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ARTICLE TYPE

Modifying the phenyl group of PUGNAc: Reactivity tuning to deliver selective inhibitors for *N*-acetyl-D-glucosaminidases†‡

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The synthesis of analogues of the potent *N*-acetylhexosaminidase inhibitor PUGNAc are described. These compounds were assayed against a set of biologically important *N*-acetyl-D-glucosaminidases and were found to vary in both potency and selectivity.

Carbohydrate-processing enzymes are an important class of proteins found in nature involved in an array of biological processes including cell signalling, inflammation and protein quality control.¹ Inhibitors are widely useful in helping to understand this class of enzymes and especially the glycoside hydrolases (glycosidases).^{2–5} These inhibitors, including both natural products and unprecedented synthetic compounds enable researchers to understand catalysis by glycosidases, the tertiary structures of these enzymes, and their functional roles in biological systems. Indeed these compounds have also formed the basis for the development of therapeutics.^{6–10}

One sub-class of glycosidases of significant interest are the *exo-N*-acetyl-D-glucosaminidases which process *N*-acetylglucosamine residues from glycoconjugates. These enzymes are ubiquitous in all domains of life where, for example, they play roles in peptidoglycan recycling,¹¹ bacterial virulence¹² and competition¹³, as well as diverse roles in eukaryotes.¹⁴ As a result of the biological importance of these enzymes various different classes of inhibitor have been investigated.

One set of compounds which stand out as useful inhibitors of glycosidases are the hydroxylactones of which *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino *N*-phenylcarbamate (PUGNAc, Figure 1A) is the best known member.^{15,16} This compound is a potent inhibitor of *exo-N*-acetyl-D-glucosaminidases.^{15,17–21} The structural basis for the potency of hydroxylactones including PUGNAc lies, in part from these compounds possessing an *sp*²-hybridised carbon at C-1 which is thought to constrain these compounds to adopt a conformation resembling the geometry of the dissociative SN₂-like transition state of the reaction catalysed by glycosidases (Figure 1B). Another important feature is the oxime substituent, which mimics the aglycon of a natural substrate to harness additional binding energy.¹⁵

Despite PUGNAc having the benefits of high potency and membrane permeability, which make it suitable for cell-based studies, it lacks the selectivity,¹⁷ which is critically important

especially in eukaryotic systems containing multiple *exo-N*-acetyl-D-glucosaminidases. This selectivity problem is also shared by other glycosidase inhibitors, including various azas and iminosugars.^{22,23} In an effort to address the problems associated with selectivity, inhibitors have been designed where the compound scaffold is retained but elaborated with various chemical moieties. This method has met with success for various glycosidase inhibitors.^{17,24–27}

Some efforts have been made to improve the selectivity of PUGNAc toward specific *exo-N*-acetyl-D-glucosaminidases. For example, the C-4 epimer of PUGNAc, termed Gal-PUGNAc, has been shown to be selective for the lysosomal *exo-N*-acetyl-D-glucosaminidases²⁸ and the *N*-acyl group of PUGNAc has been modified to obtain modest selectivity for a nucleocytoplasmic *exo-N*-acetyl-D-glucosaminidase found in multicellular eukaryotes termed *O*-GlcNAcase (OGA) (Figure 1C).^{29,30}

Figure 1. (A) The chemical structure of PUGNAc and hydroxylactone LOGNAc. (B) The carbohydrate portion of the putative transition state of *exo-N*-acetyl-D-glucosaminidases. (C) Modifications of PUGNAc to induce selectivity.

One part of the chemical structure of PUGNAc which has not received much attention in terms of developing potent and selective inhibitors for *exo-N*-acetyl-D-glucosaminidases, is modification of the phenyl moiety (Figure 1C). This is despite this feature being important to binding as manifested by the markedly tighter binding of PUGNAc than 2-acetamido-2-deoxy-D-glucosaminohydroxyl-1,5-lactone (LOGNAc), to various

exo-N-acetyl-D-glucosaminidases.^{15,19} Also relevant is that *exo-N*-acetyl-D-glucosaminidases have different substrate specificities³¹⁻³³ which is reflected in different active site architectures in the region bound by the phenylcarbamate group.^{18,20,34,35} Based on these considerations we felt that modification of the phenylcarbamate group would yield new inhibitors of *exo-N*-acetyl-D-glucosaminidases that might possess selectivity for one enzyme over another. Here we present a divergent synthesis for preparing analogues of PUGNAc where the carbamoyl moiety is varied in terms of size, shape and hydrophobicity. Using enzymatic assays, with well characterized human *exo-N*-acetyl-D-glucosaminidases, we evaluate these molecules for potency and selectivity.

Scheme 1. a) 4-nitrophenyl chloroformate, DIPEA, THF. b) Aniline, DIPEA. c) Amine or aniline-based compound, DIPEA. d) NH₃, MeOH.

