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Small Molecule Regulators Targeting NAD+ Biosynthetic Enzymes

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Abstract

Nicotinamide adenine dinucleotide (NAD^+) is a key player in many metabolic pathways as an activated carrier of electrons. In addition to being the cofactor for redox re-actions, NAD^+ also serves as the substrate for various enzymatic transformations such as adenylation and ADP-ribosylation. Maintaining cellular NAD^+ homeostasis has been suggested as an effective anti-aging strategy. Given the importance of NAD^+ in regulating a broad spectrum of cellular events, small molecules targeting NAD^+ metabolism have been pursued as therapeutic interventions for the treatment of mitochondrial disorders and age-related diseases. In this article, small molecule regulators of NAD^+ biosynthetic enzymes will be reviewed. The focus will be given to the discovery and development of these molecules, the mechanism of action as well as their therapeutic potentials.

Keywords

Nicotinamide adenine dinucleotide (NAD^+); metabolic pathways; electrons; redox reactions; enzymatic transformations; therapeutic potentials

1. INTRODUCTION

Nicotinamide adenine dinucleotide (NAD^+), best known for its role in redox biology, serves as the substrate for a group of NAD^+-utilizing enzymes such as sirtuins (NAD^+-dependent protein deacylases) [1], poly(ADP-ribose) polymerases (PARPs) [2, 3], and ADP-ribosyl

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CONFLICT OF INTEREST

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cyclases [4]. These enzymes catalyze the cleavage of the *N*-glycosidic bond to remove the nicotinamide (NAM) moiety and subsequently transfer the ADP-ribosyl group to other small molecules or protein targets. NAD^+-utilizing enzyme-mediated cellular processes play important roles in maintaining genomic integrity [5], regulating gene transcription [6], promoting DNA repair, as well as controlling cell proliferation and differentiation [7, 8]. Additionally, NAD^+ and its reduced form, NADH, have been suggested as inhibitors of mitochondrial permeability transition pore (mPTP), the opening of which leads to the disruption of mitochondrial membrane homeostasis and triggers cell death through apoptosis or necrosis [9–11]. Both matrix [12, 13] and external NAD(H) [9] suppressed the opening of mPTP at millimolar concentrations to maintain the integrity of the mitochondrial membrane.

Given the importance of NAD^+ in a myriad of cellular events, it is not surprising that the intracellular NAD^+ content needs to be tightly and precisely regulated. In mammalian cells, NAD^+ level is maintained through the coordinated actions of complementary *de novo* [14], salvage [15], and nicotinamide riboside (NR) [16] biosynthetic pathways. The *de novo* pathway (Fig. 1), also known as the kynurenine pathway, starts with tryptophan which can be oxidized to *N*-formylkynurenine by either tryptophan 2,3-dioxygenase (TDO) [17] or indoleamine 2,3-dioxygenase (IDO) [18]. Further hydrolysis of *N*-formylkynurenine by arylformamidase leads to the formation of L-kynurenine. Three consecutive reactions catalyzed by kynurenine 3-monooxygenase (KMO), kynureninase (KYU), and 3-hydroxyanthranilate 3,4-dioxygenase (HAO) are required to convert kynurenine to 2-amino-3-carboxymuconate semialdehyde, which undergoes spontaneous cyclization to quinolinic acid [19]. Subsequently, quinolinic acid is coupled with 5-phosphoribosyl-1-pyrophosphate (PRPP) by the action of quinolinate phosphoribosyl transferase (QPRTase) to produce nicotinic acid mononucleotide (NaMN) [20]. The adenylation of NaMN catalyzed by nicotinamide/nicotinic acid mononucleotide adenylyltransferase (NMNAT) forms nicotinic acid adenine dinucleotide (NaAD). Completion of NAD^+ synthesis is accomplished by amidation of NaAD to form NAD^+ catalyzed by NAD^+ synthetase.

The salvage pathway recycles NAD^+ degradation products to reconstitute NAD^+ (Fig. 2). NAM is coupled with PRPP by nicotinamide phosphoribosyl transferase (NAMPT), the rate-limiting enzyme of the pathway, to form nicotinamide mononucleotide (NMN). Ultimately, NMN can be adenylated to NAD^+ by NMNAT. Similarly, nicotinic acid (NA) is avidly recycled by nicotinic acid phosphoribosyl transferase (NaPRTase) to NaMN through the Preiss-Handler pathway [21]. NaMN thus formed can then enter the *de novo* pathway to be further transformed.

The NR pathway was discovered more than a decade ago (Fig. 3) [16, 22]. NR, a natural metabolite and an ingredient in milk, is phosphorylated by nicotinamide riboside kinases (NRK1/NRK2) to NMN and eventually to NAD^+. Recent studies suggest that NR is one of the most potent agents to stimulate NAD^+ production in cells [23–25]. NR is water-soluble and cell-permeable with no apparent toxicity [26]. All of these features render NR an ideal candidate for the NAD^+ boosting campaign.

There is a renewed interest in understanding NAD^+ metabolism because increasing cellular NAD^+ contents has been suggested as a potential anti-aging strategy [27–29]. During

the normal aging process, the NAD^+ level decreases as a result of oxidative stress, reduced mitochondrial biogenesis, and imbalanced synthesis and consumption of NAD^+ [30–32]. This decline has been associated with age-related diseases such as metabolic disorders, neurodegenerative diseases, and cancer. Exercise, calorie restriction (CR), and supplementation with NAD^+ precursors are known to augment intracellular NAD^+ concentrations [33–37], which may slow down or even reverse the aging process and delay the progression of age-associated disorders. From herein, we will provide an overview of the discovery and development of small molecule modulators targeting NAD^+ biosynthetic enzymes. The design and optimization of some representative cases and their potential therapeutic applications will also be discussed.

2. MOLECULES TARGETING NAD^+ BIOSYNTHETIC PATHWAYS

Maintaining NAD^+ homeostasis is essential for cell survival and growth. Pharmacological interventions of the above-mentioned NAD^+ biosynthetic pathways have been intensely pursued as therapeutic treatments for mitochondrial dysfunction and age-related disorders. It is important to point out that although NR has attracted great interest as a potent NAD^+ boosting agent, there have not been many advances in identifying small molecule regulators of NRKs. Our discussion will be focusing on *de novo* and salvage pathway enzymes, particularly those with known regulators.

2.1. Compounds targeting *de novo* NAD^+ biosynthesis

2.1.1. IDO Inhibitors—The heme enzyme IDO catalyzes the oxidation of tryptophan by molecular oxygen to form *N*-formylkynurenine [38, 39]. IDO is distinct from TDO in its tissue expression pattern, substrate specificity, and inducibility. It is highly expressed in non-hepatic tissues [40] and has a broader specificity as it triggers the oxidative cleavage of indole moiety in tryptophan, 5-hydroxytryptophan, and serotonin [41]. IDO can be induced by inflammatory stimuli such as interleukin-1 (IL-1) [42], tumor necrosis factor (TNF) [43], and bacterial lipopolysaccharide (LPS) [44]. It has been implicated in mediating tumor immune escape and suggested as an oncology target [45, 46].

Most of the IDO inhibitors are substrate analogs. For example, 1-methyltryptophan, β-(3-benzofuranyl) alanine, and β–(3-benzothienyl)alanine (Fig. 4), discovered in the early 1990s, were competitive inhibitors of IDO [47]. Using purified rabbit IDO, the K_i values were determined to be 6.6 ± 0.6 μM for 1-methyltryptophan, 70 ± 4 μM for β-(3-benzofuranyl) alanine, and 25 ± 2 μM for β-(3-benzothienyl)alanine, respectively [47]. The D-isomer of 1-methyltryptophan, indoximod (D-1MT/NLG-8189), was one of the first IDO inhibitors entering clinical trials as a combination therapy for breast cancer [48], prostate cancer [49], and melanoma [50]. Although the mechanism of action (MOA) of indoximod remains elusive, it has been suggested that it acts as a tryptophan mimic to restore tryptophan signaling to the mTOR pathway after tryptophan depletion by IDO [51].

