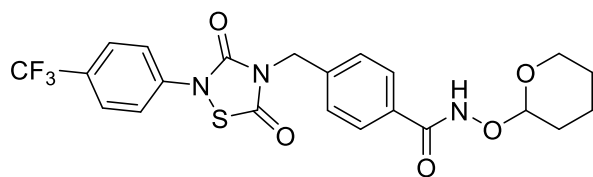
(0.055 g, 0.47 mmol), EDCI (0.180 g, 0.94 mmol) and HOBt (0.064 g, 0.47 mmol). Obtained 0.110 g of a red solid (51% yield). ^1H NMR (400 MHz, DMSO) δ 10.81 (s, 1H), 6.95 –

6.86 (m, 2H), 6.49 – 6.37 (m, 7H), 3.92 (s, 2H), 3.07 (t, J = 7.0 Hz, 2H), 2.08 (t, J = 6.9 Hz, 2H), 0.95 – 0.85 (m, 3H), 0.77 – 0.67 (m, 3H). ¹³C NMR (100 MHz, DMSO) δ 166.27, 152.78, 139.57, 137.99, 129.30, 128.90, 128.00, 127.77, 127.07, 101.49, 61.87, 45.83, 45.12, 34.32, 28.35, 25.17, 18.76.



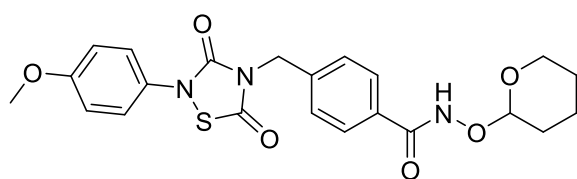
4-((3,5-dioxo-2-(p-tolyl)-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (120). Synthesized from **111** (0.125 g, 0.365 mmol), O-(tetrahydro-2H-pyran-2-

yl)hydroxylamine (0.043 g, 0.365 mmol), EDCI (0.140 g, 0.73 mmol) and HOBt (0.050 g, 0.365 mmol). Obtained 0.098 g of a red solid (61% yield). ¹H NMR (400 MHz, DMSO) δ 11.63 (s, 1H), 7.79 – 7.72 (m, 2H), 7.48 – 7.41 (m, 4H), 7.31 – 7.23 (m, 2H), 4.88 (s, 2H), 2.32 (s, 3H), 1.72 (s, 3H), 1.54 (s, 3H).



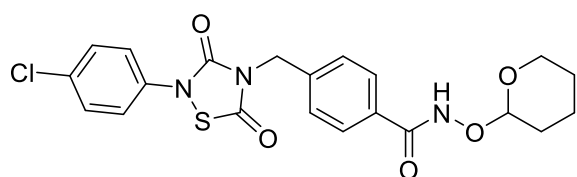
4-((3,5-dioxo-2-(4-(trifluoromethyl)phenyl)-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (121). Synthesized from **112** (0.570 g, 1.44 mmol), O-(tetrahydro-2H-

pyran-2-yl)hydroxylamine (0.170 g, 1.44 mmol), EDCI (0.552 g, 2.88 mmol) and HOBt (0.194 g, 1.44 mmol). Obtained 0.347 g of a red solid (70% yield).



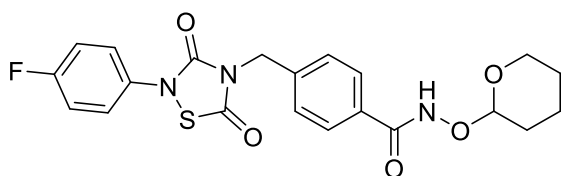
4-((2-(4-methoxyphenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (122). Synthesized from **113** (0.240 g, 0.67 mmol), O-(tetrahydro-2H-pyran-2-

yl)hydroxylamine (0.078 g, 0.67 mmol), EDCI (0.257 g, 1.34 mmol) and HOBt (0.090 g, 0.67 mmol). Obtained 0.110 g of a red solid (36% yield).



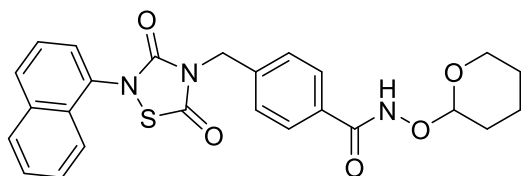
4-((2-(4-chlorophenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (123). Synthesized from **114** (0.105 g, 0.29 mmol), O-(tetrahydro-2H-pyran-2-

yl)hydroxylamine (0.034 g, 0.29 mmol), EDCI (0.122 g, 0.58 mmol) and HOBt (0.039 g, 0.29 mmol). Obtained 0.087 g of a red solid (65% yield). ^1H NMR (400 MHz, DMSO) δ 11.65 (s, 1H), 7.78 – 7.72 (m, 2H), 7.61 (d, J = 9.0 Hz, 2H), 7.55 (d, J = 8.9 Hz, 2H), 7.51 – 7.42 (m, 2H), 4.88 (s, 2H), 1.71 (s, 3H), 1.54 (s, 3H).



4-((2-(4-fluorophenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (124). Synthesized from **115** (0.055 g, 0.16 mmol), O-(tetrahydro-2H-pyran-2-

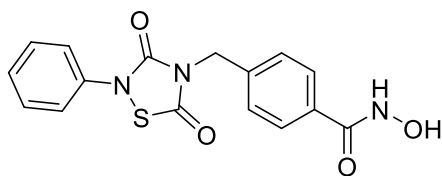
yl)hydroxylamine (0.018 g, 0.16 mmol), EDCI (0.060 g, 0.32 mmol) and HOBt (0.021 g, 0.16 mmol). Obtained 0.033 g of a red solid (47% yield).



4-((2-(naphthalen-1-yl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)-N-((tetrahydro-2H-pyran-2-yl)oxy)benzamide (125). Synthesized from **116** (0.213 g, 0.56 mmol), O-(tetrahydro-2H-pyran-2-

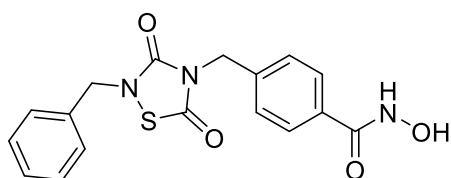
yl)hydroxylamine (0.065 g, 0.56 mmol), EDCI (0.214 g, 1.12 mmol) and HOBt (0.076 g, 0.56 mmol). Obtained 0.160 g of a red solid (60% yield).

General procedure for the synthesis of 39-47. To a solution of **117-125** in DCM, TFA was added and the mixture was stirred for 12 h. The solvent was concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of DCM / methanol to give the corresponding **39-47** as red solid.



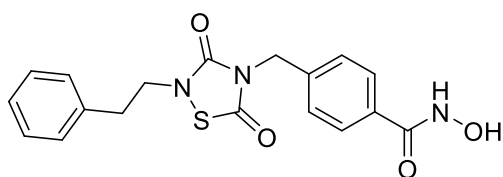
4-((3,5-dioxo-2-phenyl-1,2,4-thiadiazolidin-4-yl)methyl)-N-hydroxybenzamide (39). Synthesized from **117** (0.120 g, 0.20 mmol) and TFA (2 ml). Obtained 0.037 g of red solid (53% yield). ^1H NMR (400 MHz, DMSO) δ 11.17 (s, 1H), 7.72 (d, J

= 7.9 Hz, 2H), 7.38 (dd, J = 17.4, 7.9 Hz, 4H), 7.29 – 7.15 (m, 2H), 6.94 – 6.84 (m, 1H), 4.34 (s, 2H). ^{13}C NMR (100 MHz, DMSO) δ 164.58, 155.72, 144.23, 140.88, 131.65, 129.08, 127.37, 127.32, 121.55, 118.15, 42.89.

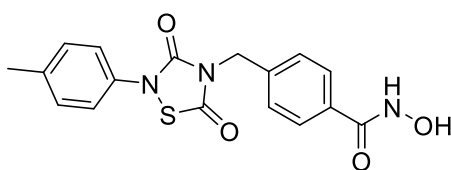


4-((2-benzyl-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)-N-hydroxybenzamide (40). Synthesized from **118** (0.300 g, 0.68 mmol) and TFA (2 ml). Obtained 0.113 g of red solid (47% yield). ^1H NMR (400 MHz, DMSO) δ 10.36 (s, 1H), 8.20 (s, 1H), 6.88 (d, J = 8.1 Hz, 2H), 6.59 – 6.39 (m, 7H), 3.97 (s, 4H). ^{13}C NMR (100 MHz, DMSO) δ

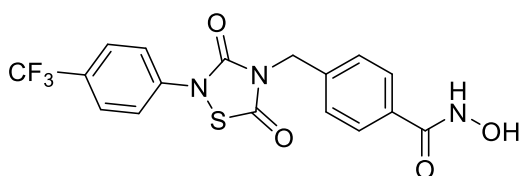
165.83, 163.92, 152.72, 138.73, 135.47, 132.36, 128.84, 128.31, 128.15, 127.50, 127.29, 47.66, 44.92.



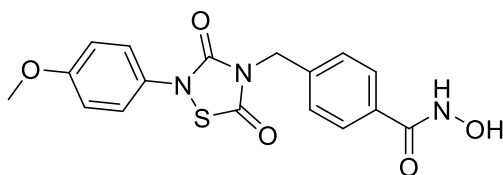
4-((3,5-dioxo-2-phenethyl-1,2,4-thiadiazolidin-4-yl)methyl)-N-hydroxybenzamide (41). Synthesized from **119** (0.110 g, 0.24 mmol) and TFA (2 ml). Obtained 0.029 g of red solid (33% yield).



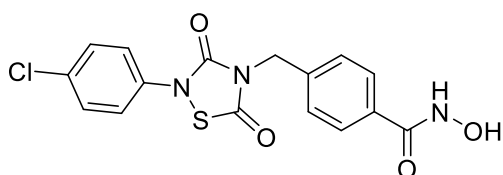
4-((3,5-dioxo-2-(p-tolyl)-1,2,4-thiadiazolidin-4-yl)methyl)-N-hydroxybenzamide (42). Synthesized from **120** (0.098 g, 0.22 mmol) and TFA (2 ml). Obtained 0.040 g of red solid (22% yield).



4-((3,5-dioxo-2-(4-(trifluoromethyl)phenyl)-1,2,4-thiadiazolidin-4-yl)methyl)-N-hydroxybenzamide (43). Synthesized from **121** (0.347 g, 0.70 mmol) and TFA (2 ml). Obtained 0.017 g of red solid (14% yield).

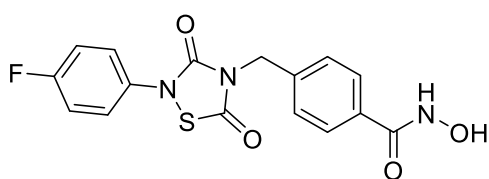


N-hydroxy-4-((2-(4-methoxyphenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzamide (44). Synthesized from **122** (0.110 g, 0.24 mmol) and TFA (2 ml). Obtained 0.045 g of red solid (50% yield).

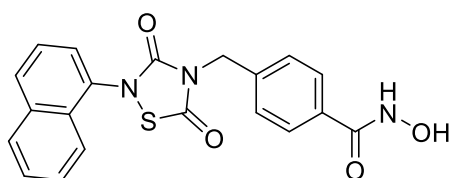


4-((2-(4-chlorophenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)-N-hydroxybenzamide (45). Synthesized from **123** (0.087 g, 0.19 mmol) and TFA (2 ml). Obtained 0.050 g of red solid (70% yield). ^1H NMR (400 MHz, DMSO) δ

11.21 (s, 1H), 9.04 (s, 1H), 7.74 (d, J = 7.9 Hz, 2H), 7.61 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 7.43 (d, J = 7.9 Hz, 2H), 4.87 (s, 2H).



4-((2-(4-fluorophenyl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)-N-hydroxybenzamide (46). Synthesized from **124** (0.033 g, 0.07 mmol) and TFA (2 ml). Obtained 0.016 g of red solid (60% yield).



N-hydroxy-4-((2-(naphthalen-1-yl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzamide (47). Synthesized from **125** (0.160 g, 0.34 mmol) and TFA (2 ml). Obtained 0.100 g of red solid (75% yield). ^1H NMR (400 MHz, DMSO) δ

11.24 (s, 1H), 8.09 (t, J = 8.4 Hz, 2H), 7.94 – 7.89 (m, 1H), 7.80 (t, J = 7.1 Hz, 3H), 7.67 – 7.57 (m, 3H), 7.49 (d, J = 7.9 Hz, 2H), 4.95 (s, 2H). ^{13}C NMR (100 MHz, DMSO) δ 166.55, 162.75, 152.40, 139.15, 134.44, 132.80, 131.50, 130.58, 130.55, 129.04, 128.38, 128.09, 128.02, 127.76, 127.44, 126.25, 122.87, 45.80.

3 DEGRADATION OF GLYCOGEN SYNTHASE KINASE 3 β

3.1 Proteolysis Targeting Chimeras (PROTACs) mediated GSK-3 β degradation

As previously discussed, GSK-3 β regulates several signalling pathways, such as PI3K, Wnt, Hedgehog, Notch and is involved in a series of disease such as cancer, diabetes, inflammatory, immune and neuronal disorders.^{88 95, 114} Considering the last, it is found that GSK-3 β is hyperactivated in the brain of AD patients, where it participates in tau hyperphosphorylation and A β formation, contributing also to oxidative stress, inflammation, memory formation and synaptic plasticity.¹¹⁴ For its activity, GSK-3 β is a promising drug target, as shown by considering inhibitors already described.¹⁰⁹ All those drugs are small molecules which modulate the enzyme activity through classical inhibition. Despite being the absolute foundation of drug research up to now, small molecules drugs present several critical issues; indeed, several proteins lack catalytic activity and/or have catalytic independent functions and their modulation could be difficult to achieve. Consequently, more than 80% of proteins are actually “undruggable”, comprising critical targets such as transcription factors, scaffolding proteins, or non-enzymatic proteins inside the cell.¹⁹⁵

For these reasons, in recent years innovative therapeutic approaches were developed. Some of them aim at modifying gene expression through messenger RNAs (mRNAs) cleavage or modulation: a) Antisense oligonucleotides (ASO) are single-stranded deoxyribonucleotides complementary to mRNA target, which form an RNA-DNA heteroduplex. In this way, activity of RNase H endonuclease is induced, cleaving mRNA complexes and reducing gene translation. Other ASO-induced effects are alteration of splicing processes, inhibition of 5' cap formation and steric hindrance of ribosomal activity; b) small interfering RNAs (siRNAs) are fragments of double-stranded RNA, with two nucleotides overhanging the 3' end of each strand; they cleave mRNA by binding to a protein complex, RISC (RNA induced silencing complex), which separates the double strand. The single strand with the more stable 5' end stays integrated into the complex and guides it to the target mRNA, which is cleaved;¹⁹⁶ c) CRISPR/CAS-9 is the more efficient method of genomic editing and it is the mechanism of adaptive immunity of many prokaryotes, to defend themselves against viruses and bacteriophages.¹⁹⁷ It consists of three phases in which the target sequence is recognized by a guide RNA (gRNA), CAS-9 breaks the double-strand and cleaves the RNA-DNA heterodimer freshly formed. The last phase is the reparation of the double-strand. This technology is still in its early phases, so it has to be better developed to be more accurate and avoid undesired consequences.¹⁹⁸

Beside these approaches, other techniques were developed, based on immunity acquisition. This category includes monoclonal antibodies and CAR-T cell therapy. The first is one of the most promising new technologies, which managed to bring new drugs approval for AD since Memantine in 2003.^{54, 55} Monoclonal antibodies are artificial immune system proteins, built for contrasting a specific biological target. They activate immunity response by interacting with the antigen which they are produced for. This allows a great amount of possible target, but in some cases problems of selectivity may occur.¹⁹⁹ Regarding CAR-T therapy, CAR are chimeric antigen receptors which are developed to interact high specifically with their target. Once inserted into T-cells, they redirect them to surface antigen towards desired target. This technology showed great successes in cancer therapy, in particular against hematologic cancers like acute and chronic B cell leukemias, lymphoma and myeloma.²⁰⁰ Like monoclonal antibodies, lack of selectivity is the main problem of CAR-T cells: for example, CD19-targeting CAR T cells effectively kill both CD19+ tumor cells and CD19+ normal B cells, causing prolonged B cell aplasia.²⁰¹

However, the development of these methods has faced many challenge that limits their applicability. Therefore, there is an urgent need for new paradigms and technologies that can allow to approach this issue in unconventional prospective.²⁰²

Among novel technologies, a new paradigm which introduce a new way to induce enzymatic activity reduction is represented by molecules called Proteolysis Targeting Chimeras (PROTACs). These are small molecules which regulates protein levels rather than controlling their function. This effect is achieved not by classical inhibition, but by removal of the enzyme trough recruitment of ubiquitin-proteasome system (UPS). Indeed, PROTACs can bind a protein of interest (POI) and, at the same time, the E3 ubiquitin ligase, leading to ubiquitination and degradation of the target.²⁰³ This process is performed catalytically, so low concentration of PROTAC can remove sub-stoichiometric quantities of target by repeating several cycles of activity. In this way, many doses dependent issues, typical of small molecules inhibitors, can be avoided.²⁰⁴ In addition to catalytic activity, PROTACs can extend their effects on cells, whose response go beyond pharmacokinetic profile of classical inhibitors, depending entirely on synthesis ratio of the POI. This is because the cell needs to synthesize POI again, which requires longer time than recovering from inhibition.

Given the great variability of ligands which can be included in PROTAC and the promising characteristics of the technique, in 20 years since the first report almost 1000 different PROTAC were reported. Some of them, also, reached clinical trials.²⁰⁵

3.2 Medicinal chemistry of PROTAC

PROTACs are bifunctional molecules constituted by 3 parts: a ligand that recognize the POI, a linker and a E3 ligase recruitment moiety.²⁰⁶ Concerning POI, a great range of possible ligands are available,²⁰⁷ distinguished by mechanism of action in non-covalent, irreversible, reversible or allosteric.²⁰⁸ Over 100 targets were identified able to be modulated by PROTACs, including transcriptional factors like bromodomain-containing protein 4 (BRD4),²⁰⁹ cytoplasmic hormonal receptors, like estrogen and androgen receptors (ER and AR),^{210, 211} regulatory proteins and tyrosine-kinase receptors (EGFR,²¹² HER3,²¹³ FLT3).²¹⁴ The structure of the linker plays an important role. It can be represented by a flexible aliphatic or polyethyleneglycolic (PEG) chain or by a more rigid chain made up of piperazine or piperidine. Its composition and length influence the flexibility, hydrophobicity and solubility of the whole PROTAC and are important factors for the formation of ternary complex, for the degrading ability and selectivity towards the POI.²¹⁵ The choice of E3 ligase ligand is another crucial point. More than 600 E3 ligase are encoded by human genome,²¹⁶ but only few of these were successfully exploited in PROTAC design. Some examples are mouse double minute 2 homologue (MDM2), von Hippel Lindau (VHL), cereblon (CRBN) and cellular inhibitor of apoptosis protein (cIAP). Depending on which ligase is recruited, differences in degradation profile as well as tissues selectivity can be observed.²¹⁷ A great help in this was given by the discover of thalidomide as a CRBN E3 ligase ligand. Thalidomide is immunomodulating drug (IMiD) sadly known for its teratogenic effects; indeed, it was available over the counter in 50s and 60s as sedative, hypnotic and anti-sickness drug.²¹⁸ Subsequently it was suspected of teratogenicity, after several cases of birth defects in women who were using it in pregnancy. After the obvious removal from the market, thalidomide and its derivatives, lenalidomide and pomalidomide, emerged as promising treatment for some types of cancer and inflammatory disorders. Studies on their mechanism of action showed that these drugs target a CRBN complex, formed by regulator of cullin 1 (ROC1), Cullin-4A (Cul4A), damage specific DNA binding-protein 1 (DDB1), known as CRL4-CRBN and redirect its activity. In this way, CRL4-CRBN complex targets IKZF1 and IKZF3, two fundamental lymphoid transcriptional factors, which are ubiquitinated and degraded, leading to death of myeloma cells. This evidence promoted the use of IMiDs in patient with myeloma.^{219, 220}

3.3 PROTAC pharmacology

Despite the promising properties and revolutionary mechanisms, PROTACs presents some criticism when it comes to pharmacokinetics. Most of the problems are due to their large structure, which can make very hard to cross cell membrane and enter the cell. In comparison to classical inhibitors, they

have higher molecular weight (MW) and an increased number of hydrogen bond donors and acceptors.²²¹ However, PROTACs are reported to penetrate in different cell types through a passive process, which is still not fully understood.²²² Further proof to this was given by two studies of Mattson and Pay, in which it is stated that molecules with high MW and a sufficiently flexible structure can fold to be both water soluble and lipophilic enough to cross cell membrane.^{223, 224} Moreover, due to their catalytic action, it is not required a high intracellular concentration to achieve protein degradation. Once inside the cell, a PROTAC will form a binary complex with one between POI or E3 ligase. The novel complex will search the other target, forming a ternary complex and making POI and E3 ligase closer. In this way, interactions between these two molecules can occur, even which normally cannot happen. The degradation mechanism starts with ubiquitination of POI by E3 ligase. More than one ubiquitin can be bound in subsequent sites on POI. Then, ubiquitinated POI is carried to proteasome 26S, which degrades the protein and sets PROTAC free, able to restart the process (Figure 48).

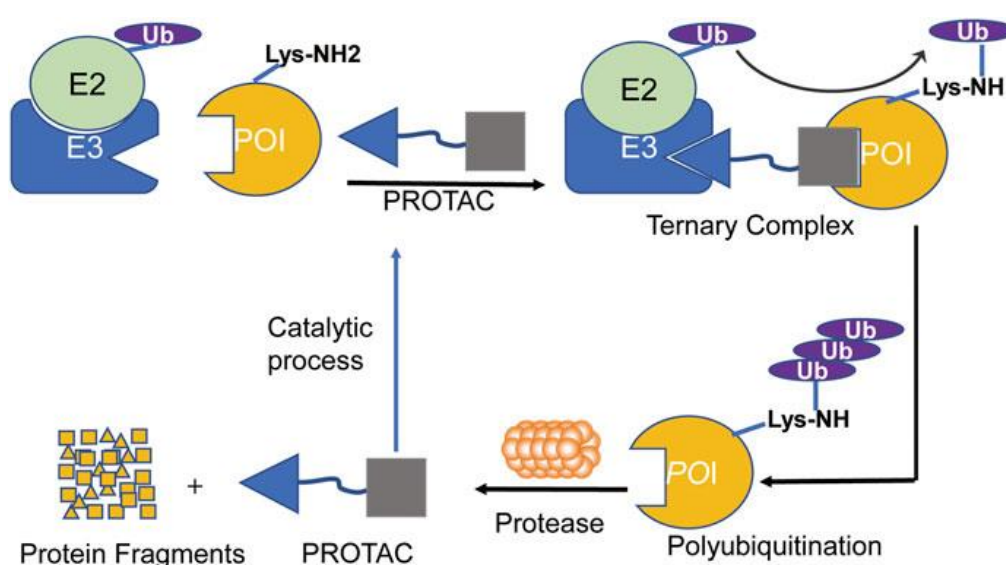


Figure 48. Catalytic mechanism of PROTACs.²²⁵

This PROTAC-induced degradation occurs through a bell-shaped concentration mechanism, called Hook Effect (Figure 49). Indeed, high concentration of PROTAC can saturate either the POI or E3 ligase binding, failing to form the ternary complex and, consequently, degrade the POI. This can lead to two possible adverse effects: a) the first is the degradation of off-target molecules with lower affinity for PROTAC, by binding of the complex PROTAC-E3 ligase to these molecules instead of the POI, which is bound to PROTAC itself; b) the second one can be the possible interaction of

PROTAC-POI complex with other mechanism different from degradation.²²⁶ In particular, this undesired action can occur when the complex acts as an agonist, like in mutated AR, which are activated by molecules that normally are receptor antagonists.²²⁷ On the other hand, the catalytic amount of PROTAC needed to trigger the degradation of POI can help to avoid the Hook effect.

