HHS Public Access

Author manuscript

Eur J Med Chem. Author manuscript; available in PMC 2024 January 15.

Published in final edited form as:

Eur J Med Chem. 2023 January 15; 246: 114998. doi:10.1016/j.ejmech.2022.114998.

Design, synthesis, and pharmacological evaluations of pyrrolo[1,2-*a*]quinoxaline-based derivatives as potent and selective sirt6 activators

Jimin Xu[a,1], **Shuizhen Shi**[b,1], **Gang Liu**[a], **Xuping Xie**[c], **Jun Li**[a], **Andrew A. Bolinger**[a], **Haiying Chen**[a], **Wenbo Zhang**[b], **Pei-Yong Shi**[c], **Hua Liu**[b,*], **Jia Zhou**[a,*]

[a]Chemical Biology Program, Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas 77555, United States

[b]Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, Texas 77555, United States

[c]Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas 77555, United States

Abstract

Sirt6 activation has emerged as a promising drug target for the treatment of various human diseases, while only limited Sirt6 activators have been reported. Herein, a series of novel pyrrolo[1,2-*a*]quinoxaline-based derivatives have been identified as potent and selective Sirt6 activators with low cytotoxicity. Sirt6-knockdown findings have validated the on-target effects of this class of Sirt6 activators. Docking studies indicate the protonated nitrogen on the side chain of **38** forms π-cation interactions with Trp188, further stabilizing it into this extended binding pocket. New compounds **35**, **36**, **38**, **46**, **47**, and **50** strongly repressed LPS-induced proinflammatory cytokine/chemokine production, while **38** also significantly suppressed SARS-CoV-2 infection with an EC_{50} value of 9.3 μM. Moreover, compound **36** significantly inhibited the colony formation of cancer cells. These new molecules may serve as useful pharmacological tools or potential therapeutics against cancer, inflammation, and infectious diseases.

Graphic Abstract

[*]**Corresponding author:** Jia Zhou, PhD, Chemical Biology Program, Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas 77555, United States, Tel.: +1-409-772-9748, jizhou@utmb.edu; Hua Liu, PhD, Department of Ophthalmology and Visual Sciences, University of Texas Medical Branch, Galveston, Texas 77555, United States, Tel.: +1-409-742-3793, hualiu@utmb.edu.

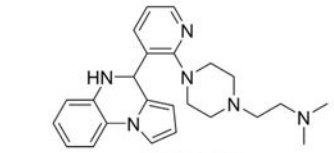
[1]These authors contribute equally to this work.

Declaration of Interest Statement

The authors have no conflict of interest to disclose.

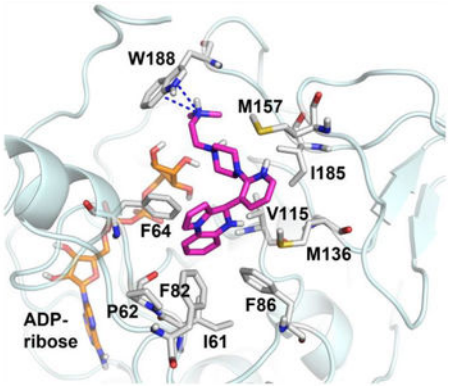
Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/XXX



38 (GL0710)

- Sirt6 activation: 7.38-fold at 100 μM
  3.83-fold at 30 μM
- On-target effects validated by Sirt6 knockdown
- Significantly suppress LPS-induced inflammatory genes
- Anti-SARS-CoV-2: EC_{50} = 9.3 μM



Keywords

Sirt6 activator; Deacetylation; Anti-inflammation; Anti-SARS-CoV-2; Drug discovery

1. Introduction

The sirtuin (Sirt) family is a class of enzymes that possess NAD^+-dependent either deacylase or mono-ADP-ribosyltransferase activity, regulating many cellular processes such as energy metabolism, stress responses, and genomic stability [1–4]. Currently, there are seven mammalian sirtuins (Sirt1-7), characterized by a highly conserved catalytic core, but differing in their cellular localization and substrate preferences [5–7]. Of these members, Sirt6 specifically catalyzes the deacetylation of N^{ε}-acetyl-lysines 9, 18, and 56 of histone H3 (H3K9ac, H3K18ac, and H3K56ac, respectively) and associates with chromatin to modulate DNA repair, gene expression, and telomeric maintenance [8–13]. In addition, Sirt6 can hydrolyze long-chain acylated substrates and catalyze mono-ADP-ribosylation [14, 15]. Sirt6 knockout mice display smaller body size, shortened lifespan, and aging-associated degenerative phenotypes such as cancers and metabolic defects, while Sirt6 overexpression impairs the development of several cancer types and prolongs the lifespan of male mice [16–19]. It was reported that siRNA-mediated Sirt6-knockdown led to a significant increase in the yield of human cytomegalovirus (HCMV), herpes simplex virus 1 (HSV-1), human adenovirus 5 (HAdV-5), and influenza virus H1N1 in cultured cells, suggesting an antiviral role of Sirt6 [20]. Sirt6 inhibits dengue virus (DENV)-induced proinflammatory cytokine and chemokine production via RIG-I-like receptor (RLR) and Toll-like receptor 3 (TLR3) signaling pathways [21]. Besides, Sirt6 was also found associated with many other human diseases including osteoarthritis (OA), diabetes, heart diseases, neurodegenerative diseases, etc., and its emerging role in a variety of human diseases has been comprehensively reviewed recently by our group [5]. Thus, targeting Sirt6 activation has been considered a promising approach for the treatment of Sirt6-related human conditions such as cancers, inflammation, viral infections, and aging-related diseases [22].

Despite the great clinical application prospect, only several Sirt6 activators have been reported so far [2, 5]. Long-chain fatty acids (containing 14–18 carbons) were found to stimulate Sirt6 deacetylation, represented by myristic acid (**1**, Fig. 1) which increases the activity of Sirt6 deacetylation up to 10.8-fold, with an EC_{50} value of 246 μM [23].

Oleoylethanolamide (**2**) is an ethanolamide analog of oleic acid which displays a 2.1-fold maximum activation (E_{max}) on Sirt6 deacetylation with an EC_{50} of 3.1 μM [24]. Flavonoids could increase Sirt6 deacetylation activity at high concentrations, and cyanidin (**3**) shows the most potent activating effect with a 55-fold E_{max} and an EC_{50} of 460 μM [25, 26]. Moreover, cyanidin could ameliorate the aggressive course of OA in surgical destabilization of the medial meniscus mouse models, possibly exerting the protective effect through regulating the Sirt6/NF-κB signaling axis [27]. UBCS039 (**4**) is the first synthetic Sirt6 activator that increases Sirt6 deacetylation activity in a dose-dependent manner with a ~2-fold E_{max} and an EC_{50} of 38 μM. Meanwhile, it displays no obvious effects on basal Sirt1, 2, and 3 deacetylation activities [28]. Through activity-based screening and subsequent chemical optimization, CL5D (**5**) was identified as a potent Sirt6 activator, inducing 4-fold activation at a low concentration of 3 μM [29]. MDL-800 (**6**), a Sirt6 allosteric activator, increases the deacetylation activity of Sirt6 up to 22-fold with an EC_{50} of 10.3 μM and shows more than 10-fold selectivity over Sirt1-5, Sirt7, and HDAC1–11 [30]. It significantly promotes H3K9 and H3K56 deacetylation dose-dependently in human hepatocellular carcinoma (HCC) cells, consequently inhibits their proliferation through Sirt6-mediated cell cycle arrest, and suppresses HCC tumor growth in mouse xenograft models [30]. Compared to MDL-800, MDL-811 (**7**) with an extra water-soluble fragment shows improved Sirt6 deacetylation activity (EC_{50} = 5.3 μM) and bioavailability (F = 92.96%) in mice. Notably, MDL-811 displays broad antiproliferative effects on various colorectal cancer (CRC) cell lines and *in vivo* anti-tumor efficacy in cell line- and patient-derived xenografts as well as in the $APC^{min/+}$ spontaneous CRC model [31]. The quinoline derivative **8** was reported to activate Sirt6-dependent peptide deacetylation and demyristoylation (EC_{50} = 5.35 μM and 8.91 μM, respectively) and exhibit weak or no activity against other HDAC family members [32]. In addition, it inhibits the proliferation and migration of pancreatic ductal adenocarcinoma (PDAC) cells, decreases the levels of H3K9ac, H3K18ac, and H3K56ac in PDAC cell lines (PANC-1 and BXPC-3), and significantly suppresses tumor growth in a PDAC tumor xenograft model. However, it has several drawbacks such as limited aqueous solubility, poor absorption, and low oral bioavailability (F = 4.24%) [32]. Therefore, it remains an unmet need to develop more effective and selective Sirt6 activators with favorable druglike properties.

Based on the lead compound UBCS039, herein, we further explored the structure-activity relationship (SAR) studies by introducing a functional hydrophilic side chain at the 2-position of the 3-pyridyl moiety assisted by molecular modeling/docking and identified a series of novel pyrrolo[1,2-*a*]quinoxaline-based derivatives as potent and selective Sirt6 activators with improved efficacy and low cytotoxicity. The Sirt6-knockdown experiment has further validated the on-target effects of this class of Sirt6 activators. Molecular docking studies indicate that the protonated nitrogen on the side chain of compound **38** forms π-cation interactions with Trp188, further stabilizing it into this extended binding pocket. This functional hydrophilic side chain provides us a useful moiety for designing novel and potent Sirt6 activators with new scaffolds while also tuning their druglike properties such as aqueous solubility. New compounds **35**, **36**, **38**, **46**, **47**, and **50** strongly repressed lipopolysaccharide (LPS)-induced proinflammatory cytokine/chemokine production, while **38** also significantly suppressed severe acute respiratory syndrome coronavirus 2 (SARS-

CoV-2) infection with an EC_{50} value of 9.3 μM, indicating the great potential of this class of Sirt6 activators to be developed as anti-inflammatory and anti-SARS-CoV-2 agents. Moreover, compound **36** significantly inhibited the colony formation of cancer cells. These new molecules may serve as powerful pharmacological tools for elucidating the role of Sirt6 in various human conditions or as potential therapeutics against such relevant disorders including cancer, inflammation, and infectious diseases.

2. Results and discussion

2.1. Chemistry

The general synthesis of new derivatives with different amino side chains on the pyridine ring is outlined in Scheme 1. Substitution of commercially available 2-chloronicotinaldehyde (**9**) with various amines gave the intermediates **11a-o**, and the subsequent condensation of **9** or **11a-o** with 2-(1*H*-pyrrol-1-yl)aniline (**10**) in the presence of acetic acid in ethanol afforded derivatives **14–29**. 2-Fluoronicotinaldehyde (**12**) was treated with *N*-Boc-ethanolamine to generate the intermediate **13**, which was then coupled with **10**, followed by acidic Boc-deprotection, to provide compound **30**.

Compounds **35**-**50** with different substituted piperazine moieties were synthesized following similar procedures to that of preparing **14**-**30** (Scheme 2). Direct substitution of **9** with appropriately substituted piperazines or with piperazine followed by a second substitution with bromide or chloride afforded the key intermediates **32a-i**, which were then condensed with **10** to yield new compounds **35–40**, **42**, **44**, and **48**. Amide **41** was accessed from nitrile **40** via hydrolysis. Substitution of **42** with dimethylamine produced compound **43**. Chlorination of **32a** using $SOCl_2$ gave chloride **33**, which was then treated with different amines to yield the intermediates **34a-e**. Condensation of **34a-e** with **10** afforded new derivatives **45–47**, **49**, and **50**, respectively.

As described in Scheme 3, the substitution of aldehydes **51a-d** with substituted piperazine **52** produced the intermediates **53a-d**, which were then converted into compound **60–63** by similar condensations with 2-(1*H*-pyrrol-1-yl)aniline **10**. Acylation of **38** provided compound **64**. Compound **65** was formed by a reaction of **32c** with **10** and acetic acid in ethanol at a higher temperature of 70 °C overnight. The starting material **54** was treated with 2-methyl-1*H*-imidazole to give the nitro intermediate **55**, which was transformed to **67** via hydrogenation using Pd/C and subsequent condensation with **32c**.

Compounds **66** and **68–76** were prepared according to the general synthesis outlined in Scheme 4. Treatment of commercially available 2-aminophenols **57a-e** with 2,5-dimethoxytetrahydrofuran provided the intermediates **59a-e**. The intermediates **59f** and **59g** were synthesized by hydrolysis of **57e** and subsequent condensations with NH_4Cl or 2-aminoethylmethylsulfone. Condensations of **59a-g** with **32c** produced compounds **66**, **68–71**, **73**, and **74**, respectively. Reduction of **71** using $LiAlH_4$ yielded compound **72**. Formylation of **66** followed by reduction using $NaBH_4$ afforded compound **75**. Compound **76** was synthesized by the reaction of **66** with *S*-(trifluoromethyl)dibenzothiophenium triflate.

2.2. In vitro Evaluation of Sirt6 deacetylation activation

All newly synthesized compounds were first screened by Fluor de Lys (FDL) assay at 100 μM [33], and the fold-changes of Sirt6-dependent peptide deacetylation activity were quantified, using the lead compound UBCS039 as the reference control for comparison. The active compounds screened out (activation fold > 2) were further evaluated for their activation effects on Sirt6 at 30 μM. Previous studies showed that the 3-pyridyl group of UBCS039 was critical to maintaining its activating activity on Sirt6 and shifting the pyridine nitrogen from the *meta* to *ortho* position led to a significant decrease in Sirt6 activation [28]. Starting from the lead compound UBCS039, we first attempted to introduce additional functional groups and side chains at the 2-position of the 3-pyridyl moiety to form potential important interactions between the compound and the Sirt6 protein. As shown in Table 1, inserting 2-Cl (**14**), 2-dimethylamino (**15**), 2-(azetidin-1-yl) moieties (**16–17**), and 2-(pyrrolidin-1-yl) (**18**) impacted the potency of Sirt6 deacetylation significantly to a small extent, displaying activation folds of 1.04 ~ 1.47 at 100 μM, compared to UBCS039 (1.12-fold at 100 μM in our assay). Surprisingly, compounds with heterocycles bearing terminal -$NHCH_3$- group (**19–21**) displayed dramatically enhanced Sirt6 deacetylation activities by more than 4-fold at 100 μM and 2-fold at 30 μM. Among them, **21** with 2-(4-methylpiperazin-1-yl) potently activated Sirt6 deacetylation by 4.62- and 2.44-fold at 100 μM and 30 μM, respectively. Further replacing its terminal methyl with acetyl (**22**) or methylsulfonyl (**23**) resulted in a slight decrease in potency with activation folds of 2.74 and 3.63 at 100 μM, respectively. Compound **24** with morpholinyl did not activate Sirt6, while compounds with terminal -CF_2- (**25**), -SO_2- (**26**), and -CHOH- (**27**) exhibited decreased activating effects of ~2-fold at 100 μM, in comparison with compound **21**. Substituting the terminal methyl (**21**) with pyridin-2-yl (**28**) also led to a significant loss in activation potency. Interestingly, compound **29** with 4-(pyrrolidin-1-yl)piperidin-1-yl moiety showed obvious Sirt6 deacetylation activity with 3.44-fold at 100 μM. The short side chain of 2-aminoethoxy (**30**) was not tolerated at the 2-position of the 3-pyridyl moiety.

Through the above initial SAR investigation, compound **21** with the 4-methylpiperazin-1-yl moiety was found to be the most potent compound to activate Sirt6. To further explore the effect of different side chains linked to the terminal nitrogen of piperazinyl moiety, compounds **35–50** were designed, synthesized, and pharmacologically evaluated (Table 2). The alkyl side chains bearing terminal hydroxyl (**35**), ether (**36** and **37**), dimethylamino (**38**), and dimethylcarbamoyl (**39**) were all well tolerated, presenting significantly improved activating effects (5.02 ~ 7.38-fold at 100 μM and 2.92 ~ 3.83-fold at 30 μM). In contrast to compound **21**, compound **40** with cyanomethyl maintained the same level of potency, while its reduced amide analog **41** exhibited slightly decreased Sirt6 deacetylation activity. The substituted acyl groups (**42** and **43**) were unfavorable for the activating effects. Excitingly, the carbamide analog **44** bearing a 3-hydroxyazetidine moiety also showed potent activity in activating Sirt6 deacetylation (5.26-fold at 100 μM and 2.67-fold at 30 μM). Introducing additional heterocycles with a two-carbon linker yielded compounds **45**-**50**. Notably, all these compounds displayed excellent activating activity on Sirt6, with activation folds of 5.38~6.30 and 2.81~4.80 at 100 μM and 30 μM, respectively.

Next, we explored other positions of the pyridyl moiety and the tricyclic ring while retaining the side chain of 4-(2-(dimethylamino)ethyl)piperazin-1-yl of compound **38** which displayed an excellent activating effect on Sirt6. As listed in Table 3, moving the nitrogen from the *ortho* to *para* position of the side chain (**60**) or inserting an additional nitrogen (**61**) or 5-Cl (**62**) on the pyridine ring led to a complete loss in activating Sirt6 deacetylation. Intriguingly, in contrast to compound **38**, the substitution of the pyridine ring with the benzene ring (**63**) only resulted in a slight decrease in potency, exhibiting the activation fold of 5.43 and 2.62 at 100 μM and 30 μM, respectively. Either acetylating the -NH- group of the tricyclic ring (**64**) or dehydrogenating the quinoxaline ring (**65**) was unfavorable, and not active up to 100 μM. Interestingly, replacing the -NH- group of the quinoxaline ring with an oxygen atom (**66**) slightly diminished the activating effect (4.23-fold at 100 μM and 2.67-fold at 30 μM). The ring-opened derivative **67** did not activate Sirt6.

To facilitate a quick investigation of the effect of substituents on other positions of the tricyclic system, we kept the tricyclic ring of derivative **66** of 4*H*-benzo[*b*]pyrrolo[1,2-*d*][1,4]oxazine intact to prepare and evaluate compounds **68–76** (Table 4). As compared with compound **66**, insertion of 8-CH_3 (**68**), 8-OCH_3 (**69**), 7-$COOCH_3$ (**71**), or 1-Br (**76**) on the tricyclic ring maintained the same level of potency with activation folds of 4.20~5.65 and 2.69~4.51 at 100 μM and 30 μM, respectively. Compounds with 8-F (**70**) and 7-CH_2OH (**72**) showed a decrease in activating Sirt6 deacetylation (2.53-fold and 2.69-fold at 100 μM, respectively) while the substituents 7-$CONH_2$ (**73**), 7-$CONH(CH_2)_2SO_2CH_3$ (**74**), and 1-CH_2OH (**75**) resulted in a dramatic loss of potency. These results indicated that the hydrophobic groups such as methyl and trifluoromethyl on the tricyclic ring are beneficial for the activating effect on Sirt6, while the hydrophilic substituents such as hydroxyl and sulfonyl are unfavorable.

2.3. Evaluation of selected compounds on Sirt activities

We next selected six potent and representative compounds (**35**, **36**, **38**, **46**, **47**, and **50**), evaluated their activating effects on Sirt6 deacetylation at different time points, and compared their activities with that of lead compound UBCS039. As shown in Figs. 2A and 2B, these selected compounds exhibited a declining trend in promoting Sirt6 activity from 15 min to 125 min at concentrations of both 100 μM and 30 μM, which is a typical phenomenon of enzyme kinetics when the substrate is consumed over time. The maximum activating effects were observed at 15 min for these compounds, displaying ~5–8-fold and ~3–5-fold activation at 100 μM and 30 μM, respectively, which is much higher than the activity of UBCS039. Moreover, these selected compounds **35**, **36**, **38**, **46**, **47**, and **50** showed dose-dependent increases in Sirt6 activities, and the E_{max} and EC_{50} values of each compound were presented in Fig. 2C.

To detect whether the activating effect on Sirt6 deacetylation of this class of derivatives is selective, we also measured their effects on the activities of other Sirt family members. As expected, compounds **35**, **36**, **38**, **46**, **47**, and **50** specifically promoted Sirt6 activity (Fig. 3A), but showed no significant effects on Sirt1, Sirt2 and Sirt3 deacetylation activities and Sirt5 desuccinylation activity (Figs. 3B–3E), in agreement with the reported selectivity of the lead compound UBCS039 [28].

2.4. Docking analysis of compound 38 with Sirt6 protein

A docking study of compound **38** with Sirt6/ADP ribose structure (PDB ID: 5MF6) demonstrated that the core of **38** binds to Sirt6 at the hydrophobic pocket formed by F64/82/86, I61, P62, and M136, the binding site where UBCS039 binds at, in a slightly different conformation (Figs 4A and 4B). The tricyclic moiety of compound **38** is anchored at the hydrophobic pocket through hydrophobic interaction with F64/82/86, I61, and V115, while the pyridine ring interacts with M136 through hydrophobic interaction, consistent with previous SAR results that the hydrophobic substituents on the tricyclic ring are beneficial and introducing the benzene ring instead of the pyridine ring is well tolerated. The additional piperazinyl moiety of the side chain extended to the exit of the acyl channel, into a site formed by L186, D187, W188, M157, and W71. The protonated nitrogen of the dimethylamino group on the side chain formed π cation interactions with W188 (Figs 4A and 4C), further stabilizing compound **38** into this extended binding pocket.

2.5. Evaluation of selected compounds on the deacetylation of H3K9 in nucleosomes

Next, we explored the effects of selected compounds on Sirt6-dependent deacetylation of complete nucleosomes extracted from human embryonic kidney (HEK) 293T cells, as nucleosomes represent more physiologically relevant substrates. As shown in Fig. 5A, compounds **35**, **36**, **38**, **46**, and **50** significantly decreased the acetylation of H3K9 in the nucleosome test, while no effect was observed for compound **47**, likely due to poor aqueous solubility. More importantly, no obvious toxicity on retinal precursor cells (R28), which is a kind of neurons and sensitive to compound toxicity, was observed for these compounds at 30 μM, and even at 100 μM for compounds **35** and **36** (Figs. 5B and 5C), indicating their great safety advantages.

2.6. Evaluation of compounds 35 and 36 in cancer cells

Considering the strong activating effects on the deacetylation of H3K9 in nucleosomes as well as the low toxicity on R28 cells even at 100 μM, compounds **35** and **36** were further selected to evaluate their effects on Sirt6 deacetylation in cancer cells. Briefly, we treated cancer cells H1299 and PLC/PRF/5 with compounds **35** or **36** at the indicated concentrations for 24 hours, then calculated the ratio of acetylated H3K9 to total histone H3. Compound **36** significantly activated Sirt6 to deacetylate H3K9 at 30 μM in both H1299 and PLC/PRF/5 cells, while no obvious influence was observed for compound **35** (Figs. 6A and 6B). When Sirt6 was knockdown by siRNA, the decrease of acetylated H3K9 by compound **36** was abolished in both H1299 and PLC/PRF/5 cells (Figs. 6C and 6D), suggesting that the activity of compound **36** depends on Sirt6. As Sirt6 acts as a tumor suppressor in some cancer types, such as non-small cell lung carcinoma and hepatoma,[5, 34–37] we further evaluated the effects of compounds **35** and **36** on the clonogenicity of H1299 and PLC/PRF/5 cells in a colony formation. As shown in Figs. 6E and 6F, about half of colony formation of H1299 and PLC/PRF/5 cells was blocked by **36** at 30 μM, but only a slight inhibitory effect was observed for **35**.

