Brain Penetrable Inhibitors of Ceramide Galactosyltransferase for the Treatment of Lysosomal Storage Disorders

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ABSTRACT: Metachromatic leukodystrophy (MLD) is a rare, genetic lysosomal storage disorder caused by the deficiency of arylsulfatase A enzyme, which results in the accumulation of sulfatide in the lysosomes of the tissues of central and peripheral nervous systems, leading to progressive demyelination and neurodegeneration. Currently there is no cure for this disease, and the only approved therapy, hematopoietic stem cell transplant, has limitations. We proposed substrate reduction therapy (SRT) as a novel approach to treat this disease, by inhibiting ceramide galactosyltransferase enzyme (UGT8). This resulted in the identification of a thienopyridine scaffold as a starting point to initiate medicinal chemistry. Further optimization of hit compound **1** resulted in the identification of brain penetrable, orally bioavailable compound **19**, which showed efficacy in the *in vivo* pharmacodynamic models, indicating the potential to treat MLD with UGT8 inhibitors.

KEYWORDS: Arylsulfatase A enzyme, lysosomal storage disorder, metachromatic leukodystrophy, uridine diphosphate-galactose galactosyltransferase 8

Uridine diphosphate-galactose ceramide galactosyl-transferase (CGT or UGT8) is an enzyme within the UDP-glycosyltransferase enzyme family which catalyzes the transfer of galactose from UDP-galactose to ceramide¹ to make galactosylceramide (GalCer), a key enzymatic step in the sphingolipid biosynthetic pathway (Figure 1). GalCer is a substrate for galactose-3-O-sulfotransferase 1 (GAL3ST1) or cerebroside sulfotransferase (CST), which transfers sulfate to GalCer, yielding 3-O-sulfogalactosylceramide (sulfatide, SFT). SFT is a major component of the myelin sheath in the central nervous system (CNS) and peripheral nervous systems (PNS). The inappropriate recycling of SFT can result in the alteration and destruction of the myelin structure, degrading the normal physiological transmission of electrical impulse between nerve cells.

Arylsulfatase A enzyme (ARSA; EC 3.1.6.8), a lysosomal sulfatase, hydrolyzes the 3-O sulfate ester bond from SFT in the presence of activator protein, Saposin B (Figure 1). A total of over 250 mutations in arylsulfatase A (ARSA) gene have been reported in different populations. Mutations^{2,3} in ARSA gene cause functional deficiency of ARSA, resulting in the accumulation of SFT in the lysosomes of the tissues in the CNS and PNS. This leads to progressive demyelination and neurodegeneration causing metachromatic leukodystrophy (MLD).^{4–7} MLD is a rare, genetic, pan-ethnic lysosomal storage disorder (LSD),^{8–10} which affects approximately one in 40,000 to 100,000 people worldwide, primarily children,

causing destruction of myelin leading to gross motor deterioration, cognitive failure, and eventually death.

Based on the age of onset of first symptoms, MLD is classified into three main clinical manifestations - late infantile, juvenile, and adult forms. The late infantile form, which is the most common and aggressive form affecting ~50–60% of the patients with MLD, has its onset before four years of age. The disease progresses rapidly with a severe phenotype involving the CNS and PNS such as developmental delay, psychomotor deterioration, dysphagia, blindness, deafness, seizures, inability to coordinate voluntary muscular movements, weakness with absence of reflexes, and eventually resulting in death within 5–6 years of age. The juvenile form, with a disease onset between the ages of 4 and 15 years, is further classified into early juvenile and late juvenile. The adult form of the disease is very rare and refers to the variant with onset after early teens.^{4,7}

Enzyme replacement therapy (ERT), gene therapy (GT), chaperone therapy, and hematopoietic stem cell transplant (HSCT) have been investigated as potential treatments for LSDs but preclinical and clinical studies have shown only

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Figure 1. Biosynthetic pathway of sulfatide formation.

limited efficacy in some cases.^{11,12} Despite the awareness and research conducted on MLD, currently there are no curative treatments for this fatal and devastating disease. HSCT, GT, and ERT have been extensively tested in animal models, and the positive results reported have led to clinical trials investigating the efficacy of these approaches.^{13–15} Among these approaches, ERT¹⁶ and hematopoietic stem cell based gene therapy¹⁷ have reached late stage clinical trials. However, HSCT is the only therapy currently approved for MLD, but the outcome depends on the disease variant and the stage of the disease at the time of transplant. In addition, HSCT also involves high risk of complications and mortality related to the procedure. Thus, identification of new therapeutic approaches including intervention with small molecule drugs is needed to treat MLD.

