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Structure-activity analysis and cell-based optimization of human galactokinase inhibitors

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Abstract

Classic Galactosemia is a rare human disease associated with the accumulation of toxic level of galactose-1-phosphate (gal-1P) caused by the inherited deficiency of galactose-1-phosphate uridyltransferase (GALT) activity. To reduce the toxic level of gal-1P in the patients, we have identified, *via* high-throughput screening, over 200 small molecule GALK inhibitors. We selected a 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile scaffold for further structure-activity relationships characterization, lead optimization with regards to potency and efficacy to reduce gal-1P accumulation in patient cells.

Keywords

galactokinase; galactose-1-phosphate; dihydrothiazinone; GHMP kinases; galactosemia

Classic Galactosemia (CG) is an inherited metabolic condition caused by deficiency of galactose-1-phosphate uridyltransferase (GALT, EC 2.7.7.12) activity.[1] GALT is the second enzyme in the evolutionarily conserved Leloir pathway of galactose metabolism, and facilitates the simultaneous conversion of uridine diphosphoglucose (UDP-glucose) and galactose-1-phosphate (gal-1P) to uridine diphosphogalactose (UDP-galactose) and glucose-1-phosphate.[2] Consequently, GALT deficiency leads to the accumulation of gal-1P and deficiency of UDP-galactose in patient cells.[3,4] If untreated, CG can result in a lethal disease in the affected newborn.[5] Ever since most states in the USA included this disorder in the newborn screening panel, neonatal morbidity and mortality have decreased considerably. The current mainstay of treatment is the withdrawal of (ga-)lactose from the diet.[5] However, it has become clear that despite optimal dietary management, chronic complications such as IQ deficits, ataxia, speech dyspraxia, premature ovarian insufficiency, and decreased bone mineralization persist in many affected adults.[6,7] In the past few years alone, at least six groups of investigators reported that the health-related quality of life consequences of galactosemic patients and their parents were worse than generally thought.[8-13] Such outcry of concerns for a relatively rare disease suggested that the stressful conditions suffered by the patients and their family members have been under-estimated for too long, and swift actions are required to improve the current situation. Moreover, little can we deny that the failure of galactose-restricted diet to prevent secondary complications has slowly eroded the

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SUPPORTING INFORMATION AVAILABLE: Experimental procedures and spectral data for the test compounds are available free of charge *via* the Internet at http://pubs.acs.org.

early success of newborn screening of this genetic disorder, and the medical community is yearning for a more effective therapy. To develop a more effective therapy, one must elucidate the pathogenic mechanisms of the disease and identify useful therapeutic targets. Although the precise pathophysiology of CG remain unexplained, decades of clinical observations,[14,15] confirmed recently by prospective outcome study,[16] showed that patients with galactokinase (GALK) deficiency rarely manifest the chronic complications seen in GALT-deficient patients. Note that GALK-deficient patients do NOT accumulate 10-20 fold increases of gal-1P over their lifetime as do GALT-deficient patients even when on galactose-restricted diets. Others and we confirmed these clinical findings in a *Saccharomyces cerevisiae* (Baker's yeast) model for CG. While a GALT-deficient mutant yeast was sensitive to galactose in growth medium, disruption of GALK function in this mutant reversed its susceptibility to galactose toxicity.[17-19] These findings support the pathological role of gal-1P in GALT deficiency in humans and yeast models for CG, but also raise the question about the origin of gal-1P, the enzymatic product of GALK on galactose, in a galactosemic patient who refrains from dairy products. It has been found that galactose moieties converted to gal-1P can also come from non-dairy sources, e.g., galactose-containing fruits and vegetables amounting to as much as 30mg/day.[20] However, galactose moieties can also be produced endogenously from UDP-glucose *via* the UDP-4-galactose epimerase (GALE) reaction, as well as from the natural turnover of glycolipids and glycoproteins. In fact, using isotopic labeling, Berry *et al*. elegantly demonstrated that a 50-kg adult male could produce up to 1.2 grams of galactose per day, which is many times of the amount of exogenous galactose potentially present in galactose-restricted diets.[21] Therefore, endogenous synthesis of galactose is likely to undermine the efficacy of dietary management as standard therapy. Since endogenous galactose production is not amenable to dietary manipulation, there is a need for innovative, non-dietary therapy. Since gal-1P, enzymatic product of GALK, is a major culprit for the complications seen in CG patients and GALK deficiency is more manageable than GALT deficiency, a few investigators have advocated the inhibition of human GALK as an innovative approach to treat CG.[16,22]