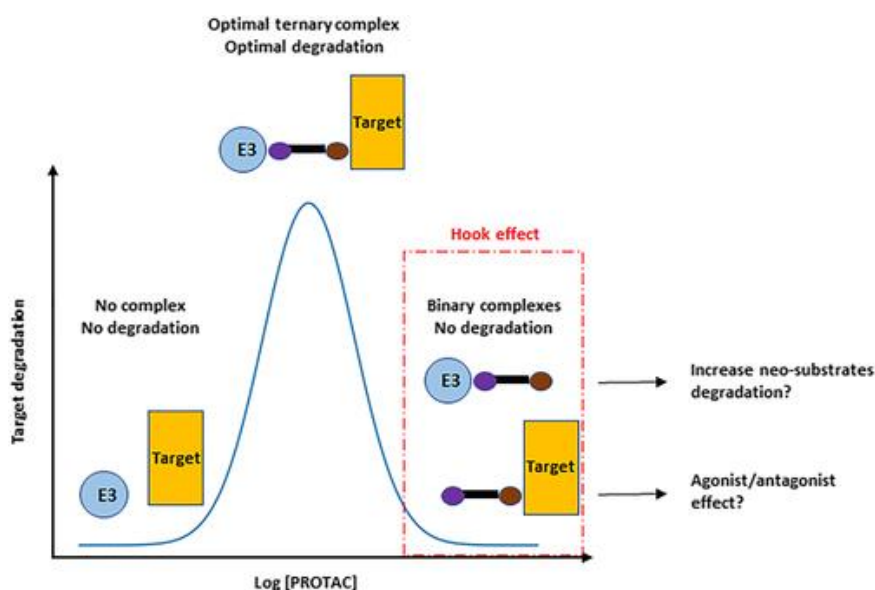


Figure 49. The Hook effects in PROTAC pharmacology.²²⁶

Another aspect to consider is the possible resistance acquisition through genomic alteration in E3 ligase complex, which may become resistant to PROTAC binding. In a study, Zhang and co-workers showed that chronic treatment with VHL- and CRBN- based PROTACs in cancer cells led to acquired resistance through a genomic modification of the core components of E3 ligase.²²⁸ Selectivity is also an aspect not to be underestimated, as PROTACs can degrade undesired protein (off-target effects) or the target protein in wrong tissues (on-target effects). An example is given by CRBN-PROTACs that degrade IKF1 and IKF3,²¹⁹ as well as some transcriptional factors like SALL4.²²⁹ A possible solution to this problem can be the design of a prodrug PROTAC or spatiotemporal control.¹⁹⁵

3.4 Single-target CRBN-PROTACs

To enrich what explained in previous paragraphs, some examples of developed single-target PROTAC will be briefly discussed. As already seen, different E3 ligase recruitment moieties are available when designing a PROTAC. Among these, CRBN-based PROTAC have received most attention for their promising role in therapy against several diseases, in particular in cancer.²³⁰ In

particular, ARV-110 and ARV-471 (Figure 50) developed by Arvinas, are the most advanced PROTEC reaching phase II of clinical trials.²³¹ ARV-110 is an AR-directed PROTAC which is able to reduce AR levels at concentrations lower than 1 nM and, when orally administrated (10 mg/kg), significantly inhibited the growth of tumors in a model of cancer resistant to enzalutamide, reducing AR levels by 70-90%.^{225, 232-234} On the other hand, ARV-471, a PROTAC targeting ER, degrades ER in ER-positive breast cancer cell lines with DC₅₀ around 1 nM. It also decreases the expression of classically regulated ER-target genes and suppress the growth of ER-dependent cell lines. In clinical trials, it was able to reduce ER level in tumor tissue by 62-90%.²²⁵

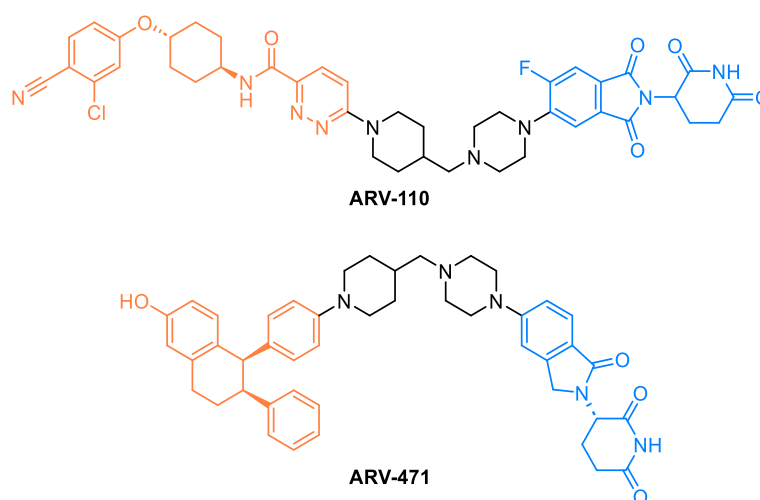


Figure 50. Structures of ARV-110 and ARV-471.

Another target on which research focused to develop PROTACs is Epidermal Growth Factor Receptor (EGFR), a factor contributing to tumor cell proliferation, apoptosis inhibition and metastasis.^{235, 236} In 2020, Zhang et al. reported the first EGFR-directed PROTAC, combining CRBN ligand thalidomide to pyrido[3,4] pyrimidine, a structure present in fourth generation EGFR inhibitors. Main compound of the series, **I**, degrade EGFR with a DC₅₀ of 45 nM, arresting tumor cells in G1 phase and inducing their apoptosis.²³⁷ After short time, Zhang et al. synthesized a series of CRBN PROTAC including **II**, which showed good inhibitory abilities in PC9 cells (IC₅₀ = 0.413 μM).²³⁸ In 2021, Jiang et al reported two PROTACs based on EGFR inhibitors canertinib and pomalidomide, **III** and **IV**. Both compounds were able to degrade EGFR at concentrations between 30-50 nM and with better activity compared to canertinib. Moreover, they induced apoptosis and cell cycle arrest in tumor cells (Figure 51).²³⁹

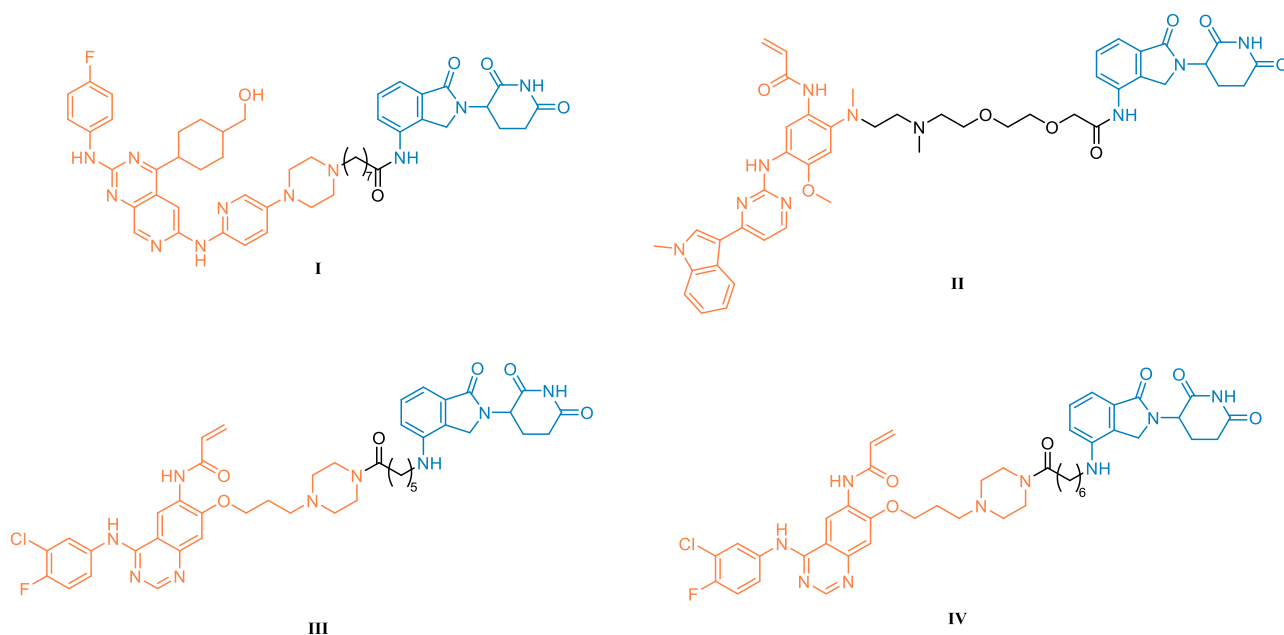


Figure 51. Structures of EGFR-directed PROTACs **I**, **II**, **III** and **IV**.

Cell proliferation is also regulated by phosphatidylinositol-3 kinases (PI3K), enzymes taking part in PI3K/Akt/mTor signalling pathway. Overexpression of PI3K is connected to tumorigenesis, but the poor selectivity of its inhibitors, along with severe side effects, limit their use in therapy.²⁴⁰ For this reason, PROTACs can be a useful alternative. In 2018, Jiang et al. synthesised a series of PI3K-directed PROTACs, which included **V**, tethering pomalidomide and PI3K inhibitor ZSTK474 (Figure 52). **V** was able to degrade PI3K level at 10 μ M, managing to downregulate phosphorylation of Akt, mTor and GSK-3 β in their signalling pathway.²⁴¹

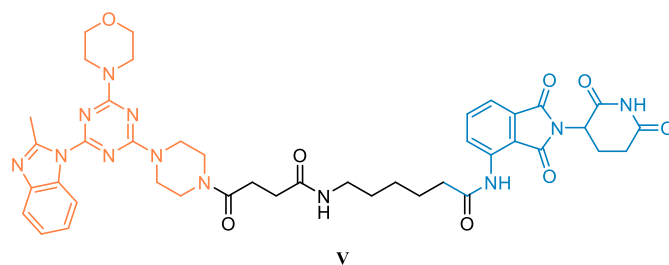


Figure 52. Structure of PROTAC **V**.

In tumorigenesis, BRD9 is a factor which, if overexpressed, contributes to it through regulation and expression of several genes and DNA replication.²⁴² In this optic, in 2017 Bradner et al reported the

first PROTAC targeting BRD9, combining known inhibitors and pomalidomide (Figure 53). Compound **VI** managed to degrade BRD9 selectively over BRD4 and BRD7 ($IC_{50} = 104$ nM and $DC_{50} = 50$ nM), 10-100 times more potent than its parent inhibitor.²⁴³

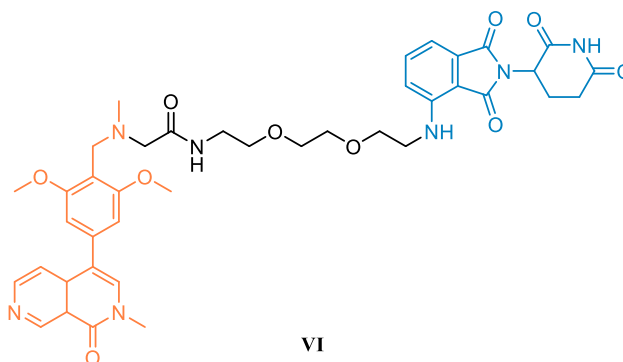


Figure 53. Structure of PROTAC **VI**.

As already discussed, HDAC6 is showed to play an important role in cancerogenesis.¹⁴¹ Moreover, the lack of selectivity of its inhibitor over HDAC1 and HDAC3 stressed the need for drugs able to selectively target it.¹⁵³ It is in this contest that, in 2019, Tang et al reported PROTAC **VII**, whose structure included selective HDAC6 inhibitor Nexturastat A. **VII** turned out to degrade HDAC6 at the concentration of 3 nM and to have a good selectivity towards HDAC1 and HDAC3.²⁴⁴ Nexturastat A was the base of another PROTAC, **VIII**, reported by Rao et al in 2019, which showed degradation ability towards HDAC at 100 nM, while it was not able to degrade other HDAC isoforms even at 10 μ M.²⁴⁵ In the same year, they reported another HDAC6 degrader, **IX**, with a DC_{50} of 3.2 nM in MM.1S cells at 24h (Figure 54).²⁴⁶

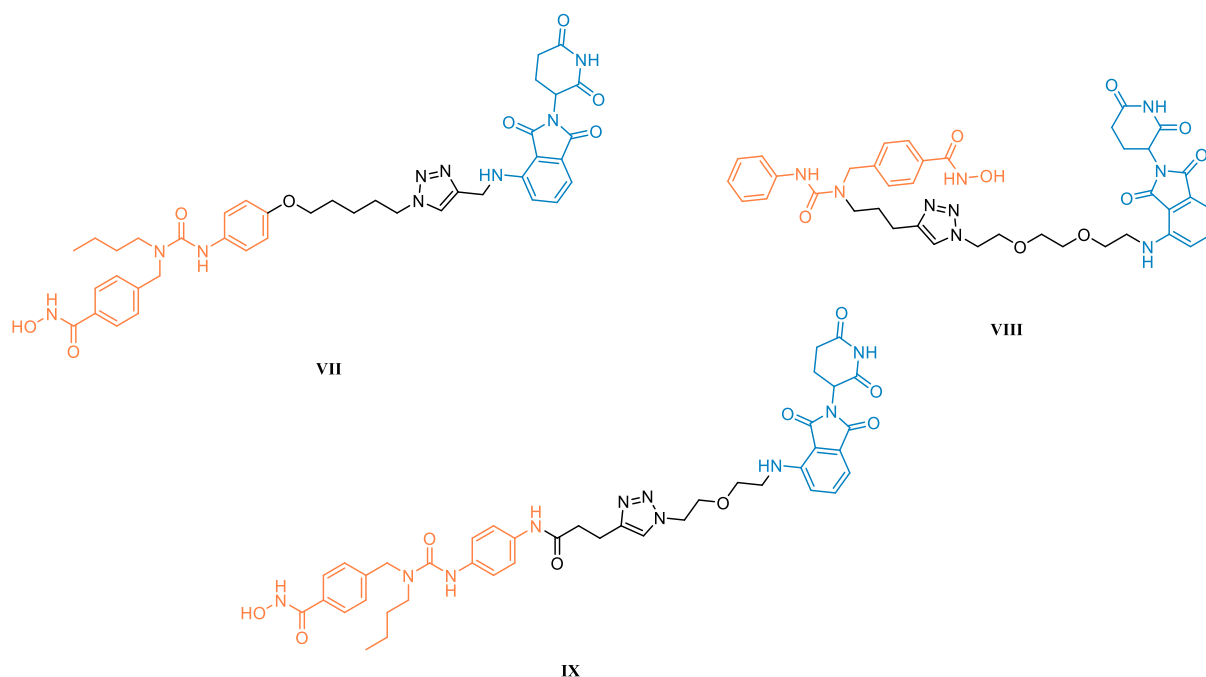


Figure 54. Structures of PROTACs **VII**, **VIII** and **IX**.

Another factor which contributes to the onset of cancer and neurodegenerative diseases is the dysregulation of microtubules and tubulin. Microtubule-stabilizing and destabilizing have been useful agents in oncology, even though drug resistance in cancer cells limits their use.²⁴⁷ The application of PROTAC technology to bypass this drug resistance led Mitchison et al to develop an α - and β -tubulin degrader, based on microtubule-destabilizing agent. Unfortunately, **X** was not able to induce any degradation of tubuline, therefore it is still valued if this strategy could be applied to this target (Figure 55).²⁴⁸

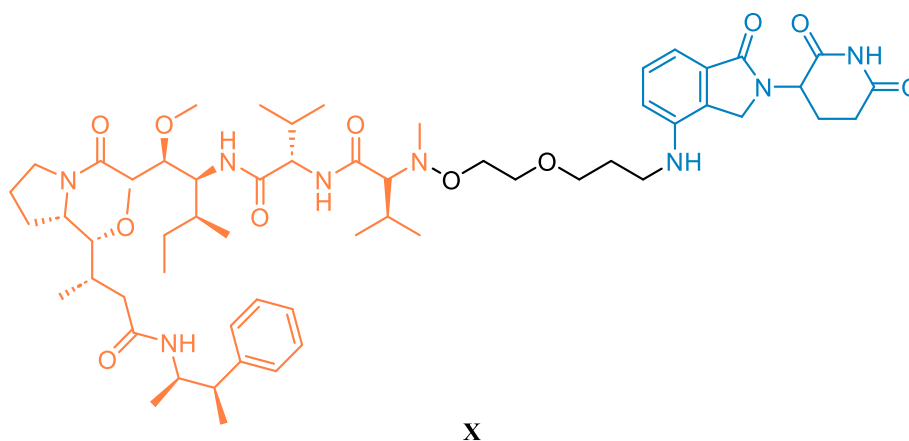
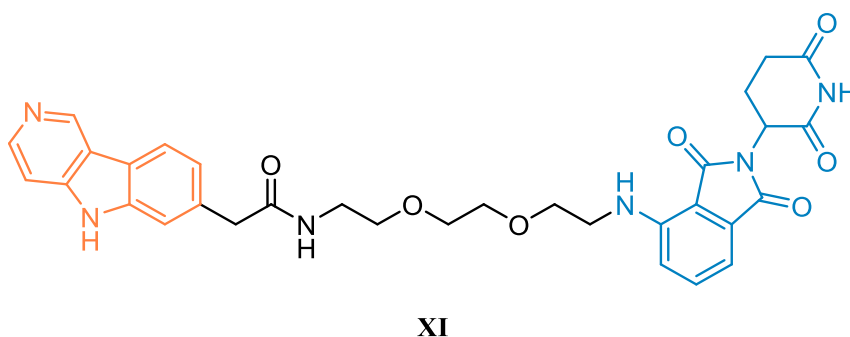


Figure 55. Structure of PROTAC **X**.

Concerning neurodegenerative diseases, one of the main contributors is tau protein, a microtubule-associated protein which play a role in axonal transport.⁷⁹ Its dysregulation represents a characteristic of several neuronal disorders, like AD and dementia, where it modulates also A β formation.^{7, 34} In 2019, Haggarty et al. reported a series of tau-directed PROTACs based on CRBN. Among them, compound **XI** emerged as tau degrader in low micromolar concentration range and more active in diseased cells rather than healthy ones. In mice model, it was able to penetrate BBB when administrated at a dose of 30 mg/Kg (Figure 56).²⁴⁹



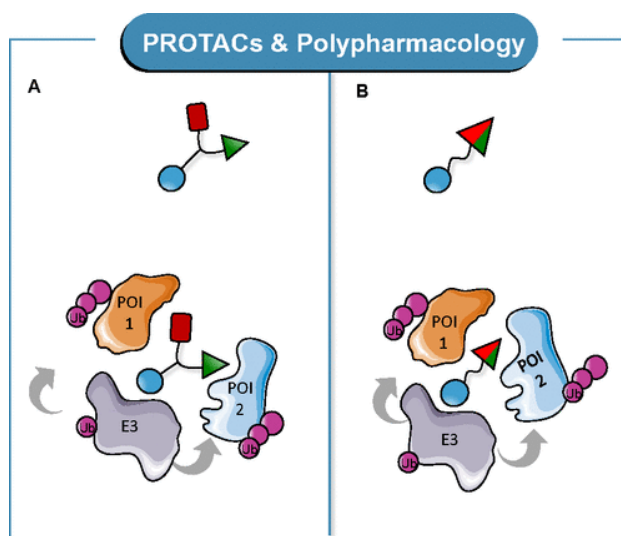


Figure 57. Multitarget PROTACs structure. A) two different ligands toward two targets; B) one ligand targeting two POI.¹⁹⁵

Recently, a multitarget PROTACs library has been reported. In particular, getifinib e olaparib were included into CRBN-PROTAC and VHL-PROTAC, to degrade two targets involved in cancer progression, EGFR and poly (ADP-ribose) polymerase (PARP). The two drugs were connected to the E3 ligase binder by a branched amino acidic core, obtaining compound DP-C-1 and DP-V-4 (Figure 58). The first one displayed better dual-degrading properties than its mono-PROTAC counterparts, while the second mitigates a better degrading effect with lower antiproliferative activity in tumor cells in comparison with corresponding inhibitors. Although engagement of each target was observed, no proof of formation of ternary complex was reported. However, this was the first work in which was reported successfully a PROTAC able to degrade two different POIs. An example of dual-targeting POI ligand is represented by DT2216 (Figure 58), a PROTAC which includes ABT-263, a potent dual inhibitor of two member of BCL-2 family proteins, BCL-2 and BCL-xL. This drug was not approved for causing thrombocytopenia, so it was designed a PROTAC hoping to avoid this adverse effect. DT2216 was the first PROTAC of his kind, even though it managed to degrade only BCL-xL. This led to molecular optimization of DT2216 in multiple steps, modifying the linker. In this way, 753b was obtained, resulting in a PROTAC able to degrade both BCL-2 and BCL-xL.

These two examples proved that a single multitarget PROTAC can provide more benefits than the coadministration of two single compounds. However, the design is way more challenging and the pharmacokinetic profile, due to higher MW and complexity, can be problematic. Nevertheless, the great advantage could be a better therapeutic window, as they are effective in lower doses and avoid drug-drug interactions, and the reduced drug resistance.

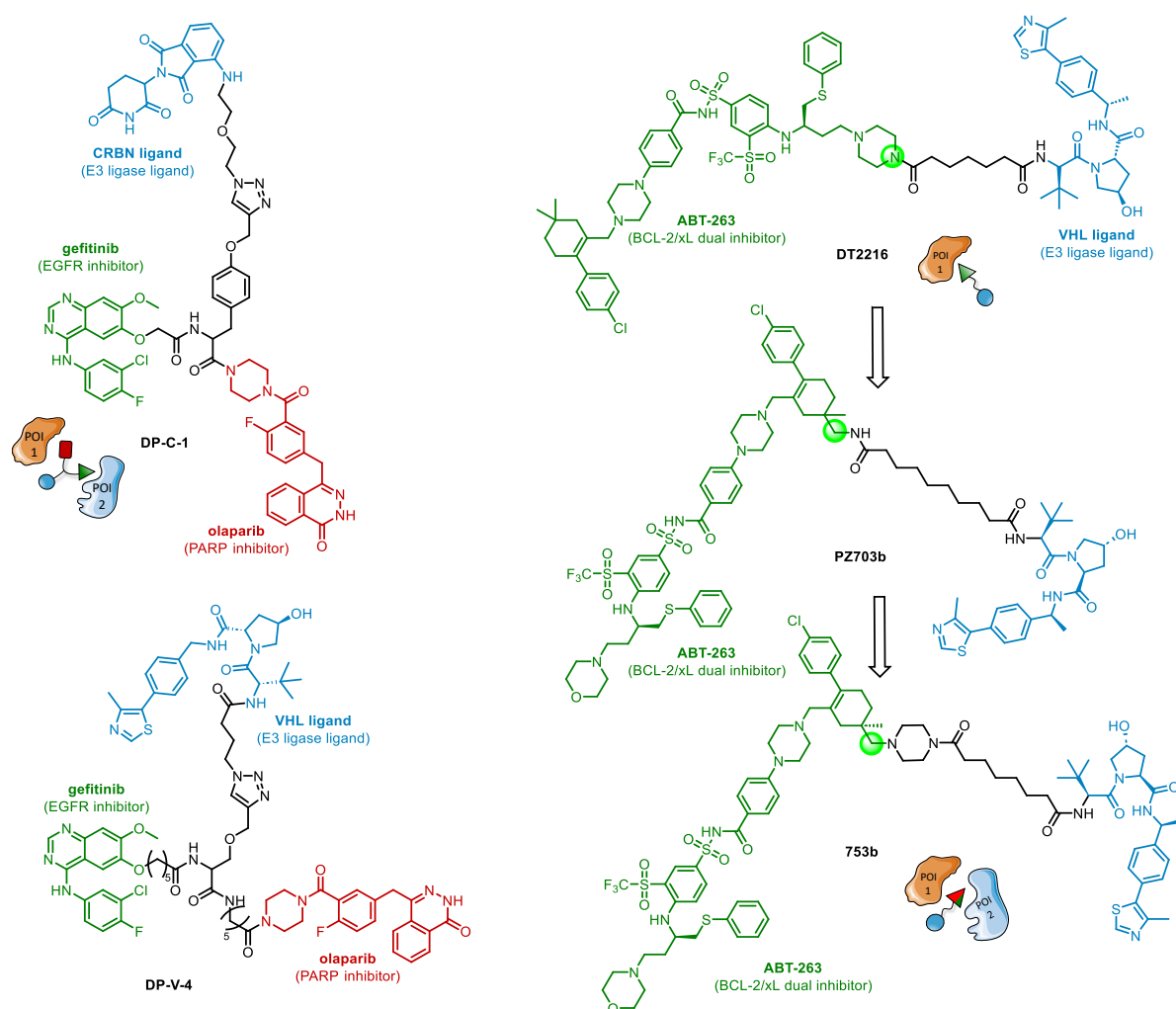


Figure 58. Design of multitarget PROTACs DP-C-1, DP-V-4 and 753b.