2.7. Anti-inflammatory activities of selected compounds

Sirt6 plays a pivotal role in regulating inflammatory diseases [38–40]. It was reported that Sirt6 interacts with the NF-κB RelA subunit and modulates NF-κB-dependent gene expression via its deacetylation of H3K9 at NF-κB target gene promoters [41]. Here, NF-κB targeted inflammatory genes (IL-1β, IL-6, MCP-1, TNFα, iNOS, CXCL10, VCAM-1 and ICAM-1) were chosen to assess the anti-inflammatory effects of this class of derivatives. BV2, a type of immortalized murine microglial cells which are widely used to determine the mechanisms of microglial activation [42], were pretreated with 30 μM compounds for 30 minutes followed by the treatment of 100 ng/mL LPS for 6 hours. The expressions of inflammatory genes, including IL-1β, IL-6, MCP-1, TNFα, iNOS, CXCL10, VCAM-1, and ICAM-1 were significantly induced by LPS treatment (Fig. 7A). Intriguingly, all selected compounds (**35**, **36**, **38**, **46**, **47**, and **50**), as well as the lead compound UBCS039, strongly inhibited LPS-induced expression of IL-1β, IL-6, MCP-1, and TNFα, and especially, compounds **46**, **47**, and **50** almost reversed LPS-induced IL-1β, IL-6, and MCP-1 response (Figs. 7B–7E). In addition, compounds **46**, **47**, and **50** significantly repressed LPS-induced iNOS and CXCL10 expression (Figs. 7F and 7G), whereas **46** and **47** could also obviously inhibit VCAM-1 induction (Fig. 7H). However, selected compounds **35**, **38**, **47**, and **50** only exhibited a slight inhibition against ICAM-1 induction (Fig. 7I). These promising data highlight the great potential of this class of Sirt6 activators to be developed as anti-inflammatory agents.

2.8. Anti-SARS-CoV-2 activities of selected compounds

SARS-CoV-2 is an enveloped and positive-sense single-stranded RNA virus that is responsible for the ongoing global pandemic of debilitating respiratory illness COVID-19 [43–45]. We further selected four potent Sirt6 activators (**35**, **36**, **38**, and **46**) along with the lead compound UBCS039 for evaluating their potentials against SARS-CoV-2. Interestingly, as shown in Figs. 8A and 8C, these compounds showed micromolar potency in inhibiting SARS-CoV-2 infection, and compound **38**, the most potent one, displayed an EC_{50} value of 9.3 μM. In addition, none of these compounds exhibited significant cytotoxicity at the highest tested concentration of 50 μM (Fig. 8B). These data indicate the therapeutic potential of Sirt6 activators as SARS-CoV-2 inhibitors. However, their exact mechanism in inhibiting SARS-CoV-2 remains to be elucidated.

CONCLUSION

In summary, a series of novel pyrrolo[1,2-*a*]quinoxaline-based derivatives have been identified as potent and selective Sirt6 activators with significantly improved efficacy and low cytotoxicity. Sirt6-knockdown abolished the decrease of acetylated H3K9 caused by compound **36** (**GL0752**) in both H1299 and PLC/PRF/5 cells, suggesting that the on-target effects of stimulating deacetylation of these derivatives depend on Sirt6. Molecular docking studies indicate that the protonated nitrogen on the side chain of compound **38** (**GL0710**) forms π-cation interactions with Trp188, further stabilizing it into this extended binding pocket. This functional hydrophilic side chain provides a useful moiety for designing novel and potent Sirt6 activators with other new scaffolds while tuning their druglike properties such as aqueous solubility. Compounds **35** (**GL0738**), **36**, **38**, **46** (**GL0817**), **47** (**GL0822**),

and **50** (**GL0819**) strongly repressed LPS-induced proinflammatory cytokine/chemokine production, while **38** also significantly suppressed SARS-CoV-2 infection with an EC_{50} value of 9.3 μM. Moreover, compound **36** significantly inhibited the colony formation of cancer cells. These new molecules may serve as useful pharmacological tools for further elucidating the role of Sirt6 in various human conditions or as potential therapeutics against relevant diseases including cancer, inflammation, and infectious diseases. Our further efforts will be focused on conducting the extensive mechanistic studies against each specific disease to understand the exact action modes of these compounds and the systematic SAR studies based on the introduction of the functional hydrophilic side chain to discover more effective Sirt6 activators with good druglike properties and low toxicity as well as the evaluation of their potential efficacy and safety in appropriate animal models which mimic human inflammatory disorders or infectious diseases.

4. Experimental section

4.1. Chemistry

All commercially available starting materials and solvents were reagent grade and used without further purification. Reactions were performed under a nitrogen atmosphere in dry glassware with magnetic stirring. Preparative column chromatography was performed using silica gel 60, particle size 0.063–0.200 mm (70–230 mesh, flash). Analytical TLC was carried out employing silica gel 60 F254 plates (Merck, Darmstadt). Visualization of the developed chromatograms was performed with detection by UV (254 nm). NMR spectra were recorded on a Brucker-300 (^{1}H, 300 MHz; ^{13}C, 75 MHz; ^{19}F, 282 MHz) spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with TMS as an internal reference. Chemical shifts were expressed in ppm, and *J* values were given in Hz. High-resolution mass spectra (HRMS) were obtained from Thermo Fisher LTQ Orbitrap Elite mass spectrometer. Parameters include the following: Nano ESI spray voltage was 1.8 kV; Capillary temperature was 275 °C and the resolution was 60,000; Ionization was achieved by positive mode. Melting points were measured on a Thermo Scientific Electrothermal Digital Melting Point Apparatus and uncorrected. Purities of final compounds were established by analytical HPLC, which was carried out on a Shimadzu HPLC system (model: CBM-20A LC-20AD SPD-20A UV/VIS). HPLC analysis conditions: Waters μBondapak C18 (300 × 3.9 mm); flow rate 0.5 mL/min; UV detection at 270 and 254 nm; linear gradient from 10% acetonitrile in water to 100% acetonitrile in water in 20 min followed by 30 min of the last-named solvent (0.1% TFA was added into both acetonitrile and water). All biologically evaluated compounds are > 95% pure.

4-(2-Chloropyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (14)—To a solution of 2-chloronicotinaldehyde (25 mg, 0.16 mmol) and 2-(1*H*-pyrrol-1-yl)aniline (22 mg, 0.16 mmol) in EtOH (5 mL) was added 3 drops of AcOH. The reaction mixture was stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **14** as a pale-yellow foam (29 mg, 69%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (dd, *J* = 4.8, 2.1 Hz, 1H), 7.44 (dd, *J* = 7.7, 1.9 Hz, 1H), 7.38 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.29 – 7.28 (m, 1H), 7.15 (dd, *J* = 7.8, 4.8 Hz, 1H), 6.98 (td, *J* = 7.5, 1.5 Hz, 1H), 6.88 (td, *J* = 7.8, 1.5 Hz, 1H), 6.74 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.36 (t, *J* = 3.3 Hz,

1H), 6.07 (s, 1H), 5.92 – 5.91 (m, 1H), 4.56 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 149.6, 148.9, 148.8, 138.1, 138.0, 136.4, 134.4, 126.1, 125.2, 125.0, 123.1, 123.0, 119.8, 115.9, 114.8, 114.6, 110.6, 110.5, 106.4, 106.1, 51.7, 51.6. HRMS (ESI) calcd for $C_{16}H_{13}ClN_3$, 282.0798 $[M + H]^+$; found, 282.0788.

3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)-N,N-dimethylpyridin-2-amine (15)—To a solution of 2-chloronicotinaldehyde (141 mg, 1.0 mmol) in toluene (5 mL) was added dimethylamine hydrochloride (162 mg, 2.0 mmol) and K_2CO_3 (276 mg, 2.0 mmol). The reaction mixture was stirred at 110 °C overnight, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/EtOAc) to give 2-(dimethylamino)nicotinaldehyde (**11a**) as yellow oil (130 mg, 86%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.92 (s, 1H), 8.27 (dd, *J* = 4.6, 2.0 Hz, 1H), 7.90 (dd, *J* = 7.6, 2.0 Hz, 1H), 6.74 (dd, *J* = 7.6, 4.7 Hz, 1H), 3.09 (s, 6H).

To a solution of **11a** (40 mg, 0.27 mmol) and 2-(1*H*-pyrrol-1-yl)aniline (48 mg, 0.3 mmol) in EtOH (5 mL) was added 3 drops of AcOH. The reaction mixture was stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **15** as a pale-yellow foam (43 mg, 74%). HPLC purity 98.7% (t_R = 10.49 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.28 (dd, *J* = 4.8, 2.1 Hz, 1H), 7.64 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.98 – 6.83 (m, 3H), 6.69 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.33 (t, *J* = 3.3 Hz, 1H), 5.89 (s, 1H), 5.80 (dd, *J* = 3.5, 1.5 Hz, 1H), 4.60 (s, 1H), 2.90 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.3, 147.3, 138.8, 136.5, 128.9, 128.4, 125.8, 124.7, 119.6, 118.5, 115.7, 114.9, 114.8, 110.0, 106.2, 50.8, 43.5. HRMS (ESI) calcd for $C_{18}H_{19}N_4$, 291.1610 $[M + H]^+$; found, 291.1599.

4-(2-(Azetidin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (16)—Compound **16** was prepared by following a procedure similar to that used to prepare compound **15**, starting from 2-chloronicotinaldehyde, azetidine hydrochloride, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (21 mg, 35%) was obtained as a pale-yellow foam. HPLC purity 98.5% (t_R = 10.73 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.42 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.34 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.96 (td, *J* = 7.5, 1.5 Hz, 1H), 6.85 (td, *J* = 7.5, 1.5 Hz, 1H), 6.74 – 6.65 (m, 2H), 6.33 (t, *J* = 3.3 Hz, 1H), 5.84 (ddd, *J* = 3.6, 1.5, 0.9 Hz, 1H), 4.44 (s, 1H), 4.28 (q, *J* = 7.5 Hz, 2H), 4.07 (q, *J* = 7.5 Hz, 2H), 2.34 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.9, 147.3, 137.6, 135.8, 128.6, 125.5, 124.7, 122.5, 119.5, 115.9, 114.9, 114.7, 114.6, 110.1, 106.4, 52.9, 50.5, 17.2. HRMS (ESI) calcd for $C_{19}H_{19}N_4$, 303.1610 $[M + H]^+$; found, 303.1598.

1-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)azetidin-3-ol (17)—Compound **17** was prepared by following a procedure similar to that used to prepare compound **15**, starting from 2-chloronicotinaldehyde, 3-hydroxyazetidine hydrochloride, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (19 mg, 30%) was obtained as a white foam. HPLC purity 95.0% (t_R = 13.61 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.15 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.47 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.34 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.23 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.96 (td, *J* = 7.6, 1.5 Hz, 1H), 6.86 (td, *J* = 7.8, 1.5 Hz, 1H), 6.73 – 6.69 (m, 2H), 6.31 (t, *J* = 3.3 Hz, 1H), 5.79 (dd, *J* = 3.3, 1.5 Hz, 1H), 5.60 (s, 1H), 4.69 (tt,

J = 6.5, 4.8 Hz, 1H), 4.50 – 4.40 (m, 2H), 4.27 (dd, *J* = 8.7, 6.5 Hz, 1H), 4.09 (dd, *J* = 8.7, 4.8 Hz, 1H), 3.89 (ddd, *J* = 8.7, 4.5, 1.2 Hz, 1H), 2.63 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.5, 147.2, 138.0, 135.8, 128.5, 125.5, 124.8, 122.8, 119.7, 115.9, 115.4, 114.8, 114.6, 110.2, 106.5, 62.7, 62.5, 62.0, 50.7. HRMS (ESI) calcd for $C_{19}H_{19}N_4O$, 319.1559 $[M + H]^+$; found, 319.1545.

4-(2-(Pyrrolidin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (18)—To a solution of 2-chloronicotinaldehyde (141 mg, 1.0 mmol) in toluene (5 mL) was added pyrrolidine (261 mg, 3.0 mmol). The reaction mixture was stirred at 110 °C overnight, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/EtOAc) to give 2-(pyrrolidin-1-yl)nicotinaldehyde (**11d**) as yellow oil (75 mg, 42%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.98 (s, 1H), 8.29 (dd, *J* = 4.6, 2.0 Hz, 1H), 7.91 (dd, *J* = 7.7, 2.0 Hz, 1H), 6.67 (dd, *J* = 7.6, 4.6 Hz, 1H), 3.56 – 3.47 (m, 4H), 2.01 – 1.90 (m, 4H).

Compound **18** was prepared by following a procedure similar to that used to prepare compound **15**, starting from **11d** and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (18 mg, 29%) was obtained as a pale-yellow foam. HPLC purity 99.3% (t_R = 11.28 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.19 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.66 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.35 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.96 (td, *J* = 7.5, 1.5 Hz, 1H), 6.85 (td, *J* = 7.5, 1.5 Hz, 1H), 6.77 – 6.70 (m, 2H), 6.33 (t, *J* = 3.2 Hz, 1H), 5.84 – 5.81 (m, 2H), 4.19 (s, 1H), 3.73 – 3.64 (m, 2H), 3.45 – 3.38 (m, 2H), 2.03 – 1.86 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.6, 146.9, 138.8, 136.5, 129.7, 125.8, 124.7, 123.7, 119.6, 115.7, 115.3, 114.8, 114.7, 110.0, 106.6, 51.1, 50.9, 25.6. HRMS (ESI) calcd for $C_{20}H_{21}N_4$, 317.1766 $[M + H]^+$; found, 317.1753.

4-(2-(4-Methyl-1,4-diazepan-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (19)—*To a solution of* 2-chloronicotinaldehyde (141 mg, 1.0 mmol) and 1-methylhomopiperazine (228 mg, 2.0 mmol) in DMF (5 mL) was added K_2CO_3 (414 mg, 3.0 mmol) and 18-crown-6 (132 mg, 0.5 mmol). The reaction mixture was stirred at 100 °C overnight. Then the mixture was diluted with EtOAc, washed with H_2O, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/EtOAc) to give 2-(4-methyl-1,4-diazepan-1-yl)nicotinaldehyde (**11e**) as yellow oil (90 mg, 41%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.90 (s, 1H), 8.29 – 8.23 (m, 1H), 7.94 – 7.86 (m, 1H), 6.75 – 6.68 (m, 1H), 3.82 – 3.72 (m, 2H), 3.60 (t, *J* = 5.9 Hz, 2H), 2.79 – 2.71 (m, 2H), 2.58 – 2.50 (m, 2H), 2.33 (s, 3H), 2.05 – 1.96 (m, 2H).

Compound **19** was prepared by following a procedure similar to that used to prepare compound **15**, starting from **11e** and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (27 mg, 37%) was obtained as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.52 (dd, *J* = 7.5, 2.1 Hz, 1H), 7.35 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.96 (td, *J* = 7.5, 1.5 Hz, 1H), 6.88 – 6.82 (m, 2H), 6.71 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.33 (t, *J* = 3.3 Hz, 1H), 5.83 – 5.81 (m, 2H), 4.83 (s, 1H), 3.71 – 3.57 (m, 3H), 3.50 – 3.42 (m, 1H), 2.95 – 2.73 (m, 4H), 2.46 (s, 3H), 2.14 – 1.96 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.1, 147.1, 138.8, 136.2, 128.4, 127.9, 125.7, 124.7, 119.4, 118.1, 115.7, 114.8, 114.8, 110.1, 106.2, 58.6, 57.3, 53.4, 52.6, 50.9, 46.8, 28.0. HRMS (ESI) calcd for $C_{22}H_{26}N_5$, 360.2188 $[M + H]^+$; found, 360.2178.

4-(2-((3aR,6aS)-5-Methylhexahydropyrrolo[3,4-c]pyrrol-2(1H)-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (20)—Compound **20** was prepared by following a procedure similar to that used to prepare compound **18**, starting from 2-chloronicotinaldehyde, cis-2-methylhexahydropyrrolo[3,4-*c*]pyrrole, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (21 mg, 28%) was obtained as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.42 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.33 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.95 (td, *J* = 7.5, 1.5 Hz, 1H), 6.88 – 6.80 (m, 2H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.34 (t, *J* = 3.3 Hz, 1H), 5.87 – 5.85 (m, 2H), 3.65 (dd, *J* = 10.5, 5.7 Hz, 1H), 3.53 (d, *J* = 10.2 Hz, 1H), 3.18 – 3.10 (m, 2H), 2.99 – 2.90 (m, 4H), 2.70 – 2.65 (m, 1H), 2.62 – 2.57 (m, 1H), 2.44 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.1, 147.2, 138.1, 136.2, 128.2, 127.9, 125.5, 124.8, 119.2, 118.2, 115.7, 114.7, 114.7, 110.0, 106.4, 62.5, 62.4, 57.1, 56.3, 51.2, 42.2, 41.2, 41.2. HRMS (ESI) calcd for $C_{23}H_{26}N_5$, 372.2188 $[M + H]^+$; found, 372.2175.

4-(2-(4-Methylpiperazin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (21)—Compound **21** was prepared by following a procedure similar to that used to prepare compound **18**, starting from 2-chloronicotinaldehyde, 1-methylpiperazine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (128 mg, 53%) was obtained as colorless oil. HPLC purity 96.0% (t_R = 10.15 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.27 (dd, *J* = 4.8, 2.0 Hz, 1H), 7.61 (dd, *J* = 7.7, 2.1 Hz, 1H), 7.33 (d, *J* = 6.2 Hz, 1H), 7.22 (d, *J* = 2.7 Hz, 1H), 6.99 – 6.90 (m, 2H), 6.89 – 6.80 (m, 1H), 6.70 (d, *J* = 6.9 Hz, 1H), 6.29 (t, *J* = 3.2 Hz, 1H), 5.80 (s, 1H), 5.73 (d, *J* = 4.1 Hz, 1H), 3.43 – 3.31 (m, 2H), 3.18 – 3.07 (m, 2H), 2.68 – 2.46 (m, 4H), 2.33 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.5, 147.7, 138.9, 136.5, 129.3, 128.8, 125.8, 124.8, 119.6, 119.3, 115.7, 115.0, 114.9, 110.2, 106.3, 55.5, 51.6, 50.6, 46.2. HRMS (ESI) calcd for $C_{21}H_{24}N_5$, 346.2032 $[M + H]^+$; found, 346.2026.

1-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)ethan-1-one (22)—Compound **22** was prepared by following a procedure similar to that used to prepare compound **18**, starting from 2-chloronicotinaldehyde, 1-acetylpiperazine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (34 mg, 46%) was obtained as a yellow foam. HPLC purity 95.2% (t_R = 10.42 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.78 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.37 (dd, *J* = 7.8, 1.2 Hz, 1H), 7.24 (dd, *J* = 3.3, 1.5 Hz, 1H), 7.05 – 6.95 (m, 2H), 6.88 (td, *J* = 7.8, 1.2 Hz, 1H), 6.73 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.29 (t, *J* = 3.3 Hz, 1H), 5.85 (s, 1H), 5.67 (dd, *J* = 3.3, 1.5 Hz, 1H), 4.46 (s, 1H), 3.81 – 3.49 (m, 4H), 3.36 – 3.22 (m, 2H), 3.13 – 3.01 (m, 2H), 2.11 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.1, 161.0, 147.8, 139.2, 136.3, 129.5, 128.8, 125.7, 124.8, 119.9, 119.7, 115.6, 115.0, 114.9, 110.1, 106.1, 51.7, 51.6, 50.4, 46.4, 41.5, 21.4. HRMS (ESI) calcd for $C_{22}H_{24}N_5O$, 374.1981 $[M + H]^+$; found, 374.1973.

4-(2-(4-(Methylsulfonyl)piperazin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (23)—Compound **23** was prepared by following a procedure similar to that used to prepare compound **18**, starting from 2-chloronicotinaldehyde, 1-methylsulfonyl-piperazine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (61 mg, 74%) was obtained as a pale-yellow foam. HPLC purity 97.7% (t_R = 10.74 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.35 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.83 (dd,

J = 7.5, 1.8 Hz, 1H), 7.39 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.07 (dd, *J* = 7.5, 4.8 Hz, 1H), 7.01 (td, *J* = 7.5, 1.5 Hz, 1H), 6.91 (td, *J* = 7.8, 1.5 Hz, 1H), 6.75 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.31 – 6.29 (m, 1H), 5.82 (s, 1H), 5.66 – 5.64 (m, 1H), 4.29 (s, 1H), 3.46 – 3.18 (m, 8H), 2.81 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.9, 147.9, 139.3, 136.4, 129.2, 128.8, 125.8, 124.8, 120.1, 119.8, 115.5, 115.0, 115.0, 110.2, 106.1, 51.2, 50.5, 45.9, 34.4. HRMS (ESI) calcd for $C_{21}H_{24}N_5O_2S$, 410.1651 $[M + H]^+$; found, 410.1635.

4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)morpholine (24)— Compound **24** was prepared by following a procedure similar to that used to prepare compound **18**, starting from 2-chloronicotinaldehyde, morpholine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (45 mg, 69%) was obtained as a pale-yellow foam. HPLC purity 97.0% (t_R = 10.49 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.76 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.38 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.04 – 6.97 (m, 2H), 6.89 (td, *J* = 7.5, 1.5 Hz, 1H), 6.74 (dd, *J* = 7.7, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.85 (s, 1H), 5.71 – 5.69 (m, 1H), 4.47 (s, 1H), 3.93 – 3.76 (m, 4H), 3.40 – 3.32 (m, 2H), 3.13 – 3.05 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.3, 147.8, 139.1, 136.4, 129.3, 128.9, 125.8, 124.8, 119.7, 119.6, 115.6, 115.0, 114.9, 110.1, 106.2, 67.1, 52.1, 50.4. HRMS (ESI) calcd for $C_{20}H_{21}N_4O$, 333.1715 $[M + H]^+$; found, 333.1705.

4-(2-(4,4-Difluoropiperidin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (25)—Compound **25** was prepared by following a procedure similar to that used to prepare compound **15**, starting from 2-chloronicotinaldehyde, 4,4-difluoropiperidine hydrochloride, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (21 mg, 29%) was obtained as a pale-yellow foam. HPLC purity 96.7% (t_R = 11.95 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.32 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.77 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.38 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.05 − 6.97 (m, 2H), 6.90 (td, *J* = 7.8, 1.5 Hz, 1H), 6.74 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.31 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.70 – 5.68 (m, 1H), 4.35 (s, 1H), 3.49 – 3.40 (m, 2H), 3.31 – 3.22 (m, 2H), 2.30 − 1.97 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.9, 147.8, 147.6, 139.2, 139.0, 136.3, 129.1, 128.8, 125.8, 125.0, 124.8, 121.8, 119.8, 119.7, 118.6, 115.6, 115.1, 115.0, 114.9, 110.2, 110.0, 106.1, 105.9, 50.7, 50.5, 48.8, 34.2 (t, *J* = 23 Hz). HRMS (ESI) calcd for $C_{21}H_{21}F_2N_4$, 367.1734 $[M + H]^+$; found, 367.1724.

4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)thiomorpholine 1,1-dioxide (26)—To a solution of 2-chloronicotinaldehyde (141 mg, 1.0 mmol) and thiomorpholine 1,1-dioxide (135 mg, 1.0 mmol) in DMF (5 mL) was added K_2CO_3 (276 mg, 2.0 mmol). The reaction mixture was stirred at 100 °C overnight. Then the mixture was diluted with EtOAc, washed with H_2O, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/EtOAc) to give 2-(1,1-dioxidothiomorpholino)nicotinaldehyde (**11l**) as a white solid (57 mg, 23%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.98 (s, 1H), 8.39 (dd, *J* = 4.8, 2.0 Hz, 1H), 8.04 (dd, *J* = 7.6, 2.0 Hz, 1H), 7.06 (dd, *J* = 7.6, 4.7 Hz, 1H), 4.02 – 3.92 (m, 4H), 3.27 – 3.20 (m, 4H).

