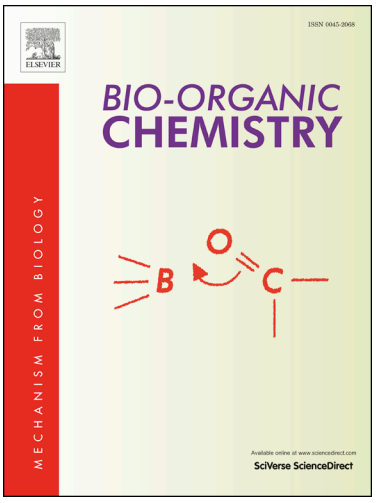

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Graphical Abstract

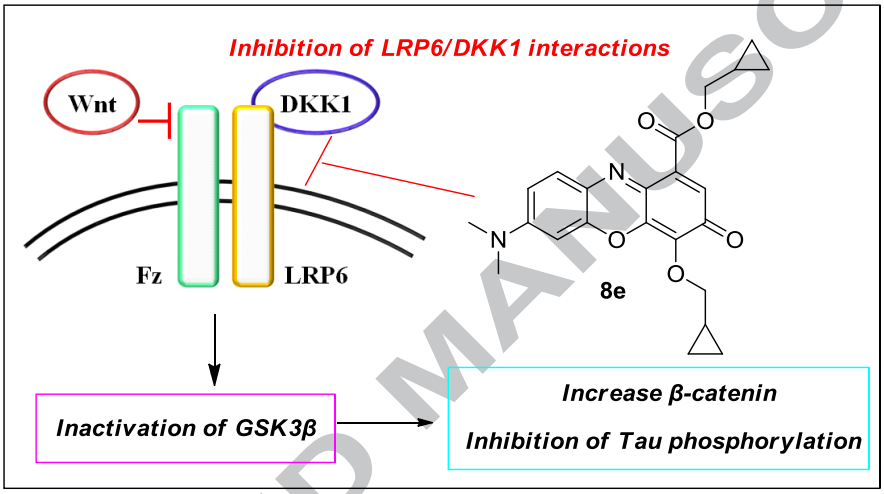
Design and Synthesis of Gallocyanine Inhibitors of DKK1/LRP6 Interactions for Treatment of Alzheimer's Disease

Savvas Thysiadis,[a] Sotirios Katsamakas,[c] Spyros Mpousis,[a] Nicolaos Avramidis,[b] Spiros Efthimiopoulos,[*b] and Vasiliki Sarli[*a]

[a] Department of Chemistry, Aristotle University of Thessaloniki, University Campus, 54124, Thessaloniki, Greece, sarli@chem.auth.gr

[b] Division of Animal and Human Physiology, Department of Biology, National & Kapodistrian University of Athens, Panepistimiopolis, Ilisia, Greece, efthis@biol.uoa.gr

[c] Department of Pharmaceutical Chemistry, School of Pharmacy, Aristotle University of Thessaloniki, University Campus, 54124, Thessaloniki, Greece.



Design and Synthesis of Gallocyanine Inhibitors of DKK1/LRP6 Interactions for Treatment of Alzheimer 's Disease

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[a] *Department of Chemistry, Aristotle University of Thessaloniki, University Campus, Thessaloniki, 54124, Greece, sarli@chem.auth.gr*

[b] *Division of Animal and Human Physiology, Department of Biology, National & Kapodistrian University of Athens, Panepistimiopolis, Ilisia, Greece, efthis@biol.uoa.gr*

c Department of Pharmaceutical Chemistry, School of Pharmacy, Aristotle University of Thessaloniki, University Campus, Thessaloniki, 54124, Greece.

Keywords: DKK1/LRP6 interactions, Tau phosphorylation, phenoxazinone, Alzheimer's disease, gallocyanine derivatives

Highlights

- A series of NCI8642 derivatives was synthesized
- The synthesized NCI8642 derivatives inhibit DKK1/LRP6 interactions
- The synthesized NCI8642 derivatives inhibit Tau phosphorylation
- Alkylated NCI8642 derivatives on C1 provided the most active compounds
- The synthesized derivatives affect the levels of pGSK3β kinase and β-catenin

ABSTRACT

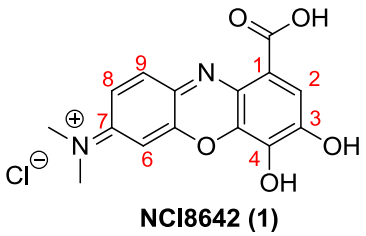
Based on NCI8642, a series of gallocyanine derivatives was synthesized with modifications of the substituent groups in position 1, 2 and 4 of the phenoxazinone scaffold. The effectiveness of gallocyanines to inhibit DKK1/LRP6 interactions and Tau phosphorylation induced by prostaglandin J2 and DKK1 was elucidated by both experimental data and molecular docking simulations. Bis-alkylated with flexible alkyl ester groups on C1 and bis-benzyl gallocyanines provided the most active inhibitors, while amino derivatives on C2 of NCI8642 that have alkoxy or benzyloxy substituents on C4, were less active. Furthermore, it is shown that treating of SHSY5Y cells with NCI8642 derivatives activates Wnt signaling and increases the levels of pGSK3β kinase and β-catenin.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease, characterized by the presence of β-amyloid plaques (Aβ protein) and neurofibrillary tangles (NFTs). Despite the research progress in the past 25 years, the pathological processes underlying AD have not been fully understood. In AD and related disorders called tauopathies, tau is abnormally hyperphosphorylated and is accumulated as intraneuronal tangles.[1] Tau was initially discovered as a microtubule-associated protein from porcine brain that promoted microtubule assembly *in vitro*.[2] Today, it is known that different kinases are involved in tau phosphorylation including glycogen synthase kinases GSK3α and GSK3β, cyclin-dependent kinase 5 (Cdk5), c-Jun N-terminal kinase (JNK) and microtubule-associated regulatory kinase (MARK).[3] In particular, GSK3β plays a key role in the pathogenesis of both sporadic and familial

forms of AD and is also related to the formation of amyloid plaques.[4,5,6,7] GSK3 is a component of Wnt/β-catenin signaling pathway, which controls a wide range of biological processes in embryonic development and in the adult tissues.[8] Wnts are an evolutionary conserved family of growth factors and so far, 19 Wnt isoforms have been identified in humans. These secreted glycoproteins activate the pathway by binding to the receptor Frizzled (Fz) and the low-density lipoprotein receptor-related protein 5/6 co-receptor (LRP5/6).[9] Upon activation, GSK3β is repressed,[10] resulting in the accumulation of β-catenin, which translocates into the nucleus and activates transcription of Wnt target genes [11 12] Wnt signaling can be inhibited by several antagonists including the secreted frizzled-related protein (sFRP), Wnt Inhibitory Factor 1 (Wif1), Xenopus Cerberus, Wise and the Dickkopf (Dkk) family of secreted proteins.[13] The Dkk family of secreted proteins includes Dkk-1-4, which bind to LRP5/6, preventing it from interacting with Wnt-Frizzled complexes.[14]

In previous studies, it is shown that small molecule inhibitors of DKK1/LRP6 interactions affect the Wnt signaling and decrease Tau hyperphosporylation.[15, 16] The first identified inhibitor was NCI8642 (gallocyanine).[17, 18] It weakly inhibits the binding of DKK1 to LRP6 with an IC_{50} of about 3 μM, but it is effective *in vivo* as it reduces basal blood-glucose concentrations and improves glucose tolerance in mice.[18] Our group has already reported on the synthesis and the biological evaluation of a series of NCI8642 derivatives as inhibitors of DKK1/LRP6 interactions. In the beginning, our SAR studies focused on the alkyl ester groups on C1 and amino-substituents on C2 of NCI8642, and identified substituents that had only a moderate effect on potency. Starting from these results and aiming both to optimize the activity of NCI8642, and to study the structure–activity relationships, we designed new gallocyanine derivatives. In particular, new compounds were synthesized and evaluated for their activity to inhibit DKK1/LRP6 interactions in order to investigate: (i) the influence of ester substituents on C1 (ii) the effect of the replacement of hydroxyl group with alkoxy or benzyloxy substituents on C4 and (iii) the activity of alkylated derivatives that bear an amine on C2 of NCI8642. Molecular docking studies were also performed to elucidate the binding mode for these compounds.



NCI8642 (1)

Figure 1. Chemical structure of NCI8642

2. Results and Discussion

2.1. Synthesis of NCI8642 derivatives

Guided by docking studies we prepared a series of phenoxazinones and evaluated their activity in modulating Wnt signaling. Phenoxazinones **4a-e** were synthesized by a similar reaction to that reported previously, more specifically by the reaction of *N*,*N*-dimethyl-4-nitrosoaniline hydrochloride salt **2** and gallamide derivatives **3**, in refluxing ethanol (Figure 2).[15,16] In order to improve their solubility in water, several ammonium salts were prepared from the corresponding amines after treatment with HCl in iPrOH.

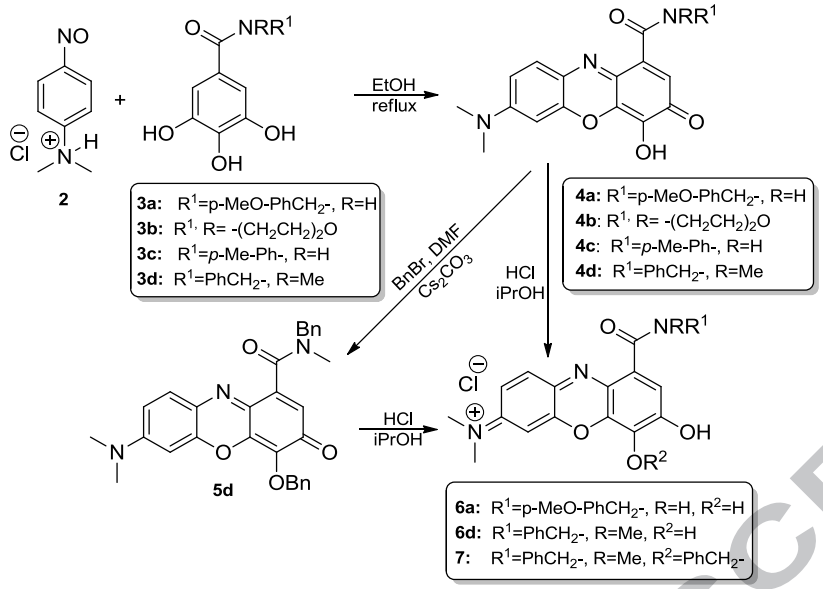


Figure 2. Synthesis of amide derivatives.

Bis-benzyl and bis-allyl phenoxazinone derivatives **8a-g** were obtained by reacting gallocyanine **1** with the corresponding benzyl bromides or allyl bromide using Cs_2CO_3 in DMF (Figure 3). Different substitution on gallocyanine scaffold was achieved by the alkylation reaction of gallocyanine esters **8** and **10** under similar conditions. In accordance, amino derivatives **14** were synthesized by alkylation of the previously reported *p*-MeO-benzylamino gallocyanine derivative **13**.[15]

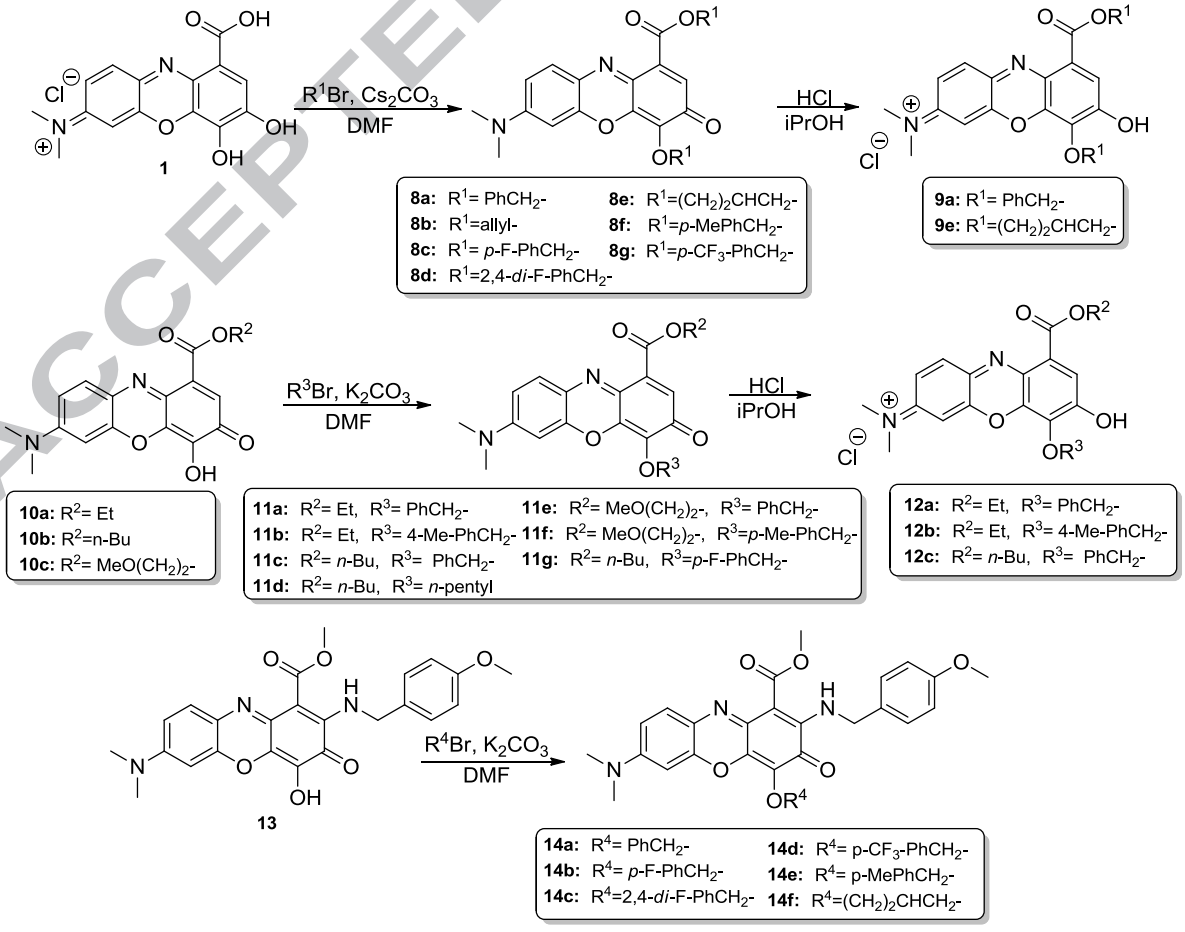


Figure 3. Synthesis of gallocyanine derivatives

2.2. Molecular docking studies

Our results from previously docking studies of ester and amino gallocyanine derivatives[15,16] have revealed important structural features for the binding of phenoxazinones on LRP6 protein. Herein, a thorough analysis of closely related compounds arising from the modification of the substitution on positions 1, 2 and 4 will be presented. All novel compounds were docked against the heterotetrameric LRP6 structure in the absence of DKK1c module (PDB entry: 3S8V).[19] The studies were focused on the LRP6-E3E4/DKK1 interface, consisting of the residues Ser851, Asp874 in interface A and Trp850, Asp874, Tyr875 in interface B, as described before. [15,16]

