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Optimization of a series of heterocycles as survival motor neuron gene transcription enhancers

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ABSTRACT

Spinal muscular atrophy (SMA) is a neurodegenerative disorder that results from mutations in the *SMN1* gene, leading to survival motor neuron (SMN) protein deficiency. One therapeutic strategy for SMA is to identify compounds that enhance the expression of the *SMN2* gene, which normally only is a minor contributor to functional SMN protein production, but which is unaffected in SMA. A recent high-throughput screening campaign identified a 3,4-dihydro-4-phenyl-2(1H)-quinolinone derivative (**2**) that increases the expression of *SMN2* by 2-fold with an EC_{50} = 8.3 µM. A structure-activity relationship (SAR) study revealed that the array of tolerated substituents, on either the benzo portion of the quinolinone or the 4-phenyl, was very narrow. However, the lactam ring of the quinolinone was more amenable to modifications. For example, the quinazolinone (**9a**) and the benzoxazepin-2(3H)-one (**19**) demonstrated improved potency and efficacy for increase in *SMN2* expression as compared to **2**. 2017 Elsevier Ltd. All rights reserved.

Spinal muscular atrophy (SMA) is a neurodegenerative disease characterized by progressive muscle wasting, loss of motor function and premature death in the most severe cases.[1-3] Two genes, *SMN1* and *SMN2*, produce survival motor neuron (SMN) protein,[4-6] which is ubiquitously expressed with the highest levels in the spinal cord[7] and functions in the assembly of spliceosomal small nuclear ribonucleoproteins.[8] The majority of SMN protein normally is produced from the *SMN1* gene, while the almost identical *SMN2* gene only produces approximately 10% of functional protein.[9,10]

SMA results from mutations within exon 7 of *SMN1*, leading to SMN protein deficiency.[11] The clinical severity of this disease is, therefore, indirectly proportional to the copy number of *SMN2* genes, the sole source of protein in these patients.[12,13] Although the threshold level of SMN protein necessary to maintain healthy motor neurons is not known, it has been estimated that increasing the amount of functional protein by only 2- or 3-fold may be clinically significant.[14] Thus, the *SMN2* gene has become a therapeutic target, in which multiple strategies (*i.e.*, small molecules, antisense oligonucleotide) work to increase the transcription of *SMN2*, increase the inclusion of exon 7 *SMN2*, stabilize the full-length exon-7 included *SMN2* mRNA, or stabilize the SMN protein.[15-17] As SMA is the leading heritable cause of infant mortality, it is critical to build upon the one recently FDA-approved treatment, Nusinersen, an antisense oligonucleotide[18] and to provide a variety of treatment options with different modes of action. Several repurposed drugs, such as riluzole, phenylbutyrate, valproic acid, albuterol and hydroxyurea, have advanced into clinical trials, but none has elicited convincing improvement in muscle function or survival in SMA. Currently, there are several small molecules in Phase I

to Phase III clinical trials for SMA, including compounds that increased exon 7 inclusion of SMN2.[19]

Previously, we reported an *SMN2*-lucifrase reporter assay for identifying compounds that increase SMN expression from the *SMN2* gene and its use in high-throughput screening.[20] Using the reporter assay, we discovered two hit compounds, **LDN-75654** (**1**) and **LDN-76070** (**2**) (**Figure 1**), that increase expression of *SMN2* by 2-fold, but that have different mechanisms of action than the small molecules already in clinical trials to treat SMA. Compound **1** increases the stability of SMN protein, whereas compound **2** acts in a transcriptional manner.[21]

We recently reported the structure-activity relationship (SAR) of analogs of the 5-isopropylisoxazole-3-carboxamide **1**.[22] In this paper, we report preliminary SAR of the 3,4-dihydro-4-phenyl-2(1H)-quinolinone **2** for increasing SMN expression.

