This is a repository copy of *Analysis of transition state mimicry by tight binding aminothiazoline inhibitors provides insight into catalysis by human : O-GlcNAcase.*

White Rose Research Online URL for this paper:
http://eprints.whiterose.ac.uk/104657/

Version: Published Version

**Article:**
Cekic, N., Heinonen, J. E., Stubbs, K. A. et al. (6 more authors) (2016) Analysis of transition state mimicry by tight binding aminothiazoline inhibitors provides insight into catalysis by human : O-GlcNAcase. Chemical Science. pp. 3742-3750. ISSN 2041-6539

https://doi.org/10.1039/c6sc00370b

**Reuse**
This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here:
https://creativecommons.org/licenses/

**Takedown**
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.
Analysis of transition state mimicry by tight binding aminothiazoline inhibitors provides insight into catalysis by human O-GlcNAcase†

N. Cekic,‡§ J. E. Heinonen,‡ a K. A. Stubbs, ab C. Roth, c Y. He, c A. J. Bennet, a E. J. McEachern,§ G. J. Davies c and D. J. Vocadlo* ad

The modification of nucleocytoplasmic proteins with O-linked N-acetylglucosamine (O-GlcNAc) plays diverse roles in multicellular organisms. Inhibitors of O-GlcNAc hydrolase (OGA), the enzyme that removes O-GlcNAc from proteins, lead to increased O-GlcNAc levels in cells and are seeing widespread adoption in the field as a research tool used in cells and in vivo. Here we synthesize and study a series of tight binding carbohydrate-based inhibitors of human OGA (hOGA). The most potent of these 2'-aminothiazolines binds with a sub-nanomolar $K_i$ value to hOGA (510 ± 50 μM) and the most selective has greater than 1 800 000-fold selectivity for hOGA over mechanically related human lysosomal β-hexosaminidase. Structural data of inhibitors in complex with an hOGA homologue reveals the basis for variation in binding among these compounds. Using linear free energy analyses, we show binding of these 2'-aminothiazoline inhibitors depends on the pKₐ of the aminothiazoline ring system, revealing the protonation state of the inhibitor is a key driver of binding. Using series of inhibitors and synthetic substrates, we show that 2'-aminothiazoline inhibitors are transition state analogues of hOGA that bind to the enzyme up to 1-million fold more tightly than the substrate. These collective data support an oxazoline, rather than a protonated oxazolinium ion, intermediate being formed along the reaction pathway. Inhibitors from this series will prove generally useful tools for the study of O-GlcNAc. The new insights gained here, into the catalytic mechanism of hOGA and the fundamental drivers of potency and selectivity of OGA inhibitors, should enable tuning of hOGA inhibitors with desirable properties.

Introduction

The modification of serine and threonine residues of nuclear and cytoplasmic proteins with terminal O-linked β-N-acetylglucosamine (O-GlcNAc) has been found on hundreds of proteins. 1-4 O-GlcNAc is present in all multi-cellular eukaryotes studied and occurs in a dynamic and reversible manner. 5 Global O-GlcNAc levels have been shown to vary in response to cellular nutrient availability and stress and in some cases has been found to influence protein phosphorylation. 6 These observations have stimulated interest in the physiological roles played by O-GlcNAc and research has implicated this modification in controlling various cellular processes including, for example, proteosomal degradation of proteins 7-9 and transcriptional regulation. 10-11 Additionally, a growing body of literature has implicated O-GlcNAcylation in chronic diseases such as neurodegeneration 12-15 and cancer. 9,16,17 Given the mounting potential in targeting protein O-GlcNAcylation for therapeutic benefit, there has been rising interest in understanding the molecular basis for inhibition of modulators of the O-GlcNAc pathway and the creation of small molecule modulators of this pathway for use in tissues. 18-20