Other pharmacophores have also been explored for the discovery of IDO inhibitors. Epacadostat (or INCB24360 (Fig. 4), with a hydroxyamidine scaffold, is a potent and selective IDO1 inhibitor with an IC_{50} of 10 nM as determined by a cell-based assay [52]. Despite lacking classical drug-like properties, epacadostat demonstrated excellent cell

permeability and pharmacokinetic (PK) profile in animal models [52]. This compound was used in combination with anti-PD1 monoclonal antibody (mAb) pembrolizumab in phase III clinical trial for the treatment of melanoma [53]. Navoximod (or NLG919, Fig. 4) was inspired by a series of structure-based drug development campaigns stemming from 4-phenylimidazole [54, 55]. Navoximod inherited phenylimidazole ability to bind to heme iron with improved active site occupancy and hydrogen bond interactions. Using cell-based assays, the EC_{50} of navoximod was determined to be 75 nM [56]. It was entered into clinical trials either as a monotherapy or combination therapy for solid tumors due to its favorable bioavailability, PK, and toxicity profiles [56–58].

2.1.2. KMO Inhibitors—KMO serves as a key branching point enzyme of the *de novo* NAD^+ biosynthetic pathway. The inhibition of this enzyme funnels L-kynurenine to kynurenic acid, a metabolite with anticonvulsant and neuroprotective properties due to its antagonism against the ionotropic receptors such as *N*-methyl-D-aspartate (NMDA) receptor and nicotinic acetylcholine receptor (nAChR) [39, 59–61]. KMO is a FAD-dependent monooxygenase that converts L-kynurenine to 3-hydroxy-L-kynurenine, a free radical generator known to increase oxidative stress and induce neuronal cell death [62, 63]. *Kmo*^{null} mice showed markedly reduced serum 3-hydroxy-L-kynurenine levels and improved phenotypes in response to extrapancreatic tissue insults, suggesting that pharmacological inhibition of KMO could serve as a novel therapeutic treatment for acute pancreatitis (AP) [64].

KMO inhibitors (Table 1) can be classified into several major categories, including substrate analogs (4-phenyl-4-oxobutanoic acids, sulfonamides, oxazolidinones), 3-oxo-propanitriles, tetrazoles, and arylpyrimidines.

2.1.2.1. Substrate Analogs

2.1.2.1.1. 4-Phenyl-4-oxobutanoic Acids: Substrate analogs were the first class of KMO inhibitors to be explored, even before the crystal structure of KMO was solved. *m*-Nitrobenzoyl-L-alanine (mNBA, Fig. 5), a close mimic of the endogenous substrate kynurenine, is a competitive inhibitor of KMO with an IC_{50} of 0.9 μM [65]. It potently increased the levels of kynurenine in the brain and blood of rats when administered at a dosage of 400 mg/kg intraperitoneally [65]. A series of structure-activity relationship (SAR) campaigns inspired by mNBA led to the identification of FCE28833A (Fig. 5), a dichloronated derivative of mNBA. It demonstrated improved potency with an IC_{50} of 0.2 μM [66]. Oral treatment of FCE28333A at 400 mg/kg resulted in a 14-fold increase of kynurenine in the rat brain [66]. A nanomolar KMO inhibitor, UPF-648 (IC_{50} = 40 nM) [67], was developed by inserting a cyclopropyl group between the carbonyl and carboxylic acid moieties in FCE28333A (Fig. 5). Significant elevation of kynurenine in the gerbil brain and plasma was observed after UPF-648 treatment [67]. The co-crystal structure of UPF-648 in complex with *S. cerevisiae* KMO was later determined [68]. The binding of UPF-648 to the active site of KMO induced a structural rearrangement to preclude substrate binding [68]. This study provided a blueprint for the future structure-based inhibitor design for the highly related human KMO.

2.1.2.1.2. Sulfonamides: Removal of the amino group in mNBA, which was known to not contribute significantly to the ligand binding, and replacing the carboxylic acid moiety with a sulfonamide isostere led to the discovery of a group of nanomolar KMO inhibitors [69]. Ro-61–8048 (Fig. 5), with an IC_{50} of 37 nM *in vitro*, increased brain kynurenine levels with an EC_{50} of 5.5 μmol/kg [69]. JM6 is an orally bioavailable prodrug of Ro-61–8048 (Fig. 5). The additional *N*-methylenepiperidine group can be cleaved off under acidic conditions to release Ro-61–8048. Chronic administration of JM6 not only raised the neuroprotective kynurenic acid levels in the brain but also ameliorated neurodegeneration in Alzheimer's (AD) and Huntington's disease (HD) mouse models [59]. Both Ro-61–8048 and JM6 showed poor blood-brain barrier (BBB) permeability, suggesting that the peripheral inhibition of KMO could serve as a therapeutic approach for neurodegenerations [59].

2.1.2.1.3. Oxazolidinones and Benzisoxazoles: GSK180 (Fig. 5), with an oxazolidinone core, is a competitive KMO inhibitor with an IC_{50} of 6 nM as determined by a recombinant KMO biochemical assay [64]. It preserves the carboxylic acid and hydrogen bond acceptor required for KMO inhibition. Moreover, GSK180 also demonstrates favorable physiochemical properties for intravenous administration [64]. Treatment with GSK180 raised the levels of kynurenine in mice and provided protective effects against multiple organ dysfunction syndrome (MODS) in a rat model of AP [64].

GSK366 is a benzisoxazole analog of GSK180 (Fig. 5). It is one of the most potent human KMO inhibitors with an IC_{50} of 2.3 nM [70]. The addition of the methylpyridazine group to the benzisoxazole core structure was thought to trap FAD in a "tilting" conformation, which further improved binding affinity and residence time with minimum peroxide formation [70]. Due to these favorable properties, GSK633 has been suggested as a suitable clinical candidate for the treatment of AP and MODS.

2.1.2.2. 3-Oxo-propanitriles: A group of tricyclic 3-oxo-propanitriles has been disclosed in a patent. They act as KMO inhibitors, among which PNU-168754 (Fig. 5) showed an IC_{50} of 40 nM [71]. Unfortunately, no additional information is available for these compounds.

2.1.2.3. Tetrazoles: Tetrazole analogs were uncovered using a high throughput mass spectrometry (MS) assay as human KMO inhibitors [72]. One representative compound, 5-(3-nitrobenzyl)-1*H*-tetrazole (Fig. 5), was reported to have an IC_{50} of 6.3 μM [72].

2.1.2.4. Arylpyrimidines: Arylpyrimidine carboxylic acids were considered as rigid cyclic analogs of kynurenine. They were designed to retain the favorable structural features of known KMO inhibitors. The structure of one of the lead compounds in this series, 6-(3-chlorophenyl)pyrimidine-4-carboxylic acid, is shown in Fig. (5). This remarkably simple compound demonstrated sub-nanomolar potency against human KMO (IC_{50} = 0.5 nM) [73]. Further elaboration of the phenyl ring with a *para*cyclopropoxy group led to the formation of CHDI-340246 (Fig. 5) as a potent KMO inhibitor both *in vitro* and *in vivo* [73]. This inhibitor showed limited ability to cross the BBB. Oral administration of CHDI-340246 at 10 mg/kg dosage caused pronounced elevation of plasma kynurenine levels and reduction of 3-hydroxy-L-kynurenine with excellent ADME and PK profiles in Sprague-Dawley rats [73]. In an HD mouse model, treatment of CHDI-340246 restored spiny projection neurons

(SPNs) membrane excitability without significant improvement in behavioral phenotypes or disease progression [74].

2.1.3. KYU Inhibitors—KYU is a PLP-dependent enzyme that facilitates the hydrolytic cleavage of the C_β-C_γ bond in either kynurenine or 3-hydroxykynurenine to generate anthranilic acid or 3-hydroxyanthranilic acid, respectively [75]. Human KYU demonstrated a 256-fold substrate preference toward 3-hydroxykynurenine [76]. When KMO is inhibited, KYU provides an alternative pathway to form quinolinic acid.