Compound **26** was prepared by following a procedure similar to that used to prepare compound **15**, starting from **11l** and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (46 mg, 61%) was obtained as a white solid. HPLC purity 95.9% (t_R = 14.92 min). ^{1}H NMR (300

MHz, $CDCl_3$) δ 8.32 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.90 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.39 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.11 (dd, *J* = 7.8, 4.8 Hz, 1H), 7.02 (td, *J* = 7.5, 1.5 Hz, 1H), 6.92 (td, *J* = 7.8, 1.5 Hz, 1H), 6.76 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.29 (t, *J* = 3.3 Hz, 1H), 5.76 (s, 1H), 5.57 (dd, *J* = 3.0, 1.5 Hz, 1H), 4.15 (s, 1H), 3.89 – 3.69 (m, 4H), 3.37 – 3.28 (m, 2H), 3.10 – 3.01 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.7, 147.8, 139.8, 136.2, 128.7, 125.7, 125.0, 120.4, 120.1, 115.6, 115.3, 115.0, 110.2, 105.7, 51.1, 50.9, 50.0. HRMS (ESI) calcd for $C_{20}H_{21}N_4O_2S$, 381.1385 $[M + H]^+$; found, 381.1371.

1-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperidin-4-ol (27)— Compound **27** was prepared by following a procedure similar to that used to prepare compound **18**, starting from 2-chloronicotinaldehyde, 4-hydroxypiperidine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (37 mg, 53%) was obtained as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.28 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.59 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.00 – 6.94 (m, 2H), 6.86 (td, *J* = 7.8, 1.5 Hz, 1H), 6.71 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.32 (t, *J* = 3.2 Hz, 1H), 5.85 (s, 1H), 5.77 (dd, *J* = 3.0, 1.5 Hz, 1H), 4.71 (s, 1H), 3.87 (tt, *J* = 9.0, 4.2 Hz, 1H), 3.53 – 3.45 (m, 1H), 3.39 – 3.31 (m, 1H), 3.18 (ddd, *J* = 12.8, 10.2, 3.0 Hz, 1H), 2.90 (ddd, *J* = 12.8, 10.2, 3.0 Hz, 1H), 2.13 – 1.95 (m, 2H), 1.85 – 1.63 (m, 3H). ^{13}C NMR (75 MHz, CDCl3) δ 161.75, 147.54, 138.68, 136.20, 129.84, 128.44, 125.68, 124.78, 119.57, 119.44, 115.64, 114.86, 114.82, 110.08, 106.11, 67.92, 50.57, 48.79, 34.96, 34.89. HRMS (ESI) calcd for $C_{21}H_{23}N_4O$, 347.1872 $[M + H]^+$; found, 347.1864.

4-(2-(4-(Pyridin-2-yl)piperazin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (28)—Compound **28** was prepared by following a procedure similar to that used to prepare compound **18**, starting from 2-chloronicotinaldehyde, 1-(2-pyridyl)piperazine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (39 mg, 47%) was obtained as a pale-yellow foam. HPLC purity 96.4% (t_R = 10.15 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (dd, *J* = 4.8, 1.8 Hz, 1H), 8.21 (dd, *J* = 5.0, 2.1 Hz, 1H), 7.73 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.50 (ddd, *J* = 9.0, 7.1, 2.1 Hz, 1H), 7.37 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.03 – 6.95 (m, 2H), 6.88 (td, *J* = 7.8, 1.5 Hz, 1H), 6.74 – 6.63 (m, 3H), 6.33 (t, *J* = 3.3 Hz, 1H), 5.92 (s, 1H), 5.76 (dt, *J* = 3.3, 1.2 Hz, 1H), 4.60 (s, 1H), 3.79 – 3.72 (m, 2H), 3.65 – 3.57 (m, 2H), 3.51 – 3.43 (m, 2H), 3.29 – 3.21 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.3, 159.6, 147.9, 147.7, 139.0, 137.5, 136.3, 129.4, 128.7, 125.7, 124.8, 119.7, 119.6, 115.6, 115.0, 114.9, 113.6, 110.1, 107.4, 106.2, 51.4, 50.5, 45.7. HRMS (ESI) calcd for $C_{25}H_{25}N_6$, 409.2141 $[M + H]^+$; found, 409.2126.

4-(2-(4-(Pyrrolidin-1-yl)piperidin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (29)—Compound **29** was prepared by following a procedure similar to that used to prepare compound **18**, starting from 2-chloronicotinaldehyde, 4-(1-pyrrolidinyl)piperidine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (28 mg, 36%) was obtained as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.26 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.48 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.33 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.97 – 6.89 (m, 2H), 6.85 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.67 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.33 (t, *J* = 3.3 Hz, 1H), 5.84 – 5.82 (m, 2H), 4.89 (s, 1H), 3.55 – 3.38 (m, 2H), 3.16 (td, *J* = 12.3, 2.4 Hz, 1H), 2.83

(td, J = 12.3, 2.4 Hz, 1H), 2.65 – 2.61 (m, 4H), 2.24 – 2.11 (m, 2H), 2.03 – 1.97 (m, 1H), 1.86 – 1.67 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.8, 147.5, 138.3, 136.2, 129.9, 128.2, 125.6, 124.8, 119.4, 119.2, 115.6, 114.8, 114.7, 110.0, 106.0, 61.6, 52.7, 51.5, 50.7, 49.1, 32.3, 32.1, 23.3. HRMS (ESI) calcd for $C_{25}H_{30}N_5$, 400.2501 $[M + H]^+$; found, 400.2488.

2-((3-(Pyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)oxy)ethan-1-amine (30)—To a solution of 2-fluoronicotinaldehyde (500 mg, 4.0 mmol) and *N*-Boc-ethanolamine (1287 mg, 8.0 mmol) in DMF (10 mL) was added Na_2CO_3 (847 mg, 8.0 mmol). The reaction mixture was stirred at 110 °C for 6 h, and then water (50 mL) was added. The mixture was extracted with EtOAc (3 × 60 mL), washed with water (3 × 20 mL), dried over Na_2SO_4, filtered, concentrated *in vacuo* and purified by silica gel column chromatography (Hex/EtOAc) to give tert-butyl (2-((3-formylpyridin-2-yl)oxy)ethyl)carbamate (**13**) as pale-yellow liquid (460 mg, 43%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.37 (d, J = 1.0 Hz, 1H), 8.34 (dd, J = 4.9, 2.1 Hz, 1H), 8.11 (dd, J = 7.5, 2.1 Hz, 1H), 7.02 (ddd, J = 7.4, 4.9, 0.9 Hz, 1H), 4.96 (s, 1H), 4.53 (t, J = 5.3 Hz, 2H), 3.59 (q, J = 5.6 Hz, 2H), 1.43 (s, 9H).

To a solution of **13** (282 mg, 1.05 mmol) and 2-(1*H*-pyrrol-1-yl)aniline (158 mg, 1.0 mmol) in EtOH (5 mL) was added 6 drops of AcOH. The reaction mixture was stirred at 50 °C for 8 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/ EtOAc) to give the crude intermediate. The intermediate was dissolved in CH_2Cl_2 (5 mL), and TFA (1.14 g, 10 mmol) was slowly added. The resulting mixture was stirred at RT overnight and then treated with saturated $NaHCO_3$ (10 mL). The mixture was extracted with CH_2Cl_2 (3 × 30 mL), dried over Na_2SO_4, filtered, concentrated, and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **30** as a pale-yellow foam (101 mg, 33% in two steps). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.99 (dd, J = 5.1, 1.8 Hz, 1H), 7.32 (dd, J = 7.8, 1.5 Hz, 1H), 7.24 (dd, J = 3.0, 1.5 Hz, 1H), 7.09 (dd, J = 7.2, 1.8 Hz, 1H), 6.89 (td, J = 7.5, 1.5 Hz, 1H), 6.81 – 6.67 (m, 3H), 6.34 (t, J = 3.0 Hz, 1H), 5.94 – 5.92 (m, 2H), 4.53 – 4.40 (m, 2H), 3.17 – 3.12 (m, 2H), 2.12 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.6, 145.8, 136.7, 135.2, 126.5, 125.2, 125.1, 124.7, 119.0, 117.2, 115.7, 114.5, 114.3, 110.2, 105.8, 67.8, 49.4, 41.2. HRMS (ESI) calcd for $C_{18}H_{19}N_4O$, 307.1559 $[M + H]^+$; found, 307.1549.

2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)ethan-1-ol (35)—To a solution of 2-chloronicotinaldehyde (141 mg, 1.0 mmol) in toluene (5 mL) was added 2-(piperazin-1-yl)ethan-1-ol (195 mg, 1.5 mmol). The reaction mixture was heated to reflux and stirred for 5 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give 2-(4-(2-hydroxyethyl)piperazin-1-yl)nicotinaldehyde (**32a**) as a yellow solid (53 mg, 23%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.99 (s, 1H), 8.37 (dd, J = 4.8, 2.1 Hz, 1H), 7.99 (dd, J = 7.5, 2.1 Hz, 1H), 6.95 (dd, J = 7.5, 4.8 Hz, 1H), 3.81 – 3.77 (m, 2H), 3.61 (t, J = 4.8 Hz, 4H), 2.92 (t, J = 4.8 Hz, 4H), 2.85 – 2.79 (m, 2H).

To a solution of **32a** (53 mg, 0.23 mmol) and 2-(1*H*-pyrrol-1-yl)aniline (45 mg, 0.28 mmol) in EtOH (2 mL) was added 1 drops of AcOH. The reaction mixture was stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **35** as yellow solid (30 mg, 35%). HPLC purity 98.2%

(t_R = 13.86 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.67 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.37 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.01 – 6.95 (m, 2H), 6.88 (td, *J* = 7.8, 1.5 Hz, 1H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.31 (t, *J* = 3.3 Hz, 1H), 5.84 (s, 1H), 5.74 (dt, *J* = 3.4, 1.2 Hz, 1H), 4.58 (s, 1H), 3.67 – 3.63 (m, 2H), 3.42 – 3.35 (m, 2H), 3.17 – 3.10 (m, 2H), 2.78 – 2.58 (m, 7H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.3, 147.7, 138.9, 136.3, 129.4, 128.7, 125.7, 124.8, 119.6, 119.4, 115.6, 114.9, 114.9, 110.1, 106.2, 59.3, 57.7, 53.1, 51.6, 50.5. HRMS (ESI) calcd for $C_{22}H_{26}N_5O$, 376.2137 $[M + H]^+$; found, 376.2123.

4-(2-(4-(2-Methoxyethyl)piperazin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (36)—Compound **36** was prepared by following a procedure similar to that used to prepare compound **35**, starting from 2-chloronicotinaldehyde, 1-(2-methoxyethyl)piperazine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (50 mg, 65%) was obtained as a pale-yellow foam. HPLC purity 96.7% (t_R = 14.59 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.66 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.23 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.99 – 6.93 (m, 2H), 6.86 (td, *J* = 7.8, 1.5 Hz, 1H), 6.71 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.74 (ddd, *J* = 3.5, 1.5, 0.9 Hz, 1H), 4.60 (s, 1H), 3.55 (t, *J* = 5.7 Hz, 2H), 3.44 – 3.35 (m, 5H), 3.19 – 3.12 (m, 2H), 2.74 – 2.59 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.41, 147.61, 138.83, 136.44, 129.02, 128.82, 125.75, 124.74, 119.56, 119.06, 115.61, 114.89, 114.84, 110.06, 106.18, 70.22, 58.93, 57.97, 53.84, 51.43, 50.51. HRMS (ESI) calcd for $C_{23}H_{28}N_5O$, 390.2294 $[M + H]^+$; found, 390.2281.

4-(2-(4-(3-Methoxypropyl)piperazin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (37)—To a solution of 2-chloronicotinaldehyde (282 mg, 2.0 mmol) in DMF (10 mL) was added piperazine (860 mg, 10.0 mmol). The reaction mixture was stirred at 90 °C overnight, Then the mixture was diluted with EtOAc, washed with H_2O, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give 2-(piperazin-1-yl)nicotinaldehyde (**31**) as a yellow solid.

To a solution of **31** (90 mg, 0.5 mmol) in 5 mL of CH_3CN was added 1-bromo-3-methoxypropane (76 mg, 0.5 mmol) and K_2CO_3 (106 mg, 1.0 mmol) The reaction mixture was stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give 2-(4-(3-methoxypropyl)piperazin-1-yl)nicotinaldehyde (**32d**) as a yellow solid (70 mg, 53%).

To a solution of **32d** (53 mg, 0.2 mmol) and 2-(1*H*-pyrrol-1-yl)aniline (32 mg, 0.2 mmol) in EtOH (2 mL) was added 1 drops of AcOH. The reaction mixture was stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **37** as yellow solid (45 mg, 56%). HPLC purity 95.5% (t_R = 14.89 min).^{1}H NMR (300 MHz, $CDCl_3$) δ 8.30 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.68 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.00 – 6.95 (m, 2H), 6.87 (td, *J* = 7.8, 1.5 Hz, 1H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.81 (s, 1H), 5.72 (dt, *J* = 3.3, 1.2 Hz, 1H), 3.46 – 3.39 (m, 4H), 3.34 (s, 3H), 3.22 – 3.14 (m, 2H), 2.77 – 2.66 (m, 4H), 2.57 (dd, *J* = 9.0, 6.3 Hz, 2H), 1.89 – 1.79 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.2, 147.6, 138.9, 136.3, 129.1, 128.7, 125.7, 124.8, 119.6,

119.3, 115.6, 114.9, 114.9, 110.1, 106.2, 70.9, 58.6, 55.2, 52.9, 51.0, 50.5, 26.3. HRMS (ESI) calcd for $C_{24}H_{30}N_5O$, 404.2450 $[M + H]^+$; found, 404.2437.

2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)-N,N-dimethylethan-1-amine (38)—Compound **38** was prepared by following a procedure similar to that used to prepare compound **35**, starting from 2-chloronicotinaldehyde, 1-(2-dimethylaminoethyl)piperazine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (39 mg, 49%) was obtained as a pale-yellow foam. HPLC purity 98.2% (t_R = 14.18 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.65 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.23 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.00 – 6.91 (m, 2H), 6.86 (td, *J* = 7.5, 1.5 Hz, 1H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.74 (dt, *J* = 3.3, 1.2 Hz, 1H), 4.60 (s, 1H), 3.42 – 3.35 (m, 2H), 3.17 – 3.10 (m, 2H), 2.73 – 2.47 (m, 8H), 2.29 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.4, 147.6, 138.8, 136.4, 129.1, 128.8, 125.7, 124.7, 119.6, 119.1, 115.6, 114.9, 114.8, 110.0, 106.2, 56.8, 56.6, 53.9, 51.5, 50.5, 45.9. HRMS (ESI) calcd for $C_{24}H_{31}N_6$, 403.2610 $[M + H]^+$; found, 403.2599.

2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)-N,N-dimethylacetamide (39)—Compound **39** was prepared by following a procedure similar to that used to prepare compound **37**, starting from compound **31**, 2-chloro-*N*,*N*-dimethylacetamide, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (37 mg, 44%) was obtained as a pale-yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.64 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.35 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.23 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.99 – 6.94 (m, 2H), 6.86 (td, *J* = 7.8, 1.5 Hz, 1H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.29 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.73 (ddd, *J* = 3.6, 1.5, 0.9 Hz, 1H), 4.61 (s, 1H), 3.42 – 3.35 (m, 2H), 3.24 (s, 2H), 3.18 – 3.11 (m, 5H), 2.95 (s, 3H), 2.79 – 2.61 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 169.4, 161.4, 147.6, 138.8, 136.3, 129.4, 128.6, 125.7, 124.8, 119.6, 119.3, 115.6, 114.9, 114.8, 110.0, 106.1, 61.0, 53.4, 51.5, 50.5, 37.2, 35.6. HRMS (ESI) calcd for $C_{24}H_{29}N_6O$, 417.2403 $[M + H]^+$; found, 417.2391.

2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)acetonitrile (40)—Compound **40** was prepared by following a procedure similar to that used to prepare compound **37**, starting from compound **31**, bromoacetonitrile, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (58 mg, 78%) was obtained as a pale-yellow solid. HPLC purity 97.8% (t_R = 14.75 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.32 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.77 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.37 (d, *J* = 7.8 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.04 – 6.97 (m, 2H), 6.89 (td, *J* = 7.5, 1.5 Hz, 1H), 6.75 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.29 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.68 (d, *J* = 3.3 Hz, 1H), 4.40 (s, 1H), 3.58 (s, 2H), 3.45 – 3.37 (m, 2H), 3.21 – 3.14 (m, 2H), 2.84 – 2.66 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.1, 147.7, 139.2, 136.4, 129.3, 128.9, 125.7, 124.9, 119.7, 119.6, 115.7, 115.0, 114.9, 114.7, 110.0, 106.1, 51.9, 51.2, 50.4, 46.0. HRMS (ESI) calcd for $C_{22}H_{23}N_6$, 378.1984$[M + H]^+$; found, 371.1972.

2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)acetamide (41)—To a solution of compound **40** (39 mg,

0.1 mmol) in 2 mL of *t*-BuOH was added KOH (23 mg, 0.4 mmol). The reaction mixture was stirred at 110 °C for 1 h. The reaction was evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **41** as yellow solid (15 mg, 39%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.32 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.70 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.37 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.08 – 6.96 (m, 3H), 6.88 (td, *J* = 7.8, 1.5 Hz, 1H), 6.73 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.84 (s, 1H), 5.71 (d, *J* = 3.6 Hz, 1H), 5.58 (s, 1H), 4.49 (s, 1H), 3.42 – 3.35 (m, 2H), 3.17 – 3.09 (m, 4H), 2.82 – 2.65 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.0, 161.3, 147.7, 139.0, 136.3, 129.7, 128.6, 125.7, 124.8, 119.7, 119.7, 115.6, 115.0, 114.9, 110.1, 106.1, 61.4, 53.8, 51.7, 50.4. HRMS (ESI) calcd for $C_{22}H_{25}N_6O$, 389.2090 $[M + H]^+$; found, 389.2075.

2-Chloro-1-(4-(3-(4,5-dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)ethan-1-one (42)—To a solution of 2-(piperazin-1-yl)nicotinaldehyde (38 mg, 0.2 mmol) in 2 mL of CH_2Cl_2 was added 2-chloroacetyl chloride (34 mg, 0.3 mmol) and K_2CO_3 (55 mg, 0.4 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give 2-(4-(2-chloroacetyl)piperazin-1-yl)nicotinaldehyde (**32g**) as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.02 (s, 1H), 8.40 (dd, *J* = 4.8, 2.1 Hz, 1H), 8.02 (dd, *J* = 7.5, 2.1 Hz, 1H), 7.00 (dd, *J* = 7.5, 4.8 Hz, 1H), 4.12 (s, 2H), 3.83 – 3.71 (m, 4H), 3.56 – 3.45 (m, 4H).

Compound **42** was prepared by following a procedure similar to that used to prepare compound **37**, starting from compound **32g** and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (41 mg, 51%) was obtained as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.81 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.38 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.07 – 6.975 (m, 2H), 6.89 (td, *J* = 7.8, 1.5 Hz, 1H), 6.74 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.85 (s, 1H), 5.66 (dt, *J* = 3.3, 1.2 Hz, 1H), 4.38 (s, 1H), 4.08 (s, 2H), 3.84 – 3.54 (m, 4H), 3.42 – 3.27 (m, 2H), 3.19 – 3.06 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.1, 160.9, 147.9, 139.3, 136.3, 129.4, 128.8, 125.7, 124.9, 120.1, 119.8, 115.6, 115.0, 114.9, 110.2, 106.1, 51.6, 51.4, 50.4, 46.4, 42.2, 40.9. HRMS (ESI) calcd for $C_{22}H_{23}ClN_5O$, 408.1591 $[M + H]^+$; found, 408.1576.

1-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)-2-(dimethylamino)ethan-1-one (43)—To a solution of compound **42** (41 mg, 0.1 mmol) in 2 mL of DMF was added dimethylamine (0.15 mL, 0.2 mmol, 2 M in THF) and K_2CO_3 (41 mg, 0.3 mmol). The reaction mixture was stirred at 50 °C for 4 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **43** as a pale-yellow foam (20 mg, 48%). HPLC purity 97.2% (t_R = 13.64 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.32 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.77 (dd, *J* = 7.56, 1.8 Hz, 1H), 7.38 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.25 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.05 – 6.96 (m, 2H), 6.89 (td, *J* = 7.8, 1.5 Hz, 1H), 6.74 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.31 (t, *J* = 3.3 Hz, 1H), 5.86 (s, 1H), 5.69 (dt, *J* = 3.6, 1.2 Hz, 1H), 4.42 (s, 1H), 3.86 – 3.63 (m, 4H), 3.37 – 3.25 (m, 2H), 3.14 – 3.05 (m, 4H), 2.29 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.6, 161.1, 147.8, 139.1, 136.3, 129.4,

128.8, 125.7, 124.8, 119.9, 119.8, 115.6, 115.0, 114.9, 110.1, 106.1, 62.7, 52.2, 51.6, 50.4, 45.7, 45.5, 41.9. HRMS (ESI) calcd for $C_{24}H_{29}N_6O$, 417.2403 $[M + H]^+$; found, 417.2391.

(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)(3-hydroxyazetidin-1-yl)methanone (44)—To a solution of 2-(piperazin-1-yl)nicotinaldehyde (**31**) (191 mg, 1.0 mmol) in 2 mL of CH_2Cl_2 was added 4-nitrophenyl carbonochloridate (201 mg, 1.0 mmol) and Et_3N (202 mg, 2.0 mmol) at 0 °C. The reaction mixture was stirred at RT overnight, and then evaporated *in vacuo* to give the intermediate 4-nitrophenyl 4-(3-formylpyridin-2-yl)piperazine-1-carboxylate as a yellow solid (160 mg, 45%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.07 (s, 1H), 8.44 (dd, *J* = 4.8, 2.1 Hz, 1H), 8.28 (d, *J* = 9.0 Hz, 2H), 8.06 (dd, *J* = 7.5, 2.1 Hz, 1H), 7.35 (d, *J* = 9.0 Hz, 2H), 7.03 (dd, *J* = 7.5, 4.8 Hz, 1H), 3.92 – 3.79 (m, 4H), 3.58 – 3.55 (m, 4H).

To a solution of this intermediate (71 mg, 0.2 mmol) in 5 mL of CH_3CN was added 3-hydroxyazetidine hydrochloride (66 mg, 0.6 mmol) and K_2CO_3 (83 mg, 0.6 mmol). The reaction mixture was stirred at 60 °C overnight, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give 2-(4-(3-hydroxyazetidine-1-carbonyl)piperazin-1-yl)nicotinaldehyde (**32h**) as yellow oil (15 mg, 26%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.01 (s, 1H), 8.38 (dd, *J* = 4.8, 2.1 Hz, 1H), 8.01 (dd, *J* = 7.5, 2.1 Hz, 1H), 6.96 (dd, *J* = 7.5, 4.8 Hz, 1H), 4.64 – 4.60 (m, 1H), 4.24 (dd, *J* = 9.1, 6.9 Hz, 2H), 3.93 – 3.88 (m, 2H), 3.55 – 3.43 (m, 9H).

Compound **44** was prepared by following a procedure similar to that used to prepare compound **37**, starting from compound **32h** and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (16 mg, 74%) was obtained as a pale-yellow solid. HPLC purity 96.5% (t_R = 13.92 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.74 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.04 – 6.95 (m, 2H), 6.88 (td, *J* = 7.5, 1.5 Hz, 1H), 6.73 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.29 (t, *J* = 3.3 Hz, 1H), 5.84 (s, 1H), 5.68 (d, *J* = 3.6 Hz, 1H), 4.61 – 4.48 (m, 2H), 4.23 – 4.18 (m, 2H), 3.90 – 3.85 (m, 2H), 3.57 – 3.36 (m, 4H), 3.31 – 3.23 (m, 2H), 3.08 – 3.01 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.4, 161.1, 147.7, 139.2, 136.3, 129.6, 128.7, 125.7, 124.8, 119.9, 119.7, 115.6, 115.0, 114.9, 110.1, 106.1, 61.8, 61.0, 51.6, 50.4, 44.8. HRMS (ESI) calcd for $C_{24}H_{27}N_6O_2$, 431.2195 $[M + H]^+$; found, 431.2184.

