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Glucose-Lowering in a *db*/*db* Mouse Model by Dihydropyridine Diacid Glycogen Phosphorylase Inhibitors

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This paper is dedicated to the memory of Chris A. Willoughby

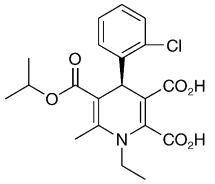
Abstract—The synthesis of a series of novel dihdyropyridine diacid glycogen phosphorylase inhibitors is presented. SAR and functional assay data are discussed, along with the effect of a single inhibitor on blood glucose in a diabetic animal model.

A recent World Health Organization report estimates that approximately 130 million people worldwide are afflicted with type 2 diabetes.[1] Patients suffer from reduced insulin sensitivity and secretion that result in glucose intolerance and postabsorptive hyperglycemia. Hepatic glucose output (HGO), a significant contributor to diabetic hyperglycemia,[2] derives from both the hydrolytic breakdown of stored glycogen (glycogenolysis), and gluconeogenesis, the latter of which is elevated in type 2 diabetics relative to normal patients.[3] Recent evidence suggests that a portion of the gluconeogenic flux cycles through the glycogenolytic pathway,[4] thereby identifying a viable intervention point for restoring glycemic control. Phosphorylated glycogen phosphorylase *a* (GPa) catalyzes the breakdown of glycogen to glucose-1-phosphate, which represents the rate determining step in glycogenolytic glucose production. Glucose lowering by inhibition of GPa with small molcule inhibitors has been reported for several unique structural classes.[5] GPa, therefore, represents an attractive therapeutic target for the treatment of type 2 diabetes.

Recent literature reported the inhibition of glycogenolysis by a dihydropyridine dicarboxylate inhibitor (**U6751**) in rat liver.[6] (Fig. 1) An enzyme-inhibitor co-crystal structure (3AMV) indicated a binding site also utilized by AMP to allosterically activate glycogen phosphorylase *b*.[7] Although AMP binding does not impact GPa activity, the spatial proximity of the aforementioned bound inhibitor to Ser14, the residue phosphorylated to activate GP, suggests an inhibitory mechanism for this compound class.

At present, SAR exploration of the dihydropyridine dicarboxylate scaffold and demonstrated efficacy in a diabetic model remain unreported. We report herein the synthesis and evaluation of racemic dihdyropyridine GPa inhibitors that address the functional role of several substituents, as well as an optimized GPa inhibitor that effected sustained glucose lowering in a *db*/*db* mouse model.

The core 1,4-dihydropyridine scaffold for all reported inhibitors derived from a Hantzsch synthesis that



U6751

Figure 1. Previously reported inhibitor U6751.

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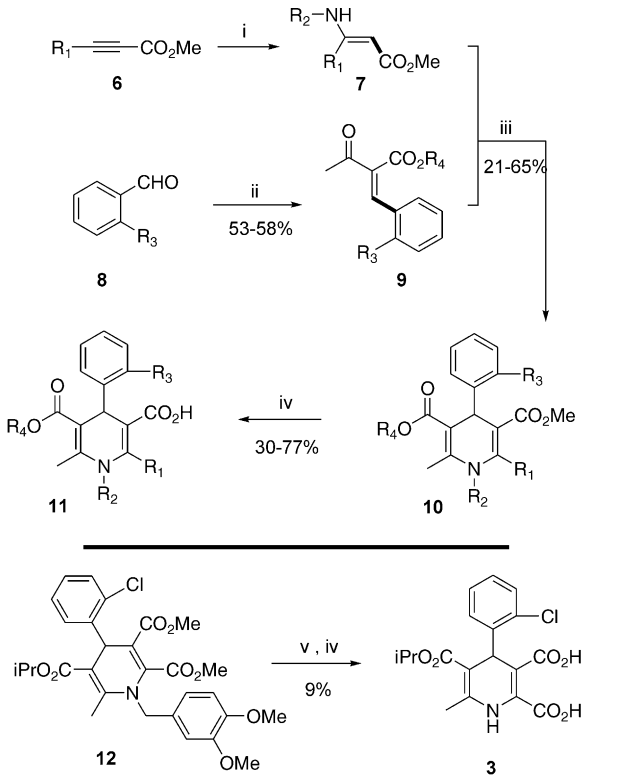