LDN-75654 (1)
EC_{50} = 1.8 µM (242%)
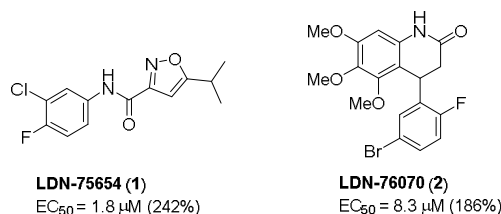
LDN-76070 (2)
EC_{50} = 8.3 µM (186%)

**Figure 1.** Two SMN mRNA expression enhancers identified following high-throughput screening.

The 3,4-dihydro-4-phenyl-2(1H)-quinolinone derivatives were prepared according to the procedure outlined in **Scheme 1**. A three-component coupling of an aniline (**3**), aromatic aldehyde (**4**) and Meldrum's acid in refluxing ethanol generated the

quinolinones **5a-m** in good yield.[23] These compounds were transformed further into the methyl substituted derivatives **6a-b**, which were isolated as mixture of *cis-* and *trans-*isomers.
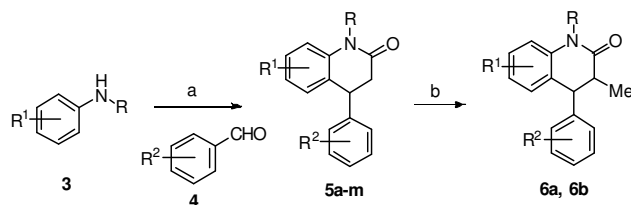

Scheme 1. (a) Meldrum's acid, EtOH, 85ºC, 16 h (55-78%); (b) BuLi, THF, 0ºC then MeI (48%).

A series of 3,4-dihydro-quinazolinone derivatives **9a-9l** was prepared according to the procedures outlined in **Scheme 2**.[24] Anilines **3** were treated with potassium isocyanate to give the ureas **8** (*e.g.*, R = H). Alternatively, phenyl isocyanates **7** were treated with primary amines to yield substituted ureas **8** (*e.g.*, R = alkyl). The ureas then were treated with catalytic methane sulfonic acid in refluxing toluene, with azeotropic removal of water, to produce the 3,4-dihydro-quinazolinones **9a-9r**.
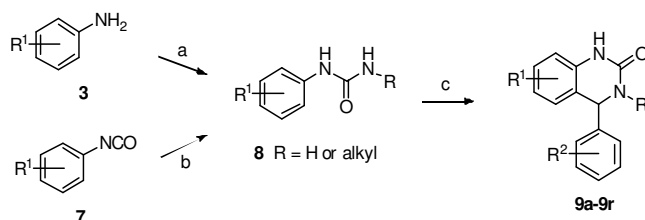
Scheme 2. (a) KNCO, AcOH, RT; (84-97%); (b) RNH2, THF, 0 ºC to RT (63-89%); (c) R²PhCHO, cat. MeSO3H, toluene, reflux (31-81%).

The synthesis of the 2-quinazolinone **13** and the seven-membered 1,4-benzodiazepin-2-one **14** is outlined in **Scheme 3**. The pivolate-protected 3,4,5-trimethoxyaniline **10** was lithiated with butyllithium in THF at -78ºC, and the resulting anion was quenched with methyl 5-chloro-2-fluorobenzoate to give the ketone **11** in modest yield. The pivolate group was cleaved with refluxing sulfuric acid and gave the versatile intermediate **12**. For example, the ketone **12** was treated with trichloroacetyl chloride, and the product, upon treatment with ammonium acetate, cyclized to **13**. On the other hand, the ketone **12** also was treated with glycine ethyl ester, with azeotropic removal of water, and gave the 1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one **14**.
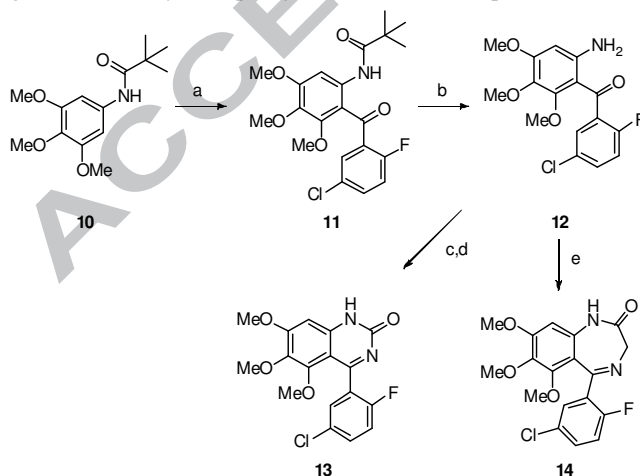
Scheme 3. (a) BuLi, THF -78ºC then methyl 5-chloro-2-fluorobenzoate (15%); (b) H2SO4, EtOH, reflux (57%); (c) Cl3CCOCl, DCM, Et3N, RT; (d) NH4OAc, DMSO, 80ºC (41% over

2-steps); (e) HCl•NH2CH2CO2Et, Py, Dean-Stark trap, reflux, 24 h (38%).

