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Exploring Artificial Metalloglycosidases as Selective Catalysts for the Recognition and Degradation of the sLe^x Tetrasaccharide

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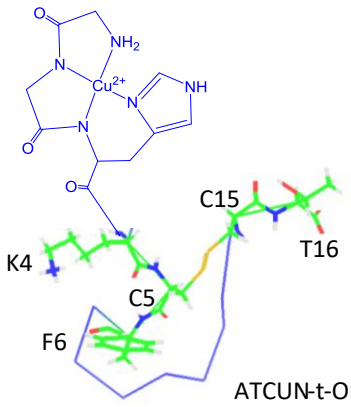
Small molecule recognition of carbohydrate moieties is a largely uncharted area of chemistry. Herein we report the use of metallopeptide-based scaffolds for sugar recognition and catalysis by application of a metalated fucose-binding peptide to remove cellular sLe^x antigen that has been linked to cellular adhesion and cancer metastasis. Catalytic assays against synthetic carbohydrates show preference of the metallopeptide for fucosyl- and galactose-containing carbohydrates, while MS-assays demonstrate metallopeptide mediated degradation of native sLe^x tetrasaccharide in solution. Cellular assays indicate that the metallopeptide can remove the antigen from the surface of an HL-60 leukemia cell line, demonstrating potential therapeutic utility for these metallopeptides as artificial metalloglycosidases.

Glycosylation of proteins and lipids is ubiquitous in biology. This process aids in altering the structural and functional properties of the tagged biomolecules. N-acetyl neuraminic acid, otherwise known as sialic acid, is one specific terminal glycosylation event that plays a critical role in cellular signalling, cell-cell adhesion, and cellular recognition[1]. While glycosylation is intrinsically important for cellular chemistry, both over-expression or aberrant sialylation of glycans have been implicated in tumorigenic cancer phenotypes, metastasis,[1, 2] and poor patient prognosis. Appending sialic acid to specific Lewis-blood antigens, namely sialyl-Lewis^a (sLe^a) and sialyl-Lewis^x (sLe^x), has also been correlated to a variety of cancer types.[2-4] Upregulation of sLe^x antigen on the tumor's cell surface enables key binding interactions with selectins, which have been well documented in cellular adhesion.[5] These interactions promote the development of aggregates comprised of leukocytes, platelets, and tumor cells, enabling evasion from the immune system and adherence to the endothelium, contributing to metastasis.[2, 6]

Interestingly, metastasis has been treated in mouse models either by the inhibition of sialyltransferases, which contribute to the terminal sialylation of these glycans,[7, 8] or by treatment of cancer cells by sialidases that remove sialic acid from the sLe^x antigen.[9] It has also been shown that removal of sialic acid from human promyelocyte leukemia cell line HL60 by reactive-oxygen species (ROS) results in inhibition of adhesion to human umbilical vein endothelial cells (HUVECs).[10]



ATCUN-t-OL GGHKCFRYPNGVLACT-NH2

ATCUN-t-OL2 GGHGKCFRYPNGVLACT-NH2

Figure 1. Structural representation of the two peptides described in this work, showing the metal binding ATCUN sequence (GGH) and carbohydrate recognition domain (t-OL). The copper bound form of the GGH ATCUN motif is explicitly shown. The structure of the t-OL sequence has been reported.[11, 16]

These previous reports suggest that removal or inhibition of the sLe^x antigen binding interaction can be viewed as a potential pathway to slow or prevent cancer cell adhesion and metastasis. In this paper we expand on our prior efforts to identify peptides capable of molecular recognition and chemical cleavage of targeted carbohydrate moieties,[11] Such peptides have been modified with an amino-terminal copper and nickel (ATCUN) metal-binding domain (Gly-Gly-His, and summarized hereafter as GGH). This binds copper with femtomolar affinity and catalytically mediates formation of metal-associated reactive oxygen species (ROS) *in vitro* when supplied with oxidizing and reducing agents such as ascorbic acid and dioxygen.[11] These metal-associated ROS are capable of acting on a variety of biological targets.[11-15]

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[#] Electronic Supplementary Information (ESI) available: Experimental methods, SPR binding plots, ESI-MS data, antigen binding data and molecular structures.