3.6 Photopharmacology and PROTAC combination

Photopharmacology is a fast-growing field which adds to the classical pharmacological approach based on small-molecule drugs the light control used in photochemistry.^{250, 251} Photopharmacology aims to control drug activity with high spatiotemporal precision, to avoid off-target problems and side effects. This includes modulation of pharmacokinetic and pharmacodynamic profile.²⁵² The two main mechanisms in which photopharmacology is expressed are photocaged and photoswitchable ligands. Photocaged compounds are irreversibly photoresponsive small molecules presenting a protecting group (the “cage group”), which masks the bioactive pharmacophore and makes the molecule inactive. Then, photocleavage of the cage group upon irradiation activates the bioactive compound, which is free to express its pharmacological activity. Photoswitchable ligands are reversibly photoresponsive small molecules, able to switch between isomeric forms. This switch is catalysed by

a light stimulus. The resulting conformational change enables on/off switching of the therapeutic action by affecting target recognition. Photopharmacology has made huge progress in the past decade, with a large library of photopharmacology small molecules directed to a wide array of biological targets.²⁵³ This technology can give a great support to PROTAC administration, to avoid toxic effects by controlling when and where activate their activity. Several PROTACs have been endowed with light-controllable release mechanism. As previously said, the main outcome of such multifunctional PROTACs is the spatiotemporal control of protein degradation, which is generally related to the formation of a productive ternary complex trough light irradiation. Light-controllable PROTACs can be divided into two categories, photocaged PROTACs and photoswitchable PROTACs (Figure 59).

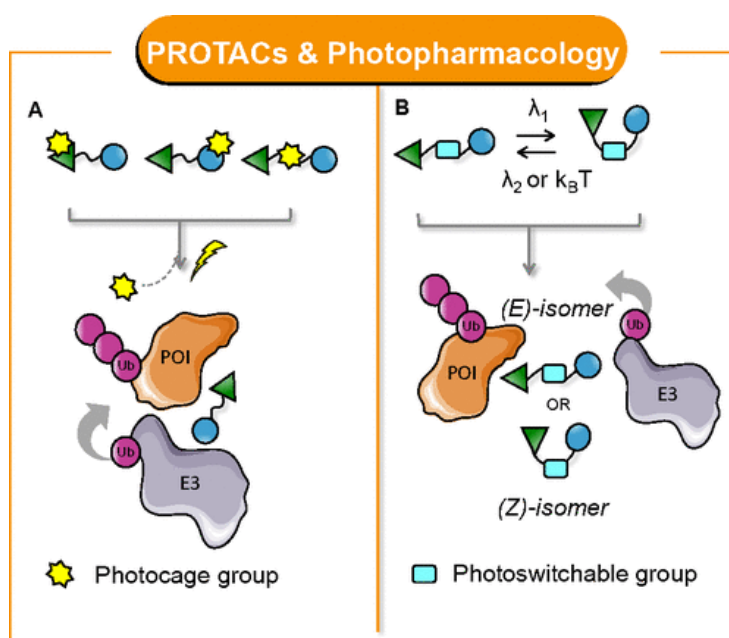


Figure 59. Structure and mechanism of action of light-controllable PROTACs.¹⁹⁵

- Photocaged PROTACs are usually designed with a protecting group which can be irreversibly cleaved through light irradiation, releasing the active PROTAC in the desired site. The “cage group” can be introduced in either the E3 ligase or POI ligand, to avoid their binding to target protein, or in the linker structure, to affect the interaction between POI and E3 ligase and prevent the formation of the ternary complex. Among the options mentioned, the most frequently developed is the insertion of the cage group on the E3 ligase ligand. By caging it, the degradation of the POI and potential off-target effects are avoided, while the POI ligand can still interact with its target and fulfil its eventual inhibitory activity. On the other hand, caging the POI ligand would still let the E3 ligase ligand interact with its target, causing potential side effects like the IKZ1/3 degradation.²⁵⁴ The first photocaged PROTACs was

reported by Xue et al., who installed a 4,5-dimethoxy-2-nitrobenzyl (DMNB) cage group on a BRD2-4 degrader, dBET1. The cage group was put either on the CRBN ligand or on the amide moiety on the linker. This latter modification gave pc-PROTAC 1 (Figure 60), whose cage group was cleaved upon 365 nm irradiation to give the active compound dBET1. This was able to bind BRD4 in the dark with more than 100-fold reduced affinity. Degradation of the POI started after a light irradiation of only 0.3 min, ending with complete degradation after 3 min. dBET1 activity was evaluated in vivo in zebrafish embryos, which confirmed its light-induced degrading activity at 50 and 100 μM .²⁵⁵ Similarly, Naro and co-workers developed a strategic way to convert any small-molecule based PROTAC into a light-controllable one by inserting cage groups into the E3 ligase ligand. In particular, they substituted the hydroxyl group of the VHL ligand of an estrogen-related receptor α (ERR α)-targeted PROTAC with diethylamino coumarin (DEACM) cage group and introduced 6-nitropiperonyloxymethyl (NPOM) moiety on the imide nitrogen of the CRBN ligand of dBET1 (Figure 60). Both photocaged PROTACs were activated by light irradiation under 405 nm, with no degradation of the target observed in dark. Moreover, the induced degradation was confirmed in MCF-7 breast cancer cells expressing ERR α transcripts, while showing no ERR α depletion in the dark.²⁵⁶

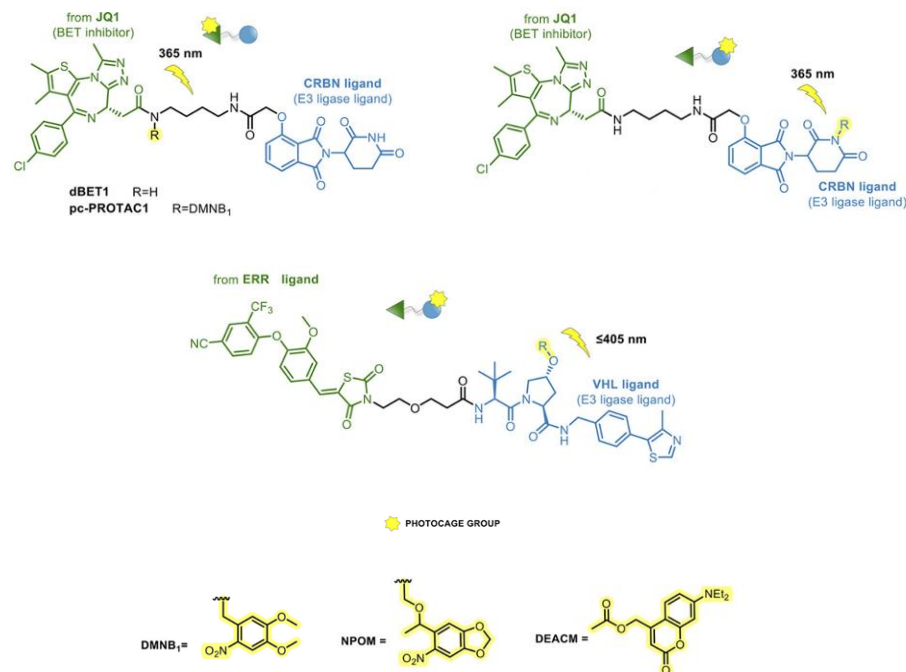


Figure 60. Design of photocaged PROTACs.

- Photoswitchable PROTACs are another modality to control where and when degradation activity should happen. In this case, light is used to change the spatial conformation of the molecule, switching on and off the degrading of the POI. In this way, the azobenzene photoswitch has been largely used for the development of photoswitchable PROTACs, given its chemical stability, predictable geometrical changes (Z/E isomers), and facile modulation of properties.²⁵⁷ In addition, insertion of azobenzene does not change drastically MW, but its conformational change from Z to E isomer affects binary and ternary complex formation. Based on previous X-ray crystal structure studies, Ju et al. designed a series of BCR-ABL directed PROTACs based on its inhibitor dasatinib (Figure 61). By bonding azobenzene to lenalidomide, AZOPROTAC-4C was synthesized. Tests on the two different E/Z isomers showed completely absence of degradation ability of Z isomer in a BCR-ABL-positive K562 cell line, while E isomer degraded BCR-ABL fusion protein and ABL. However, light - induced conformational switch was only tested under UV-light (200-280 nm), which has a poor penetrability and high harmful effects on cells.²⁵⁸ Following the same strategy, Reynders et al. inserted the azobenzene unit as part of lenalidomide on dBET1, obtaining photoswitchable PROTAC PHOTAC-I-3 (Figure 61). When tested, it was able to switch to active Z isomer conformation upon 390 nm light irradiation, and consequent BET protein degradation was detected in AML cell line RS4. Nevertheless, it was observed that only a low amount of compound switched back to inactive form in the dark, not letting an optimal control of its activity.²⁵⁹

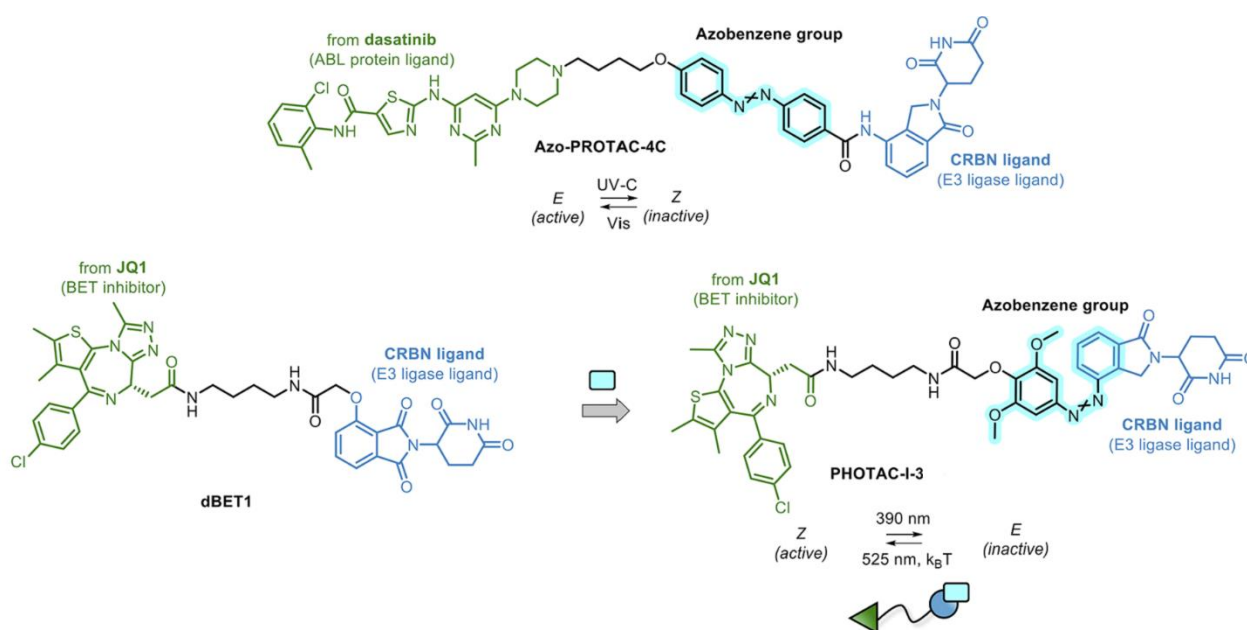


Figure 61. Design of photoswitchable PROTACs.

In conclusion, light-controllable PROTACs deal spatiotemporal control of protein degradation and side effect minimization. However, they still face clear limitations. In particular, their implication in clinical therapy may be limited by light-induced cellular damage and poor tissue penetration of the irradiation.

3.7 PROTACs and drug conjugation

In recent decades, new technologies for delivery of therapeutic agents to diseased cells and tissues, and not to healthy ones, have emerged. The main of those new approached are nanotechnology²⁶⁰ and drug conjugates featuring a targeting moiety.²⁶¹ Antibody–drug conjugates (ADCs) are generally made of an antibody (Ab) conjugated to a cytotoxic molecule via a chemical linker.²⁶² Three examples of ADC therapeutics (trastuzumab emtansine, brentuximab vedotin, and inotuzumab ozogamicin) are already available on the market, while many others are involved in clinical trials.²⁶³ More recently, small molecule–drug conjugates (SMDCs) have been developed to make therapy more accurate. SMDCs usually feature three parts, i.e., a targeting ligand, a linker and a therapeutically active small molecule. Targeted drug conjugates act by recognizing overexpressed receptors on target cells and, once inside, selectively releasing the therapeutic agent. In most cases, linker cleavage is needed to release the therapeutic agent and this can happen by reacting with intracellular thiols²⁶⁴ or by using linkers particularly sensitive to specific stimuli, such as pH, redox system, and light.²⁶⁵ As already seen, PROTAC can benefit from technologies which give them selectivity that they lack of. The possibility of designing a tissue-selective or target-selective PROTAC with reduced toxicity and side effects had gained more and more attention. It is no surprise that conjugated PROTACs started to be reported (Figure 62).

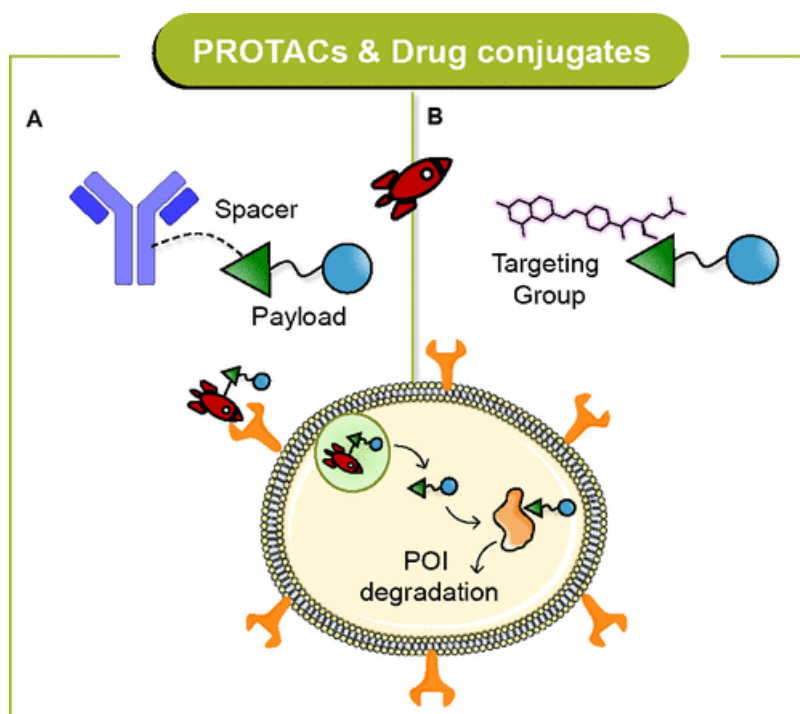


Figure 62. Conjugated PROTACs design: A) antibody-PROTACs conjugates; B) small molecules-PROTAC conjugates.

- Ab-PROTAC: ADCs are targeted drug conjugates formed by an Ab as the targeting moiety, the therapeutic agent (payload) and the linker connecting the Ab to the payload. In this way, the high toxicity of the payload is combined to the selectivity of the Ab, which recognizes the target cell and binds to it, releasing the therapeutic agent inside it. However, there are some factors to be considered when designing an Ab-PROTAC conjugate: a) the antigen targeted by the Ab should be overexpressed in desired cells, to improve selectivity and avoid off-target effects; b) consequently, the Ab should be the most specific possible for that antigen, to avoid cross-reactions;²⁶⁶ c) the linker has to be stable until target recognition, then should be cleavable inside the cell; d) the payload, in particular when the target is a cancer cell, has to be highly active (IC_{50} value in the low nanomolar or picomolar range), interact with a well-known and defined mechanism of action and be endowed with a suitable attachment point for the linker insertion.²⁶⁷ Last, a single Ab can be loaded with multiple ligands. This, obviously, modifies pharmacokinetic profile and stability, as well as toxicity of the PROTAC. Despite these factors, a crescent interest in Ab-PROTAC is developing, as can be seen from evidence in literature. Dracovich et al. developed the first reported Ab-PROTAC conjugate by attaching a BET degrader to an anti-C-type lectinlike molecule-1 (CLL1) antibody. CLL1 is a valid target in therapy for acute myeloma leukemia (AML), due to its high expression in leukemic

cells, while being absent in normal hematopoietic stem cells. The base of the Ab-PROTAC is a potent BRD4 degrader, GNE-987, synthesized by the authors by incorporating the VHL-binding moiety along with the structure of a potent BET inhibitor. It turned out to be a potent degrader, with activity at picomolar concentration in a cell model of AML (EOL-1 AML). Unfortunately, the compound was characterized by a poor pharmacokinetic profile either *in vitro* and *in vivo* in mice model. To contrast these issues, the molecule was modified by inserting the S₁ spacer, containing the cleavable disulfide group, which was conjugated to CLL1 antibody. Resulting product CLL1-XII showed high dose-dependent potency, stronger than his parental molecule one. It also displayed improved pharmacokinetic profile and *in vivo* properties.^{268, 269} Another research group developed an Ab-PROTAC conjugate, HER2-XIV, (Figure 63) by combining the S5 azido-PEG linker with the BRD4 degrader **XIII**. The resulting molecule was linked with anti-HER2 Ab through a copper-free click chemistry reaction. DAR4 HER2-XIV resulted stable in PBS and showed selective degradation of BRD4 only in HER2+ cells, leaving BRD4 levels intact in HER2- cell line. Proof of the effective mechanism of action was obtained through test with proteasome inhibitors, confirming that the degradation was proteasome-dependent.

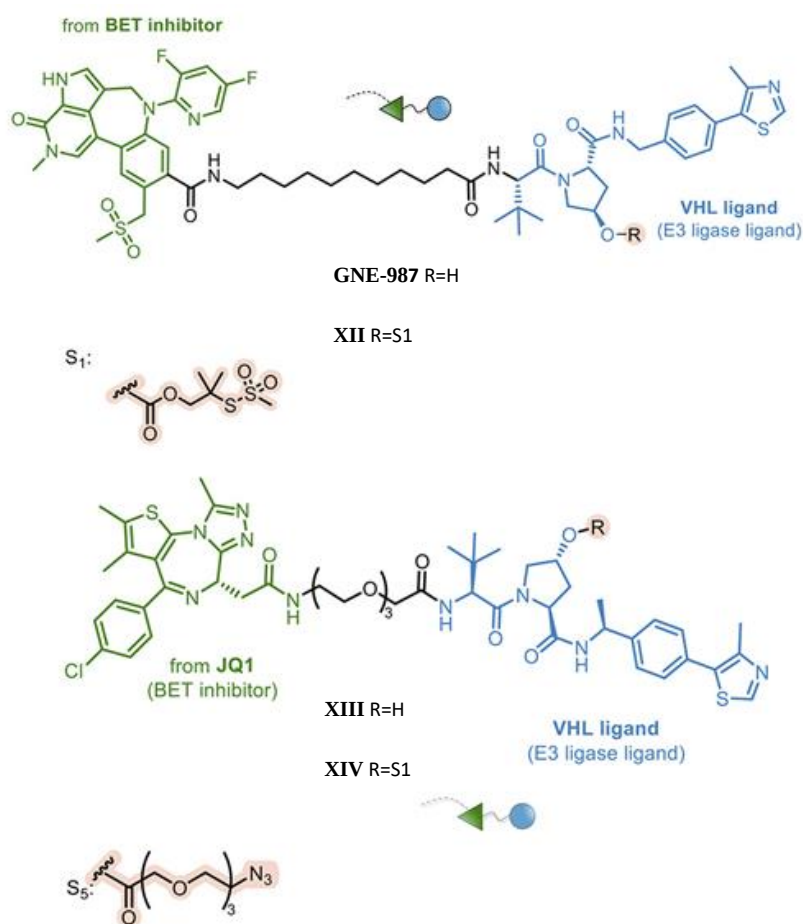


Figure 63. Design of CLL1-XII and HER-XIV.

As an evolution of the Ab-PROTAC conjugate concept for the treatment of breast cancer, compound **XIII** was encapsulated into Ab conjugate nanoparticles (ACNPs).²⁷⁰ The authors selected poly-lactic acid and polyethylenimine as building blocks for the preparation of the nanoparticles, which were loaded with **XIII** and conjugated with the anti-HER2 Ab trastuzumab via a covalent bond. The resulting ACNPs (XIII-ACNP, Figure 64) displayed an improved anti-tumoral efficacy in HER2+ overexpressing breast cancer cell lines when compared to **XIII** and exhibited high stability and controlled release over time. Mechanism of action of **XIII** was not modified by conjugation and did not increase toxicity and side effects. Importantly, XIII-ACNP displayed a strong cytotoxic effect in trastuzumab- and XIII-resistant HCC1954 cell lines, overcoming the resistance developed to this degrader. Despite good properties and therapeutical benefits, Ab-PROTACs present some issues, like relatively high tendency to aggregate, immunogenicity and instability in systemic circulation, which drastically limit their possible application in clinical therapy.²⁷¹

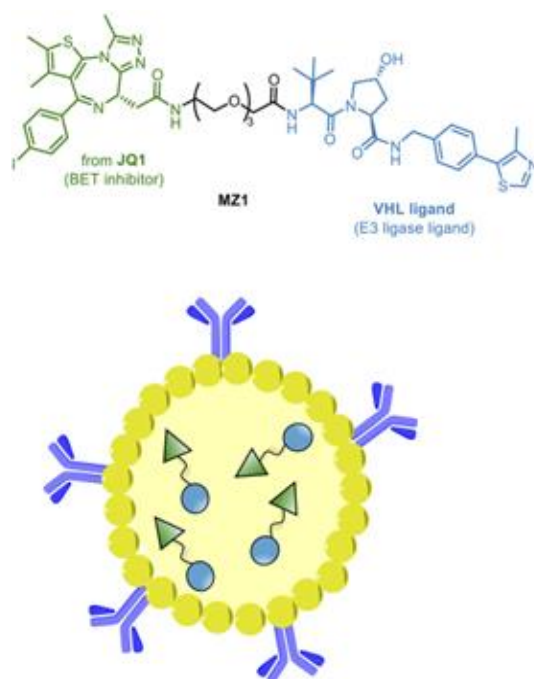


Figure 64. Design of XIII-ACNP.