Several oxygen-containing heterocycles (*e.g.*, **16**, **19-21**) also were prepared from the ketone **12** (**Scheme 4** and **Scheme 5**). Ketone **12** was reduced to the racemic alcohol **15** with sodium borohydride in ethanol. Treatment of **15** with carbonyl diimidazole (CDI) directly gave the oxazinone **16** in good yield. Acylation of **15** with 2-bromoacetyl chloride or 2-bromopropionyl chloride gave the amides **17** and **18**, respectively. Treatment of **17** with two equivalents of sodium hydride gave the 7-membered benzoxazepin-2(3H)-one **19** in moderate yield.[25] Similarly, cyclization of **18** gave the 3-methyl-benzoxazepin-2(3H)-one **20** as a mixture of diastereoisomers. Finally, treatment of **19** with methyl iodide gave the *N*-methyl amide **21**.
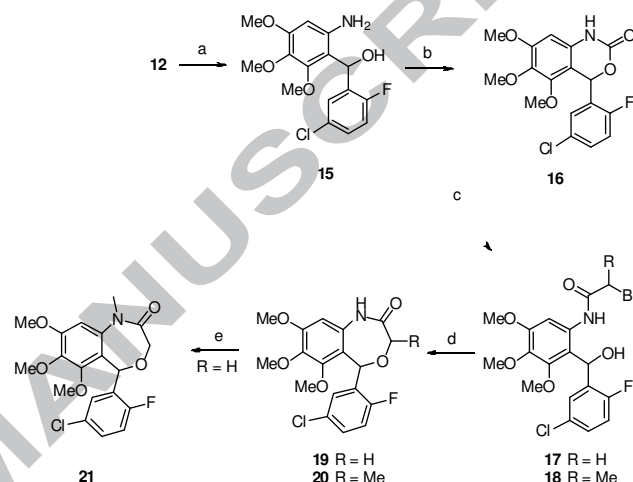
Scheme 4. (a) NaBH4, EtOH, RT (82%); (b) CDI, THF, RT (76%); (c) BrCHRC(O)Cl, Et2O, Et3N, 0ºC to RT (62-73%); (d) NaH, THF, RT then reflux (43-72%); (e) MeI, Cs2CO3, THF, RT (81%).

The synthesis of the 5-methyl substituted benzoxazepinone **23** is outlined in **Scheme 5**. Treatment of the ketone **12** with methyl magnesium bromide gave the racemic tertiary alcohol **22** in good yield. Reaction of **22** with 2-bromoacetyl chloride and cyclization gave **23**.
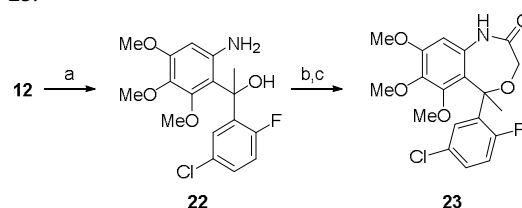
Scheme 5. (a) MeMgBr, THF, 0ºC (67%); (b) BrCH2C(O)Cl, Et2O, Et3N, 0ºC to RT; (c) NaH, THF, RT then reflux (54% over 2-steps).

Compounds were assessed for activity in the luciferase reporter assay, as previously described, at various concentrations (0.1 – 50 μM), and were compared to DMSO (0.1%) controls.[19] Dose-response curves were generated to determine EC_{50} values and percent maximum increase in *SMN2* expression. Unless otherwise stated, all values are the mean ± S.E.M. of 3 separate experiments. First, the importance of the tri-methoxy group on the aryl ring and methyl substitution at each of the 1- and 3-positions of the dihydro-quinolinone was investigated (**Figure 2**). Removal of either the 7- or 6-methoxy group (*e.g.*, compounds **5a** and **5b**) resulted in the complete loss of activity. With this information, the methoxy groups were determined to be necessary for activity, and the tri-methoxy group was retained in the remaining analogs described in this optimization study.

