The Evolution of Small Molecule Enzyme Activators

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The Evolution of Small Molecule Enzyme Activators

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Abstract

There is a myriad of enzymes within the body responsible for maintaining homeostasis by providing the means to convert substrates to products as and when required. Physiological enzymes are tightly controlled by many signaling pathways and their products subsequently control other pathways. Traditionally, most drug discovery efforts focus on identifying enzyme inhibitors, due to upregulation being prevalent in many diseases and the existence of endogenous substrates that can be modified to afford inhibitor compounds. As enzyme downregulation and reduction of endogenous activators are observed in multiple diseases, the identification of small molecules with the ability to activate enzymes has recently entered the medicinal chemistry toolbox to afford chemical probes and potential therapeutics as an alternative means to intervene in diseases. In this review we highlight the progress made in the identification and advancement of non-kinase enzyme activators and their potential in treating various disease states.

Introduction

The pursuit of small molecules that provide therapeutic effect makes up a significant part of the pharmaceutical industry, resulting in 21 of the 37 (57%) drugs approved by the U.S. Food and Drug Administration (FDA) in 2022 being part of this category. [1] Off these 21 compounds, the majority are enzyme inhibitors (8) and receptor antagonists or agonists (6) acting by positive allosteric modulation (PAM). Only one acts by activating an enzyme. [1] This trend has been consistent over the past five years with enzyme modulators being represented by more than 50 inhibitors gaining clinical approval and yet only two enzyme activators or stimulators have been approved in that same time period (**Figure 1**). [1-5]

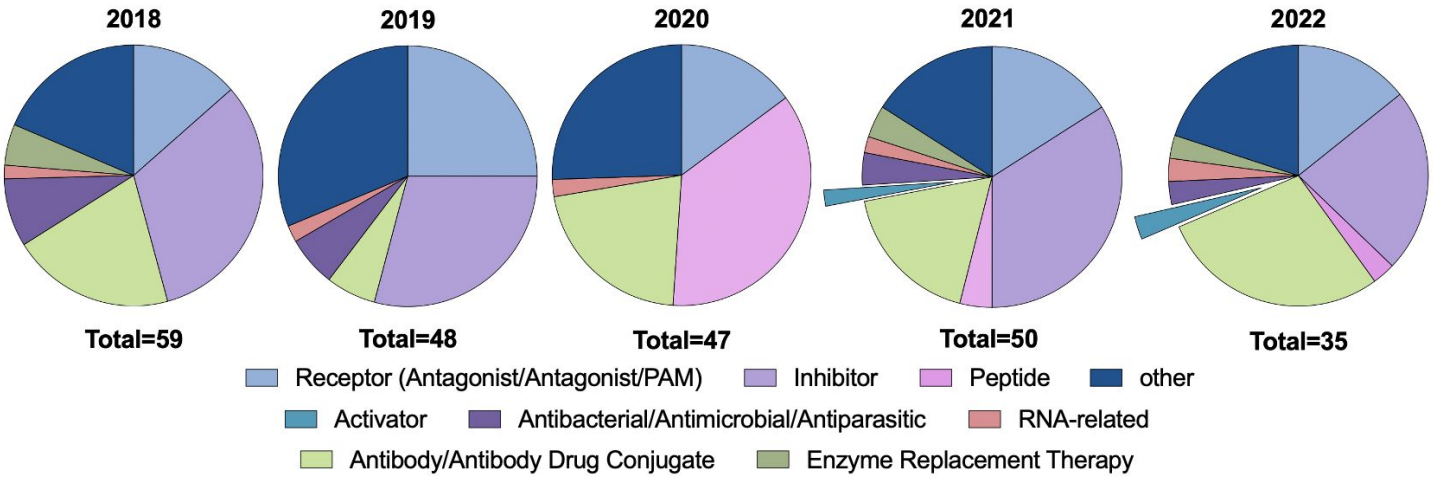


Figure 1. Distribution of FDA approved drugs from 2018 to 2022. During this period over 50 inhibitors have been approved as therapies, but only two enzyme activators have been approved during the same period.

Although enzyme activation has been slow to gain traction for therapeutic use, this method has potential in many areas of disease including; cancer, cardiovascular, neurodegeneration and aging and, represents a largely unexplored area with unexploited opportunity. [6, 7] There are two categories of enzyme activation, non-essential and essential with small molecules providing the opportunity to modulate activity through both of these methods. [8] In non-essential activation the catalytic reaction can occur with and without the presence of the activator molecule, often increasing activity beyond existing basal level however, with essential activation, the catalytic reaction will not occur without the activator. [8, 9] Essential activation occurs physiologically for some enzymes with cofactors, substrates and metal ions acting as an endogenous activator. Thus, small molecule activators can act through replacement of essential physiological molecules required for enzyme activity or by alternate mechanisms and sites on the enzyme surface.

Many prior reports focus on kinase activation, [9-12,] inhibition, [13-16] or a small cohort of kinase and non-kinase activators. [17] This review aims to highlight the potential and progress of small molecule non-kinase enzyme activators. For a detailed synopses of kinase activators, the reader is directed to an excellent recent review. [17] This review is structured to briefly describe the function and disease potential of each enzyme, the structures for the main published activators or activator chemotypes will then be discussed alongside a summary of their *in vitro* and *in vivo* activity.

Identifying Enzyme Activators

Whereas enzyme inhibitors can bind in either the active site, to hinder substrate binding, or at allosteric sites to modulate inhibition, enzyme activators can only bind to an allosteric site to achieve upregulation or by binding other regulatory subunits that interact with the enzyme to facilitate the catalytic process. Endogenously, enzymes are activated using ions, post-translational modifications (phosphorylation) and gaseous molecule binding.[18-21] Therefore, identifying both the allosteric site and a molecule that interacts with this site that provides the same activating effect as an endogenous molecule can be challenging.

Similar to inhibitors, activators can be classified by how they affect Michaelis constant (K_m, the concentration of substrate which permits the enzyme to achieve half of the maximum reaction rate) and maximum rate of reaction (V_{max}). Activators that decrease K_m are referred to as K-type and those that increased V_{max} as V-type activators and therefore both of these parameters must be assessed to characterize activators.[9, 22] Traditional methods for identifying inhibitors can also be employed for the identification of enzyme activators. Fluorescence resonance energy transfer (FRET) and other fluorescent based assay, activity-based protein profiling (ABPP) are some of the methods that have proved successful in the literature.[23-25] There are however aspects that make enzyme activation more challenging and thus require additional considerations. Firstly, there is a lack of tool compounds that can be used for the development of enzyme activation assays. [9, 19, 20] This is not always an issue for identifying inhibitors, as there are alternate ways to identify an expected decrease in reaction rate when the enzyme is fully inhibited. Secondly, as activators increase the reaction rate, the reaction curve changes; thus, the portion of the reaction curve where steady-state kinetics can be applied is smaller. [9] Therefore, this shortens the window for measuring initial rate and steady states which in turn decreases reliability. The mechanism of action is also important for understanding and identifying activators as they may bind or interact

with the enzyme before or after substrate binding. Additionally, the substrate concentration used to run the assay will affect the ability to detect compounds that activate. [9, 26] It is also possible that while an activation occurs initially, past a certain concentration point, the compound acts as an inhibitor.

Screening for Nuisance Compound

With high-throughput screening, the most common method for identify activators and inhibitors, high numbers of hit compounds are generated. Pfizer has reported that to identify one hit compound an average of 120,000 compounds need to be screened. [27] False hits can occur due to multiple reasons including activity, absorbance wavelength, aggregation and redox-cycling. [28-31] It Is important to be aware of 'promiscuous enzyme activators' when embarking on enzyme activation projects. Like pan-assay interference compounds,[32] these molecules can provide false hits in high-throughput screening that, when further investigated, provide a general activation to multiple enzymes. 'Surfactant-like' compounds were identified to possess promiscuous activation effect, possibly through the masking of hydrophobic patches on protein surfaces, by enzyme stabilization in solution, or loosening the protein's secondary structure. [33] A rough pharmacophore for these promiscuous activators was proposed; a polar or charged headgroup and a large hydrophobic tail however, these features can be included within 'drug-like' molecules. [33]

To identify these false hits before and after the initial screening a number of methods can be applied that are identical to those employed in the search for inhibitors. Firstly, by creating computational filter sets, these promiscuous compounds can be flagged and removed from the screening collections in silico. [33] Secondly, applying appropriate orthogonal assays to confirm compound activity should be standard for all campaigns for identifying both activators and inhibitors. [34]

Angiotensin-converting enzyme 2

Angiotensin-converting enzyme 2 (ACE2) is a zinc-dependent monocarboxypeptidase plays a critical role in the control of the renin-angiotensin system and in balancing overexpression of angiotensin II (Ang II). [35-37] As ACE2 possesses a highly conserved sequence identity (41%) and sequence similarity (61%) with Angiotensin-converting enzyme (ACE) in the regions surrounding the active site, supported by modeling of the ACE2 active

site, it is proposed that the catalytic mechanism of ACE2 resembles that of ACE. [38] However, due to differences in substrate specificity it is certain there are distinct differences in the active site of ACE2, one of which is the smaller pocket size. [38]

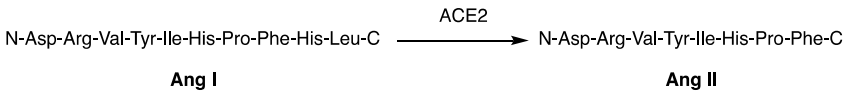


Figure 2. Hydrolysis of Ang I to Ang II catalyzed by ACE2.

The ACE2 enzyme hydrolyzes the C-terminal residue Ang I to produce the cardioprotective peptide Ang II (**Figure 2**). [39] The hydrolysis occurs through a water mediated nucleophilic attack of the peptide bond resulting in formation of a non-covalent tetrahedral intermediate. [38, 40] Activation of the water occurs by the pentacoordinate zinc ion and water molecules within the active site. Additionally, it has been shown that ACE2 activity is highly dependent on pH, hydrolysis occurs between pH 6.5 and 8, with minimal activity being observed at pH 8. [41]

Altered expression of ACE2 is associated with cardiac, vascular, and renal dysfunctions, as it has been demonstrated that ACE2 plays an essential role in anti-hypertensive, anti-fibrotic, anti-oxidant, anti-inflammatory, and anti-atherosclerosis effects. [35, 39, 42] The activation of ACE2 has also been identified to protect against amyloid-related hippocampal pathology and cognitive impairment in preclinical Alzheimer's disease models. [43]

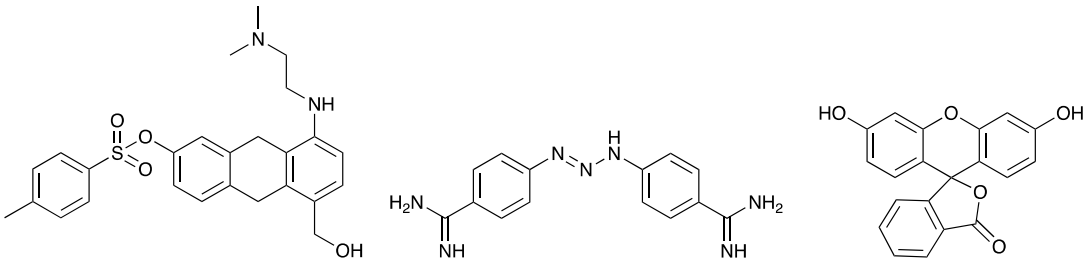


Figure 3. Chemical structures of Angiotensin Converting Enzyme 2 activators **1**-**3**, and their activities.