Metallopeptides termed ATCUN-t-OL and ATCUN-t-OL2 (Figure 1) are synthetic, truncated versions[11, 16] of a natural peptide isolated from the frog skin of *Odorrana grahami*[17] that differ by the introduction of a spacer glycine (G) between the carbohydrate recognition sequence (t-OL) and the copper-bound catalytic motif (Cu-GGH). Such metallopeptides have been reported to bind and degrade the erythrocyte H2-antigen both *in vitro* and *in vivo*.[11] While only fucose and the H2-antigen were explored in this previous report, it was of interest to determine the selectivity of this peptide against an array of fucose-containing Lewis blood antigens using surface plasmon resonance, as each carbohydrate moiety contains at least one or two fucose carbohydrate units.

In this work we first evaluated catalytic activity toward simple sugar derivatives. Subsequently, reactivity toward the sLe^x tetrasaccharide was evaluated by use of a liquid chromatography mass spectrometry (LC-MS) assay, and degradation monitored by electrospray ionization mass spectrometry (ESI-MS). As the ultimate goal was to demonstrate removal of the sLe^x antigen from the surface of cancer cells, an immunostaining assay was also performed, where catalytically active peptides were incubated with a leukemia cell line (HL-60) that has previously been shown to express the sLe^x antigen on the cell surface.[10, 18] Monitoring the removal of the sLe^x antigen was performed by use of a fluorescent antibody that selectively binds the sLe^x antigen via fluorescence-assisted cell sorting (FACS).

Initial insight on catalytic reactivity and carbohydrate recognition was obtained by use of a rapid and efficient *para*-nitrophenol (*p*NP) assay with *p*NP-conjugated carbohydrates (Figure S5). These were incubated with metallopeptide and co-reagents to facilitate catalysis. Reaction progress was monitored by following the 405 nm absorbance of the *p*-nitrophenolate product anion. Results show that the ATCUN-t-OL peptide was active against a wide array of mono- and disaccharide carbohydrate substrates (Table 1 and Figures S6). Minimal activity was observed against the control *p*NP-phosphate reagent, indicating there to be little contribution to recognition from the *p*NP-moiety of these substrates. Catalytic rate constants varied for most carbohydrate substrates, lying between 0.51 min^{-1} and 4.37 min^{-1}, with higher rate constants observed for galactose, mannose, and lactose *p*NP-substrates. The Michaelis-Menten constant also varied, with values ranging from 0.36 to 2.8 mM and the L-fucose and D-galactose substrates showing the lowest (higher affinity) values.

The disaccharide substrate *p*NP-cellobiose displayed comparable catalytic activity to the monosaccharide substrate *p*NP-glucose. These results are in agreement as the disaccharide is comprised of two glucose monomers linked in a β(1-4) fashion. The ATCUN-t-OL peptide also showed a preference for galactose, galactose-containing, and mannose carbohydrates. The highest catalytic efficiency was observed for the *p*NP-D-galactose substrate, while *p*NP-L-fucose, *p*NP-D-mannose, and *p*NP-D-lactose all displayed similar catalytic efficiencies. This ranged from 3000 to 3400 M^{-1}min^{-1}, consistent with our previous investigations into the binding behavior of a longer peptide containing an N-terminal YASP-sequence.[19] Furthermore, since the mannose substrate varies from galactose at the axial and equatorial C-2 and C-4 positions, the exchange of axial and equatorial hydroxyls at these positions could potentially result in a preferential binding conformation for the mannose substrate that is unavailable to the glucose moiety, which contains all-equatorial hydroxyl groups.