The glycosyltransferase uridine diphospho-N-acetylglucosamine:peptide β-N-acetylglucosaminyl transferase (OGT) installs O-GlcNAc residues using uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) as the sugar substrate donor. 21-22 The enzyme responsible for removing O-GlcNAc from proteins is O-GlcNAcase (OGA), 23 which is a member of glycoside hydrolase family 84 (GH84) of the CAZy classification system. 24 Consistent with the reversible nature of protein O-GlcNAcylation, inhibitors of these enzymes have been shown to induce time-dependent changes in cellular O-GlcNAc levels. 19,25-27 Small molecule
inhibitors of OGA, in particular, have emerged as commonly used research tools for evaluating the phenotypic effects of increased O-GlcNac levels in cultured cells, as well as in vivo.

Interest in inhibitors of OGA has gained increasing attention due to growing recognition of the physiological roles of O-GlcNac. Among the first reported inhibitors of OGA is O-[2-acetamido-2-deoxy-D-glucopyranosylidene]amino-N-phenylcarbamate (PUGNAc, Fig. 1, 1)\textsuperscript{18} (hOGA $K_i$ = 46 nM). This inhibitor, however, has well-described off target effects\textsuperscript{29,30} including the inhibition of the functionally related lysosomal $\beta$-hexosaminidases HEXA and HEXB from family GH20.\textsuperscript{26} These two lysosomal enzymes cleave $\beta$-linked terminal $N$-acyethylhexosamine residues from various glycoconjugates including gangliosides. Genetic deficiency of these hexosaminidases results in Tay–Sachs and Sandhoff’s disease, which stem from the accumulation of gangliosides within lysosomes. More recently identified inhibitors such as 6-acetamido-6-deoxy-castanospermine (6-Ac-Cas)\textsuperscript{29} (hOGA $K_i$ = 300 nM) (Fig. 1, 2), and 1,2-dideoxy-2'-methyl-$\alpha$-D-glucopyranosyl-[2,1-$d$]-$\Delta^2$-thiazoline (NAG-thiazoline)\textsuperscript{26} (hOGA $K_i$ = 70 nM) (Fig. 1, 3) while fairly potent, are also non-selective. Given that gangliosides play varied roles in cellular processes ranging from cell membrane structure to cell signaling, the selectivity of OGA inhibitors has emerged as being important for the creation of useful probe molecules for use in vivo.\textsuperscript{29,31,32}

Rationally designed OGA inhibitors have been pursued based on knowledge of the catalytic mechanism of hOGA. Detailed mechanistic studies\textsuperscript{26,31} coupled with structural studies of bacterial homologues of hOGA\textsuperscript{34,35} have provided clear support for a catalytic mechanism involving substrate-assisted catalysis in which the 2-acetamido group of the substrate serves as a catalytic nucleophile to generate a transient enzyme-bound oxazoline or oxazolinium intermediate (Fig. 1A) and stabilizing an oxocarbenium ion-like transition state (Fig. 1B). In this two-step catalytic mechanism, two aspartates (Asp$^{\text{D}}$ and Asp$^{\text{E}}$) play key roles as general acid/base catalytic residues.\textsuperscript{26} Asp$^{\text{D}}$ serves to orient and polarize the 2-acetamido group to aid its leaving group. (B) The proposed transition state (TS) for formation of the oxazoline intermediate. Note that the extent of proton transfer to the stabilizing residue $D_{276}/D_{254}$ is not known. (C) Some known hOGA inhibitors. Catalytic residues are $D_{276}/D_{175}$ for human OGA and $D_{254}/E_{355}$ for human lysosomal $\beta$-hexosaminidase A.