Previously, we initiated a campaign to identify small molecule inhibitors for the human GALK enzyme.[23,24] We hypothesized that GALK deficiency induced pharmacologically in GALT-deficient patients would significantly reduce gal-1P accumulation and prevent the chronic outcomes. Lastly, as for the uniqueness of the therapeutic target, human GALK is unique because even it phosphorylates galactose, it does *not* belong to the same family of other sugar kinases such as glucokinase (E.C. 2.7.1.2) or hexokinase (E.C. 2.7.1.1). Instead, it belongs to the superfamily of small molecule kinases, also known as the GHMP (**G**alactose, **H**omoserine, **M**evalonic acid, **P**hosphomevalonic acid) kinases family.[22,25]

In our previous work, we have identified over 150 small molecules inhibitors of human GALK *via* high-throughput screening (HTS).[23] We selected 34 compounds for further characterization.[24] Although their IC_{50} values were determined as 200nM to 33μM, their selectivity among the individual GHMP kinases varied and some were shown to be toxic to cells.[24] Thus, these first-generation compounds will require further optimization for therapeutic use. In this study, we chose 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile (**compound 1**) (Fig. 1) for structure activity relationship (SAR) studies because it shares similar aromatic core and functionality array with many of the positive hits.[24] The IC_{50} of compound 1 in the *in vitro* galactokinase inhibitory assay was 12μM, and therefore, our major objective is to improve its potency. We began by exploring nine commercially available compounds with structural similarities to compound **1** (Fig. 1). These compounds carried structural modifications in the A ring or the C ring with none in the central 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile core. In the *in vitro* GALK inhibitory assays, only compounds **2, 3**, and **4** possess inhibitory activities against GALK while the remaining compounds were not active below 50μM.

Based on these results, we rationalized that the presence of hydroxyl functional group(s) are necessary on the aryl ring A, since the active compounds have either a hydroxyl or a carboxylic acid group. However, not all the compounds with hydroxyl groups on the A ring displayed inhibitory activity as is the case with compound **7**. This compound shares the same structural similarity to compound **1** except that it has a thiobutyl group instead of the aromatic C ring. Therefore, the aromatic C ring is deemed necessary for activity since it was absent in compound **7**.

Another observation in our limited SAR studies revealed that compound **3** having 2-hydroxy aryl substituent in the A ring is active, whereas compound **10** with the same thiomethyl substituent at the 6^{th} position having 2,4-dichloro substituents in the A ring was inactive. This clearly suggests that electronic withdrawing hydrophobic groups at the 2- and 4-position may not be tolerated in the A ring for activity. Compound **9**, however, has a spiroketal carboxyl group instead of an aryl carboxyl group exhibited no activity. Similarly, compounds **5, 6** and **8** that do not have aromatic A ring, did not display any inhibitory activity. This further underscores the preference of an aromatic ring as the A ring adjacent to the 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile core.

Through the above SAR studies, the IC_{50} of compound **2** with two aryl hydroxyl groups from this selection was determined to be in the low micromolar range (1.4 ± 0.7 μM) for GALK in contrast to the original compound **1**, which has an IC_{50} of 12μM.