- small molecules-PROTAC conjugates (SMDCs): considering Ab-PROTACs critical aspects, SMDCs can represent a valid alternative. Compared to Abs, small molecules have better pharmacokinetic profile and no immunogenicity. Moreover, they are easily obtainable by synthesis in relatively cheap way.²⁷² Small molecules bind to specific receptors overexpressed in the diseased tissue or are sensitive to specific tissue conditions, like hypoxia. First approaches to this technology regarded vitamin B12, transferrin and folate and their respective receptors.²⁷³⁻²⁷⁵ Indeed, recently it was investigated the insertion of folate group on a PROTAC.^{276, 277} The rationale behind this is the high expression of folate α receptor (FOLR1) in cancer cells, while it is absent in normal cell. Notably, FOLR1-targeted drugs are in advanced phase clinical trials.²⁷⁸ Folate group was used as cage group in some E3 ligase masked PROTACs, to mask its activity until hydrolysis occur. Jin and colleagues inserted folate group through ester bond to VHL ligase hydroxyl of ARV-771, a BRD4-directed PROTAC (Figure 65). The resulting product was tested in three cancer cell lines overexpressing FOLR1 (HeLa, OVCAR-8, and BRCA cells) and three noncancerous cell lines (HFF-1, HK2, and 3T3). Both folate-ARV-771 and ARV-771 managed to degrade BRD4 in cancer cell, while the uncaged PROTAC ARV-771 was more efficient in degradation in normal cells not expressing FOLR1.²⁷⁶ The same group developed a folate-caged PROTAC FA-S2-MS4048, based on MS4048, a PROTAC directed towards anaplastic lymphoma kinase (ALK) in cancer cells. 5 The folate group was inserted on the glutarimide

core of pomalidomide via a self-immolative linker, initially hindering the interaction with the E3 ligase. The presence of a disulfide bond, which is cleaved by the intracellular glutathione (GSH), triggers the release of the active PROTAC, which was shown to degrade ALK fusion proteins effectively and selectively in FOLR1-expressing cells, and in a FOLR1-, CRBN-, and proteasome-dependent manner. However, the authors pointed out how the conjugation raised MW over 1000 Da, potentially compromising the pharmacokinetic properties.²⁷⁷

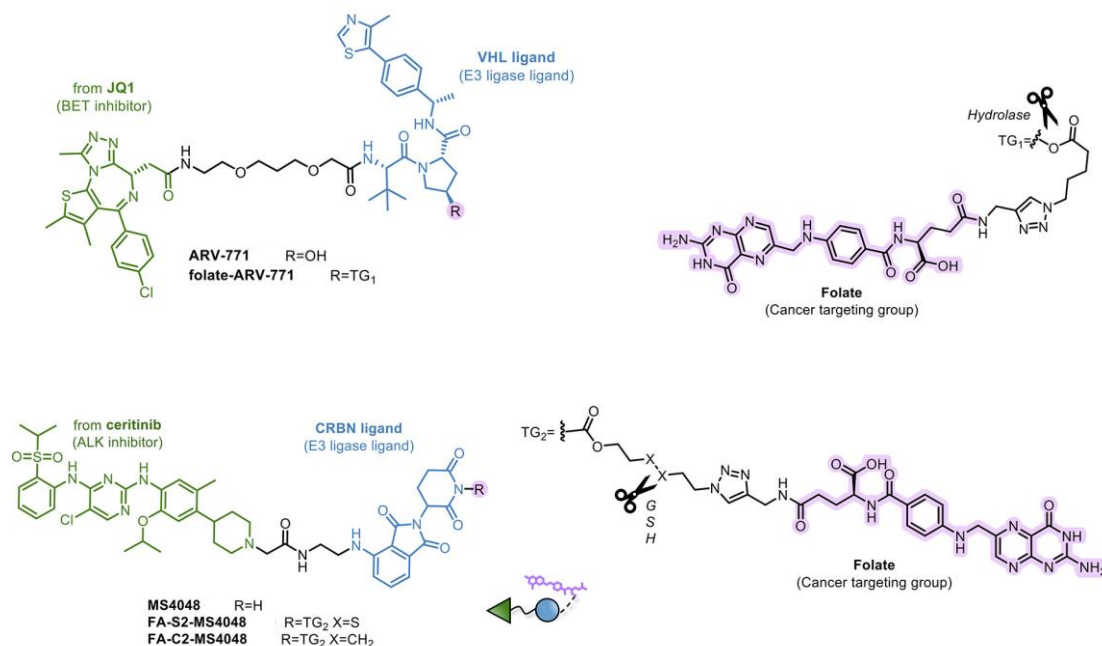


Figure 65. Design of folate-ARV-771 and FA-C2-MS4048.

Another strategy relies on cage groups which respond to specific condition. One of these are groups reacting to nitroreductase (NTR), an enzyme expressed in hypoxic cancer tissue. Nitroaromatic groups can be selectively reduced by NTRs to hydroxylamine and release the active drug after intramolecular rearrangement. In 2021, Tian and co-workers applied a hypoxia-activated prodrug strategy by inserting nitroaromatic groups into PROTAC structures. Starting from an EGFR-directed degrader, they developed two PROTAC prodrugs which were not able to recognize EGFR due to the steric hindrance (Figure 66). Once in hypoxic tissue, the active PROTAC was released by bioreduction of the nitroaromatic groups, being free to exert its activity.²⁷⁹

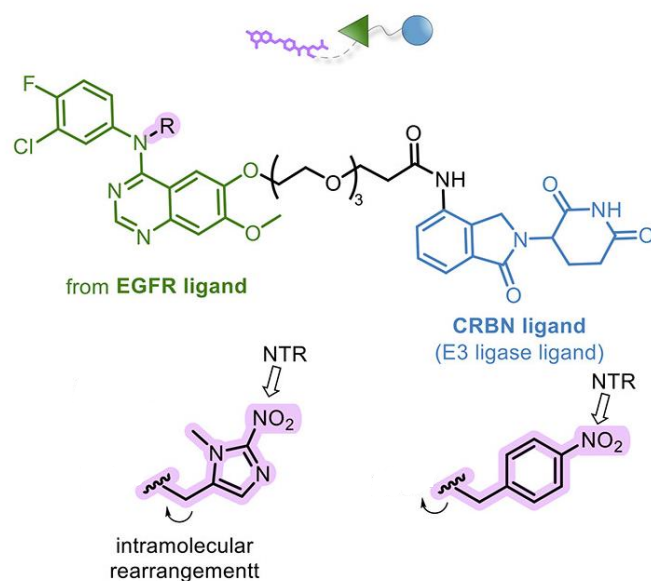


Figure 66. Design of nitroaromatic-cage-PROTACs.

Considering these examples, it should be noted that they have not been validated *in vivo*, while their proof-of-concept is usually restricted to a cellular setting. Therefore, further translational studies are needed to confirm these small molecule-PROTAC conjugates as therapeutic tools able to improve PROTAC specificity, selectivity, and potency, while decreasing toxicity.

3.8 Drug design

Despite being the predominant technology when it comes to drug design, small-molecules drugs present some critical aspects which, sometimes, limits their functionality. Those can be dose-dependent, like a narrow therapeutic window which can lead to toxic effects or poor pharmacologic effects, or structure-related, like inadequate pharmacokinetic profile or poor BBB penetrating effects. In order to contrast these problems, novel technologies were developed, often bypassing the classic small-molecule modulators paradigm. Among them, PROTACs gained several attentions for their promising characteristics, like catalytic activity and innovative mechanism of action, which manifests through target degradation rather than inhibition. Aim of this project is the design and synthesis of a new GSK-3 β -directed PROTACs.

Last year, two research groups independently reported about the first two PROTACs targeting GSK-3 β (Figure 67).^{280, 281} These two PROTACs turned out to be able to induce GSK-3 β degradation *in cell* and one of them is also effective in a mouse model of AD. In the work of Qu and co-workers,²⁸⁰ it was reported the synthesis of PT-65, a PROTAC which has GSK-3 β inhibitor AZD2858 as ligand

targeting the POI and pomalidomide as E3 ligase recruitment moiety. The resulting product was able to bind GSK-3 β with high affinity (K_D = 12.41 nM) and reduce enzymatic levels by 90% at 1 μ M when tested in SH-SY5Y cells. Moreover, it ameliorated learning and memory impairment in mice model of AD. Jiang et al. synthesized a series of PROTAC with substituted thiazole-acetylaminopyridine **XV** as GSK-3 β binding ligand and pomalidomide as E3 ligase recruitment moiety.²⁸¹ The most promising product **XVI** inhibited GSK-3 β in nanomolar concentration range (IC_{50} = 83.1 nM) and managed to reduce its levels by 44% at 2.6 μ M.

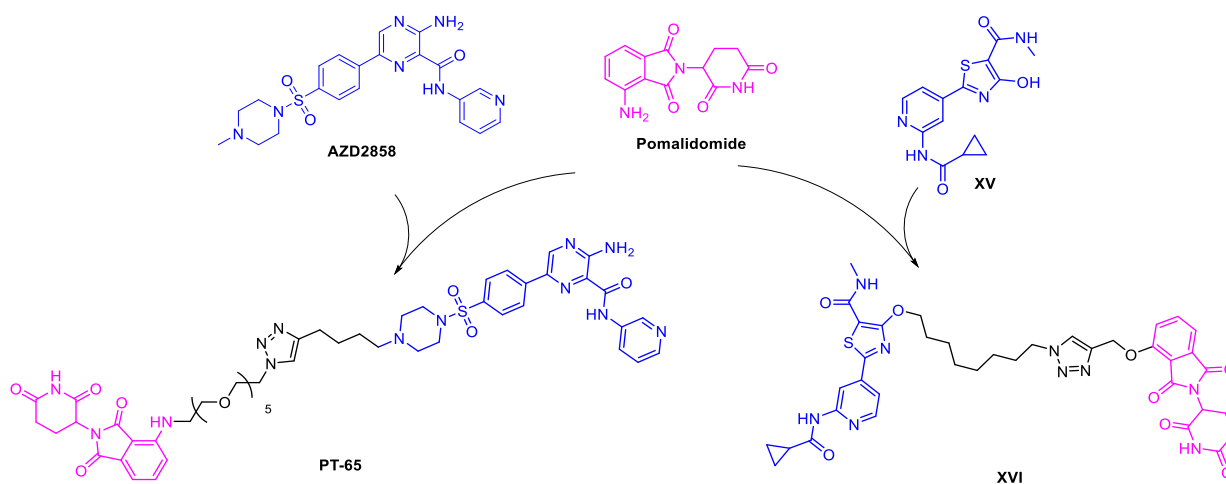


Figure 67. Design of GSK-3 β directed PROTAC PT-65 and 2.

Given the availability of an arsenal of GSK-3 β inhibitors that can be potentially explored to design new PROTACs and due to our interest in GSK-3 β as potential therapeutic target for chronic-degenerative diseases, we sought to develop two new examples of PROTACs characterized by different and previously non-reported GSK-3 β recruiting elements. Indeed, we were interested to evaluate whether other classes of GSK-3 β inhibitors may be used as recruiting elements and if any difference could be observed between a reversible and irreversible GSK-3 β engagement. Considering this insight, herein we report about the design, synthesis and preliminary biochemical evaluation of these novel GSK-3 β -directed PROTACs (Figure 68).

To develop such PROTACs we focused our attention on compounds SB-216763 and Tideglusib as GSK-3 β recruiting elements (Figure 68). We selected these two compounds because they are characterized by a different mechanism of inhibition. Indeed, SB-216763 is the prototype of

reversible maleimide-based ATP-competitive inhibitors able to block GSK-3 β with high selectivity,¹²⁷ while Tideglusib acts with non ATP-competitive mechanism, binding irreversibly Cys-199 in a site different from ATP binding pocket.¹³⁵ Analyzing the structure activity relationships regarding SB-216763 it turned out that the indolyl nitrogen may be substituted with groups bulkier than the methyl one without significant activity reduction.¹²⁰ Similarly, we observed that a chain may be introduced at the para-position of the phenyl group of Tideglusib, by means of amide-bond connection, without significant loss of the inhibitory activity (unpublished results). Therefore, we identified these two positions, i.e. the indolyl nitrogen of SB-216763 and the para-position of the phenyl ring of Tideglusib, as the tethering site to the E3 ligase recruiting element. The highly linear and flexible 3-4-3 polyethylene glycol and the pomalidomide scaffold were selected as linker and E3 ligase respectively to obtain compounds **1** and **3** (Figure 68). Lastly, we anticipated that due to the observed results, we designed and synthesized also the **1**-analogue, compound **2**, characterized by a lower PEG-homologue as linker (Figure 68).

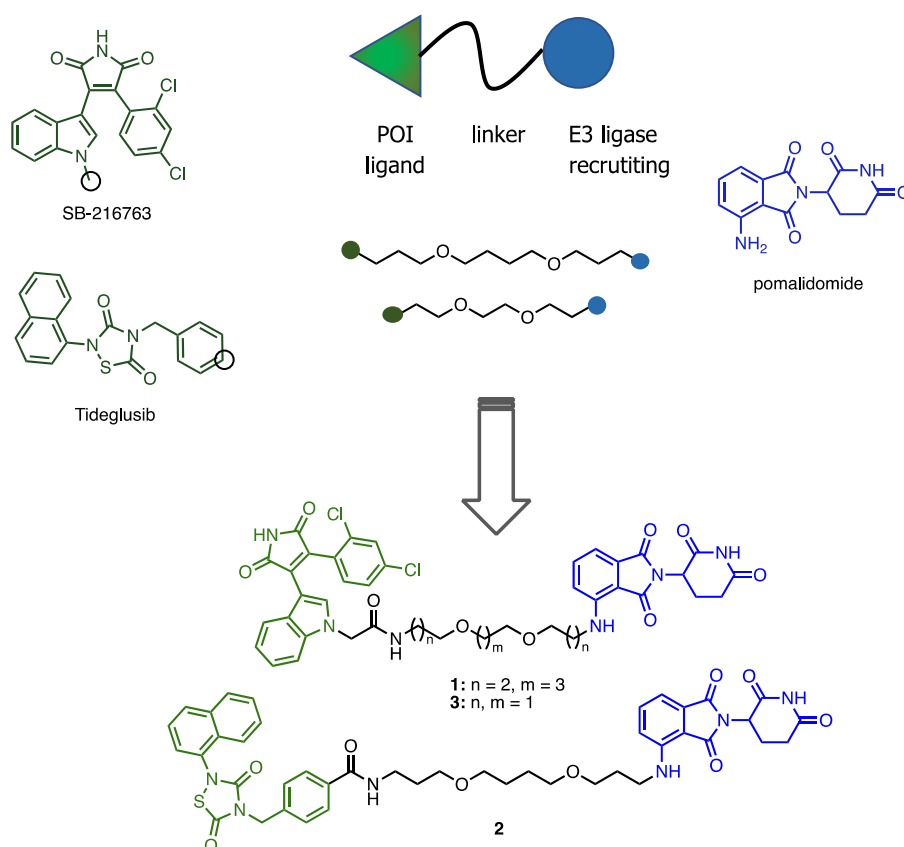
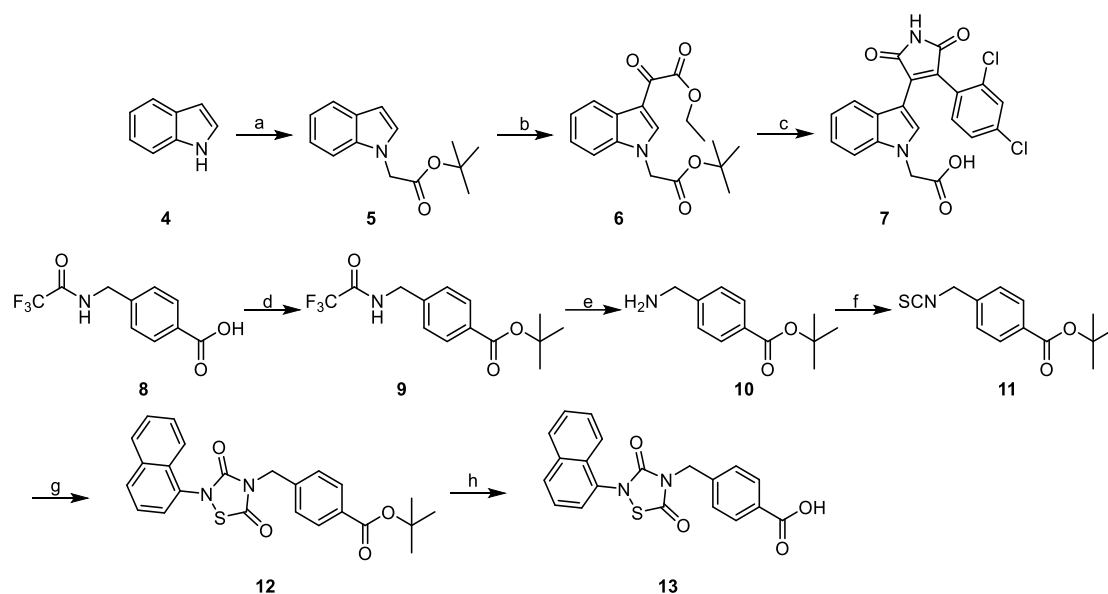


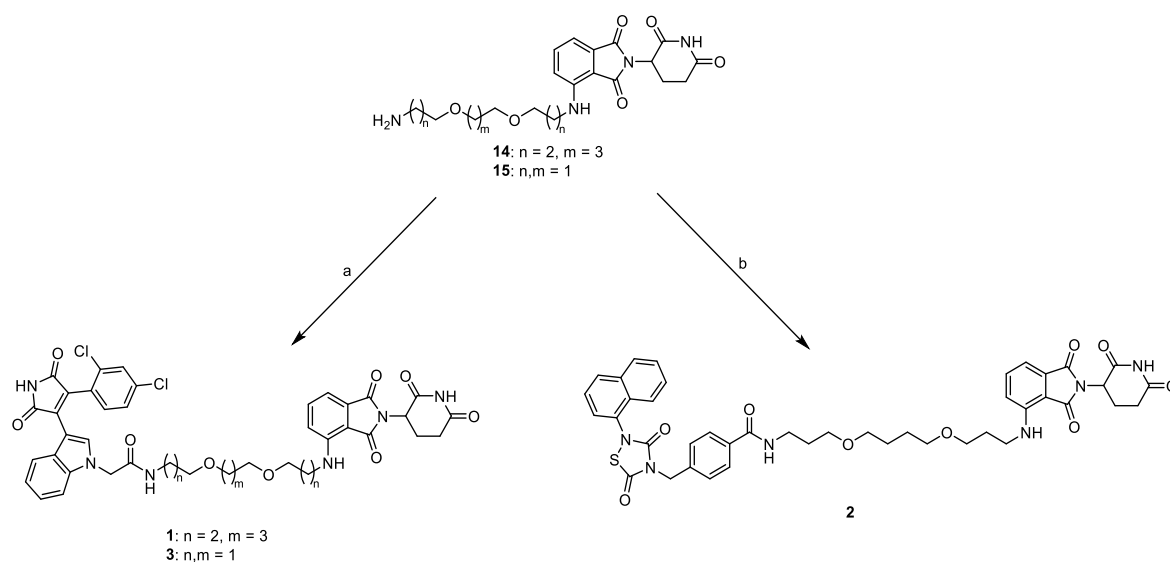
Figure 68. Design of compounds **1-3**.

3.9 Chemistry section

Target compounds have been synthesized following a sequence reported in Scheme 4 and 5. The sequence commenced with the synthesis of fragments **7** and **13** (Scheme 4). In particular, for the synthesis of SB-216763-like fragment **7** we started from commercially available indole **4**, which was alkylated with bromoacetic acid in sodium hydride to give **5** that was treated with ethyl chlorooxoacetate to give glyoxalate **6**. Perkin-type condensation between **6** and 2-(2,4-dichlorophenyl)acetamide generated, surprisingly, the desired deprotected maleimide **7**. For the synthesis of Tideglusib-like fragment **13** we started from protected-amino acid **8**, that was protected at the acidic functionality by treatment with tert-butanol to obtain **9**. Basic hydrolysis of the trifluoroacetamide protecting group free primary amino acid **10**, that was transformed in isothiocyanate for treatment with 1,1'-Thiocarbonyldi-2(1H)-pyridone to obtain **11**. The thiadiazolidinone **12** was obtained via a straightforward cyclization/oxidation sequence involving a cyclization between isothiocyanate **11** and commercially available naphthyl isocyanate in presence of sulfuryl chloride and the subsequent oxidation of the obtained product in atmospheric oxygen. The thiadiazolidinone **12** was then deprotected in trifluoroacetic acid to obtain fragment **13**, ready to be coupled via linker to the E3-ligase recruitment element. EDCI/HOBT assisted amide formation between the common intermediate **14**, **15** and the GSK-3 β recruiting elements **7** and **13** lead to the target compound **1-3**.



SCHEME 4. Synthesis of the precursors **7** and **13**. Reagents and conditions. a) tBuOH, K₂CO₃, acetone, reflux, 12h; b) ethyl chlorooxoacetate, diethyl ether, rt, 12h, N₂; c) 2-(2,4-dichlorophenyl)acetamide, KOtBu, DMF, rt, 12h, N₂; d) tBuOH, EDCI, DMAP, THF, rt, 12h; e) K₂CO₃, H₂O/MeOH, rt, 12h; f) 1,1'-thiocarbonyldi-2(1H)-pyridone, DCM, rt, 12h; g) naphthyl isocyanate, sulfonyl dichloride, THF, rt, 12h, N₂ then air, THF, rt, 30 min; h) TFA, DCM, rt, 12h.



SCHEME 5. Synthesis of the target compounds **1-3**. Reagents and conditions. a) **7**, EDCI, HOBT, DIPEA, DMF, rt, 12h; b) **13**, EDCI, HOBT, DIPEA, DMF, rt, 12h.

3.10 Results and discussions

In order to evaluate the impact of introduction of the ligase recruiting element on the inhibitors scaffold, we evaluated the ability of compound **1-3** together with the parent inhibitors SB-216763 and Tideglusib to inhibit GSK-3 β (Table 6).

Table 6. Effects of Compounds **1-3**, SB-216763 and Tideglusib on GSK-3 β .

Compound	Linker	GSK-3 β warhead	IC ₅₀ (nM) ^a
1			833 ± 62
2			79.26 ± 19.95
3			457 ± 99
SB-216763			34.4 ^b
Tideglusib			200 ± 15

^a IC₅₀ values are defined as the drug concentration that reduces by 50% the target activity and are reported as a mean value of three or more determinations. ^b data from ref.¹²⁷

Compounds displayed anti-GSK-3 β activity spanning from the micromolar to nanomolar scales and showed a clear influence based on GSK-3 β warhead and linkers. In detail, the structural modification introduced on the maleimide scaffold is highly detrimental for the GSK-3 β inhibitory activity: indeed, the PROTAC **1** and **3** are characterized by IC₅₀ values (**1**: 833 ± 62 nM; **3**: 457 ± 99 nM) one order of magnitude higher than the pure inhibitor SB-21676 (IC₅₀ = 34.41 nM). Interestingly, the length of the linker influences the interaction with GSK-3 β since compound **1** characterized by the 3-4-3 PEG linker is less active than compound **3** characterized by the shorter 2-2-2 linker. Concerning the Tideglusib-based PROTAC **2**, interestingly the introduction of the linker-E3 ligase recruiting moiety induces an increase in the inhibitory activity compared to pure inhibitor Tideglusib (**2**: 79.26 ± 19.95

nM, Tideglusib: 200 ± 15 nM). Overall, the level of target engagement reached by the three PROTACs seems sufficient to engage GSK-3 β and allowed the formation of the ternary GSK-3 β –PROTAC–E3 ligase complex necessary to trigger the process. Indeed, it is well known that different from traditional small molecule inhibitors that need to form strong interactions with their biological counterparts, PROTACs may only require smooth binding to the POI to catalytically induce its degradation.²⁸² To evaluate the cytotoxicity of compounds **1-3**, SB216763 and Tideglusib in neuronal SH-SY5Y cells, it was performed the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) reduction assay after 24 h of treatment with various concentrations of all the compounds (1.25 – 40 μ M). Compounds **1**, **3** and SB216763 showed cytotoxicity at concentration higher than 20 μ M while for compound **2** cytotoxicity was detected at 40 μ M (Figure 69). Tideglusib did not show any cytotoxicity at all the tested concentrations. To select the correct concentration to perform the degradation assay, to confirm the absence of cytotoxicity at the concentration of 10 μ M, the neuronal viability was measured after 48 h of treatment with the studied compounds in SH-SY5Y cells. The treatment with all the studied compounds did not significantly modify the cell viability (data not shown). Based on these results, we selected concentrations up to 10 μ M for all the following experiments.