Three small molecules, Xanthenone (**1**) (EC_{50} of 20.1 μM), Diminazene aceturate (**2**) (EC_{50} of 8.04 μM) and Resorcinolphthalein (**3**) (EC_{50} of 19.5 μM) (**Figure 3**), have been identified to activate ACE2. [44] These molecules have been shown to attenuate diabetes-induced cardiac dysfunction and mitigable neuroinflammatory burden in Parkinson's Disease. [35, 39, 45,] It has been suggested that **1** activates ACE2 by binding the structural

pocket in the open confirmation, whereas **2** activates ACE2 by binding Ang (1-7) with receptor Mas (MASR). [39, 45,]

In vivo assessment of **1** in spontaneously hypertensive rats (SHR) showed a reduction in blood pressure (BP) of 71 mm/Hg at 10 mg/kg, and in wistar kyoto rats (WKR) resulted in a decrease in BP of 21 ±8 mm/Hg. [35, 39] Acute administration of **1** was shown to induce a dose-dependent hypotensive response, and chronic administration results in the reversal of cardiac and renal fibrosis in SHR. This highlights the ability of these ACE2 activators to be potential antihypertensive treatment options. Furthermore, **1** can attenuate diabetes-induced cardiac dysfunction. [39]

Assessment of **2** (25 μM) *in vitro, in* 6-hydroxydopamine (6-OHDA)-induced dopaminergic neurons, enhanced glutathione (GSH) and reversed 6-OHDA-induced damage in cells and brain tissues. [45] Additionally, *in vivo* treatment with **2** (10 mg/kg) also resulted in a reduction in Glial Fibrillary acidic protein (GFAP) (in C6 cells and SNpc brain region) and IbA1 (in BV2 cells and SNpc brain region), and reduced neuroinflammation. [45] Finally, **2** activates the ACE2/Ang (1-7)/MASR axis resulting in the downregulation of the RAS pathway, and enhances the neuroprotective, antioxidative, and anti-inflammatory effects of **2**.

This assessment highlights the role of ACE2 enzyme activators in numerous disease areas. Further exploration of targeted small molecule enzyme activators can provide a potential avenue of treatment for different disease types associated with the altered expression of ACE2.

Carnitine Palmitoyltransferase One

The carnitine palmitoyltransferase (CPT) system assists in the transport of long-chain acyl-Coenzyme A (CoA) esters into the mitochondrial matrix for entry into the fatty acid b-oxidation (FAO) cycle. [46, 47] Three enzymes make up the CPT system: CPT1, CPT2 and acylcarnitine translocase (ACT). [48-50] Located on the outer mitochondrial membrane, CPT1A (one of three CPT1 isoforms) is responsible for the conversion of long-chain acyl-CoA into long-chain acylcarnitines. [51, 52] CPT1 is considered as the rate controlling enzyme for the CPT system and the FAO cycle. [53-55] Therefore, the activation of CPT1 has been identified as a mechanism to promote FAO, which results in the reduction of lipid accumulation and a decreased tendency for metabolic disorders. [56, 57]

The Catalytic mechanism of CPT1 is unknown to date as crystal structure information is not available. However, three mechanisms have been proposed with His_{473} being identified as a key residue for catalytic activity. [58-60] One proposed mechanism suggests that the $Cys_{305,}$ His_{473} and Asp_{454} residues form a catalytic triad responsible for acyl transfer. [61] Whereas another proposes His_{473} and Asp_{477} are catalytic residues. [60] The final proposed mechanism is based on the model of carnitine acetyltransferase where His_{473} deprotonates carnitine. [58]

Although there are multiple structures suggested to activate CPT1, it has been indicated that some of these activators achieve upregulation by the displacement of malonyl-CoA, a known inhibitor of CPT1 and therefore, do not act directly on the enzyme. Baicalin (**4**) and C89b (**5**) (**Figure 4**) have both been identified as CPT1 activators. [62-64] Baicalin is predicted to bind to an orthosteric site of CPT1 (**Figure 5**), with key residues Ley_{286}, Ile_{291}, Glu_{309} and His_{327} interacting with **4**. [62] Shift assays have determined that the binding of **4** affects the thermal stability of CPT1A however not actin control. The binding site for C89b remains unknown.

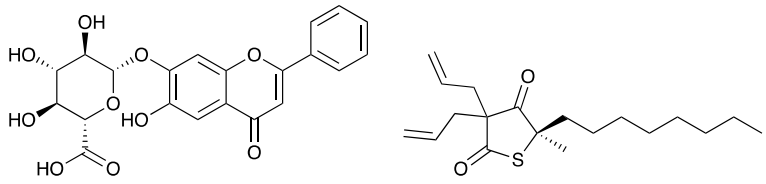


Figure 4. Chemical structures of carnitine palmitoyltransferase activators **4** and **5**, and their activities.

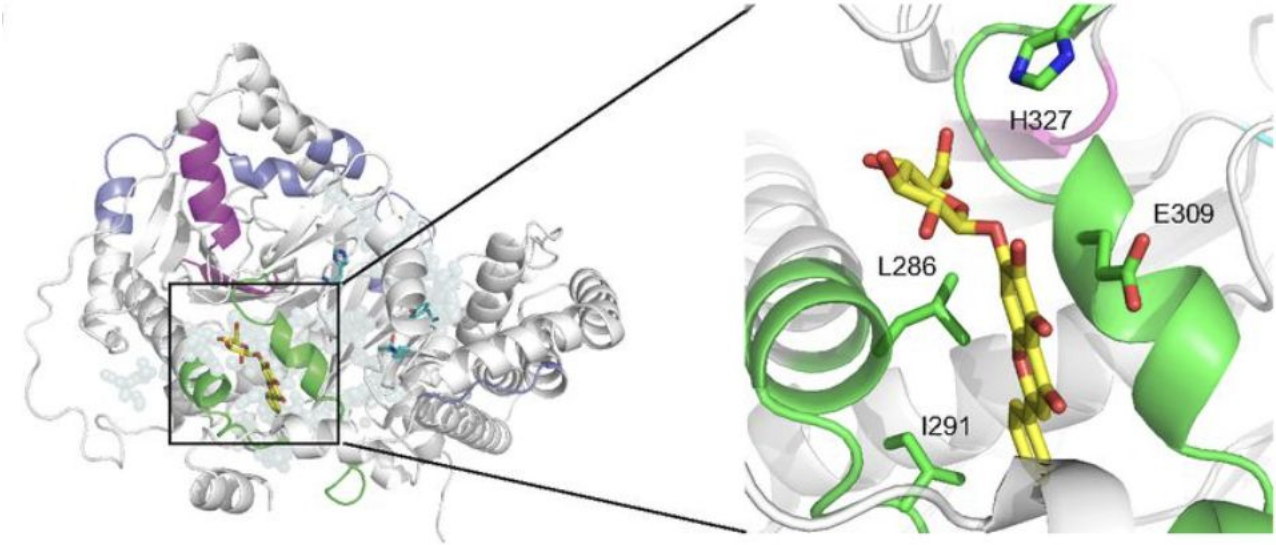


Figure 5. (Left)) Structural overview of a CPT1A–**4** predicted complex mode, residues around the predicted area are colored green. (Right) Magnified view of predicted CPT1A-**4** interface with key interface residues; Leu_{286}, Ile_{291}, Glu_{309}, and His_{327} shown (labelled by residue name and position). Reproduced from ref. 62 with permission from The U.S. National Academy of Sciences, copyright 2018.

Assessment of CPT1 activation by **4** is demonstrated by multiple *in vitro* assays resulting in an increase (7-fold at 100 μM) in CPT1A activity from the direct addition of **4** to cell lysate, and a reduction in free fatty acids (50% at 100 μM) in HeLa Cells. [62] *In vivo* studies reported (for H327E mice following a high-fat diet) a decrease in body weight (-12.5 g) and body fat percentage (-0.085%), restoration of insulin sensitivity to normal, and improvements in other diet induced obesity (DIO) markers when receiving a daily dose of **4** (400 mg/kg). [62] The results from the *in vivo* experiments suggested that more fat is used as the energy source for animals receiving chronic treatment of **4**. [62] Activation of CPT1 by **5** was measured by increased levels of radiolabelled palmitoylcarnitine in MCF-7 cells and the increased oxidation of fatty acids (177% at 31 μM, estimated EC_{50} of 3.0 μM). [63] The data surrounding these CPT1 activators have shown their potential to be used for the treatment of diabetes and obesity. However, increased potency may be required to fully explore the potential of these activators.

Caseinolytic Protease P

Caseinolytic protease P (ClpP), an Adenosine triphosphate (ATP)-dependent serine protease, is a mitochondrial protein that catalyzes the hydrolysis of peptide bonds. [65-67] Therefore, ClpP helps to maintain protein homeostasis and quality and, when misfolded and non-functional proteins accumulate, ClpP expression is increased. [65, 68,] Additionally, ClpP has been identified to regulate the production of reactive oxygen species (ROS) by association with the enzymes of the electron transport chain. [65]

Mitochondrial ClpP forms a stable heptamer ring, through hydrophobic interactions, that possesses peptidase activity. However, substrate degradation by ClpP is dependent on ClpX and ATPase. [69] Alone, ClpP with Fourteen internal catalytic cleavage sites located within the heptamer has the capability of only digesting small peptides. Binding of ClpX to ClpP, and in the presence of ATPase, the active tetradecameric state is stabilized, possessing the unfoldase activity and is able to degrade full length proteins with Ser_{153}, His_{178}, and

Asp_{227} acting as the catalytic residues within the active site. [69-72] Activation of ClpP can be achieved by the physical displacement of ClpX from ClpP whilst ClpP is maintained in its active state, this allows proteins to enter the active site non-specifically. [73-75]

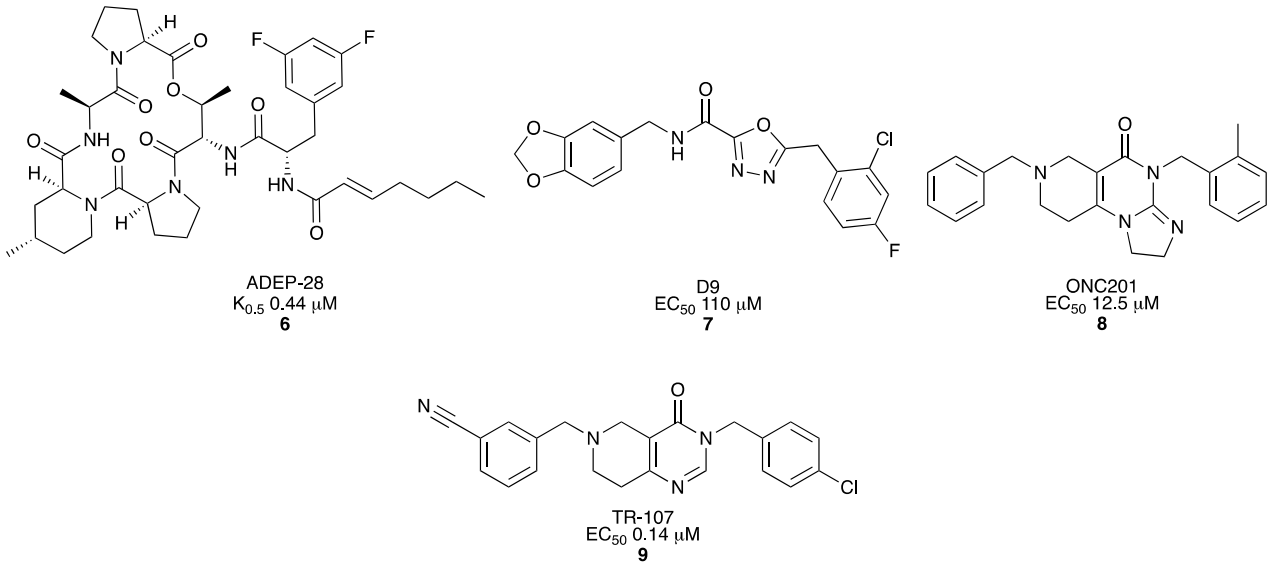


Figure 6. Chemical structures of caseinolytic protease P activators **6**-**9**, and their activities.