In order to modify compound **2** for further optimization of potency, it was considered necessary to identify the functional groups that are needed for potency. As a result, new compounds with different positions of the hydroxyl groups in the A ring were designed and synthesized by a procedure described by Yokoyama[26,27] where many commercially available aldehydes such as aldehyde **11** was treated with mercaptoacrylamide **12**[27] in the presence of catalytic HCl in MeOH under refluxing conditions and afforded solid compound **2** in 61 % yield (Scheme 1).

The IC_{50} of these compounds were subsequently determined and used to assess the efficacy of the positions of the aryl hydroxyl groups in the newly designed inhibitors (Table 1). Results showed that compound **14** with the *meta*-hydroxyl group exhibited a very high IC_{50} of 50μM. When the hydroxyl group was changed to the *para* position for compound **15**, the potency of this compound dramatically improved to an IC_{50} of 1.8μM. When this aryl hydroxyl group was substituted with $–OCH_3$ functionality in compound **16**, the GALK inhibitory activity was abolished (IC_{50} > 50μM).

Similarly, in compound **17**, the substitution for benzo[*d*][1,3]dioxole group caused an increase in the IC_{50} (23μM). The thiazinone compounds with 2,4- and 2,5- aryl hydroxyl substitutions were assayed for inhibitory activities and compound **19** with the 2,5-dihydroxy functional group was more potent than the 2,4-dihydroxy thiazione derivative **18**.

It was previously realized through computational modeling studies that the aromatic C ring of the 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile core mimics the adenine moiety of ATP within the active site of human GALK crystal structure as shown in Fig. 2. Reference protein coordinates used for structure-based lead optimization were taken from the X-ray structure of the human GALK in complex with the non-hydrolysable ATP analog, AMP-PNP (PDB: 1WUU)[28], and were used in all computational experiments using ICM suite. The active site for the GALK protein was defined as being within 8Å of AMP-PNP in the X-ray co-crystal structure. Energy grids representing the active site (*van der* Waals, hydrogen bonding, electrostatics, and hydrophobic interactions) were calculated with 0.5Å grid spacing, and docking experiments were performed using the defined AMP-PNP binding pocket with the application of our docking workflow.

The *meta* position to the thiobenzyl group of the C ring could be modified with a morpholine group to further stabilize the binding of the molecule to the adenine binding site. Computational studies suggested this group was well tolerated within the 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile scaffold and its binding mode within the ATP site of GALK. Hence, morpholine derivatives of compounds **2, 15**, and **19** were synthesized with the aim to further improve the potency (Scheme 2). We chose the 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile scaffold **2, 15** and **19** (Table 1) to prepare new morpholine derivatives **25, 27** and **29** (Scheme 2) because we had previously determined that the compounds **2, 15** and **19** had IC_{50} values of 1.4, 1.8 and 0.7μM respectively (Table 1), which were among the lowest IC_{50} values in our *in vitro* GALK assays. As a result, the preparation of their morpholine derivatives was pursued by the condensation of acrylamide **23** with aldehydes **24, 26** and **28** (Scheme 2).

The synthesis of morpholine **25** (Scheme 2) began with the transformation of benzylic alcohol **20** to bromide **21** by the addition of NBS/DMS cocktails.[29] A slow addition of bromide **21** to the solution of bis(ammoniothio)-2-cyanoacrylamide **22** selectively afforded acrylamide **23** in 55% from alcohol **20**. The synthesis of morpholine **25** was completed by the treatment of acrylamide **23** with aldehyde **24** under acid catalyzed conditions in MeOH with a fair yield of 35%.

We determined the IC_{50} values of the morpholine derivatives and the results were shown in Table 2.