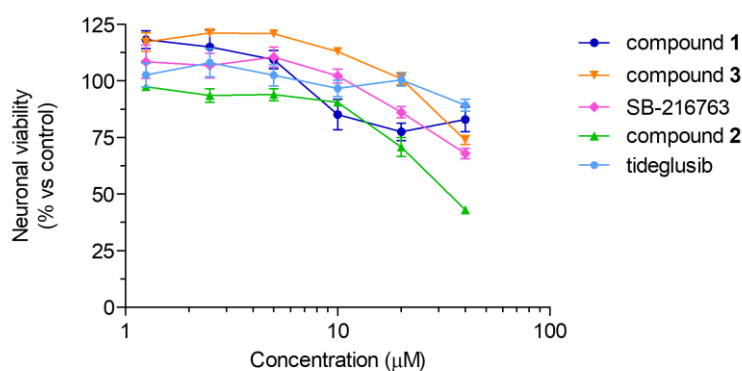


Figure 69. Neurotoxicity of compounds **1-3**, SB216763 and Tideglusib in SH-SY5Y cells. Cells were incubated for 24 h with different concentrations of the studied compounds (1.25 – 40 μ M). At the end of incubation, the cell viability was measured by MTT assay as described in the materials and methods section. Data are reported as mean $\pm$ SEM of three independent experiments.

Then, the ability of compounds **1-3**, SB216763 and Tideglusib to induce the degradation of GSK-3 β , in terms of decrease of total GSK-3 β protein level, was evaluated in SH-SY5Y cells by western blotting. Cells were treated for 48 h with PROTACs **1-3** at the concentration of 10 μ M. As shown in Figure 70, the treatment of 48 h with compounds **2**, SB216763 and Tideglusib did not modify the basal level of GSK-3 β protein. At the same experimental conditions, compounds **1** and **3** significantly decreased the total level of GSK-3 β protein suggesting the specific ability of these two compounds to degrade GSK-3 β protein. In particular, PROTAC **1** showed a higher activity in comparison to compound PROTAC **3** suggesting the ability of this compound to induce specific and reversible mechanisms of degradation. The inability of compound **2** to induce GSK-3 β degradation could be associated to the covalent interaction established by Tideglusib residue with the target, which could hamper the engagement of the catalytic PROTAC mechanism.

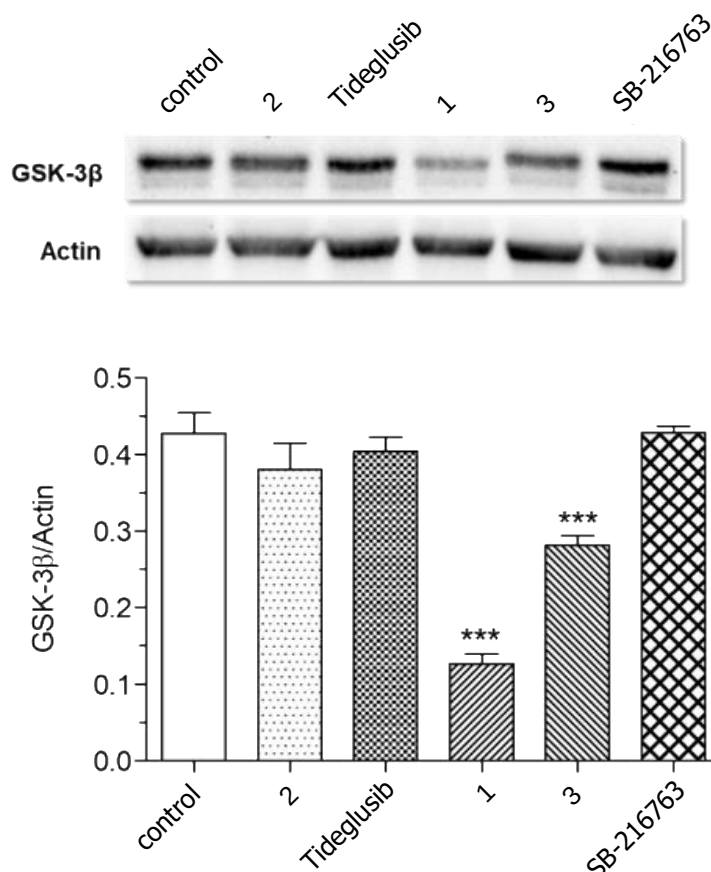


Figure 70. GSK-3 β protein level after 48 h of treatment with compounds **1-3**, SB216763 and Tideglusib in SH-SY5Y cells. Cells were treated for 48 h with the studied compounds at the concentration of 10 μ M. At the end of incubation, GSK-3 β protein level were measured by western blotting. Data are expressed as mean $\pm$ SEM of three independent experiments (***p<0.001 vs untreated cells at one-way ANOVA with Dunnett post-hoc test).

Driven by these evidences, we further investigated the GSK-3 β degradation activity of compound **1** using concentrations lower than 10 μ M in SH-SY5Y cells. In this case cells were treated for 48 h with compound **1** at different concentrations (0.5 – 1 – 5 – 10 μ M) and, at the end of incubation, the total GSK-3 β level were measured by western blotting. As shown in Figure 71, compound **1** significantly decreased GSK-3 β protein level at all the concentrations tested in a dose dependent manner suggesting a high selectivity for GSK-3 β protein.

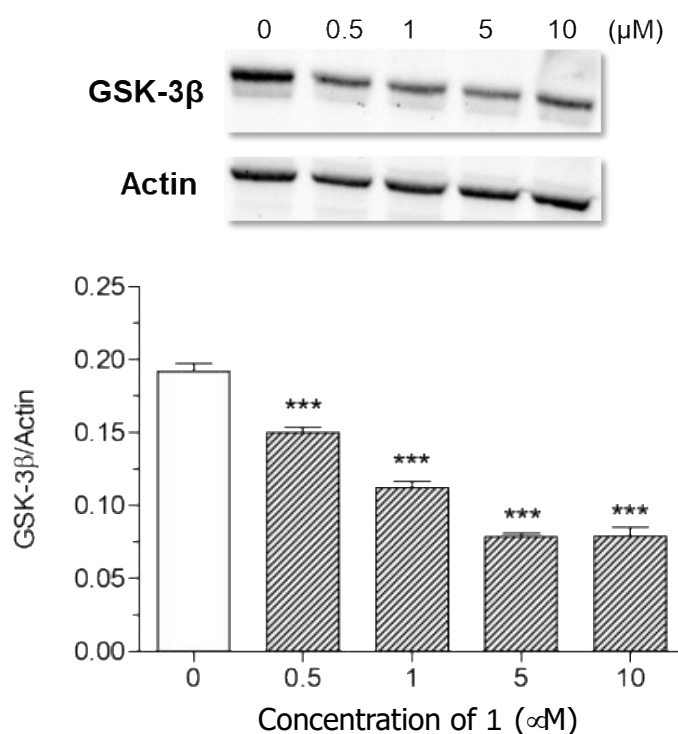


Figure 71. GSK-3 β protein level after 48 h of treatment with different concentrations of compound **1** in SH-SY5Y cells. Cells were treated for 48 h with compound **1** at different concentrations (0.5 – 1 – 5 – 10 μ M). At the end of incubation, GSK-3 β protein level were measured by western blotting. Data are expressed as mean $\pm$ SEM of three independent experiments (***p<0.001 vs untreated cells at one-way ANOVA with Dunnett post-hoc test).

Given those interesting results, the mechanism of action of compound **1** was investigated to confirm the involvement of ubiquitin-proteasome system in the GSK-3 β protein degradation. SH-SY5Y cells were treated for 24 h with compound **1** (10 μ M) in the presence of increased concentration of lactacystin (1.25 – 2.5 – 5 μ M), a potent and selective irreversible 20S proteasome inhibitor. As shown in Figure 72, the treatment with compound **1** in the presence of lactacystin at the concentration of 5 μ M did not have effects on the total GSK-3 β protein level suggesting that the GSK-3 β degradation induced by compound **1** involves the ubiquitin-proteasome system. Interestingly, the GSK-3 β degradation induced by compound **1** was directly proportional to the dose of lactacystin used, confirming the involvement of the ubiquitin-proteasome system in the mechanism of degradation.

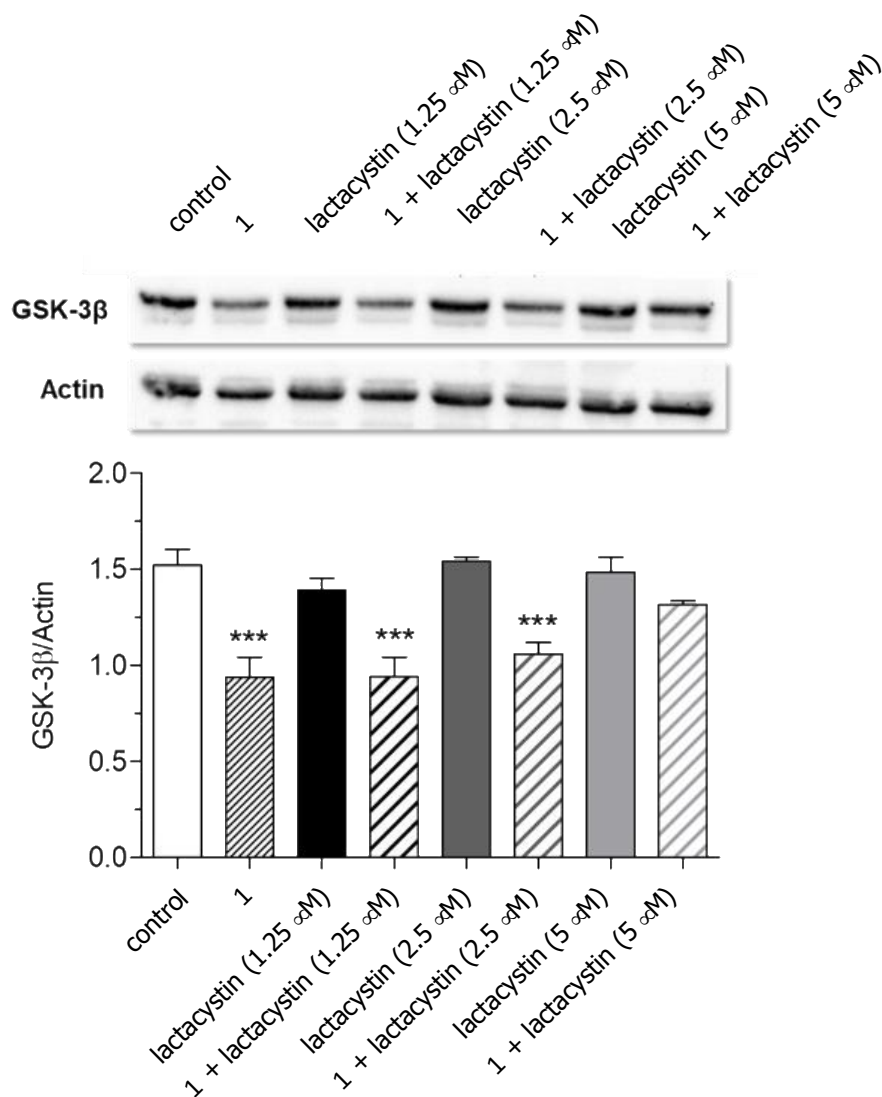


Figure 72. GSK-3β protein level after 24 h of treatment with compound **1** in the presence of different concentrations of lactacystin in SH-SY5Y cells. Cells were treated for 24 h with compound **1** at the concentration of 10 μM in the presence of lactacystin (1.25 – 2.5 – 5 μM). At the end of incubation, GSK-3β protein level were measured by western blotting. Data are expressed as mean ± SEM of three independent experiments (***p<0.001 vs untreated cells at one-way ANOVA with Bonferroni post-hoc test).

Based on the evidence that copper induce neuronal death by exacerbating tau phosphorylation, to assess the neuroprotective effects of compound **1**, SH-SY5Y cells were incubated for 24 h with compound **1** (0.5 – 1 μM) in the presence of copper sulphate (CuSO₄, 150 μM) and the cell viability was measured by MTT assay. As shown in Figure 73a, compound **1** significantly counteracted the neurotoxicity induced by CuSO₄ at 1 μM. In parallel, the neuroprotective activity of compound **1** was

also evaluated against A β ₂₅₋₃₅ peptide, the neurotoxic fragment of A β involved in the neuropathology of AD. SH-SY5Y cells were incubated for 2 h with compound **1** (0.5 – 1 μ M) and further 3 h in the presence of A β ₂₅₋₃₅ peptide (10 μ M). At the end of incubation, the cell viability was evaluated by MTT assay. The treatment with all the tested concentrations of compound **1** significantly reduced the neurotoxicity induced by A β ₂₅₋₃₅ peptide, in a dose dependent manner (Figure 73b).

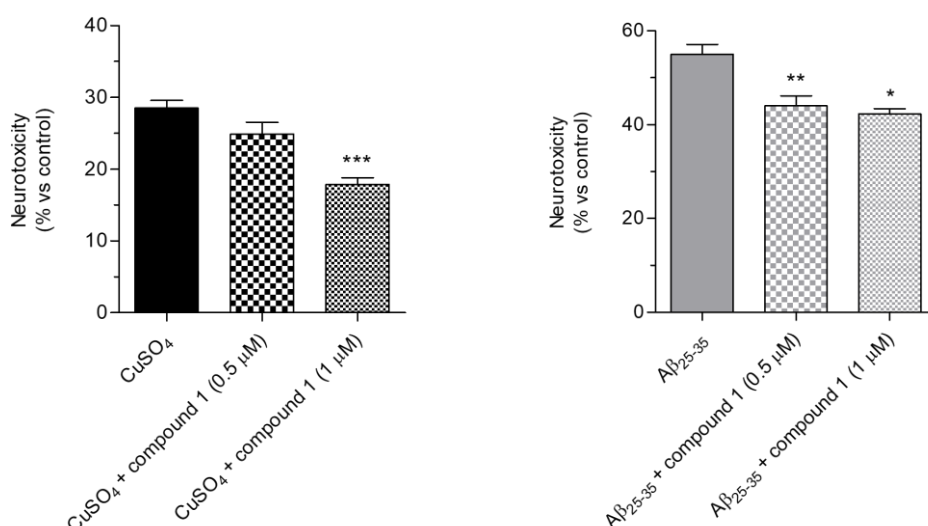


Figure 73. Compound **1** reduced the neurotoxicity induced by CuSO₄ and A β ₂₅₋₃₅ in SH-SY5Y cells. (A) Cells were incubated for 24 h with compound **1** in the presence of CuSO₄; (B) cells were incubated for 2 h with compound **1** and further 3 h in the presence of A β ₂₅₋₃₅. At the end of incubation, the cell viability was measured by MTT as-say as described in the materials and methods section. Data are re-reported as mean $\pm$ SEM of three independent experiments (***p<0.001 vs cells treated with CuSO₄; *p<0.05 and **p<0.01 vs cells treated with A β ₂₅₋₃₅ at one-way ANOVA with Dunnett post-hoc test).

GSK-3 β and other kinases present a β -hairpin loop (residues 35–41) in the N-lobe that connects the β -strands 3 and 4. In an CK-1 α X-ray ternary complex with cereblon (CRBN) and lenalidomide, inducing kinase degradation (PDB ID: 5fqd)²⁸³, CK-1 α was found to bind the carbon terminal domain (CTD) of CRBN via this loop. We, thus, supposed that the β -hairpin loop could play a similar role also for GSK-3 β . CK-1 α and GSK-3 β kinases present a low sequence identity (25%) but a high 3D structure similarity, in particular for the key β -hairpin loop, for which both structures share the glycine

signature (Gly79 for GSK-3 β and Gly40 for CK-1 α), suggested by several studies to be at the basis of the degradation mechanism of different CRBN substrates (e.g. kinases, CK-1 α , E3 ubiquitin-protein ligase ZFP91 and GSTP1 or eukaryotic peptide chain release factor GTP-binding subunit ERF3A).^{283, 284} The **1**-GSK-3 β and **3**-GSK-3 β complexes obtained by docking simulations were submitted to standard MD simulations to verify and compare the ligand behavior, according to the different linker length. As shown in Figure 74, the kinase inhibitor is quite stable within the kinase binding site for both compounds **1** and **3**. In particular, a well conserved bidentate H-bond is formed by the ligand core with Val135 and Asp133, and hydrophobic interactions are made by the ligand core with Ile62, Val70, Lys85, Val110, Leu188 and Cys199. The linker gains additional contacts with Tyr134 for compound **1** and with Pro136 and Arg141 for compound **3** (Figure 74). Moreover, it is possible to highlight a higher propensity for compound **1**, because of the longer linker, to orient the pomalidomide moiety towards the kinase β -hairpin loop, with respect to compound **3**. Compound **1** docking pose seems to be stabilized in MD simulations by pomalidomide H-bonds with Lys74 and with Gly79 backbone (Figure 74 left panel). In contrast, compound **3** shorter linker does not allow the pomalidomide moiety to reach the kinase interacting loop, remaining for most of the simulation trapped in a cavity made up by Arg141, His145 and Arg148 (Figure 74 right panel). This behavior was confirmed by measuring the distance between the kinase loop key residues and pomalidomide. Indeed, only compound **1** pomalidomide NH (and not compound **1**'s one) resulted stably H-bonded to Gly79 carbonyl in both replicas, reaching a N---O minimum distance of 2.6 Å.

Altogether, these results could support the compound **1** higher degradation activity observed from *in vitro* experiments.

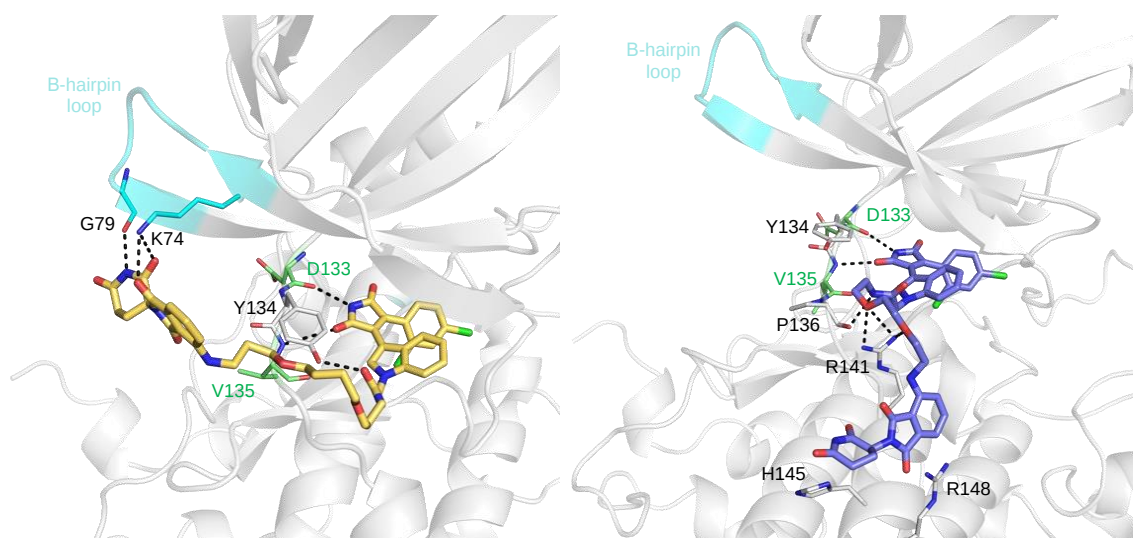


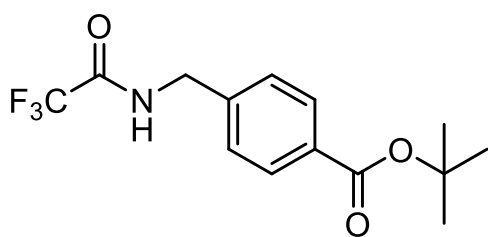
Figure 74. Computational analysis of the **1**-GSK-3 β (left) and **3**-GSK-3 β (right) complexes (PDB ID: 1q4l).

3.11 Conclusions

Concluding, we applied the PROTAC technology to the development of GSK-3 β degraders based on the structure of two mechanistic different GSK-3 β inhibitors, SB-216763 and Tideglusib. The obtained compound **1-3**, which employs pomalidomide as CRBN E3 ligase targeting element, showed a good level of target engagement (IC_{50} in the nanomolar range of concentration). Compound **1**, characterized by the SB-216763 recruiting moiety and the 3-4-3 PEG linker, is not toxic in SH-SY5Y cells up to 20 μ M and is the most potent degrader of the series inducing significant SB-216763 degradation already at 0.5 μ M and in a dose-dependent manner. Moreover, we demonstrated that the GSK-3 β degradation is mediated by the E3 ubiquitin ligase component CRBN. Compound **1** is able to counteract in SH-SY5Y cells toxic insults induced by Cu^{2+} and $A\beta_{25-35}$ at the low concentration of 1 and 0.5 μ M, respectively. Computational analysis revealed that the minor activity of the lower **1**-analogue **3** is due to the nature of the linker and the role it plays in the formation of the ternary complex. On the other hand, the absence of degradation ability demonstrated by compound **2** could be ascribed to the irreversible interaction that Tideglusib established with GSK-3 β . Above all, these results demonstrated that SB-216763-based PROTACs are potent GSK-3 β degraders.

3.12 Experimental section

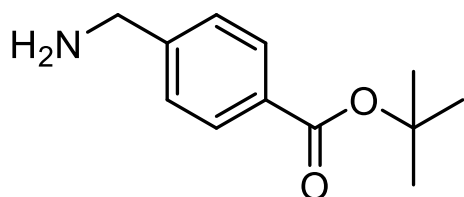
Chemistry



tert-butyl 4-((2,2,2-trifluoroacetamido)methyl)benzoate (9).

To a solution of **8** (1 g, 4.0 mmol) in anhydrous THF under stream of nitrogen, DMAP (0.49 g, 4.0 mmol) and tert-Butanol (10 ml) were added at 0 °C. Shortly after EDCI (2.31 g, 12.0 mmol) and the reaction was allowed to reach rt

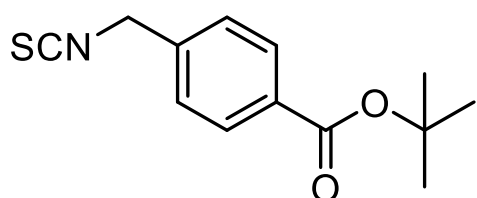
and stirred overnight. Solvents were removed in vacuo and the resulting residue was taken up with DCM and washed with HCl aq 1N, water and brines. Organic phase was dried over Na₂SO₄ and concentrated to give **9** (0.960 g, 77% yield) as yellow oil. ¹H-NMR (400 MHz, CDCl₃) δ 7.94-7.91 (dd, 2H, J = 8.4, 2.0 Hz), 7.31-7.27 (dd, 2H, J = 8.0, 0.4 Hz), 4.54 (d, 2H, J = 6.4 Hz), 1.57 (s, 9H); ¹³C-NMR (100 MHz, CDCl₃) δ 165.5, 157.0-158.2 (q, J = 37.4, 37.4 Hz), 140.7, 130.0, 129.9, 127.6, 111.6-121.2 (q, J = 285.6, 276.9 Hz), 81.4, 43.4, 28.1; ESI-MS = 304 (M+H)+.



tert-butyl 4-(aminomethyl)benzoate (10).

To a solution of **9** (0.885 g, 2.9 mmol) in a mixture of methanol / water (30 ml, 17:1 ratio), potassium carbonate (1.61 g, 12 mmol) was added at 0 °C and the reaction was allowed to reach rt and stirred

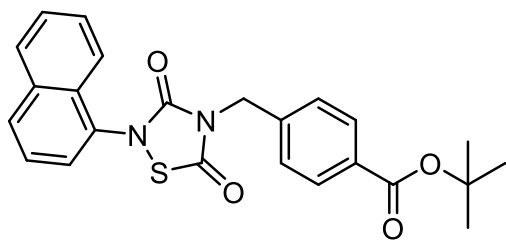
overnight. Methanol was removed in vacuo and the aqueous phase was extract with DCM. Organic phases were collected, dried over Na₂SO₄ and concentrated to give **10** (0.480 g, 79% yield) as yellow oil. ¹H-NMR (400 MHz, CDCl₃) δ 7.88 (d, 2H, J = 8.4 Hz), 7.33 (d, 2H, J = 8.4 Hz), 3.95 (s, 2H), 1.58 (s, 9H); ¹³C-NMR (100 MHz, CDCl₃) δ 165.4, 131.4, 129.8, 127.6, 127.0, 81.1, 43.6, 28.1; ESI-MS = 208 (M+H)+.



tert-butyl 4-(isothiocyanatomethyl)benzoate (11).