So far, only a handful of potent and specific KYU inhibitors have been discovered, most of which are transition state (TS) mimics of this enzyme. (4*S*)- and (4*R*)-dihydro-L-kynurenines (Fig. 6) are competitive inhibitors of KYU with the K_i values of 0.3 and 1.4 μM, respectively [77]. Based on this work, a group of *S*-aryl-L-cysteine *S,S*-dioxides were also developed as KYU inhibitors. The most potent compound in this group, *S*-(2-aminophenyl)-L-cysteine *S,S*-dioxide (Fig. 6), has a K_i of 70 nM against *P. fluorescens* KYU [78]. Chemically stable TS mimics are powerful inhibitors by trapping catalysis energy as binding energy. A couple of phosphinic acid analogs (Fig. 6) were thus designed and synthesized with the K_i values in the micromolar range [79]. A nanomolar human KYU inhibitor, 2-amino-4-(3'-hydroxyphenyl)-4-hydroxybutanoic acid (Fig. 6), was identified in 2002. It inhibited rat, human, and bacterial KYU with K_i values of 130 nM, 100 nM, and 10 μM, respectively [80].

2.1.4. QPRTase Inhibitors—In mammals, QPRTase is primarily localized in the liver and kidney, and to a lesser extent, the brain and spleen [81]. As the rate-limiting enzyme of the *de novo* pathway, QPRTase converts quinolinic acid to NAD^+ even after the salvage pathway is inhibited [82]. QPRTase has been suggested as a therapeutic target for the treatment of gliomas [83]. Overexpression of QPRTase was thought to confer poor prognosis and increase resistance to oxidative stress through stimulating NAD^+ *de novo* production [83].

The only known QPRTase inhibitor is phthalic acid. This compound structurally mimics the native substrate and serves as a competitive inhibitor of human QPRTase with a K_i of 2.8 μM as determined by a continuous UV assay [84]. Treatment of phthalic acid in primary astrocytes and neurons decreased intracellular NAD^+ levels in a concentration-dependent manner, and in turn, reduced NAD^+-dependent SIRT1 activity, both of which were thought to compromise cell viability [85].

2.1.5. NMNAT Modulators—NMNAT is a common enzyme involved in all three NAD^+ biosynthetic pathways. In mammals, there are three NMNAT isoforms with distinct subcellular localizations: NMNAT1 is mainly localized in the nucleus, NMNAT2 resides primarily in the Golgi complex, and NMNAT3 is a mitochondrial protein [86]. The three isoforms also demonstrated differential substrate selectivity, with NMNAT3 having the highest substrate tolerance [86]. The NMNATs were thought to play key roles in regulating compartment-specific "NAD pools" [86]. Overexpression of NMNATs extended lifespans with improved health profiles in model organisms [87–89]. More strikingly, NMNATs also exhibited neuronal protective effects, presumably through stimulating NAD^+-

dependent sirtuin activity [90–92]. Recently, NMNAT2 has been suggested as an anti-cancer therapeutic target. Overexpression of NMNAT2 in colorectal cancer (CRC) patients showed positive correlations with tumor invasiveness and p53 expression [93]. At the transcriptional level, NMNAT2 expression can be induced by p53 in response to DNA damage [94]. At the protein level, NMNAT2 is deacetylated and activated by SIRT3 [95]. The SIRT3-NMNAT2 axis has been proposed as the key regulator of cell growth and proliferation in non-small cell lung cancer (NSCLC) cells [95].

Natural products were first explored for the identification of NMNAT modulators. Gallotannin (Fig. 7), a known PARP inhibitor, inhibited all three NMNAT isoforms with IC_{50} values of 10, 55, and 2 μM [86]. Epigallocatechin gallate (EGCG, Fig. 7), at 50 μM concentration, doubled NMNAT2 activity and also activated NMNAT3 to a lesser extent [86]. Vacor adenine dinucleotide (VAD, Fig. 7), a metabolite of Vacor and an NAD^+ analog, inhibited NMNAT2 and NMNAT3 with IC_{50} values of 20 and 463 μM, respectively [82]. Unfortunately, VAD also inhibited other NAD^+-dependent dehydrogenases with micromolar potency [82]. Treatment of VAD in NMNAT2-expressing cancer cells caused significant NAD^+ depletion, reduced glycolysis, and energy metabolism, and ultimately necrosis [82].

2.2. Compounds Targeting NAD^+ Salvage Pathway

2.2.1. NAMPT Modulators—NAMPT is the rate-limiting enzyme of the salvage pathway that recycles NAM directly to NMN. Mammalian NAMPT is expressed in intracellular and extracellular forms [96]. Unlike the intracellular NAMPT (iNAMPT), which has been confirmed as an NAD^+ biosynthetic enzyme, the extracellular form of NAMPT is a multifaceted protein. It serves as a cytokine named pre-B cell colony enhancing factor (PBEF) [97], and as an insulin-like adipocytokine termed visfatin [98] in addition to its role in extracellular NAD^+ biosynthesis (eNAMPT). eNAMPT demonstrates robust NAD^+ biosynthetic activity, even higher than that of iNAMPT, and acts coordinately with iNAMPT to regulate pancreatic insulin secretion [99]. Overexpression of NAMPT has been closely correlated with poor prognosis in astrocytoma/glioblastoma [100], increased tumorigenesis and metastases in melanoma [101], and increased aggressiveness in malignant lymphoma [102]. All of these suggested NAMPT as an anti-cancer therapeutic target.

Co-crystal structures of NAMPT in complex with known inhibitors, GMX1778 and FK866, suggested that an ideal NAMPT inhibitor should be a heterocyclic NAM analog conjugated to a tail group through a hydrogen bond-forming spacer and a narrow hydrophobic linker (Fig. 8) [103, 104]. FK866 (Fig. 8), initially designed as a substrate mimic, represents the first noncompetitive yet highly specific NAMPT inhibitor with nanomolar potency as determined by cell viability assays [105, 106]. It induced apoptosis in cancer cells *via* blockade of the NAD^+ salvage pathway [105, 106]. FK866 is one of the few NAMPT inhibitors that advanced into clinical trials for the treatment of T-cell lymphoma [107], advanced melanoma (ClinicalTrials.org identifier: NCT00432107), and B-cell chronic lymphocytic leukemia (ClinicalTrials.org identifier: NCT00435084). GMX1778 (Fig. 8), structurally distinct from FK866, is a competitive NAMPT inhibitor [108, 109]. It exhibited low nanomolar cytotoxicity, which can be rescued by exogenously added NA or NAM [108, 109]. To improve the water solubility of GMX1778, a polyethylene glycol (PEG) linker

was tethered to the parent compound through an esteraselabile carbonate, leading to the formation of GMX1777 (Fig. 8) [110]. Intravenous administration of GMX1777 at 75 mg/kg led to tumor regression in several models [110]. FK866 and GMX1778/GMX1777 laid the foundation for further medicinal chemistry campaigns of NAMPT inhibitors. The following discussion will be grouped into three different sections: 1) FK866 and GMX1778 inspired inhibitors; 2) Inhibitors with novel pharmacophores; and 3) Dual NAMPT inhibitors. All of these NAMPT inhibitors are summarized in Table 2.

2.2.1.1. FK866 and GMX1778 Inspired NAMPT inhibitors

2.2.1.1.1. NAM Mimic Modifications: The pyridine moiety is the common structural feature of FK866 and GMX1778. Attempts have been made to replace it with other nitrogen-containing heterocycles such as pyrimidine, pyrrole, and indole with very little success [111–113]. A pyrrole-based inhibitor (27, Fig. 8) exhibited comparable or mildly improved cytotoxicity in MCF7 and K562 cells [111]. 2-Aminopyridine analog (28, Fig. 8) demonstrated nanomolar IC_{50}s in both recombinant NAMPT (3 nM) and A2780 cell proliferation (70 nM) assays with good PK profile and efficacy in mouse models [112]. Accumulating data suggested that the pyridine ring is still the preferred NAM mimic for a potent NAMPT inhibitor.