Similarly, the 1- and 3-methyl analogs (*e.g.*, **6a** and **6b**) were inactive, and these two positions were left unsubstituted in


5a Inactive   **5b** Inactive   **6a** R_1 = Me, R_2 = H Inactive
6b R_1 = H, R_2 = Me Inactive
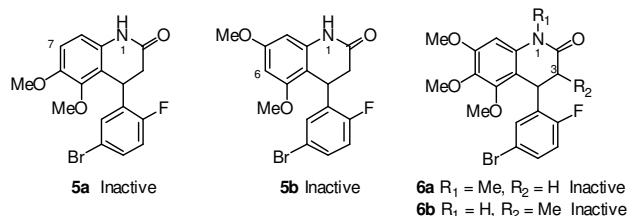
subsequent analogs.

Figure 2. Effects of deletion of individual methoxy substituents and methyl substitution to the dihydro-quinolinone core.

We next investigated the effects on *SMN2* expression of substituents on the 4-aryl ring (**Table 1**). Compounds were prepared following the cyclization procedure outlined in **Scheme 1**. Removal of either the 5-bromo (*e.g.*, **5c**) or the 2-flouro (*e.g.*, **5d**) led to a complete loss in activity, indicating the importance of a substituent at both positions. Therefore, we retained the 2-flouro substituent and varied the 5-substituent. Replacing the 5-bromo by 5-chloro gave an analog (**5e**) of similar activity to **2**, but other electron withdrawing groups were not active (*e.g.*, **5f**, **5g**, **5h** and **5i**). Several other halogenated analogs also were found to be inactive (*e.g.*, **5j**, **5k**, **5l** and **5m**).

Table 1. Effects of substitution on the aryl ring of the 3,4-dihydro-4-aryl-2(1H)-quinolinone core.

2, **5a-5m**
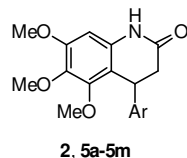
#	Ar	EC$_{50}$ (μM)	#	Ar	EC$_{50}$ (μM)
2	2-F,5-Br	8.3 (186%)	**5c**	2-F	IA
5d	3-Br	IA	**5e**	2-F,5-Cl	12 (150%)
5f	2-F,5-F	IA	**5g**	2-F,5-CN	IA
5h	2-F,5-NO$_2$		**5i**	2-F,5-CONH$_2$	IA
5j	2-Cl,3-Cl	IA	**5k**	2-Cl,4-Cl	IA
5l	3-F,4-F	IA	**5m**	3-Cl,4-Cl	IA

After the disappointing finding that limited substitution was tolerated on the 4-aryl ring, we chose to explore other cyclic cores. The goal was to identify a more potent core and then to revisit the SAR on the 4-aryl ring. Although the 5-bromo-2-fluoro aryl analog **2** was the most active compound, for chemical stability reasons, we chose to retain the 5-chloro-2-fluoro aryl ring at the benzylic position of the new cores (**Table 2**). The 3,4-dihydro-quinazolinone derivative **9a** had very promising activity, although the *N*-methyl (**9b**) was less active, and both the *N*-propyl (**9c**) and the 2-quinazolinone (**13**) were inactive. The seven-membered benzodiazepin-2-one **14** retained potency similar to the lead **2**, although its efficacy was reduced. Several of the oxygen containing heterocycles had interesting activity.

The benzoxazin-2-one **16** had encouraging activity, and the 7-membered benzoxazepin-2(3H)-ones, **19** and **20**, had similar activities as **9a**. Interestingly, the 3-methyl analog **20** was tested as a mixture of diastereoisomers, and it is likely that one isomer will retain all activity. *N*-Methylation of the amide (**21**) was not

tolerated, and introduction of a methyl group at the benzylic position (*e.g.*, **23**) also was not tolerated.

Table 2. Effects of the heterocyclic core (Ar = 2-F,5-Cl-phenyl)

#	Compound	#	Compound
9a	EC$_{50}$ = 4.1 μM (263%)	**9b**	EC$_{50}$ = 6 μM (180%)
9c	Inactive	**13**	Inactive
14	EC$_{50}$ = 8.4 (125%)	**16**	EC$_{50}$ =? (125%)
19	EC$_{50}$ = 5.9 (150%)	**20**	EC$_{50}$ = 2.6 (125%)
21	Inactive	**23**	Inactive