**Fig. 1** Catalytic mechanism of OGA and lysosomal $\beta$-hexosaminidases and some known hOGA inhibitors. (A) hOGA uses a substrate-assisted catalytic mechanism involving two key catalytic aspartate residues that enable the transient formation of an oxazoline intermediate. R = leaving group. (B) The proposed transition state (TS) for formation of the oxazoline intermediate. Note that the extent of proton transfer to the stabilizing residue $D_{276}/D_{254}$ is not known. (C) Some known hOGA inhibitors. Catalytic residues are $D_{276}/D_{175}$ for human OGA and $D_{254}/E_{355}$ for human lysosomal $\beta$-hexosaminidase A.
Results and discussion

Early studies showed that varying the 2′-alkyl substituent of NAG-thiazoline resulted in increased selectivity for OGA over the lysosomal hexosaminidases at the slight expense of NAG-thiazoline. However, cell-penetrant glucoimidazole inhibitors including the hOGA inhibitors GlcNAcstatin G (hOGA) have only modest selectivity. Exploiting a similar approach of increasing steric bulk of the acetamido group, however, cell-penetrant glucoimidazole inhibitors including the hOGA inhibitors GlcNAcstatin G (hOGA) showed only modest selectivity.

Scheme 1. Synthesis of OGA inhibitors 11a–b and 15a–h from 8 and 12, respectively. (A) 11a: (a) 1. NEt$_3$, DCM; 2. Fmoc-NCS, pyridine, NEt$_3$; (b) SnCl$_4$, pyridine, NET$_3$. (c) 1. NaOMe, MeOH; (ii) AcOH; 2. piperidine, DMF. (B) 11b: (a) alkyl-NCS (2 eq.), NET$_3$ (2 eq.), CH$_2$CN; (c) TFA (7.5 eq.), DCM; (d) Fmoc-NCS, pyridine, NEt$_3$. (C) 15a–h: (a) NHR$_2$, HCl (1.25 eq.), NET$_3$ (2 eq.), CH$_2$CN; (h) TFA (7.5 eq.), DCM; (i) K$_2$CO$_3$, MeOH. 15a–15d: R$_1$ = H, R$_2$ = CH$_3$; 15e–15f: R$_1$ = H, R$_2$ = (CH$_2$)$_2$F; 15g–15h: R$_1$ = H, R$_2$ = CH$_2$CH$_2$F; 15i–15j: R$_1$ = H, R$_2$ = CH$_2$CF$_2$. 15k: R$_1$ = H, R$_2$ = (CH$_2$)$_2$CH$_2$; 15l: R$_1$ = H, R$_2$ = (CH$_2$)$_2$CF$_2$.
assumption that the free inhibitor concentration is equal to the total inhibitor concentration.

\[
v_i = \frac{1}{1 - \left( \frac{[E_i] + [I]_T + K_i^{app}}{2[E_i][I]_T} \right) - \sqrt{\left( \frac{[E_i] + [I]_T + K_i^{app}}{2[E_i][I]_T} \right)^2 - 4[E_i][I]_T}}
\]

(1)

Further, \( K_i \) values can be accurately determined over a wide range of enzyme concentrations using this method, which enabled us to use hOGA concentrations that permit accurate initial rate determinations. We followed existing guidance\(^7\) to select inhibitor concentrations for our \( K_i \) value determinations and confirmed the accuracy of this method by showing the \( K_i \) values for inhibitor 11b were in reasonable accord when using either the Michaelis–Menten or Morrison method (ESI Fig. S2f).