Initially, the intracellular inhibition of GALK and the subsequent lowering of gal-1P for compound **2** with an IC_{50} value of 1.4μM could not be determined in patient cells because of its toxicity. In the subsequent cell-based studies, compound **2** in its protected form as diacetate **13** (Scheme 1) continued to be toxic to the human fibroblast cells at either 50μM or higher concentrations and resulted in total cell lethality within hours. However, compound **25** with an IC_{50} value of 1.2μM (Table 2) was tolerated at 50μM by the cells without visible signs of lethality. Therefore, we proceeded to determine the intracellular inhibitory activity of morpholine derivative **25** against GALK by measuring the reduction of gal-1P accumulation. This compound inhibited GALK in patient fibroblast cells and lowered cellular gal-1P by 16% at 50μM.

Additionally, morpholine derivative **27** was also well tolerated in cells at up to 100μM and reduced cellular gal-1P by 16% at 100μM without gross cell lethality, while morpholine derivative **29** was proved to be lethal to cells at 100μM or higher. However at 50μM, this compound was well-tolerated. Unlike compound **25**, neither **27** nor **29** was able to inhibit GALK or lowered gal-1P at 50μM.

Unknown cellular side reactions with 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile scaffold might have limited the inhibitory potential of these morpholine derivatives to further lower the cellular gal-1P below 16% at 50μM or 100μM. Based on this observation we have considered the design and synthesis of new 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile scaffold containing compounds that might be much less prone to cellular side-reactions and our on-going efforts will be reported in the near future.

In summary, we have conducted limited SAR studies and optimization on a selected 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile chemotype identified previously as a GALK inhibitor and was initially toxic. The new series of compounds showed improved *in vitro* potency, improved cell tolerance and efficacy to partially inhibit GALK in cell-based assays. Since the accumulation of gal-1P plays a pathological role in the disorder CG, the discovery of the oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile scaffold substituted with morpholine

is first in the class of new compounds that can be explored as therapeutic agents to lower cellular gal-1P in patient cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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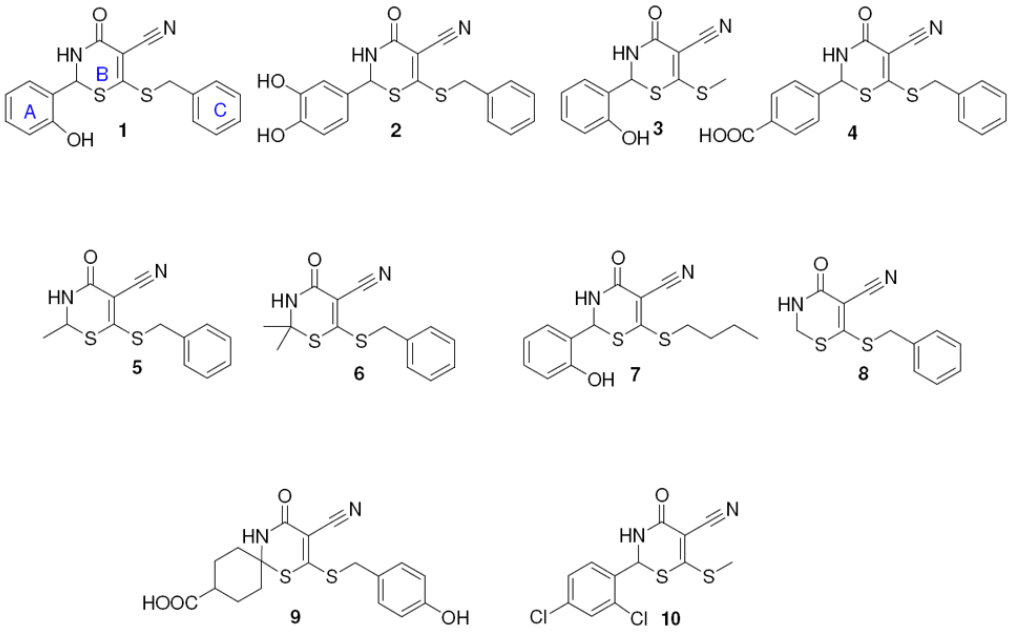


Fig. 1.
Lead compounds selected for SAR studies.