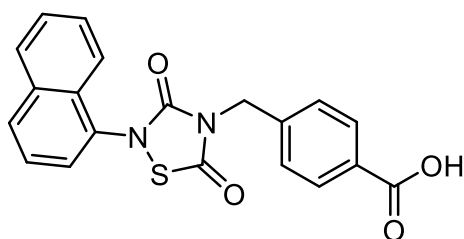
To a solution of **10** (0.480 g, 2.32 mmol) in DCM under stream of nitrogen, 1,1'-Thiocarbonyldi-2(1H)-pyridone (0.533 g, 2.32 mmol) in DCM was added dropwise and the resulting mixture was stirred overnight at rt. The solvent was removed in vacuo

and the resulting residue was purified via flash chromatography using as mobile phase a mixture of petroleum ether / ethyl acetate (9.8:0.2) to give **11** (0.320, 55% yield) as yellow oil. ¹H-NMR (400 MHz, CDCl₃) δ 8.01 (d, 2H, J = 8.0 Hz), 7.36 (d, 2H, J = 8.0 Hz), 4.77 (s, 2H), 1.59 (s, 9H); ¹³C-NMR (100 MHz, CDCl₃) δ 165.1, 138.5, 132.1, 130.1, 126.5, 81.3, 48.4, 28.1; ESI-MS = 250 (M+H)+.



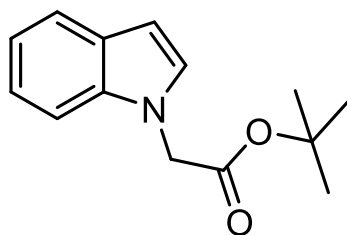
tert-butyl 4-((2-(naphthalen-1-yl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoate (12).

A stirred solution of **11** (0.5 g, 2 mmol) and 1-naphthyl isocyanate (0.34 ml, 0.372 g, 2 mmol) under nitrogen in THF was cooled to 0 °C. Sulfuryl chloride in 1M solution of DCM (4 ml) was added slowly and the mixture was allowed to warm to room temperature and stirred overnight. The reaction was then opened to the air and stirred for 30 minutes before the solvent was removed under vacuo and the obtained residue was purified via flash chromatography using as mobile phase a mixture of petroleum ether / ethyl acetate (9.5:0.5 to 9:1) to give **12** (0.5 g, 57% yield) as yellow oil. ¹H NMR (400 MHz, DMSO) δ 8.09 (t, J = 8.8 Hz, 2H), 7.97 – 7.90 (m, 3H), 7.80 (dd, J = 7.4, 1.2 Hz, 1H), 7.69 – 7.61 (m, 3H), 7.53 (d, J = 8.4 Hz, 2H), 4.97 (s, 2H), 1.55 (s, 9H). ESI-MS = 436 (M+H)+.



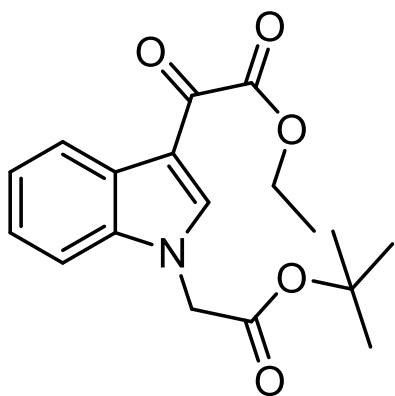
4-((2-(naphthalen-1-yl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzoic acid (13).

In a stirred solution of **12** (0.50 g, 1.15 mmol) in anhydrous DCM at 0°C under stream of nitrogen, trifluoroacetic acid was added (2 ml). The reaction was allowed to reach rt and stirred overnight. The reaction mixture was washed with water and the organic phase was dried over Na₂SO₄ and concentrated to give **13** (0.459 g, 81% yield) as yellow oil. ¹H-NMR (400 MHz, CDCl₃) δ 8.11-8.07 (m, 2H), 7.98 (d, 2H, J = 6.4 Hz), 7.94-7.92 (m, 1H), 7.82-7.80 (m, 1H), 7.67-7.61 (m, 3H), 7.53 (d, 2H, J = 8.8 Hz), 4.97 (s, 2H); ESI-MS = 377 (M-H)⁻.



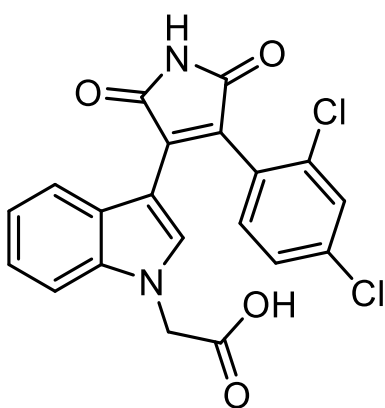
tert-butyl 2-(1H-indol-1-yl)acetate (5).

To a solution of indole **4** (2.00 g, 17.00 mmol) in acetone potassium carbonate (4.71 g, 34.18 mmol) and tert-Butyl bromoacetate (5.00 g, 25.6 mmol) were added and the reaction was refluxed for 12h. The solvent was removed under vacuo and the residue was taken up with ethyl acetate and washed with sodium bicarbonate, brine. The organic phase was dried over Na₂SO₄ and concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of petroleum ether / ethyl acetate (9.5:0.5) to give **5** (1.95 g, 50% yield) as yellow oil. ¹H-NMR (400 MHz, DMSO) δ 7.55-7.53 (m, 1H), 7.34-7.30 (m, 2H), 7.14-7.10 (m, 1H), 7.05-7.00 (m, 1H), 6.43 (dd, 1H, J = 3.2, 0.8 Hz), 4.99 (s, 2H), 1.42 (s, 9H); ¹³C-NMR (100 MHz, CDCl₃) δ 168.5, 136.8, 130.1, 128.5, 121.6, 120.7, 119.6, 110.1, 101.4, 81.8, 48.1, 28.1; ESI-MS = 232 (M+H)+.



ethyl 2-(1-(2-(tert-butoxy)-2-oxoethyl)-1H-indol-3-yl)-2-oxoacetate (6). To a solution of **5** (1.00 g, 4.33 mmol) in diethyl ether at 0°C and under a stream of nitrogen, ethyl chlorooxoacetate (2.40 ml, 2.94 g, 21.65 mmol) was added over 15 minutes and the reaction was allowed to reach rt and stirred for 2h. The reaction was filtered and the solid was washed with diethyl ether and water to give **6** (0.60 g, 42% yield). ¹H-NMR (400 MHz, CDCl₃) δ 7.58-7.50 (m, 1H), 7.38-7.32 (m, 2H), 7.11-7.16 (m, 1H), 6.99-7.08 (m, 1H),

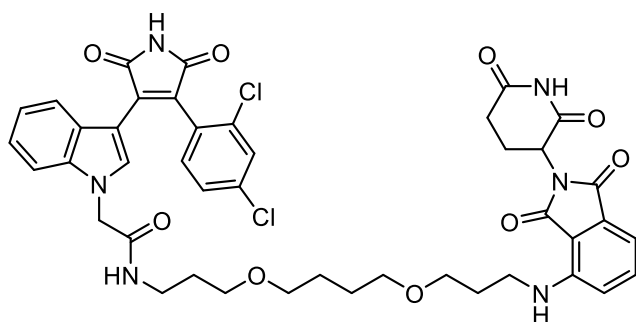
6.47 (dd, 1H, J = 3.2, 0.8 Hz), 5.03 (s, 2H), 4.31 (q, 2H, J = 7.0 Hz), 1.51 (t, 3H, J = 7.1 Hz), 1.42 (s, 9H).



2-(3-(4-(2,4-dichlorophenyl)-2,5-dioxopyrrolidin-3-yl)-1H-indol-1-yl)acetic acid (7). To a solution of **6** (0.535 g, 1.60 mmol) and 2-(2,4-dichlorophenyl)acetamide (0.363 g, 1.78 mmol) in THF at 0°C and under a stream of nitrogen, potassium tert-butoxide (0.448 g, 4.0 mmol) in DMF was added and the mixture was stirred at rt for 4h. HCl 1N was added and the mixture was extracted with ethyl acetate and washed with brine. The organic phase was dried over Na₂SO₄ and concentrated to give **7** as free acid (0.257 g, 39%).

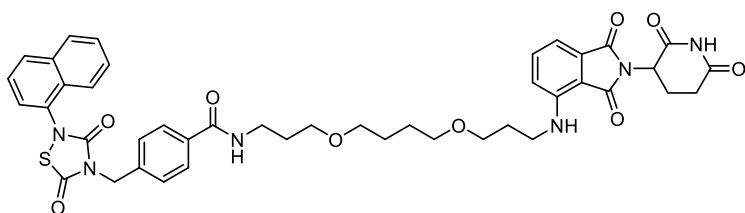
¹H-NMR (400 MHz, CDCl₃) δ 8.10 (s, 1H), 7.70 (d, 1H, J = 2.4 Hz), 7.50-7.28 (m, 3H), 7.04 (t, 1H, J = 7.2 Hz), 6.71 (t, 1H, J = 7.6 Hz), 6.29 (d, 1H, J = 8.0 Hz), 4.75 (s, 2H).

General procedure for the synthesis of 1-3. To the corresponding acid **7**, **13** and amine **14**, **15** in DMF, DIPEA, EDCI and HOBT were added at 0°C and under a stream of nitrogen. Then the mixture was allowed to warm to room temperature and stirred overnight. Ethyl acetate and water were added, and the organic phase was washed with brine, dried over Na₂SO₄ and concentrated to give a residue that was purified via flash chromatography using as mobile phase a mixture of petroleum ether / ethyl acetate to give the corresponding PROTACs **1-3** as yellow oil.



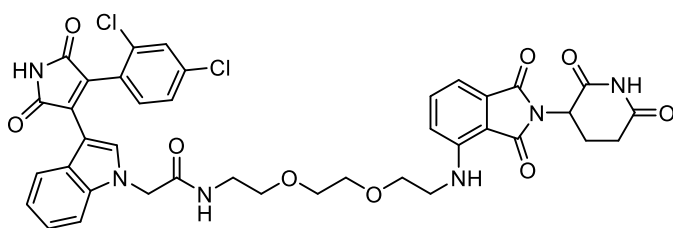
2-(3-(4-(2,4-dichlorophenyl)-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-1H-indol-1-yl)-N-(3-(4-(3-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)propoxy)butoxy)propyl)acetamide (1). Synthesized from **7** (0.095 g, 0.23 mmol), **14** (0.155 g, 0.23 mmol), DIPEA (0.12 ml, 0.089 g,

0.69 mmol), EDCI (0.087 g, 0.46 mmol), HOBT (0.031 g, 0.23 mmol). Obtained 0.074 g of yellow oil (32% yield). $^1\text{H-NMR}$ (400 MHz, DMSO) δ 11.19 (s, 2H), 11.08 (s, 1H), 8.27 (t, 1H, $J = 5.6$ Hz), 8.12 (s, 1H), 7.72 (d, 1H, $J = 2.0$ Hz), 7.58-7.52 (m, 2H), 7.48-7.46 (dd, 1H, $J = 8.4, 2.0$ Hz), 7.41-7.35 (m, 2H), 7.12-7.06 (m, 2H), 7.02-7.00 (d, 1H, $J = 6.8$ Hz), 6.77 (t, 1H, $J = 7.6$ Hz), 6.65 (t, 1H, $J = 6.0$ Hz), 6.38-6.36 (d, 1H, $J = 8.0$ Hz), 5.06-5.02 (dd, 1H, $J = 12.8, 5.6$ Hz), 4.94 (s, 2H), 3.45-3.42 (m, 1H), 3.35-3.29 (m, 11H), 3.14-2.92 (m, 2H), 2.03-1.99 (m, 1H), 1.82-1.76 (m, 2H), 1.67-1.61 (m, 2H), 1.53-1.48 (m, 4H); $^{13}\text{H-NMR}$ (100 MHz, DMSO) δ 172.81, 171.78, 171.14, 170.09, 168.83, 167.32, 166.40, 146.41, 136.91, 136.24, 135.80, 135.22, 134.65, 134.35, 133.53, 132.21, 129.75, 129.06, 127.30, 125.76, 125.17, 122.47, 120.63, 120.02, 117.03, 110.74, 110.38, 109.08, 104.38, 70.03, 69.87, 67.86, 67.46, 49.04, 48.53, 36.09, 30.98, 29.21, 28.88, 26.04, 25.97, 22.15; HRMS (ESI): $\text{C}_{43}\text{H}_{43}\text{Cl}_2\text{N}_6\text{O}_9$ $[\text{M} + \text{H}]^+$: calcd 858.7495, found 858.7485.



N-(4-(3-(3-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)propoxy)propoxy)butyl)-4-((2-(naphthalen-1-yl)-3,5-dioxo-1,2,4-thiadiazolidin-4-yl)methyl)benzamide

(2). Synthesized from **13** (0.112 g, 0.29 mmol), **14** (0.186 g, 0.27 mmol), DIPEA (0.42 ml, 0.313 g, 0.81 mmol), EDCI (0.204 g, 0.54 mmol), HOBT (0.039 g, 0.29 mmol). Obtained 0.110 g of yellow oil (48% yield). $^1\text{H-NMR}$ (400 MHz, DMSO) δ 11.06 (brs, 1H), 8.41 (t, 1H, $J = 8.0$ Hz), 8.09-8.05 (m, 3H), 7.90-7.88 (m, 1H), 7.84-7.82 (d, 2H, $J = 8.0$ Hz), 7.77-7.76 (d, 1H, $J = 4.0$ Hz), 7.63-7.60 (m, 5H), 7.47-7.45 (d, 1H, $J = 8.0$ Hz), 7.07-7.05 (d, 1H, $J = 8.0$ Hz), 7.0-6.98 (d, 1H, $J = 8.0$ Hz), 6.63 (t, 1H, $J = 8.0$ Hz), 5.04-5.00 (dd, 1H, $J = 12.8, 5.6$ Hz), 4.92 (m, 2H), 3.44-3.27 (m, 10H), 2.99-2.81 (m, 2H), 2.01-1.97 (m, 2H), 1.81-1.71 (m, 5H), 1.53-1.52 (m, 5H); $^{13}\text{H-NMR}$ (100 MHz, DMSO) δ 172.79, 170.07, 168.82, 167.30, 166.06, 165.79, 151.94, 146.40, 138.55, 136.23, 134.22, 133.99, 132.20, 131.04, 130.13, 130.08, 128.59, 127.91, 127.63, 127.54, 127.46, 127.22, 126.99, 126.85, 125.80, 122.39, 117.03, 110.36, 109.07, 70.04, 69.88, 67.84, 48.52, 45.34, 36.70, 30.97, 29.41, 28.87, 26.08, 25.98, 22.15; HRMS (ESI): $\text{C}_{43}\text{H}_{45}\text{N}_6\text{O}_9\text{S}$ $[\text{M} + \text{H}]^+$: calcd 821.9269, found 821.9281.



2-(3-(4-(2,4-dichlorophenyl)-2,5-dioxo-2,5-dihydro-1H-pyrrol-3-yl)-1H-indol-1-yl)-N-(2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethyl)acetamide

(3). Synthesized from **7** (0.236 g, 0.97 mmol), **15** (0.394 g, 0.97 mmol), DIPEA (0.32 ml, 0.241 g,

1.87 mmol), EDCI (0.236 g, 1.24 mmol), HOBT (0.084 g, 0.62 mmol). Obtained 0.210 g of yellow oil (25% yield). $^1\text{H-NMR}$ (400 MHz, DMSO) δ 11.12 (m, 2H), 8.39 (t, 1H, $J = 6.0$ Hz), 8.12 (s, 1H), 7.72 (d, 1H, $J = 2.0$ Hz), 7.59-7.56 (m, 2H), 7.48-7.46 (dd, 1H, $J = 8.4, 2.0$ Hz), 7.39-7.35 (m, 2H), 7.15-7.08 (m, 2H), 7.04-7.03 (d, 1H, $J = 4.0$ Hz), 6.76 (t, 1H, $J = 7.6$ Hz), 6.61 (t, 1H, $J = 6.0$ Hz), 6.37-6.35 (d, 1H, $J = 8.0$ Hz), 5.07-5.02 (dd, 1H, $J = 14.0, 6.0$ Hz), 4.96 (s, 2H), 3.63-3.61 (m, 2H), 3.59-3.52 (m, 4H), 3.48-3.44 (m, 4H), 3.28-3.23 (m, 2H), 2.90-2.81 (m, 1H), 2.58-2.51 (m, 2H); $^{13}\text{C-NMR}$ (100 MHz, DMSO) δ 172.79, 170.07, 168.95, 167.27, 166.62, 146.39, 136.90, 136.23, 135.81, 135.20, 134.64, 134.35, 133.53, 132.09, 129.74, 129.05, 127.30, 125.76, 125.16, 122.46, 120.62, 119.99, 117.43, 110.75, 110.69, 109.25, 104.35, 69.70, 69.61, 68.97, 68.89, 54.91, 48.96, 48.55, 41.69, 30.96, 22.12; HRMS (ESI): $\text{C}_{39}\text{H}_{35}\text{Cl}_2\text{N}_6\text{O}_9$ $[\text{M} + \text{H}]^+$: calcd 802.6420, found 802.6404.