ADEP-28 (**6)** ($K_{0.5}$ = 0.44 μM), D9 (**7**) (EC_{50} = 110 μM), ONC201 (**8**) (EC_{50} = 12.5 μM) and TR-107 (**9**) (EC_{50} = 0.14 μM) (**Figure 6**) have all been identified to bind ClpP by the same mechanism and activate ClpP in a dose-dependent manner. [76-79] These activators all fit into a hydrophobic pocket of the enzyme. The opposing hydrophobic difluorophenyl moiety and the hydrophobic tail of **6** along with the far left and right aryl portions of **7**, **8** and **9** are buried in ClpP while the main part of **6**, and the mid sections of **7**, **8** and **9** rest horizontally or close to the surface of the enzyme. [79] Due to the suggestion that these activators have the potential to be used for the treatment of cancer, the apoptotic nature of these compounds have been assessed in various cancer cell lines; HEK293 T-REx (**6** IC_{50} = 0.36 μM, **8** IC_{50} = 1 μM), TEX (**8** IC_{50} = 0.7 μM), OCI-AML3 (**8** IC_{50} = 1.2 μM), Z-138 (**8** IC_{50} = 1.0 μM), SUM159 (**8** IC_{50} = 0.3 μM, **9** IC_{50} = 12 nM), MDA-MB-231 (**9** IC_{50} = 3.0 nM). [76, 78-80] It has been highlighted that apoptosis occurs via intrinsic caspase-dependent apoptosis, which is ClpP dependent, due to both **6** and **8** showing no effect on cell viability in HEK293 T-REx $CLPP^{-/-}$ cells. [80] Furthermore, oral gavage of **8**, a close analogue, and **9**, have shown both a time- and dose-dependent reduction of tumor burden in mice xenograft models using OCI-AML2, Z138 and MDA-MB-2 cells, respectively. [78, 80] In addition to anti-cancer

therapies, these compounds can also be employed as antibiotics due to **6** exhibiting bactericidal effects against Neisseria Meningitidis H44/76 (Minimum inhibitory concentration of 0.125 mg/mL). [81]

Cyclic adenosine monophosphate response element-binding protein binding protein

Histone acetyltransferases (HATs) are vital in the regulation of gene transcription by making epigenetic modifications. [82] Consequently, the dysregulation of HAT's is observed across multiple neurodegenerative diseases including: Huntington's disease, amyotrophic lateral sclerosis, and Parkinson's disease, which suggests a causative role. [83]

Cyclic adenosine monophosphate response element-binding protein-binding protein (CBP), also known as CREB-binding protein or CREB-BP, is a transcriptional co-activating protein that contains a HAT domain, a bromodomain (BRD) and a plant homeodomain (PHD) finger motif. [84, 85] Both transcription factors and DNA histones are acetylated by CBP. [86-90] The regulation of gene expression by CBP occurs by two mechanisms; acetylation of histones and by acting as a protein scaffold, recruiting, and constructing the complexes required for transcription or modification of chromatin. [91-93] It is the HAT domain of CBP that catalyzes the transfer of an acetyl group from acetyl-Co-A to lysine side chains found on proteins. In the absence of a cofactor the acetyl-Co-A binding site remains stable however, the BRD is essential for histone H3 acetylation. [94] Both the Really Interesting New Gene (RING) domain, located within the second cysteine/histidine-rich domain, and autoinhibitory loop (AIL) are identified to regulate the access to the active site. [94-96] The RING domain restricts bulky substrates accessing the active site. The AIL, when hypoacetylated, occupies the active site but on hyperacetylation is displaced leaving the active site accessible to substituents.

Current data shows that gene expression is promoted by the CBP enzyme and that the cyclic adenosine monophosphate (cAMP)/CBP signaling pathway is strongly associated with long term memory and other learning processes through promotion of neuron survival and synaptic plasticity. [97-100] Furthermore, CBP is critical in hippocampal long-term potentiation and the overexpression of CBP in neurodegenerative models delays cell death. [101, 102] *In vitro* data suggests mutant α-synuclein exerts some toxicity by the direct inhibition of histone acetylation, further supporting the activation of HATs for neurodegenerative diseases. [103]

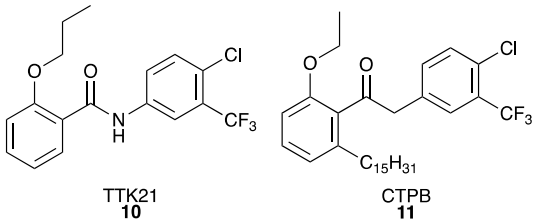


Figure 7. Chemical structures of cyclic adenosine monophosphate response element-binding protein-binding protein activators **10** and **11**.

Two activators of CBP, analogues TTK21 (**10**) and CTPB (**11**) (**Figure 7**) have been identified, however **10** requires conjugation to a glucose-derived carbon nanosphere (CSP) for cell permeation. [104, 105] Both **11** and the **10**-CSP conjugate achieve significant acetylation of histone H3, however the mechanism to achieve this increased acetylation is yet to be elucidated. [105, 106] Increased gene expression of neuroD1 (1.34-fold) and Brain Derived Neurotrophic Factor (>two-fold) is observed with the **10**-CSP conjugate alongside increased histone acetylation of their respective promoter regions, [105] suggesting histone acetylation is associated with transcription of genes required for neuron growth and survival. The *in vitro* treatment of **11** showed promotion of neurite growth, indicating an increase of neuronal growth factors. [106] Furthermore, *in vivo* treatment of the **10**-CSP conjugate in B57BL6/6J male mice resulted in increased dendritic branching. However, this may be a result of increased gene expression of neural growth factors. [105] *In vivo* assessment of the **10**-CSP conjugate in a behavioral assay using B57BL6/6J male mice showed an improvement in memory duration but no improvement in learning or retention. This improvement has been associated with neuroprotection from cell death rather than the promotion of cell proliferation. [105] Finally, qRT-PCR experiments revealed a significant increase in DCX and TUC4 mRNA, which suggests the induction of maturation in progenitor cells, however, immunohistochemistry contradicted this. [105] This data shows that activation of CBP may provide a way to treat the degradation of neurons associated with aging and neurodegenerative diseases.

Glucose-6-Phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) is a rate-limiting, ubiquitous cytoplasmic enzyme that converts glucose-6-phosphate into 6-phosphogluconate (G6P) in the pentose phosphate pathway (PPP) (**Figure**

8). [107-109] The enzyme achieves the catalytic oxidation of G6P with coenzyme $NADP^+$ which results in the formation of nicotinamide adenine dinucleotide phosphate (NADPH).

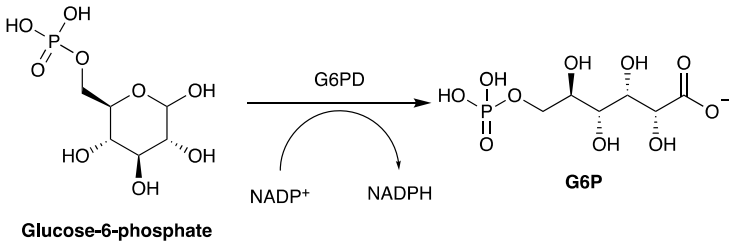


Figure 8. Conversion of glucose-6-phosphate to G6P Catalyzed by G6PD and coenzyme $NADP^+$.

The enzyme exists in an equilibrium of tetramer (dimer of dimers) and dimer with the equilibrium position shifting to the tetramer at low pH. [109] X-ray crystallography has shown that $NADP^+$ binds to each of the dimers, providing structural stability to the enzyme, additionally these two $NADP^+$ molecules also allosterically modulate the enzyme. [110, 111] An additional NADP+ binding site is present on each dimer near to the active site which acts as the coenzyme for the catalytic reaction. Density functional theory experiments have identified that proton extraction from His_{309} by Asp_{247} is crucial and responsible for a step-wise catalytic process. [112] Studies show that G6DP deficiencies are related to mutations of the enzyme affecting the catalytic and NADP+ sites. [113]

Since G6PD uses $NADP^+$ as a coenzyme, resulting in the formation of NADPH, it therefore plays an important role in redox homeostasis and maintaining the NADPH levels which are essential for numerous cellular systems, including antioxidant pathways, nitric oxide synthase, NADPH oxidase, and the cytochrome p450 system. [114-118] The cofactor NADPH is also crucial in counterbalancing cells from oxidative stress and is important in glutathione regeneration, an essential component for the reduction of hydrogen peroxide to balance oxidative stress. [107, 114] A deficiency in G6DP is detrimental in erythrocytes and is the most common human enzyme defect with a prevalence of more than 400 million people worldwide. [108, 115] Essential for cell survival, G6PD is highly regulated by numerous signals that affect transcription, post-translation, intracellular location, and interactions with other proteins. [114, 119]

The pathophysiological role of G6PD has been identified in numerous acute and chronic diseases associated with oxidative stress including hemolytic anemia, bilirubin-induced neurological damage, diabetes mellitus, viral infections, and cardiovascular diseases; such as coronary heart disease, cerebrovascular disease, peripheral arterial disease, and aortic atherosclerosis. [114, 120-122] Furthermore, G6PD inhibition in neurons

increases (ROS), induces apoptosis, and induces neurodegeneration in aging and neurodegenerative disease models. [120] There is no current treatment for G6PD deficiency; however, small molecule activators have been studied for the regulation of G6PD. [123]

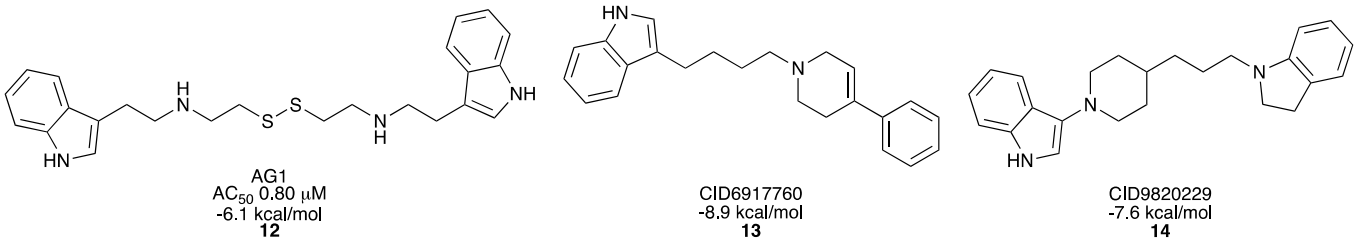


Figure 9. Chemical structures of Glucose-6-phosphate dehydrogenase activators **12**-**14**, and their activities.