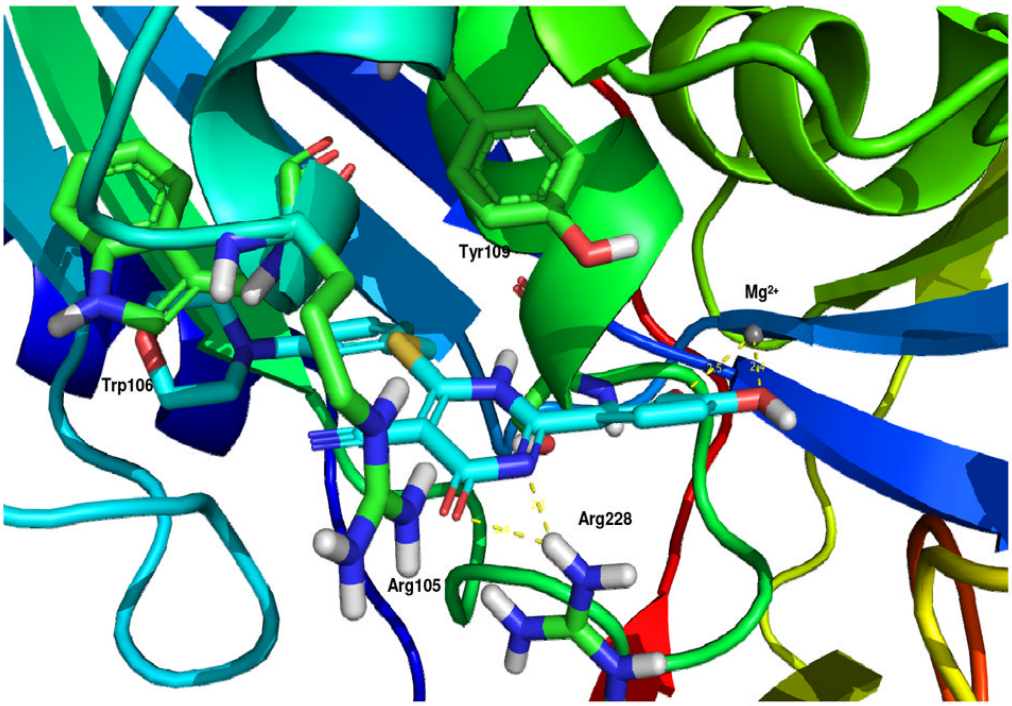
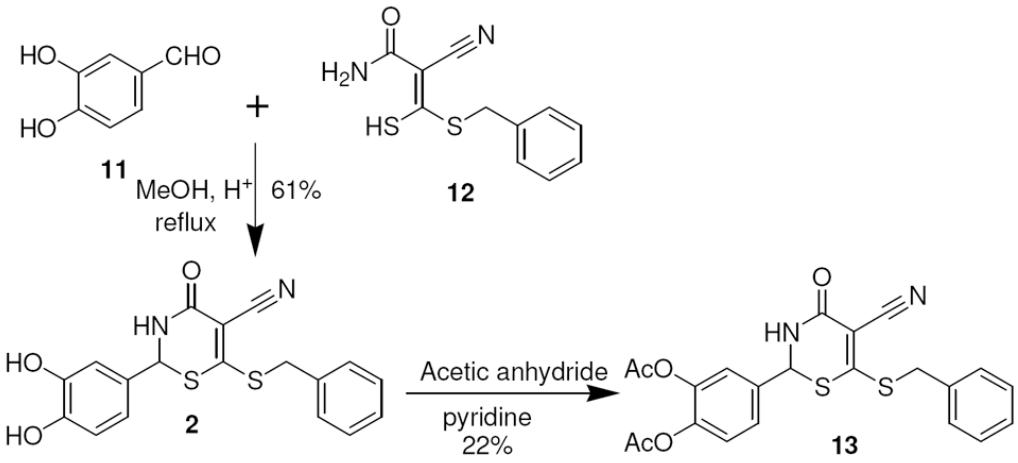