Based on their structure, the synthesized derivatives can be categorized in three major groups: the first comprises the amides **4**; the second group consists of alkylated ester or amide derivatives (**5, 11**); and the third refers to alkylated amino compounds **14**. Our previous docking studies revealed that NCI8642 with flexible alkyl substituents on C1, binds to the same cavity on LRP6 interface as the parent compound NCI8642, enclosed by residues Trp850B, Ser851A, Asp874A, Asp874B and Tyr875B. The LRP6/DKK1 interface is characterized by a hydrophobic surface on LRP6, which interacts with DKK1 residues 226–231 (KGSHGL).[19] Thus, several hydrophobic and/or aromatic substituents were added on gallocyanine scaffold in order to increase the hydrophobic and/or π stacking interactions. The docking results show that ester derivatives with flexible alkyl chains on position 1 engage the typical binding site of DKK1, providing potent inhibitors. This is demonstrated by the measured activities and the predicted binding mode of the most active compounds of this screen, i.e. **8e** and **11b** (Figures 4A, 4B). Several HBs are formed in each case but **11b** seems to form an important cation-π interaction[20, 21] with residue Arg853A inside a rich Arg region confined by residues Arg638B, Arg639B, Arg853A, Asp874B and Tyr875B. On the other hand, when benzyl substituents are incorporated into C1, the molecules appear to move down to the cleft and away from the initial binding cavity of NCI8642. Some derivatives like **8f** lose activity, while others like bis-benzyl derivatives **9a**, **8c**, **8d** were potent inhibitors. The third group of this study (**14**) while exhibited the highest scoring functions (See Supporting Information Table S1) failed to reside inside the cavity, indicating that only the benzylamine on C2 can be tolerated on the scaffold of NCI8642 and the introduction of bulky substituents on **13** decreased activity. As shown in Figure 4C, compound **14f** is also oriented towards the rich Arg region and interacts with Tyr875B and Ser851A.

2.3 Inhibition of DKK1 binding to LRP6 by gallocyanine derivatives

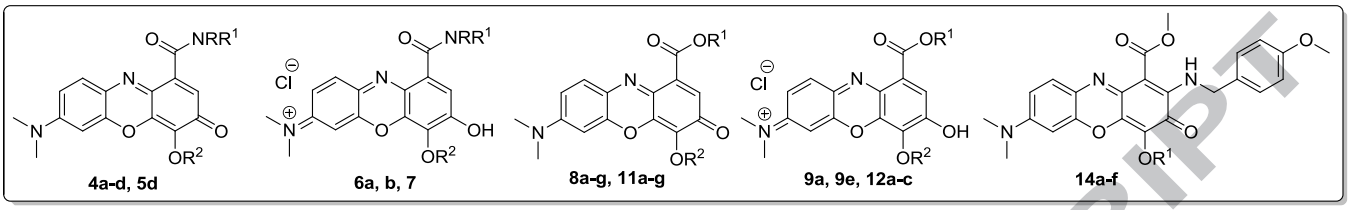
Initial screening of the activities of the synthesized compounds, to inhibit the binding of DKK1 to LRP6, was assessed as described previously.[17] HEK293 cells overexpressing LRP6 were incubated with DKK1 conditioned medium (DKK1-CM) plus DMSO, NCI8642 or NCI8642 derivatives at a concentration of 100 μM. Cell surface binding of DKK1 was visualized by immunocytochemistry, using a primary anti-DKK1 antibody and fluorescent secondary antibody. Fluorescence images were analyzed using Zen software and quantified results are presented in Table 1. When NCI8642 was incubated together with DKK1 at a concentration of 100 μM, a 47.8±2.0% binding of DKK1 was observed. Further detailed testing on its half inhibitory concentration (IC_{50}) showed that compound NCI8642 inhibits the binding of DKK1 to LRP6 with an IC_{50} value of 6.38 μM.[15] Ethyl gallate did not alter the binding of DKK1 and was used as negative control.

In this study, our experiments showed that changing the carboxylic group of NCI8642 to an amide led to a decrease in affinity. Gallamide derivatives **4** and **5** were found less active than the parent NCI8642, but also displayed poor

solubility in water. In an attempt to improve solubility the hydrochloride salt of **4a** was prepared and tested. Indeed, the inhibitory activity was significantly enhanced and **6a** exhibited 27.4±1.5% DKK1 binding. Overall, enhanced activities were recorded for compounds **8** and **11**, with a range of substituents to be tolerated on the ester group. Bis-benzyloxy derivative (**8a**) was equipotent to NCI8642, whereas the bis-allyl gallocyanine (**8b**) displayed decreased activity. Concerning the benzyl substitution, a slightly higher inhibitory activity was observed when a fluorine group was introduced in para position (**8c)** and even higher when two fluorine groups were introduced in ortho and meta positions of the benzyl group (**8d**). In para position of the benzyl group, CH_3 and CF_3 groups seem to be deleterious (i.e. **8a** vs **8f** vs **8g**), while very good activity was observed with the cyclopropylmethyl group (**8e**). As indicated in Table 1, alkylation of compound **13** lowers its activity, except for the case of the cyclopropylmethyl group (**14f**) which slightly improves potency with 31.4±1.3% binding of DKK1.

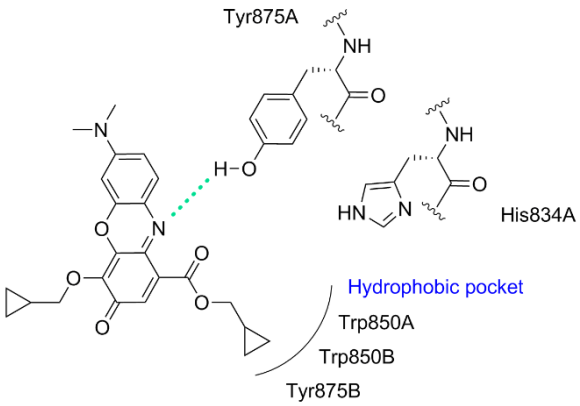
For the synthesized compounds several PK properties were calculated and are summarized in Table S2 (See Supporting Information). A few Lipinski's rule of five violations (MW, clogP and/or HBDs) are witnessed for some of the compounds. The polar surface area varied from approximately 75 to 105, HBD from 0 to 3, HBA from 7 to 9 and the flatness index (Fsp3 values) are ranging from 0.14 to 0.43. A correlation between the Fsp3 value and the inhibitory effect of compounds is observed with a minimum threshold of 0.24. Hence, flexible aliphatic substituents are of great importance. Moreover, lipophilicity values close to 4±0.5 seem to be optimum.

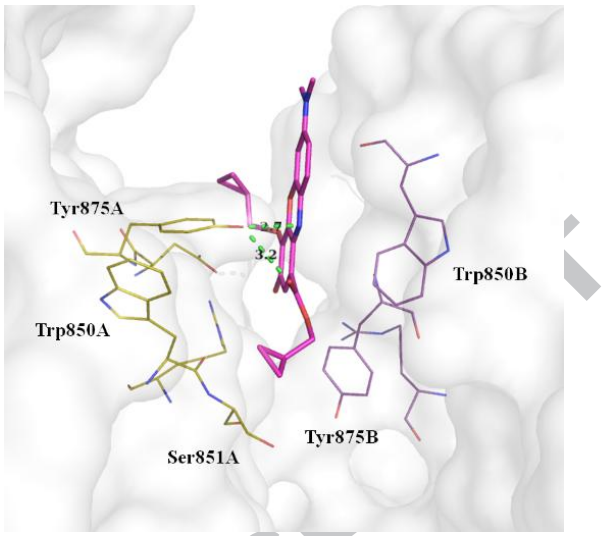
Table 1. DKK1 binding % to the surface of HEK-293 cells overexpressing LRP6 (HEK-293LRP6). The results are expressed as percentage of DKK1 binding (DKK1 binding %). Each experiment was repeated three times in duplicates and 750 cells were analyzed in each case. The differences are statistically significant (p value<0.001). If not, they are indicated as (+).



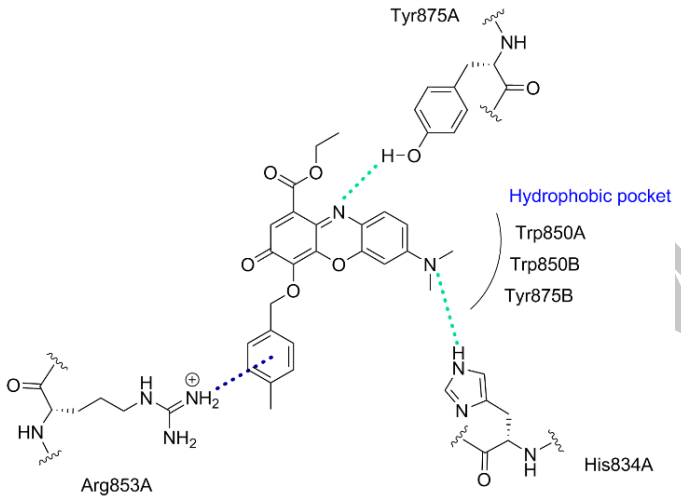
Entry	Substituents		DKK1 binding %	Entry	Substituents		DKK1 binding %
NCI8642	R^1= OH	R^2= H	47.8±2.0	**13**	-	-	32.1±1.1
Ethyl gallate	-	-	97.2±2.1$^{(+)}$	**8g**	R^1=*p*-CF_3-$PhCH_2$-	R^2= *p*-CF_3-$PhCH_2$-	65.1±2.4
4a	R^1=*p*-MeO-$PhCH_2$-, R=H	R^2= H	81.1±2.7	**11a**	R^1= Et	R^2= $PhCH_2$-	25.7±1.8
6a	R^1=*p*-MeO-$PhCH_2$-, R=H	R^2= H	27.4±1.5	**12a**	R^1= Et	R^2= $PhCH_2$-	32.7±1.1
4b	R^1, R= -$(CH_2CH_2)_2O$	R^2= H	45.4±2.0	**11b**	R^1= Et	R^2= 4-Me-$PhCH_2$-	16.5±1.0
4c	R^1=*p*-Me-Ph-, R=H	R^2= H	50.2±2.9	**11c**	R^1= *n*-Bu-	R^2= $PhCH_2$-	22.3±1.5
4d	R^1=$PhCH_2$-, R=Me	R^2= H	61.3±3.0	**12c**	R^1= *n*-Bu-	R^2= $PhCH_2$-	35.5±1.6
6d	R^1= $PhCH_2$-, R=Me	R^2= H	81.3±3.4	**11d**	R^1= *n*-Bu-	R^2= *n*-pentyl-	45.2±2.2
5d	R^1= $PhCH_2$-, R=Me	R^2= $PhCH_2$-	64.3±2.6	**11e**	R^1= $MeO(CH_2)_2$-	R^2= $PhCH_2$-	26.4±1.3
7	R^1= $PhCH_2$-, R=Me	R^2= $PhCH_2$-	67.6±2.4	**11f**	R^1= $MeO(CH_2)_2$-	R^2=*p*-Me-$PhCH_2$-	31.2±1.4
8a	R^1= $PhCH_2$-	R^2= $PhCH_2$-	47.4±1.0	**11g**	R^1= *n*-Bu-	R^2=*p*-F-$PhCH_2$-	46.8±1.7
9a	R^1= $PhCH_2$-	R^2= $PhCH_2$-	26.1±1.0	**14a**	R^1= $PhCH_2$-	-	78.1±3.6
8b	R^1=allyl-	R^2=allyl	71.9±4.0	**14b**	R^1= *p*-F-$PhCH_2$-	-	74.3±3.0
8c	R^1= *p*-F-$PhCH_2$-	R^2= *p*-F-$PhCH_2$-	35.9±2.5	**14c**	R^1=2,4-*di*-F-$PhCH_2$-	-	66.8±2.6
8d	R^1=2,4-*di*-F-$PhCH_2$-	R^2=2,3-*di*-F-$PhCH_2$-	21.3±1.4	**14d**	R^1= *p*-CF_3-$PhCH_2$-	-	68.6±1.5
8e	R^1=$(CH_2)_2CHCH_2$-	R^2=$(CH_2)_2CHCH_2$-	20.7±1.3	**14e**	R^1= *p*-Me$PhCH_2$-	-	65.5±2.3
8f	R^1=*p*-Me$PhCH_2$-	R^2= *p*-Me$PhCH_2$-	54.3±1.1	**14f**	R^1=$(CH_2)_2CHCH_2$-	-	31.4±1.3

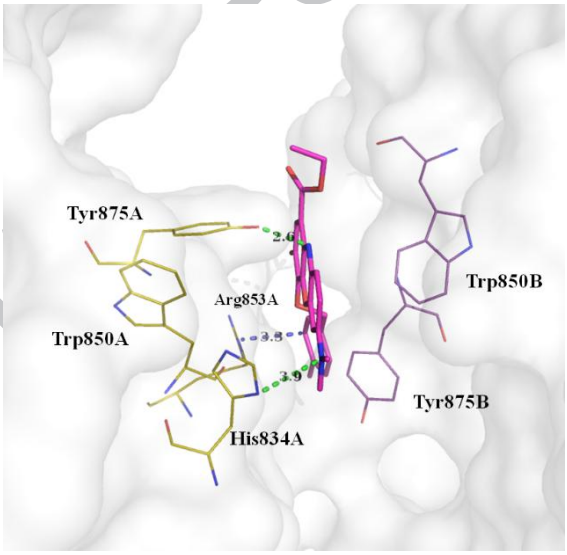
A)



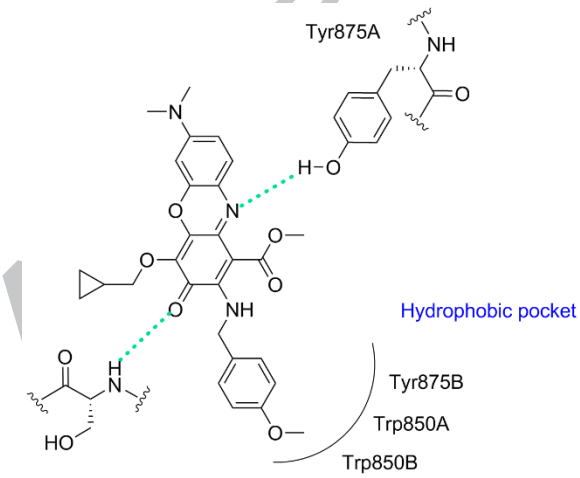


B)