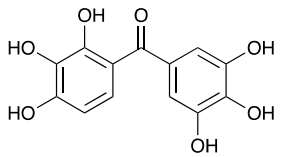
G6PD activator AG1 (**12**) (**Figure 9**), AC_{50} = 0.80 μM, was identified using high-throughput screening. [124] Subsequently, by using machine learning approaches of the AG1 pharmacophore, multiple additional activators were identified including CID6917760 (**13**) and CID9820229 (**14**) (**Figure 9**). These new activators had improved binding energies over **12** (-6.1 kcal/mol) achieving binding energies of -8.9 kcal/mol and -7.6 kcal/mol for **13** and **14,** respectively. [107, 123] These small molecules activate G6PD by binding to the active site of the dimer, and consequently bridging the dimer interface at $NADP^+$ binding sites. [107] Compound **12** was able to selectively promote G6PD dimerization in a G6PD deficient cell model (HG 02367), resulting in an EC_{50} of 3 mM, and, in G6PD deficient lymphocytes, **12** showed an increase in lysate activity of 78%. Furthermore, *in vivo* assessment of **12** with zebrafish embryos has been shown to decrease the level of reduction in relevant ROS. Similar results are observed in human erythrocytes, providing translational validity of **12**. [123]

Histone Deacetylase I

Histone deacetylases (HDACs) are a class of enzymes that act on both histone and non-histone proteins by catalyzing lysine residue deacetylation. [125-127] The acetylation and deacetylation of lysine residues is a dynamic, post-translational modification that is required for the regulation of various cellular processes. [128, 129] Primarily located in the nucleoplasm, HDAC1 acts on lysine residues within histone tails resulting in the suppression of transcription and also regulates enzyme activity through direct deacetylation. [130-132]

Belonging to Class I of the HDAC family, HDAC1 shares a common catalytic mechanism with the other members of HDAC Class I, II and IV, requiring a zinc ion for catalysis. [133] Zinc ion access is essential for activity,

with identified small molecule inhibitors exerting their effects by either binding to the zinc ion or spanning the channel to the catalytic site thereby bocking substituent access. [134] Structural studies of HDLP has conferred information into the catalytic mechanism of HDAC1 as they have nearly the same catalytic domain structure with a conservation of resides in the active site. Mutation studies identified that amino acids lining the active site (14 Å) are not only critical for the activity of the enzyme but also acetate competition. [135] Additionally, HDAC1 enzyme activity is enhanced when incorporated with silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), a co-repressor, to form a complex and regulated by inositol-tetraphosphate. [136]



Exifone
EC_{50} 0.045 μM
15

Figure 10. Chemical structure of histone deacetylase class I activator **15**, and its activity.

Exifone (**15**) (**Figure 10**), was identified as an activator of HDAC1 from *in vitro* enzymatic activity assay (EC_{50} = 0.045 μM), where the activation was identified to occur dose-dependently. [137] Furthermore, it was identified that **15** acts as a mixed, non-essential activator, such that it has the ability to bind both the free and substrate-bound enzyme. [137] Pre-treatment of **15** in induced pluripotent stem cell (iPSC)-derived neuronal cells from a frontotemporal dementia patient, followed by stress induction by rotenone, resulted in the rescue of cell viability to approximately 80%. [137] It was also identified that **15** is capable of partially reversing HDAC1 inhibition. [137] The results suggest that HDAC1 activators may provide protection against neurotrophic stress and have the potential to be developed for central nervous systems disorders.

Indoleamine 2,3-dioxygenase 1

The intracellular heme-containing enzyme indoleamine 2,3-dioxygenase (IDO1), is one of three enzymes present in the kynurenine pathway, which plays a dominant role in the conversion of tryptophan (trp), an essential amino acid in mammals, to *N*-formylkynurine (*N*-formyl Kyn) (**Figure 11**). [138-141] The rate-limiting first step in the break-down of trp is controlled by IDO1 and, this trp depletion combined with kynurenine production is involved

in the conversion of $CD4^+$ T-cells into $Foxp3^+$ regulatory T-cells. [142, 143] Due to defective IDO1 occurring in autoimmune and neuroinflammatory diseases, it is possible this enzyme provides a druggable target for these diseases. However, IDO1 inhibitors are more commonly studied for their effectiveness in cancer therapy.

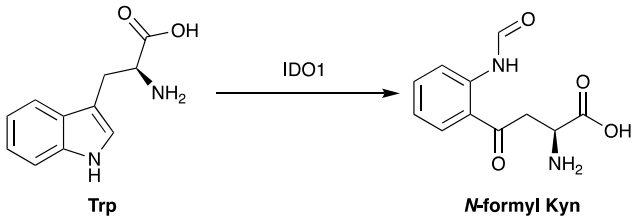


Figure 11. Conversion of trp to *N*-formyl Kyn catalyzed by IDO1.

Crystal structure data has shown IDO1 is folded into two domains, a large catalytic C terminal domain and small N-terminal domain that is not catalytically active. [144] In the large domain, IDO1 converts trp to *N*-formyl kyn using its dioxygenase activity to oxidatively cleave the 2,3 double bond of the indole moiety present in trp. [144] The literature for this enzymatic mechanism is conflicting as mechanisms propose both sequential and concerted addition of two atoms of oxygen from one oxygen molecule. [144, 145] Additionally, some literature proposed base assisted abstraction of the indole NH of trp by the protein structure whereas mutational analysis shows that no polar or charged protein side chains act as a catalytic base and therefore, proton abstraction occurs by the iron bound oxygen. [146] The small non-catalytic domain plays a part in regulation of the enzyme. Within this domain two immunoreceptor tyrosine-based inhibitory motifs (ITMs) exist which, on phosphorylation can result in upregulation of enzyme expression or enzyme proteasomal degradation. [144] The reader is directed to an excellent review covering IDO1 and its regulatory role. [147]

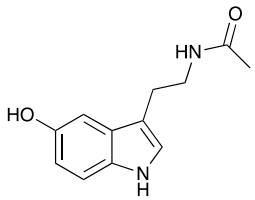


Figure 12. Chemical structure of indoleamine 2,3-dioxygenase 1 activator **16**, and its activity.

The trp metabolite, *N*-acetylserotonin (**16**) (**Figure 12**) was identified as a PAM of IDO1 (mouse IDO1 EC_{50} = 19.56 μM, human IDO1 EC_{50} = 20.95 μM) without increasing enzyme affinity for trp. [148] Furthermore, it was identified that **16** exhibits anti-ischemic and antioxidant effects and restrains neuroinflammation in mice. However, in mice lacking IDO1 expression, these neuroprotective effects were lost. [148] Although **16** up-regulates IDO1 catalytic activity in $CD11c^+$ dendritic cells, resulting in immunosuppressive effects, it does not modulate IDO1 transcripts and protein expression. [148]

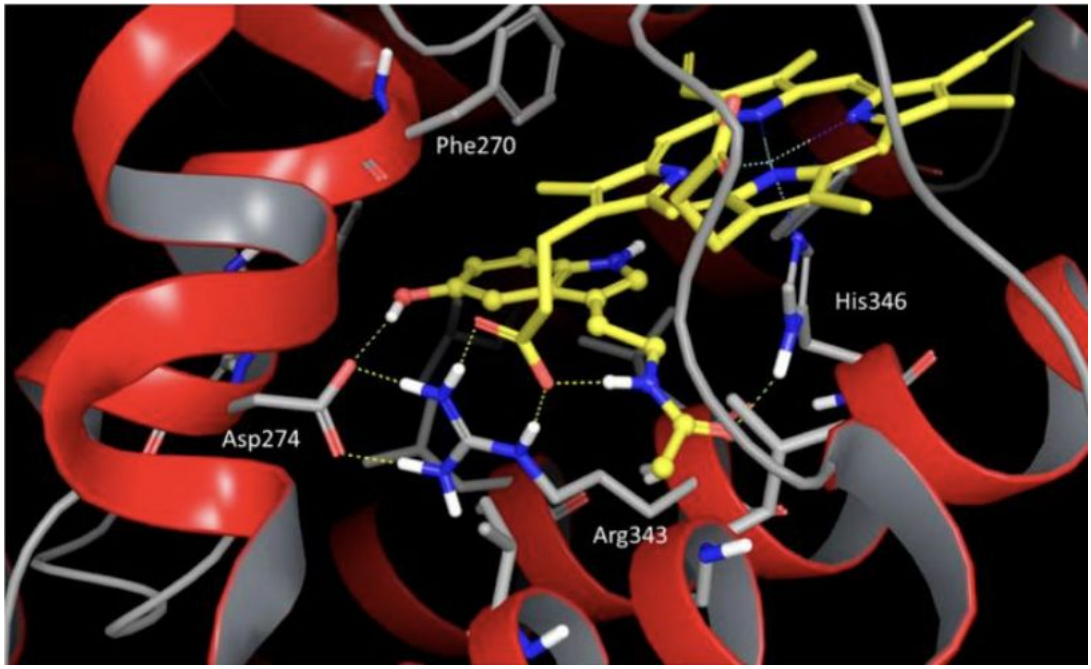


Figure 13. Best energy-scored binding mode of **16** in the allosteric pocket of IDO1 (ligand and protein are colored by atom types, with yellow carbon the former and gray carbon the latter). Key hydrogen-bond interactions are shown with yellow dashed lines. Reproduced from ref. 148 with permission from The U.S. National Academy of Sciences, copyright 2020.

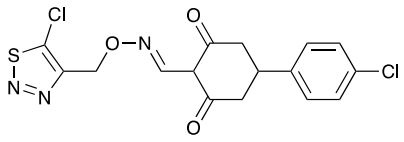
It is suggested from docking studies that **16** could bind to the allosteric pocket of IDO1 with Phe_{270} interacting through a $\pi-\pi$ edge-to-face with the indole ring and Asp_{274} by hydrogen bonding with the phenol hydroxy (**Figure 13**). [148] Due to the inactivity of similar analogues it was identified that the Asp_{274} hydrogen bond to **16** is fundamental for IDE activity. It was also identified that this Phe_{270} interaction is within the binding pocket and important for the binding of **16**, as a Phe_{270} mutant mouse model showed loss of enzyme activating effect. [148] These results highlight the potential for using IDO1 activators to treat neuroinflammation and autoimmune diseases.

Insulin-Degrading Enzyme

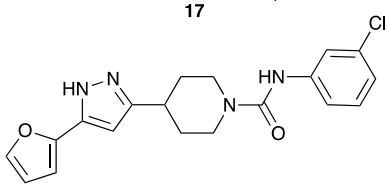
Insulin-degrading enzyme (IDE), belonging to the M16A family of metallopeptidases, is a ubiquitous, endogenous, multifunctional and multi-substrate protease. [149-151] Differentially distributed within cells and tissues, IDE primarily degrades insulin, glucagon and the protein amyloid beta (Aβ). [152-155]

Crystal structures of IDE have identified the enzyme adopting two conformations, open and closed, for the internalization, catalysis of substrates at the active site, and release of products. [156] A secondary binding site is located 30 Å away from the active site and plays a role to suitably arrange the substrates for cleavage within the active site. [157, 158] This is not the case for substrates of less than 12 amino acids as they are unable to reach between both sites. Mutagenic experiments established that chelation of zinc occurs with His_{108}, His_{112} and Glu_{189} and that Glu_{111} is responsible for the deprotonation of water in the active site to complete the coordination sphere of zinc. [159] Like many zinc metallopeptidases the activated water molecule (hydroxide ion) is used for the nucleophilic attack of the targeted peptide bond carbonyl, resulting in weakening of the peptide C-N bond, leading to peptide bond cleavage. [160-163] Computational analysis provides a four-step mechanism for IDE's degradation of Aβ40 and it is likely this mechanism is consistent for insulin and glucagon. [157] Additional to the internal active site, IDE has an allosteric site allowing ATP to attach which accelerates short peptide degradation. [164, 165] Conflictingly, ATP has also been shown to inhibit IDE. [166]

Due to the importance of IDE in the degradation of Insulin and Aβ, there is interest in identifying small molecules that activate the enzyme. Furthermore, an increase in IDE expression has been linked to tissue remodeling. [167]



LDN-1487
500% increase at 200 μM
17



LDN-1844
60% increase at 6.25 μM
18

Figure 14. Chemical structures of insulin degrading enzyme activators **17** and **18**, and their activities.