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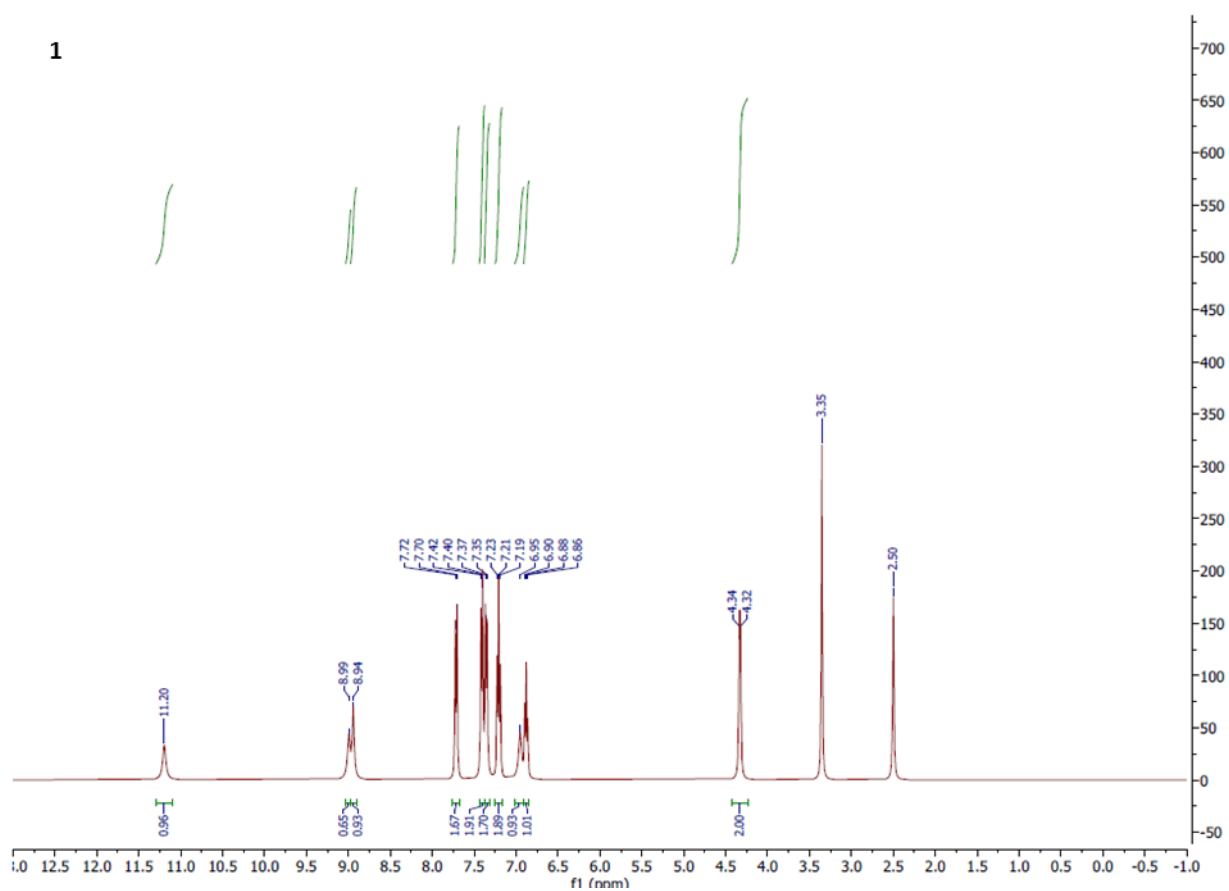
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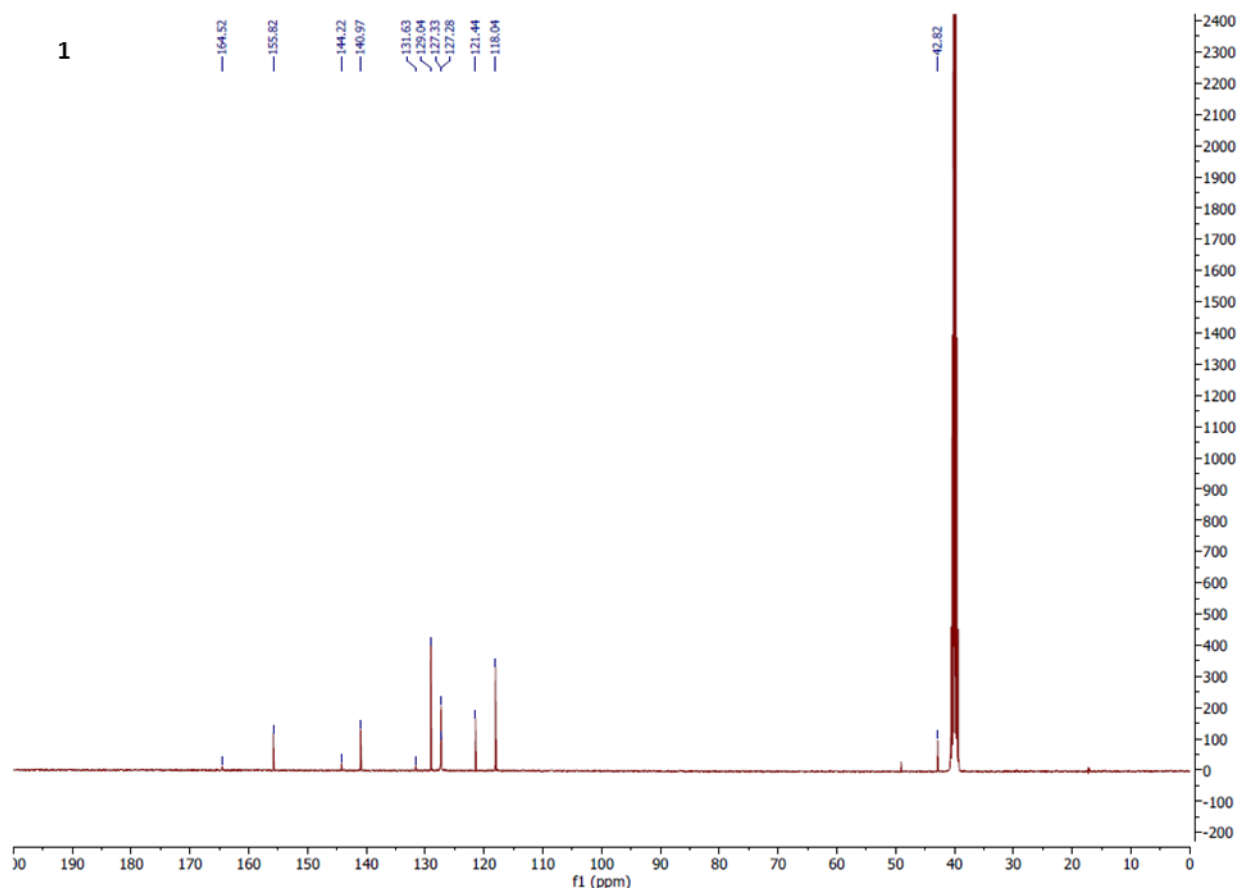
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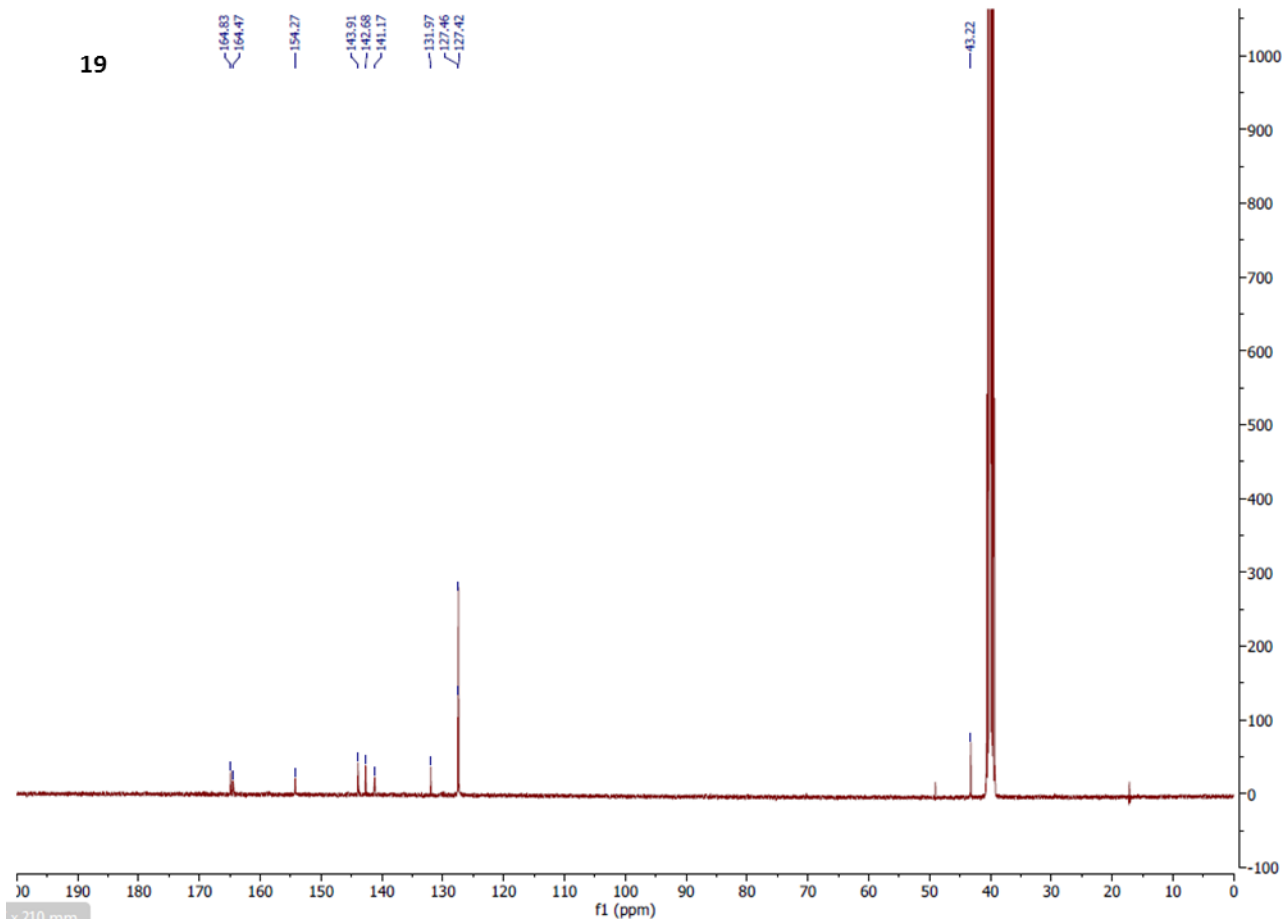
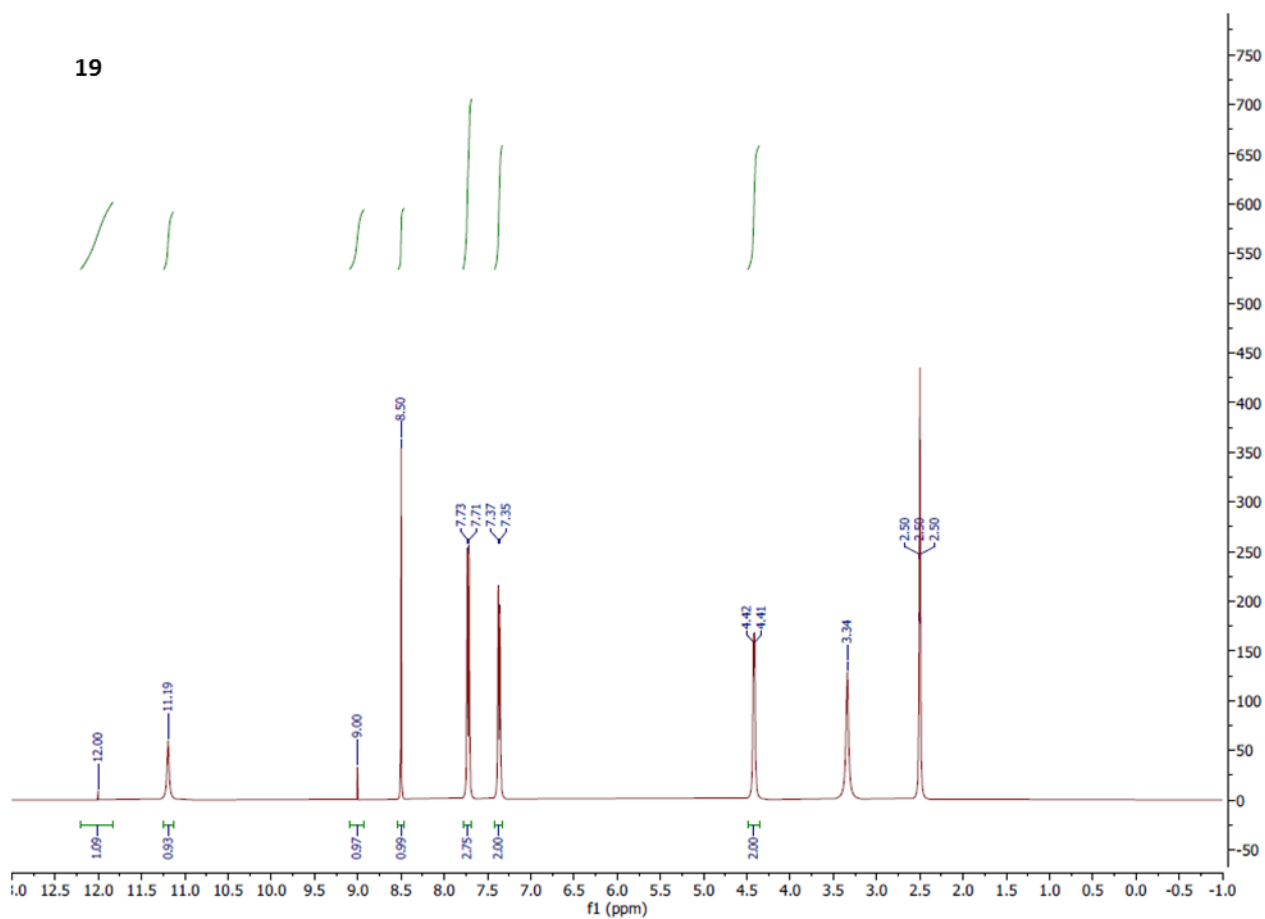
Appendix A – NMR spectra

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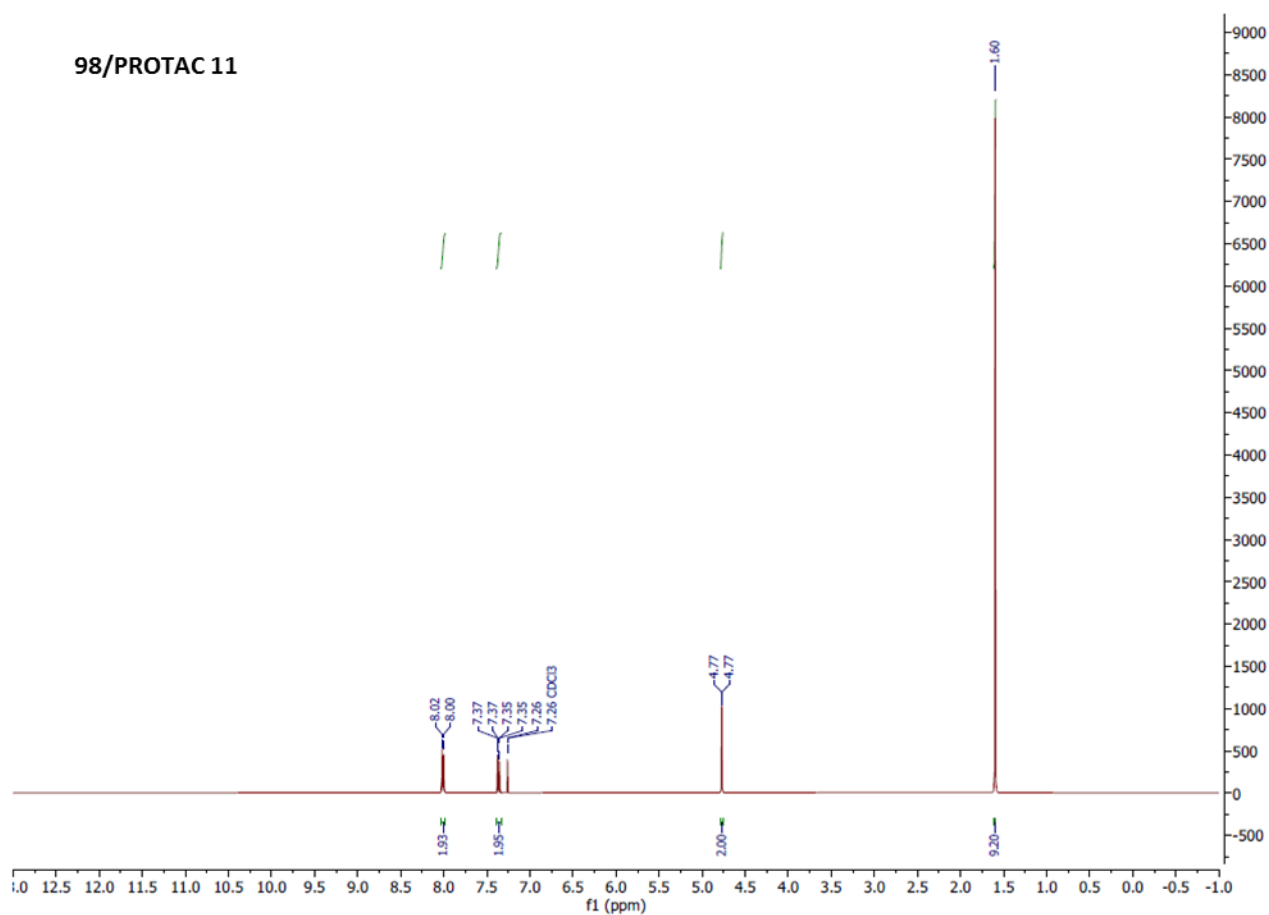


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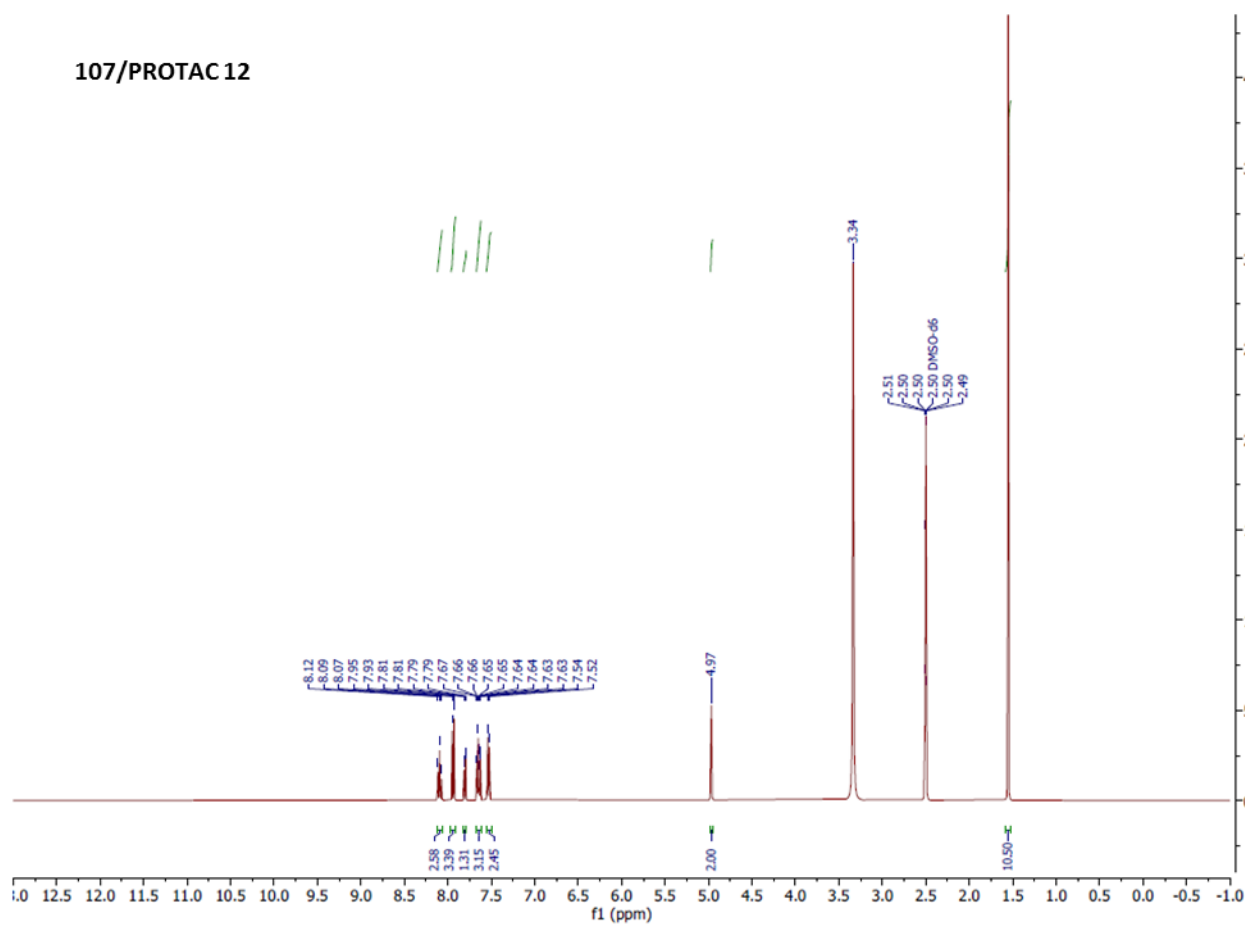




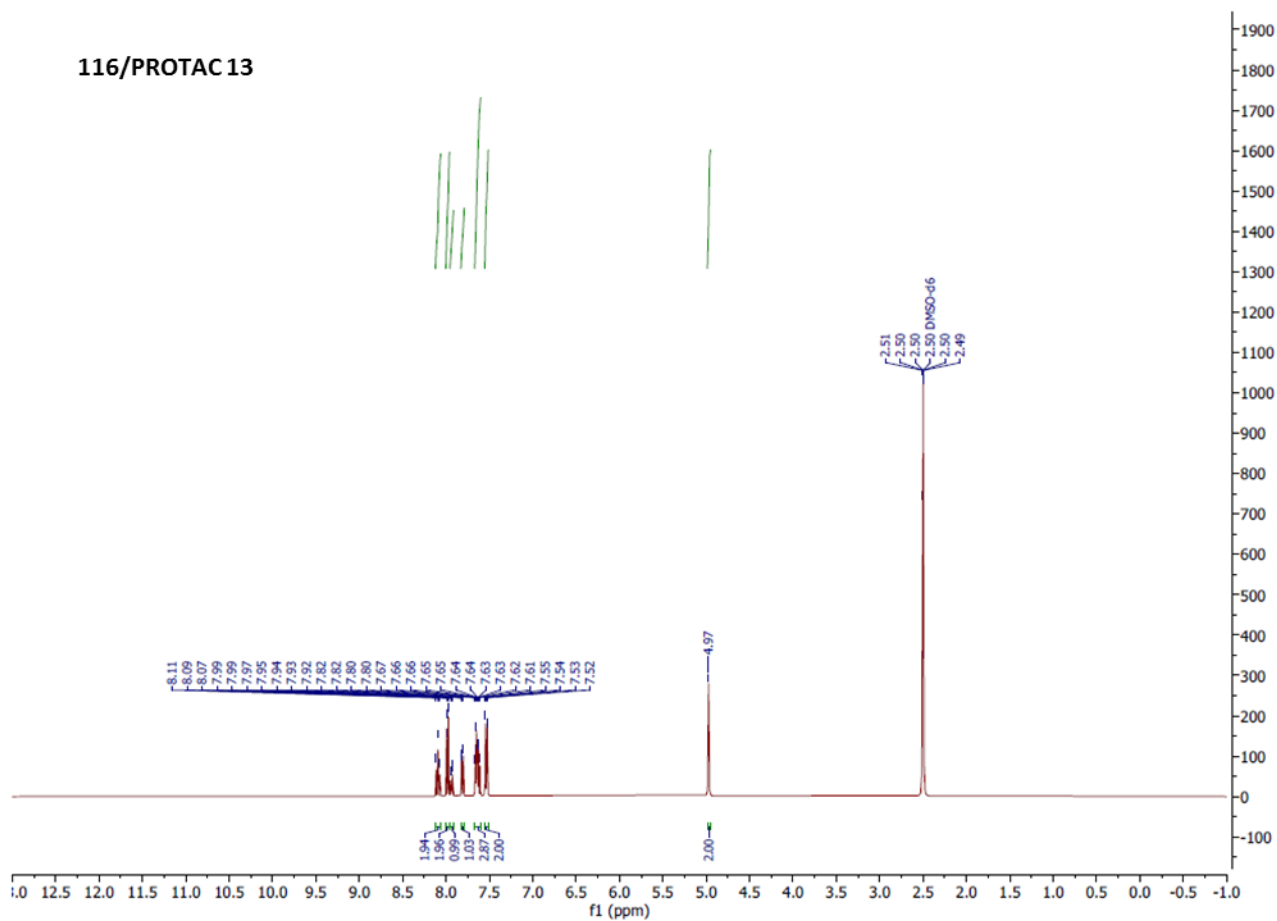
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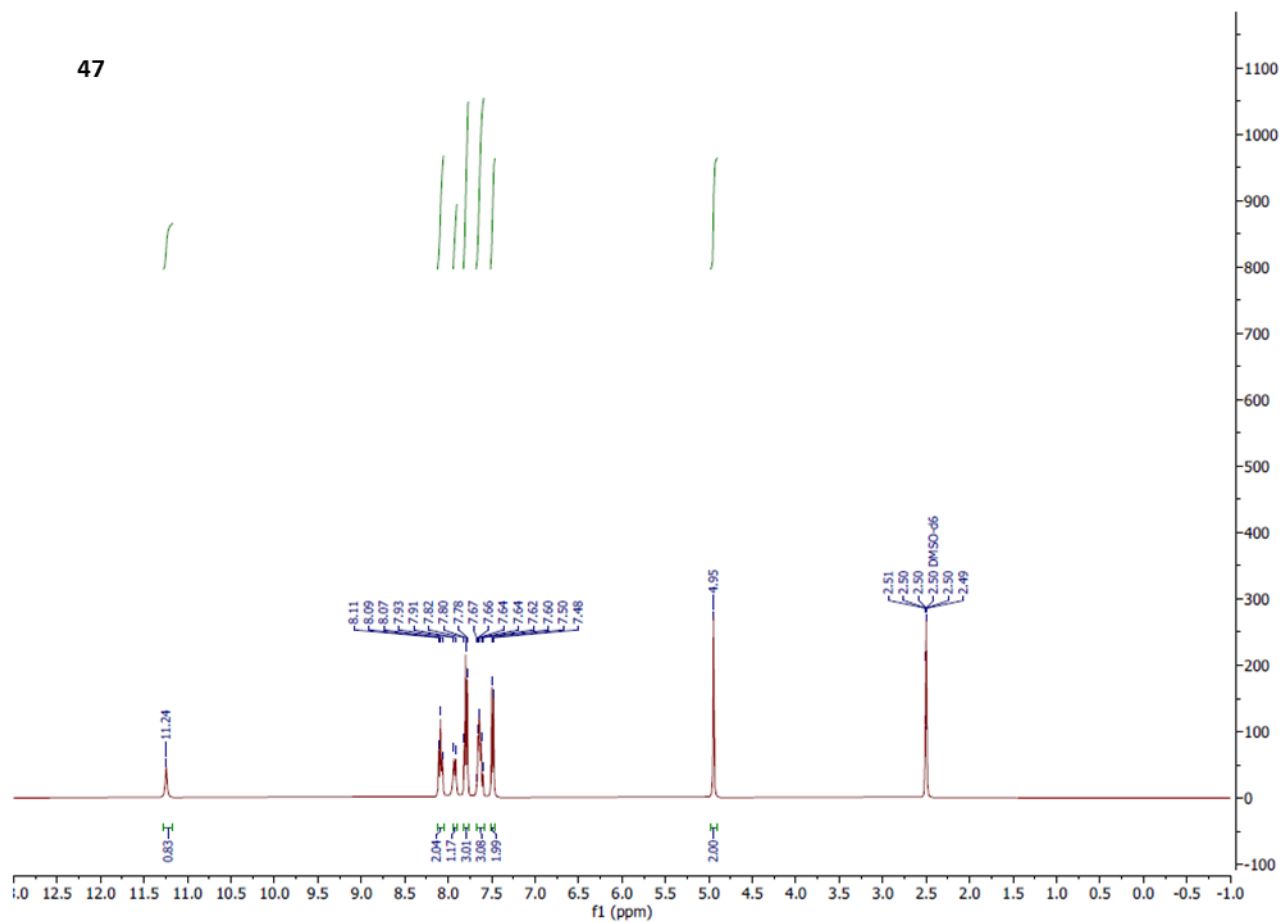
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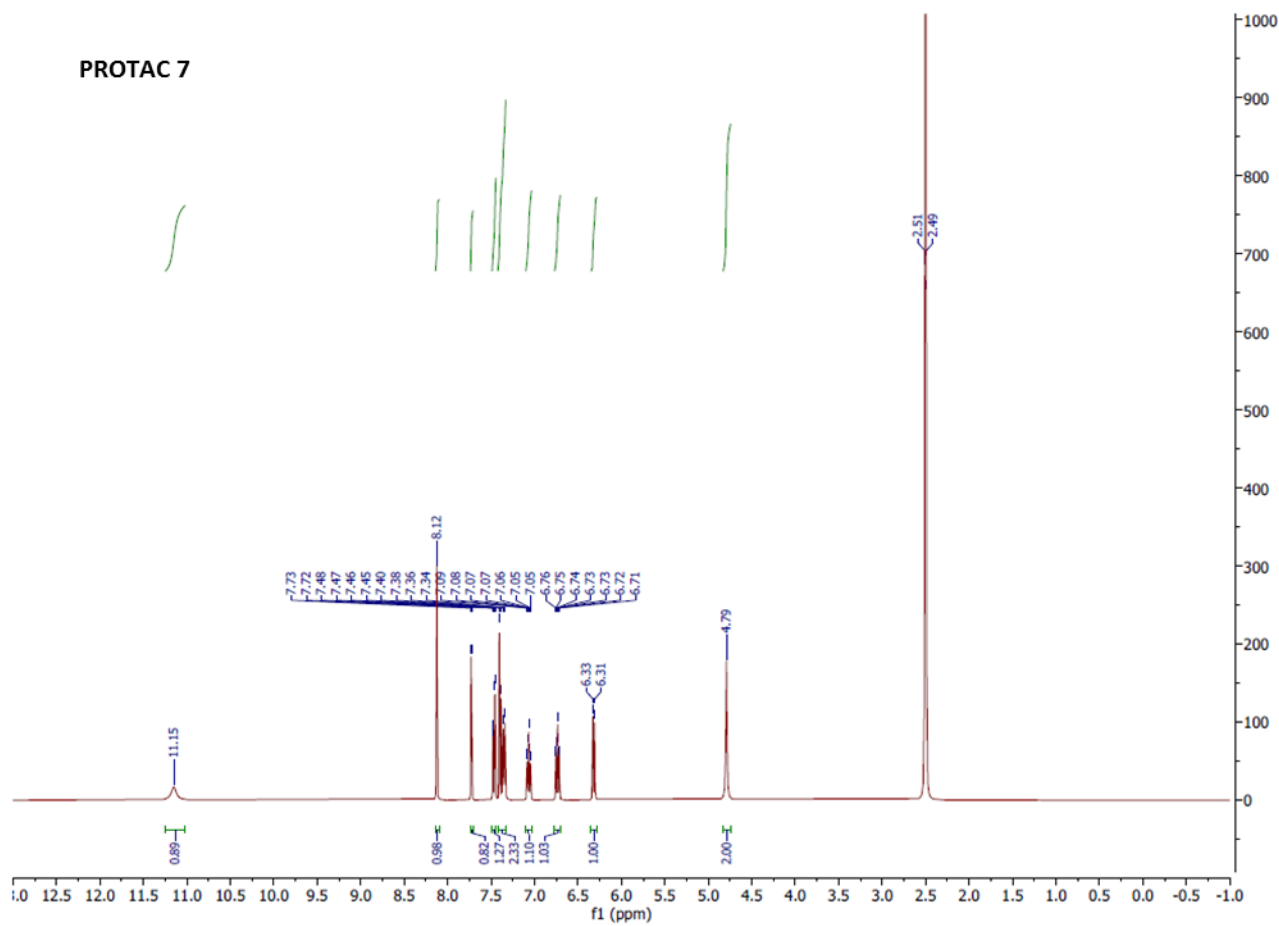
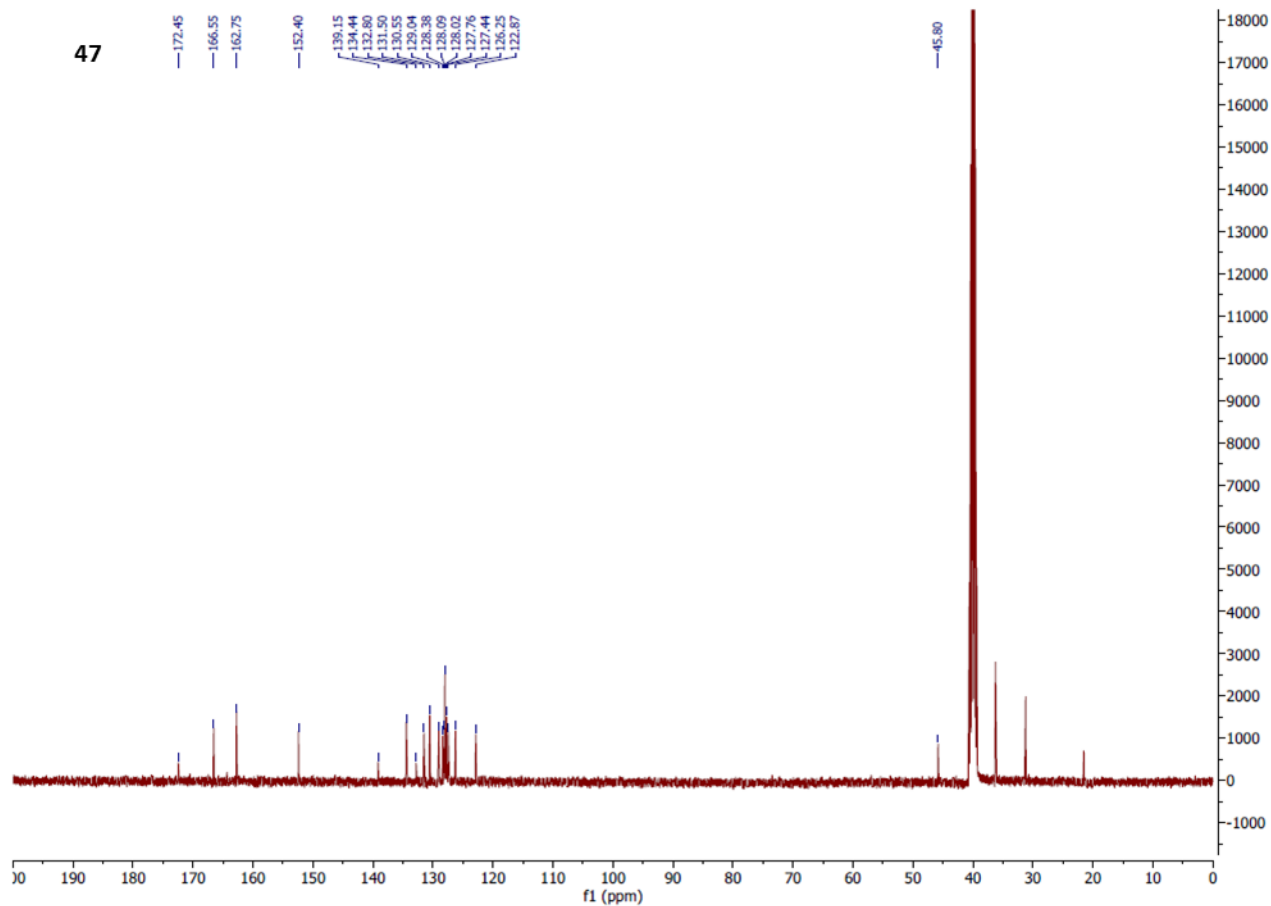