2.2.1.1.2. Spacer Modifications: "Click" chemistry-mediated library construction has led to the development of a group of NAMPT inhibitors with triazole-containing spacers [114]. One representative compound from this library, GPP78 (Fig. 8), significantly reduced intracellular NAD^+ content with an IC_{50} of 3 nM and induced cytotoxicity with an IC_{50} of 3.8 nM in SH-SY5Y cells [114]. In the same study, linker length was also investigated. Compounds with seven carbon linkers were the most potent ones across the panels [114]. In addition to vinylogous amide (such as the one in FK866), cyanoguanidine (such as the one in GMX1778) and triazole, amide, urea, thiourea spacers can also be tolerated by NAMPT [112, 115, 116].

2.2.1.1.3. Tail Group Modifications: CB30865 (Fig. 8), a quinazolinone derivative, was uncovered as a potent NAMPT inhibitor in a chemical proteomics study [117]. The methylpiperazinyl analog of CB30865, MPI-0479626 (Fig. 8), showed sub-nanomolar potency in a recombinant NAMPT inhibition assay as well as an HCT116 cell cytotoxic assay [117]. MPI-0479626 treatment caused remarkable NAD^+ depletion and subsequent PARP inhibition in response to oxidative damage [117].

An extensive SAR study resulted in compound 32 (Fig. 8) featuring a sulfonamide tail group [113]. In addition to its nanomolar IC_{50}s in *in vitro* assays, this inhibitor also demonstrated excellent ADME profiles [113]. Also noteworthy in this study was that the classic long hydrophobic linker was condensed to a benzene ring without significant loss of binding affinity and potency [113].

Diphenylsulfone can also be used as the tail group in NAMPT inhibitors. For example, GNE-617 (Fig. 8) possessed robust NAMPT inhibitory activity and *in vivo* efficacy [118]. It was proposed that the intracellular metabolism of GNE-617 produced a phosphoribosylated

GNE-617, the true tight-binding inhibitor of NAMPT [118]. This was further confirmed by structural biology analysis.

Interestingly, FK866 analogs with carborane tail groups were explored as NAMPT inhibitors, taking advantage of carborane's hydrophobic nature and its potential to form hydrogen bond interactions with the protein target [119]. MC4-PPEA (Fig. 8), in particular, exhibited an IC_{50} of 410 pM in A549 cells, four times more potent than FK866 [119].

2.2.1.2. Inhibitors with Novel Pharmacophores: In a recent study, fragment-based NMR and FRET screenings were coupled with structure-based drug design for the discovery of NAMPT inhibitors [120]. From two libraries of roughly 15,200 fragments, 6 ligands were initially identified as strong NAMPT binders [120]. Subsequently, structural biology-guided inhibitor design led to the synthesis and characterization of compound 35 (Fig. 9) with a K_i of 80 nM as determined by FRET assay [120].

High-throughput screenings (HTSs) have also yielded some potent NAMPT inhibitors with novel pharmacophores. An HTS using recombinant NAMPT uncovered several non-classical NAMPT inhibitors (compounds 36 to 38, Fig. 9), all of which had nanomolar potency [115]. These compounds clearly provided new templates for future inhibitor optimizations. OT-82 (Fig. 9), a lead compound discovered in a cell-based HTS, showed nanomolar potency in a panel of hematological malignancies and non-hematological malignancies derived cell lines [121]. No apparent toxicity of OT-82 at 55 mg/kg was observed in multiple mouse models. Currently, OT-82 has entered a Phase I clinical trial for the treatment of relapsed or refractory lymphoma (Clinical-Trials.gov identifier: NCT03921879) [121].

2.2.1.3. Dual NAMPT Inhibitors: Dual inhibitors are small molecule inhibitors targeting two protein targets simultaneously. The power of dual inhibitors has been increasingly appreciated because of their improved therapeutic efficacy and reduced risk of drug-drug interactions [122, 123]. KPT-9274 (Fig. 10) is a dual inhibitor of NAMPT and p21-activated kinase 4 (PAK4), both of which are essential for renal cell carcinoma (RCC) [124]. KPT-9274 not only reduced invasiveness and metastases of RCC cell lines but also inhibited tumor growth in a concentration-dependent manner in a human RCC xenograft model [124]. STF-31 (Fig. 10) serves as a dual inhibitor of both NAMPT and glucose transporter 1 (GLUT1) [125]. It inhibited glucose uptake and cell proliferation with distinct dosage windows in tumor cells [125]. Inhibitors targeting NAMPT and epigenetically important histone deacetylase (HDAC) have been developed using Cu(I)-catalyzed "click" reaction [126, 127]. A representative compound from these studies is shown in Fig. (10) (compound 42). It features a classical NAMPT inhibitor structure as well as the hydroxamate "warhead" targeting HDACs. It exhibited strong inhibitory effects on both targets with IC_{50} of 15 nM against NAMPT and 2 nM against HDAC1, respectively [127]. At 25 mg/kg intraperitoneal dosage, this compound potently inhibited tumor growth in an HCT116 xenograft mouse model, outperforming FK866 and SAHA (a known HDAC inhibitor) [127].

2.2.1.4. NAMPT Activators: The first purported NAMPT activator was discovered through an *in vivo* screen for the identification of chemicals that could elevate hippocampal neurogenesis in mice [128]. The initial screening of a compound library of 1,000 chemicals

produced eight compounds with neurogenic properties, out of which one had a promising pharmacological profile. The lead compound, P7C3 (Fig. 11), was found to reduce the death of newborn neurons rather than stimulating neuron birth. Using shotgun mass spectrometry, NAMPT was determined to be the cellular target of P7C3 [129]. The same study further examined the effects of P7C3 on the NAD^+ levels in living cells. NAD^+ deficiency caused by doxorubicin was alleviated with P7C3 treatment, presumably through NAMPT activation to stimulate NAD^+ biosynthesis [129]. The direct activation of NAMPT by P7C3 has been controversial due to its inability to shift the melting temperature of NAMPT [130].

A second NAMPT activator was identified through HTS using a protein thermal shift assay. The lead compound, SBI-136892 (Fig. 11), was able to increase the melting temperature of NAMPT in a dose-dependent manner [130]. Intriguingly, the structure of SBI-136892 was similar to a known NAMPT inhibitor, GNI-50. Based on the lead compound, an improved analog was generated. The analog, SBI-797812 (Fig. 11), had an EC_{50} of 0.37 ± 0.06 μM and could increase the formation of NMN by 2.1-fold compared to the control [130]. It was observed that NAMPT activation by SBI-797812 required ATP and could be nullified through the addition of NAMPT inhibitors. SBI-797812 was shown to increase the affinity of NAMPT for ATP, stabilize the pHis247 intermediate, reduce the levels of product pyrophosphate, and diminish the NAD^+ feedback loop [130]. Treatment of mice with SBI-797812 led to a 1.3-fold increase of NAD^+ levels in the liver, despite mouse NAMPT having an 8-fold lower affinity for the drug than human NAMPT [130].

2.2.2. NaPRTase Inhibitors—NaPRTase catalyzes the phosphoribosylation of NA to form NaMN, merging the salvage pathway with the *de novo* NAD^+ synthesis (Fig. 2). NaPRTase is ubiquitously expressed in normal tissues such as heart, kidney and pancreas [131], but is significantly downregulated in certain tumors, including neuroblastoma, glioblastoma [109], and lymphomas [102]. On the other hand, overexpression of NaPRTase was observed in ovarian cancer and CRC [132, 133].

Although human NaPRTase shares a high degree of structural similarity with human NAMPT [134], it cannot be inhibited by FK866 [135]. NaPRTase lacks the “tunnel” structure in NAMPT to accommodate FK866 [134]. It is not surprising that most classical NAMPT inhibitors failed to demonstrate any appreciable inhibitory effects on NaPRTase. It was reported that NA analogs such as 2-pyrazinoic acid, 2-hydroxynicotinic acid and 2-fluoronicotinic acid inhibited NaPRTase activity in human platelet lysate with apparent K_i values in the micromolar range [136]. 2-Hydroxynicotinic acid (Fig. 12), in particular, sensitized ovarian cancer cells to FK866 treatment both *in vitro* and *in vivo* through NaPRTase inhibition [132]. A group of non-steroidal anti-inflammatory drugs (NSAIDs) was suggested as competitive NaPRTase inhibitors [137]. The most potent one in the group, flufenamic acid (Fig. 12), showed a K_i of 46 μM [137].