Using these methods we found the \( K_i \) values for inhibition of hOGA by compounds 11a-b and 15a-h ranged from the high sub-nanomolar to low nanomolar range (Table 1). Notably, we find that the \( K_i \) value for ThiamEt-G (Fig. 1 and 2) was 10-fold lower (\( K_i = 2.1 \) nM) than that previously determined\(^9\) using the Michaelis–Menten method (\( K_i = 21 \) nM). This makes ThiamEt-G over 100-fold more potent than the isosteric NBButGT (\( K_i = 230 \) nM). The most tight-binding compound with a \( K_i \) of 510 ± 50 pM (Fig. 2) is 15a (ThiamMe-G, Scheme 1), which ranks this compound among the most potent glycoside hydrolase inhibitors known, as well as the most potent selective hOGA inhibitor reported. Interestingly, we note that there is only a slight decrease in potency for hOGA upon increasing the volume of the 2'-aminobutythiazoline 15e, which are products of the highly homologous HEXA and HEXB genes. Using purified human hexosaminidase B (hHexB) we determined the approximate \( K_i \) values for compounds 11a to 15h using Dixon plot analysis and found remarkably high inhibitor selectivities ranging from 1100- to 1 850 000-fold preference for hOGA (Table 1). We confirmed these Dixon plot analyses for the two most potent compounds, 11a and 15a, by determining full \( K_i \) values for their inhibition of hHexB (ESI Fig. S4f). Remarkably, inhibitors 11a and 15a still retain 1100 and 3300-fold selectivity for hOGA despite their similarity in size to NAG-thiazoline, which itself demonstrated no selectivity.\(^{26}\) Accordingly, the presence of the 2'-amino substituent, on its own, confers at least 1000-fold selectivity for hOGA over hHexB.

Table 1. \( K_i \) selectivity ratios of inhibitors 11a to 15h for hOGA over hHexB and the \( pK_a \) values for compounds 15c, 15f–h.

| Inhibitor | hOGA \( K_i \) (nM) | hHexB \( K_i \) (µM) | (hHexB/hOGA) | \( pK_a \) | Fraction protonated at pH 7.4 |
|-----------|----------------|----------------|----------------|---------|-----------------|
| 11a: \( R_1 = R_2 = H \) | 4.7 ± 0.3 | 5.0 ± 0.6\(^d\) | 1100 | | |
| 15a: \( R_1 = H, R_2 = CH_3 \) | 0.51 ± 0.05 | 1.7 ± 0.19\(^d\) | 3300 | | |
| 15b: \( R_1 = R_2 = CH_3 \) | 2.4 ± 0.2 | 13.0 ± 3.8 | 5400 | | |
| 15c: \( R_1 = H, R_2 = CH_2CH_2CH_3 \) | 2.1 ± 0.3 | 740 ± 60 (ref. 19) | 350 000 | 7.68 | 0.66 |
| 11b: \( R_1 = H, R_2 = CH_2CH_2CH_3 \) | 3.2 ± 0.4 | 2850 ± 570 | 950 000 | | |
| 15d: \( R_1 = H, R_2 = (CH_2)_2CH_3 \) | 2.0 ± 0.2 | 3700 ± 670 | 1 850 000 | | |
| 15e: \( R_1 = H, R_2 = (CH_2)_3CH_3 \) | 350 ± 90\(^d\) | 4800 ± 763 | 13 700 | | |
| 15f: \( R_1 = H, R_2 = (CH_2)_2F \) | 15 ± 5\(^d\) | 180 ± 44 | 12 000 | 6.92 | 0.25 |
| 15g: \( R_1 = H, R_2 = CH_2CHF_2 \) | 60 ± 10\(^d\) | 150 ± 50 | 2500 | 6.18 | 0.06 |
| 15h: \( R_1 = H, R_2 = CH_2CF_3 \) | 1000 ± 200\(^d\) | 4200 ± 1525 | 4200 | 5.33 | 0.01 |

\(^a\) Determined using the Morrison \( K_i \) fit if the values are below 5 mM. \(^b\) Determined using Dixon plot analysis. \(^c\) Selectivity ratios representing the favored selectivity for hOGA compared to hHexB. \(^d\) Determined using Michaelis–Menten inhibition analysis. \(^e\) See ESI for full details, \( pK_a \) [NBButGT] = 4.65.
Preparation of 2'-alkylaminothiazoline OGA inhibitors for evaluating electronic effects in OGA inhibition