The therapeutic strategy we chose for MLD was predicated on small molecule mediated “substrate reduction therapy” (SRT). This approach, which corrects the defect by decreasing the biosynthesis of the accumulating substrate instead of correcting the enzymatic defect, was initially proposed for the treatment of Gaucher disease¹⁸ and subsequently explored for other LSDs either on its own, as a monotherapy, or in combination with other treatments.^{19–22} In addition, SRT with a brain penetrable small molecule might overcome the CNS challenges inherent to other therapeutic modalities such as ERT. By applying this concept to MLD, we reasoned that suppression of GalCer biosynthesis could be used to indirectly slow or block the accumulation of the downstream metabolite, SFT, brought about by insufficient degradation by ARSA.

Another attraction of SRT at this point in the glycosphingolipid (GSL) pathway is its possible application to another LSD, Krabbe disease (KD or globoid cell leukodystrophy). Similar to MLD, KD^{23–26} is characterized by abnormally low levels of galactosylceramidase (GALC, EC 3.2.1.46, Figure 1) enzyme caused by mutations to the *GALC*

gene. Both GalCer and psychosine are degraded by GALC and the patients with KD are unable to break down GalCer and psychosine, resulting in their accumulation which also results in demyelination. Identification of inhibitors of UGT8 could prevent the accumulation of GalCer and psychosine and offer a potential therapeutic mechanism to counter the fundamental pathology of this disorder. In addition to LSDs, recent studies suggest that inhibition of UGT8 could also offer a promising strategy for treating other challenging diseases such as baseline-like breast cancer.²⁷

To identify inhibitors of UGT8, a medium throughput screening of a diverse set of around 30,000 in-house compounds was performed using a mass spectrometry-based assay with OE19 cells. Several hit series with a wide range of potencies for inhibition of GalCer and SFT were identified, and thienopyridine (TP) compound **1** (Figure 2), identified as

Figure 2. Thienopyridine piperazines as UGT8 inhibitors.

a singleton, was selected for further optimization of physicochemical properties, pharmacokinetics, and brain penetration. Potent compounds from this TP series which inhibit UGT8, demonstrated reduction of SFT accumulation both *in vitro* and *in vivo*. These orally bioavailable compounds penetrated the blood brain barrier (BBB) and have the potential to be used as novel treatments for MLD and KD.

Due to the presence of an ester moiety, compound **1** was metabolically unstable in human and rat liver microsomes. Attempts to replace the ester group resulted in the identification of the secondary amide **2** (Table 1) with comparable potency to that of **1**, but with improved metabolic stability in human and rat liver microsomes (Cl_{int} 43 and 48 mL/min.mg, respectively). Tertiary amide **3** was less active but had better solubility (>960 μ M) compared to the secondary amide **2** (40 μ M), probably due to the high polarity of the amide resulting from lack of intramolecular hydrogen bonding with the pyridine nitrogen in the TP and a less planar conformation compared to compound **2**. Our initial exploration of the 3-position of the TP ring also revealed the importance of a properly positioned carbonyl group and the presence of small lipophilic terminal groups while polar groups are not favored. The good potency of the secondary amide (**2**) compared to tertiary amide (**3**) was attributed to the restricted conformation of the secondary amide due to potential intramolecular H-bonding of the amide N–H with the pyridine nitrogen atom of the TP. Other groups such as –CO₂H (IC₅₀ = 3.61 μ M), reverse-amide (CH₃CONH–, IC₅₀ = 0.33 μ M) and alkyl groups resulted in less potent compounds. Compounds with bio isosteric replacement of the amide (e.g., 4-methyloxazol-2-yl, Supporting Information, Table S1) were also less active, presumably due to lack of intramolecular H-bond orientating the carbonyl group.

Since the TP piperazine analogs were highly lipophilic and poorly soluble, attempts to improve solubility by replacing the CF₃ group at the 7-position of the TP ring resulted in limited success. Replacement with small lipophilic groups (e.g., Cl, Me, or CHF₂) led to less potent compounds compared to

Table 1. SAR of Thienopyridine Piperazines

Cpd	R ₃	R ₅	SFT nM ^a	IC ₅₀ nM ^a	GalCer IC ₅₀ nM ^a
2	CONHMe		67	90	
3			640	320	
4			97	120	
5	CONHMe		170	360	
6	CONHMe		660	450	
7	CONHMe		610	1400	
8	CONHMe		330	200	
9	CONHMe		190	350	

^aAll values are geometric means of at least two determinations.

compound 2. Electron donating groups (e.g., OMe or NMe₂) improved solubility but resulted in less potent compounds (Supporting Information, Table S2). After establishing the *N*-methyl amide and CF₃ as preferred substituents on the C3 and C7 positions of the TP ring, we began to explore other substituents on the TP and piperazine rings. However, none of these modifications gave any significant improvement of potency (Supporting Information, Tables S3 and S4).