CONCLUSION AND PERSPECTIVES

NAD^+ is an essential metabolite that couples cellular energy status to signaling. Intracellular NAD^+ levels have been linked to organismal health and are maintained by a complex network of biosynthetic pathways [15]. There have been many excellent review articles on

the physiological relevance of NAD^+ metabolism in normal biology as well as disease pathogenesis [138–141]. In this article, our discussion focuses on the small molecule regulators targeting NAD^+ biosynthetic enzymes, especially the enzymes involved in the *de novo* and salvage pathways.

De novo pathway generates NAD^+ from an amino acid precursor, tryptophan. IDO catalyzes the first and rate-limiting step in this pathway. It has been suggested as a therapeutic target for cancer immunotherapy [142]. Earlier inhibitors targeting IDO are mainly tryptophan mimics with micromolar potency [47]. Recently, several IDO inhibitors with novel pharmacophores and nanomolar potency have entered clinical trials [53, 56–58].

KMO serves as the other important control point of the *de novo* pathway. Inhibition of KMO leads to the reduction of the free radical generator 3-hydroxy-L-kynurenine. As such, KMO has been implicated as a viable target for the treatment of neurodegenerative diseases such as Alzheimer's Disease and Huntington's Disease [59]. Given the physiological relevance of KMO, intense efforts have been made to the development of several series of KMO inhibitors, ranging from substrate analogs to compounds with novel chemical entities. The discovery of KMO inhibitors has also been facilitated by the advance in structural biology [68], which guided the design of some of the most potent inhibitors known so far. The limitation of the currently available KMO inhibitors is their inability to pass through the blood-brain barrier (BBB). New drug delivery technologies, diverse formulations as well as structural modifications have been proposed as effective strategies to improve the BBB permeability of KMO inhibitors.

In humans, the salvage pathway is the predominant NAD^+ biosynthetic pathway in which NAMPT acts as the rate-limiting enzyme [143]. Consequently, NAMPT has been intensively studied for the regulation of intracellular NAD^+ levels. FK866, GMX1778, and GMX1777, three nanomolar NAMPT inhibitors, have advanced into clinical trials as cancer therapeutics [107, 110]. However, all of them demonstrated dose-limiting toxicities such as thrombocytopenia. Despite their limitations, they provided the blueprint for the development of new generations of NAMPT inhibitors. Most recently, co-administration of NA, the substrate of NAPRTase, has emerged as an alternative approach to mitigate NAMPT inhibitor toxicity in normal cells [109, 110].

Elevating intracellular NAD^+ concentrations has provided therapeutic benefits in various pre-clinical studies [27–29, 144, 145]. Although small molecule activators targeting NAD^+ biosynthetic enzymes hold great therapeutic potentials, the reports of these compounds have been rather sporadic. Unlike inhibitors, there are no general and systematic strategies to identify activators. Furthermore, the discovery of the NR pathway is relatively new, and our understanding of NRKs is still in its infancy. Detailed interrogation of the biological functions and cellular regulations of these NAD^+ biosynthetic enzymes will open new avenues for the development of selective and potent small-molecule regulators.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
BBB	Blood-brain Barrier
CR	Calorie Restriction
CRC	Colorectal Cancer
GLUT1	Glucose Transporter 1
HAO	3-hydroxyanthranilate 3,4-dioxygenase
HD	Huntington's Disease
HDAC	Histone Deacetylase
HTSs	High-throughput Screenings
IDO	Indoleamine 2,3-dioxygenase
KMO	Kynurenine 3-monooxygenase
KYU	Kynureninase
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MOA	Mechanism of Action
mPTP	Mitochondrial Permeability Transition Pore
MS	Mass Spectrometry
NA	Nicotinic Acid
NaAD	Nicotinic Acid Adenine Dinucleotide
nAChR	Nicotinic Acetylcholine Receptor
NAD^+	Nicotinamide Adenine Dinucleotide
NAM	Nicotinamide

NaMN	Nicotinic Acid Mononucleotide
NAMPT	Nicotinamide Phosphoribosyl Transferase
NaPRTase	Nicotinic Acid Phosphoribosyl Transferase
NMDA	*N*-methyl-D-aspartate
NMN	Nicotinamide Mononucleotide
NMNAT	Nicotinamide/Nicotinic Acid Mononucleotide Adenylyltransferase
NR	Nicotinamide Riboside
NRK1/NRK2	Nicotinamide Riboside Kinases
NSAIDs	Non-steroidal Anti-inflammatory Drugs
NSCLC	Non-small Cell Lung Cancers
PARPs	Poly(ADP-ribose) Polymerases
PBEF	Pre-B Cell Colony Enhancing Factor
PK	Pharmacokinetic
PRPP	5-phosphoribosyl-1-pyrophosphate
QPRTase	Quinolinate Phosphoribosyl Transferase
RCC	Renal Cell Carcinoma
TDO	Tryptophan 2,3-dioxygenase
TNF	Tumor Necrosis Factor
TS	Transition State

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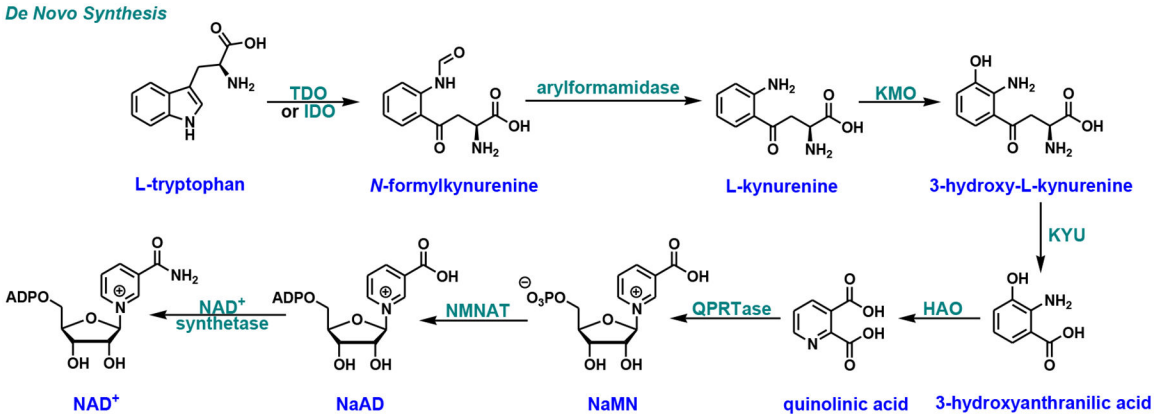


Fig. (1).

De novo NAD^+ biosynthetic pathway in mammals. TDO, tryptophan 2,3-dioxygenase; IDO, indoleamine 2,3-dioxygenase; KMO, kynurenine 3-monooxygenase; KYU, kynureninase; HAO, 3-hydroxyanthranilate 3,4-dioxygenase; QPRTase, quinolinate phosphoribosyl transferase; NMNAT, nicotinamide/nicotinic acid mononucleotide adenylyltransferase; NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide.