Notably, ThiamEt-G ($K_i = 2.1$ nM) binds over 100-fold more tightly than NBuGT ($K_i = 230$ nM) and is 25-fold more selective for hOGA. Detailed mechanistic studies in combination with pH-rate profiles of wild-type and mutant hOGA revealed the key catalytic residue Asp$^{174}$ in the OGA catalytic site (Asp$^{42}$ in BhGH84) acts as a general base to assist the attack of the substrate 2-acetamido group onto the anomeric center. The kinetic $pK_a$ of this residue was determined to be 5.2 so that at physiological pH this residue is expected to be in its carboxylate form$^{13}$ and therefore suitably ionized in the resting enzyme to facilitate catalysis. Given that aminothiazolines are known to be more basic than thiazolines, it was speculated that installation of the 2'-alkylamino group would increase the basicity of ThiamEt-G as compared to NBuGT and thereby contribute to its enhanced potency relative to inhibitors bearing 2'-alkyl groups at physiological pH. Structural data of ThiamEt-G bound within the active site of BhGH84 is consistent with this proposal, revealing that both the endo and exocyclic amines engaged Asp$^{42}$ of BhGH84.$^{19}$

We set out to evaluate this proposal and assess the importance of the inhibitor $pK_a$ on potency by studying a series of 2'-aminoethylthiazoline inhibitors with increasing fluorine substitution at the terminal methyl group (15f-h). Evaluation of the potency of these compounds revealed a progressive increase in $K_i$ value upon increasing substitution with fluorine ($K_i = 2.1$ (CH$_3$, 15e) 15 (CH$_3F$, 15f), 60 (CHF$_3$, 15g) and 1000 (CF$_3$, 15h) nM) (Table 1). To clearly understand the relationship between inhibitor basicity and potency we used $^{13}$C NMR titration to determine the $pK_a$ values of conjugate acids of these inhibitors (15e, 15f-h). NMR methods are highly accurate and can be used to determine relative $pK_a$ values between one or more compounds having an unknown $pK_a$ and a reference compound having a well established $pK_a$ value.$^{19}$

Practically, two advantages of this relative measurement approach is that it does not require repeated pH measurements throughout the titration and it can be used to evaluate small changes in $pK_a$ values. In this way, a non-linear plot of the difference between NMR resonance frequencies for a compound of interest and those for a standard, such as 3-nitrophenol, can be used to determine the ratio ($R$) of the acid
dissociation constants between these two materials. A typical plot for the difference in $^{13}$C chemical shifts is shown in Fig. 4 (see ESI† methods for a full discussion).

To assess the extent to which inhibitor potency depends on its $pK_a$ value, we plotted the $pK_a$ value of inhibitors (15c, 15f–h and NBuTG, 4) with the corresponding log $K_i$, which are both free energy terms. The resulting linear free energy relationship (LFER) shows a linear correlation ($R^2 = 0.9876$) with a slope of $-1.12 \pm 0.09$ (Fig. 5), which is consistent with the $pK_a$ value of inhibitors mostly dominating the effect of binding as compared to steric effects associated with increasing fluorine substitution. Notably, we also find that NBuTG, which is isosteric to Thia-mEt-G (15c), matches reasonably well within this correlation, supporting the electronic effects dominating this correlation. While the affinities of each protonation state of these inhibitors for hOGA (Fig. 5) cannot be readily determined because the enzyme itself has various ionization states, these data collectively suggest that the $pK_a$ value of the inhibitor, either by favoring the protonated inhibitor form or by optimizing hydrogen bonding strength, plays a key role in binding of these 2′-aminothiazoline inhibitors.