C)



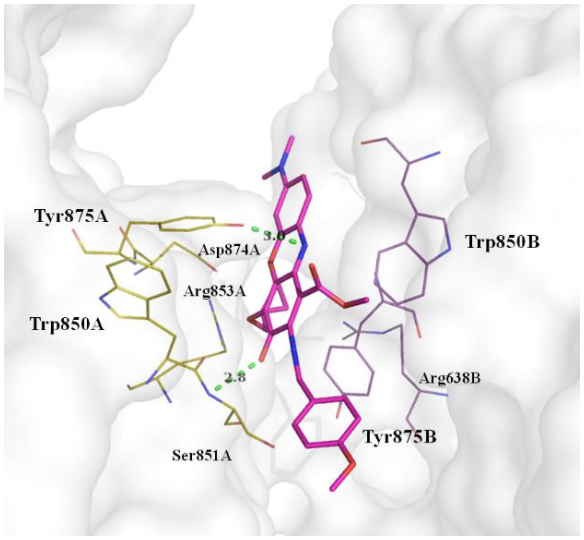


Figure 4. Schematic representation of the hydrogen bonding network with green dashed lines between compounds **8e** (A), **11b** (B), **14f** (C) and the neighboring residues (left), and the predicted docking conformations of **8e** (A), **11b** (B), **14f** (C in the X-ray structure of LRP6 (right). The cavity surface is depicted as the gray mesh. Drawings were prepared by PYMOL version 1.4.1.19.[22]

2.4. Cytotoxicity of the synthesized compounds

The possible cytotoxic effects of the synthesized compounds were investigated using the colorimetric MTT assay. The compounds exhibited no detectable toxic effects on neuronal cells after 24 h of cell culture at concentrations up to 100 μM (See Supporting Information Figure S1 and S2).[23]

2.5. Inhibition of Tau phosphorylation

Previous studies have demonstrated that prostaglandins like PGJ2 and PGE2 inactivate GSK3 by phosphorylation.[24,25] In a recent work, our group has showed that prostaglandin J2 (PGJ2) induces Tau phosphorylation in mouse cortex primary neurons. Thus, treatment of primary neurons with 10 or 20 μM PGJ2 resulted in a significant increase in Tau phosphorylation at serine 396 and Tau hyperphosphorylation at other residues including serine 404, serine 199 and threonine 205. In this study, selected NCI8642 derivatives were examined for their effect on inhibition of PGJ2-induced Tau phosphorylation at serine 396. Primary cortical neurons were incubated with 20 μM PGJ2 plus DMSO, NCI8642 (10 μM) or NCI8642 derivatives (10 μM) for 1 hour at 37 °C and the phosphorylation of Tau was assessed by western blot using a pSer396 antibody. Quantification of the results normalized for actin is shown in Figure 5 (Figure S3). Compound **11d** exhibited 50.12±5.4% of Tau phosphorylation and was equipotent to NCI8642 (55±2%). The other compounds were better inhibitors, with **11g** being the most active demonstrating approximately 21.4±3.86% of Tau phosphorylation at concentration of 10 μM.

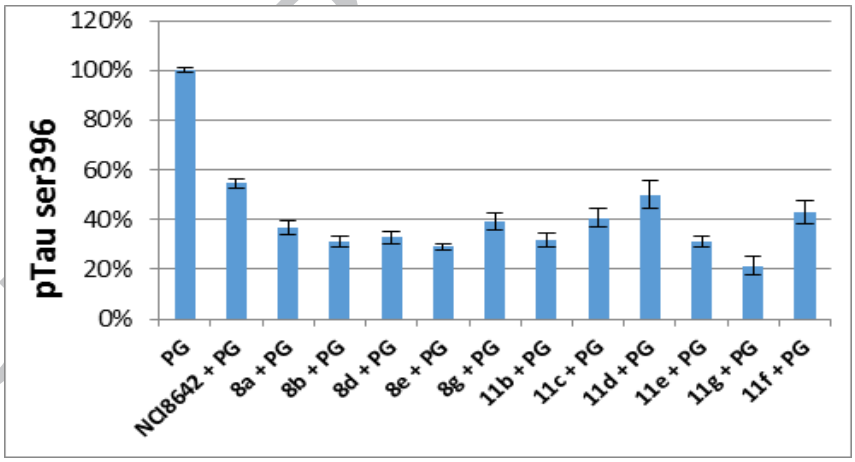


Figure 5. Tau phosphorylation upon treatment of primary mouse cortical neurons with 20 μM PGJ2 plus DMSO, 10 μM NCI8642 or 10 μM NCI8642 derivatives are shown, as a percentage of Tau phosphorylation upon treatment only with PGJ2. The levels of phosphorylation upon combined treatment with PGJ2 plus the various NCI8642 derivatives are depicted as percent of tau phosphorylation upon treatment only with PGJ2. The experiments were repeated at least three times. The differences are statistically significant (p value <0.01)

Dkk1 has been demonstrated to induce hyperphosphorylation of Tau protein,[26,27] thus the inhibition of DKK1-induced Tau phosphorylation at serine 396 by gallocyanine derivatives was also examined. SH-SY5Y cells were treated with DKK1-CM plus NCI8642 or **9e**, **12b** at three different concentrations. Cell lysates were analyzed by western blot using antibodies against Tau phosphorylated at serine 396 or actin. Western blot analysis data (Figure 6)

indicated that NCI8642, **9e** and **12b** dose dependently caused a decrease in the levels of phosphorylated Tau in comparison to cells treated with DKK1 alone. The most potent compound in this assay was **12b**.

2.6. Activation of WNT-β-catenin signaling

Since gallocyanine inhibited the interactions of DKK1 to LRP6, the activation of Wnt signaling was studied by examination of the levels of GSK3β and β-catenin (Figure 6, Figure S4). Upon binding of Wnt to Fz and LRP5/6, GSK3β is inactivated by phosphorylation.[10] This prevents GSK3β from phosphorylating β-catenin and induces its degradation. Thus, β-catenin is accumulated in the cytoplasm and is available to enter the nucleus and interact with TCF/LEF transcription factors.

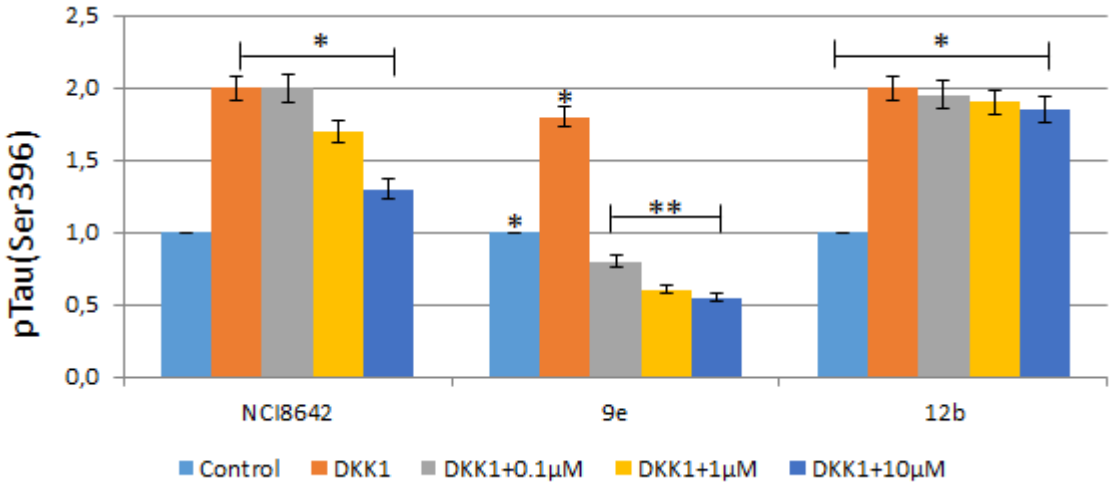


Figure 6. NCI8642 and NCI8642 derivatives decrease DKK1-induced Tau phosphorylation at serine 396. SH-SY5Y cells were treated with DKK1-CM plus DMSO or DKK1-CM plus NCI8642 or NCI8642 derivatives at 10 µM concentration. Cell lysates were analyzed by western blot using antibodies against Tau phosphorylated at serine 396 or actin. The experiments were repeated at least three times. The differences are statistically significant (*p value <0.01, **p value < 0.1).

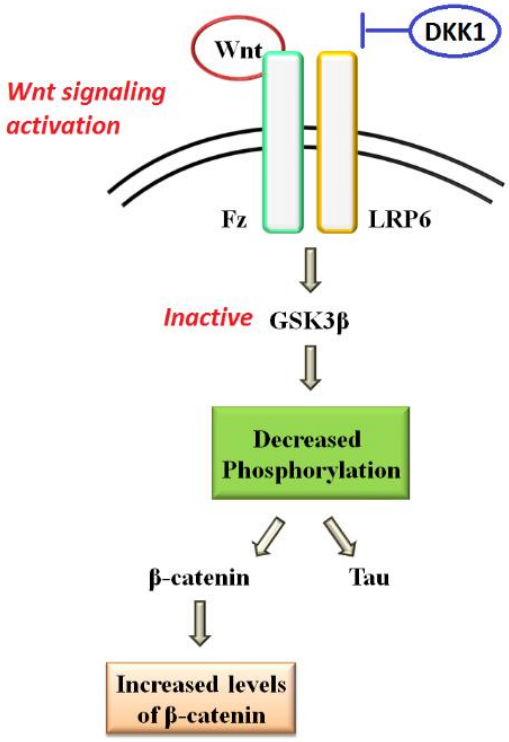


Figure 7. Simplified representation of Wnt signalling pathway

As shown in Figures 8 and 9, NCI8642 and **9e** caused dose dependently an increase in the levels of *p*GSK3β and β-catenin, while a minor increasing effect was observed in the case of **12b** (Figure S5, S6). SHSY5Y cells were incubated for 1 h at 37 °C with DKK1-CM plus DMSO, DKK1-CM plus NCI8642 or DKK1-CM plus **9e** or **12b** at different concentrations. Protein extracts were analyzed by western blot using an antibody against *p*GSK3b and against β-catenin.

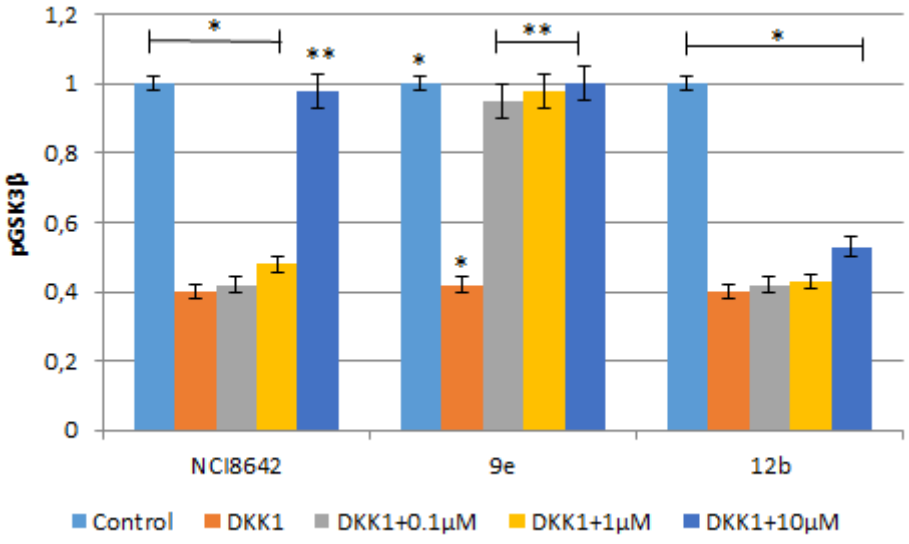


Figure 8. NCI8642, **9e** and **12b** increase the levels of *p*GSK3β. SHSY5Y cells were incubated for 1 h at 37 °C with DKK1-CM plus DMSO, DKK1-CM plus NCI8642 or DKK1-CM plus NCI8642 derivatives at a concentration of 0 μM, 0.1 μM, 1 μM and 10 μM. Protein extracts were analyzed by western blot using an antibody against *p*GSK3β. The experiments were repeated at least three times. The differences are statistically significant (*p value <0.01, **p value < 0.1).

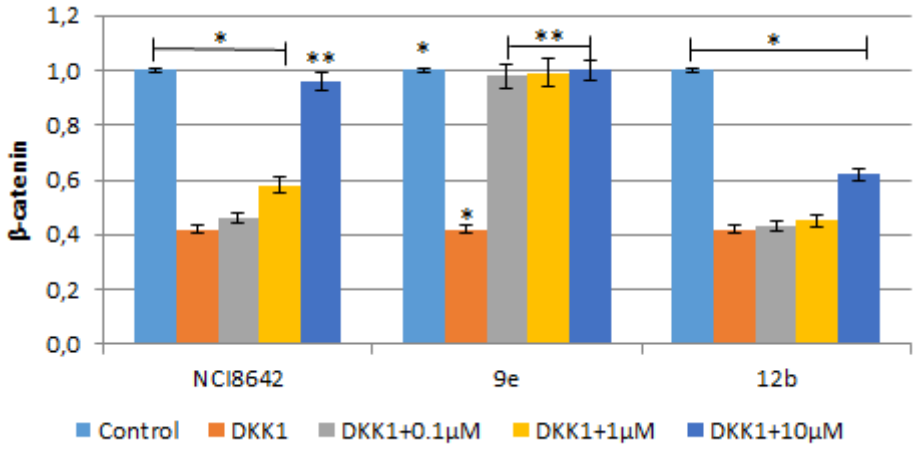


Figure 9. NCI8642, **9e** and **12b** increase the levels of β-catenin. SHSY5Y cells were incubated for 1 h at 37 °C with DKK1-CM plus DMSO, DKK1-CM plus NCI8642 or DKK1-CM plus NCI8642 derivatives at a concentration of 0 µM, 0.1 µM, 1 µM, 10 µM. Protein extracts were analyzed by western blot using an antibody against β-catenin. The experiments were repeated at least three times. The differences are statistically significant (*p value <0.01, **p value < 0.1).