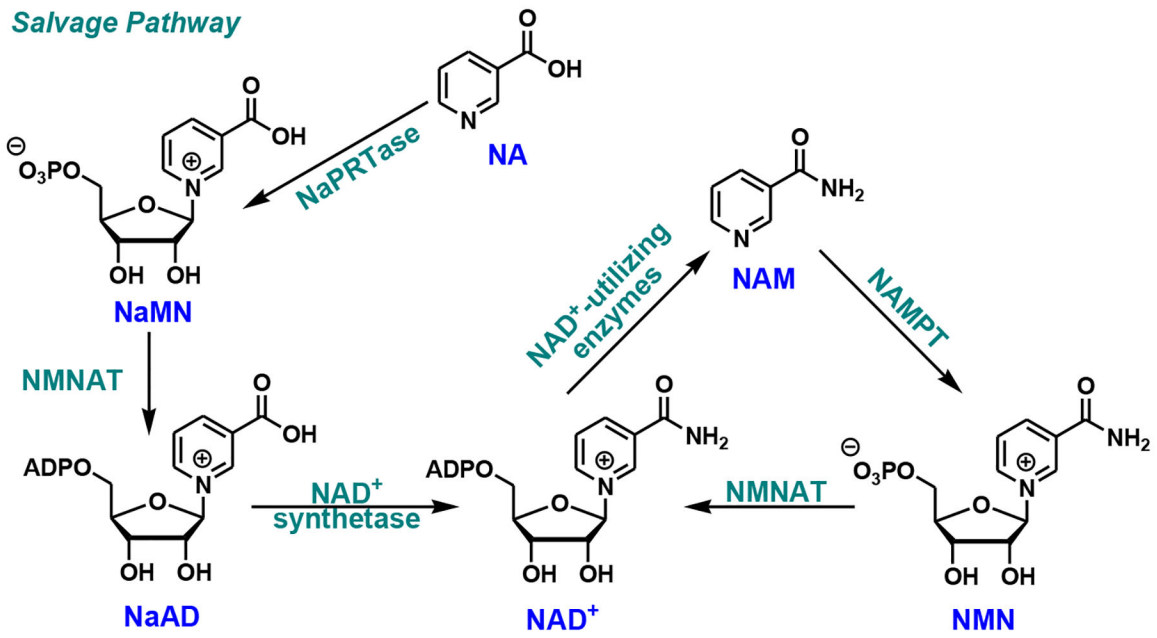


Fig. (2).

Mammalian salvage pathway. NAMPT, nicotinamide phosphoribosyl transferase; NaPRTase, nicotinic acid phosphoribosyl transferase; NAM, nicotinamide; NA, nicotinic acid.

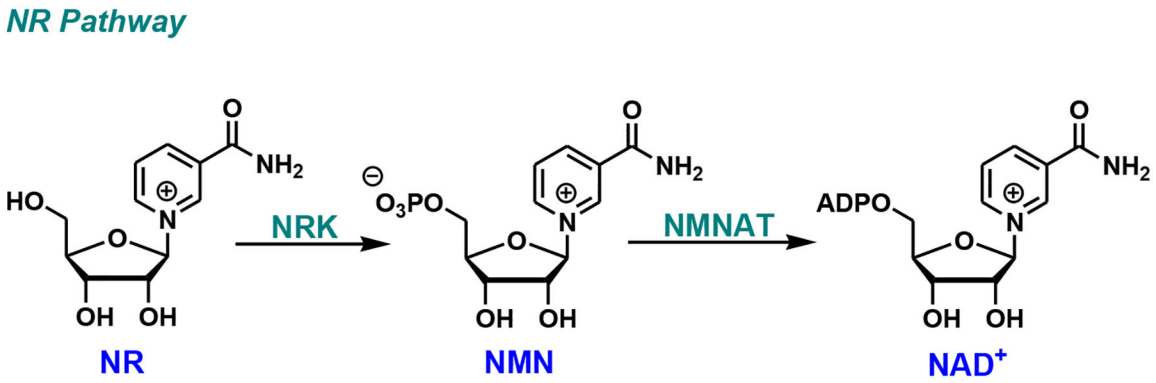


Fig. (3).
NR pathway. NRK, nicotinamide riboside kinase; NR, nicotinamide riboside.

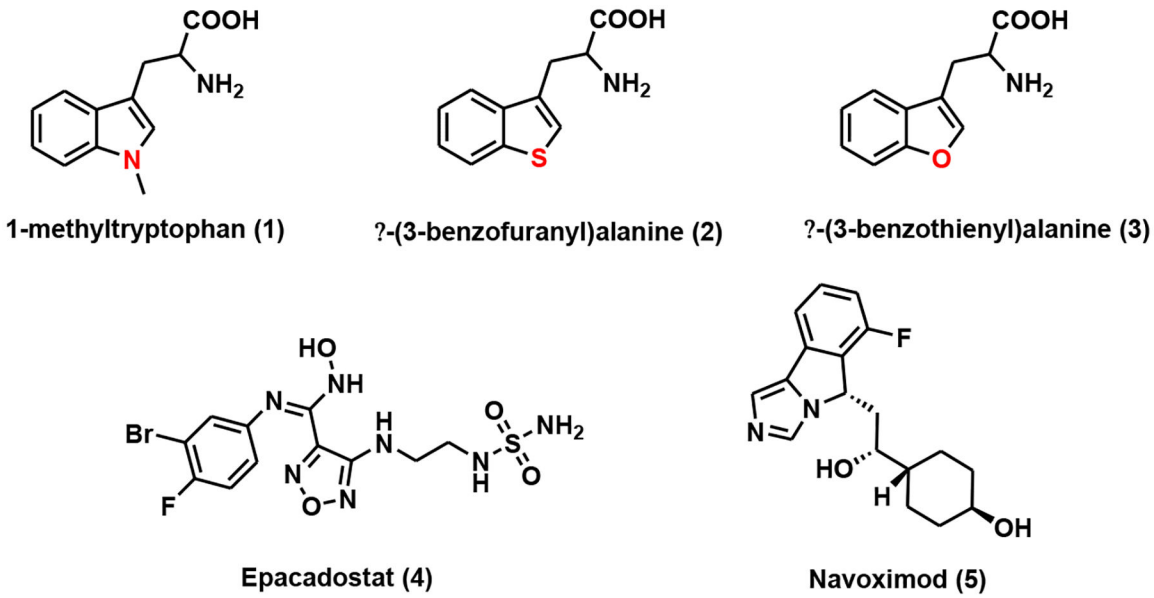


Fig. (4).
IDO inhibitors.

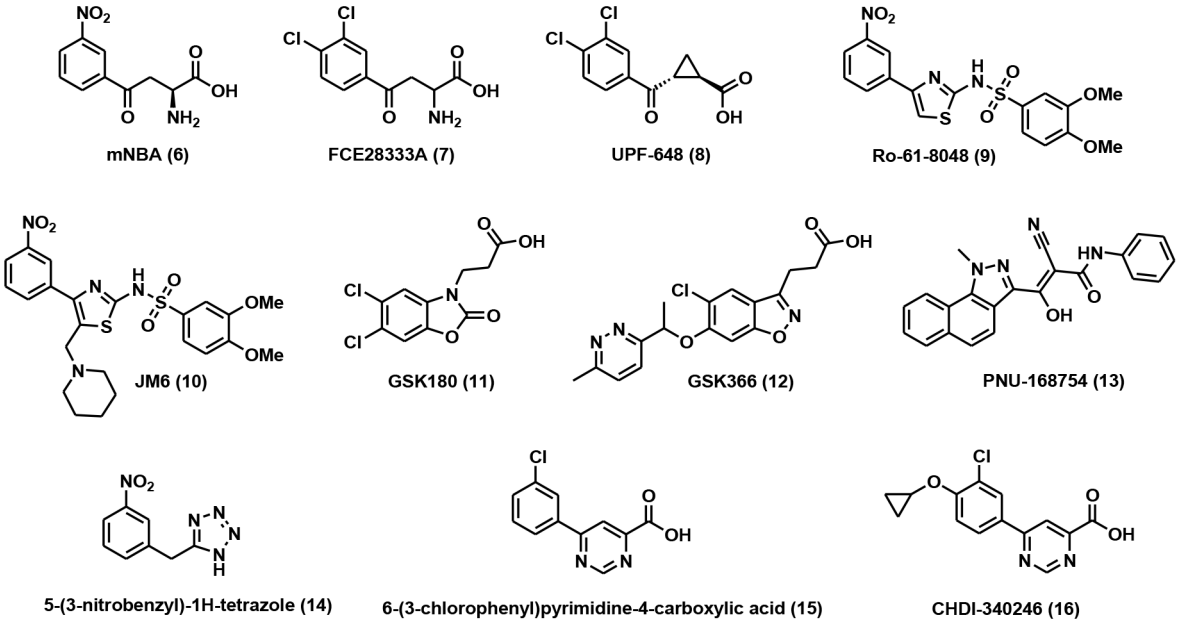


Fig. (5).
KMO inhibitors.