Given the promising activity of the 3,4-dihydro-quinazolinone core (*e.g.*, **9a**), we evaluated the SAR for aryl substitution with this heterocyclic core (**Table 3**). Removal of either the 5-chloro (*e.g.*, **9b**) or the 2-flouro (*e.g.*, **9c**) led to a partial, but not a complete, loss in activity. Other mono-halogenated analogs retained some activity (*e.g.*, **9d**, **9e** and **9g**), although the methoxy analogs (**9h-9j**) were inactive. In the next set of analogs, the 2-fluoro substituent was retained, and the 5-substituent was varied. The 5-methyl analog **9k** had similar potency, and the 5-fluoro **9n** was less active. The other 5-substituted analogs (**9l**, **9m** and **9o**) were inactive but moving the 5-chloro to the 4-position (**9p**) was tolerated. Although the 2-methoxy analog (**9h**) activity was restored on incorporation of a 4-chloro (**9q**) or 5-chloro (**9r**).

Table 3. Effects of substitution on the aryl ring of the 3,4-dihydro-4-aryl-quinazolin-2(1*H*)-one core.

9a-9l
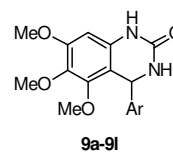
#	Ar	EC$_{50}$ (μM)	#	Ar	EC$_{50}$ (μM)
9a	2-F,5-Cl	4.1 (263%)	**9b**	2-F	10 (100%)
9c	3-Cl	5.2 (100%)	**9d**	3-F	25 (150%)
9e	4-F	14.0 (150%)	**9f**	2-Cl	IA
9g	4-Cl	6.6 (150%)	**9h**	2-OMe	IA

9i	3-OMe	18 (100%)	**9j**	4-OMe	IA
9k	2-F,5-Me	4.3 (125%)	**9l**	2-F,5-Ph	IA
9m	2-F,5-CN	IA	**9n**	2-F,5-F	17 (100%)
9o	2-F,5-OMe	IA	**9p**	2-F,4-Cl	6.3 (150%)
9q	2-OMe,4-Cl	7.8 (200%)	**9r**	2-OMe,5-Cl	3.6 (225%)

In the preliminary SAR of the quinazolinone series, we did not establish the importance of the 5-methoxy group on SMN enhancing activity (*e.g.*, deletion of the 5-OMe). We did, however, establish the importance of a methoxy group at the analogous position in the benzoxazepin-2(3H)-one series. The synthesis of **26** is outlined in **Scheme 6**. Palladium-catalyzed addition of the commercially available 5-chloro-2-fluorophenylboronic acid and 2-amino-4,5-dimethoxybenzonitrile **24** gave the corresponding ketone, which then was reduced with sodium borohydride in ethanol to give the racemic alcohol **25**. Reaction of **25** with 2-bromoacetyl chloride, and cyclization as described previously, gave the 7,8-dimethoxy analog **26**. Compound **26** was determined to be inactive in the luciferase reporter assay. Combined, the lack of activity for **5a**, **5b**, and **26** establishes the importance of all three methoxy groups for
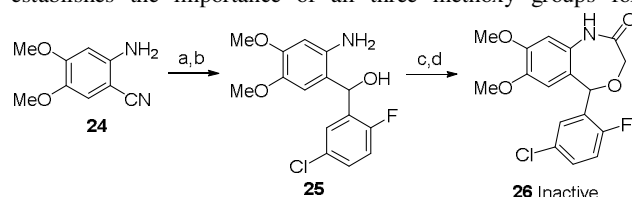

activity.

Scheme 6. (a) 2-F,5-Cl-PhB(OH)$_2$, Pd(OAc)$_2$, DMSO, 24 h, 90°C (79); (b) NaBH$_4$, EtOH, RT (77%); (c) BrCH$_2$C(O)Cl, Et$_2$O, Et$_3$N, 0°C to RT (67%); (d) NaH, THF, RT to reflux (71%).

Two promising analogs, the quinazolinone (**9a**) and the benzoxazepin-2(3H)-one (**19**), were evaluated for aqueous solubility and stability in mouse microsomes (**Figure 3**). Both **9a** and **19** had improved aqueous solubility, **9a** had promising stability in mouse microsomes although **19** was poor.