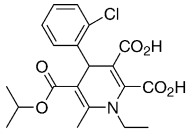
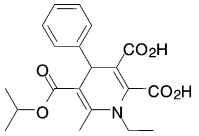
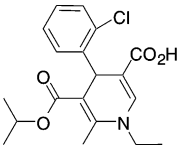
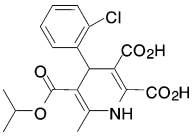
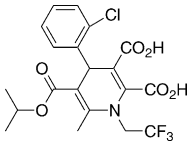
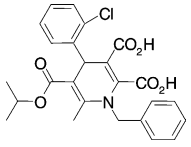
Scheme 1. Reagents and conditions: (i) R_2NH_2, MeOH, rt (crude product >95% pure by ^1H NMR); (ii) cat *p*TSA, $CH_3C(O)CH_2CO_2R_4$, benzene, reflux; (iii) 140 °C, neat; (iv) NaOH, (1:1) dioxane/H_2O, 50 °C; (v) TFA.

involved regio-selective condensation of enamine substrates with α-carboxy-α,β-unsaturated ketones at elevated temperature[8] (Scheme 1). Cyclization was followed by methyl ester hydrolysis, which proceeded cleanly at 50 °C overnight with no hydrolysis of the C5-isopropyl ester observed. Isolation of the C2,C3-dicarboxylate as the bis-sodium salt yielded the desired product.[9] The synthesis of inhibitor **3** required TFA deprotection 3,4-dimethoxybenzyl group (**12**) prior to ester hydrolysis.

In vitro analysis of ***rac*-U6751** and inhibitors **1–5** provided broad SAR for the dihydropyridine scaffold. Qualitative analysis of the 3AMV structure suggested that the C4-*o*-chlorophenyl substituent from **U6751** oriented the C4-aryl group perpendicular to the dihydropyridine ring system.[7] Assay of the C4-phenyl derivative (**1**) resulted in a 10-fold increase in IC_{50}, thereby supporting the aforementioned structure–function hypothesis (Table 1). Removal of the C2-carboxylate (**2**) verifed the necessity for the C2,C3-diacid functionality, as the potency fell by >200-fold. Clearly, disruption of salt bridges formed with one or more of the proximal arginine residues in 3AMV imparted grave consequences on GP inhibition.

Regarding the N1-substituent, the N1-desalkyl inhibitor (**3**) was significantly less active, and although the physical underpinnings for this effect were unclear, productive van der Waals interactions between the ethyl group and proximal GP*a* residues must clearly exist. N1-trifluoroethyl

Table 1. In vitro liver enzyme, muscle enzyme and hepatocyte inhibition assay results for compounds **1–3**[10,11]

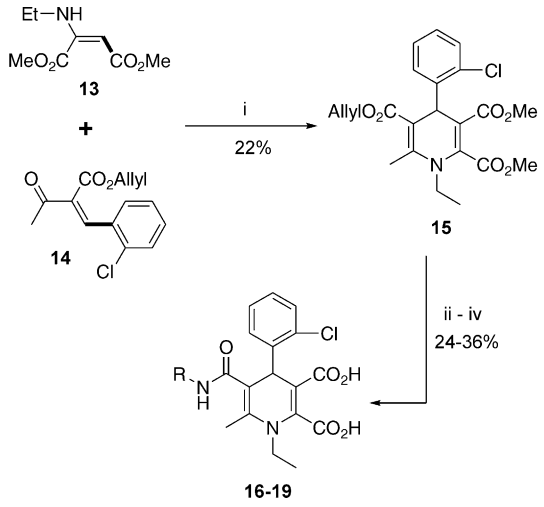
Compd	Structure	HLGPa IC_{50} (nM)	HMGPa IC_{50} (nM)	Cell EC_{50} (μM)
***rac*-U6751**		39	138	2.23
1		395	1195	6.50
2		15,600	15,000	NA
3		692	172	11.0
4		43	52	1.89
5		11	26	1.13

substitution (**4**) had little effect on potency relative to ***rac*-U6751**, but the N1-benzyl inhibitor (**5**) was distinctly more potent against GP*a*, both in vitro and in the hepatocyte cell-based assay as well. In order to evaluate the potential for this inhibitor series, subsequent SAR of the corresponding N1-benzylic moiety was pursued and will be discussed later.