To realise a divergent synthetic pathway we hypothesized that the colourigenic carbonate **1** would serve as a useful intermediate because formation of the carbamate can be indirectly monitored by observation of the liberated 4-nitrophenolate anion. Thus, treatment of the readily prepared hydroxylactone **2**, which is prepared using either the *N*-acetyl^{16,36} or Boc-protected²⁹ synthetic methodologies, with 4-nitrophenyl chloroformate gave the desired carbonate **1** in good yield (Scheme 1). To test whether **1** would be suitably reactive with either substituted anilines or amines, we reacted **1** with aniline and, quite gratifyingly, obtained the phenyl carbamate **3**³⁶ in good yield. To further improve the efficiency of the method we felt that the reaction to give the carbamate **3** from the carbonate **2** could work in a one-pot strategy. Thus treatment of **2** to give the presumed carbonate *in situ* followed by addition of aniline gave the carbamate **3** in good overall yield (Scheme 1).

Using this one-pot methodology we treated the hydroxylactone **2** with a variety of aniline and amine derivatives that were comprised of a broad spectrum of

materials with different size, shape and hydrophobic/hydrophilic properties to yield a suite of acetylated analogues **4-33** (Scheme 1). Reaction times to prepare the carbamates were variable, and were mainly dependent on the nucleophilicity of the amine utilised. Of interest is that the yields of the reactions were not generally affected by the nucleophilicity of the amine. Generally, the primary amines reacted completely with the *in situ* formed carbonate **1**, as judged by TLC, within one hour, however anilines required up to four days for complete reaction at room temperature. Removal of the *O*-acetyl protecting groups was achieved using ammonia in methanol to give the desired triols **34-63** in good overall yield.

With these compounds in hand we evaluated them as potential inhibitors of three well characterised human enzymes, β -hexosaminidase B (HexB), NAGLU and OGA. These three enzymes, despite all being *exo-N*-acetyl-D-glucosaminidases, act on different substrates and their loss of function within cells give rise to distinct cellular phenotypes. For HexB and NAGLU, which are both lysosomal enzymes, their respective natural substrates are glycosphingolipids, bearing either *N*-acetylglucosamine or *N*-acetylgalactosamine,³¹ and heparan sulfate.³³ Dysfunction of either of these enzymes leads to the lysosomal storage disorders Tay-Sachs and Sandhoff diseases³⁷ for HexB and Mucopolysaccharidosis IIIB for NAGLU.³⁸ The enzyme OGA acts on different substrates by removing *N*-acetylglucosamine residues from post-translationally modified nucleocytoplasmic glycoproteins³² and dysfunction of this enzyme has been linked to a variety of diseases.³⁹ PUGNAc inhibits these enzymes^{15,18,40} with respective *K_i* values of 36 nM¹⁷, 7.1 ± 0.3 μ M and 46 nM¹⁷. Using an appropriate substrate (MU- β -D-GlcNAc for HexB and OGA and MU- α -D-GlcNAc for NAGLU, see Supporting Information) we screened compounds **34-63** at two concentrations (100 nM and 10 μ M) to gain a sense of the overall potency and selectivity of the compounds. We observed that these compounds are all inhibitors of these enzymes to varying extents (Figure 2).

Analysis of the phenyl derivatives reveals **34-37**, unsurprisingly, that this group of analogues generally shows similar potency to that of PUGNAc. Of note is the benzyl derivative **37**, even though the molecule has increased rotation of the phenyl ring due to the methylene spacer, the potency against the enzymes was not severely affected. The electronics of the phenyl ring seems to be important for inhibition for all the enzymes tested with the electron donating methoxy group on **35** reducing the potency of the scaffold relative to PUGNAc, whilst electron deficient **36** showed similar potency for HexB and OGA relative to PUGNAc. A potential reason for this observation lies in the binding interactions the phenyl ring makes in the +1 subsites of OGA and HexB.^{35,41}

Generally the potency of the cyclic aliphatic derivatives **38-42**, decreased with increasing ring size with the exception of the *N*-cyclohexyl derivative **38**, as it was more potent for HexB and OGA than the *N*-cyclopentyl derivative **40**. Interestingly, both **38** and **40** but not the cyclobutyl derivative **39** demonstrated selectivity for HexB over OGA. The percentage inhibition of the amino acid derived analogues

Phenyl derivatives

Cyclic aliphatic and amino acid derivatives

N,N'-disubstituted derivatives

Aliphatic derivatives

Figure 2. Percentage inhibition assays of the suite of PUGNAc-like compounds against three well characterized human enzymes HexB, NAGLU and OGA, all of which have been demonstrated to be inhibited by PUGNAc, using the substrates MU- β -D-GlcNAc for HexB and OGA and MU- α -D-GlcNAc for NAGLU. All assays were carried out in triplicate with the error representing the standard error in the average of the measurements.