Since MLD is a disease of the CNS and PNS, and most of the accumulation of SFT occurs in these regions, we needed brain penetrable compounds to target this disease. Calculation of central nervous system multiparameter optimization (CNS MPO) score for a set of 101 TP piperazines revealed that many of the compounds had undesirable calculated parameters for CNS penetration compared to optimal values reported previously (clogP 2.8, clogD 1.7, TPSA 44.8, MW 305.3, pK_a 8.4).^{28,29} However, the experimental LogD values for many of the TP piperazines were lower than the calculated LogDs obtained using the ACD lab software, sometimes up to 3 log units lower, indicating an overestimation of calculated LogDs for this series of compounds.

Despite the lower scores of CNS MPO, in order to fully understand and evaluate the compounds, a diverse set of piperazine analogs with good eADME profile was assessed in an *in vivo* mouse brain uptake study to determine their ability to penetrate the BBB (Table 2). Since the TP piperazines showed high plasma protein binding (>99%), we decided to use total brain and plasma concentrations to rank order compounds, even though unbound brain concentration was likely the most pharmacologically relevant parameter for assessing the pharmacodynamic response in the CNS.³⁰ Regardless of their CNS MPO scores, only those compounds

Table 2. *In Vitro* eADME Profile and *in Vivo* Mouse Brain Uptake of Piperazine Analogs

Cpd	HLM ^a	P _{app} ^b	Log D ^c	C _b @ 5 min/1 h ^d	C _{b/p} @ 5 min/1 h ^e
3	27	660	2.7	0/0	0/0
4	36	>800	3.6	1400/270	0.83/0.72
5	34	750	3.3	43/0.001	0.01/0.00
6	42	690	3.1	228/8.0	0.07/0.06
7	5	>800	3.3	1100/170	0.81/0.54
8	96	>800	3.6	3000/1700	1.8/1.0
9	51	780	3.7	1700/210	0.93/0.35

^aIntrinsic clearance in human liver microsomes in $\mu\text{L}/(\text{min}\cdot\text{mg})$.

^bPassive permeability measured using parallel artificial membrane permeability assay at pH 6.5–7.4 expressed in $\times 10^{-7}$ cm/s.

^cExperimental distribution coefficient at pH 7.4. ^dBrain concentration (ng/mL) after *iv* dosing of compound at 3 mg/kg. ^eBrain to plasma ratio.

without the bis-amide moiety (compounds 4 and 7–9) showed signs of brain penetration, determined by the brain concentration and the brain to plasma ratio at five minutes after *iv* dosing of the compounds. Despite having a lower value for CNS MPO of 2.1, compound 9 showed better brain penetration than the bis-amide 3 which had a higher CNS MPO score (4.6). This indicated to us the necessity of identifying potent compounds without a bis-amide moiety in order to optimize brain penetration.

The outcome from the early mouse brain uptake studies, coupled with the moderate activity of the piperazine analogs indicated the need for other scaffolds/compounds without the bis-amide. Hence, we investigated the impact of changing the trajectory of the terminal lipophilic tail on potency, by replacing the piperazine ring with other 4/5/6 and spirocyclic ring systems.³¹ Among the ring systems explored, the TP piperidine analogs were more potent for inhibition of both SFT and GalCer compared to the closely related 4 and 5 membered analogs, suggesting that the trajectory and rigidity of the lipophilic tail were important for improving the potency (Table 3). Encouraged with the identification of the piperidine carbamates 10 and 11 with low nM IC₅₀ values, we focused on further exploration of piperidine analogs by varying the substituents on the piperidine ring (Table 4). Compounds with lipophilic terminal groups (16–20) gave good potencies for inhibition of SFT and GalCer, but they suffered from poor solubility and had relatively low desirability scores for CNS MPO. Introduction of basic terminal groups (21–22) increased solubility unfortunately at the expense of potency.