4-(2-(4-(2-(Azetidin-1-yl)ethyl)piperazin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (45)—To a solution of 2-(4-(2-hydroxyethyl)piperazin-1-yl)nicotinaldehyde (**32a**) (1.31 g, 5.57 mmol) in 60 mL of CH_2Cl_2 was added $SOCl_2$ (796 mg, 6.69 mmol). The reaction mixture was stirred at 50 °C for 2 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/EtOAc) to give 2-(4-(2-chloroethyl)piperazin-1-yl)nicotinaldehyde (**33**) as a yellow oil (560 mg, 40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.02 (s, 1H), 8.38 (dd, *J* = 4.8, 2.1 Hz, 1H), 7.99 (dd, *J* = 7.5, 2.1 Hz, 1H), 6.92 (dd, *J* = 7.5, 4.8 Hz, 1H), 3.63 (t, *J* = 6.9 Hz, 2H), 3.53 – 3.49 (m, 4H), 2.81 (t, *J* = 6.9 Hz, 2H), 2.72 – 2.69 (m, 4H).

To a solution of compound **33** (51 mg, 0.2 mmol) in 2 mL of EtOH was added azetidine hydrochloride (56 mg, 0.6 mmol) and Cs_2CO_3 (196 mg, 0.6 mmol). The reaction mixture

was stirred at 70 °C for 2 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give 2-(4-(2-(azetidin-1-yl)ethyl)piperazin-1-yl)nicotinaldehyde (**34a**) as yellow oil (41 mg, 74%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.01 (s, 1H), 8.37 (dd, *J* = 4.8, 2.1 Hz, 1H), 7.98 (dd, *J* = 7.5, 2.1 Hz, 1H), 6.90 (dd, *J* = 7.5, 4.8 Hz, 1H), 3.73 – 3.70 (m, 4H), 3.51 – 3.48 (m, 4H), 2.70 – 2.49 (m, 10H).

To a solution of **34a** (41 mg, 0.15 mmol) and 2-(1*H*-pyrrol-1-yl)aniline (36 mg, 0.23 mmol) in EtOH (4 mL) was added 2 drops of AcOH. The reaction mixture was stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **45** as a pale-yellow foam (40 mg, 64%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.30 (dt, *J* = 4.8, 1.5 Hz, 1H), 7.66 (dt, *J* = 7.5, 1.5 Hz, 1H), 7.36 (d, *J* = 7.8 Hz, 1H), 7.24 (dt, *J* = 3.0, 1.5 Hz, 1H), 7.00 – 6.95 (m, 2H), 6.90 – 6.84 (m, 1H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.31 (td, *J* = 3.3, 1.2 Hz, 1H), 5.82 (s, 1H), 5.74 (d, *J* = 3.3 Hz, 1H), 4.59 (s, 1H), 3.72 (t, *J* = 4.8 Hz, 4H), 3.41 – 3.34 (m, 2H), 3.20 – 3.16 – 3.09 (m, 2H), 2.74 – 2.49 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.4, 147.6, 138.8, 136.4, 129.2, 128.7, 125.7, 124.7, 119.6, 119.2, 115.6, 114.9, 114.8, 110.1, 106.2, 67.0, 56.3, 55.7, 54.2, 53.9, 51.6, 50.5. HRMS (ESI) calcd for $C_{25}H_{31}N_6$, 415.2610 $[M + H]^+$; found, 415.2598.

1-(2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)ethyl)azetidin-3-ol (46)—Compound **46** was prepared by following a procedure similar to that used to prepare compound **45**, starting from compound **33**, 3-hydroxyazetidine hydrochloride, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (11 mg, 20%) was obtained as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.65 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.00 – 6.94 (m, 2H), 6.87 (td, *J* = 7.8, 1.5 Hz, 1H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.81 (s, 1H), 5.73 (dd, *J* = 3.0, 1.8 Hz, 1H), 4.59 (s, 1H), 4.47 (p, *J* = 5.7 Hz, 1H), 3.75 (td, *J* = 6.3, 1.8 Hz, 2H), 3.52 – 3.49 (m, 1H), 3.40 – 3.33 (m, 2H), 3.15 – 3.04 (m, 4H), 2.72 – 2.58 (m, 6H), 2.48 – 2.44 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.3, 147.6, 138.8, 136.3, 129.3, 128.7, 125.7, 124.7, 119.6, 119.3, 115.6, 114.9, 114.8, 110.1, 106.2, 64.7, 62.7, 56.3, 56.3, 53.7, 51.5, 50.5. HRMS (ESI) calcd for $C_{25}H_{31}N_6O$, 431.2559 $[M + H]^+$; found, 431.2546.

4-(2-(4-(2-(Pyrrolidin-1-yl)ethyl)piperazin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (47)—Compound **47** was prepared by following a procedure similar to that used to prepare compound **45**, starting from compound **33**, pyrrolidine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (47 mg, 54%) was obtained as a pale-yellow foam. HPLC purity 97.1% (t_R = 13.60 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.65 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.5, 1.2 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.00 – 6.93 (m, 2H), 6.87 (td, *J* = 7.8, 1.5 Hz, 1H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.75 – 5.74 (m, 1H), 4.60 (s, 1H), 3.42 – 3.35 (m, 2H), 3.17 – 3.09 (m, 2H), 2.75 – 2.53 (m, 12H), 1.83 – 1.74 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.42, 147.62, 138.80, 136.38, 129.16, 128.74, 125.73, 124.74, 119.56, 119.13, 115.61, 114.88, 114.83, 110.05, 106.18, 57.71, 54.65, 53.87, 53.69, 51.56, 50.49, 23.40. HRMS (ESI) calcd for $C_{26}H_{33}N_6$, 429.2767 $[M + H]^+$; found, 429.2755.

1-(2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)ethyl)pyrrolidin-2-one (48)—To a solution of 2-(piperazin-1-yl)nicotinaldehyde (**31**) (77 mg, 0.4 mmol) in 5 mL of toluene was added 1-(2-chloroethyl)pyrrolidin-2-one (89 mg, 0.6 mmol) and Et_3N (81 mg, 0.8 mmol). The reaction mixture was heated to reflux and stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give 2-(4-(2-(2-oxopyrrolidin-1-yl)ethyl)piperazin-1-yl)nicotinaldehyde (**32i**) as yellow oil (45 mg, 37%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.01 (s, 1H), 8.36 (dd, *J* = 4.8, 2.1 Hz, 1H), 7.98 (dd, *J* = 7.5, 2.1 Hz, 1H), 6.90 (dd, *J* = 7.5, 4.8 Hz, 1H), 3.49 – 3.42 (m, 8H), 2.66 – 2.63 (m, 4H), 2.57 (t, *J* = 6.6 Hz, 2H), 2.37 (t, *J* = 8.1 Hz, 2H), 2.02 (tt, *J* = 8.1, 6.6 Hz, 2H).

Compound **48** was prepared by following a procedure similar to that used to prepare compound **37**, starting from compound **32i** and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (45 mg, 68%) was obtained as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.64 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.35 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.23 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.99 – 6.93 (m, 2H), 6.86 (td, *J* = 7.8, 1.5 Hz, 1H), 6.72 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.73 (dt, *J* = 3.6, 1.2 Hz, 1H), 4.62 (s, 1H), 3.50 – 3.31 (m, 6H), 3.13 – 3.06 (m, 2H), 2.73 – 2.55 (m, 6H), 2.38 (t, *J* = 8.1 Hz, 2H), 2.06 – 1.96 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.0, 161.4, 147.6, 138.8, 136.3, 129.5, 128.6, 125.7, 124.8, 119.6, 119.4, 115.6, 114.9, 114.8, 110.1, 106.2, 55.6, 53.3, 51.6, 50.4, 47.8, 39.6, 30.9, 18.0. HRMS (ESI) calcd for $C_{26}H_{31}N_6O$, 443.2559 $[M + H]^+$; found, 443.2549.

4-(2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)ethyl)morpholine (49)—Compound **49** was prepared by following a procedure similar to that used to prepare compound **45**, starting from compound **33**, morpholine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (30 mg, 54%) was obtained as a pale-yellow foam. HPLC purity 97.2% (t_R = 13.47 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.29 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.66 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.00 – 6.94 (m, 2H), 6.86 (td, *J* = 7.5, 1.5 Hz, 1H), 6.71 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.74 – 5.72 (m, 1H), 4.60 (d, *J* = 1.6 Hz, 1H), 3.72 – 3.70 (m, 4H), 3.41 – 3.34 (m, 2H), 3.16 – 3.08 (m, 2H), 2.74 – 2.49 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.4, 147.6, 138.9, 136.4, 129.2, 128.8, 125.7, 124.7, 119.6, 119.2, 115.6, 114.9, 114.8, 110.1, 106.2, 67.0, 56.3, 55.7, 54.2, 53.9, 51.6, 50.5. HRMS (ESI) calcd for $C_{26}H_{33}N_6O$, 445.2716 $[M + H]^+$; found, 445.2703.

4-(2-(4-(2-(1H-Imidazol-1-yl)ethyl)piperazin-1-yl)pyridin-3-yl)-4,5-dihydropyrrolo[1,2-a]quinoxaline (50)—1*H*-imidazole (41 mg, 0.6 mmol) was dissolved in 5 mL of toluene, NaOH (12 mg, 0.6 mmol) and TBAB (65 mg, 0.2 mmol) were added. The mixture was stirred for 15 min followed by adding 2-(4-(2-chloroethyl)piperazin-1-yl)nicotinaldehyde **33** (51 mg, 0.2 mmol). The reaction mixture was stirred 110 °C for 6 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give 2-(4-(2-(1H-imidazol-1-yl)ethyl)piperazin-1-yl)nicotinaldehyde (**34e**) as yellow oil (40 mg, 70%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.96 (s, 1H), 8.33 (dd, *J* = 4.8, 2.0 Hz, 1H), 7.94

(dd, *J* = 7.6, 2.0 Hz, 1H), 7.56 (s, 1H), 7.09 – 6.92 (m, 2H), 6.88 (dd, *J* = 7.5, 4.8 Hz, 1H), 4.05 (t, *J* = 6.4 Hz, 2H), 3.51 – 3.39 (m, 4H), 2.73 (t, *J* = 6.4 Hz, 2H), 2.66 – 2.55 (m, 4H).

Compound **50** was prepared by following a procedure similar to that used to prepare compound **45**, starting from compound **34e** and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (21 mg, 25%) was obtained as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.31 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.68 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.55 (s, 1H), 7.37 (dd, *J* = 7.8, 1.2 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.06 (s, 1H), 7.01 – 6.96 (m, 3H), 6.91 – 6.85 (m, 1H), 6.73 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.31 (t, *J* = 3.3 Hz, 1H), 5.82 (s, 1H), 5.74 – 5.72 (m, 1H), 4.55 (s, 1H), 4.07 (t, *J* = 6.3 Hz, 2H), 3.40 – 3.34 (m, 2H), 3.15 – 3.10 (m, 2H), 2.79 – 2.57 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.3, 147.7, 138.9, 137.3, 136.3, 129.3, 129.3, 128.7, 125.7, 124.8, 119.6, 119.5, 119.3, 115.6, 114.9, 114.9, 110.1, 106.2, 58.7, 53.5, 51.6, 50.4, 44.7. HRMS (ESI) calcd for $C_{25}H_{28}N_7$, 426.2406 $[M + H]^+$; found, 426.2400.

2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-4-yl)piperazin-1-yl)-N,N-dimethylethan-1-amine (60)—To a solution of 4-chloronicotinaldehyde (70 mg, 0.5 mmol) and *N,N*-dimethyl-2-(piperazin-1-yl)ethan-1-amine (118 mg, 0.75 mmol) in 5 mL of toluene was stirred at 110 °C for 4 h. The reaction mixture was evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give the intermediate 4-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**51a**) as pale yellow oil (53 mg, 20%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.99 (s, 1H), 8.70 (s, 1H), 8.40 (d, *J* = 6.0 Hz, 1H), 6.79 (d, *J* = 6.0 Hz, 1H), 3.34 – 3.26 (m, 4H), 2.72 – 2.63 (m, 4H), 2.58 – 2.53 (m, 2H), 2.49 – 2.44 (m, 2H), 2.25 (s, 6H).

To a solution of compound **51a** (53 mg, 0.2 mmol) and 2-(1*H*-pyrrol-1-yl)aniline (47 mg, 0.3 mmol) in EtOH (5 mL) was added 1 drops of AcOH. The reaction mixture was stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **36** as a pale-yellow foam (53 mg, 67%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.59 (s, 1H), 8.44 (d, *J* = 5.4 Hz, 1H), 7.37 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.23 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.01 – 6.85 (m, 3H), 6.74 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.29 (t, *J* = 3.3 Hz, 1H), 5.84 (s, 1H), 5.69 (dt, *J* = 3.5, 1.2 Hz, 1H), 4.41 (s, 1H), 3.30 – 3.23 (m, 2H), 3.01 – 2.94 (m, 2H), 2.65 – 2.51 (m, 6H), 2.46 – 2.41 (m, 2H), 2.25 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.0, 152.4, 150.2, 136.5, 129.8, 128.7, 125.8, 124.7, 119.7, 115.5, 115.0, 114.9, 113.5, 110.1, 106.2, 56.9, 56.5, 53.6, 52.4, 49.1, 45.9. HRMS (ESI) calcd for $C_{24}H_{31}N_6$, 403.2610 $[M + H]^+$; found, 403.2599.

2-(4-(3-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyrazin-2-yl)piperazin-1-yl)-N,N-dimethylethan-1-amine (61)—Compound **61** was prepared by following a procedure similar to that used to prepare compound **60**, starting from 3-chloro-2-pyrazinecarboxaldehyde, *N,N*-dimethyl-2-(piperazin-1-yl)ethan-1-amine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (32 mg, 39%) was obtained as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.20 (q, *J* = 2.4 Hz, 2H), 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.23 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.99 – 6.85 (m, 2H), 6.77 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.30 (t, *J* = 3.3 Hz, 1H), 5.94 (d, *J* = 2.1 Hz, 1H), 5.73 (dt, *J* = 3.6, 1.2 Hz, 1H), 4.45 (s, 1H), 3.43 – 3.38 (m, 2H), 3.26 – 3.19 (m,

2H), 2.69 – 2.36 (m, 8H), 2.27 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.2, 147.9, 141.2, 138.3, 135.7, 127.2, 126.3, 124.6, 119.9, 116.4, 114.8, 114.7, 110.3, 105.7, 56.9, 56.7, 53.5, 52.5, 50.8, 46.0. HRMS (ESI) calcd for $C_{23}H_{30}N_7$, 404.2563 $[M + H]^+$; found, 404.2544.

2-(4-(5-Chloro-3-(4,5-dihydropyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)-N,N-dimethylethan-1-amine (62)—Compound **62** was prepared by following a procedure similar to that used to prepare compound **60**, starting from 2,5-dichloronicotinaldehyde, *N,N*-dimethyl-2-(piperazin-1-yl)ethan-1-amine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (32 mg, 37%) was obtained as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (d, *J* = 2.7 Hz, 1H), 7.66 (d, *J* = 2.7 Hz, 1H), 7.37 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 6.98 (td, *J* = 7.5, 1.5 Hz, 1H), 6.89 (td, *J* = 7.8, 1.5 Hz, 1H), 6.73 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.31 (t, *J* = 3.3 Hz, 1H), 5.77 – 5.74 (m, 2H), 4.57 (s, 1H), 3.40 – 3.33 (m, 2H), 3.15 – 3.08 (m, 2H), 2.71 – 2.44 (m, 8H), 2.27 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.7, 146.2, 138.5, 136.1, 130.3, 128.0, 126.7, 125.7, 124.8, 119.8, 115.6, 115.1, 114.9, 110.2, 106.4, 56.9, 56.7, 53.7, 51.5, 50.4, 46.0. HRMS (ESI) calcd for $C_{24}H_{30}ClN_6$, 437.2220 $[M + H]^+$; found, 437.2209.

2-(4-(2-(4,5-Dihydropyrrolo[1,2-a]quinoxalin-4-yl)phenyl)piperazin-1-yl)-N,N-dimethylethan-1-amine (63)—Compound **63** was prepared by following a procedure similar to that used to prepare compound **60**, starting from 2-fluorobenzaldehyde, *N,N*-dimethyl-2-(piperazin-1-yl)ethan-1-amine, and 2-(1*H*-pyrrol-1-yl)aniline. The title compound (44 mg, 55%) was obtained as a pale-yellow foam. HPLC purity 95.0% (t_R = 10.06 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.28 – 7.21 (m, 4H), 7.06 (ddd, *J* = 8.1, 6.9, 1.5 Hz, 1H), 6.94 (td, *J* = 7.5, 1.5 Hz, 1H), 6.83 (td, *J* = 7.5, 1.5 Hz, 1H), 6.67 (dd, *J* = 7.8, 1.5 Hz, 1H), 6.32 (t, *J* = 3.3 Hz, 1H), 6.06 (s, 1H), 5.76 (ddd, *J* = 3.6, 1.5, 0.9 Hz, 1H), 4.80 (s, 1H), 3.24 – 3.17 (m, 2H), 2.96 – 2.89 (m, 2H), 2.67 – 2.46 (m, 8H), 2.29 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.4, 137.1, 136.5, 129.6, 129.2, 128.8, 125.7, 124.8, 124.6, 120.5, 119.0, 115.4, 114.7, 114.4, 110.0, 105.8, 57.0, 56.7, 54.2, 53.5, 50.2, 46.0. HRMS (ESI) calcd for $C_{25}H_{32}N_5$, 402.2658 $[M + H]^+$; found, 402.2646.

1-(4-(2-(4-(2-(Dimethylamino)ethyl)piperazin-1-yl)pyridin-3-yl)pyrrolo[1,2-a]quinoxalin-5(4H)-yl)ethan-1-one (64)—To a solution of compound **38** (40 mg, 0.1 mmol) in 2 mL of CH_2Cl_2 was added acetyl chloride (12 mg, 0.15 mmol) and Et_3N (21 mg, 0.2 mmol). The reaction mixture was stirred at RT for 30 min, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **64** as a white foam (27 mg, 61%). HPLC purity 98.9% (t_R = 13.28 min). ^{1}H NMR (300 MHz, CD_3OD) δ 8.07 (dd, *J* = 4.5, 2.4 Hz, 1H), 7.63 (dd, *J* = 8.1, 1.2 Hz, 1H), 7.53 (s, 1H), 7.44 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.34 (ddd, *J* = 10.2, 7.5, 2.1 Hz, 2H), 7.13 (td, *J* = 78, 1.2 Hz, 1H), 6.73 – 6.65 (m, 2H), 6.37 (t, *J* = 3.3 Hz, 1H), 6.26 (dd, *J* = 3.6, 1.5 Hz, 1H), 3.27 – 3.13 (m, 4H), 2.85 – 2.78 (m, 4H), 2.71 – 2.56 (m, 4H), 2.34 (s, 6H), 2.18 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 170.5, 161.4, 147.1, 137.7, 131.6, 128.6, 128.5, 127.7, 127.5, 127.1, 124.0, 119.1, 115.9, 115.1, 110.5, 106.6, 55.8, 55.7, 53.4, 50.6, 44.5, 21.3. HRMS (ESI) calcd for $C_{26}H_{33}N_6O$, 445.2716 $[M + H]^+$; found, 445.2703.

N,N-Dimethyl-2-(4-(3-(pyrrolo[1,2-a]quinoxalin-4-yl)pyridin-2-yl)piperazin-1-yl)ethan-1-amine (65)—To a solution of 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**) (53 mg, 0.2 mmol) and 2-(1*H*-pyrrol-1-yl)aniline (47 mg, 0.3 mmol) in EtOH (5 mL) was added 1 drop of AcOH. The reaction mixture was stirred at 70 °C overnight, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **65** as a yellow solid (30 mg, 38%). HPLC purity 98.8% (t_R = 11.94 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (dd, *J* = 4.8, 2.1 Hz, 1H), 8.08 – 7.98 (m, 2H), 7.93 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.82 (dd, *J* = 7.5, 2.1 Hz, 1H), 7.61 – 7.48 (m, 2H), 6.94 – 6.84 (m, 2H), 6.78 (dd, *J* = 4.2, 1.2 Hz, 1H), 3.32 (t, *J* = 4.9 Hz, 4H), 2.46 – 2.09 (m, 15H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.6, 154.5, 148.5, 140.1, 136.0, 130.1, 127.7, 127.1, 125.4, 124.7, 121.6, 114.8, 114.5, 113.9, 113.7, 109.1, 56.7, 53.4, 48.2, 45.8. HRMS (ESI) calcd for $C_{24}H_{29}N_6$, 401.2454 $[M + H]^+$; found, 401.2443.

2-(4-(3-(4H-Benzo[b]pyrrolo[1,2-d][1,4]oxazin-4-yl)pyridin-2-yl)piperazin-1-yl)-N,N-dimethylethan-1-amine (66)—To a solution of 2-aminophenol (500 mg, 4.6 mmol) in AcOH (7.5 mL) was added 2,5-dimethoxytetrahydrofuran (608 mg, 4.6 mmol) slowly. The mixture was stirred at 110°C for 15 min, and then concentrated. 30 mL of $NaHCO_3$ (aq.) was added to the mixture. The resulting mixture was extracted with CH_2Cl_2 (3 × 50 mL), dried over Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography (Hex/ EtOAc) to give 2-(1*H*-pyrrol-1-yl)phenol (**59a**) as brown oil (340 mg, 46%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.30 – 7.20 (m, 2H), 7.06 – 6.93 (m, 2H), 6.85 (t, *J* = 2.1 Hz, 2H), 6.39 (t, *J* = 2.1 Hz, 2H), 5.28 (s, 1H).

To a solution of 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**) (53 mg, 0.2 mmol) and compound **59a** (32 mg, 0.2 mmol) in 2 mL of $CHCl_3$ was added TFA (23 mg, 0.2 mmol). The reaction mixture was stirred at 60 °C for 24 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **66** as pale-yellow oil (37 mg, 46%). HPLC purity 99.4% (t_R = 9.52 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.39 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.86 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.47 – 7.41 (m, 1H), 7.24 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.13 – 7.01 (m, 4H), 6.34 – 6.31 (m, 2H), 5.68 (dt, *J* = 3.5, 1.2 Hz, 1H), 3.41 – 3.24 (m, 4H), 2.62 – 2.59 (m, 4H), 2.55 – 2.42 (m, 4H), 2.25 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.9, 148.6, 146.5, 139.2, 127.7, 126.9, 125.1, 122.6, 118.4, 118.1, 115.4, 114.9, 110.7, 107.2, 107.2, 71.7, 56.9, 56.8, 53.7, 51.4, 46.0, 46.0. HRMS (ESI) calcd for $C_{24}H_{30}N_5O$, 404.2450 $[M + H]^+$; found, 404.2440.

(E/Z)-N,N-Dimethyl-2-(4-(3-(((2-(2-methyl-1H-imidazol-1-yl)phenyl)imino)methyl)pyridin-2-yl)piperazin-1-yl)ethan-1-amine (67)—To a solution of 1-fluoro-2-nitrobenzene (423 mg, 3.0 mmol) and 2-methyl-1*H*-imidazole (246 mg, 3.0 mmol) in 20 mL of CH_3CN was added K_2CO_3 (828 mg, 6.0 mmol). The reaction mixture was heated to reflux and stirred for 24 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/hexane) to give 2-methyl-1-(2-nitrophenyl)-1-Himidazole (**55**) as pale-yellow oil (200 mg, 33%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.77 (td, *J* = 7.8, 1.5 Hz, 1H), 7.68 (td, *J* = 7.8, 1.5 Hz, 1H), 7.45 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.08 (s, 1H), 6.93 (s, 1H), 2.24 (s, 3H).

To a solution of compound **55** (200 mg, 1.0 mmol) in 5 mL of EtOH was added 10% Pd/C (20 mg), the reaction mixture was stirred under hydrogen atmosphere for 4 h and then filtered and concentrated to afford 2-(2-methyl-1H-imidazol-1-yl)aniline (**56**) as pale-yellow oil (140 mg, 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25 (ddd, *J* = 8.1, 7.5, 1.5 Hz, 1H), 7.09 – 7.05 (m, 2H), 6.93 (d, *J* = 1.5 Hz, 1H), 6.86 – 6.78 (m, 2H), 3.61 (s, 2H), 2.25 (s, 3H).