Assessment of 2′-aminothiazoline inhibitors as transition state analogs

The tight binding of these 2′-aminothiazoline inhibitors prompted us to consider their potency in the context of their size. One widely used parameter to understand the efficiency of binding as a function of molecular weight is to consider the ligand efficiency (LE) of a ligand. This measure provides the binding affinity of the compound as a measure of the number of heavy atoms. We calculate a remarkably high LE of 0.88 kcal per mol per heavy atom for compound 15a. Such a LE is comparable to some of the highest ever observed LEs observed for compounds in the size range of between 10–50 heavy atoms and suggests to us that these compounds could well be TS analogues, as had been observed for the related thiazoline inhibitors. Tight-binding inhibitors that bear resemblance to enzyme substrates or intermediates have often been considered to be TS analogues simply by virtue of their potency. However, because enzymes are thought to catalyze reactions by tightly binding the TS, for genuine TS analogues changes in free energies of binding of a series of TS analogues ($\log K_i$) should parallel changes in the free energies of a series of related transition states TS ($\log K_{cat}/K_{m}$). Bartlett has formalized these concepts and methods to quantitatively assess whether compounds are TS analogues using LFERs. Using this method, genuine TS analogues yield linear correlations having a slope of unity. Furthermore, $\log K_{cat}/\log K_i$ are not correlated for TS analogues but do correlate for substrate analogues.

Previous studies showed that NAG-thiazoline analogues are TS analogues despite their obvious resemblance to the oxazoline intermediate, perhaps due to the longer C–S bonds altering the thiazoline ring to resemble a late TS. Given the greater than 100-fold increase in potency we observe for the 2′-aminothiazoline inhibitors over their thiazoline counterparts and their structural resemblance to the oxazoline intermediate found along the reaction coordinate of hOGA, we wanted to assess whether incorporation of the 2′-amino group benefitted binding through serendipitous interactions, or whether the presence of the charge included in this class of inhibitors also made them TS analogues. We therefore turned to using the Bartlett LFER approach. With the series of inhibitors in hand we synthesized a series of fluorogenic 4-methylumbelliferyl 2-deoxy-2-urea-β-D-glucopyranoside substrates (18a–e, Scheme 2) bearing N-alkyl substituents on the terminal urea nitrogen that correspond to those alkyl groups present on the series of 2′-aminothiazoline inhibitors (11a, 15a, 15c–e). We started from 4-methylumbelliferyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride (16) as a common

---

**Fig. 4** A representative example for $^{13}$C-NMR determination of the $pK_a$ value for ThiamEt-G (15c) with chemical shifts of the 2′-C of the thiazoline ring resonances (Δ ppm) for compounds 15c as a function of the fractional protonation ($n$) of the reference compound, 3-nitrophenol ($pK_a$ of 8.42). The solid line is the best non-linear least squares fit to eqn (S1) (ESI†).
intermediate.\textsuperscript{24} Per-O-acetylated urea substrates 17a–e were prepared by reacting 16 with the appropriate alkylisocyanate in the presence of triethylamine, followed by Zemplen de-O-acetylation. With this series of substrates (18a–e) in hand we determined the $k_{\text{cat}}/K_m$ values governing their hOGA catalyzed hydrolysis (ESI Table S1\textsuperscript{†}). Plotting these data to assess TS analogy, we observe (Fig. 6) a clear correlation ($R^2 = 0.9950$) with a slope of 1.08 ± 0.04 between log $K_i$ values for the inhibitors versus the log $K_m/k_{\text{cat}}$ values for the corresponding series of urea substrates (Fig. 6A). In contrast, we find no correlation between log $K_i$ values for the inhibitors and log $K_m$ values for the series of substrates (Fig. 6B). These results indicate the 2′-aminothiazoline inhibitors are TS analogues for the hOGA catalyzed hydrolysis of urea substrates.

We recognized that our observations showed 2′-aminothiazoline inhibitors are TS analogues for the hOGA-catalyzed hydrolysis of unnatural urea substrates (18a–e), however, we were curious as to whether these inhibitors would also be TS analogues for the hOGA-catalyzed hydrolysis of the more natural N-acyl substrates. To address this question, we examined the correlation between log $K_i$ values for the same series of 2′-aminothiazoline inhibitors and the series of N-acyl substrates (Fig. 6C) having the analogous structural changes for which $k_{\text{cat}}/K_m$ values are reported (Fig. 6D).\textsuperscript{33} We find a fair correlation ($R^2 = 0.9768$) with a slope of 2.3 ± 0.3. For this analysis we excluded the 2′-aminothiazoline (11a) because of its unexpectedly poor inhibition of hOGA (Table 1).