5 **43-47** demonstrated that the glycine derivative **43** was selective for HexB over OGA. Notably, the serine derivative **45** showed completely reversed selectivity as compared to PUGNAc and was selective for NAGLU over the the other two enzymes. This may be due to the polar serine group
10 mimicking elements of the natural substrate of NAGLU which is the polar molecule heparan sulfate. The additional hexyl chain of **47** proved to increase the potency of the parent scaffold **43** for HexB and particularly OGA, suggesting that

these enzymes require some degree of hydrophobic character
15 in the +1 subsite. However, this substituent was deleterious for inhibition of NAGLU.

The aliphatic derivatives **48-58** were all less potent inhibitors than PUGNAc against the three *N*-acetylhexosaminidases. The *N*-2-chloroethyl derivative **58** did
20 not show significant differences compared to the *N*-ethyl and *N*-propyl analogues **49** and **50**, which may be of interest in terms of enabling further functionalisation.

The *N*-isobutyl derivative **54** showed selectivity for HexB over OGA and the unsaturated derivatives **56** and **57** also showed similar selectivity when compared to **50**. The compounds were not as potent as expected despite the extra electron density located in the aglycone portion of these molecules. The alkyne **57** was selective for HexB over OGA and so sets the stage for this molecule potentially being used as a synthon in click chemistry based library approaches to generate triazole-based inhibitors of *exo-N*-acetyl-D-glucosaminidases. The *N,N*-disubstituted derivatives **59–63**, which lack one less hydrogen bond donor, were prepared to investigate the role that this H-bond may play in potency and selectivity. These compounds were inhibitors of all the enzymes tested, with **59**, **60** and **63** showing selectivity for HexB over OGA.

With these activity assays in hand several inhibitors were carried forward to the next stage of evaluation, where K_i values were obtained against the enzymes (Table 1). The trends observed in the rapid screen assay were consistent with the K_i values obtained. Inhibitors lacking the phenyl ring were significantly less potent than PUGNAc. The assay results were consistent with **35** being a worse inhibitor

Table 1. Inhibition constants of inhibitors against HexB, NAGLU and OGA.

Compound	K_i (μ M)		
	HexB	NAGLU	OGA
PUGNAc	0.036 ¹⁷	7.1 $\pm$ 0.3	0.046 ¹⁷
34	0.021 $\pm$ 0.005	39 $\pm$ 3.4	0.028 $\pm$ 0.007
35	0.111 $\pm$ 0.019	19 $\pm$ 2.1	0.178 $\pm$ 0.042
36	0.047 $\pm$ 0.002	21 $\pm$ 4.3	0.056 $\pm$ 0.016
40	14 $\pm$ 3.5	122 $\pm$ 19	230 $\pm$ 78
43	1.3 $\pm$ 0.17	8.6 $\pm$ 0.9	78 $\pm$ 26
45	45 $\pm$ 16	4.3 $\pm$ 1.4	420 $\pm$ 140
54	17 $\pm$ 4	105 $\pm$ 15	220 $\pm$ 75
55	161 $\pm$ 8	195 $\pm$ 19	188 $\pm$ 72
63	0.77 $\pm$ 0.031	222 $\pm$ 28	11 $\pm$ 4.8

compared to PUGNAc while **34** and **36** showed similar potency for HexB and OGA relative to PUGNAc. Consistent with the assay results, good selectivity was observed for the amino acid analogues **43** and **45**, with **43** having over 50-fold selectivity for HexB and ~10-fold for NAGLU, against OGA. This presents potentially a useful scaffold for synthetic elaboration. The serine analogue **45** is potent against NAGLU, relative to PUGNAc, but is more selective for this enzyme against HexB compared to **43**. As a result, compound **45** could serve as an efficient starting point when preparing potential selective inhibitors for NAGLU. Interestingly the morpholine derivative **63** is >10-fold more selective for HexB over the other enzymes and the cyclopentyl derivative **40**, whilst having lost potency compared to PUGNAc, is ~10-fold selective for HexB over the other enzymes.

In conclusion we have developed a divergent synthetic route that enables the rapid synthesis of a series of PUGNAc-like inhibitors bearing different carbamate groups. Using

assays that allow for rapid screening we demonstrated that all the compounds prepared were inhibitors against the *exo-N*-acetyl-D-glucosaminidases tested, HexB, NAGLU and OGA. Difference in potencies is consistent with differences in the active site architectures between these enzymes and suggests that modification of PUGNAc at this position warrants further investigation. Indeed these further investigations, using the strategy outlined here may reveal even more selective inhibitors for these enzymes. Finally, we anticipate that these observations will, in combination with other structural variations, lead to highly selective tool compounds for these enzymes that could be used in cells and tissues.

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Notes and references

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