To a solution of 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**) (39 mg, 0.15 mmol) and compound **56** (39 mg, 0.23 mmol) in EtOH (4 mL) was added 2 drops of AcOH. The reaction mixture was stirred at 50 °C for 3 h, and then evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **67** as a pale-yellow foam (32 mg, 41%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.31 (dd, *J* = 4.8, 2.1 Hz, 1H), 7.96 (dd, *J* = 7.5, 2.1 Hz, 1H), 7.53 – 7.47 (m, 1H), 7.34 – 7.31 (m, 2H), 7.12 (dt, *J* = 7.8, 0.9 Hz, 1H), 7.00 (d, *J* = 1.5 Hz, 1H), 6.93 – 6.89 (m, 2H), 3.32 – 3.29 (m, 4H), 2.66 – 2.50 (m, 8H), 2.32 (s, 6H), 2.25 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.1, 159.1, 150.4, 148.3, 145.7, 137.5, 131.3, 129.9, 127.8, 127.4, 126.3, 121.4, 121.0, 119.2, 117.7, 56.7, 56.5, 53.6, 51.3, 45.8, 13.6. HRMS (ESI) calcd for $C_{24}H_{32}N_7$, 418.2719 $[M + H]^+$; found, 418.2707.

N,N-Dimethyl-2-(4-(3-(8-methyl-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazin-4-yl)pyridin-2-yl)piperazin-1-yl)ethan-1-amine (68)—Compound **68** was prepared by following a procedure similar to that used to prepare compound **66**, starting from 2-amino-p-cresol, 2,5-dimethoxytetrahydrofuran, and 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**). The title compound (42 mg, 51%) was obtained as a pale-yellow foam. HPLC purity 99.8% (t_R = 14.58 min). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.39 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.85 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.25 – 7.21 (m, 2H), 7.03 (dd, *J* = 7.5, 4.8 Hz, 1H), 6.94 – 6.88 (m, 2H), 6.32 – 6.29 (m, 2H), 5.68 – 5.66 (m, 1H), 3.40 – 3.23 (m, 4H), 2.62 – 2.43 (m, 8H), 2.40 (s, 3H), 2.26 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.9, 148.6, 144.3, 139.2, 132.3, 127.8, 126.6, 125.6, 125.2, 118.4, 117.8, 115.4, 115.3, 110.5, 107.1, 71.7, 56.8, 56.7, 53.7, 51.4, 45.9, 21.0. HRMS (ESI) calcd for $C_{25}H_{32}N_5O$, 418.2607 $[M + H]^+$; found, 418.2597.

2-(4-(3-(8-Methoxy-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazin-4-yl)pyridin-2-yl)piperazin-1-yl)-N,N-dimethylethan-1-amine (69)—Compound **69** was prepared by following a procedure similar to that used to prepare compound **66**, starting from 2-amino-4-methoxyphenol, 2,5-dimethoxytetrahydrofuran, and 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**). The title compound (55 mg, 64%) was obtained as a pale-yellow foam. HPLC purity 99.5% (t_R = 14.18 min). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.38 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.85 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.20 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.05 – 6.97 (m, 3H), 6.65 (dd, *J* = 9.0, 2.7 Hz, 1H), 6.31 (t, *J* = 3.0 Hz, 1H), 6.26 (s, 1H), 5.68 (dt, *J* = 3.6, 1.2 Hz, 1H), 3.86 (s, 3H), 3.40 – 3.23 (m, 4H), 2.62 – 2.43 (m, 8H), 2.26 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.9, 155.2, 148.6, 140.5, 139.2, 128.0, 127.4, 125.1, 118.5, 118.4, 115.4, 110.7, 109.6, 107.2, 101.5, 71.7, 56.8, 56.7, 55.9, 53.7, 51.4, 45.9. HRMS (ESI) calcd for $C_{25}H_{32}N_5O_2$, 434.2556 $[M + H]^+$; found, 434.2546.

2-(4-(3-(8-Fluoro-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazin-4-yl)pyridin-2-yl)piperazin-1-yl)-N,N-dimethylethan-1-amine (70)—Compound **70** was prepared by following a procedure similar to that used to prepare compound **66**, starting from 2-amino-4-fluorophenol, 2,5-dimethoxytetrahydrofuran, and 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**). The title compound (26 mg, 30%) was obtained as a pale-yellow foam. HPLC purity 95.2% (t_R = 14.19 min) ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.40 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.83 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.17 – 7.13 (m, 2H), 7.06 – 6.98 (m, 2H), 6.80 (td, *J* = 8.7, 3.0 Hz, 1H), 6.34 (t, *J* = 3.3 Hz, 1H), 6.29 (s, 1H), 5.70 (dt, *J* = 3.6, 1.2 Hz, 1H), 3.40 – 3.22 (m, 4H), 2.63 – 2.43 (m, 8H), 2.26 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.9, 159.7 (d, *J* = 238.7 Hz), 148.7, 142.5 (d, *J* = 2.7 Hz), 139.2, 127.7, 127.4 (d, *J* = 10.8 Hz), 124.8, 118.9 (d, *J* = 9.0 Hz), 118.5, 115.6, 111.3 (d, *J* = 23.0 Hz), 111.2, 107.6, 102.6 (d, *J* = 27.5 Hz), 71.8, 56.9, 56.7, 53.7, 51.4, 45.9. HRMS (ESI) calcd for $C_{24}H_{29}FN_5O$, 422.2356 $[M + H]^+$; found, 422.2344.

Methyl 4-(2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)pyridin-3-yl)-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazine-7-carboxylate (71)—Methyl 3-hydroxy-4-(1*H*-pyrrol-1-yl)benzoate (**59e**) was synthesized as yellow oil (970 mg, 74%) by following a procedure similar to that used to prepare compound **59a**, starting from methyl 4-amino-3-hydroxybenzoate and 2,5-dimethoxytetrahydrofuran. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (d, *J* = 1.8 Hz, 1H), 7.66 (dd, *J* = 8.3, 1.8 Hz, 1H), 7.31 (d, *J* = 8.2 Hz, 1H), 6.99 (t, *J* = 2.2 Hz, 2H), 6.41 (t, *J* = 2.2 Hz, 2H), 6.11 (s, 1H), 3.94 (s, 3H).

The synthesis of compound **71** was conducted by following a procedure similar to that of compound **66**, starting from compound **59e** and 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**). The title compound (38 mg, 42%) was obtained as a pale-yellow foam. HPLC purity 97.8% (t_R = 14.49 min). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.40 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.83 – 7.78 (m, 2H), 7.73 (d, *J* = 1.8 Hz, 1H), 7.47 (d, *J* = 8.4 Hz, 1H), 7.26 (dd, *J* = 3.0, 1.4 Hz, 1H), 7.03 (dd, *J* = 7.5, 4.8 Hz, 1H), 6.37 – 6.35 (m, 2H), 5.73 (dt, *J* = 3.6, 1.2 Hz, 1H), 3.92 (s, 3H), 3.42 – 3.22 (m, 4H), 2.63 – 2.43 (m, 8H), 2.26 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.2, 162.0, 148.8, 146.0, 139.2, 130.4, 127.7, 126.8, 124.8, 124.4, 119.6, 118.5, 115.8, 114.6, 111.7, 108.1, 71.9, 56.9, 56.7, 53.7, 52.2, 51.4, 45.9. HRMS (ESI) calcd for $C_{26}H_{32}N_5O_3$, 462.2505 $[M + H]^+$; found, 462.2496.

(4-(2-(4-(2-(Dimethylamino)ethyl)piperazin-1-yl)pyridin-3-yl)-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazin-7-yl)methanol (72)—To a solution of compound **71** (46 mg, 0.1 mmol) in 2 mL of THF was added $LiAlH_4$ (8 mg, 0.2 mmol). The reaction mixture was stirred at RT for 2 h, and then quenched with ice water. The mixture was filtered, and then the solvent was evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **72** as pale-yellow oil (29 mg, 69%). HPLC purity 99.6% (t_R = 12.73 min). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.39 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.83 (dd, *J* = 7.5, 2.1 Hz, 1H), 7.42 (d, *J* = 8.1 Hz, 1H), 7.23 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.12 – 7.08 (m, 2H), 7.03 (dd, *J* = 7.5, 4.8 Hz, 1H), 6.33 – 6.31 (m, 2H), 5.68 (dt, *J* = 3.5, 1.2 Hz, 1H), 3.40 – 3.24 (m, 4H), 2.60 – 2.43 (m, 8H), 2.25 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.9, 148.6, 146.5, 139.2, 138.4, 127.5,

126.2, 125.2, 121.1, 118.5, 116.7, 115.4, 114.9, 110.7, 107.2, 71.8, 64.7, 56.8, 56.7, 53.7, 51.4, 45.9. HRMS (ESI) calcd for $C_{25}H_{32}N_5O_2$, 434.2556 $[M + H]^+$; found, 434.2545.

4-(2-(4-(2-(Dimethylamino)ethyl)piperazin-1-yl)pyridin-3-yl)-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazine-7-carboxamide (73)—To a solution of methyl 3-hydroxy-4-(1*H*-pyrrol-1-yl)benzoate (**59e**) (217 mg, 1.0 mmol) in 3 mL of EtOH was added LiOH monohydrate (210 mg, 5 mmol, in 3 mL of H_2O). The mixture was stirred at 50 °C for 4 h and then cooled to RT. The pH of the mixture was adjusted to ~7 with 1 M HCl (aq.). The mixture was extracted with EtOAc, dried over Na_2SO_4, and concentrated to give 3-hydroxy-4-(1*H*-pyrrol-1-yl)benzoic acid (195 mg, 96%) as a pale solid.
To a solution of 3-hydroxy-4-(1*H*-pyrrol-1-yl)benzoic acid (102 mg, 0.5 mmol) and NH_4Cl (133 mg, 2.5 mmol) in 5 mL of DMF was added HATU (210 mg, 0.55 mmol) and Et_3N (101 mg, 1.0 mmol). The mixture was stirred at RT overnight. Then the mixture was diluted with EtOAc, washed with H_2O, dried over Na_2SO_4, and concentrated. The residue was purified by silica gel column chromatography to afford 3-hydroxy-4-(1*H*-pyrrol-1-yl)benzamide (**59f**) as a yellow solid (60 mg, 59%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46 – 7.42 (m, 1H), 7.33 – 7.29 (m, 2H), 7.11 (t, *J* = 2.2 Hz, 2H), 6.34 (t, *J* = 2.2 Hz, 2H), 2.96 (s, 3H).

The synthesis of compound **73** was conducted by following a procedure similar to that of compound **66**, starting from compound **59f** and 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**). The title compound (29 mg, 34%) was obtained as a pale-yellow foam. HPLC purity 99.0% (t_R = 12.46 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.81 (dd, *J* = 7.5, 1.8 Hz, 1H), 7.61 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.51 – 7.47 (m, 2H), 7.26 (dd, *J* = 3.0, 1.5 Hz, 1H), 7.04 (dd, *J* = 7.5, 4.8 Hz, 1H), 6.38 – 6.36 (m, 2H), 5.99 – 5.72 (m, 3H), 3.39 – 3.22 (m, 4H), 2.61 – 2.47 (m, 8H), 2.26 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.0, 162.0, 148.9, 146.1, 139.2, 130.0, 129.7, 127.6, 124.8, 122.2, 118.5, 117.4, 115.7, 114.9, 111.7, 108.0, 71.9, 56.8, 56.7, 53.7, 51.4, 45.9. HRMS (ESI) calcd for $C_{25}H_{31}N_6O_2$, 447.2508 $[M + H]^+$; found, 447.2499.

4-(2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)pyridin-3-yl)-N-(2-(methylsulfonyl)ethyl)-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazine-7-carboxamide (74)—Compound **74** was prepared as a pale-yellow foam by following a procedure similar to that used to prepare compound **73**, starting from of methyl 3-hydroxy-4-(1*H*-pyrrol-1-yl)benzoate (**59e**), 2-(methylsulfonyl)ethanamine hydrochloride, and 2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)nicotinaldehyde (**32c**). HPLC purity 98.2% (t_R = 12.73 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.38 (dd, *J* = 4.9, 1.9 Hz, 1H), 7.78 (dd, *J* = 7.6, 1.8 Hz, 1H), 7.55 – 7.42 (m, 3H), 7.25 – 7.20 (m, 1H), 7.04 – 6.97 (m, 2H), 6.36 – 6.31 (m, 2H), 5.70 (d, *J* = 3.8 Hz, 1H), 4.03 – 3.95 (m, 2H), 3.39 – 3.28 (m, 4H), 3.27 – 3.19 (m, 2H), 2.99 (s, 3H), 2.63 – 2.55 (m, 4H), 2.54 – 2.42 (m, 4H), 2.24 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.6, 162.1, 149.0, 146.4, 139.3, 130.5, 129.8, 127.8, 125.0, 121.8, 118.7, 117.3, 115.9, 115.0, 111.8, 108.2, 72.1, 57.0, 56.8, 54.0, 53.9, 51.6, 46.1, 41.8, 33.7. HRMS (ESI) calcd for $C_{28}H_{37}N_6O_4S$, 553.2597 $[M + H]^+$; found, 553.2586.

(4-(2-(4-(2-(Dimethylamino)ethyl)piperazin-1-yl)pyridin-3-yl)-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazin-1-yl)methanol (75)—$POCl_3$ (23 mg,

0.15 mmol) was added dropwise to DMF (17 mg, 2.3 mmol) at 0 °C and stirred for another 20 min at 0 °C. A solution of compound **66** (40 mg, 0.1 mmol) in DMF (1 mL) was added dropwise and the mixture was stirred at 50 °C overnight. Then the mixture was diluted with water (10 mL), extracted with CH_2Cl_2 (3 × 20 mL), dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by silica gel chromatograph (CH_2Cl_2/MeOH) to give 4-(2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)pyridin-3-yl)-4*H*-benzo[*b*]pyrrolo[1,2-*d*][1,4]oxazine-1-carbaldehyde (38 mg, 88%) as a pale-yellow foam. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.71 (s, 1H), 8.42 – 8.34 (m, 1H), 8.24 – 8.16 (m, 1H), 7.80 – 7.69 (m, 1H), 7.21 – 7.12 (m, 3H), 7.12 – 7.00 (m, 2H), 6.17 (s, 1H), 5.82 (d, *J* = 4.1 Hz, 1H), 3.37 – 3.27 (m, 2H), 3.25 – 3.16 (m, 2H), 2.61 – 2.53 (m, 4H), 2.51 – 2.41 (m, 4H), 2.23 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.3, 162.1, 149.2, 147.7, 139.5, 139.1, 131.7, 128.0, 127.2, 126.5, 124.0, 123.1, 121.4, 118.7, 118.0, 108.5, 72.1, 56.9, 56.8, 53.7, 51.5, 46.0.

To a solution of compound 4-(2-(4-(2-(dimethylamino)ethyl)piperazin-1-yl)pyridin-3-yl)-4*H*-benzo[*b*]pyrrolo[1,2-*d*][1,4]oxazine-1-carbaldehyde (35 mg, 0.08 mmol) in 2 mL of dry EtOH was added $NaBH_4$ (7 mg, 0.16 mmol). The reaction mixture was stirred at RT for 6 h, and then quenched with ice water. The mixture was evaporated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH) to give compound **75** as a pale white foam (30 mg, 85%). HPLC purity 99.7% (t_R = 12.93 min). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.36 (dd, *J* = 4.8, 2.0 Hz, 1H), 8.18 – 8.11 (m, 1H), 7.81 (dd, *J* = 7.6, 1.9 Hz, 1H), 7.17 – 6.98 (m, 4H), 6.24 (d, *J* = 3.6 Hz, 1H), 6.13 (s, 1H), 5.56 (d, *J* = 3.6 Hz, 1H), 4.82 (d, *J* = 13.1 Hz, 1H), 4.66 (d, *J* = 13.1 Hz, 1H), 3.38 – 2.97 (m, 5H), 2.61 – 2.36 (m, 8H), 2.19 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.7, 148.5, 147.7, 139.1, 131.2, 130.6, 127.4, 125.5, 124.8, 123.0, 118.5, 118.4, 118.0, 112.4, 105.8, 71.8, 56.8, 56.7, 56.5, 53.6, 51.3, 45.8. HRMS (ESI) calcd for $C_{25}H_{32}N_5O_2$, 434.2556 $[M + H]^+$; found, 434.2547.

N,N-Dimethyl-2-(4-(3-(1-(trifluoromethyl)-4H-benzo[b]pyrrolo[1,2-d][1,4]oxazin-4-yl)pyridin-2-yl)piperazin-1-yl)ethan-1-amine (76)—To a mixture of compound **66** (25 mg, 0.06 mmol) and K_2CO_3 (22 mg, 0.16 mmol) in 1 mL of DMF was added *S*-(trifluoromethyl)dibenzothiophenium triflate (120 mg, 0.30 mmol). The mixture was stirred at 80 °C for 48 h. Then the mixture was diluted with EtOAc, washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by preparative TLC (CH_2Cl_2/MeOH) to afford compound 48 as a pale-yellow foam (6 mg, 21%). HPLC purity 95.4% (t_R = 15.54 min). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 8.42 (dd, *J* = 4.8, 1.8 Hz, 1H), 7.87 – 7.76 (m, 2H), 7.25 – 7.06 (m, 4H), 6.79 (d, *J* = 3.9 Hz, 1H), 6.15 (s, 1H), 5.70 (d, *J* = 3.9 Hz, 1H), 3.35 – 3.20 (m, 4H), 2.60 – 2.48 (m, 8H), 2.29 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.9, 148.9, 147.8, 139.0, 134.4, 134.4, 126.8, 126.4, 124.1, 123.1, 123.0, 119.9, 119.4, 118.6, 118.5, 118.4, 118.4, 118.4, 115.2, 115.1, 115.0, 115.0, 106.2, 71.7, 56.6, 56.4, 53.6, 51.4, 45.8. ^{19}F NMR (282 MHz, $CDCl_3$) δ −56.1. HRMS (ESI) calcd for $C_{25}H_{29}F_3N_5O$, 472.2324 $[M + H]^+$; found, 472.2313.

4.2. Cell culture and transfection

H1299, PLC/PRF/5 and HEK 293T cells were purchased from ATCC and cultured with DMEM supplemented with 10% FBS and 1% streptomycin-penicillin. BV2 cells were cultured with DMEM/F12 supplemented with 10% FBS, 2 mM Glutamine and 1%

streptomycin-penicillin. R28 cells were cultured with DMEM supplemented with 10% calf serum, 2mM Glutamine, 1% MEM non-essential amino acids, 1% MEM vitamin and 100 μg/mL Gentamicin. H1299 and PLC/PRF/5 cells were seeded in 6-well plates overnight and transfected with 20 nM control siRNA or Sirt6 siRNA (Dharmacon) by Lipofectamine RNA iMAX according to the manufacturer's instruction (Invitrogen). The knockdown efficiency of Sirt6 was verified by Western blot.

4.3. MTT assay

R28 cells were seeded in 96 well plates at a density of 1×10^4/well overnight, then treated with compounds for 24 h. MTT (MilliporeSigma) was added to cells (20 μL /well) and incubated for 3.5 h. After medium was removed, 100 μL dimethyl sulfoxide (DMSO) was added into each well. Absorbance was measured at 540 nm using a microplate reader.

4.4. Sirt6/Sirt1/Sirt2/Sirt3 activity assay

Sirt6/Sirt1/Sirt2/Sirt3 activity was tested by Activity Assay Kits (Abcam) according to the manufacturer's instruction with minor modification. Briefly, compounds of indicated concentrations were added into the reaction system, 2.5 μL 10X buffer + 2.5 μL Fluoro-substrate peptide (0.1 mM) + 0.5 μL NAD^+ (8 mM) + 2.5 μL Developer + 1 μL recombinant Sirt6/Sirt1/Sirt2/Sirt3, and the mixture was brought up to 25 μL with ddH_2O. Fluorescence intensity was detected using a microplate reader at Ex/Em = 490 nm/530 nm for Sirt6/Sirt2 activity or at Ex/Em = 350 nm/450 nm for Sirt1/Sirt3 activity for 125 minutes. All experiments were conducted at room temperature.

4.5. Sirt5 activity assay

Sirt5 activity was tested by Fluorogenic Sirt5 Assay Kit (BPS Bioscience) according to the manufacturer's instruction. Briefly, 100 μM compounds were incubated with Sirt5 substrate, cosubstrate NAD^+ and Sirt5 enzyme at 37 °C for 30 min, followed by incubation with Sirt Developer (containing Nicotinamide) at room temperature for 15 min. Fluorescence intensity was detected using a microplate reader at Ex/Em = 350 nm/450 nm. The fold-change of Sirt5 activity in the presence of selected compounds was calculated as compared to vehicle group.

4.6. Molecular docking method

The molecular docking study was performed using Schrödinger Small-Molecule Drug Discovery Suite (Schrödinger, LLC, New York, NY, 2020). Human Sirt6 in complex with an activator UBCS039 crystal structure (PDB ID: 5MF6) was downloaded from RCSB PDB bank [28]. The structure was prepared using Protein Preparation Wizard with default settings. During the preparation, hydrogens were added, crystal waters beyond 3 Å from existing ligand were removed, partial charges were assigned, and structure was minimized. 3D-structure of compound **38** (GL0710) was generated using Maestro and further prepared with LigPrep using OPLS3 forcefield. Compound 38 was ionized at target pH 7.4, desalted and tautomers were generated using Epik, and a low energy conformation was calculated. The grid box in size of 24 Å on each side was created with Glide. The grid center was chosen on the center of the existing ligand based on the binding site of crystal

structure. Docking was employed with Glide using the SP protocol. Docked poses were incorporated into Schrödinger Maestro for a ligand-receptor interactions analysis. The final pose selected was among the best scoring poses. The selected pose was superimposed with Sirt6-UBCS039 complex structure for an overlay analysis.

4.7. Nucleosome test

Nucleosome was extracted from HEK 293T cells with Nucleosome Preparation Kit (Active Motif) according to the manufacturer's instruction. Briefly, HEK 293T cells in 100 mm dish were lysed with lysis buffer supplemented with PIC and PMSF for 30 min. After centrifugation for 10 min at 5000 rpm at 4 °C, supernatant was removed and nuclei pellet was resuspended with digestion buffer supplemented with PIC and PMSF for 5 min at 37 °C. Working enzymatic sharing cocktail was added to digest the chromatin, and EDTA was added after incubation to stop the digestion. Digested nucleosome was obtained in the supernatant after centrifugation for 10 min at 15000 rpm at 4 °C and the concentration was quantified by measuring the absorbance at 230 nm. 20 μg nucleosome was incubated in solution (1X buffer, 0.5 μL recombinant Sirt6 and 160 μM NAD, from Sirt6 Activity Assay Kit) for 60 min. To test the effects of the compounds on Sirt6 activity, corresponding compound or DMSO control was added before incubation. The reaction mixture was analyzed by Western blot.