Notably, previous studies have shown $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values for the OGA-catalyzed hydrolysis of aryl 2-acetamido-2-deoxyglucopyranosides, including 19a, vary in according to the $pK_a$ value of the phenolic leaving group.\textsuperscript{35} Additionally, such substrates bearing different sized N-acyl groups, including 19a–d (ref. 51) coupled with the large $\alpha^\text{-}(V)$-KIE ($k_{\text{th}}/k_0 = 1.14 ± 0.02$) value observed for the OGA-catalyzed hydrolysis of p-nitrophenol 2-acetamido-2-deoxy-glucopyranoside\textsuperscript{35} all support $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ values reflect a chemical step being rate limiting for human OGA, which supports the validity of this TS analogy study. The steep slope observed for this series of 2-acyl substrates in correlation with the aminothiazoline inhibitors is surprising.
However, slopes other than unity in Bartlett LFER plots are pre-
cededent though not often rationalized. Here, we interpret this
steep slope as indicating that the TS for the hOGA catalyzed
hydrolysis of N-acyl substrates bears less positive charge in the
forming oxazoline ring system. This may arise because the amide
proton is in flight in the TS, as compared to the N-urea substrates
(Fig. 6E and F), which are expected to be more basic and therefore
likely lead to the formation of 2’-amino-oxazolinium ion inter-
mediates that retain their proton. Accordingly, the TS leading to
such aminooxazolinium ion intermediates is expected to have
more positive charge than the corresponding transition state
leading to the oxazoline intermediate (Fig. 6E and F). In keeping
with this proposal, it is notable that site-directed deletion of the
side chain of Asp174, which is the catalytic general acid/base
catalytic residue that interacts with the acetamido group of the
substrate, leads to a similar drop of between 150 to 750-fold in
second order rate constant as seen on going from N-acyl to N-
urea substrates (250-fold). Accordingly, these data indicate that
2’-aminothiazoline inhibitors are TS analogues for hOGA, by
virtue of both their shape and general charge distribution.
However, the steep slope observed for the LFER between log K_sp/
k_cat values observed for N-acyl substrates and the log K_i values
seen for the 2’-aminothiazoline inhibitors suggests that this
feature lends improved binding over the corresponding partial
charge that likely develops for the TS found for the hOGA cata-
lyzed processing of natural N-acyl-containing substrates. These
data support a catalytic mechanism in which residue D174 of
hOGA acts as a general acid/base catalytic residue rather than
simply stabilizing an oxazolinium ion intermediate as proposed
for GH20 β-hexosaminidases.

Conclusion

In summary, we describe a series of aminothiazoline inhibitors
for human OGA having picomolar and low nanomolar K_i values.
The great potency of this inhibitor family is in large part attrib-
utable to their pK_a values since a clear correlation was observed
between the pK_a and log K_i of a series of these compounds.
Structures of these inhibitors in complex with BtOGA reveal the
molecular basis for the trends in observed inhibitor potencies
and selectivities. Using quantitative methods we find that these
2’-aminothiazoline inhibitors are tight-binding TS analogues for
hOGA. These inhibitors benefit from their formal positive charge
at physiological pH, harnessing favorable interactions that are
only partly realized within the transition state for the natural 2-
acyl-containing substrates. These observations should permit the
design of more potent and selective inhibitors, not only in this
class of inhibitor but also using other inhibitor scaffolds. Finally,
the great potencies and selectivities of these inhibitors reveal
a series of useful tool compounds that can be used to manipulate
hOGA activity in vivo.