High-throughput screening identified compounds possessing the ability to interact with the allosteric site of IDE, resulting in LDN-1487 (**17**) and LDN-1844 (**18**) (**Figure 14**) being identified as activators. These activators were assessed in fluorogenic peptide substrate assay and increased activity of IDE by 500% at 200 μM and 60% at 6.25 μM for **17** and **18,** respectively. [168] It was identified that although these activators bind to the same allosteric site used by ATP, they provide a synergistic activation effect with ATP, resulting in an increase in IDE activity to ~1000% with **17**. [168] Disappointingly, these activators failed to increase the degradation of Aβ alone or in combination with ATP, however, when in combination with a short fluorogenic substrate, degradation of Aβ is increased. [168] Although it is unknown how these small molecules are activating IDE and the optimum concentration of activators are yet to be elucidated, these molecules provide a possibility for therapeutic development in Alzheimer's disease.

Lysophospholpase-like 1

Lysophospholpase-like-1 (LYPLAL1), a serine hydrolase, which although belonging to a different subclass of the lysophospholpase family, contains the same α/β hydrolase fold and catalytic triad observed in acyl protein thioesterase one and two. [169-171] Acyl protein thioesterase one and two are responsible for cleaving lipid modifications of cystine residues in proteins and it is thought that LYPAL1 has a comparable cellular function. [171, 172] Mutagenic experiments have identified that $Ser_{119,}$ Asp_{174}, and His_{208} are not only found within the active site but are essential for activity. [173, 174] These residues, although separated significantly in LYPAL1's protein sequence, orientate themselves in the ternary structure in such a way that they provide a charge relay required for catalysis. Interestingly, although LYPAL1 shows no regiospecificity for substrates, the enzyme does have size specificity due to the closed tunnel of the active site, resulting in a preference for short chain substrates. [171, 174] Genetic variants in 19 loci near the LYPLAL1 gene were associated with fat distribution, waist-to-hip ratio, and nonalcoholic fatty liver disease. [175-177]

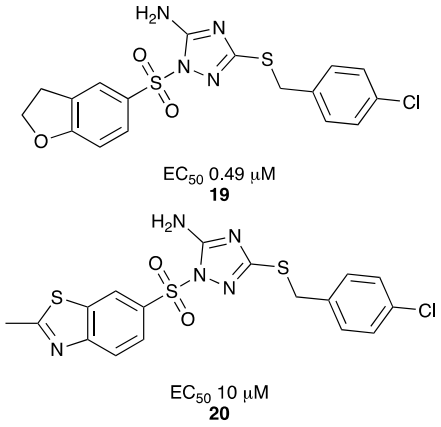


Figure 15. Chemical structures of lysophospholpase-like-1 activators **19** and **20**, and their activities.

Activators of LYPLAL1 were identified using a kinetically controlled ABPP-based fluorescence polarization assay. [24] The bidirectional assay provided the ability to identify both activators and inhibitors of LYPLAL1. Compounds showing an increase in the fluorescence polarization signal were identified as activators. The ABPP assay led to the identification of **19** (EC_{50} = 0.49 μM) (**Figure 15**), which was used as the starting point for a structure-activity relationship study to identify more potent activators, leading to the identification of **20** (EC_{50} = 0.10 μM) (**Figure 15**). [24] Activity was confirmed using a HEK293 cell-based assay (shown to over express LYPLAL1) and it was identified that the compounds enhanced activity more effectively in the complex proteome than the purified enzyme. [24] Gel filtration studies were used to identify the reversible nature of the LYPLAL1 activators. Furthermore, *in vivo* studies on DIO mice, resulted in enhanced glucose tolerance and increased insulin sensitivity, showing that these LYPLAL1 activators could be utilized for the treatment of metabolic disorders. [24]

Nicotinamide Phosphoribosyltransferase

Nicotinamide phosphoribosyltransferase (NAMPT) is responsible for the condensation of nicotinamide (NAM) and phosphoribosyl pyrophosphate (PRPP) into nicotinamide mononucleotide (NMN) (**Figure 16**), which is then converted further to nicotinamide adenine dinucleotide (NAD). [178-180] Widely distributed within the body, NAD acts as a coenzyme for hundreds of enzymes, resulting in the movement of electrons around the body via NADH. [181-186] Therefore, NAMPT is a vital enzyme to ensure sufficient levels of NAD within the body. It was

suggested that NAMPT activators can upregulate NAD presence improving neuronal cell health, memory, and cognitive function. [187-190]

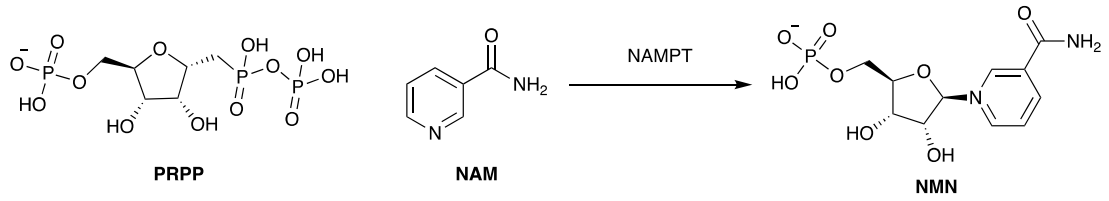


Figure 16. Conversion of PRPP and NAM to form NMN catalyzed by NAMPT.

Structurally NAMPT exists as a dimer with the active site located on the contact surface of the two monomers (known as A and B). [191] Through $\pi-\pi$ stacking NAM is positioned between $Phe_{193(A)}$ and $Tyr_{18(B)}$ of the different monomers and forms hydrogen bonds between $Asp_{219(A)}$ and $Arg_{311(A)}$, of one monomer, with the N and O atoms of the amide respectively. [191, 192] Stabilization of PRPP within the active site occurs through Ionic interactions through $Arg_{171(A)}$ and $Lys_{396(B)}$, and hydrogen bonding through $Arg_{318(A)}$ and $Ser_{214(A)}$. Through these interactions, NAM is positioned with is pyridine N towards the ribose C of PRPP for nucleophilic substitution with the assistance of the enzyme's catalytic triad residues $Ser_{280(A)}$, $His_{247(A)}$ and $Asp_{313(A)}$. [180]

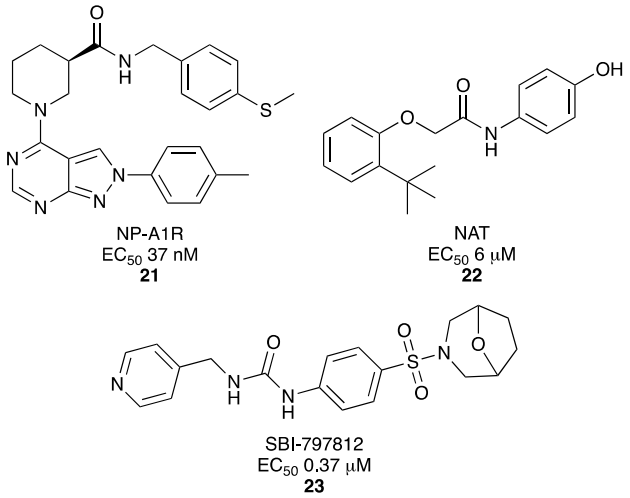


Figure 17. Chemical structures of nicotinamide phosphoribosyltransferase activators **21**-**23**, and their activities.

Compounds NP-A1R (**21**) (EC_{50} = 37 nM), NAT (**22**) (EC_{50} = 6 μM) and SBI-797812 (**23**) (EC_{50} = 0.37 μM) (**Figure 17**) have been identified as dose-dependent NAMPT activators. [193-197] High-throughput fluorescent *in vitro* screening was used to identify both **21** and **22,** whereas **23** was first identified to bind NAMPT using a

protein thermal shift assay. An additional secondary fluorometric assay identified that **23** has the potential to both activate and inhibit NAMPT, with its function dependent on concentration. [194] Furthermore, a cytotoxicity assay in U2OS cells was used to assess **22** in the presence of a NAMPT inhibitor (80% viability at 10 mM). [193] Additionally, treatment of T98G, SH-SY5Y and HepG2 cells with **22** at concentrations between 0.1-10 μM resulted in no change in cell viability and therefore, were non-toxic at the effective concentration. [195] Assessment for the ability of activator **23** to increase NAD^+ and NMN levels was undertaken in A549 human lung carcinoma cells and a dose-dependent increase was observed for both NAD^+ and NMN.

In vivo assessment of **22** (30 mg/Kg) in C57BL/6 J mice revealed that pre-treatment of **22**, prior to treatment of paclitaxel, resulted in restoration of myelin density when compared with just paclitaxel and significantly increased levels of NAD^+ in the sciatic nerve. [194, 195] Despite the high potency, tissue homogenates from C57BL/6 J mice treated with **23** (20 mg/Kg) by intraperitoneal injection only showed a significant increase in NAD^+ concentration in the liver, with other tissues showing no change in concentration. [194] It has been identified that ATP is required for **23** to activate NAMPT, and it additionally increases the affinity of NAMPT for ATP resulting in the increased efficiency of substrate capture and processing. [194]

Through crystal structures, it has been identified that these activators, similar to NAMPT inhibitors, bind to the rear channel of the active site (**Figure 18**), and that **23** may be activated by other binding mechanisms. [196] However, the Arg_{311}/Phe_{193} cation π-clamp required for inhibition is not engaged to achieve activation. [196] Additionally, hydrogen bonding (H-bonding) interaction with Lys_{189} is common for activators while NAM binding to the NAMPT active site is not required. [196]

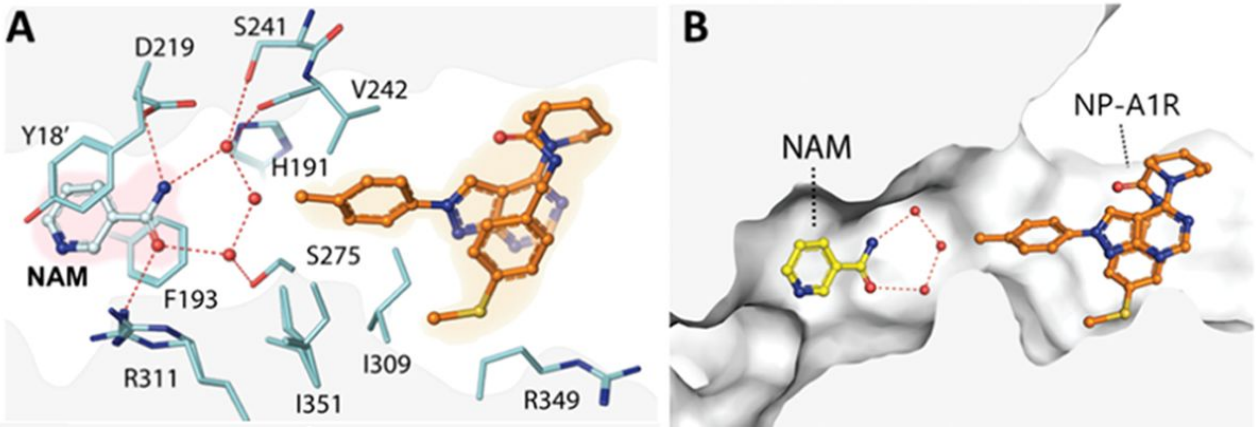


Figure 18. Crystal structure of **21** and NAMPT (A) **21** (gold) and NAM showing key residues and H-bonding network. B) **21** bound to NAMPT with NAM showing key water molecules. Reproduced from ref. 196 with permission from The American Chemical Society, copyright 2023.

These NAMPT activators may provide a means to treat diseases related to the reduction of NAD^+ which are associated with ageing and neurodegeneration. However, to date, there is yet to be data presented past simply EC_{50} values of the most potent activator **21** and further changes will be required to improve on the mediocre pharmacokinetic profiles of **23**.