Encouraged by the glycosidase activity observed for copper-bound ATCUN-t-OL peptide, it was of interest to determine if catalytic activity could be observed for the natural sLe^x tetrasaccharide. Liquid-chromatography mass spectrometry (LC-MS) was a convenient analytical method to monitor the reaction progress for copper ATCUN-t-OL promoted cleavage of the 3'-sLe^x tetrasaccharide following the addition of co-reagents (ascorbic acid and hydrogen peroxide) to initiate catalytic activity.[12, 13] Reactions were incubated over the time span of 0 to 165 min and aliquots were removed, quenched, and chilled until they were analyzed via LC-MS. Reaction progress was monitored by use of the base peak current (BPC) at specific mass-to-charge ratios corresponding to the sLe^x tetrasaccharide reactant. Both the [M+H] and [M+Na] peaks at m/z values of 821.3 and 843.29, respectively, were summed and the integrated peak area was used to quantitate the amount of reactant left within the reaction mixture. A control reaction was performed with an α2-3,6,8 neuraminidase enzyme that specifically cleaves terminal sialic acids from proteins in order to validate our methodology (Figure S7-S8).

Table 1. Michaelis-Menten Parameters for metallopeptide ATCUN-t-OL.

substrate	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (M^{-1} min^{-1})
pNP-α-L-fucose[11]	0.36 ± 0.08	1.2 ± 0.17	3400 ± 310
pNP-β-D-glucose[11]	2.25 ± 0.62	0.51 ± 0.08	230 ± 30
pNP-β-D-galactose	0.81 ± 0.11	3.29 ± 0.12	4100 ± 570
pNP-α-D-mannose	1.36 ± 0.22	4.37 ± 0.22	3200 ± 540
pNP-phosphate	1.9 ± 0.28	0.21 ± 0.01	110 ± 20
pNP-β-D-cellobiose	2.8 ± 0.38	1.01 ± 0.05	360 ± 50
pNP-β-D-lactose	1.07 ± 0.09	3.2 ± 0.08	3000 ± 270

Following validation of the LC methodology, the metallopeptide ATCUN-t-OL was incubated with the sLe^x tetrasaccharide and relevant co-reagents. The degradation of substrate is shown in Figures 2. Control reactions were performed with no metallopeptide and only co-reagents, in addition to the Cu-GGH tripeptide and co-reagents (Figures S9-S11). As the method of separation for the sialidase control reaction shows clear evidence that sialic acid and the Le^x trisaccharide could be detected, it was of interest to see if these products were produced for the metalloglycosidase reaction. Indeed, both sialic acid and the Le^x carbohydrates were observed, albeit at lower levels than the sialidase reaction (Figure 2C).

It was also of interest to determine if the addition of a spacer glycine residue between the ATCUN motif and the

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binding moiety in ATCUN-t-OL2 could introduce additional flexibility into the catalytic domain that might enhance reactivity and/or change the principal reaction site. While similar robust catalytic activity was observed, as well as the sialic acid and the Le^x trisaccharide products, in this case the additional flexibility of the glycine linker did not enhance reactivity nor yield additional products.

We then moved to assess binding efficiency. Previous reports have shown that the binding affinity for this metallopeptide towards L-fucose was 61 μM.[13] SPR experiments against biotinylated Lewis blood antigens demonstrate a clear preference for the sLe^x tetrasaccharide with a K_D of 77 μM (Table 2 and Figures S1-S4). Interestingly, no binding was observed for the Le^x trisaccharide which contains fucose but lacks sialic acid. An unexpected trend was observed in comparing the sLe^a and Le^a blood antigens, insofar as the preferred binding was found to be for the non-sialylated Le^a trisaccharide rather than the sialylated sLe^a tetrasaccharide, suggesting that not only are the individual carbohydrate units important, but also their orientation and appropriate structural linkages. Only the di-fucosylated Le^y antigen showed any measurable binding, which may be due to the nature of branching and connectivity of fucose within the tetrasaccharide.