3. Conclusions

NCI8642 derivatives with different substituents on C1, C2 and C4 of the phenoxazinone scaffold were prepared and evaluated for their ability to inhibit DKK1/LRP6 interactions and Tau phosphorylation induced by prostaglandin J2 or DKK1. In this effort molecular docking experiments have supported the experimental data and provided useful insight for the analysis of the binding of NCI8642 derivatives on LRP6. Alkylated gallocyanines with flexible alkyl ester groups on C1 provided the most active inhibitors, while amino derivatives on C2 of NCI8642 that have alkoxy of benzyloxy substituents on C4 were less active. Additionally, it is shown that treatment of SHSY5Y cells with DKK1-CM and the salts **9e** or **12b** affect Wnt signaling and increase the levels of *p*GSK3β kinase and β-catenin. The synthesized compounds were not cytotoxic up to a concentration of 100 µM. Therefore, modulators of Wnt signaling hold promise for the treatment of Alzheimer's disease and other neurodegenerative diseases.

4. Experimental Section

General Experimental Details. All reactions were carried out under an atmosphere of Ar unless otherwise specified. Commercial reagents of high purity were purchased and used without further purification, unless otherwise noted. Reactions were monitored by TLC and using UV light as a visualizing agent and aqueous ceric sulfate/phosphomolybdic acid, ethanolic *p*-anisaldehyde solution, potassium permanganate solution, and heat as staining agents. The ^{1}H and ^{13}C NMR spectra were recorded at 500, 300 and 125, 75 MHz, and tetramethylsilane was used as an internal standard. Chemical shifts are indicated in δ values (ppm) from internal reference peaks (TMS ^{1}H 0.00; $CDCl_3$ ^{1}H 7.26, ^{13}C 77.00; DMSO-d_6 ^{1}H 2.50, ^{13}C 39.51, pyridine-d_5 ^{1}H 8.74, 7.58, 7.22; ^{13}C 150.35, 135.91,

123.87). Melting points (mp) are uncorrected. High-resolution mass spectra (HRMS) were recorded by direct injection of a 2 μM (2 μL) solution of the compounds in water–acetonitrile (1/1; v/v) and 0.1% formic acid on a mass spectrometer (hybrid ion trap-orbitrap mass spectrometer) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 °C) with resolution R = 60.000 at m/z = 400 (mass range = 150–2000) and dioctylphthalate (m/z = 391.28428) as the "lock mass".

The polyclonal/monoclonal Rabbit Anti-Mouse antibody kindly provided from Dr. Luc Buee (Insern, Fac de Medicine, Univ. Lille 2, Lille France) directed against the N-terminus of tau and the polyclonal Rabbit Anti-Mouse antibody detecting tau phosphorylated at serine396 were purchased from Acris Antibodies GmbH (Germany). Goat Anti-Rabbit IgG-HRP secondary antibody was purchased from Jackson ImmunoResearch (Baltimore Pike, USA). The monoclonal Mouse antibody used to detect actin by immunoblot was purchased from Merk Millipore (Darmstatd, Germany). Goat Anti-Mouse IgG-HRP secondary antibody was purchased from Santa Cruz Biotechnology (Dallas/Texas, USA). The polyclonal Rabbit Anti-Human antibody against DKK1 was obtained from Santa Cruz Biotechnology (Dallas/Texas, USA). Goat anti-Rabbit IgG (H+L) Alexa Fluor 488-conjugated and/or Goat anti-Rabbit IgG (H+L) Cy 3-conjugated secondary was purchased from Jackson ImmunoResearch (Baltimore Pike, USA). B27 and Neurobasal used to culture mouse primary neuronal culture were purchased from Life Technologies (Grand Island/NY, USA). Rabbit anti-Human, Mouse, Rat, Monkey (Zebrafish, Bovine) p-GSK3-beta (S9) was purchased from Cell Signaling Technology. Prostaglandin J2 was purchased from Santa Cruz Biotechnology (Dallas/Texas, USA). Common chemicals were from Sigma Aldrich (Athens, Greece).

3,4,5-*Tris*((*tert*-butyldimethylsilyl)oxy)-*N*-(4-methoxybenzyl)benzamide, (S1): To a solution of 3,4,5-*tris*((*tert*-butyldimethylsilyl)oxy) benzoic acid[28] (250 mg, 0.488 mmol) in 3 mL of dry DCM were added 76.5 μL of *p*-methoxy-benzylamine (0.586 mmol), 151 mg DCC (0.732 mmol) and 9.5 mg of DMAP (0.078 mmol) at 0 °C. The reaction mixture was warmed to rt and stirred overnight. Then, the reaction mixture was filtrated to remove impurities and was concentrated. The mixture was extracted with CH_2Cl_2, and the organic layers were dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 8/1) to afford 208 mg of **S1** in 68% yield. **S1**: mp=155-158 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27 (2H overlapping with $CDCl_3$), 6.92 (s, 2H), 6.88 (d, *J* = 8.7 Hz, 2H), 4.54 (d, *J* = 5.6 Hz, 2H), 3.80 (s, 3H), 0.98 (s, 9H), 0.93 (s, 18H), 0.21 (s, 15H), 0.11 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.9, 159.0, 148.6, 141.8, 130.5, 129.0, 126.6, 114.1, 113.2, 55.3, 43.5, 26.2, 26.1, 18.7, 18.5, -3.7, -3.9; FT-IR (KBr): 3309, 2955, 2930, 2894, 2858, 1630, 1569, 1546, 1512, 1485, 1418, 1361, 1343, 1253, 1080, 889 cm^{-1}; HRMS m/z for $C_{33}H_{58}NO_5Si_3$ $[M + H]^+$ calcd 632.3623, found 632.3625.

3,4,5-Trihydroxy-*N*-(4-methoxybenzyl)benzamide, (3a): To a solution of 3,4,5-*tris*((*tert*-butyldimethylsilyl)oxy)-*N*-(4-methoxybenzyl)benzamide (70 mg, 0.111 mmol) in 1.8 mL of THF and 0.5 mL pyridine at 0 °C were added dropwise 0.7 mL of HF in pyridine solution (70% pyridine). The reaction mixture was stirred at rt overnight and then was quenched with sat. $NaHCO_3$. The mixture was extracted with ethyl acetate (5 × 10 mL) and the combined organic extracts were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (ethyl acetate) to afford 66 mg of **3a** (85% yield) as a white solid. **3a:** mp=97-100°C; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 8.95 (br, 3H), 8.54 (t, *J* = 5.9 Hz, 1H), 7.20 (d, *J* = 8.5 Hz, 2H), 6.87 (d, *J* = 8.5 Hz, 2H), 6.84 (s, 2H), 4.31 (d, *J* = 6.0 Hz, 2H), 3.72 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 166.2, 158.0, 145.4, 136.2, 132.1,

128.4, 124.8, 113.5, 106.7, 55.0, 41.9; FT-IR (KBr): 3420, 2927, 2846, 1615, 1508, 1457, 1340, 1247, 1177, 1033 cm^{-1}; HRMS m/z for $C_{15}H_{16}NO_5$ $[M + H]^+$ calcd 290.1028, found 290.1027.

7-(Dimethylamino)-4-hydroxy-*N*-(4-methoxybenzyl)-3-oxo-3*H*-phenoxazine-1-carboxamide, (4a): 3,4,5-trihydroxy-*N*-(4-methoxybenzyl)benzamide (70 mg, 0.242 mmol) and freshly prepared *N*,*N*-dimethyl-4-nitrosoaniline hydrochloride (54 mg, 0.29 mmol) were added in 0.8 mL of ethanol. The reaction mixture was refluxed for 1 hour, while the solution was turning into purple color. Then, the reaction mixture was cooled, the crystals were collected by suction filtration on a filter paper and were washed with chilled EtOH. The solid was dried under reduced pressure to give 63 mg of **4a** as dark purple solid (62% yield). **4a**: mp=255-258 °C; ^{1}H NMR (500 MHz, pyridine-d_5) δ 10.25 (d, *J* = 5.1 Hz, 1H), 8.21 (s, 1H), 7.49 (d, *J* = 9.0 Hz, 1H), 7.10 (d, *J* = 8.6 Hz, 2H), 6.62 (d, *J* = 9.1 Hz, 1H), 6.53 (d, *J* = 1.8 Hz, 1H), 4.90 (d, *J* = 5.5 Hz, 2H), 3.72 (s, 3H), 2.90 (s, 6H), 2H are missing due to overlapping with pyridine-*d*5; ^{13}C NMR (126 MHz, pyridine-d_5) δ 179.5, 164.1, 159.8, 154.6, 147.5, 141.2, 137.7, 132.9, 132.8, 132.5, 131.9, 131.7, 129.7, 125.4, 114.8, 111.0, 97.3, 55.6, 43.9, 40.2; FT-IR (KBr): 3300, 2926, 1655, 1591, 1545, 1513, 1481, 1460, 1426, 1382, 1302, 1278, 1261, 1240, 1197, 1144, 1023 cm^{1-}; HRMS m/z for $C_{23}H_{22}N_3O_5$ $[M + H]^+$ calcd 420.1559, found 420.1560.

7-(Dimethylamino)-4-hydroxy-*N*-(4-methoxybenzyl)-3-oxo-3*H*-phenoxazine-1-carboxamide hydrochloride, (6a): 36 µL of HCl in iPrOH (5N) solution were added dropwise in a suspension of **4a** (15 mg, 0.036 mmol) in 0.7 mL of ethanol. The mixture was stirred at room temperature for 30 min. Then, the mixture was concentrated to furnish **6a** (quantitative yield) as a dark blue-green solid. **6a**: mp >300 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.53 (t, *J* = 5.2 Hz, 1H), 7.64 (d, *J* = 9.2 Hz, 1H), 7.36 (d, *J* = 8.6 Hz, 2H), 7.27 (s, 1H), 7.13 (d, *J* = 8.9 Hz, 1H), 6.93 (d, *J* = 8.6 Hz, 2H), 6.80 (s, 1H), 4.50 (d, *J* = 5.2 Hz, 2H), 3.75 (s, 3H), 3.26 (s, 6H); FT-IR (KBr): 3446, 2933, 2849, 1654, 1591, 1541, 1513, 1456, 1383, 1300, 1279 cm^{1-}.

Morpholino(3,4,5-*tris*((*tert*-butyldimethylsilyl)oxy)phenyl)methanone, (S2): To a solution of 3,4,5-tris((tert-butyldimethylsilyl)oxy)benzoic acid (100 mg, 0.195 mmol) in 1.2 mL of dry DCM were added 34 mg of morpholine (0.39 mmol), 61 mg DCC (0.293 mmol) and 3.8 mg of DMAP (0.031 mmol) at 0 °C. The reaction mixture was warmed to rt and stirred overnight. Then, the reaction mixture was filtrated to remove impurities and was concentrated. The mixture was extracted with CH_2Cl_2, and the organic layers were dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 40/1) to afford 89 mg of **S2** in 78% yield. **S2**: mp=125-128 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.55 (s, 2H), 3.63 (br, 8H), 0.99 (s, 9H), 0.93 (s, 18H), 0.21 (s, 6H), 0.12 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.3, 148.7, 140.1, 127.0, 113.3, 66.9, 26.2, 26.1, 18.8, 18.4, -3.6, -3.9; FT-IR (KBr): 2957, 2930, 2894, 2857, 1641, 1602, 1575, 1497, 1473, 1457, 1421, 1343, 1252, 1230, 1115, 1093, 904, 889, 830 cm^{-1}; HRMS m/z for $C_{29}H_{56}NO_5Si_3$ $[M + H]^+$ calcd 582.3466, found 582.3466.

Morpholino(3,4,5-trihydroxyphenyl)methanone, (S3): To a solution of morpholino(3,4,5-tris((*tert*-butyldimethylsilyl)oxy)phenyl)methanone (188 mg, 0.324 mmol) in 5.4 mL of THF and 1.3 mL pyridine at 0 °C were added dropwise 2 mL of HF in pyridine solution (70% pyridine). The reaction mixture was stirred at rt overnight and then was quenched with sat. $NaHCO_3$. The mixture was extracted with ethyl acetate (5 × 10 mL) and the combined organic extracts were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 1/2) to afford 66 mg of **S3** (85% yield) as a white solid. **S3**:

mp=247-250 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.00 (s, 2H), 8.39 (s, 1H), 6.33 (s, 2H), 3.57 (m, 4H), 3.47 (m, 4H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.39, 145.56, 134.63, 125.19, 106.49, 66.09; FT-IR (KBr): 3328, 2971, 2926, 2852, 2676, 1617, 1570, 1476, 1459, 1448, 1430, 1377, 1345, 1311,1253, 1221, 1107, 1065, 1031, 970 cm^{-1}; HRMS m/z for $C_{11}H_{14}NO_5$ $[M + H]^+$ calcd 240.0872, found 240.0875.

7-(Dimethylamino)-4-hydroxy-1-(morpholine-4-carbonyl)-3*H*-phenoxazin-3-one, (4b): Compound **S3** (40 mg, 0.167 mmol) and freshly prepared *N*,*N*-dimethyl-4-nitrosoaniline hydrochloride (37 mg, 0.2 mmol) were added in 0.6 mL of ethanol. The reaction mixture was refluxed for 1 hour, while the solution was turning into purple color. Then, the reaction mixture was cooled, the crystals were collected by suction filtration on a filter paper and were washed with chilled EtOH. The solid was dried under reduced pressure to give 26 mg of **4b** as dark purple solid (42% yield). **4b:** mp> 300 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.58 (d, J = 9.3 Hz, 1H), 7.02 (d, J = 9.3 Hz, 1H), 6.78 (s, 1H), 6.76 (d, J = 2.4 Hz, 1H), 3.76 (s, 1H), 3.69 (s, 2H), 3.56 (d, J = 4.9 Hz, 1H), 3.52 – 3.42 (m, 2H), 3.23 (s, 6H), 3.20 (m, 2H); ^{13}C-NMR (500 MHz, pyridine-d_5, 40 °C) 179.7, 165.8, 154.9, 147.6, 141.1, 139.6, 136.5, 132.7, 132.3, 127.1, 126.7, 110.8, 97.4, 67.3, 67.1, 40.2; FT-IR (KBr): 3422, 2924, 2847, 1604,1558, 1488, 1381, 1317, 1270, 1201, 1139, 1106 cm^{1-}; HRMS m/z for $C_{11}H_{14}NO_5$ $[M + H]^+$ calcd 370.1403, found 370.1405.