Neurolysin

Peptidase neurolysin (Nln), a zinc metallopeptidase located within the mitochondria, is a member of the M3 metallopeptidases and is responsible for hydrolyzing neuropeptide bonds, specifically neurotensin to form shorter inactive fragments. [198-201] The enzyme is structured in an ellipsoid shape with a long channel running through the molecule to the active site. [201] Three residues, His_{474}, His_{478}, and Glu_{503}, within the active site co-ordinate the zinc ion. [202] Additionally, Glu_{475} provides a hydrogen bond to the zinc which assist in polarizing the catalytic quantity of water (coordinated to the zinc), to enable nucleophilic attack to the peptide bond and additionally donates a proton to the leaving chain nitrogen.

Despite the prevalence of Nln, the molecular signaling pathway responsible for the translocation of cytosolic neurolysin to the cell membranes and the mitochondrial function following stroke, remains unknown. [203] Whilst the inhibition of Nln is a mechanism that has been exploited for the design of therapeutic agents to target acute myeloid leukemia, [204] it has recently been identified that the activation of Nln may provide a route to identify therapeutics for the treatment of neurodegenerative diseases, including ischemic stroke. [203, 205, 206,

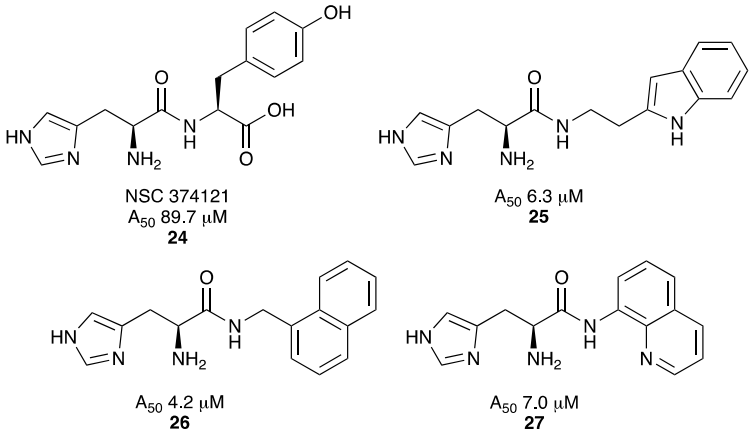


Figure 19. Chemical structures of neurolysin activators **24**-**27**, and their activities.

Activator NSC 374121 (**24**) (**Figure 19**) was identified as a hit from a virtual screen with an A_{50} value of 89.7 μM. Detailed SAR studies identified analogues **25-27** with increased activities over the original hit (AC_{50} of 6.5 μM, 4.2 μM and 7.0 μM for compounds **25**, **26** and **27** respectively). [207-210] *In vitro* metabolic stability studies reported that each of the three analogues had significantly increased half-lives in mouse plasma compared to the original hit, which reported a half-life of 34.19 minutes, in comparison to compounds **25** and **26**, which presented the best half-lives of >1000 minutes. [205] The permeabilities of each of the compounds were obtained within the MDR1-MDCA cell line, in which **26** and **27** presented the best permeability. Compound **26** presented a permeability greater than 15 $x10^{-6}$ cm/s, while **27** presented a permeability of 20 $x10^{-6}$ cm/s in the basolateral to apical direction, suggesting a higher efflux ratio, allowing for increased drug concentrations within the brain. The identified compounds were also assessed for their neurolysin selectivity over the related peptidases thimet oligopeptidase (TOP), neprilysin, and ACE/ACE2, in which all three showed high selectivity towards the target enzyme neurolysin.

Furthermore, *in vivo* assessment on C57BL/6 (stroke-induced) mice brain tissue samples, identified a higher concentration of **27** within damaged brain tissues than in undamaged and a higher localized drug concentration in the ischemic hemisphere over the non-ischemic hemisphere and control tissue samples. [209] It was hence determined that compound **27** would advance to further development. This data highlights the potential for NIn activators to be used for the treatment of stroke and potentially other neurodegenerative diseases.

8-Oxoguanine DNA glycosylase

The bifunctional enzyme 8-oxoguanine DNA glycosylase (OGG1) catalyzes both glycosylase and abasic (AP) lyase reactions, with the latter being the rate-limiting step. [211-213] Mutagenic experiments have identified key residues in the active site that are crucial for activity, resulting in multiple postulated mechanisms. [214] Firstly, Lys_{249} acts as a catalytic nucleophile in the glycosylase reaction, this allows for a covalent enzyme-DNA interaction which can undergo rearrangement. [215] Additionally, Asp_{268}, Cys_{146} and Cys_{255} Cys_{140}, Cys_{163}, Cys_{241}, and Cys_{253} have also all been identified as key residues for enzyme activity. [214, 216, 217]

The enzyme is responsible for performing the first rate-limiting step in the DNA damage control pathway that replaces the premutagenic base, 8-oxo-7,8-dihydroguanine (8-oxo-G) with guanine and protecting against

the transversion of G-C base pair to T-A within DNA. [218, 219] It has been suggested that OGG1 activation may result in enhanced base excision repair in cells that are undergoing repetitive oxidative stress.

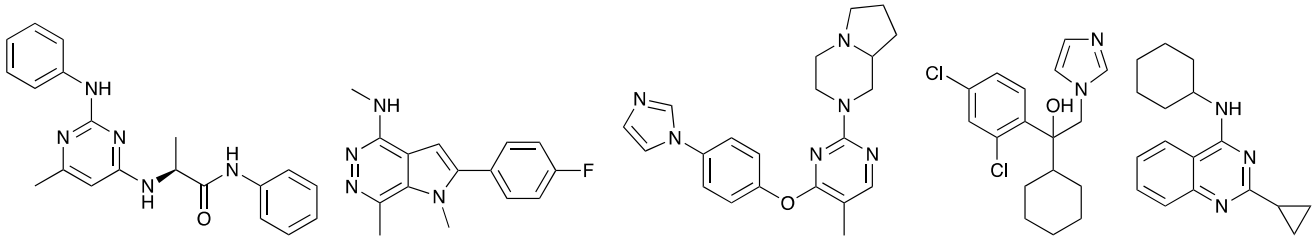


Figure 20. Chemical structures of 8-oxoguanine DNA glycosylase activators **28**-**32**, and their activities.

Several diverse small molecules **28-32** (**Figure 20**) have been identified as activators of OGG1 in a fluorescent kinetic assay with EC_{50}'s of 4.1 μM, 5.1 μM, 4.6 μM, and 5.0 μM respectively, for **28-31,** and an AC_{50} of 0.78 μM for **32**. [213, 220, 221] These compounds were also assessed for their ability to reduce A549 cell injury caused under oxidative challenge, accumulation of 8-oxo-G, and associated induced cytotoxicity. This experiment identified compounds **29** and **31** can reduce cell injury by oxidative damage (by 200% and 650%, respectively). These compounds were also identified to reduce cytochrome c concentration (reduction of mean nuclear intensity by 550 for both **30** & **31** at 30 μM), which is associated with mitochondrial membrane integrity and DNA damage. [213] It has further been reported that mitochondrial DNA levels in mouse embryonic fibroblasts were recovered at a faster rate when treated with **31** than alone when cells are subjected to hydrogen peroxide. [221] To identify the mechanism in which **32** activates OGG1 *in vitro,* DNA repair studies and cocrystal structure analysis were undertaken. These studies found that **32** binds to the catalytic site to achieve this activation, and that **32** acts as an essential cofactor by increasing the enzyme activity through stimulation of substrate turnover. [221] The OGG1-**32** co-crystal structure shows the cyclopropyl ring sitting within a shallow pocket of the site, π-stacking interactions between the quinazoline ring and Phe_{319}, and hydrogen bonding interactions between both the secondary amine and Gly_{42}, and pyrimidine-N and Asp_{268}. Finally, **32** activates OGG1 by enhancing β-elimination and inducing δ-elimination, which allows for apurinic/apyrimidinic sites as new substrates. [221] These OGG1 activators could be used to better understand or treat diseases associated with increased oxidative stress

or reduced OGG1 function, including Alzheimer's disease, atherosclerosis and chronic obstructive pulmonary disease. [222-224]

Phosphodiesterase-4

Phosphodiesterase-4 (PDE4) is one of 11 different cyclic nucleotide phosphodiesterase (PDE) families. [225] PDE4 is constituted by four genes and, due to the various splicing sites, both long and multiple short PDE4 proteins can be formed, resulting in at least 20 different isozymes that differ in their N-terminus. [226, 227] These enzymes are responsible for specifically hydrolyzing cyclic adenosine monophosphate (cAMP) to Adenosine monophosphate (AMP) (**Figure 21**), thereby controlling cAMP homeostasis in the body. [226] Therefore, this enzyme plays an important role in regulating many key physiological processes in the body. [228-230]

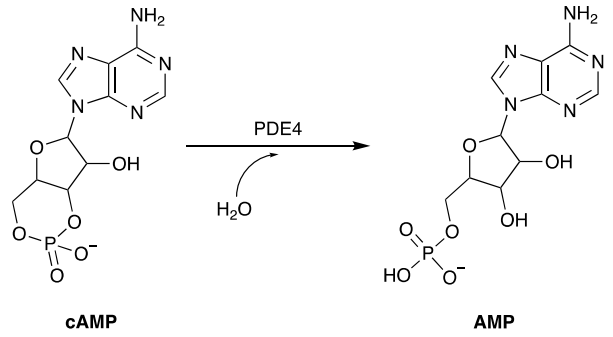


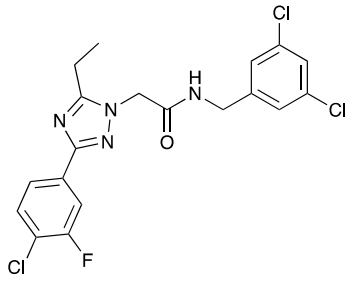
Figure 21. Hydrolysis of cyclic adenosine monophosphate to Adenosine monophosphate catalyzed by PDE4.

All PDE4 isoforms show high conservation in the catalytic domain, however isoforms are categorized by the prescence of absence of upstream regulatory conserved regions one (URC1) and two (URC2). The super short isoforms contain only part of URC2, short contain only URC2 and long isoforms possess both URC1 and URC2. [231] The amount of the UCR regions present in the isoforms provide for functional differences between them. For long form PED4, the UCR regions allow for the regulation of the enzyme through phosphorylation by protein kinases. [228, 232] The interaction between the PDE4 URC2 region and the catalytic domain regulates activity by either limiting access to the active site, altering the product release, or both. [231]

The catalytic pocket of PDE4 can be split into two sub-pockets: one for binding of bivalent metals and the other for substrates. The bivalent metal sub-pocket is occupied by two ions, zinc plus another believed to be magnesium. [233, 234] This additional ion is shown to be important for the enzyme catalytic activity. However, while

the zinc ion co-ordinates four amino acid residues of the protein, His_{164}, His_{200}, Asp_{201} and Asp_{318}, plus two water molecules, the other ion interacts with only one of the amino acid residues, Asp_{201}, and the five remaining interactions are with water molecules. Co-ordination between the metal ions of the active site, and the two phosphate oxygen atoms of AMP, results in the displacement of two water molecules from the active site. [234] Additionally, AMP forms hydrogen bonds in the active site with His_{160}, Asp_{201}, and Asp_{318} residues.

It has been proposed that catalytic activity occurs through the nucleophilic attack of the phosphodiester bond by a water molecule or a hydroxide ion. [235] The phosphodiester bond can become polarized through a hydrogen bonding interaction with a nearby His_{160} residue thereby facilitating the nucleophilic attack.



MR-L2
EC_{50} 1.2 μM
33

Figure 22. Chemical structure of phosphodiesterase-4 activator **33**, and its activity.