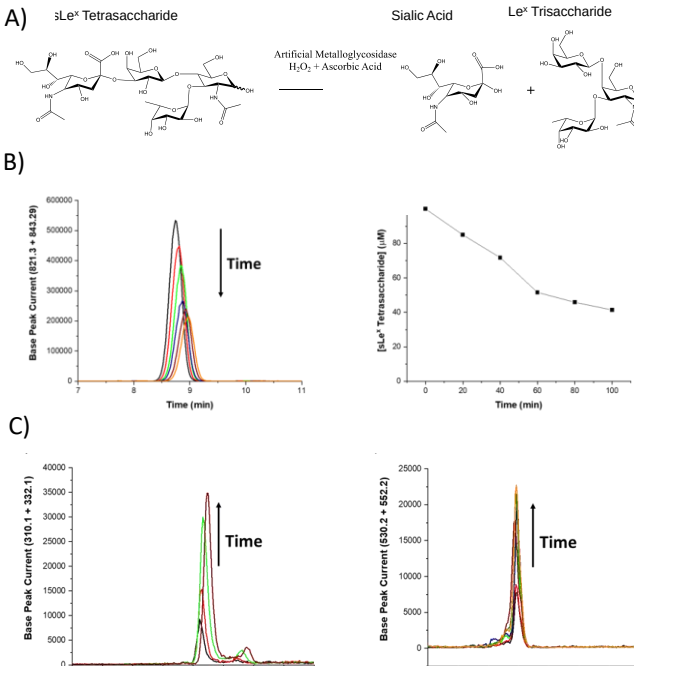
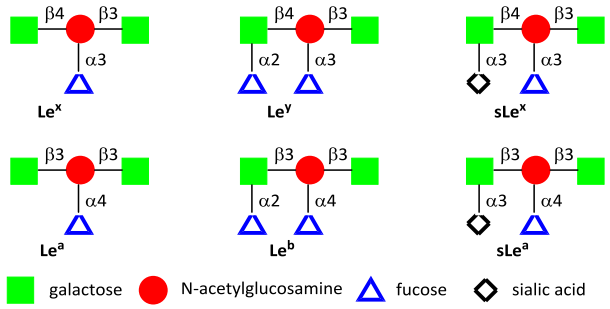


Figure 2. Metallopeptide reaction scheme and base peak current (BPC) chromatograms. A) Proposed reaction mechanism. B) Reactant BPC chromatogram (left) and reaction progress (right) for sLe^x substrate for the Cu-ATCUN-tOL peptide reaction, C) BPC chromatogram for sialic acid (left) and Le^x trisaccharide (right) products.

As a clear preference for binding the sLe^x epitope had been established through SPR, and the designed metallopeptides demonstrated the capacity for cleavage of the sLe^x tetrasaccharide in LC-MS reaction assays, we then evaluated activity against cancer cells that have been shown to express the sLe^x determinant that is important for cellular adhesion.[3, 10, 20] The removal or inhibition of this interaction has been shown to be critical for preventing these cancer cells from adhering to endothelial cells and could be envisioned as a vehicle for development of potential anti-metastatic therapeutics.[6, 10, 21, 22] Monitoring the removal of carbohydrate determinants is readily facilitated through immunostaining methodologies and has been previously used to detect the removal of the H-Type-II blood antigens from red blood cells.[11]

Table 2. Binding affinities for ATCUN-t-OL peptide against biotinylated-Lewis antigens as determined by SPR (NB = no binding observed). Scheme below shows the tetrasaccharides studied and their components

Lewis Blood Antigens	K_D (μM)
sLe^x (sialyl $Lewis^x$)	77.2 ± 3.5
Le^x ($Lewis^x$)	NB
sLe^a (sialyl $Lewis^a$)	3030 ± 400μM
Le^a ($Lewis^a$)	150.2 ± 6.9μM
Le^b ($Lewis^b$)	NB
Le^y ($Lewis^y$)	179.0 ± 2.5



Both metallopeptides, ATCUN-t-OL and ATCUN-t-OL-2 were incubated with a human leukemia cell line (HL-60) that expresses the sLe^x epitope important for cell adhesion. Control experiments were performed with the same α2-3,6,8 sialidase enzyme described earlier in LC-MS experiments. These experiments show a concentration dependence, whereby at 25 U of sialidase approximately 30 % of the sLe^x antigen is removed, while at 250 U of sialidase virtually all of the antigen is eliminated (Figure S12). Additional controls were performed for both untreated, and the CU-ATCUN motif (Cu-GGH) lacking the sugar recognition domain, to demonstrate that reactivity requires the binding moiety. Another factor that is generally required for these types of reactions is the requirement for reducing reagents; therefore cells were incubated both with and without ascorbic acid.