4.8. Real-time quantitative PCR

Total mRNA of untreated and treated BV2 cells was isolated by Trizol reagent and chloroform, quantified by NanoDrop (ThermoFisher Scientific), and converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Quantitative PCR was performed with SYBR Green Master Mix (Applied Biosystems) using a PCR system (StepOnePlus; Applied Biosystems). Primer sequences for mouse transcripts were as follows: CXCL10 For-5′-GGA CGG TCC GCT GCA A-3′; CXCL10 Rev-5′-CCC TAT GGC CCT CAT TCT CA-3′; MCP-1 For-5′-GGC TCA GCC AGA TGC AGT TAA-3′; MCP-1 Rev-5′-CCT ACT CAT TGG GAT CAT CTT GCT-3′; ICAM-1 For-5′-CAG TCC GCT GTG CTT TGA GA-3′; ICAM-1 Rev-5′-CGG AAA CGA ATA CAC GGT GAT-3′; IL-1β For-5′-AGT TGA CGG ACC CCA AAA GA-3′; Il-1β Rev-5′-GGA CAG CCC AGG TCA AAG G-3′; IL-6 For-5′-CCA CGG CCT TCC CTA CTT C-3′; IL-6 Rev-5′-TTG GGA GTG GTA TCC TCT GTG A-3′; iNOS For-5′-GGC AGC CTG TGA GAC CTT TG-3′; iNOS Rev-5′-TGC ATT GGA AGT GAA GCG TTT-3′; TNFα For-5′-GGT CCC CAA AGG GAT GAG AA-3′; TNFα Rev-5′-TGA GGG TCT GGG CCA TAG AA-3′; VCAM-1 For-5′-ACA AGT CTA CAT CTC TCC CAG GAA TAC-3′; VCAM-1 Rev-5′-CAC AGC ACC ACC CTC TTG AA-3′. Hprt For-5′-GAA AGA CTT GCT CGA GAT GTC ATG-3′; Hprt Rev-5′-CAC ACA GAG GGC CAC AAT GT-3′. Data were normalized to internal control Hprt and the fold difference in different transcripts was calculated by the ΔΔCT method.

4.9. Western blot

Proteins were extracted from cells with Pierce RIPA Buffer (89901, ThermoFisher Scientific), and protein concentration was determined using Pierce BCA Protein Assay kit (23225, ThermoFisher Scientific). Equal amounts of protein were loaded and separated

by SDS-PAGE gel. Next, separated proteins were transferred onto Nitrocellulose Blotting Membranes (A29457237, GE Healthcare Life science), and then the membranes were blocked by 5% non-fat dry milk. After incubation with corresponding primary antibodies against H3K9ac, H3 and Sirt6 at a 1:1000 dilution, or α-tubulin at a 1:5000 dilution, goat anti-mouse or goat anti-rabbit secondary antibodies at a 1:5000 dilution were applied. Finally, membranes were incubated with enhanced chemiluminescent and protein bands were detected by ChemiDoc imaging system (Bio-Rad). Image-Lab software was used to analyze the intensities of the protein bands.

4.10. Colony formation assay

H1299 and PLC/PRF/5 were seeded into 6-well plates and cultured for one week with or without indicated compounds treatment. After obvious colonies were formed, culture medium was removed and colonies were fixed with 4% paraformaldehyde for 15 min and followed by staining with 1% crystal violet (C6158, Sigma-Aldrich) for 30 min at room temperature. After washing and drying, the stained colonies were photographed by ChemiDoc Imaging System (Bio-Rad) and analyzed by Image J.

4.11. SARS-CoV-2-nluc antiviral assay

The antiviral activities were evaluated in A549-hACE2 cells using a protocol described previously [46]. In brief, 12,000 cells per well in phenol-red free medium containing 2% FBS were plated into a white opaque 96-well plate (Corning). On the next day, 2-fold serial dilutions of compounds were prepared in DMSO. The compounds were further diluted 100-fold in the phenol-red free culture medium containing 2% FBS. Cell culture fluids were removed and incubated with 50 μL of diluted compound solutions and 50 μL of SARS-CoV2-Nluc viruses (MOI 0.05). At 48 h post-infection, 50 μL Nano luciferase substrates (Promega) were added to each well. Luciferase signals were measured using a Synergy™ Neo 2 microplate reader. The relative luciferase signals were calculated by normalizing the luciferase signals of the compound-treated groups to that of the DMSO-treated groups (set as 100%). The relative luciferase signal (Y-axis) versus the log 10 values of compound concentration (X-axis) was plotted in software Prism 9. The EC_{50} (compound concentration for reducing 50% of luciferase signal) was calculated using a nonlinear regression model (four parameters). Two experiments were performed with technical duplicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported in part by the National Institutes of Health (NIH) grant EY031054 (to H.L.). Jia Zhou was partly supported by the John D. Stobo, M.D. Distinguished Chair Endowment Fund. W.Z. was supported by NIH grants EY022694 and EY026629, and UT System Faculty STARs Award. P.-Y.S. was supported by NIH grants U19AI171413 and U01AI151801, and awards from the Sealy & Smith Foundation, the Kleberg Foundation, the John S. Dunn Foundation, the Amon G. Carter Foundation, the Gilson Longenbaugh Foundation, and the Summerfield Robert Foundation. The BV2 microglial cells were kindly provided by Prof. Linda J. Van Eldik (University of Kentucky, USA) and Prof. Elisabetta Blasi (University of Modena and Reggio Emilia, Italy). We thank Drs. Lawrence C. Sowers and Jason Herring at the Department of Pharmacology as well as Dr. Tianzhi Wang at the NMR core facility of UTMB for the NMR spectroscopy assistance, and Dr. William Russell at the UTMB mass spectrometry core with funding support from UT system proteomics network for the HRMS analysis.

Abbreviations

Sirt	sirtuin
NAD	nicotinamide adenine dinucleotide
OA	osteoarthritis
HCC	hepatocellular carcinoma
CRC	colorectal cancer
SAR	structure-activity relationship
HDAC	histone deacetylase
PDAC	pancreatic ductal adenocarcinoma
HEK	human embryonic kidney
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
COVID-19	coronavirus disease 2019
LPS	lipopolysaccharide

References

[1]. Finkel T, Deng C-X, Mostoslavsky R, Recent progress in the biology and physiology of sirtuins, Nature, 460 (2009) 587–591. [PubMed: 19641587]

[2]. Fiorentino F, Mai A, Rotili D, Emerging therapeutic potential of SIRT6 modulators, J. Med. Chem, 64 (2021) 9732–9758. [PubMed: 34213345]

[3]. Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, Kim J, Woo J, Kim JH, Choi BH, He B, Chen W, Zhang S, Cerione RA, Auwerx J, Hao Q, Lin H, Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase, Science, 334 (2011) 806–809. [PubMed: 22076378]

[4]. Rack Johannes Gregor M., Morra R, Barkauskaite E, Kraehenbuehl R, Ariza A, Qu Y, Ortmayer M, Leidecker O, Cameron David R., Matic I, Peleg Anton Y., Leys D, Traven A, Ahel I, Identification of a Class of Protein ADP-Ribosylating Sirtuins in Microbial Pathogens, Mol. Cell, 59 (2015) 309–320. [PubMed: 26166706]

[5]. Liu G, Chen H, Liu H, Zhang W, Zhou J, Emerging roles of SIRT6 in human diseases and its modulators, Med. Res. Rev, 41 (2021) 1089–1137. [PubMed: 33325563]

[6]. Sauve AA, Wolberger C, Schramm VL, Boeke JD, The biochemistry of sirtuins, Annu. Rev. Biochem, 75 (2006) 435–465. [PubMed: 16756498]

[7]. Chalkiadaki A, Guarente L, The multifaceted functions of sirtuins in cancer, Nat. Rev. Cancer, 15 (2015) 608–624. [PubMed: 26383140]

[8]. Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M, Cheung P, Kusumoto R, Kawahara TLA, Barrett JC, Chang HY, Bohr VA, Ried T, Gozani O, Chua KF, SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin, Nature, 452 (2008) 492–496. [PubMed: 18337721]

[9]. Michishita E, McCord RA, Boxer LD, Barber MF, Hong T, Gozani O, Chua KF, Cell cycle-dependent deacetylation of telomeric histone H3 lysine K56 by human SIRT6, Cell Cycle, 8 (2009) 2664–2666. [PubMed: 19625767]

[10]. Yang B, Zwaans BMM, Eckersdorff M, Lombard DB, The sirtuin SIRT6 deacetylates H3 K56Ac *in vivo* to promote genomic stability, Cell Cycle, 8 (2009) 2662–2663. [PubMed: 19597350]

[11]. Tasselli L, Xi Y, Zheng W, Tennen RI, Odrowaz Z, Simeoni F, Li W, Chua KF, SIRT6 deacetylates H3K18ac at pericentric chromatin to prevent mitotic errors and cellular senescence, Nat. Struct. Mol. Biol, 23 (2016) 434–440. [PubMed: 27043296]

[12]. Mao Z, Hine C, Tian X, Meter MV, Au M, Vaidya A, Seluanov A, Gorbunova V, SIRT6 promotes DNA repair under stress by activating PARP1, Science, 332 (2011) 1443–1446. [PubMed: 21680843]

[13]. Kugel S, Mostoslavsky R, Chromatin and beyond: the multitasking roles for SIRT6, Trends Biochem. Sci, 39 (2014) 72–81. [PubMed: 24438746]

[14]. Jiang H, Khan S, Wang Y, Charron G, He B, Sebastian C, Du J, Kim R, Ge E, Mostoslavsky R, Hang HC, Hao Q, Lin H, SIRT6 regulates TNF-α secretion through hydrolysis of long-chain fatty acyl lysine, Nature, 496 (2013) 110–113. [PubMed: 23552949]

[15]. Liszt G, Ford E, Kurtev M, Guarente L, Mouse Sir2 homolog SIRT6 is a nuclear ADP-ribosyltransferase, J. Biol. Chem, 280 (2005) 21313–21320. [PubMed: 15795229]

[16]. Mostoslavsky R, Chua KF, Lombard DB, Pang WW, Fischer MR, Gellon L, Liu P, Mostoslavsky G, Franco S, Murphy MM, Mills KD, Patel P, Hsu JT, Hong AL, Ford E, Cheng H-L, Kennedy C, Nunez N, Bronson R, Frendewey D, Auerbach W, Valenzuela D, Karow M, Hottiger MO, Hursting S, Barrett JC, Guarente L, Mulligan R, Demple B, Yancopoulos GD, Alt FW, Genomic instability and aging-like phenotype in the absence of mammalian SIRT6, Cell, 124 (2006) 315–329. [PubMed: 16439206]

[17]. Tasselli L, Zheng W, Chua KF, Sirt6: Novel mechanisms and links to aging and disease, Trends Endocrinol. Metab, 28 (2017) 168–185. [PubMed: 27836583]

[18]. Kanfi Y, Naiman S, Amir G, Peshti V, Zinman G, Nahum L, Bar-Joseph Z, Cohen HY, The sirtuin SIRT6 regulates lifespan in male mice, Nature, 483 (2012) 218–221. [PubMed: 22367546]

[19]. Sebastián C, Zwaans Bernadette M.M., Silberman Dafne M., Gymrek M, Goren A, Zhong L, Ram O, Truelove J, Guimaraes Alexander R., Toiber, Cosentino C, Greenson Joel K., MacDonald Alasdair I., McGlynn L, Maxwell F, Edwards J, Giacosa S, Guccione E, Weissleder R, Bernstein Bradley E., Regev A, Shiels Paul G., Lombard David B., Mostoslavsky R, The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism, Cell, 151 (2012) 1185–1199. [PubMed: 23217706]

[20]. Koyuncu E, Budayeva HG, Miteva YV, Ricci DP, Silhavy TJ, Shenk T, Cristea IM, Griffin DE, Sirtuins are evolutionarily conserved viral restriction factors, mBio, 5 (2014) e02249–02214. [PubMed: 25516616]

[21]. Li P, Jin Y, Qi F, Wu F, Luo S, Cheng Y, Montgomery RR, Qian F, SIRT6 acts as a negative regulator in dengue virus-induced inflammatory response by targeting the DNA binding domain of NF-κB p65, Front. Cell. Infect. Microbiol, 8 (2018) 113. [PubMed: 29686974]

[22]. Klein MA, Denu JM, Biological and catalytic functions of sirtuin 6 as targets for small-molecule modulators, J. Biol. Chem, 295 (2020) 11021–11041. [PubMed: 32518153]

[23]. Feldman JL, Baeza J, Denu JM, Activation of the protein deacetylase SIRT6 by long-chain fatty acids and widespread deacylation by mammalian sirtuins, J. Biol. Chem, 288 (2013) 31350–31356. [PubMed: 24052263]

[24]. Rahnasto-Rilla M, Kokkola T, Jarho E, Lahtela-Kakkonen M, Moaddel R, *N*-acylethanolamines bind to SIRT6, ChemBioChem, 17 (2016) 77–81. [PubMed: 26607666]

[25]. You W, Zheng W, Weiss S, Chua KF, Steegborn C, Structural basis for the activation and inhibition of Sirtuin 6 by quercetin and its derivatives, Sci. Rep, 9 (2019) 19176. [PubMed: 31844103]

[26]. Rahnasto-Rilla M, Tyni J, Huovinen M, Jarho E, Kulikowicz T, Ravichandran S, Bohr VA, Ferrucci L, Lahtela-Kakkonen M, Moaddel R, Natural polyphenols as sirtuin 6 modulators, Sci. Rep, 8 (2018) 4163. [PubMed: 29515203]

[27]. Jiang C, Sun ZM, Hu JN, Jin Y, Guo Q, Xu JJ, Chen ZX, Jiang RH, Wu YS, Cyanidin ameliorates the progression of osteoarthritis via the Sirt6/NF-κB axis in vitro and in vivo, Food Funct, 10 (2019) 5873–5885. [PubMed: 31464310]

[28]. You W, Rotili D, Li T-M, Kambach C, Meleshin M, Schutkowski M, Chua KF, Mai A, Steegborn C, Structural basis of Sirtuin 6 activation by synthetic small molecules, Angew. Chem. Int. Ed, 56 (2017) 1007–1011.

[29]. Klein MA, Liu C, Kuznetsov VI, Feltenberger JB, Tang W, Denu JM, Mechanism of activation for the sirtuin 6 protein deacylase, J. Biol. Chem, 295 (2020) 1385–1399. [PubMed: 31822559]

[30]. Huang Z, Zhao J, Deng W, Chen Y, Shang J, Song K, Zhang L, Wang C, Lu S, Yang X, He B, Min J, Hu H, Tan M, Xu J, Zhang Q, Zhong J, Sun X, Mao Z, Lin H, Xiao M, Chin YE, Jiang H, Xu Y, Chen G, Zhang J, Identification of a cellularly active SIRT6 allosteric activator, Nat. Chem. Biol, 14 (2018) 1118–1126. [PubMed: 30374165]

[31]. Shang J, Zhu Z, Chen Y, Song J, Huang Y, Song K, Zhong J, Xu X, Wei J, Wang C, Cui L, Liu CY, Zhang J, Small-molecule activating SIRT6 elicits therapeutic effects and synergistically promotes anti-tumor activity of vitamin D(3) in colorectal cancer, Theranostics, 10 (2020) 5845–5864. [PubMed: 32483423]

[32]. Chen X, Sun W, Huang S, Zhang H, Lin G, Li H, Qiao J, Li L, Yang S, Discovery of potent small-molecule SIRT6 activators: Structure–activity relationship and anti-pancreatic ductal adenocarcinoma activity, J. Med. Chem, 63 (2020) 10474–10495. [PubMed: 32787077]

[33]. Schlicker C, Boanca G, Lakshminarasimhan M, Steegborn C, Structure-based development of novel sirtuin inhibitors, Aging, 3 (2011) 852–872. [PubMed: 21937767]

[34]. Zhang ZG, Qin CY, Sirt6 suppresses hepatocellular carcinoma cell growth via inhibiting the extracellular signal-regulated kinase signaling pathway, Molecular medicine reports, 9 (2014) 882–888. [PubMed: 24366394]

[35]. Wang Y, Pan T, Wang H, Li L, Li J, Zhang D, Yang H, Overexpression of SIRT6 attenuates the tumorigenicity of hepatocellular carcinoma cells, Oncotarget, 8 (2017) 76223–76230. [PubMed: 29100306]

[36]. Cai Y, Sheng ZY, Liang SX, Radiosensitization effect of overexpression of adenovirus-mediated SIRT6 on A549 non-small cell lung cancer cells, Asian Pac. J. Cancer Prev, 15 (2014) 7297–7301. [PubMed: 25227832]

[37]. Han Z, Liu L, Liu Y, Li S, Sirtuin SIRT6 suppresses cell proliferation through inhibition of Twist1 expression in non-small cell lung cancer, Int. J. Clin. Exp. Pathol, 7 (2014) 4774–4781. [PubMed: 25197348]

[38]. Mendes KL, D.d.F. Lelis, S.H.S. Santos, Nuclear sirtuins and inflammatory signaling pathways, Cytokine Growth Factor Rev, 38 (2017) 98–105. [PubMed: 29132743]

[39]. Lee HS, Ka SO, Lee SM, Lee SI, Park JW, Park BH, Overexpression of sirtuin 6 suppresses inflammatory responses and bone destruction in mice with collagen-induced arthritis, Arthritis. Rheum, 65 (2013) 1776–1785. [PubMed: 23553536]

[40]. Hou KL, Lin SK, Chao LH, Hsiang-Hua Lai E, Chang CC, Shun CT, Lu WY, Wang JH, Hsiao M, Hong CY, Kok SH, Sirtuin 6 suppresses hypoxia-induced inflammatory response in human osteoblasts via inhibition of reactive oxygen species production and glycolysis-A therapeutic implication in inflammatory bone resorption, Biofactors, 43 (2017) 170–180. [PubMed: 27534902]

[41]. Kawahara TLA, Michishita E, Adler AS, Damian M, Berber E, Lin M, McCord RA, Ongaigui KCL, Boxer LD, Chang HY, Chua KF, SIRT6 links histone h3 lysine 9 deacetylation to NF-κB-dependent gene expression and organismal life span, Cell, 136 (2009) 62–74. [PubMed: 19135889]

[42]. Blasi E, Barluzzi R, Bocchini V, Mazzolla R, Bistoni F, Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus, J. Neuroimmunol, 27 (1990) 229–237. [PubMed: 2110186]

[43]. Xu J, Shi P-Y, Li H, Zhou J, Broad spectrum antiviral agent niclosamide and its therapeutic potential, ACS Infect. Dis, 6 (2020) 909–915. [PubMed: 32125140]

[44]. Xu J, Xue Y, Zhou R, Shi P-Y, Li H, Zhou J, Drug repurposing approach to combating coronavirus: Potential drugs and drug targets, Med. Res. Rev, 41 (2021) 1375–1426. [PubMed: 33277927]

[45]. Samrat SK, Xu J, Xie X, Gianti E, Chen H, Zou J, Pattis JG, Elokely K, Lee H, Li Z, Klein ML, Shi P-Y, Zhou J, Li H, Allosteric inhibitors of the main protease of SARS-CoV-2, Antiviral Res, 205 (2022) 105381. [PubMed: 35835291]

[46]. Xie X, Muruato AE, Zhang X, Lokugamage KG, Fontes-Garfias CR, Zou J, Liu J, Ren P, Balakrishnan M, Cihlar T, Tseng C-TK, Makino S, Menachery VD, Bilello JP, Shi P-Y, A

nanoluciferase SARS-CoV-2 for rapid neutralization testing and screening of anti-infective drugs for COVID-19, Nat. Commun, 11 (2020) 5214. [PubMed: 33060595]

Highlights:

- A series of novel pyrrolo[1,2-*a*]quinoxaline-based derivatives have been identified as potent and selective Sirt6 activators.
- The on-target effects of these Sirt6 activators were validated by Sirt6-knockdown experiments.
- Selected derivatives repressed LPS-induced proinflammatory cytokine/chemokine production.
- Compound **36** significantly inhibited the colony formation of cancer cells.
- Compound **38** significantly suppressed SARS-CoV-2 infection.

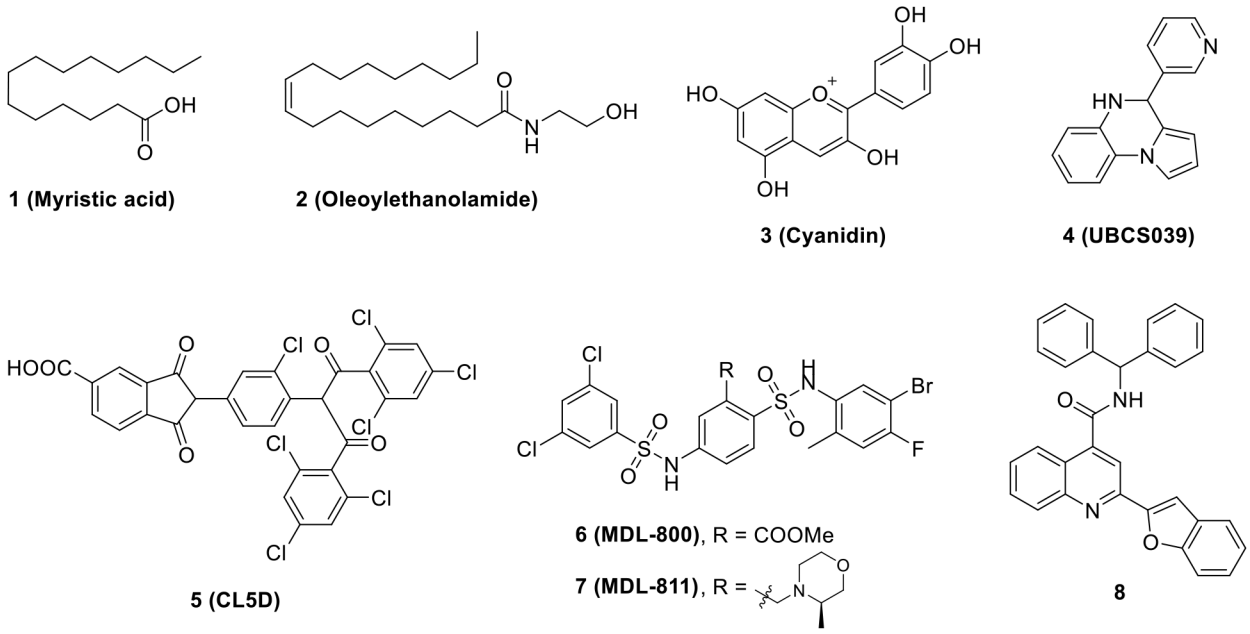
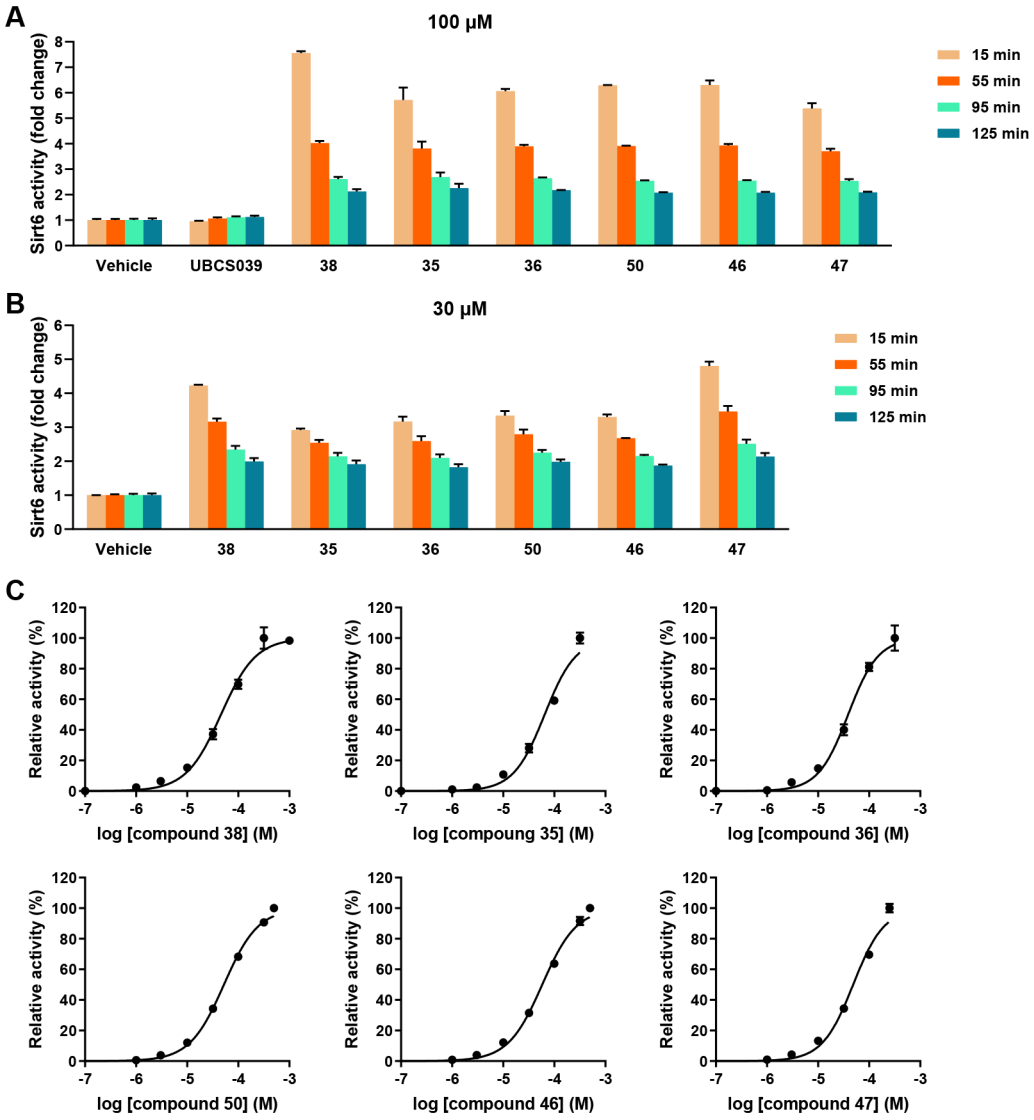


Fig. 1.
The representative Sirt6 activators.