Acknowledgements

The authors thank Diamond Light Source for beamtime
(proposals mx-1221, mx-7864 and mx-9948) and the staff of
beamlines i02, i03 and I24 for assistance with crystal testing and
data collection. We are also grateful to Johan Turkenburg and
Sam Hart for help during data collection. We thank Dr. Michael
Tropak and Prof. Donald Mahuran, Sick Children’s Hospital
Toronto, for the samples of human hexosaminidase B. This
research was supported by funding from Canadian Institutes of
Health Research for support (MOP-123341) (DJV), Biotechnology
and Biological Sciences Research Council (BB/K003836/1) (GJD
and CR), and the Natural Sciences and Engineering Research
Council (NSERC, DG-121348) of Canada (AJB). KAS thanks the
Australian Research Council (FT100100291). DJV also thanks
Brain Canada, Genome British Columbia, and the Michael Smith
Foundation for Health Research for support of this research and
the Canada Research Chairs program for support as a Tier I
Canada Research Chair in Chemical Glycobiology.

References

1 C. R. Torres and G. W. Hart, J. Biol. Chem., 1984, 259, 3308–
3317.
2 N. Khidekel, S. B. Ficarro, E. C. Peters and L. C. Hsieh-
Wilson, Proc. Natl. Acad. Sci. U. S. A., 2004, 101, 13132–13137.
3 S. A. Whelan and G. W. Hart, Circ. Res., 2003, 93, 1047–
1058.
4 J. C. Trinidad, D. T. Barkan, B. F. Gulledge, A. Thalhammer,
A. Sali, R. Schoepfer and A. L. Burlingame, Mol. Cell.
Proteomics, 2012, 11, 215–229.
5 G. W. Hart, Front. Endocrinol., 2014, 5, 183.
6 Z. Wang, M. Gucek and G. W. Hart, Proc. Natl. Acad. Sci. U. S.
A., 2008, 105, 13793–13798.
7 H. B. Ruan, Y. Nie and X. Yang, Mol. Cell. Proteomics, 2013,
12, 3489–3497.
8 Y. Zhu, T. W. Liu, Z. Madden, S. A. Yuzwa, K. Murray,
S. Cecioni, N. Zachara and D. J. Vocadlo, J. Mol. Cell Biol.,
2016, 8, 2–16.
9 C. M. Ferrer, T. P. Lynch, V. L. Sodi, J. N. Falcone,
L. P. Schwab, D. L. Peacock, D. J. Vocadlo, T. N. Seagroves
and M. J. Reginato, Mol. Cell, 2014, 54, 820–831.
10 S. Ozcan, S. S. Andrali and J. E. Cantrell, Biochim. Biophys.
Acta, 2010, 1799, 353–364.
11 S. M. Ranuncolo, S. Ghosh, J. A. Hanover, G. W. Hart
and B. A. Lewis, J. Biol. Chem., 2012, 287, 23549–23561.
12 F. Liu, J. Shi, H. Tanimukai, J. Gu, J. Gu, I. Grundke-Iqbal,
K. Iqbal and C. X. Gong, Brain, 2009, 132, 1820–1832.
13 F. Liu, K. Iqbal, I. Grundke-Iqbal, G. W. Hart and C. X. Gong,
Proc. Natl. Acad. Sci. U. S. A., 2004, 101, 10804–10809.
14 S. A. Yuzwa, X. Shan, M. S. Macauley, T. Clark,
Y. Skorobogatko, K. Vosseller and D. J. Vocadlo, Nat. Chem.
Biol., 2012, 8, 393–399.
15 S. A. Yuzwa and D. J. Vocadlo, Chem. Soc. Rev., 2014, 43,
6839–6858.
16 Z. Ma and K. Vosseller, Amino Acids, 2013, 45, 719–733.
17 W. Yi, P. M. Clark, D. E. Mason, M. C. Keenan, C. Hill,
W. A. Goddard 3rd, E. C. Peters, E. M. Driggers and
L. C. Hsieh-Wilson, Science, 2012, 337, 975–980.
18 R. F. Ortiz-Meoz, J. Jiang, M. B. Lazarus, M. Osman,
J. Janetzko, C. Fan, D. Y. Duveaux, Z. W. Tan, C. J. Thomas
and S. Walker, ACS Chem. Biol., 2015, 10, 1392–1397.