Analysis of the CNS MPO values of potent TP piperidines (IC₅₀ < 100 nM) revealed that CNS MPO varied in our data set of 265 compounds from 1.8 (10th percentile) to 2.9 (90th percentile) with a median value of 2.2, resulting from higher values for molecular mass, TPSA, clogP, clogD, and HBD, but a lower average value for pK_a, compared to optimal values reported previously from the data for CNS drugs and candidates. However, unlike the traditional CNS drugs used in the derivatization of the desirable CNS drug candidate profile which targeted GPCRs, ion channels, and transporters, our novel TP piperidines targeting UGT8 potentially occupy a physicochemical property space outside the range of the traditional CNS drugs and candidates.

Our newer, TP piperidine analogs (16–19) with excellent potency and eADME profile were tested in the *in vivo* mouse brain uptake study to determine their ability to penetrate the

Table 3. SAR of N-Linked Thienopyridines

Cpd	R ₅	SFT IC ₅₀ nM ^a	GalCer IC ₅₀ nM ^a
10		2	2
11		1	3
12		18	37
13		610	--
14		48	43
15		200	--

^aAll values are geometric means of at least two determinations.

Table 4. SAR of Piperidine Analogs

Cpd	R ₆	SFT IC ₅₀ nM ^a	GalCer IC ₅₀ nM ^a	Sol. μM ^b	CNS MPO ^c
16		0.9	0.4	<0.5	2.1
17		0.9	1.1	<0.5	2.0
18		0.4	0.6	<0.5	1.5
19		0.2	0.2	<0.5	1.5
20		1.7	2.9	<0.5	2.0
21		510	--	430	1.4
22		980	--	420	2.9

^aAll values are geometric means of at least two determinations.^bThermodynamic solubility in pH 7.4 buffer, determined from dried DMSO sample. ^cCalculated according to ref 28.

BBB. Despite having low CNS MPO scores and physicochemical properties outside the desirable range for brain penetration, TP piperidine analogs showed good brain concentration and brain to plasma ratio (Table 5). At this point, it became clear to us that using the CNS MPO method described in refs 28 and 29 did not provide us with a useful

Table 5. *In Vitro* eADME Profile and *In Vivo* Mouse Brain Uptake of Piperidine Analogs^a

Cpd	HLM	P _{app}	Log D	C _b @ 5 min/1 h	C _{b/p} @ 5 min/1 h
10	49	>800	4.35	2700/302	1.88/1.03
11	56	>800	4.20	2140/132	0.91/0.31
16	—	—	4.56	2370/192	1.45/0.76
17	70	>800	4.29	1480/143	0.76/0.42
18	—	—	4.70	1640/128	0.59/0.24
19	73	>800	5.09	2050/148	0.69/0.46
20	41	>800	5.13	3430/316	1.67/0.59

^aSee Table 2 for definitions.

way to predict the CNS penetration of our TP UGT8 inhibitors.

Compound 19 with excellent *in vitro* potency for inhibition of SFT and GalCer was selective for inhibition of UGT8 compared to other UGT enzymes (IC₅₀ > 10 μM for UGT1A1, UGT1A6, UGT2B7, UGT2B15, and UGT2B17) and a Cerep panel of around 60 kinases. It demonstrated good brain penetration in mice (Table 5) and oral bioavailability across species (Table 6). This compound was then profiled in

Table 6. Pharmacokinetic Properties of Compound 19

	<i>iv</i> (1 mg/kg)			Oral (3 mg/kg)			
	Cl ^a	V _{dss} ^b	T _{1/2} ^c	AUC ^d	C _{max} ^e	T _{max} ^f	F% ^g
Rat ^h	6.52	1520	4.3	3060	405	0.83	40
Mice ⁱ	24.7	1640	1.13	784	306	0.5	34
Dog ^j	6.32	3320	13.6	4190	693	1.33	52

^aClearance (mL/(min.kg)). ^bVolume of distribution (mL/kg). ^cHalf life (h). ^dArea under the curve, 0-last (ng·h/mL). ^eMaximum concentration (ng/mL). ^fTime at maximum concentration (h). ^gOral bioavailability. ^hSprague–Dawley rat. ⁱC57BL/6 mice. ^jBeagle dog.

the *in vivo* pharmacodynamic (PD) models assessing target engagement and inhibition of *de novo* synthesis of GalCer and SFT in mice kidney and brain. The high turnover rate of GalCer and SFT synthesis in the kidney enabled the development of an acute one-day kidney PD model to assess the efficacy of compounds for inhibition of *de novo* synthesis by measuring the incorporation of ¹³C-Galactose (¹³C-Gal) into GalCer and SFT. Compound 19 was dosed orally, 1 h prior to *ip* injection of ¹³C-Gal (3 g/kg), and tested for incorporation of ¹³C-Gal into GalCer and SFT in the kidneys 5 h after compound dosing. Consistent with its high *in vitro* potency (mice SFT IC₅₀ = 0.3 nM) and pharmacokinetic profile, compound 19 demonstrated dose dependent inhibition of incorporation of ¹³C-Gal into GalCer and SFT in adult mice kidney with an estimated ED₅₀ of 1 mg/kg for inhibition of both SFT and GalCer.