Compound	38	35	36	50	46	47
E_{max} (fold change)	10.19 ± 0.65	8.89 ± 0.29	6.38 ± 0.44	8.59 ± 0.13	7.9 ± 0.10	7.33 ± 0.18
EC_{50} (μM)	48.75 ± 3.31	64.74 ± 0.67	38.77 ± 1.83	52.53 ± 2.66	58.01 ± 0.06	48.55 ± 1.39

Fig. 2.

The effects of selected compounds on Sirt6 activities. (A), (B) Sirt6 activities of lead compound UBCS039 and 6 selected compounds **35**, **36**, **38**, **46**, **47**, and **50** were assessed by the Sirt6 Activity Assay Kit at 100 μM and 30 μM at indicated time points. (C) Concentration–response curves of compounds **35**, **36**, **38**, **46**, **47**, and **50** for Sirt6 activities. Data were presented as mean ± SEM. Experiments were repeated at least twice.

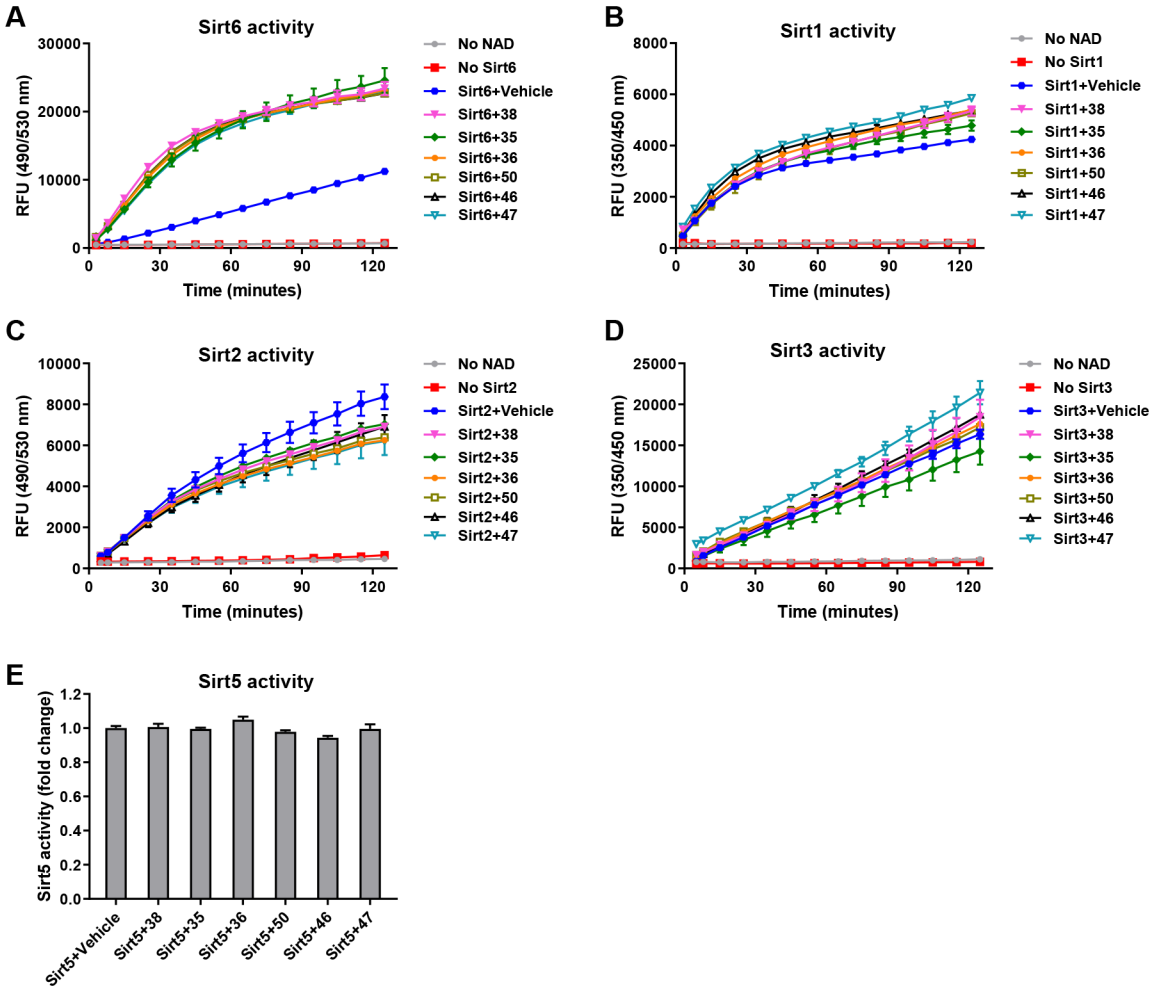


Fig. 3.

The effects of selected compounds on Sirt1, Sirt2, Sirt3, Sirt5 and Sirt6 activities. (A-D) Time courses of Sirt6, Sirt1, Sirt2 and Sirt3 deacetylation activities in response of selected compounds. (E) Fold change of Sirt5 desuccinylation activity in response of selected compounds. Data were presented as mean ± SEM. Experiments were performed at least twice.

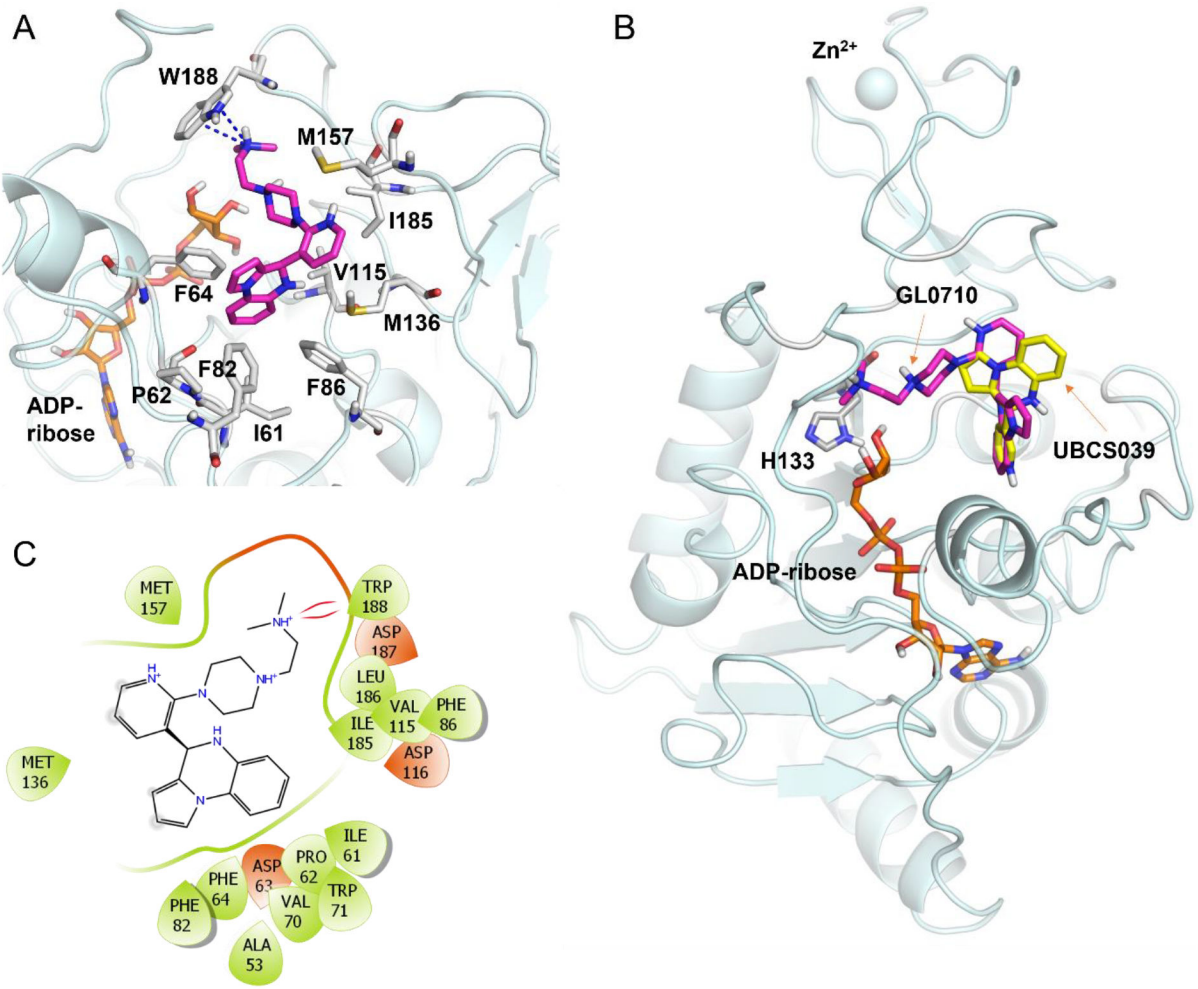


Fig. 4.

Docking analysis of compound **38** (**GL0710**) for the binding mode with Sirt6 protein. (A) **GL0710** (magenta sticks) docked into Sirt6/ADP ribose structure (PDB ID: 5MF6). ADP-ribose is shown as orange sticks and binding site residues are shown as gray sticks. π-cation interaction is shown as blue dashed lines. (B) Overlay of Sirt6/ADP ribose/UBCS039 complex with **GL0710** docked pose (**GL0710** in magenta sticks, **UBCS039** in yellow sticks, and ADP ribose in orange sticks). The catalytic residue H133 indicates the active site. (C) Interaction diagram of Sirt6-GL0710 binding site. π-Cation interaction is shown as red lines.

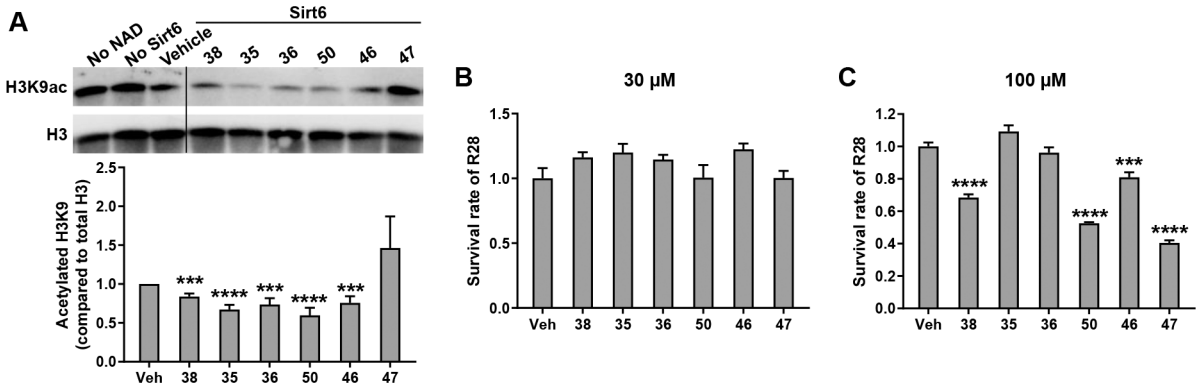


Fig. 5.

Deacetylation effect and cytotoxicity of selected compounds. (A) The deacetylation effects on H3K9 of six selected compounds were detected in nucleosomes. Nucleosomes extracted from HEK293T cells were incubated with 100 μM of selected compounds or Vehicle at 30 °C for 60 minutes. Acetylated H3K9 and total H3 were detected by Western blot. The level of acetylated H3K9 was quantified by Image Lab software. (B), (C) Cell viability was assessed by MTT assay. R28 cells were treated with 30 μM or 100 μM compounds for 24 hours. Data were presented as mean ± SEM. The experiment was repeated at least three times. ***p<0.001; ****p<0.0001.

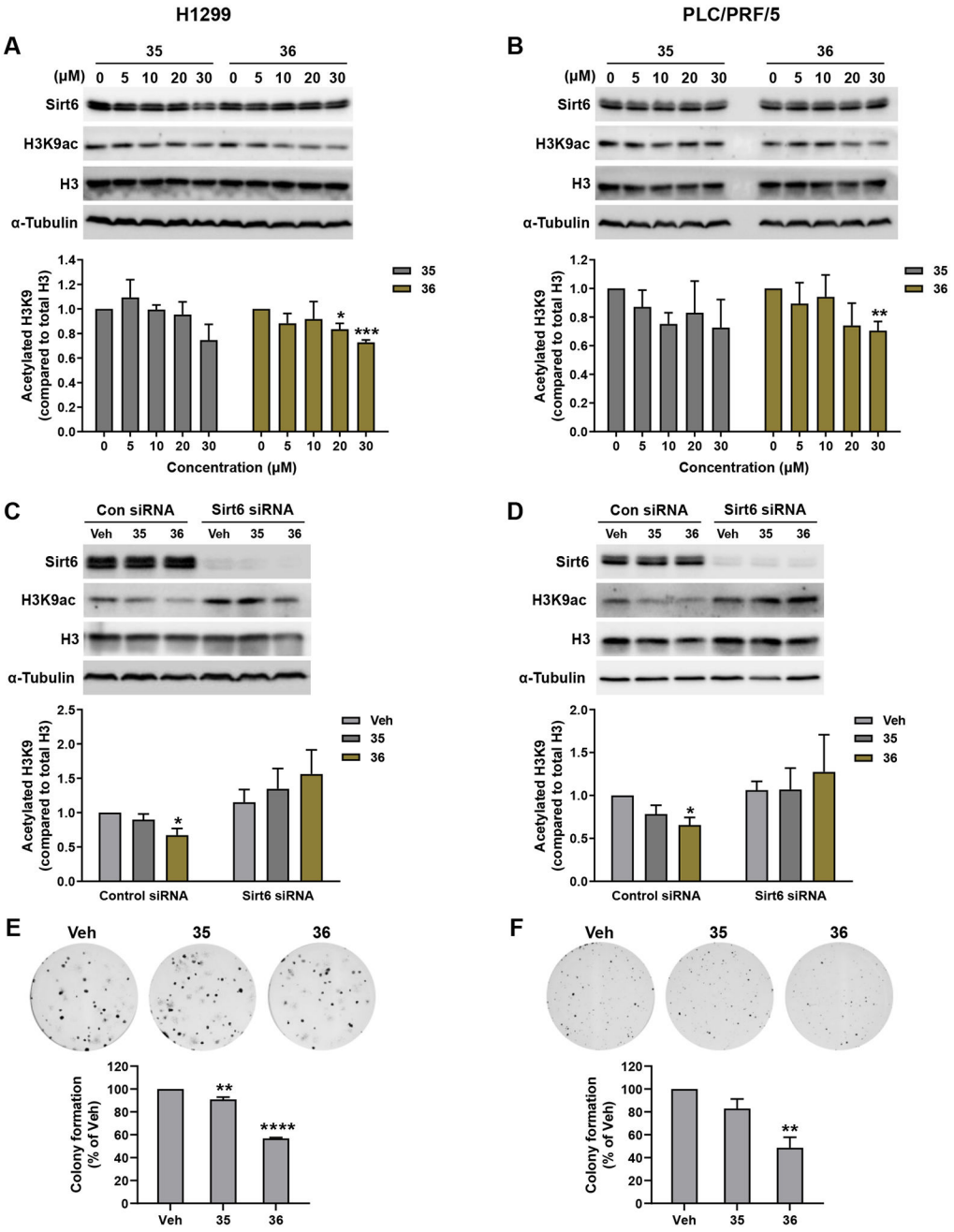


Fig. 6.

The deacetylation effects of compounds **35** and **36** in cancer cells. (A), (B) H1299 and PLC/PRF/5 cells were treated with compounds **35** and **36** at indicated concentrations for 24 hours, and the expression of Sirt6, acetylated H3K9, and total H3 was detected by Western blot and quantified. (C), (D) H1299 and PLC/PRF/5 cells were transfected with control or Sirt6 siRNA, followed by the treatment with 30 μM compounds **35** and **36** for 24 hours. The expression of Sirt6, acetylated H3K9, and total H3 was detected by Western blot and quantified. (E), (F) Colony formation assay was performed in H1299 and PLC/PRF/5 cells which were exposed to 30 μM compounds **35** and **36** or Vehicle for one week. Colony

formation was calculated as the ratio of the area by colonies to total plate area using Image J. All experiments were repeated three times. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to relevant controls.

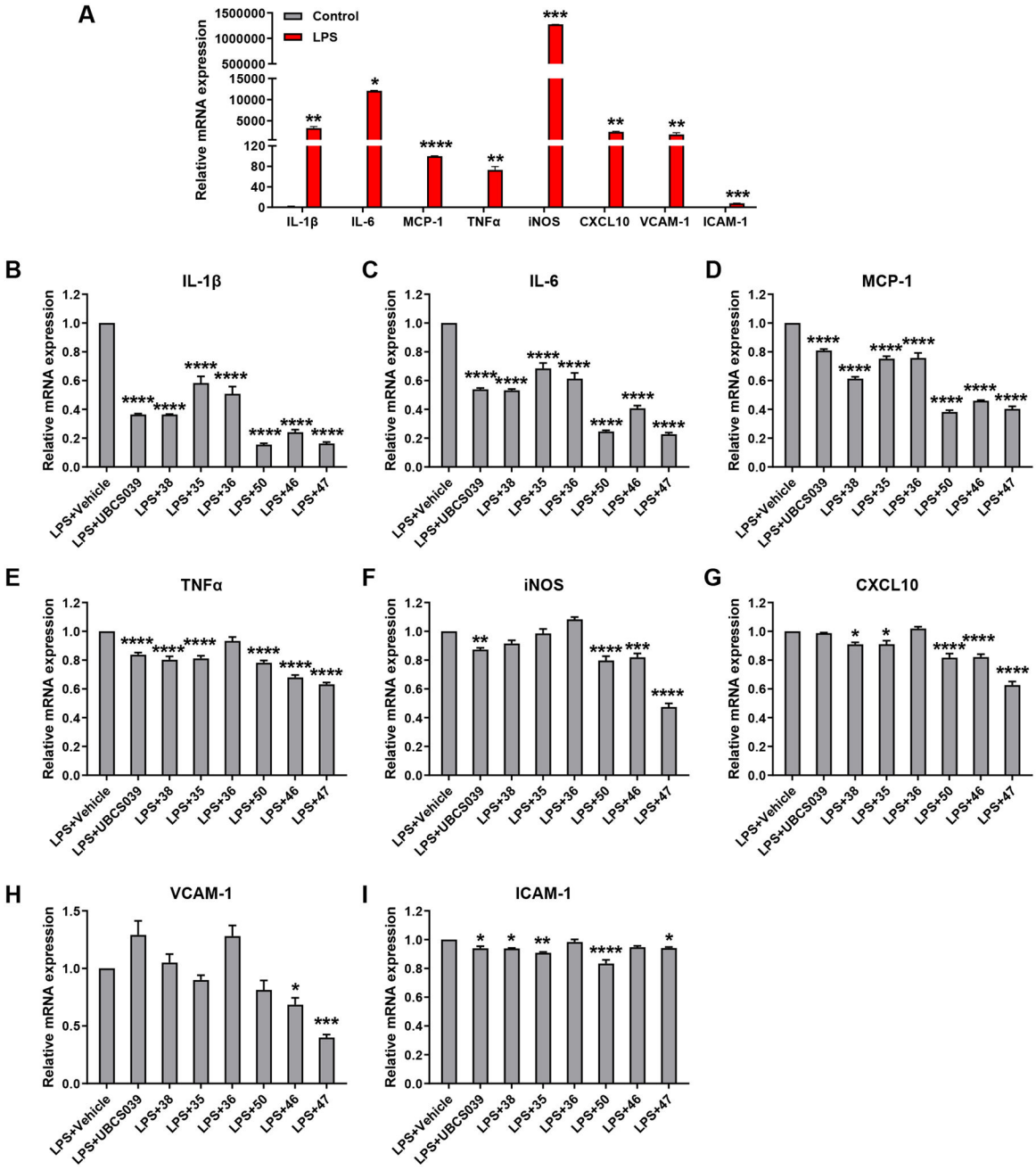
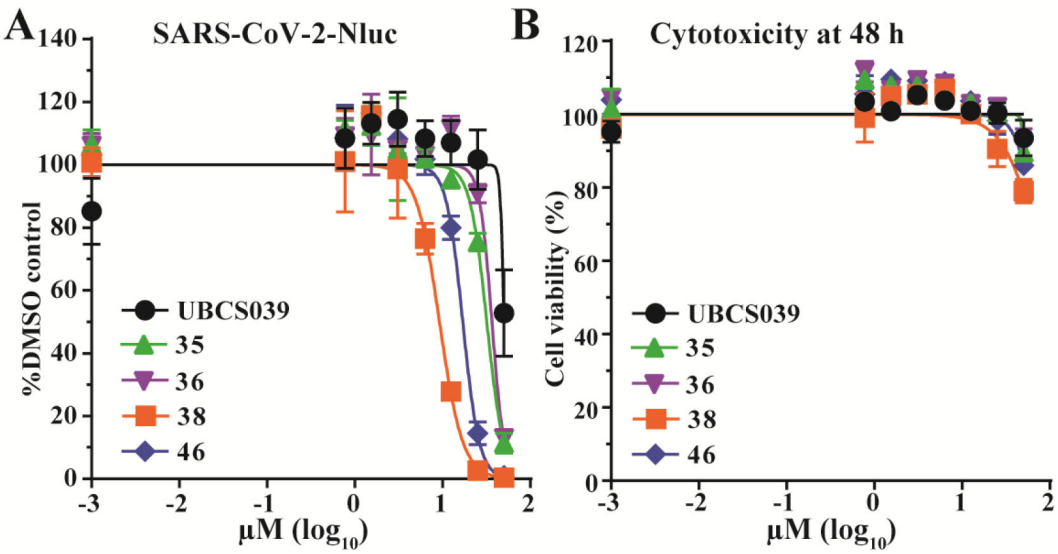


Fig. 7.