MR-L2 (**33**) (**Figure 22**) was identified as an activator of specifically long PDE4 isoforms in a dose-dependent manner, exhibiting an EC_{50} of 1.2 μM for PDE4D5, and increased potency for the PDE4A4, PDE4B1, PDE4C3 isoforms. [236] The activation of PDE4 elicited by **33** is comparable to that of protein kinase A (PKA) phosphorylation, suggesting that **33** and its analogues phenocopy the PKA activation of PDE4. It is important to note this activation is not additive to PKA activation. [236, 237] Furthermore, **33** was unable to activate short PDE4 isoforms which lack the UCR1 domain, suggesting that the activation occurs due to the interaction with this segment of the enzyme. [236] Confirmation that the activation occurs at the UCR1 domain of the enzyme was achieved as a peptide of amino acids 80-136 from PDE4D long form denoted UCR1C was able to activate long form PDE4. [238]

This PDE4 activator has been shown to decrease intracellular cAMP concentrations in MDCK cells. [238] Long form PDE4 activators have the potential to be used in diseases where cAMP elevation is important in the

molecular pathology and where PKA phosphorylation is diminished such as, autosomal dominant polycystic kidney disease. [238]

Procaspase-3

Procaspase-3 is a zymogen homodimer of caspase-3 which, is responsible for the hydrolysis of many cellular substrates rapidly inducing apoptotic cell death. Proteolysis of Procaspase-3 results in activation of the enzyme within the cell to form caspase-3. [239, 240] An initiator caspase (caspase-8 or caspase-9) achieves this activation by internal cleavage to separate the large and small subunits and is subject to strict cellular regulation. [239, 240]

The structure of procaspase-3 is very similar to mature caspase-3 enzyme however, it is the position of the inter-subunit linker (L2) in procaspase-3 that prevents a key residue, Asp169, from its active caspase-3 conformation which stabilizes the 'loop bundle' (an allosteric activator of caspase-3). [241] In mature caspase-3 the His-Cys dyad is responsible for procaspase activity and yet stimulation of procaspase-3 activity occurs at slightly acidic pH. This is somewhat contradictory as at higher pH protonation of the dyad would occur thereby limiting its participation in the hydrolysis.

In certain cell types isolated from cancerous tissue, procaspase-3 concentration is elevated; this provides a means for these cancer cells to evade apoptosis and proliferate, specifically lung, melanoma, renal and breast cancers. [242-245] Therefore, reactivation of apoptotic cascades by procaspase-3 small molecule activators could provide an alternative cancer therapy. [246, 247]

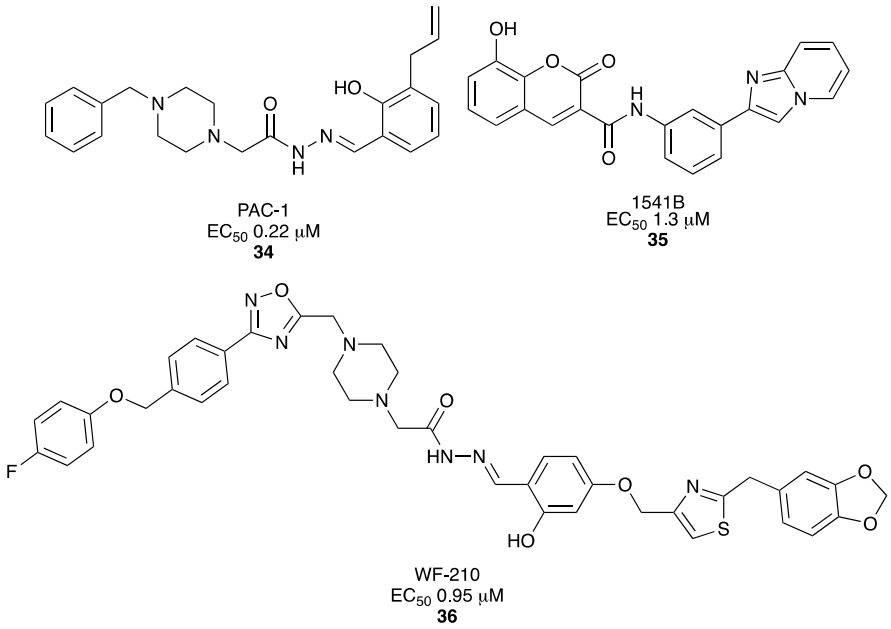


Figure 23. Chemical structures of procaspase-3 activators **34**-**36**, and their activities.

Compounds **34** (EC_{50} = 0.22 μM), **35** (EC_{50} = 1.3μM) and **36** (EC_{50} = 0.95μM) (**Figure 23**) have all been identified as activators of procaspase-3. [247-249] Both **34** and **36** can achieve procaspase-3 activation by the sequestration of inhibitory zinc ions. [249, 250] However, coumarin derivative **35** activates by promoting the auto maturation of procaspase-3 to caspase-3. [248] Structural studies have identified that for **34** and its analogues three moieties are critical for activity: the allyl group, phenol hydroxy and benzyl moiety. Analogues of **35** indicated that the imidazo[1,2-a]pyridine moiety was important for selectivity of procaspase-3 over procaspase-6.

All three of these small molecule activators display anticancer activity by inducing apoptosis in cell culture (**34** assessed in U-937, NCI-H226, HL-60, SK-N-SH and PC-12 cells, **35** assessed in BT549, MDA-MB361, HEK293, HeLa and HCC1954 cells and **36** assessed in A549, COLO 205, DU-145, NCI-H226, MCF-7, Hep 3B, HGC-27, K562, Hep-G2, U87, GBC-SD, MDA-MB-435, PC-3, U-937, HL-60, MCF-10A, PBL, L-02 and HUVEC cells) with an effect directly related to the concentration of procaspase-3. [243, 247-249] Furthermore, mouse xenograft models showed that **35** and **36** significantly inhibited the growth of Hep3B, ACHN, and NCI-H226 cancerous cell lines *in vivo*. [247, 249] Finally, it has been shown that **34** and **35**, which activate procaspase-3 by different mechanisms, can be combined to synergistically increase apoptosis in U-937, BT-549, A549, HL-60, Hs578T, U-87, and EL4 cells. [251] Following on from positive results in mice, **34** has been assessed and approved for the treatment of cancer in dogs. Treatment of **34** (25 mg/kg) or in combination with doxorubicin (Dox) (10.9-12.5/25

mg/kg **34**/Dox) was well tolerated for the treatment of canine lymphoma and metastatic osteosarcoma resulting in tumor regression in 1/6 patients (sole treatment of **34**) and 2/3 patients (oral daily treatment of **34** in combination with Dox every 14 days). [252, 253] The promising results in cells, rodents and canines has resulted in the assessment of **34** in multiple human clinical trials. The phase I dose escalation study (NCT02355535) on 48 patients with neuroendocrine tumors identified an optimum dose of 750 mg/day. [254, 255] Additionally, positive responses were observed during the trial with two out of five patients achieving a durable partial response and therefore warranting further investigation of **34**. [255] Following on from this, a second study (NCT03332355) was undertaken to assess **34** in combination with temozolomide on 18 patients with high grade glioma: glioblastoma multiforme (GBM) or anaplastic astrocytoma. [256] This study was terminated in 2021 and no results have been released. Finally, an additional clinical trial pilot study (NCT03927248) was initiated to assess the combination of **34** and nivolumab in patients suffering with metastatic renal cell carcinoma. [257] However, recruitment was withdrawn due to funding issues.

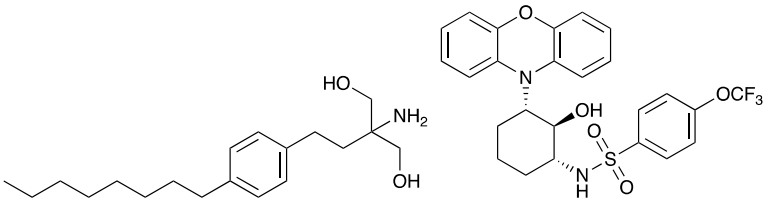
Protein Phosphatase 2A

Protein phosphatase 2A (PP2A), a heterotrimeric serine-threonine phosphatase, is responsible for the controlled activity of serine-threonine phosphorylation within eukaryotic cells and suppressing cell transformation. [258] There are 80 predicted structural variations of PP2A found throughout all cell types. [259] This is due to the heterotrimeric enzyme being composed of a scaffold subunit A, regulatory subunit B, and a catalytic subunit C, where subunits A and C can adopt two distinct isoforms and B can adopt five distinct isoforms. [260] The core dimmer of PP2A isoform consists of the catalytic subunit and scaffold subunit, the role of the scaffold subunit in this dimer is to mediate formation of the holoenzyme through recruiting additional subunits that dictate the function of each PP2A variation. Within the active site of the enzyme are two manganese ions. To accommodate different proteins PP2A's holoenzyme adapts its scaffold by recruiting different regulatory subunits which results in a variation in the active site for the required protein substrates. [261]

It is recognized that PP2A plays a pivotal role in maintaining normal homeostasis as it is involved in various signaling pathways. Furthermore, PP2A is a tumor suppressor and, therefore, the over-inhibition or inactivation of this enzyme leads to an uncontrolled level of cell transformations, which ultimately accounts for the development of multiple cancers, such as colorectal, breast, and skin cancer. [259, 262, 263] The proposed

mechanisms of PP2A inactivation include phosphorylation, methylation, or even mutations to subunit C, preventing catalytic activity, as well as the upregulation of PP2A endogenous inhibitors. [264] Therefore, the identification of PP2A activators provides an opportunity for cancer therapeutic discovery.

While the mechanism of PP2A activation is still debated, it has been proposed that through the direct binding of small-molecule activators to subunit A, a conformational change is induced, thus allowing access to the catalytic subunit C. [265]



FTY720
CIP2A IC_{50} 2.9. μM
37

DT-061
CIP2A IC_{50} 8.5 μM
38

Figure 24. Chemical structures of protein phosphatase 2A activators **37** and **28**, and their activities (represented as the inhibition of phosphorylation and CIP2A expression).

Both FTY720 (**37**) and DT-061 (**38**) (**Figure 24**) have been identified as activators of PP2A. [260] The treatment of MDA-MB-231 and BT-474 cells with **37**, both of which naturally express an endogenous PP2A inhibitor, can restore the activity of PP2A resulting in the inhibition of phosphorylation and CIP2A expression (IC_{50}'s of 2.9 and 8.5 μM respectively), which in turn reduces cell growth and increases caspase-dependent apoptosis. [266] Treatment of H3255 cells with **38** produced an IC_{50} of 12.4 μM, whereas treatment of the HCC837 cell line produced an IC_{50} of 14.3 μM. [260] Furthermore, treating tyrosine kinase inhibitor-resistant cell lines with these PP2A inhibitors resulted in an IC_{50} of 10.6 μM which was validated by the decreased ability for colonies to form. It was also identified that the PP2A activation with **37** induced caspase-dependent apoptosis, proven by a five-fold increase in caspase activity.