C5-carboxamide inhibitors (**16–19**) were synthesized to examine the SAR of the hydrophobic pocket occupied by the isopropyl ester in 3AMV. The syntheses of **16–19** followed from *tert*-butyl ester deprotection of **10** to afford an intermediate C5-acid (Scheme 2). Amide formation under standard conditions, followed by diester hydrolysis yielded the corresponding inhibitors.

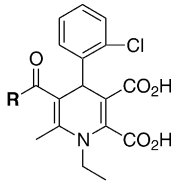
Unfortunately, carboxamide replacement of the C5-ester yielded very poor GP*a* inhibitors (Table 2). Somewhat surprising was the potency loss for amide **16** compared to isosteric ***rac*-U6751** (>200-fold), thereby suggesting that the isopropyl amide unable to adopt a productive conformation.

In order to expand on the SAR at N1, several analogues of N1-benzyl inhibitor **5** were made utilizing the



Scheme 2. Reagents and conditions: (i) 140 °C, neat; (ii) $Pd(PPh_3)_4$, dimedone; (iii) R-NH_2, EDC, HOBt; (iv) NaOH, (1:1) dioxane/H_2O, 50 °C.

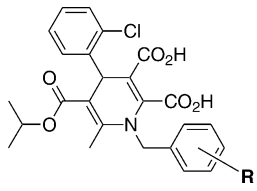
Table 2. In vitro liver and muscle enzyme inhibition assay data for compounds **16**–**19**[10]



Compd	R	HLGPa IC_{50} (μM)	HMGPa IC_{50} (μM)
16	*i*PrNH–	9.10	12.9
17	EtNH–	12.4	19.9
18	*i*BuNH–	7.88	8.28
19	BnNH–	10.90	10.61

Hantzsch chemistry outlined in Scheme 1. A comparison of mono-substituted chlorobenzyl inhibitors (**20**–**22**) revealed a general preference for *meta*- and *para*-phenyl ring substitution over *ortho* substitution. The preference against *ortho* subsituents was corroborated by dichlorobenzyl inhibitors **23**–**25**, as the addition of an *o*-Cl substituent afforded decreased potency relative to the parent mono-substituted benzyl inhibitors, **21**–**22**. Assay of this inhibitor series in rat hepatocytes supported the general trend established by the in vitro assay. Noteworthy was the relative activity of *p*-Cl-benzyl substituted inhibitor **22** and *meta*-substituted inhibitor **21**, in which presently unidentified factors related to the primary hepatocytes differentially impacted inhibitor potency in a cell assay. A second *m*-Cl substituent blunted this effect (**27**), yielding a 2-fold improvement in cell potency despite a similar 2-fold reduction in enzyme potency. *para* Substitution was still preferred as seen from a comparison of equipotent GPa inhibitors **26** and **27**. Lastly, a comparison of 3,4-Cl_2-inhibitor, **26**, with the 3,4-dimethoxybenzyl inhibitor, **29**,[12] indicated a preference for the latter substituent that was exceptionally potent in the cell assay, as well (Table 3).

Table 3. In vitro liver enzyme, muscle enzyme and hepatocyte inhibiton assay data for compounds **20**–**29**.[10,11]



Compd	R	HLGPa IC_{50} (nM)	HMGPa IC_{50} (nM)	Cell EC_{50} (μM)
5	H	11	26	1.13
20	2-Cl	79	178	3.71
21	3-Cl	8	29	4.01
22	4-Cl	2	6	0.48
23	2,3-Cl_2	109	233	6.19
24	2,4-Cl_2	104	185	7.13
25	2,5-Cl_2	65	91	6.71
26	3,4-Cl_2	13	41	1.06
27	3,5-Cl_2	15	37	2.22
28	3-NO_2	5	10	0.44
29	3,4-$(OMe)_2$	4	11	0.27