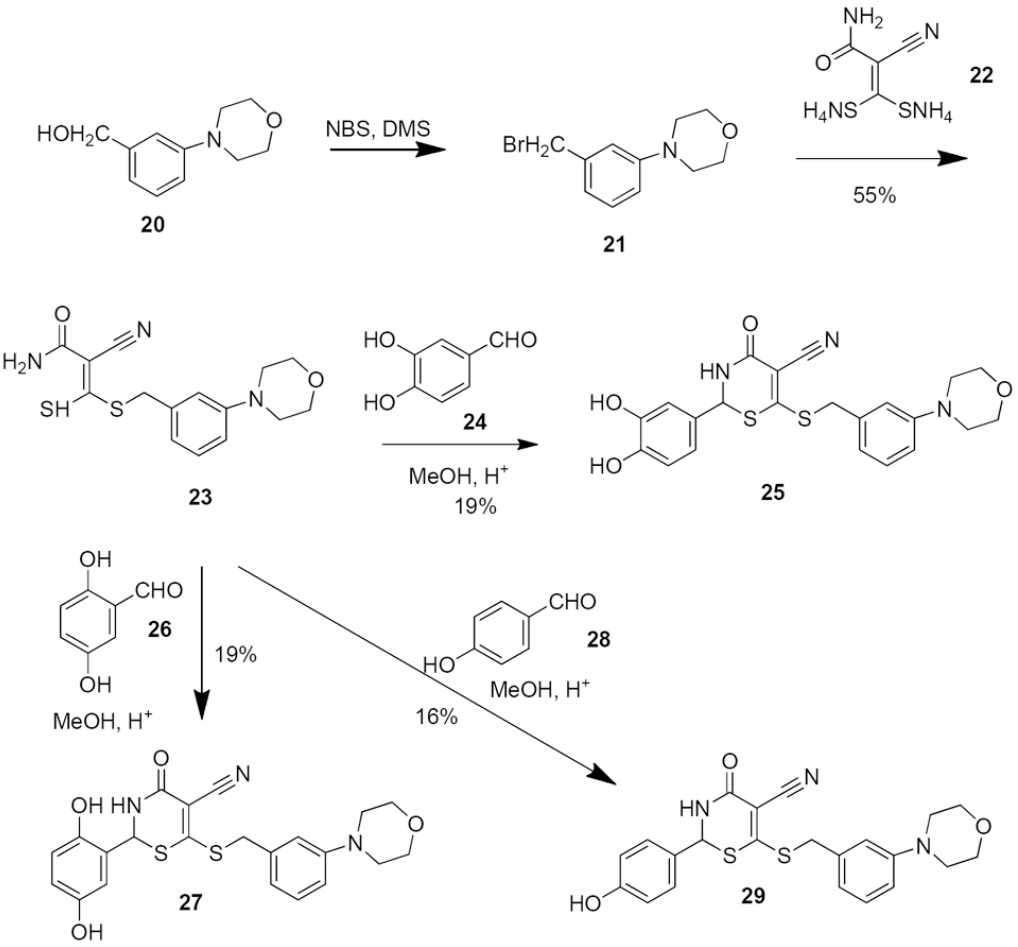