Results shown in Figure 3 indicate that in the absence of ascorbic acid there is minimal activity of all metallopeptides tested, including the control Cu-GGH. Upon the addition of 500 μM ascorbic acid, both Cu-ATCUN-t-OL and Cu-ATCUN-t-OL-2 peptides display an approximately 20 % removal of the sLe^x antigen from the surface of HL-60 cells, while at 50 μM Cu-peptide there is about a 35-40 % removal of the sLe^x antigen. The concentrations required are slightly higher than needed for the removal of the H-Type-II trisaccharide, but encouraging, nonetheless.

Investigation into the feasibility of designing metallopeptides as artificial glycosidases is extremely important, as it allows for new ways in which to treat diseases and yields new tools and probes for use in molecular biology,[11-15] Previous exploration has shown that the Cu-ATCUN motif is capable of mediating selective cleavage of carbohydrates.[11, 16] Our results show that the ATCUN-t-OL peptide is a selective molecule for a variety of Lewis blood carbohydrate determinants as evidenced by SPR. The overall binding selectivity for sLe^x over other Le^a and Le^y carbohydrates is 2:1, while others show minimal or no binding. The ATCUN-t-OL peptide also demonstrates a preference for fucose, galactose-containing, and mannose conjugated-carbohydrates via the *p*NP UV-Vis assay, further highlighting catalytic selectivity.

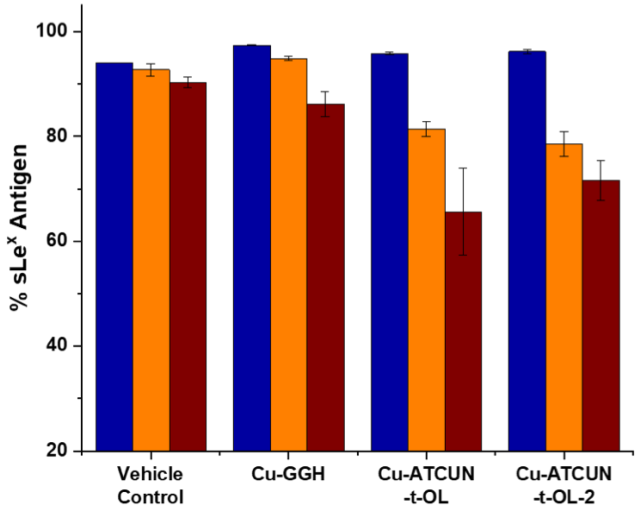


Figure 3. sLe^x Antigen Removal from HL-60 Cells by metallopeptides. A) Comparison of reaction conditions with 25 µM Cu-peptides and either no ascorbic acid (blue) or 500 µM ascorbic acid (orange), or 50 µM Cu-peptides and 500 µM ascorbic acid (red).

LC-MS reactions on the natural sLe^x tetrasaccharide show that both ATCUN-t-OL peptide variants can degrade the carbohydrate target and produce sialic acid and the Le^x trisaccharide. Control reactions with coreagents only, or with Cu-GGH and coreagents, show minimal reactivity over time. Additional aliquots of coreagents also readily facilitate the cleavage chemistry observed for both ATCUN-t-OL peptides, further highlighting their ability to turnover and serve as efficient catalysts. Experiments with HL-60 cells that express the sLe^x epitope show that in the absence of catalyst, or with only the Cu-GGH metal-binding motif, very little antigen removal is observed. In the absence of co-reagents minimal activity is also observed. However, following the addition of ascorbic acid approximately 25% or more (depending on reaction conditions) of the antigen is removed. These results showcase the potential of artificial metalloglycosidases and underscore their potential importance and the need for further development. Additional exploration into other peptides and small molecules that contain catalytic domains are warranted in order to explore the value of such catalysts as potent therapeutics or tools for use in molecular biology.