A pharmacodynamic study treating *db*/*db* mice with **29** resulted in a dose dependent effect on plasma glucose concentration that was clearly evident at six h post-dose[13] (Fig. 2). In addition, a 100 mpk dose of **29** yielded glucose levels statistically similar to lean animals. Of particular interest was the observed efficacy of **29** despite its sub-optimal pharmacokinetic profile.[14] Rough calculations involving c_{max} and $t_{1/2}$ combined with the observed EC_{50} for **29** do not predict blood levels commensurate with the observed efficacy at 6 h. Several potential explanations exist, in addition to the potential species difference between the hepatocyte assay and PK/PD models, which will be discussed in a subsequent communication. Initial tissue analysis indicated that glycogen accumulation occurred exclusively in the liver and not in peripheral skeletal muscle (data not shown), the implications of which in relation to McArdle's disease[15] will be discussed in the aforementioned future publication. This represents the first

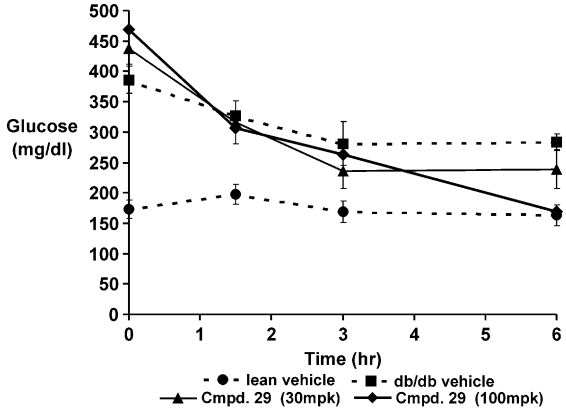


Figure 2. Blood glucose effect of compound **29** in *db*/*db* mice. *x*-Axis represents time post-dose or vehicle administration. Each datapoint represents an $n = 7$.

demonstrated glucose normalization by this inhibitor class in a relevant diabetic animal model.

In conclusion, a series of dicarboxylate GP inhibitors provided SAR for the central dihydropyridine scaffold, which led to the synthesis of compound **29**. Oral administration of **29** to *db*/*db* mice led to glucose normalization not previously reported in a diabetic model.

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9. Treatment of the concentrated diacid compound with 2 equiv NaOH(aq) precluded formation of the maleic anhydride.
10. IC_{50} values derived from inhibiting phosphorolysis of glycogen using recombinant human liver or muscle enzyme. The standard error for this assay (15%) arose from titration of an internal standard. Glucose-1-phosphate production was monitored via an enzymatic assay involving phosphoglucomutase/glucose-6-phosphate dehydrogenase-mediated NADH production (ex. 340 nm, em. 465 nm).
11. Inhibition of glucagon-stimulated glycogenolysis in primary rat hepatocytes. The standard error for this assay (15%) derived from an internal standard. Inhibition of glucose production determined by digesting remaining glycogen and quantifying by monitoring glucose dehydrogenase-mediated NADPH production (ex. 340 nm, em. 465 nm).
12. Characterization for compound **29**: ^{1}H NMR (500 MHz, CD_3OD) δ 7.15 (3H, m), 6.96 (1H, ddd, $J=7.6$, 7.5, 1.5 Hz), 6.92 (1H, m), 6.85 (2H, m), 5.50 (1H, s), 4.87 (2H, m), 4.78 (1H, d, $J=16.0$ Hz), 3.72 (3H, s), 3.65 (3H, s), 2.36 (3H, s), 1.21 (3H, d, $J=6$ Hz), 1.05 (3H, d, $J=6.5$ Hz). LC/MS ESI ($C_{27}H_{28}ClNNaO_8$) calcd for MNa^+: 552.14; found: 552.1.
13. Glucose levels determined at indicated times post-dosing of either compound **29** or vehicle. Each curve represents a single group of $n=7$ animals, and timepoints therein an average of the group.
14. Pharmacokinetic data from C57BL/6J mice (3/pt), 1 mpk po: $t_{1/2}=1.4$ h, $C_{max}=12$ nM, $\%F=4.5$.
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