3,4,5-Tris((*tert*-butyldimethylsilyl)oxy)-*N*-(*p*-tolyl)benzamide, (S4): To a solution of 3,4,5-tris((tert-butyldimethyl silyl)oxy)benzoic acid (300 mg, 0.586 mmol) in 3.5 mL of dry DCM were added 125 mg of *p*-toluidine (1.172 mmol), 181 mg DCC (0.879 mmol) and 12 mg of DMAP (0.094 mmol) at 0 °C. The reaction mixture was warmed to room temperature and stirred overnight. Then, the reaction mixture was filtrated to remove impurities and was concentrated. The mixture was extracted with DCM, and the organic layers were dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 35/1) to afford 256 mg of **S4** in 73% yield. **S4:** mp=139-141 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.48 (d, J = 8.3 Hz, 2H), 7.16 (d, J = 8.2 Hz, 2H), 7.01 (s, 2H), 2.34 (s, 3H), 1.01 (s, 9H), 0.97 (s, 18H), 0.25 (s, 12H), 0.16 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.2, 148.9, 142.2, 135.6, 133.9, 129.6, 127.2, 120.1, 113.2, 26.2, 26.2, 20.8, 18.8, 18.5, -3.6, -3.9. FT-IR (KBr): 3447, 2962, 2930, 2859, 1654, 1647, 1570, 1541, 1489, 1473, 1415, 1337, 1256, 1085 cm^{1-}; HRMS m/z for $C_{32}H_{55}NNaO_4Si_3$ $[M + Na]^+$ calcd 624.3337, found 624.3335.

3,4,5-Trihydroxy-*N*-(*p*-tolyl)benzamide, (S5): To a solution of 3,4,5-tris((tert-butyldimethylsilyl)oxy)-*N*-(*p*-tolyl)benzamide (355 mg, 0.591 mmol)) in 9.8 mL of THF and 2.5 mL pyridine at 0 °C were added dropwise 3.7 mL of HF in pyridine solution (70% pyridine). The reaction mixture was stirred at room temperature overnight and then was quenched with sat. $NaHCO_3$. The mixture was extracted with ethyl acetate (5 × 10 mL) and the combined organic extracts were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 1/1) to afford 120 mg of **S5** (78% yield) as a white solid. **S5**: mp= 209-212 °C, ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.75 (s, 1H), 7.61 (d, J = 8.4 Hz, 2H), 7.10 (d, J = 8.3 Hz, 2H), 6.94 (s, 2H), 2.26 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6)) δ 165.3, 145.4, 136.9, 136.5, 131.8, 128.7, 125.1, 120.1, 107.1, 20.3; HRMS m/z for $C_{14}H_{13}NNaO_4$ $[M + Na]^+$ calcd 282.0742, found 282.0741.

7-(Dimethylamino)-4-hydroxy-3-oxo-*N*-(p-tolyl)-3*H*-phenoxazine-1-carboxamide, (4c): 3,4,5-trihydroxy-*N*-(*p*-tolyl)benzamide (102 mg, 0.394 mmol) and freshly prepared *N*,*N*-dimethyl-4-nitrosoaniline hydrochloride (88 mg, 0.473 mmol) were added in 1.3 mL of ethanol. The reaction mixture was refluxed for 1 hour, while the solution was turning into purple color. Then, the reaction mixture was cooled, the crystals were collected by suction filtration on a

filter paper and were washed with chilled EtOH. The solid was dried under reduced pressure to give 78 mg of **4c** as dark purple solid (53% yield). **4c**: mp> 300 °C ^{1}H NMR (500 MHz, pyridine-d_5) δ 12.09 (s, 1H), 8.17 (s, 1H), 8.04 (d, *J* = 8.3 Hz, 2H), 7.61 (d, *J* = 9.0 Hz, 1H), 7.22 (d, *J* = 8.2 Hz, 2H), 6.68 (dd, *J* = 9.0, 2.2 Hz, 1H), 6.50 (d, *J* = 2.2 Hz, 1H), 2.87 (s, 6H), 2.22 (s, 3H); ^{13}C NMR (126 MHz, pyridine) δ 179.3, 161.9, 154.8, 147.6, 140.9, 137.9, 137.5, 134.1, 133.1, 133.0, 131.9, 131.8, 130.2, 125.1, 120.9, 111.2, 97.3, 40.3, 21.1; FT-IR (KBr): 3448, 2966, 2926, 1662, 1596, 1662, 1596, 1544, 1489, 1412, 1382, 1300, 1258,1198, 1135, 1026 cm^{1-}; HRMS m/z for $C_{22}H_{20}N_3O_4$ $[M + H]^+$ calcd 390.1454, found 390.1453.

***N*-benzyl-3,4,5-tris((*tert*-butyldimethylsilyl)oxy)-*N*-methyl benzamide, (S6)**: To a solution of 3,4,5-tris((*tert*-butyldimethylsilyl)oxy)benzoic acid (300 mg, 0.586 mmol) in 3.5 mL of dry DCM were added 151 µL of *N*-methyl-1-phenylmethanamine (1.171 mmol), 181 mg DCC (0.879 mmol) and 12 mg of DMAP (0.094 mmol) at 0 °C. The reaction mixture was warmed to rt and stirred overnight. Then, the reaction mixture was filtrated to remove the impurities and was concentrated. The mixture was extracted with CH_2Cl_2, and the organic layers were dried over Na_2SO_4. The solvent was evaporated, and the residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 15/1) to afford 184 mg of **S6** in 51% yield. **S6**: mp=102-104 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.50 – 7.03 (m, 5H), 6.63 (s, 2H), 4.61 (s, 2H), 2.94 (s, 3H), 0.95 (s, 9H), 0.91 (s, 18H), 0.13 (s, 12H), 0.13 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.6, 148.6, 140.0, 137.0, 128.8, 127.9, 127.5, 127.2, 113.3, 53.9, 26.2, 26.1, 18.7, 18.4, -3.7, -3.9; FT-IR (KBr): 2956, 2931, 2897, 2858, 1636, 1560, 1496, 1472, 1458, 1417, 1400, 1362, 1341, 1257, 1226, 1086 cm^{1-}; HRMS m/z for $C_{33}H_{58}NO_4Si_3$ $[M + H]^+$ calcd 616.3674, found 616.3678.

***N*-benzyl-3,4,5-trihydroxy-*N*-methylbenzamide, (S7)**: To a solution of *N*-benzyl-3,4,5-*tris*((*tert*-butyldimethylsilyl)oxy)-*N*-methylbenzamide (405 mg, 0.658 mmol) in 11 mL of THF and 2.8 mL pyridine at 0 °C were added dropwise 4.1 mL of HF in pyridine solution (70% pyridine). The reaction mixture was stirred at rt overnight and then was quenched with sat. $NaHCO_3$. The mixture was extracted with ethyl acetate (5 × 10 mL) and the combined organic extracts were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 1/1) to afford 160 mg of **S5** (86% yield) as a white solid. **S5:** mp=102-105 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.37 (m, 2H), 7.27 (m, 3H), 6.37 (s, 2H), 4.57 (s, 2H), 2.82 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.9, 145.7, 137.5, 134.6, 128.6, 127.1, 126.0, 106.3, 62.8, 28.9; FT-IR (KBr): 3382, 2954, 2922, 2851, 1594, 1576, 1489, 1451,1405, 1339, 1233, 1188, 1079, 1033, 957 cm^{1-}; HRMS m/z for $C_{15}H_{16}NO_4$ $[M + H]^+$ calcd 274.1079, found 278.1081.

***N*-benzyl-7-(dimethylamino)-4-hydroxy-*N*-methyl-3-oxo-3*H*-phenoxazine-1-carboxamide, (4d):** *N*-benzyl-3,4,5-trihydroxy-*N*-methylbenzamide (85 mg, 0.311 mmol) and freshly prepared *N*,*N*-dimethyl-4-nitrosoaniline hydrochloride (70 mg, 0.373 mmol) were added in 1 mL of ethanol. The reaction mixture was refluxed for 1 hour, while the solution was turning into purple color. Then, the reaction mixture was cooled, the crystals were collected by suction filtration on a filter paper and were washed with chilled EtOH. The solid was dried under reduced pressure to give 47 mg of **4d** as dark purple solid (38% yield). **4d** (rotamers): mp=183-186 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.58-7.49 (m, 2H), 7.44 (dd, *J* = 14.5, 8.1 Hz, 2H), 7.38 – 7.21 (m, 2H), 6.92 (m, 1H), 6.68 (m, 2H), 4.40 (s, 2H), 3.18 (s, 6H), 2.75 (s, 3H); FT-IR (KBr): 3448, 2929, 1603, 1541, 1486,1411, 1379, 1312, 1191, 1136 cm^{1-}; HRMS m/z for $C_{23}H_{22}N_3O_4$ $[M + H]^+$ calcd 404.1610, found 404.1614.

***N*-benzyl-7-(dimethylamino)-4-hydroxy-*N*-methyl-3-oxo-3*H*-phe-noxazine-1-carboxamide hydrochloride, (6d):** 25 µL of HCl in iPrOH (5N) solution were added dropwise in a suspension of **4d** (10 mg, 0.025 mmol) in 0.5 mL of ethanol. The mixture was stirred at rt for 30 min. Then, the mixture was concentrated to furnish **6d** (quantitative yield) as a dark blue-green solid. **6d:** mp=173-176 °C, ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.58-7.49 (m, 2H), 7.49-7.34 (m, 2H), 7.35-7.18 (m, 2H), 6.93 (d, *J* = 9.3 Hz, 1H), 6.75-6.62 (m, 2H), 4.39 (s, 2H), 2.93 (s, 3H), 2.74 (s, 6H); FT-IR (KBr): 3448, 2932, 1603, 1545, 1484, 1412, 1380, 1311, 1191, 1136 cm^{1-}.

***N*-benzyl-4-(benzyloxy)-7-(dimethylamino)-*N*-methyl-3-oxo-3*H*-phenoxazine-1-carboxamide,(5d)**: Compound **4d** (15 mg, 0.037 mmol) were dissolved in 0.3 mL of dry DMF. Caesium carbonate (7 mg, 0.023 mmol) and benzyl bromide (5.3 µL, 0.045 mmol) were added into the above solution respectively, and the mixture was stirred at rt overnight. Then the mixture was concentrated and was purified by flash column chromatography (eluent; ethyl acetate/Hexane = 1/1) to afford 14 mg of **5d** in 77% yield. **5d** (mixture of rotamers): mp=102-105 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.30 (s, 1H), 7.72 – 7.11 (m, 5H), 6.93 (m, 1H), 6.75-6.53 (m, 2H), 5.15 (s, 2H), 4.39(s, 2H), 3.18 (s, 6H), 2.72 (s, 3H); FT-IR (KBr): 2922, 2849, 1718, 1700, 1685, 1637, 1600, 1560, 1541, 1508, 1490, 1437, 1368, 1255, 1201, 1130, 1094 cm^{1-}; HRMS m/z for $C_{30}H_{28}N_3O_4$ $[M + H]^+$ calcd 494.2080, found 494.2081.

***N*-benzyl-4-(benzyloxy)-7-(dimethylamino)-*N*-methyl-3-oxo-3*H*-phenoxazine-1-carboxamide hydrochloride, (7):** 16 µL of HCl in iPrOH (5N) solution were added dropwise in a suspension of *N*-benzyl-4-(benzyloxy)-7-(dimethylamino)-*N*-methyl-3-oxo-3*H*-phenoxazine-1-carboxamide (8 mg, 0.016 mmol) in 0.4 mL of ethanol. The mixture was stirred at rt for 30 min. Then, the mixture was concentrated to furnish **7** (quantitative yield) as a dark blue-green solid. **7**: mp= 148-152 °C, ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.72-7.18 (m, 7H), 7.09 (m, 1H), 6.86-6.66 (m, 1H), 5.18 (s, 2H), 4.27(s, 2H), 3.25 (s, 6H), 2.7 (s, 3H), FT-IR (KBr): 3438, 2925, 1629, 1596, 1551, 1486, 1453, 1419, 1374, 1293, 1188, 1132 cm^{1-}.

Benzyl 4-(benzyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazine-1-carboxylate, 8a: Gallocyanine hydrochloride (100 mg, 0.297 mmol) was charged into a round bottom flask and dissolved in 1 mL dry DMF. The reaction mixture was cooled at 0 °C and 97 mg of Cs_2CO_3 (0.297 mmol) was added. After the addition of the base 71 µL of BnBr (0.594 mmol) was added at 0 °C. Then the reaction mixture was warmed up at rt and stirred overnight. After removal of DMF under reduced pressure the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/3 gradient to EA, then MeOH/EA=5/95) to afford **8a** as dark blue solid (71 mg, 50%). **8a:** mp=185-187 °C**;** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (d, *J* = 9.1 Hz, 1H), 7.51 (d, *J* = 7.2 Hz, 2H), 7.48 (d, *J* = 7.3 Hz, 2H), 7.42-7.27 (m, 6H), 7.09 (s, 1H), 6.71 (dd, *J* = 9.2, 2.6 Hz, 1H), 6.41 (d, *J* = 2.6 Hz, 1H), 3.16 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.28, 164.79, 154.23, 146.29, 140.26, 138.07, 137.34, 135.72, 135.42, 133.77, 132.47, 131.47, 128.57, 128.54, 128.35, 128.25, 128.23, 128.08, 126.70, 111.07, 96.36, 74.09, 67.44, 40.51; FT-IR: 2924, 1735, 1599, 1490, 1449, 1370, 1242, 1200, 1163, 1102, 904; HRMS *m/z* for $C_{29}H_{25}N_2O_5$ $[M + H]^+$ calcd 481.1763, found 481.1763.

Benzyl 4-(benzyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazine-1-carboxylate hydrochloride, (9a): 42 µL of HCl in iPrOH (5N) solution were added dropwise in a suspension of **8a (**20 mg, 0.042 mmol) in 1 mL of ethanol. The mixture was stirred at rt for 30 min. Then, the mixture was concentrated to furnish **9a** (quantitative yield) as a dark blue-green solid. **9a**: mp=169-171 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58 (d, *J* = 8.9 Hz, 1H), 7.49 (dd, *J* = 15.4, 7.2 Hz, 4H), 7.35 (ddd, *J* = 29.3, 14.9, 7.4 Hz, 6H), 7.12 (s, 1H), 6.74 (d, *J* = 9.5 Hz, 1H), 6.44 (s, 1H), 5.41 (s, 2H), 5.31

(s, 2H), 3.19 (s, 6H); FT-IR (KBr): 3436, 3056, 3027, 2924, 2853, 2739, 2553, 2498, 1730, 1637, 1591, 1568, 1547, 1485, 1452, 1421, 1380, 1310, 1282, 1231, 1194, 1141 cm^{1-}.