Since SFT biosynthesis and turnover in the myelin of adult mice is very low and most developmental myelination in rodents occurs between postnatal day 14 and 22,^{32,33} we developed a short-term acute brain model for assessing the inhibition of *de novo* synthesis in juvenile mice.

Compound 19 was dosed orally twice a day for 3 days and tested for the incorporation of ¹³C-Gal, given as *ip* injection (3 g/kg) once a day for 3 days. In this study, compound 19 showed ≥90% inhibition of incorporation of ¹³C-Gal into GalCer and SFT, at all three doses tested, with the estimated ED₅₀s of <3 mg/kg for inhibition of both SFT and GalCer

Figure 3. (A) Efficacy and exposure of compound **19** in an acute one-day kidney PD study in adult C57Bl/6 mice. (B) Efficacy and exposure of compound **19** in a three-day brain PD study in juvenile mice.

(Figure 3B). Because compound **19** has very high plasma protein and tissue binding (>99%), it was difficult to accurately determine unbound brain concentration. However, analysis of compound exposure indicated that even at the lowest dose, the total brain concentration was over 900-fold of the IC_{50} values for inhibition of m-SFT (0.3 nM) and m-GalCer (0.2 nM), further supporting the compound related effect in the brain PD study.

Compound **19** was synthesized as shown in Scheme 1. Ethyl 4,4,4-trifluoroacetate was reacted with methyl 4-aminothiophene-3-carboxylate to provide the thienopyridone **24**. Chlorination with phosphorus oxychloride followed by amidation with methylamine resulted in the secondary amide **25**. Subsequent reaction with 4-hydroxy-piperidine formed **26**. Amine **30** was synthesized via silver mediated oxidative trifluoromethylation of alcohol **28**. Formation of the imidazolyl carbamate **27**, followed by the reaction of the amine **30** resulted in compound **19**.

The excellent efficacy of compound **19** for inhibiting the *de novo* synthesis of both SFT and GalCer in mice brain and kidney suggests the potential to treat LSDs such as MLD and KD where accumulation of SFT and GalCer cause demyelination. However, it should be noted that maintaining the physiological levels of SFT is crucial since it is the main GSL of the myelin sheath, and complete inhibition of the biosynthesis may cause disruption of the stability of myelin. Hence, determining an optimal therapeutic dose will be crucial when using small molecule SRT for MLD to avoid any adverse effects. Human studies in MLD and other LSDs will be needed to understand the proper doses of compounds like **19** in treating those diseases. Compound **19** and related analogs have been selected to be profiled in two-week toxicology studies in rats and dogs to further evaluate their profile, and additional *in*

Scheme 1. Synthesis of Compound **19**^a

^aReagents and conditions, yield: (a) 130 °C, 74%; (b) POCl_3 , 85%; (c) Methylamine in methanol, 73%; (d) *N*-methylpyrrolidinone, diisopropyl ethylamine, 135 °C, 90%; (e) CDI, DCM, 100%; (f) SelectFluor, silver trifluoromethanesulfonate, KF, ethyl acetate, trifluoromethyltrimethylsilane, 2-fluoropyridine, 32%; (g) hydrogen chloride, ethyl acetate, 74%; (h) *N*-hydroxy succinimide, triethylamine, acetonitrile, 50 °C, 67%.

in vivo PK studies have been planned to address dose selection for human trials. The results of these studies will be disclosed in due course.

In conclusion, starting with a thienopyridine lead, we have optimized an inhibitor for UGT8, which may be useful as a small molecule substrate reduction therapy for treating lysosomal storage diseases such as metachromatic leukodystrophy. Further reports from this research will be forthcoming.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmmedchemlett.0c00120>.

Structure activity relationship tables, protocols for *in vitro* assay and *in vivo* models, and synthetic procedure of compound **19** (PDF)

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Notes

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■ ABBREVIATIONS

ARSA, arylsulfatase A enzyme; CST, cerebroside sulfotransferase; GalCer, galactosylceramide; LSD, lysosomal storage disorder; MLD, metachromatic leukodystrophy; SFT, sulfatide; SRT, substrate reduction therapy; UGT8, uridine diphosphate-galactose glycosyltransferase 8

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