Fig. 2. Mode of binding of compound 25 in complex with GALK

The active site ATP pocket is shown with critical residues which are in contact with central 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile. The endocyclic amide-Arg228 interactions are depicted as dashed yellow lines. Similarly the dihydroxy-substituted aryl ring is positioned to γ-phosphate binding site and positioned within the distance to Mg^{2+}. The Tyr109 is also with in the distance for wedge-face ππ interactions with morpholine-substituted aryl ring.



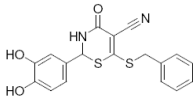
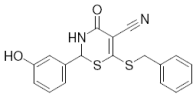
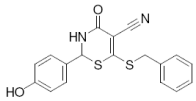
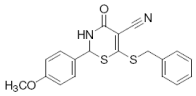
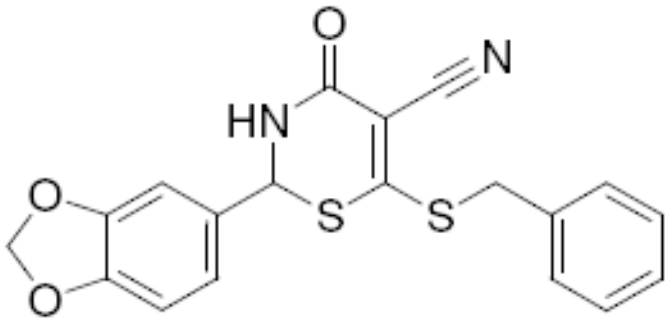
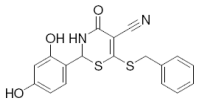
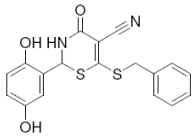
Scheme 1.
General synthesis of 4-oxo-3,4-dihydro-2*H*-1,3-thiazine-5-carbonitrile by refluxing with acidic MeOH.



Scheme 2.
Synthesis of substituted morpholine derivatives of compounds **2, 15, 19**.

Table 1

The IC_{50} values of synthesized thiazinones derivatives of compound **1**.[a,b]

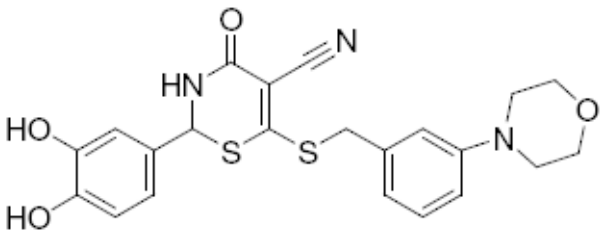
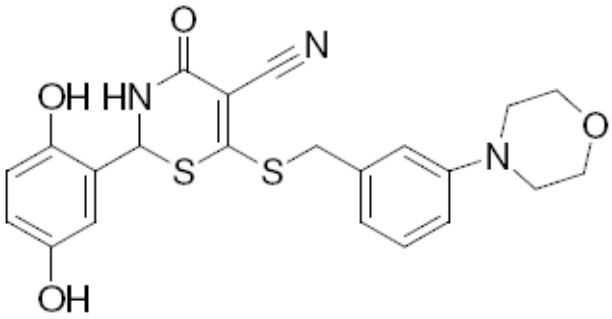
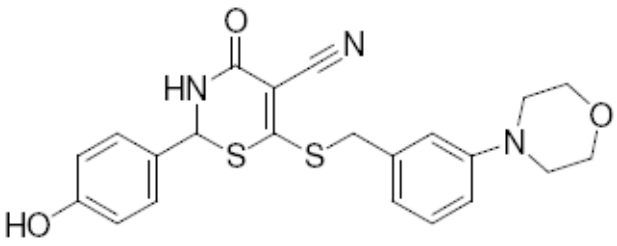
Compound	Structure	IC_{50} (μM)
1		1.4 ± 0.7
14		>50
15		1.8 ± 0.3
16		>50
17		22.5 ± 0.7
18		1.2 ± 0.7
19		0.7 ± 0.1

[a] Experimental conditions for IC_{50} determination are provided in the section of Supporting Information.

[b] Standard deviations were calculated from 3 replicate measurements

Table 2

The IC_{50} values of synthesized morpholine derivatives 25, 27, 29.[a,b]

Compound	Structures	IC_{50} (μM)
25		1.2 ± *0.1*
27		0.9 ± *0.4*
29		2.4 ± *0.8*

[a] Experimental conditions for IC_{50} determination are provided in the section of Supporting Information.

[b] Standard deviations were calculated from 3 replicate measurements