Allyl 4-(allyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazine-1-carboxylate, 8b: Gallocyanine hydrochloride (100 mg, 0.297mmol) was charged into a round bottom flask and dissolved in 1ml dry DMF. The reaction mixture was cooled at 0 °C and 97 mg of Cs_2CO_3 (0.297 mmol) was added. After the addition of the base 51,4 µL of allyl bromide (0.594 mmol) was added at 0 °C. Then the reaction mixture warm up at rt and stirred overnight. After removal of DMF under reduced pressure the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/4) to afford **8b** as dark blue solid (48 mg, 43%). **8b:** mp= 101-104 °C**;** ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.62 (d, *J* = 9.1 Hz, 1H), 7.08 (s, 1H), 6.73 (d, *J* = 9.1 Hz, 1H), 6.55 (s, 1H), 6.05 (m, 2H), 5.48 (d, *J* = 17.2 Hz, 1H), 5.33 (dd, *J* = 17.6, 14.5 Hz, 2H), 5.21 (d, *J* = 10.3 Hz, 1H), 4.87 (d, *J* = 5.5 Hz, 2H), 4.79 (d, *J* = 6.1 Hz, 2H), 3.18 (s, 6H); ^{13}C NMR (126 MHz, cdcl$_3$) δ 179.30, 164.62, 154.27, 146.38, 140.32, 138.19, 135.53, 133.94, 133.81, 132.59, 131.56, 131.30, 126.70, 118.89, 118.77, 111.05, 96.52, 73.20, 66.26, 40.53; FT-IR (KBr): 2923, 2853, 1724, 1641, 1596, 1490, 1449, 1368, 1257, 1178, 1135, 1114, 988 cm^{1-}; HRMS *m/z* for $C_{21}H_{21}N_2O_5$ $[M + H]^+$ calcd 381.1450, found 381.1451.

4-Fluorobenzyl 7-(dimethylamino)-4-((4-fluorobenzyl)oxy)-3-oxo-3*H*-phenoxazine-1-carboxylate, 8c: Gallocyanine hydrochloride (100 mg, 0.297 mmol) was charged into a round bottom flask and dissolved in 1mL dry DMF. The reaction mixture was cooled at 0 °C and 145 mg of Cs_2CO_3 (0.446 mmol) was added. After the addition of the base 74 µL of *p*-fluorobenzyl bromide (0.594 mmol) was added at 0 °C. Then the reaction mixture warm up at room temperature and stirred overnight. After removal of DMF under reduced pressure the residue was purified with flash column chromatography (eluent; ethyl acetate/hexane = 1/3) to obtain **8c** as dark blue-black solid (100 mg, 65%). **8c**: mp=189-191 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (d, *J* = 9.2 Hz, 1H), 7.47 (m, 4H), 7.08 (m, 3H), 7.00 (t, *J* = 8.6 Hz, 2H), 6.74 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.43 (d, *J* = 2.6 Hz, 1H), 5.37 (s, 2H), 5.26 (s, 2H), 3.19 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.2, 164.7, 163.7, 161.7, 154.3, 146.3, 140.3, 137.9, 135.5, 133.7, 133.2, 132.5, 131.4, 131.3, 130.5, 130.3, 126.7, 115.5, 115.1, 111.2, 96.3, 73.3, 66.7, 40.6; FT-IR (KBr): 2955, 2925, 2850, 1700, 1637, 1593, 1544, 1511, 1486, 1447, 1365, 1273, 1223, 1201, 1181, 1120 cm^{1-}; HRMS *m/z* for $C_{29}H_{23}F_2N_2O_5$ $[M + H]^+$ calcd 517.1575, found 517.1578.

2,4-Difluorophenyl 4-((2,4-difluorobenzyl)oxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazine-1-carboxylate, 8d: Gallocyanine hydro-chloride (100 mg, 0.297 mmol) were dissolved in 1 mL of dry DMF. Caesium carbonate (145 mg, 0.446 mmol) and 2,4-difluorobenzyl bromide (76 µL, 0.594 mmol) were added into the above solution respectively, and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/3) to afford 82 mg of **8d** in 50% yield. **8d:** mp=184-189 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.60-7.48 (m, 3H), 7.12 (s, 1H), 6.99-6.68 (m, 5H), 6.42 (d, *J* = 2.6 Hz, 1H), 5.42 (s, 2H), 5.31 (s, 2H), 3.19 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.7, 164.4, 163.1, 162.8, 161.2, 161.0, 154.3, 146.2, 140.3, 137.8, 135.3, 133.6, 132.4, 132.2, 131.7, 131.2, 126.7, 120.5, 118.7, 111.2, 111.0, 109.2, 103.9, 103.4, 96.2, 66.9, 60.7, 40.3. FT-IR (KBr) =2960, 2925, 1716, 1593, 1545, 1507, 1488, 1449, 1367, 1255, 1237, 1201, 1174, 1139, 1109 cm^{1-}, HRMS *m/z* for $C_{29}H_{21}F_4N_2O_5$ $[M + H]^+$ calcd 553.1387, found 553.1391.

Cyclopropylmethyl 4-(cyclopropylmethoxy)-7-(dimethyl amino)-3-oxo-3*H*-phenoxazine-1-carboxylate, 8e: Gallocyanine hydrochloride (50 mg, 0.149 mmol) was dissolved in 0.5 mL of dry DMF. Caesium carbonate (73 mg,

0.223 mmol) and bromomethyl cyclopropane (29 μL, 0.297 mmol) were added into the above solution respectively, and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 3/2) to afford 22 mg of **8e** in 36% yield. **8e:** mp=87-88 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (d, *J* = 9.1 Hz, 1H), 7.07 (s, 1H), 6.71 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.54 (d, *J* = 2.7 Hz, 1H), 4.21 (d, *J* = 7.3 Hz, 2H), 4.07 (d, *J* = 7.2 Hz, 2H), 3.17 (s, 6H), 1.36-1.11 (m, 2H), 0.66-0.60 (m, 2H), 0.58-0.52 (m, 2H), 0.43 – 0.37 (m, 2H), 0.36 – 0.29 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.6, 165.1, 154.2, 146.4, 140.4, 138.5, 136.2, 134.3, 132.6, 131.1, 126.7, 110.9, 96.5, 77.3, 70.5, 40.5, 10.9, 9.8, 3.4, 3.1; FT-IR (KBr): 3083, 2998, 2922, 2852, 1731, 1600, 1556, 1488, 1448, 1368, 1246, 1182, 1135,1113, 981cm^{1-}; HRMS *m/z* for $C_{23}H_{25}N_2O_5$ $[M + H]^+$ calcd 409.1763, found 409.1763.

Cyclopropylmethyl 4-(cyclopropylmethoxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazine-1-carboxylate hydrochloride, (9e): 14 μL of HCl in iPrOH (5N) solution were added dropwise in a suspension of **8e** (6 mg, 0.014 mmol) in 0.3 mL of ethanol. The mixture was stirred at rt for 30 min. Then, the mixture was concentrated to furnish **9e** (quantitative yield) as a dark blue-green solid. **9e:** mp=156-160 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.65 (d, *J* = 9.4 Hz, 1H), 7.27 (d, *J* = 8.6 Hz, 1H), 7.09 (s, 2H), 6.94 (s, 2H), 4.17 (d, *J* = 7.2 Hz, 2H), 3.98 (d, *J* = 7.1 Hz, 2H), 3.35 (s, 6H), 1.23 (m, 2H), 0.58 (m, 2H), 0.51 (m, 2H), 0.39 (m, 2H), 0.29 (m, 2H).

4-Methylbenzyl 7-(dimethylamino)-4-((4-methylbenzyl)oxy)-3-oxo-3*H*-phenoxazine-1-carboxylate, 8f: Gallocyanine hydrochloride (50 mg, 0.149 mmol) were dissolved in 0.5 ml of dry DMF. Caesium carbonate (72.5 mg, 0.223 mmol) and 4-methylbenzyl bromide (55 mg, 0.297 mmol) were added into the above solution respectively, and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/1) to afford 53 mg of **8f** in 70% yield. **8f**: mp=138-141 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, *J* = 9.2 Hz, 1H), 7.37 (m, 4H), 7.19 (d, *J* = 7.8 Hz, 2H), 7.10 (d, *J* = 7.8 Hz, 2H), 7.05 (s, 1H), 6.68 (dd, *J* = 9.1, 2.7 Hz, 1H), 6.42 (d, *J* = 2.7 Hz, 1H), 5.36 (s, 2H), 5.28 (s, 2H), 3.15 (s, 6H), 2.36 (s, 3H), 2.29 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.3, 164.8, 154.2, 146.3, 140.2, 138.2, 138.1, 137.8, 135.6, 134.2, 133.8, 132.4, 132.4, 131.4, 129.2, 128.9, 128.6, 128.4, 126.6, 110.9, 96.3, 73.9, 67.4, 40.5, 21.2, 21.2; FT-IR (KBr): 2922, 2852, 1733, 1646, 1600, 1541, 1488, 1456,1372,1236, 1170, 1111; HRMS *m/z* for $C_{31}H_{29}N_2O_5$ $[M + H]^+$ calcd 509.2076, found 509.2077.

4-(Trifluoromethyl)benzyl 7-(dimethylamino)-3-oxo-4-((4-(trifluo-romethyl)benzyl)oxy)-3*H*-phenoxazine-1-carboxylate, 8g: Gallocyanine hydrochloride (100 mg, 0.297 mmol) were dissolved in 1mL of dry DMF. Caesium carbonate (145 mg, 0.446 mmol) and 4-(trifluoromethyl)benzyl bromide (142 mg, 0.594 mmol) were added into the above solution respectively, and the mixture was stirred at room temperature overnight. Then the mixture was concentrated and was purified by flash column chromatography (eluent; DCM/acetone = 20/1) to afford 122 mg of **8g** in 67% yield. **8g**: mp=182-184 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77-7.58 (m, 8H), 7.55 (d, *J* = 9.2 Hz, 1H), 7.11 (s, 1H), 6.76 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.42 (d, *J* = 2.6 Hz, 1H), 5.47 (s, 2H), 5.37 (s, 2H), 3.19 (s, 6H); FT-IR (KBr): 2924, 2852, 2697, 1735, 1643, 1600, 1560, 1530, 1489, 1458, 1371, 1329, 1243, 1204, 1180, 1116, 1067, 1007, 822 cm^{1-}; HRMS *m/z* for $C_{31}H_{23}F_6N_2O_5$ $[M + H]^+$ calcd 617.1511, found 617.1512.

Ethyl 4-(benzyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxa zine-1-carboxylate, (11a): Ethyl 7-(dimethylamino)-4-hydroxy-3-oxo-3*H*-phenoxazine-1-carboxylate (20 mg, 0.061 mmol) was dissolved in 0.5 mL of dry DMF. Caesium carbonate (11 mg, 0.034 mmol) and benzyl bromide (8.7 μL, 0.073 mmol) were added and the mixture was stirred at rt

overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 2/1) to afford 27 mg of **11a** in 86% yield. **11a**: mp= 161-163 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.60 (d, *J* = 9.1 Hz, 1H), 7.52 (d, *J* = 7.2 Hz, 2H), 7.32 (t, *J* = 7.4 Hz, 2H), 7.28 (s, 1H), 7.06 (s, 1H), 6.71 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.44 (d, *J* = 2.6 Hz, 1H), 5.32 (s, 2H), 4.43 (q, *J* = 7.1 Hz, 2H), 3.18 (s, 6H), 1.40 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.5, 164.9, 154.2, 146.3, 140.3, 138.3, 137.4, 135.7, 134.2, 132.6, 131.2, 128.6, 128.2, 128.1, 126.7, 110.9, 96.4, 74.1, 61.9, 40.5, 14.2; FT-IR (KBr): 2925, 1730, 1637,1596, 1560, 1541, 1489, 1456, 1423, 1374, 1231, 1205, 1171, 1137, 1108 cm^{1-}; HRMS *m/z* for $C_{24}H_{23}N_2O_5$ $[M + H]^+$ calcd 419.4607, found 419.4605.

Ethyl 4-(benzyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxa zine-1-carboxylate hydrochloride, (12a): 24 μL of HCl in iPrOH (5N) solution were added dropwise in a suspension of **11a** (10 mg, 0.024 mmol) in 0.4 mL of ethanol. The mixture was stirred at rt for 30 min. Then, the mixture was concentrated to furnish **12a** (quantitative yield) as a dark blue-green solid. **12a:** mp=162-165 °C, ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.57 (d, *J* = 9.3 Hz, 1H), 7.49 (d, *J* = 7.1 Hz, 2H), 7.34 (t, *J* = 7.4 Hz, 2H), 7.28 (t, *J* = 7.4 Hz, 1H), 7.06 (m, 1H), 6.94 (m, 1H), 6.76 (s, 1H), 5.18 (s, 2H), 4.32 (q, *J* = 7.1 Hz, 2H), 3.25 (s, 6H), 1.30 (t, *J* = 7.1 Hz, 3H); FT-IR (KBr): 3448, 2924, 2844, 1700, 1637, 1593, 1543, 1489, 1458, 1424, 1379, 1296, 1193, 1134, 1102, 1017 cm^{1-}.

Ethyl 7-(dimethylamino)-4-((4-methylbenzyl)oxy)-3-oxo-3*H*-phenoxazine-1-carboxylate, 11b: Ethyl 7-(dimethylamino)-4-hydroxy-3-oxo-3H-phenoxazine-1-carboxylate (20 mg, 0.061 mmol) was dissolved in 0.2 mL of dry DMF. Caesium carbonate (10 mg, 0.03 mmol) and 4-methylbenzyl bromide (11 mg, 0.061 mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 3/2) to afford 19 mg of **11b** in 72% yield. **11b**: mp= 194-200 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.58 (d, *J* = 9.2 Hz, 1H), 7.39 (d, *J* = 7.9 Hz, 2H), 7.11 (d, *J* = 7.8 Hz, 2H), 7.03 (s, 1H), 6.69 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.44 (d, *J* = 2.6 Hz, 1H), 5.28 (s, 2H), 4.42 (q, *J* = 7.1 Hz, 2H), 3.16 (s, 6H), 2.30 (s, 3H), 1.40 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.2, 164.9, 154.3, 146.4, 140.3, 138.3, 137.8, 135.6, 134.3, 134.1, 132.6, 131.0, 128.9, 128.7, 126.8, 111.1, 96.4, 73.9, 61.8, 40.6, 21.2, 14.2; FT-IR (KBr): 2960, 2923, 2853, 1176, 1638, 1591, 1535, 1488,1475, 1455, 1368, 1230, 1200, 1174, 1136, 1115, 1043 cm^{1-}; HRMS *m/z* for $C_{25}H_{25}N_2O_5$ $[M + H]^+$ calcd 433.1763, found 433.1765.