Compound **9a** was further characterized in secondary assays. We observed a ~2-fold increase in total SMN protein as well as a 2-fold increase in the number of gems in SMA derived fibroblasts following treatment with **9a**. Consistent with the earlier discovery that compound **2** acts in a transcriptional manner,[21] compound **9a** increased SMN-luciferase protein expression from both the SMN1 and SMN2-luciferase constructs. qRT-PCR analysis revealed that compound **9a** increased the amount of total SMN-luciferase reporter transcripts with a corresponding increase in the amount of exon 7 included transcripts. This effect required 24 hours for full activation and is consistent with the hypothesis that this series is regulating SMN2 expression at the
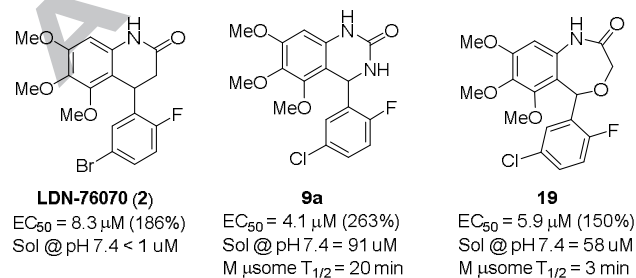
transcriptional level.

Figure 3. Solubility and stability of **9a** and **19**.

Compound **9a** was next tested in a preliminary study in SMNΔ7 mice. Animals were injected with 20 mg/kg of compound **9a** in DMSO by intraperotineal injection once daily starting on PND 2. Compound **9a** gave a significant increase in lifespan (*e.g.*, 15-day median survival) compared to vehicle treated animals (*e.g.*, 6-day median survival). Compound **9a** treated animals also displayed an increase in the ability to right themselves when compared to vehicle treated animals.

Racemic **9a** was next separated by supercritical fluid chromatography, using a ChiralPak IC column, into its two individual enantiomers in > 99% enantiomeric excess. Testing of the individual enantiomers revealed that the fastest eluting enantiomer was inactive, whereas the second enantiomer retained all activity in the SMN2 luciferase reporter assay (EC$_{50}$ = 1.3 μM, 185%).

In summary, we established preliminary SAR of the quinazolinone series and discovered that most changes to the aryl ring substituents led to a loss in activity. We discovered several alternative cores (*e.g.*, quinazolinone the benzoxazepin-2(3H)-one) that had similar or better activity than **2** and the SAR of the aryl ring was expanded. Compound **9a** was identified as a more active analog with improved solubility and stability in mouse microsomes. In an efficacy study in SMNΔ7 mice, a 2-fold extension in survival was observed following daily treatment with **9a**. The synthesis, determination of absolute stereochemistry and biological evaluation of single enantiomers of **9a** and related analogs is in progress and will be reported in due course.

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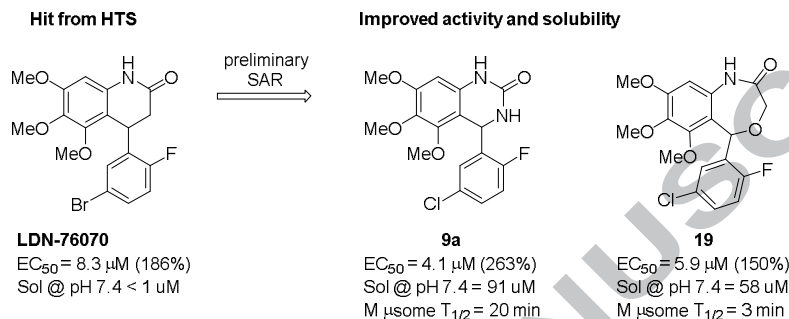
Graphical Abstract

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Optimization of a series of heterocycles as survival motor neuron gene transcription enhancers

Sungwoon Choi[a], Alyssa N. Calder[a], Eliza H. Miller[a], Kierstyn P. Anderson[a], Dawid K. Fiejtek[a], Anne Rietz[b], Hongxia Li[b], Jonathan J. Cherry[b], Kevin M. Quist[b], Xuechao Xing[a], Marcie A. Glicksman[a], Gregory D. Cuny[a], Christian L. Lorson[c], Elliot A. Androphy[b] and Kevin J. Hodgetts[a*]

Hit from HTS

preliminary SAR

Improved activity and solubility

LDN-76070
$EC_{50} = 8.3\ \mu M$ (186%)
Sol @ pH 7.4 < 1 uM

9a
$EC_{50} = 4.1\ \mu M$ (263%)
Sol @ pH 7.4 = 91 uM
M μsome $T_{1/2}$ = 20 min

19
$EC_{50} = 5.9\ \mu M$ (150%)
Sol @ pH 7.4 = 58 uM
M μsome $T_{1/2}$ = 3 min

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