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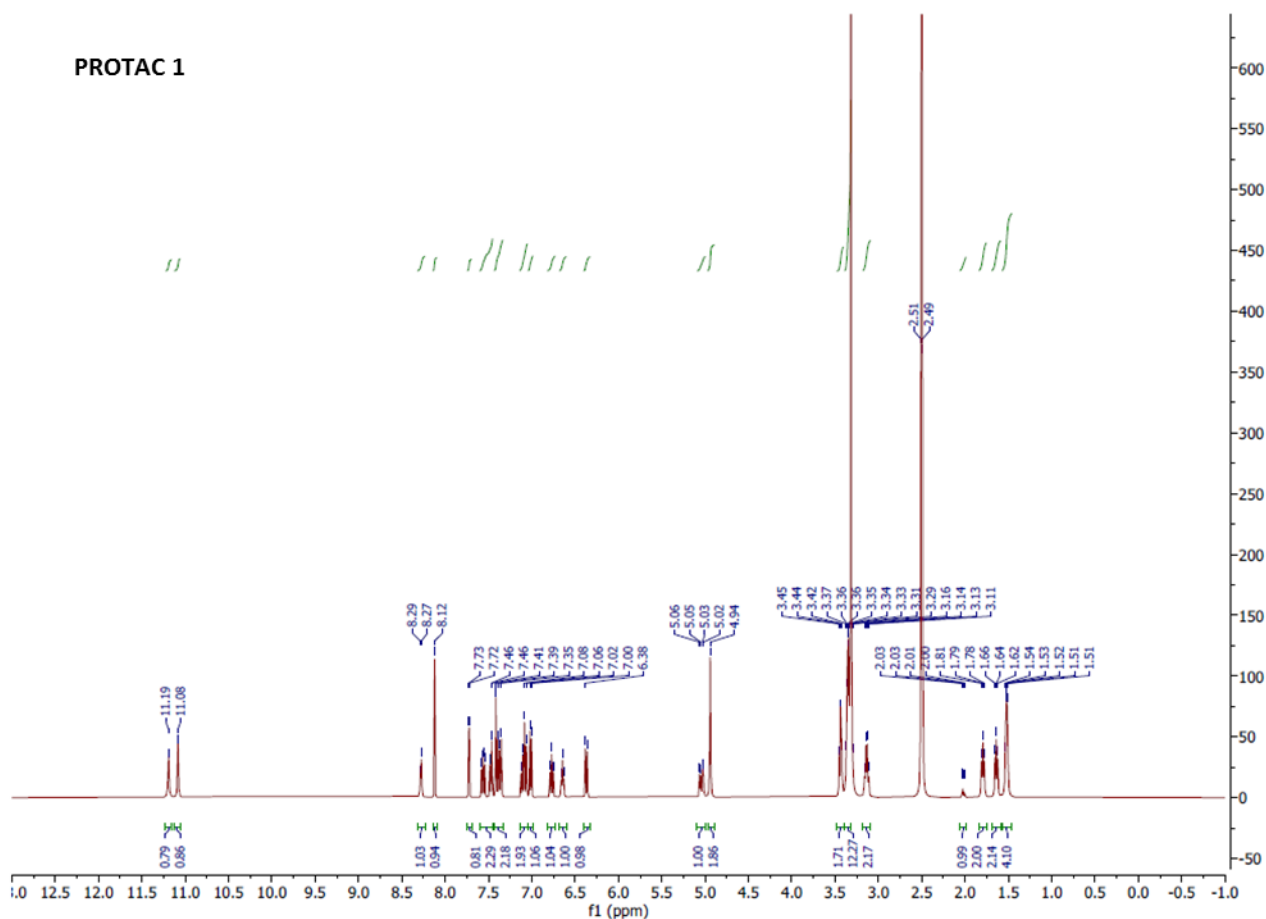


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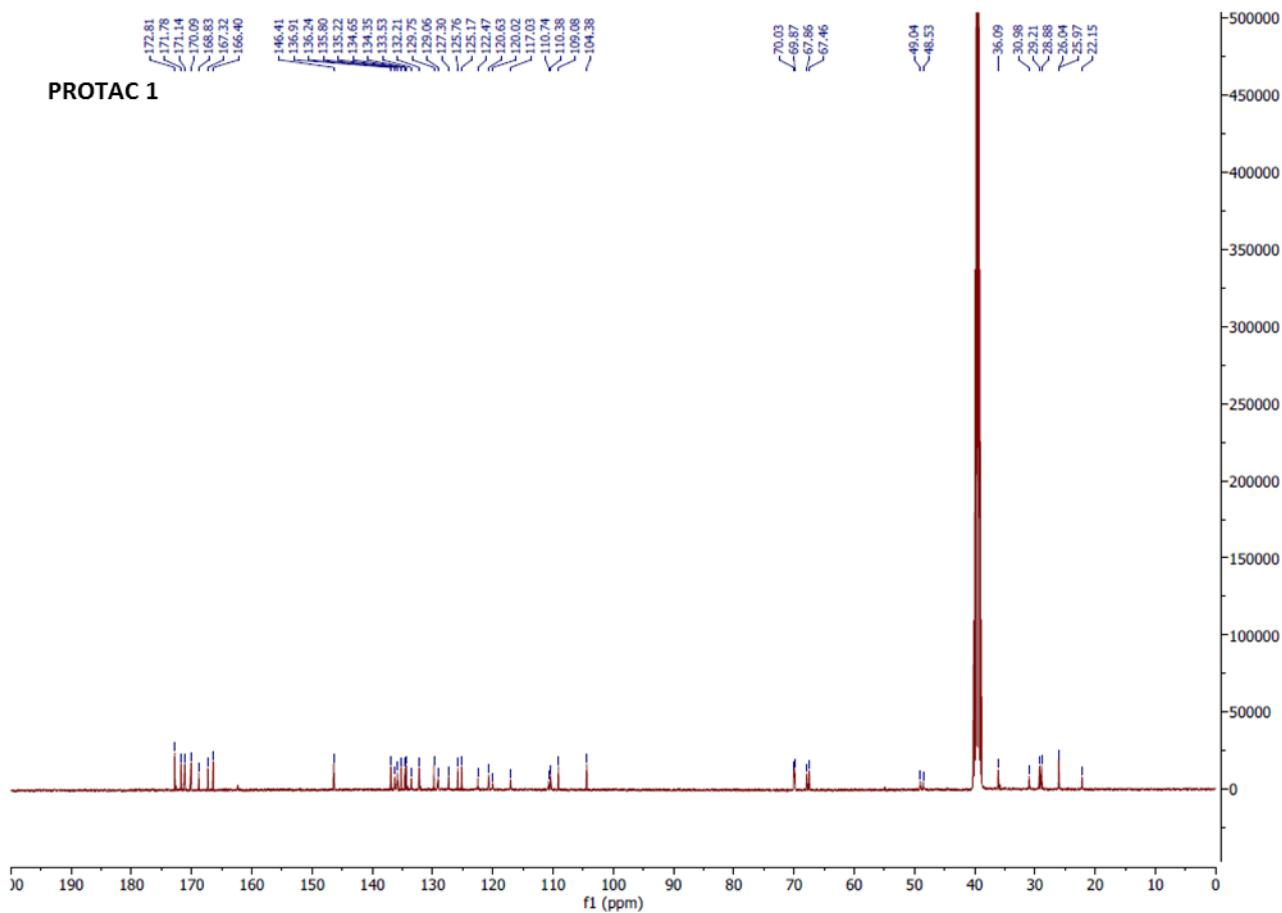




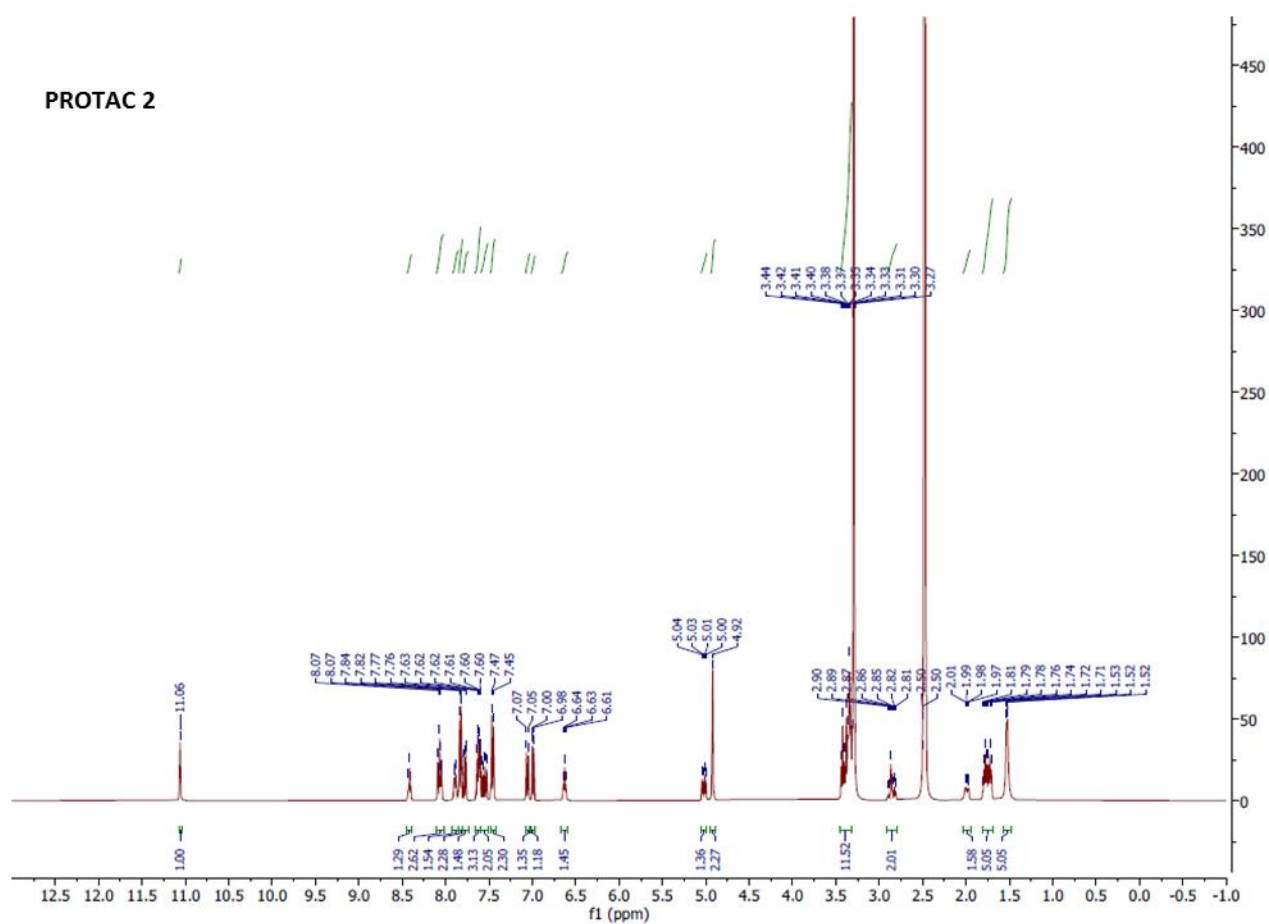
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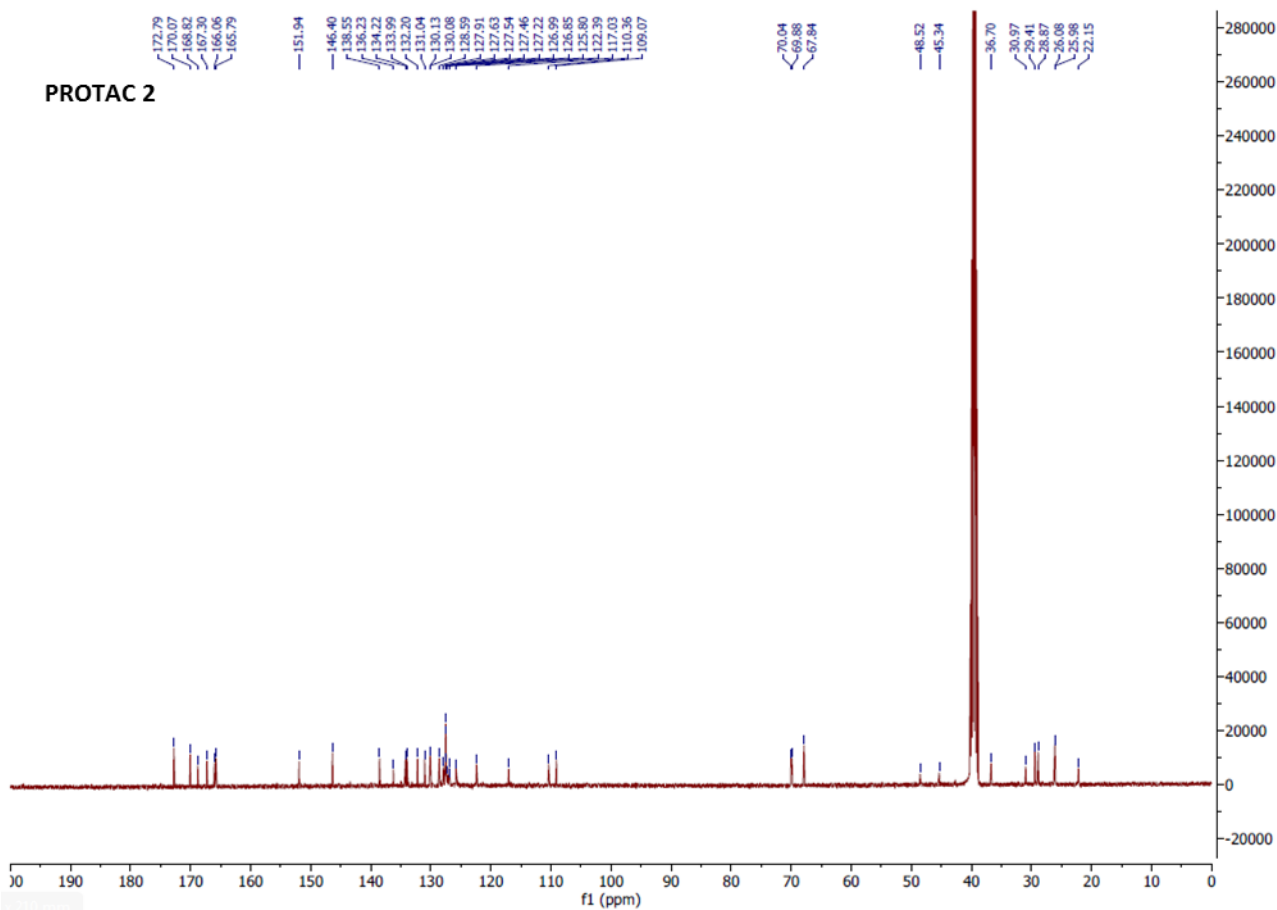
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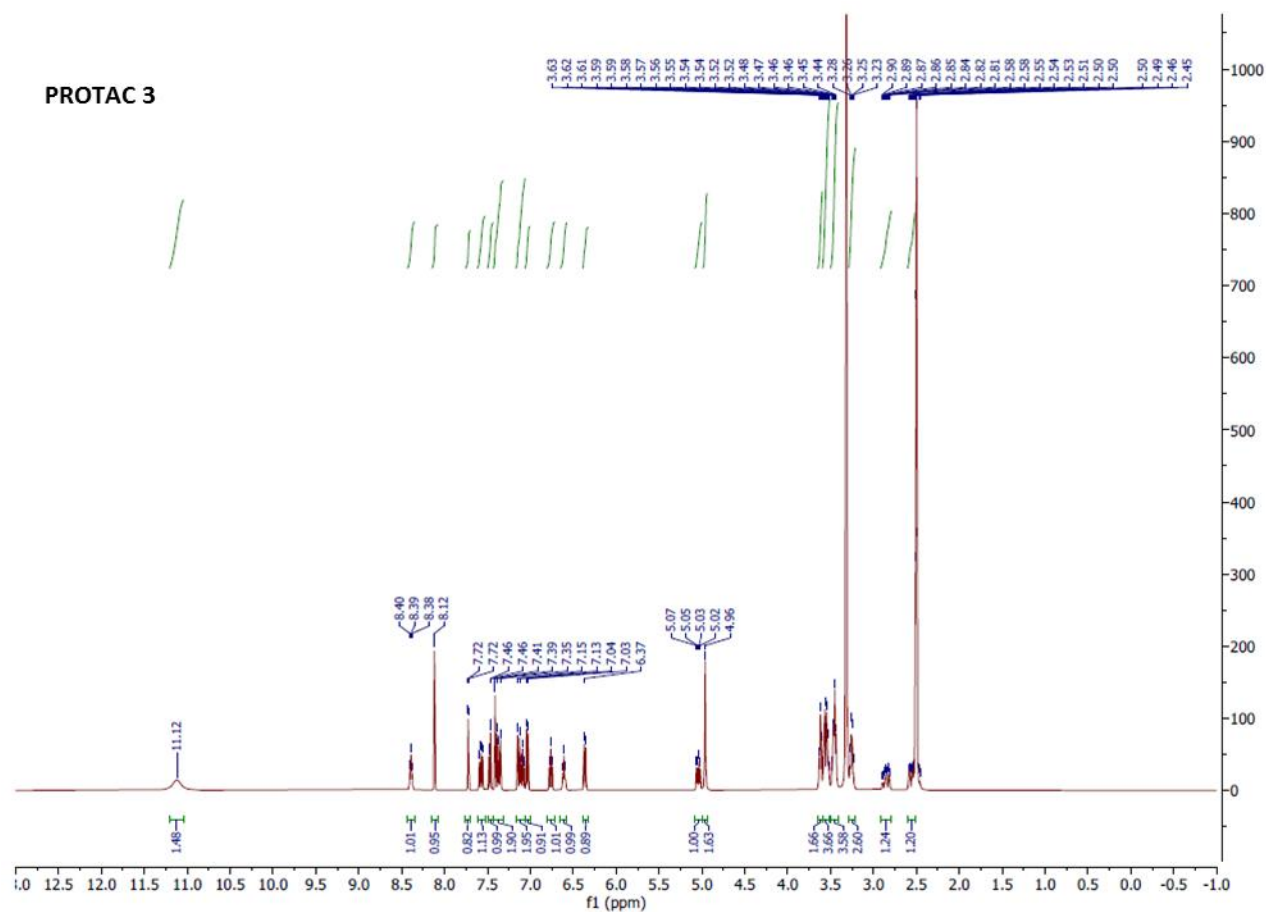
PROTAC 2



PROTAC 2



PROTAC 3



PROTAC 3