Ethyl 7-(dimethylamino)-4-((4-methylbenzyl)oxy)-3-oxo-3*H*-phenoxazine-1-carboxylate, (12b): 11 μL of HCl in iPrOH (5N) solution were added dropwise in a suspension of **11b** (5 mg, 0.012 mmol) in 0.5 mL of ethanol. The mixture was stirred at rt for 30 min. Then, the mixture was concentrated to furnish **12b** (quantitative yield) as a dark blue-green solid. **12b:** mp=153-157 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.59 (d, *J* = 9.3 Hz, 1H), 7.38 (d, *J* = 7.5 Hz, 2H), 7.16 (d, *J* = 7.5 Hz, 2H), 7.10 (s, 2H), 6.97 (s, 1H), 6.80 (s, 1H), 5.16 (s, 2H), 4.34 (dd, *J* = 14.1, 7.1 Hz, 2H), 3.28 (s, 6H), 2.27 (s, 3H), 1.31 (t, *J* = 7.1 Hz, 2H).

Butyl 4-(benzyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazine-1-carboxylate, (11c): Butyl 7-(dimethylamino)-4-hydroxy-3-oxo-3H-phenoxazine-1-carboxylate (30 mg, 0.084 mmol) was dissolved in 0.8 mL of dry DMF. Caesium carbonate (15 mg, 0.046 mmol) and benzylbromide (10.5 μL, 0.088 mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 2/1) to afford 27 mg of **11c** in 71% yield. mp= 111-113 °C ;^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (d, *J* = 9.1 Hz, 1H), 7.52 (d, *J* = 7.2 Hz, 2H), 7.32 (t, *J* = 7.3 Hz, 2H), 7.27 (m, 1H overlapping with $CDCl_3$), 7.06 (s, 1H), 6.71 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.43 (d, *J* = 2.6 Hz, 1H), 5.31 (s, 2H), 4.38 (t, *J* = 6.5 Hz, 2H),

3.17 (s, 6H), 1.86 – 1.70 (m, 2H), 1.50 (m, 2H), 0.98 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.5, 165.1, 154.2, 146.3, 140.3, 138.3, 137.4, 135.6, 134.3, 132.5, 131.2, 128.6, 128.2, 128.1, 126.7, 110.9, 96.4, 74.1, 65.7, 40.5, 30.6, 19.2, 13.7; FT-IR (KBr):2954, 2924, 2847, 1717, 1637, 1597, 1550, 1489, 1451, 1367, 1261, 1203, 1173, 1136, 1107 cm^{-1}; HRMS *m/z* for $C_{26}H_{27}N_2O_5$ $[M + H]^+$ calcd 447.1920, found 447.1919.

Butyl 4-(benzyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxa zine-1-carboxylate hydrochloride, (12c): 23 μL of HCl in iPrOH (5N) solution were added dropwise in a suspension of ethyl 4-(benzyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazi ne-1-carboxylate (10 mg, 0.022 mmol) in 0.5 mL of ethanol. The mixture was stirred at rt for 30 min. Then, the mixture was concentrated to furnish **12c** (quantitative yield) as a dark blue-green solid. **12c:** mp=156-159 °C, ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.58 (d, *J* = 8.9 Hz, 1H), 7.52 (d, *J* = 7.2 Hz, 2H), 7.37 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.3 Hz, 1H), 7.21 (br, 1H), 7.03 (br, 1H), 6.85 (br, 1H), 5.21 (s, 2H), 4.31 (t, *J* = 6.4 Hz, 2H), 3.32 (s, 6H), 1.85 – 1.61 (m, 2H), 1.45 (dq, *J* = 14.7, 7.4 Hz, 2H), 0.94 (t, *J* = 7.4 Hz, 3H); FT-IR (KBr): 3448, 3043, 2958, 2931, 2871, 1701, 1637, 1594, 1571, 1560, 1487, 1458, 1423, 1392, 1378, 1300, 1283, 1255, 1192, 1140 cm^{1-}.

Butyl 7-(dimethylamino)-3-oxo-4-(pentyloxy)-3H-phenoxazine-1-carboxylate, (11d): Butyl 7-(dimethylamino)-4-hydroxy-3-oxo-3*H*-phenoxazine-1-carboxylate (30 mg, 0.084 mmol) was dissolved in 0.8 mL of dry DMF. Potassium carbonate (6.5 mg, 0.046 mmol) and pentyl bromide (9 μL, 0.089 mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/Hexane = 3/1) to afford 25.6 mg of **11d** in 72% yield. **11d**: mp= 138-141 °C ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (d, *J* = 9.1 Hz, 1H), 7.04 (s, 1H), 6.72 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.52 (d, *J* = 2.6 Hz, 1H), 4.38 (t, *J* = 6.5 Hz, 2H), 4.21 (t, *J* = 6.8 Hz, 2H), 3.17 (s, 6H), 1.95-1.72 (m, 4H), 1.63 – 1.45 (m, 4H), 1.38 (m, 2H), 0.98 (t, *J* = 7.4 Hz, 3H), 0.93 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.6, 165.2, 154.1, 146.4, 140.2, 138.6, 136.3, 134.4, 132.5, 131.1, 126.6, 110.9, 96.5, 72.9, 65.7, 40.5, 30.6, 29.8, 28.1, 22.5, 19.2, 14.2, 13.7; FT-IR (KBr): 2956, 2919, 2855, 1734, 1637, 1600, 1559, 1490, 1457, 1372, 1248, 1203, 1181, 1135, 1112 cm^{1-}; HRMS *m/z* for $C_{24}H_{31}N_2O_5$ $[M + H]^+$ calcd 427.2233, found 427.2237.

2-Methoxyethyl 4-(benzyloxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazine-1-carboxylate, (11e): 2-Methoxyethyl 7-(dimethylamino)-4-hydroxy-3-oxo-3*H*-phenoxazine-1-carboxylate (25 mg, 0.07 mmol) was dissolved in 0.5 mL of dry DMF. Caesium carbonate (13 mg, 0.039 mmol) and benzylbromide (10 μL, 0.084 mmol) were added into the above solution respectively, and the mixture was stirred at room temperature overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 2/1) to afford 15 mg of **11e** in 47% yield. **11e**: mp= 119-122 ^{o}C ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57 (d, *J* = 9.2 Hz, 1H), 7.51 (d, *J* = 7.3 Hz, 2H), 7.32 (t, *J* = 7.3 Hz, 2H), 7.29 – 7.24 (m, 1H), 7.09 (s, 1H), 6.70 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.41 (d, *J* = 2.7 Hz, 1H), 5.31 (s, 2H), 4.65 – 4.41 (t, *J* = 5.4 Hz, 2H), 3.72 (t, *J* = 5.4 Hz, 2H), 3.42 (s, 3H), 3.16 (s, 6H); ^{13}C NMR (126 MHz,$CDCl_3$) δ 179.3, 164.8, 154.2, 146.3, 140.3, 138.1, 137.3, 135.7, 133.7, 132.5, 131.4, 128.5, 128.2, 128.1, 126.7, 111.0, 96.3, 74.1, 70.3, 64.7, 59.1, 40.5; FT-IR (KBr):2924, 2847, 2801, 1727, 1638, 1600, 1541, 1491, 1458, 1422, 1380, 1252, 1202, 1171, 1111, 1033, 903 cm^{1-}; HRMS *m/z* for $C_{25}H_{25}N_2O_6$ $[M + H]^+$ calcd 449.1713, found 449.1716.

2-Methoxyethyl 7-(dimethylamino)-4-((4-methylbenzyl)oxy)-3-oxo-3*H*-phenoxazine-1-carboxylate, 11f: 2-Methoxyethyl 7-(dimethyl amino)-4-hydroxy-3-oxo-3*H*-phenoxazine-1-carboxylate (20 mg, 0.056 mmol) was dissolved in 0.5 mL of dry DMF. Caesium carbonate (10 mg, 0.031 mmol) and 4-methylbenzylbromide (13 mg, 0.067

mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 2/1) to afford 12 mg of **11f** in 46% yield. **11f**: mp= 113-116 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58 (d, *J* = 9.1 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 2H), 7.11 (d, *J* = 7.8 Hz, 1H), 7.08 (s, 1H), 6.70 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.44 (d, *J* = 2.7 Hz, 1H), 5.29 (s, 2H), 4.51 (t, *J* = 5.5 Hz, 2H), 51 (t, *J* = 5.5 Hz, 2H), 3.42 (s, 3H), 3.17 (s, 6H), 2.30 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 179.4, 164.9, 154.2, 146.3, 140.3, 138.2, 137.8, 135.6, 134.2, 133.7, 132.5, 131.5, 128.9, 128.7, 126.7, 110.9, 96.4, 73.9, 70.3, 64.7, 59.1, 59.1, 40.5; FT-IR (KBr): 3026, 2923, 2843, 1737, 1720, 1637, 1601, 1541, 1489, 1457, 1377, 1258, 1200, 1175, 1113, 1029 cm^{1-}; HRMS *m/z* for $C_{26}H_{27}N_2O_6$ $[M + H]^+$ calcd 463.1869, found 463.1865.

Butyl 4-((4-fluorobenzyl)oxy)-7-(dimethylamino)-3-oxo-3*H*-phenoxazine-1-carboxylate, 11g: Butyl 7-(dimethylamino)-4-hydroxy-3-oxo-3H-phenoxazine-1-carboxylate (18 mg, 0.05 mmol) was dissolved in 0.2 mL of dry DMF. Caesium carbonate (9 mg, 0.028 mmol) and 4-fluorobenzyl bromide (7 µl, 0.056 mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/3) to afford 18 mg of **11g** in 77% yield. **11g**: mp= 77-80 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58 (d, *J* = 9.1 Hz, 1H), 7.49 (dd, *J* = 8.3, 5.6 Hz, 2H), 7.05 (s, 1H), 7.00 (t, *J* = 8.7 Hz, 2H), 6.72 (dd, *J* = 9.2, 2.5 Hz, 1H), 6.41 (d, *J* = 2.5 Hz, 1H), 5.26 (s, 2H), 4.38 (t, *J* = 6.5 Hz, 2H), 3.17 (s, 6H), 1.86-1.64 (m, 2H), 1.50 (m, 2H), 0.98 (t, *J* = 7.4 Hz, 3H); FT-IR (KBr): 2957, 2925, 2852, 1716, 1683, 1636, 1604, 1558, 1541, 1457, 1378, 1270, 1174, 1113 cm^{1-}; HRMS *m/z* for $C_{26}H_{26}FN_2O_5$ $[M + H]^+$ calcd 465.1826, found 465.1830.

Methyl 4-(benzyloxy)-7-(dimethylamino)-2-((4-methoxybenzyl)amino)-3-oxo-3*H*-phenoxazine-1-carboxylate, 14a: Methyl 7-(dime thylamino)-4-hydroxy-2-((4-methoxybenzyl) amino)-3-oxo-3*H*-phenoxazine-1-carboxylate (15 mg, 0.033 mmol) was dissolved in 0.1 mL of dry DMF. Caesium carbonate (5 mg, 0.017 mmol) and benzyl bromide (4 µL, 0.033 mmol) were added, and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/2) to afford 18 mg of **14a** in 97% yield. **14a**: mp= 160-162 °C ;^{1}H NMR (500 MHz, $CDCl_3$) δ 7.62 – 7.47 (m, 3H), 7.34 (t, *J* = 7.4 Hz, 2H), 7.27 (m, 2H), 6.89 (d, *J* = 8.5 Hz, 2H), 6.71 (dd, *J* = 9.1, 2.5 Hz, 1H), 6.50 (d, *J* = 2.5 Hz, 1H), 6.21 (t, *J* = 4.9 Hz, 1H), 5.23 (s, 2H), 4.37 (d, *J* = 5.1 Hz, 2H), 3.90 (s, 3H), 3.81 (s, 3H), 3.10 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.2, 168.5, 159.3, 151.6, 144.6, 140.2, 139.7, 139.1, 137.4, 132.9, 130.3, 129.4, 129.3, 128.5, 128.3, 128.1, 126.4, 114.2, 110.6, 102.7, 96.7, 74.5, 55.3, 52.3, 47.3, 40.4; FT-IR (KBr): 3308, 2954, 2924, 2852, 1724, 1637, 1605, 1565, 1508, 1430, 1353, 1315, 1247, 1200, 1173, 1117 cm^{1-}; HRMS *m/z* for $C_{31}H_{30}N_3O_6$ $[M + H]^+$ calcd 540.2135, found 540.2136.

Methyl 7-(dimethylamino)-4-((4-fluorobenzyl)oxy)-2-((4-methoxybenzyl)amino)-3-oxo-3*H*-phenoxazine-1-carbo xylate, 14b: methyl 7-(dimethylamino)-4-hydroxy-2-((4-methoxy benzyl)amino)-3-oxo-3*H*-phenoxazine-1-carboxylate (20 mg, 0.045 mmol) was dissolved in 0.15 mL of dry DMF. Caesium carbonate (7.2 mg, 0.0222 mmol) and 4-fluorobenzyl bromide (6 µL, 0.045 mmol) were added, and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/Hexane = 1/2) to afford 25 mg of **14b** in 98% yield. **14b**: mp= 187-190 °C ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (d, *J* = 9.1 Hz, 1H), 7.51 (dd, *J* = 8.4, 5.6 Hz, 2H), 7.25 (1H, overlapping with $CDCl_3$), 7.01 (t, *J* = 8.7 Hz, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 6.72 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.49 (d, *J* = 2.6 Hz, 1H), 6.19 (t, *J* = 5.2 Hz, 1H), 5.19 (s, 2H), 4.37 (d, *J* = 5.3 Hz, 2H), 3.90 (s, 3H), 3.81 (s, 3H), 3.11 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.1, 168.4, 162.6, 159.3, 151.7, 144.6, 140.0, 139.7, 139.1, 133.2, 132.7, 130.4, 130.3, 129.4, 129.3, 126.4, 115.1, 114.2, 110.7, 102.8, 96.5, 73.6, 55.3,

52.36, 47.3, 40.4; FT-IR (KBr): 3306, 2924, 2846, 1724, 1637, 1605, 1566, 1508, 1442, 1431, 1350, 1319, 1249, 1200, 1189, 1176, 1119cm^{1-}; HRMS *m/z* for $C_{31}H_{29}FN_3O_6$ $[M + H]^+$ calcd 558.2040, found 558.2044.