Anti-inflammatory effects of the selected compounds. (A) The inflammatory genes were induced by LPS stimulation in BV2 cells. (B-I) BV2 cells were pretreated with 30 μM selected compounds or Vehicle and followed by 100 ng/mL LPS for 6 hours. The mRNA expression of inflammatory genes was examined by q-PCR. Data were presented as mean ± SEM. The experiment was repeated three times. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

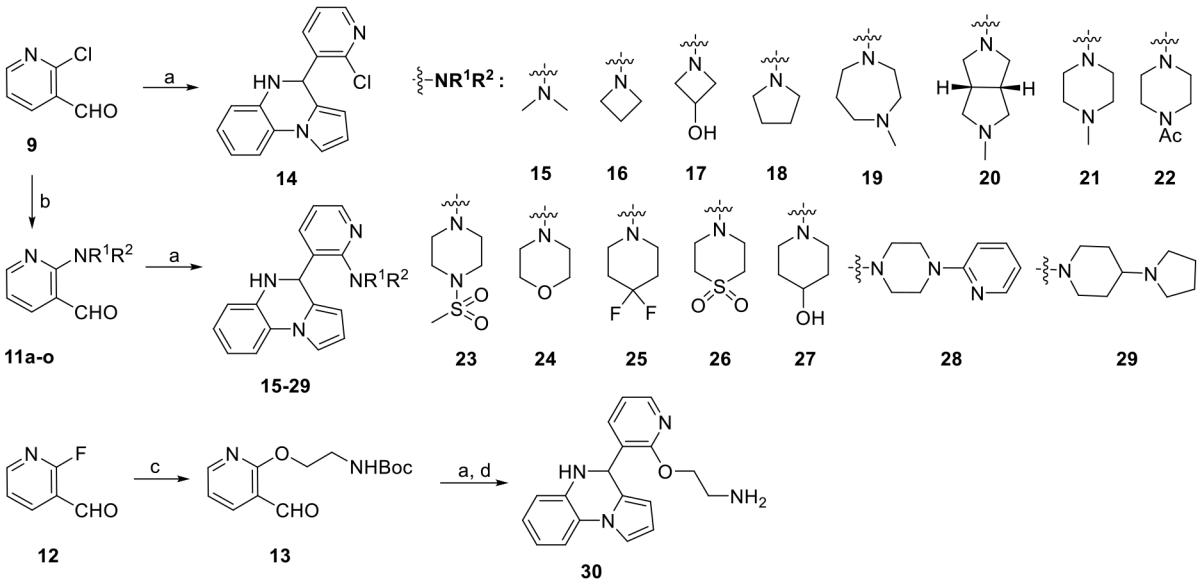


C

Compound	UBCS039	35	36	38	46
EC_{50}	50	32.1	36.7	9.3	17.1
CC_{50}	>50	>50	>50	>50	>50

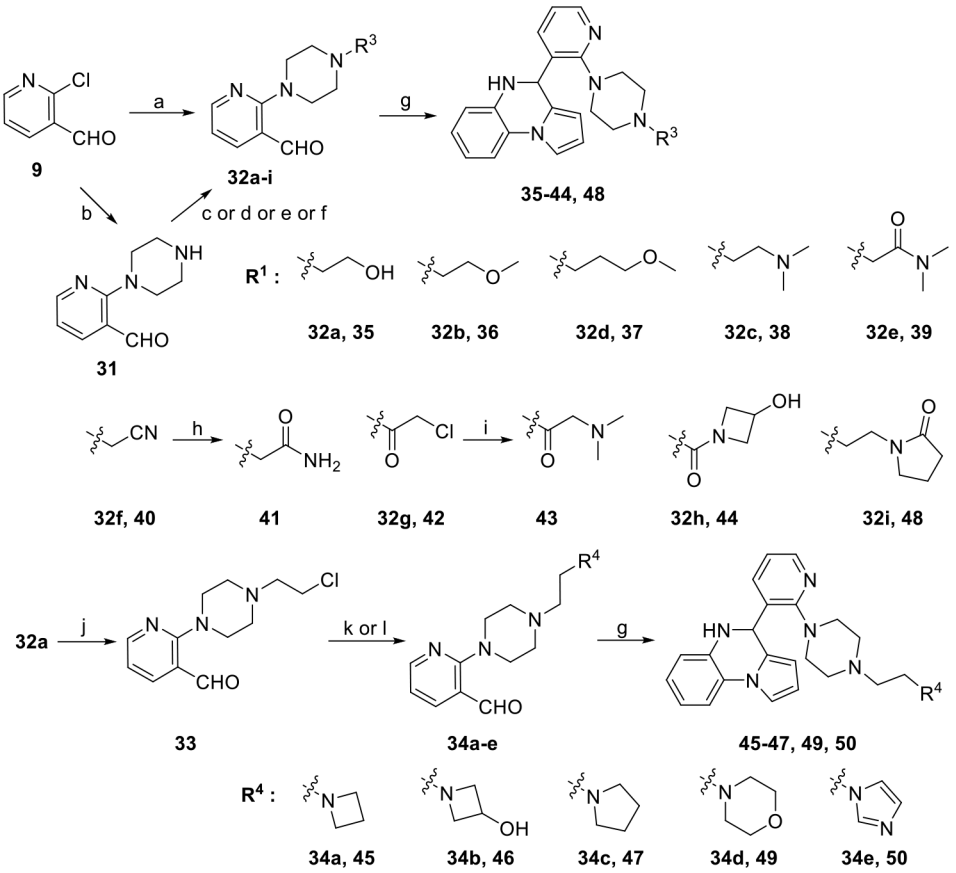
Fig. 8.

The antiviral activities of selected compounds against SARS-CoV-2. (A) Dose-dependent inhibition of SARS-CoV-2-Nluc by selected compounds. (B) Cell viability assay. A549-hACE2 cells were incubated with various concentrations of selected compounds and then assayed for viability at 48 h post-incubation. (C) Summary of EC_{50} and CC_{50} of selected compounds.



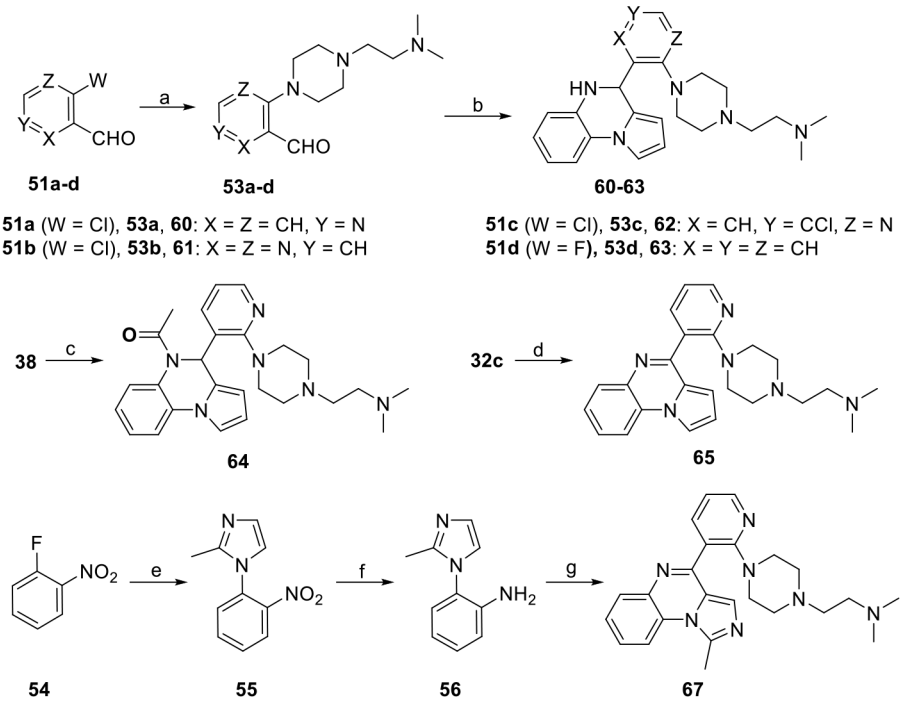
Scheme 1.

Synthesis of Compounds **14–30**. Reagents and conditions: (a) 2-(1*H*-pyrrol-1-yl)aniline (**10**), EtOH, AcOH, 50 °C, 3 h. (b) NHR^1R^2, or NHR^1R^2 hydrochloride and K_2CO_3, toluene or DMF, 100–110 °C, overnight. (c) *N*-Boc-ethanolamine, Na_2CO_3, DMF, 110 °C, 6 h. (d) TFA, CH_2Cl_2, room temperature (RT), 3 h.



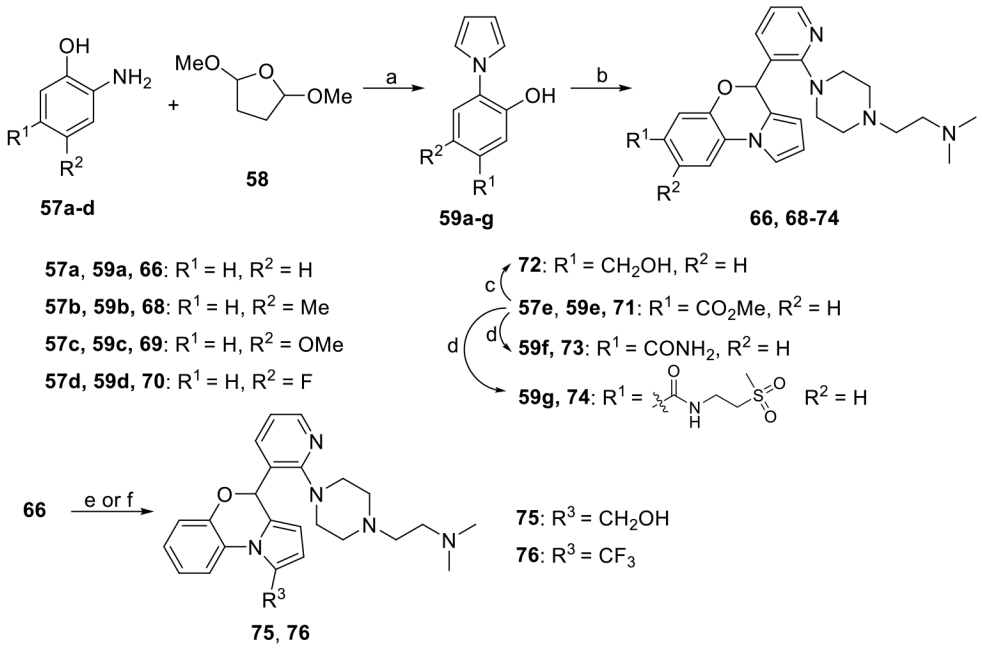
Scheme 2.

Synthesis of Compounds **35–50**. Reagents and conditions: (a) *N*-R^3 substituted piperazine (for compounds **32a-c**), toluene, 110 °C, 5 h. (b) piperazine, K_2CO_3, DMF, 90 °C, overnight. (c) R^3Cl or R^3Br (for compounds **32d-f**), K_2CO_3, CH_3CN, 50 °C, 3 h. (d) 2-chloroacetyl chloride, K_2CO_3, CH_2Cl_2, 0 °C, 1 h. (e) (1) 4-nitrophenyl chloroformate, Et_3N, CH_2Cl_2, 0 °C to RT, overnight; (2) 3-hydroxyazetidine hydrochloride, K_2CO_3, CH_3CN, 60 °C, overnight. (f) 1-(2-chloroethyl)pyrrolidin-2-one, Et_3N, toluene, 110 °C, overnight. (g) **10**, EtOH, AcOH, 50 °C, 3 h. (h) KOH, *t*-BuOH, 110 °C, 1 h. (i) dimethylamine, K_2CO_3, DMF, 50 °C, 4 h. (j) $SOCl_2$, CH_2Cl_2, 50 °C, 2 h. (k) R^4H or R^4H hydrochloride, Cs_2CO_3, EtOH, 70 °C, 2 h. (l) imidazole, NaOH, TBAB, toluene, RT to 110 °C, 6 h.



Scheme 3.

Synthesis of Compounds **60–65** and **67**. Reagents and conditions: (a) 1-(2-dimethylaminoethyl)piperazine (**52**), toluene (for **53a,c**), 110 °C, 4 h; or **52**, K_2CO_3, dioxane (for **53b**), reflux, 4 h; or **52**, K_2CO_3, DMF (for **53d**), reflux, 5 h. (b) **10**, EtOH, AcOH, 50 °C, 3 h. (c) acetyl chloride, Et_3N, CH_2Cl_2, RT, 30 min. (d) **10**, EtOH, AcOH, 70 °C, overnight. (e) 2-methyl-1*H*-imidazole, K_2CO_3, CH_3CN, reflux, 24 h. (f) Pd/C, H_2, EtOH, RT, 4 h. (g) **32c**, EtOH, AcOH, 50 °C, 3 h.

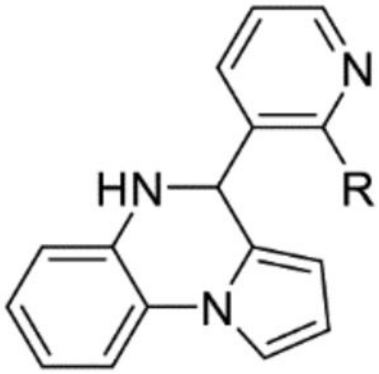


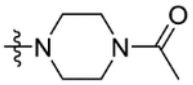
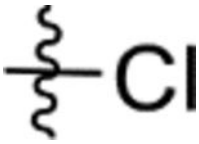
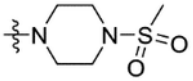
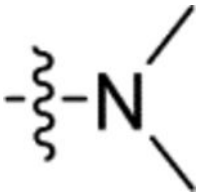
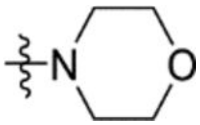
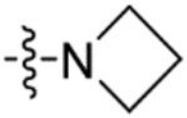
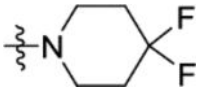
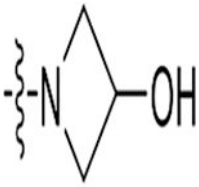
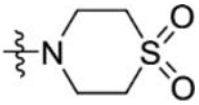
Scheme 4.

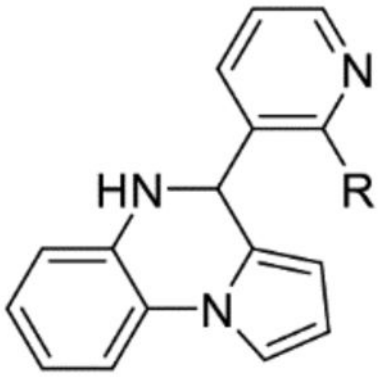
Synthesis of Compounds **66** and **68–76**. Reagents and conditions: (a) AcOH, 110 °C, 15 min. (b) **32c**, TFA, $CHCl_3$, 60 °C, 24 h. (c) $LiAlH_4$, THF, 0 °C to RT, 2 h. (d) (1) LiOH, EtOH, H_2O, RT to 50 °C, 4 h; (2) NH_4Cl (for **59f**) or 2-aminoethylmethylsulfone hydrochloride (for **59g**), HATU, Et_3N, DMF, RT, overnight. (e) (1) $POCl_3$, DMF, 0 °C to 50 °C, overnight; (2) $NaBH_4$, CH_3OH, 0 to RT, 1 h. (f) *S*-(trifluoromethyl)dibenzothiophenium triflate, K_2CO_3, DMF, 80 °C, 48 h.

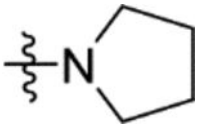
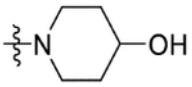
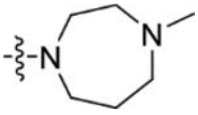
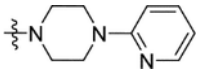
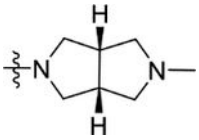
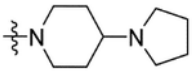
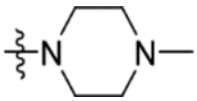
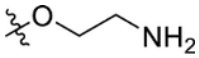
Table 1.

Effect of Compounds 14–30 on Sirt6-dependent Peptide Deacetylation Activation[a]



Entry	R	Activation Fold[a]		Entry	R	Activation Fold	
		100 μM	30 μM			100 μM	30 μM
UBCS039	H	1.12 ± 0.08	NT[b]	**22**		2.74 ± 0.10	1.60 ± 0.01
14		1.04 ± 0.02	NT	**23**		3.63 ± 0.10	2.03 ± 0.02
15		1.08 ± 0.09	NT	**24**		1.00 ± 0.07	NT
16		1.38 ± 0.11	NT	**25**		1.54 ± 0.03	NT
17		1.47 ± 0.10	NT	**26**		2.24 ± 0.09	1.32 ± 0.01



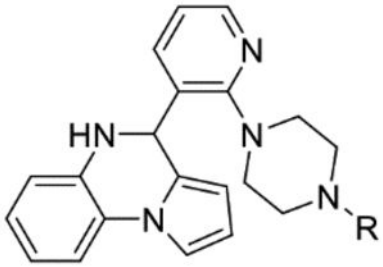
Entry	R	Activation Fold[a] 100 μM	Activation Fold[a] 30 μM	Entry	R	Activation Fold 100 μM	Activation Fold 30 μM
18		1.22 ± 0.03	NT	**27**		2.38 ± 0.15	1.29 ± 0.05
19		4.23 ± 0.06	2.18 ± 0.02	**28**		1.82 ± 0.11	NT
20		4.24 ± 0.04	2.00 ± 0.11	**29**		3.44 ± 0.07	1.89 ± 0.01
21		4.62 ± 0.12	2.44 ± 0.04	**30**		0.89 ± 0.11	NT

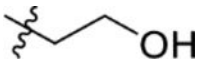
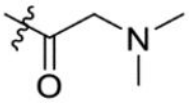
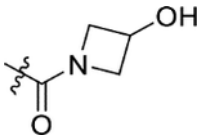
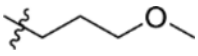
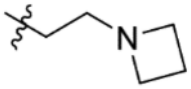
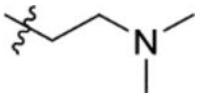
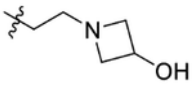
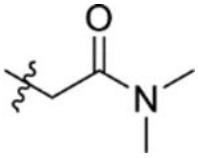
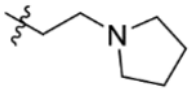
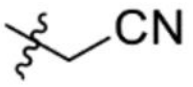
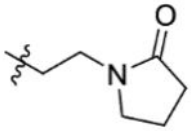
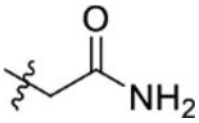
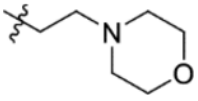
[a]Activation folds on Sirt6 activity of compounds at 100 μM and 30 μM were determined in an assay using a Sirt6 fluorometric activity kit.

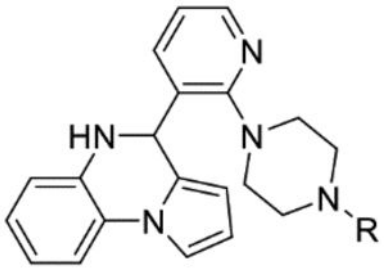
[b]NT: not tested. The results represent mean ± SD from at least two independent experiments.

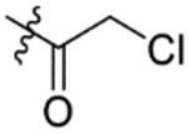
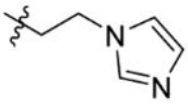
Table 2.

Effect of Compounds 35–50 on Sirt6-dependent Peptide Deacetylation Activation[a]



Entry	R	Activation Fold[a]		Entry	R	Activation Fold	
		100 μM	30 μM			100 μM	30 μM
35		5.72 ± 0.69	2.92 ± 0.06	**43**		3.78 ± 0.15	2.31 ± 0.07
36		6.06 ± 0.13	3.17 ± 0.21	**44**		5.26 ± 0.13	2.67 ± 0.19
37		5.02 ± 0.25	3.13 ± 0.14	**45**		5.85 ± 0.01	2.81 ± 0.01
38		7.38 ± 0.6	3.83 ± 0.46	**46**		6.30 ± 0.25	3.30 ± 0.11
39		6.60 ± 0.22	3.21 ± 0.06	**47**		5.38 ± 0.29	4.80 ± 0.19
40		4.74 ± 0.26	2.37 ± 0.04	**48**		5.64 ± 0.43	3.48 ± 0.02
41		3.43 ± 0.48	1.73 ± 0.12	**49**		5.99 ± 0.21	3.02 ± 0.04

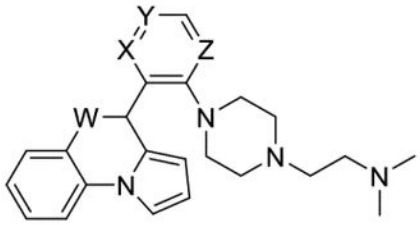


Entry	R	Activation Fold[a]		Entry	R	Activation Fold	
		100 μM	30 μM			100 μM	30 μM
42		2.84 ± 0.28	1.93 ± 0.02	**50**		6.29 ± 0.02	3.34 ± 0.19

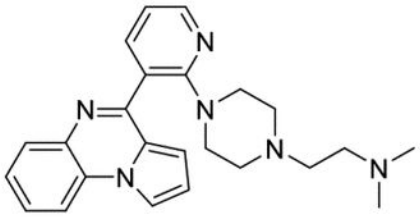
[a]Activation folds on Sirt6 activity of compounds at 100 μM and 30 μM were determined in an assay using a Sirt6 fluorometric activity kit. The results represent mean ± SD from at least two independent experiments.

Table 3.

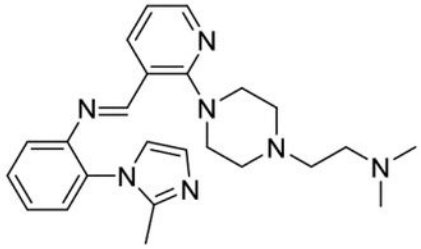
Effect of Compounds 60–67 on Sirt6-dependent Peptide Deacetylation Activation[a]



60-64, 66



65



67

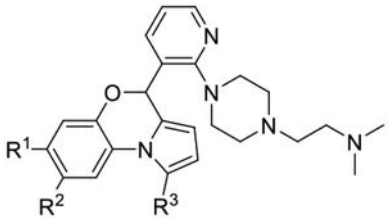
Entry	W	X	Y	Z	Activation Fold[a]	
					100 μM	30 μM
60	NH	CH	N	CH	1.07 ± 0.18	NT[b]
61	NH	N	CH	N	0.97 ± 0.05	NT
62	NH	CH	CCl	N	0.98 ± 0.01	NT
63	NH	CH	CH	CH	5.43 ± 0.21	2.62 ± 0.34
64	$NCOCH_3$	CH	CH	N	0.94 ± 0.03	NT
65					0.99 ± 0.01	NT
66	O	CH	CH	N	4.23 ± 0.22	2.67 ± 0.01
67					0.92 ± 0.03	NT

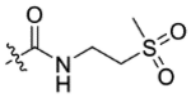
[a]Activation folds on Sirt6 activity of compounds at 100 μM and 30 μM were determined in an assay using a Sirt6 fluorometric activity kit.

[b]NT: not tested. The results represent mean ± SD from at least two independent experiments.

Table 4.

Effect of Compounds 68–76 on Sirt6-dependent Peptide Deacetylation Activation[a]



Entry	R^1	R^2	R^3	Activation Fold[a]	
				100 μM	30 μM
68	H	CH_3	H	4.56 ± 0.04	2.99 ± 0.05
69	H	OCH_3	H	5.65 ± 0.22	4.51 ± 0.06
70	H	F	H	2.53 ± 0.05	1.84 ± 0.04
71	$COOCH_3$	H	H	4.82 ± 0.14	4.16 ± 0.18
72	CH_2OH	H	H	2.69 ± 0.03	1.59 ± 0.03
73	$CONH_2$	H	H	0.83 ± 0.01	NT[b]
74		H	H	0.96 ± 0.02	NT
75	H	H	CH_2OH	1.16 ± 0.06	NT
76	H	H	CF_3	4.20 ± 0.06	2.69 ± 0.02

[a]Activation folds on Sirt6 activity of compounds at 100 μM and 30 μM were determined in an assay using a Sirt6 fluorometric activity kit.

[b]NT: not tested. The results represent mean ± SD from at least two independent experiments.