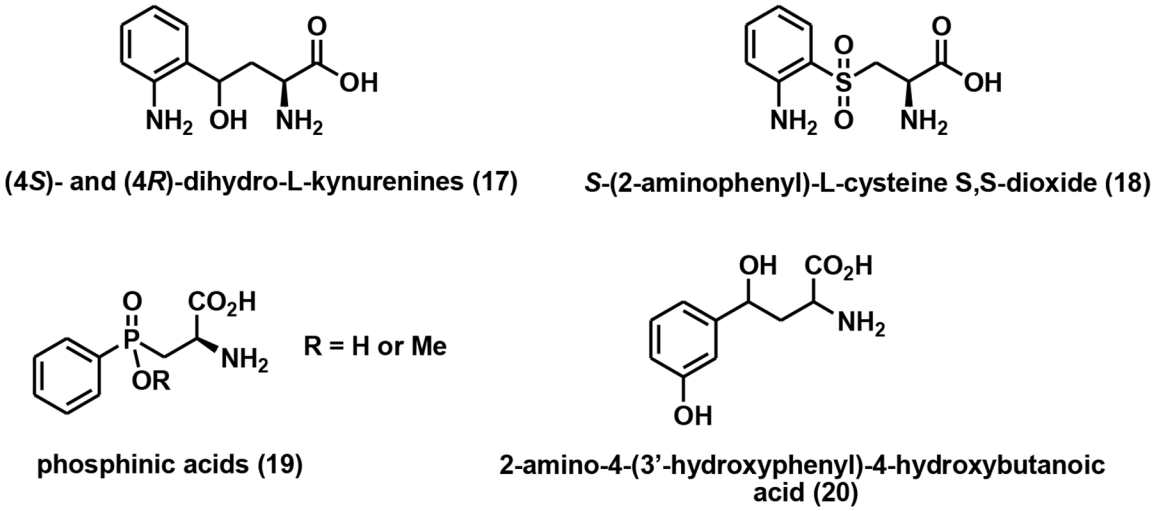


Fig. (6).
KYU inhibitors.

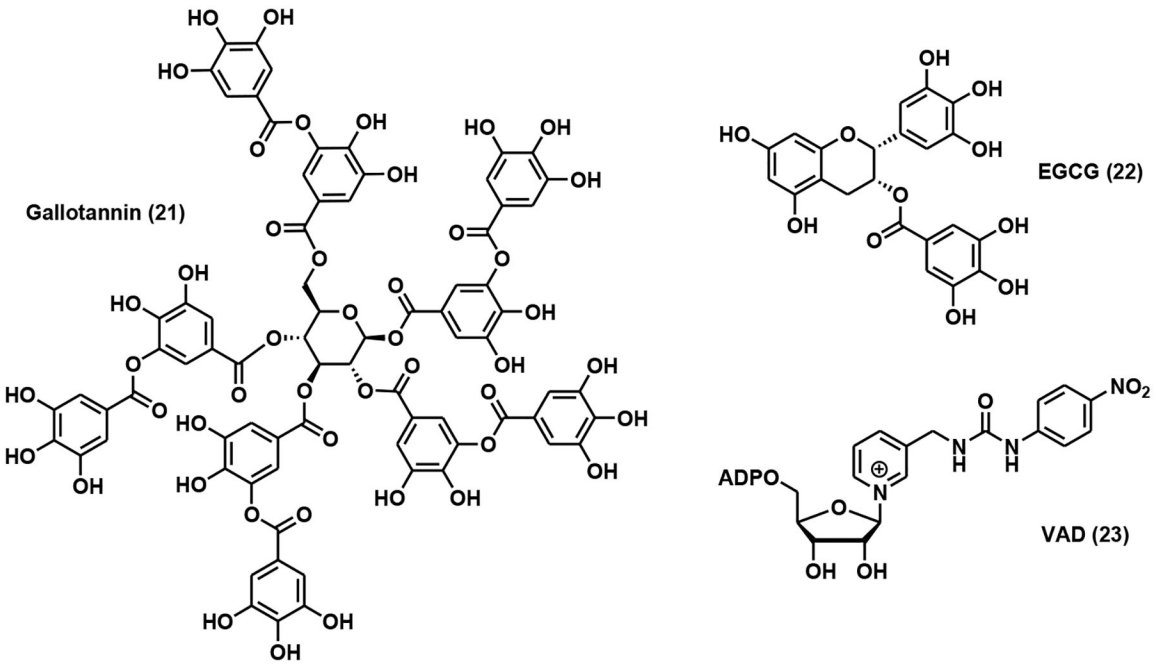


Fig. (7).
NMNAT modulators.

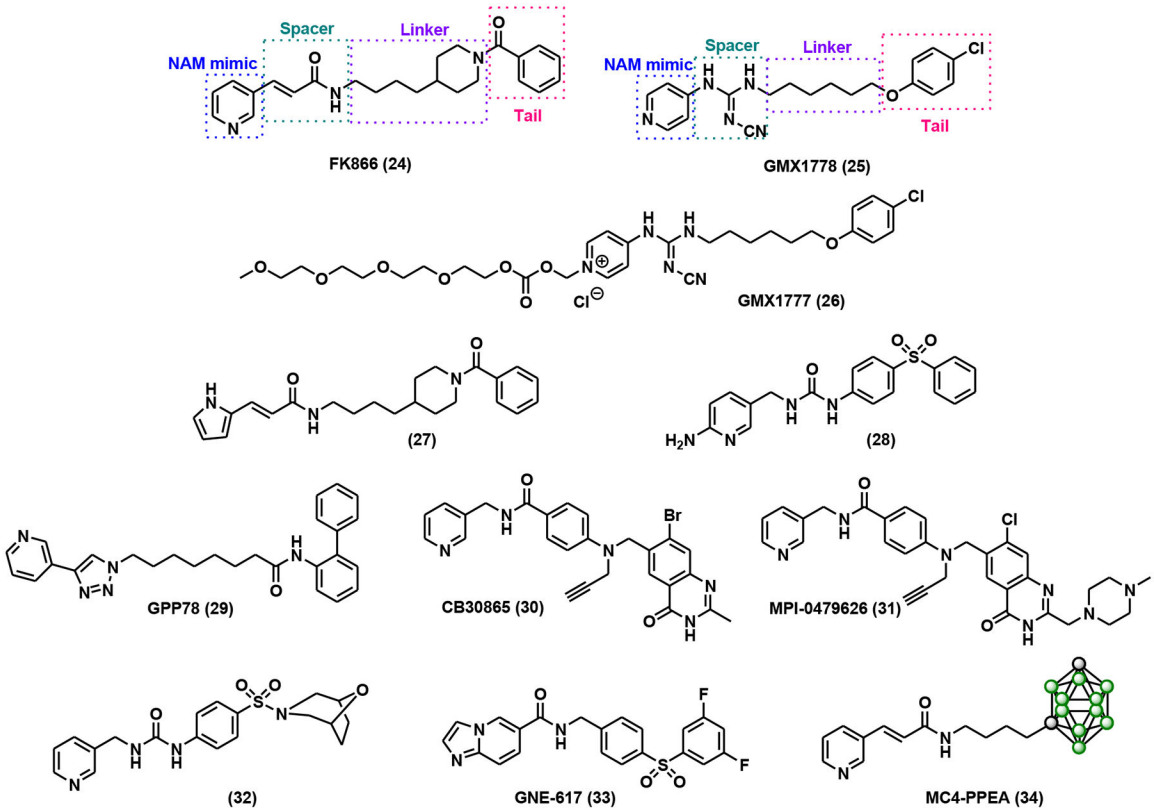


Fig. (8).
FK866 and GMX1778 inspired NAMPT inhibitors.