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References

1. S. S. Pinho and C. A. Reis, *Nat Rev Cancer*, 2015, **15**, 540-555.
2. M. J. Schultz, A. F. Swindall and S. L. Bellis, *Cancer Metastasis Rev*, 2012, **31**, 501-518.
3. A. Takada, K. Ohmori, T. Yoneda, K. Tsuyuoka, A. Hasegawa, M. Kiso and R. Kannagi, *Cancer Res*, 1993, **53**, 354-361.
4. J. X. Liang, Y. Liang and W. Gao, *Onco Targets Ther*, 2016, **9**, 3113-3125.
5. Y. Geng, J. R. Marshall and M. R. King, *Ann Biomed Eng*, 2012, **40**, 790-805.
6. H. Laubli and L. Borsig, *Semin Cancer Biol*, 2010, **20**, 169-177.
7. N. B. Drinnan, J. Halliday and T. Ramsdale, *Mini Rev Med Chem*, 2003, **3**, 501-517.
8. J. Y. Chen, Y. A. Tang, S. M. Huang, H. F. Juan, L. W. Wu, Y. C. Sun, S. C. Wang, K. W. Wu, G. Balraj, T. T. Chang, W. S. Li, H. C. Cheng and Y. C. Wang, *Cancer Res*, 2011, **71**, 473-483.
9. R. S. Bresalier, R. W. Rockwell, R. Dahiya, Q. Y. Duh and Y. S. Kim, *Cancer Res*, 1990, **50**, 1299-1307.
10. H. Eguchi, Y. Ikeda, T. Ookawara, S. Koyota, N. Fujiwara, K. Honke, P. G. Wang, N. Taniguchi and K. Suzuki, *Glycobiology*, 2005, **15**, 1094-1101.
11. Z. Yu and J. A. Cowan, *Angew Chem Int Ed Engl*, 2017, **56**, 2763-2766.
12. J. A. Cowan, *Pure Appl Chem*, 2008, **80**, 1799-1810.
13. J. C. Joyner and J. A. Cowan, *Braz J Med Biol Res*, 2013, **46**, 465-485.
14. Z. Yu and J. A. Cowan, *Chemistry*, 2017, **23**, 14113-14127.
15. Z. Thompson and J. A. Cowan, *Small*, 2020, **16**, e2000392.
16. T. Peng, Z. Wooke and N. L. B. Pohl, *Carbohydr Res*, 2018, **458-459**, 85-88.
17. J. Li, H. Wu, J. Hong, X. Xu, H. Yang, B. Wu, Y. Wang, J. Zhu, R. Lai, X. Jiang, D. Lin, M. C. Prescott and H. H. Rees, *PLoS One*, 2008, **3**, e2381.
18. T. Fujita, K. Murayama, T. Hanamura, T. Okada, T. Ito, M. Harada, A. Komatsu, H. Koyama, T. Kanai, K. Maeno, Y. Mochizuki, Y. Hama, K. Ito, J. Amano and M. Fujimori, *Jpn J Clin Oncol*, 2011, **41**, 394-399.
19. M. C. Rodriguez, A. B. Yongye, M. Cudic, K. Martinez Mayorga, E. Liu, B. M. Mueller, J. Ainsley, T. Karabencheva-Christova, C. Z. Christov, M. Cudic and P. Cudic, *Amino Acids*, 2017, **49**, 1867-1883.
20. Y. Ogasawara, T. Namai, F. Yoshino, M. C. Lee and K. Ishii, *FEBS Lett*, 2007, **581**, 2473-2477.
21. P. Borentain, S. Carmona, S. Mathieu, E. Jouve, A. El-Battari and R. Gerolami, *Cancer Chemother Pharmacol*, 2016, **77**, 847-856.
22. A. Natoni, M. L. Farrell, S. Harris, C. Falank, L. Kirkham-McCarthy, M. S. Macauley, M. R. Reagan and M. O'Dwyer, *Haematologica*, 2020, **105**, 457-467.

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