These activators have been assessed for their anti-cancer properties *in vivo*. Tumor growth was inhibited by 24% upon treatment of an EGFR/CCSP mouse model with **37** (100 mg/kg/48h). This was confirmed by immunohistochemical studies on the isolated tumor tissue, which identified increased apoptosis, decreased proliferation, and increased dephosphorylation of phosphorylated Protein Kinase B (pAKT). [266] Furthermore, these PP2A activators have been evaluated in combination with the chemotherapeutic agents Doxorubicin and

Afatinib. The H1975 xenograft mouse model experienced over a 2-fold decrease in tumor growth, compared to control when **38** was used in combination with Afatinib, showing the additive potential of **38** with existing chemotherapeutic treatments. Similar effects were also reported when used in combination with 5-fluorouracil, Irinotecan, and Oxaliplatin, for the treatment of colorectal cancer. [262]

It is important to highlight that although these PP2A activators show potential in the treatment of numerous cancers, the overexpressed levels of PP2A is also linked to the hyperphosphorylation of tau, which is a hallmark of Alzheimer's disease. [258] Furthermore, increased PP2A expression has also been suggested to have an active role in the development of Parkinson's and dementia with Lewey bodies. [267]

Soluble Guanylate Cyclase

Soluble guanylate cyclase (sGC), a heme-binding $\alpha\beta$-heterodimer, is a nitric oxide (NO) sensor that, when in the presence of NO, becomes activated resulting in the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (**Figure 25**). [268-271]

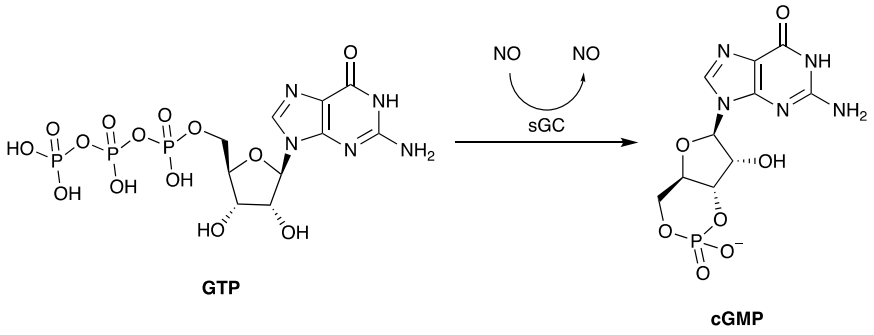


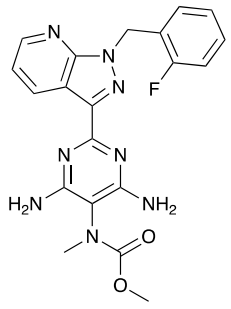
Figure 25. Conversion of GTP to cGMP catalyzed by sGC in the presence of NO.

The enzyme contains four domains: NO-sensor domain, Per-ARNT-Sim domain (PAS), coiled-coil domain, and catalytic domain. [272-274] The NO-sensor domain contains a heme cofactor to sense the ligand, His_{105}-Fe binding is severed on NO binding to Fe resulting in activation of response proteins. The PAS domain passes signals to other domains by shifts in its conformation and the coiled-coil domain acts as a scaffold for other domains and assist in heterodimerization of the enzyme. [275] The Catalytic domain contains the active site and on binding of NO to the NO-sensor domain Fe, the binding cleft is opened. [273, 276] Mechanisms for activation have been proposed but to date remain unknown. One proposed mechanism is that after substrate binding the active

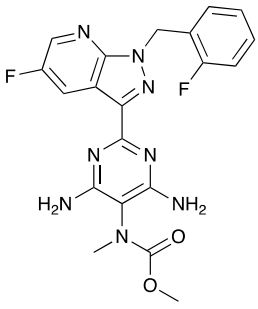
site closes allowing the amino acid residues required for catalysis to be aligned with the substrate, and that during the closing of the active site discrimination between ATP and GTP occurs. [274] Structural studies have identified that sGC has the ability to bind two NO molecules, at low NO concentrations the enzyme exhibits minimal or no activity; however, on increasing NO concentration the enzyme becomes fully active. [277]

The importance of cGMP as a messenger that regulates through the activation and alteration of specific ion channels is well documented. [278-280] The levels of cGMP are critical in homeostasis, and reductions of cGMP has been linked to multiple diseases including cardiovascular disease, [279, 281, 282] kidney disease, [283] preeclampsia, [284] heart failure, [285, 286] and erectile dysfunction. [287, 288] Therefore, methods to restore or increase cGMP levels through the modulation of enzymes is an area of interest for pharmaceutical research. [289]

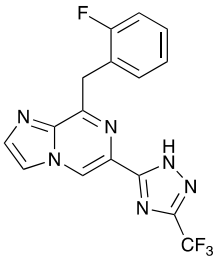
Decreased NO availability is a contributing factor to neurodegenerative disease and consequently results in cGMP deficiency. [290] To reverse this deficiency caused by NO dysregulation, drugs targeting sGC are a promising solution. There are two methods to increase sGC enzymatic conversion of cGMP to GTP; stimulation, and activation with the difference being haem dependence. [291] Stimulators bind directly to sGC acting independently but synergistically of NO. [291] Whereas activators of sGC bind to the unoccupied haem-binding pockets mimicking the NO-bound haem. [291]



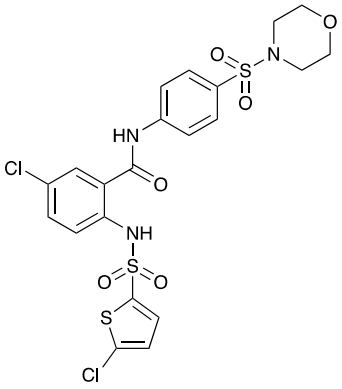
Riociguat
73-fold increase in activity
39



Vericiguat
EC_{50} 1005 nM
40



CY6463
EC_{50} 66 nM
41



Ataciguat
EC_{50} 0.5-1 μM
42

Figure 26. Chemical structures of soluble guanylate cyclase activators **39**-**42**, and their activities.

The two sGC stimulators, Riociguat (**39**) (up to 73-fold increase in sGC activity) and Vericiguat (**40**) (EC_{50} of 1005 nM) (**Figure 26**), have been approved by the FDA for treating cardiovascular indications. [292-295] These two analogues were identified to activate sGC independently of NO however, the haem component of the enzyme was key for these compounds to possess stimulation activity. [296] Following on from the identification of **39** and **40**, CY6463 (**41**) (**Figure 26**) was also identified as a novel CNS-penetrant sGC stimulator (EC_{50} = 66 nM). [297]

Due to the links between sGC and neurodegenerative diseases several *in vivo* experiments were undertaken to probe the relationship and the ability of **41** to be used for the treatment of various neurodegenerative diseases. [298, 299] Functional magnetic resonance imaging studies *in vivo* (Sprague Dawley rats) using a blood-oxygen-level-dependent contrast reveal that **41** (1.0 mg/kg) produces increased blood flow in areas associated with memory and arousal; nuclei, midbrain dopaminergic system, the hippocampal complex and anterior cerebellum, indicating an increase in neuronal activity. [297] Furthermore, treatment with **30** (3 mg/kg and 10 mg/kg) showed a significant increase in cGMP (4-fold). [297] In the Huntington's Disease mouse model R6/2, tissue slices treated with **41** (46 nM or 308 nM, 15 minutes of exposure) completely restored the long-term potentiation deficits, highlighting the ability of **41** to modulate synaptic plasticity in a diseased state. [297] The stimulator also restored the levels of *N*-acetylaspartate-glutamate, which is known to decrease in those suffering from neurodegenerative diseases. [297] Compound **41** also reduced hippocampal dendritic spine loss in an Alzheimer's disease mouse model (APP/PS1). [297]

In a screen for novel sGC activating compounds Ataciguat (**42)** (**Figure 26**) was identified as a sGC activator with an EC_{50} = 0.5–10 μM. [300] Furthermore, **42** showed an increase in efficacy when in the presence of NO-sensitive sGC inhibitors suggesting this small molecule activates the NO-insensitive heme-oxidized form of sGC. [300, 301] Four clinical trials have been conducted for **42** investigating its efficacy and safety for the treatment of calcific aortic valve stenosis (CAVS) (NCT02481258, NCT02049203), stage II peripheral arterial disease (NCT00443287), neuropathic pain (NCT00799656). [302-305] To date, only results from the CAVS study has been reported indicating an improvement of disease symptoms.

Although stimulators of sGC are favored over activators, as they act upon the reduced form of sGC which is critical in cGMP products, both have provided desirable therapeutic outcomes. [297] The FDA approval of sGC stimulator Vericiguat, to reduce the risk of cardiovascular death and heart failure, and Riociguat, to treat two forms of pulmonary hypertension, shows the potential of these molecules across multiple diseases.

Conclusion

Despite the challenges in identifying small molecule enzyme activators over inhibitors, a detailed analysis of the literature highlights the potential of this method of modulation to provide not only new methods for treating diseases but also new targets. A search of the Scifinder database for the term 'small molecule enzyme activators' demonstrates the evolution of small molecule enzyme activators has been gaining pace over the past two decades, with particularly high activity occurring from 2020 onwards. This trend is observed for both journal articles (**Figure 27A**) and patents (**Figure 27B**), indicating the potential that these compounds have for use in the future. Perhaps, it is the potential observed by some of the early activators that has resulted in increased focus on the area.

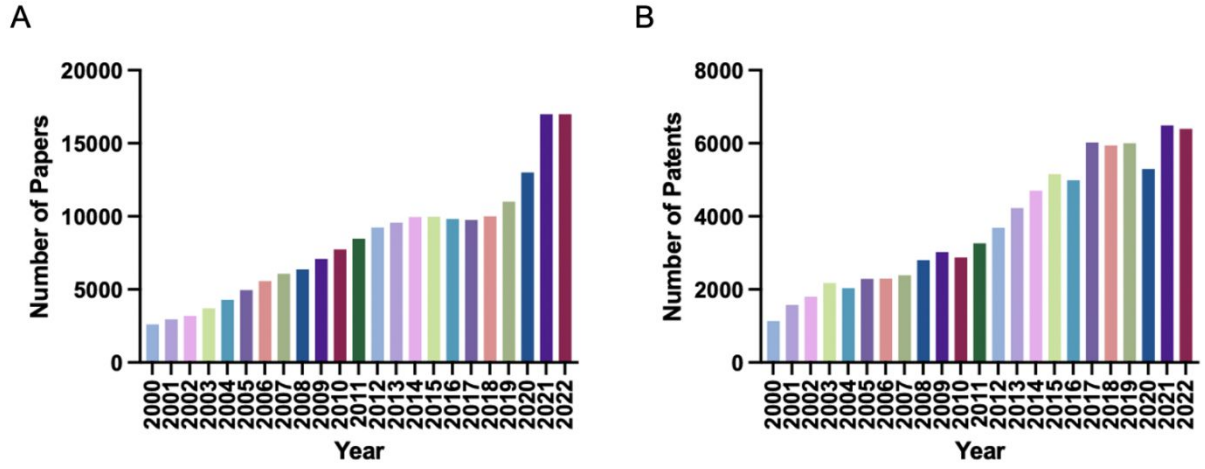


Figure 27. Graphs showing the trend in journal articles and patents featuring the search term 'small molecule enzyme activators' from 2000 to 2022. A) Publication of journal articles, excluding reviews articles. B) Publications of Patents (Note: both graphs include research on kinase activators that have not been discussed in this review).

Notwithstanding the compounds that have already received clinical approval, many of the described non-kinase enzyme activators provide clear potential for development into therapeutics due to the promising *in vitro*

and *in vivo* results that have been described. Specifically, the procaspase 3 activator PAC-1 (**23)** has progressed to clinical trials for human cancer therapies (while being deployed in canine patients) and the sGC activators Riociguat (**28**) and Vericiguat (**29**) are approved for the treatment of cardiovascular ailments. Still other compounds discussed herein represent critical chemical probes and tool compounds for the elucidation of signaling pathways in healthy and diseased models that represent potential for further development.

We have highlighted a wide range of diseases that these activators have the potential to be used for including diabetes, cardiovascular, cancer and neurodegeneration. Due to the saturation of inhibition research and development with much of the low handing fruit already picked, we expect more small molecule activators to progress through pipelines to clinical trials providing new means to combat disease. This review has described early successes of small molecule enzyme activators, yet there is still much to learn about the art of identifying, optimizing, and deploying these types of compounds into the pharmaceutical arsenal and their continued evolution.

Author Contributions

The idea was conceived by PCT. The manuscript has been written and edited by the combined effort of all authors.

Conflicts of Interest

PCT is a named inventor on US Patent App. 17/186,489 describing neurolysin activators discussed herein.

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