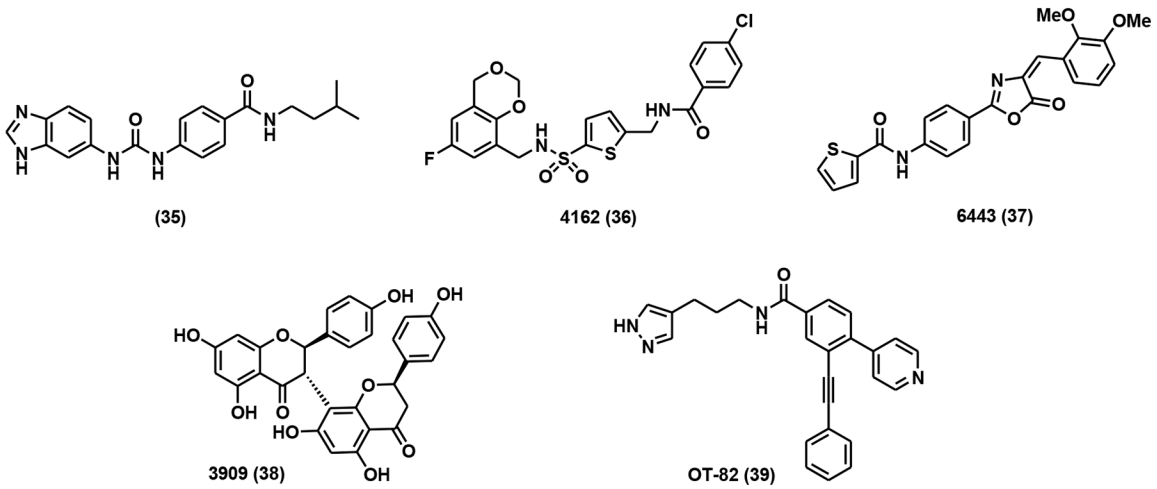


Fig. (9).
NAMPT inhibitors with novel pharmacophores.

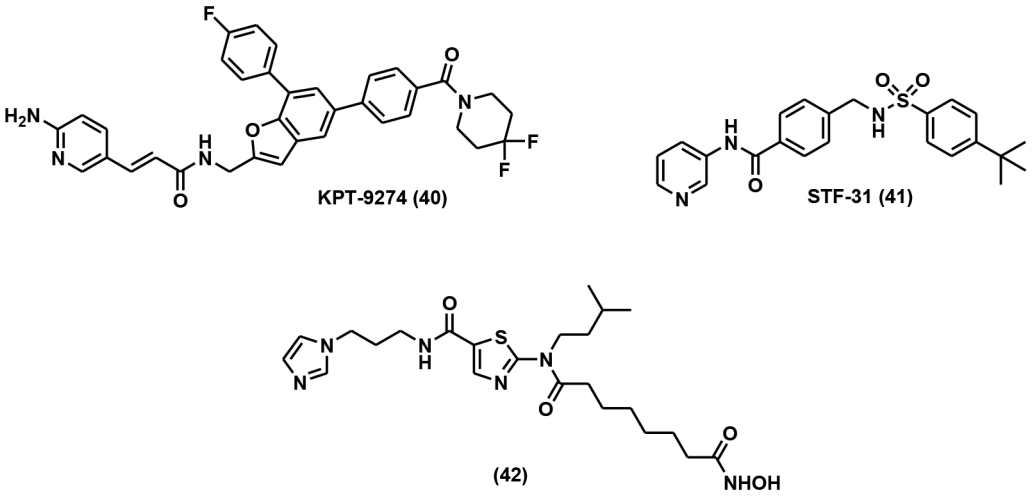
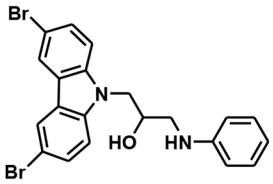
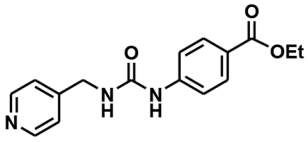


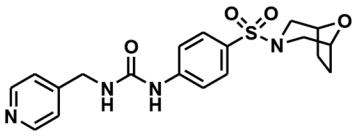
Fig. (10).
Dual NAMPT inhibitors.



P7C3 (43)

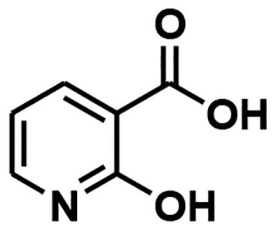


SBI-136892 (44)

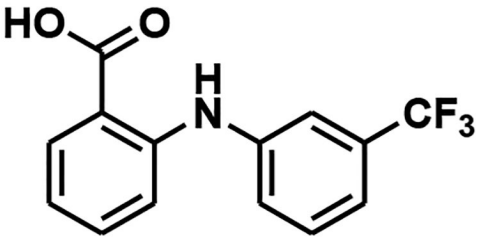


SBI-797812 (45)

Fig. (11).
NAMPT activators.



2-hydroxynicotinic acid (46)



flufenamic acid (47)

Fig. (12).
NaPRTase inhibitors.

Table 1.

KMO inhibitors.

Compound	Name	IC_{50} (nM)	Assay	References
6	mNBA	900 ± 100	competitive inhibition assay (rat tissues)	[65]
7	FCE28333A	200 ± 20	inhibition assay (rat brain tissue)	[66]
8	UPF-648	40[a]	recombinant human KMO assay	[67]
9	Ro-61–8048	37 ± 3	radioenzymatic assay (rat kidney mitochondria)	[69]
10	JM6	~4000	enzyme coupled assay (mouse brain homogenate)	[59]
11	GSK180	6	recombinant human KMO assay	[64]
12	GSK366	2.3	Rapidfire MS assay (recombinant human KMO)	[70]
13	PNU-168754	40	radiometric assay (rat liver mitochondrial extract)	[71]
14	5-(3-nitrobenzyl)-1*H*-tetrazole	6300	Rapidfire MS assay (recombinant human KMO)	[72]
15	6-(3-chlorophenyl)pyrimidine-4-carboxylic acid	0.5	human KMO assay	[73]
16	CHDI-340246	0.5	human KMO assay	[73]

[a] IC_{50} for the racemic mixture.

Table 2.

NAMPT inhibitors

Compound	Name	IC_{50} (nM)	Assay	References
24	FK866	4.90 ± 0.55	MTT assay (NIH-3T3 cells)	[146]
		1.60 ± 0.32	human NAMPT assay	[147]
25	GMX1778	< 25	coupled enzyme NAMPT assay	[109]
		K_d = 120 nM	recombinant human NAMPT	[109]
27		1290±50	SRB assay	[111]
		20300	HPLC-based NAMPT assay	[111]
28		3	NAMPT biochemical inhibition assay	[112]
		70	SRB assay (A2780 cells)	[112]
29	GPP78	3.8 ± 0.3	MTT assay (SH-SY5Y cells)	[114]
30	CB30865	2.1 ± 0.44	MTT assay (CH1 cells)	[148]
31	MPI-0479626	0.38 ± 0.08	NAD depletion assay	[117]
		0.23 ± 0.12	NAMPT inhibition assay	[117]
32		7	NAMPT biochemical inhibition assay	[113]
		32	SRB assay (A2780 cells)	[113]
33	GNE-617	5	NAMPT biochemical inhibition assay	[118]
		2	SRB assay (A2780 cells)	[118]
34	MC4-PPEA	0.31	MTT assay (DLD1 cells)	[119]
35		K_i = 80 nM	TR-FRET assay	[120]
36	4162	777 ± 84	NAMPT biochemical inhibition assay	[115]
37	6443	352 ± 45	NAMPT biochemical inhibition assay	[115]
38	3909	504 ± 124	NAMPT biochemical inhibition assay	[115]
39	OT-82	41	NAMPT biochemical inhibition assay	[121]
40	KPT-9274	120	NAMPT biochemical inhibition assay	[124]
		570	MTT assay (Caki-1 cells)	[124]
41	STF-31	19	NAMPT biochemical inhibition assay	[149]
		120	SRB assay (A2780 cells)	[149]

Compound	Name	IC_{50} (nM)	Assay	References
42		15	NAMPT biochemical inhibition assay	[127]
		5200 ± 480	MTT assay (A549 cells)	[127]