Methyl 4-((2,4-difluorobenzyl)oxy)-7-(dimethylamino)-2-((4-methoxybenzyl)amino)-3-oxo-3*H*-phenoxazine-1-carboxylate, 14c: Methyl 7-(dimethylamino)-4-hydroxy-2-((4-methoxybenzyl) amino)-3-oxo-3*H*-phenoxazine-1-carboxylate (20 mg, 0.045 mmol) was dissolved in 0.15 mL of dry DMF. Caesium carbonate (7 mg, 0.0222 mmol) and 2,4-difluorobenzyl bromide (6 µL, 0.045 mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/2) to afford 22 mg of **14c** in 86% yield. **14c**: mp=176-178 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (dd, *J* = 15.1, 8.3 Hz, 1H), 7.55 (d, *J* = 9.1 Hz, 1H), 7.25 (d, *J* = 8.6 Hz, 2H), 6.89 (d, *J* = 8.6 Hz, 2H), 6.85 (td, *J* = 8.3, 1.7 Hz, 1H), 6.79 (td, *J* = 9.6, 2.4 Hz, 1H), 6.72 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.49 (d, *J* = 2.6 Hz, 1H), 6.19 (t, *J* = 5.1 Hz, 1H), 5.25 (s, 2H), 4.37 (d, *J* = 5.1 Hz, 2H), 3.90 (s, 3H), 3.81 (s, 3H), 3.11 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.0, 168.4, 162.9, 161.2, 159.3, 151.7, 144.5, 139.9, 139.7, 139.3, 132.5, 132.2, 130.3, 129.4, 129.3, 126.4, 120.6, 114.2, 111.1, 110.7, 103.6, 102.8, 96.5, 67.4, 55.3, 52.4, 47.3, 40.4; FT-IR (KBr): 3350, 2950, 2952, 2852, 1731, 1637, 1615, 1569, 1506, 1468, 1432, 1354, 1312, 1277, 1198, 1171, 1121, 1106 cm^{1-}; HRMS *m/z* for $C_{31}H_{28}F_2N_3O_6$ $[M + H]^+$ calcd 576.1946, found 576.1946.

Methyl 7-(dimethylamino)-2-((4-methoxybenzyl)amino)-3-oxo-4-((4-(trifluoromethyl)benzyl)oxy)-3*H*-phenoxazine-1-carboxylate, 14d: Methyl 7-(dimethylamino)-4-hydroxy-2-((4-methoxy-benzyl)amino)-3-oxo-3*H*-phenoxazine-1-carboxy late (20 mg, 0.045 mmol) was dissolved in 0.15 ml of dry DMF. Caesium carbonate (7 mg, 0.022 mmol) and 4-(trifluoromethyl)benzyl bromide (10 µL, 0.045 mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/2) to afford 25 mg of **14d** in 92% yield. **14d**: mp= 146-149 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.67 (d, *J* = 8.1 Hz, 2H), 7.61 (d, *J* = 8.1 Hz, 2H), 7.55 (d, *J* = 9.1 Hz, 1H), 7.25 (1H overlapping with $CDCl_3$), 6.89 (d, *J* = 8.6 Hz, 2H), 6.72 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.47 (d, *J* = 2.6 Hz, 1H), 6.18 (t, *J* = 5.2 Hz, 1H), 5.28 (s, 2H), 4.37 (d, *J* = 5.3 Hz, 2H), 3.90 (s, 3H), 3.81 (s, 3H), 3.10 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.9, 168.3, 159.4, 151.8, 144.5, 141.6, 139.9, 139.7, 138.9, 132.9, 130.4, 130.1, 129.4, 129.3, 128.2, 126.4, 125.2, 124.8, 114.3, 110.8, 102.9, 96.5, 73.5, 55.3, 52.3, 47.3, 40.3; FT-IR (KBr): 3318, 2925, 2852, 1724, 1638, 1608, 1569, 1508, 1431, 1355, 1326, 1200, 1173, 1123, 1064 cm^{1-}; HRMS *m/z* for $C_{32}H_{29}F_3N_3O_6$ $[M + H]^+$ calcd 608.2008, found 608.2010.

Methyl 7-(dimethylamino)-2-((4-methoxybenzyl)amino)-4-((4-methylbenzyl)oxy)-3-oxo-3*H*-phenoxazine-1-carboxylate, 14e: Methyl 7-(dimethylamino)-4-hydroxy-2-((4-methoxybenzyl)amino)-3-oxo-3*H*-phenoxazine-1-carboxylate (20 mg, 0.045 mmol) were dissolved in 0.15 mL of dry DMF. Caesium carbonate (7 mg, 0.022 mmol) and 4-methylbenzyl bromide (8 mg, 0.045 mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/2) to afford 22 mg of **14e** in 91% yield. **14e**: mp= 122-125 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, *J* = 9.0 Hz, 1H), 7.41 (d, *J* = 8.0 Hz, 2H), 7.26 – 7.19 (1H, overlapping with $CDCl_3$), 7.13 (d, *J* = 7.7 Hz, 2H), 6.96 – 6.84 (m, 2H), 6.71 (dd, *J* = 9.1, 2.7 Hz, 1H), 6.52 (d, *J* = 2.7 Hz, 1H), 6.21 (t, *J* = 5.3 Hz, 1H), 5.21 (s, 2H), 4.37 (d, *J* = 5.3 Hz, 2H), 3.90 (s, 3H), 3.81 (s, 3H), 3.10 (s, 6H), 2.31 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.2, 168.5, 159.3, 151.6, 144.6, 140.3, 139.7, 139.1, 137.8, 134.3, 132.9, 130.2, 129.4, 129.3, 128.9, 128.6, 126.4, 114.2, 110.5, 102.7, 96.7, 74.3, 55.3, 52.3, 47.3, 40.4, 21.21; FT-IR (KBr): 3298, 2924, 2857, 1728, 1641, 1618, 1572, 1511, 1457, 1429, 1356, 1308, 1243, 1200, 1174, 1131, 816 cm^{1-}; HRMS *m/z* for $C_{32}H_{32}N_3O_6$ $[M + H]^+$ calcd 554.2291, found 554.2290.

Methyl 4-(cyclopropylmethoxy)-7-(dimethylamino)-2-((4-methoxybenzyl)amino)-3-oxo-3*H*-phenoxazine-1-carboxylate, 14f: Compound **13** (20 mg, 0.045 mmol) was dissolved in 0.15 mL of dry DMF. Caesium carbonate (7.2 mg, 0.022 mmol) and bromomethyl cyclopropane (4 μL, 0.045 mmol) were added and the mixture was stirred at rt overnight. Then, the mixture was concentrated and the residue was purified by flash column chromatography (eluent; ethyl acetate/hexane = 1/2) to afford 12 mg of **14f** in 54% yield. **14f**: mp= 189-192 °C ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (d, *J* = 9.0 Hz, 1H), 7.25 (d, *J* = 8.6 Hz, 1H), 6.93 – 6.83 (m, 2H), 6.72 (dd, *J* = 9.1, 2.7 Hz, 1H), 6.62 (d, *J* = 2.7 Hz, 1H), 6.18 (t, *J* = 5.3 Hz, 1H), 4.37 (d, *J* = 5.3 Hz, 2H), 4.00 (d, *J* = 7.2 Hz, 2H), 3.90 (s, 3H), 3.81 (s, 3H), 3.11 (s, 6H), 1.32 (m,1H), 0.65 – 0.35 (m, 2H), 0.43 – 0.12 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.3, 168.5, 159.3, 151.6, 144.7, 140.4, 139.7, 139.2, 133.3, 130.3, 129.4, 129.3, 126.4, 114.2, 110.5, 102.7, 96.8, 77.6, 55.3, 52.3, 47.3, 40.4, 10.9, 3.2; FT-IR (KBr): 3360, 2925, 2847, 1719, 1638, 1611, 1571, 1504, 1429, 1348, 1309, 1246, 1175, 1202, 1124 cm^{1-}; HRMS *m/z* for $C_{28}H_{30}N_3O_6$ $[M + H]^+$ calcd 504.2135, found 504.2138.

Molecular Docking Analysis: New derivatives based on the hit compound NCI8642 were designed and rationalized *in silico* prior to synthesis. These compounds form a library of a smile for matted file (*.smi) with the use of the program Open Babel, generating their conformers with the Omega v.2.5.1.4 software (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com).[29] All the docking experiments were performed on a typical desktop pc running Windows 7 64-bit operating system (Dual Core Intel Pentium 3.2 GHz CPU processors, RAM 8 GB), using the OEDocking suite v. 3.2.0.2 programs (OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com).[30] Based on the fact that the programs run with different algorithms, a greater probability of the predict model is offered. The visualization of the docking solutions is given by the software PyMol v. 1.4.1.[22]

Calculation of pharmacokinetic properties: Molecular descriptor values of the synthesized compounds were also calculated in an effort to export the drug-like profile for them (**Table S2**). Those include the factor of sp^3 molecular orbitals (Fsp^3), resembling the compound saturation levels (flatness), Lipinski's rule of five descriptors (where calculated logarithmic lipophilicity "clogPMB" was achieved with the use of program Marvin Beans v. 14.9.29) and spatial characteristics (volume and polar surface area) were calculated online with the freely available Molinspiration property calculation service (www.molinspiration.com).

Cell culture and preparation of mouse primary cortical neurons: HEK-293 human embryonic kidney cells overexpressing LRP6 (HEK-293LRP6) or DKK1 (HEK-293DKK1) and SH-SY5Y human neuroblastoma cells were cultured in a 5% CO_2 atmosphere at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, Athens, Greece) supplemented with 10% heat inactivated fetal bovine serum (FBS) (PAA Laboratories GmbH, Linz, Austria), 1% penicillin/streptomycin (Sigma Aldrich, Athens, Greece) and 10 μg/ml Blasticidine for HEK-293DKK1 and 100 μg/ml Zeocine for HEK-293LRP6. Confluent HEK-293DKK1 cells were cultured for 72 hours and the conditioned medium was collected, cleared from cell debris and floating cells by centrifugation and stored at -80 °C until use. Primary mouse cortex neuronal cultures were prepared from 16 days embryos as described and were treated with PG J2 after 6 days *in vitro*.[31]

DKK1 binding assay: Binding of DKK1 was performed as previously described by Iozzi and coworkers.[17] Briefly, HEK-293-LRP6 cells were seeded onto poly-D-lysine-coated coverslips 24 hours before the experiment and were incubated with DKK1 conditioned media (DKK1-CM) plus DMSO, DKK1-CM plus NCI8642 (100 μM) or DKK1-CM plus NCI8642 derivatives (100 μM) for 2 hours at 4 °C. After washing, the cells was fixed in 3%

paraformaldehyde (15 min, room temperature), blocked with PBS/0.1% BSA, and incubated with the primary rabbit-anti-DKK1 antibody, and fluorescent secondary antibody Alexa488 diluted into blocking solution. This antibody which emits fluorescence at 519 nM was very carefully chosen following the observation that some of the NCI8642 derivatives emit fluorescence at 570 nM. Cell nuclei were counterstained with DAPI. Images were collected using a fluorescence microscope and 750 cells were analysed each time using ZEN software.

Cell treatments and cell lysis: Mouse cortex primary neurons were treated with 20 μM PGJ2 and DMSO, 10 μM NCI8642 or 10 μM NCI8642 derivatives for 1 hour at 37 °C. Following treatment, cells were lysed at 4 °C in lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, pH 7.6, 1% Triton X-100 (v/v)] supplemented with complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Applied Science, Manheim, Germany). SH-SY5Y cells grown in Dulbecco's Modified Eagle's Medium (DMEM) that contained 10% fetal bovine serum, 1% of penicillin, and streptomycin in a 37 °C humidified incubator consisting of 5% CO_2 were treated with DKK1-CM plus DMSO, DKK1-CM plus NCI8642 (10 μM) or DKK1-CM plus NCI8642 derivatives (10 μM) for 1 h or 24 h. Following treatment, cells were lysed at 4 °C in lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, pH 7.6, 1% Triton X-100 (v/v)] supplemented with complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Applied Science, Manheim, Germany). Cell lysates were left on ice for 30 minutes and then centrifuged at 14,000 g for 12 min at 4 °C. Protein concentrations in supernatants were determined by using the Bradford protein assay.

Western blot and densitometry: Appropriate protein concentrations of cell lysates were prepared in Laemmli solubilisation buffer containing β-mercaptoethanol and analyzed by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis. Separated proteins were transferred to polyvinyldiene difluoride membranes (Roth GmbH, Karlsruhe, Germany) and immunoblotted with the appropriate dilutions of the primary antibody overnight at 4 °C. Primary antibodies were: Polyclonal rabbit antibody detecting mouse Tau phosphorylated at serine396 (1:1000) purchased from Acris Antibodies GmbH (Herford, Germany), and a polyclonal antibody directed against the N-terminus of Tau (1:1000) that was a kind gift of Dr. Luc Buee (Inserm, Lille, France), a polyclonal antibody phospho-GSK3β (Tyr216, Tyr279) from invitrogen and a polyclonal antibody against β-catenin (Acris Antibodies GmbH, Herford, Germany). The membranes were washed with Tris-buffered Saline-Tween 20 (TBS-T) three times for 5min and incubated with a 1:5000 dilution of goat anti-rabbit or anti-mouse horseradish peroxidise-conjugated secondary antibodies for 1 h at room temperature. Following washing with TBS-T, proteins were detected by chemiluminescence using the enhanced chemiluminescence system (GenScript Piscataway, NJ, USA) on a Fluorochem 8800 imaging station (AlphaInnotech, CA, USA). TIFF files of the images were viewed in ImageJ and band intensity was quantified with ImageJ software. The intensity measurements of the bands were normalized to the levels of actin. The results from three independent experiments were presented in graphs as mean±SD.

MTT assay: SH-SY5Y cultures were seeded in 96-well plates at a density of 25000 cells/well and 15000 cells/well. The cultures were grown for 6 days at 37 °C with 5% CO_2. Then, medium was changed to that containing various concentrations (10 μM or 100 μM) of NCI8642 and NCI8642 derivatives and incubated for 24 h at 37 °C with 5% CO_2. In all cases the final concentration of DMSO was 0.1%. 20 μL of MTT reagent (2.5 mg/mL MTT in PBS) was added to each well and incubated for 4 h. The resulting formazan dye was extracted with 100 μL isopropanol/HCl (100 mL isopropanol + 833 μL HCl) and the absorbance was measured spectrophotometrically at a wavelength of 545 nm.

Statistical analysis: All experiments were repeated three times. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to evaluate the statistical significance of the differences. Statistical significance was defined as $p<0.05$.

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Supplementary Material

Supplementary data (characterization data of the described compounds (^{1}H NMR and ^{13}C NMR spectra), predicted pharmacokinetic properties and docking results) associated with this article can be found, in the online version, at

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