Article

The application of N-acetylmannosamine to the mammalian cell culture production of recombinant human glycoproteins

Selwyn C Yorke

New Zealand Pharmaceuticals Limited, PO Box 1869, Palmerston North 4440
(e-mail: *selwyn.yorke@nzp.co.nz*)

Keywords: N-acetylmannosamine, sialylation, glycoproteins.

This mini-review covers recent developments in the understanding, role and value of N-acetylmannosamine (ManNAc) as a compound that can improve sialylation during the production of recombinant human glycoproteins.

Introduction

Sialic acid (N-acetylneuraminic acid, NeuAc, Neu5Ac) is an essential terminal sugar on the glycan moieties of many functional and structural human glycoproteins. A key intermediate in the biochemical process to form sialic acid is the monosaccharide, ManNAc, which is formed by the bifunctional enzyme UDP- N-acetylglucosamine / N-acetylmannosamine epimerase kinase (GNE). ManNAc is an intermediate in the formation of the final product of the GNE enzymatic transformation, ManNAc-6-phosphate, which is subsequently condensed with pyruvate to form the 9-phosphate of sialic acid. At this point the sialic acid is either modified to form other sialic acids or is activated in the cell nucleus to form the nucleotide CMP-sialic acid prior to bonding to galactose (the intermediate terminal sugar of the glycan in glycoprotein formation, by means of a sialyl transferase,[1] Fig. 1). This is normally the last step in the biosynthesis of a wide range of glycoproteins which are found in all human tissues.

The functional quality of the GNE enzyme is now recognised as one of the defining features in the efficient production of glycoprotein therapeutic drugs.[2] Incomplete sialylation can lead to poor therapeutic efficacy and short half-lives along with the possible formation of antibodies;[3] hence, there has been considerable research in recent years to optimise the sialylation process.

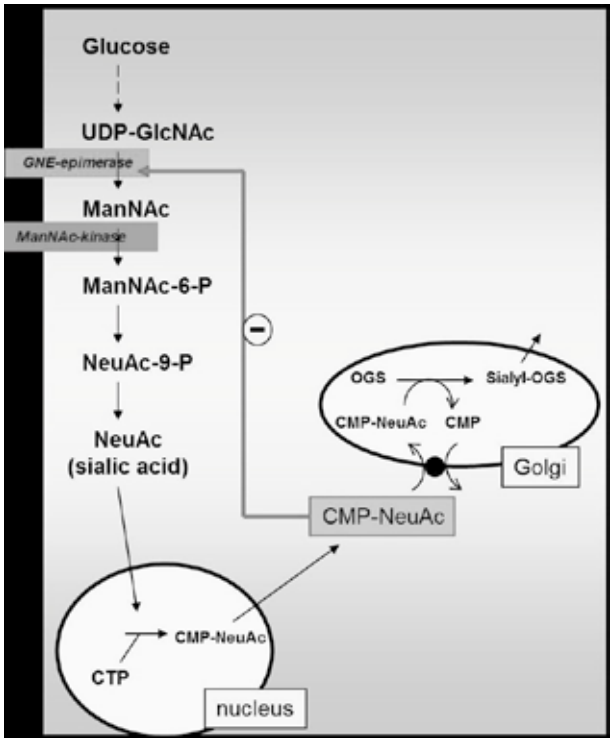

Fig. 1. Sialic acid biosynthesis and conjugation

The failure of the GNE enzyme is also the cause of "Hereditary Inclusion Body Myopathy" (HIBM), also known as "GNE Myopathy", which is a rare genetic disorder with no available therapy.[4] Disease symptoms emerge in adulthood and slowly lead to progressive muscle weakness. There is evidence that HIBM is caused by hyposialylated muscle proteins. There is also evidence that the malfunction of other sialylation-pathway enzymes could contribute to several glomerular kidney diseases[5] owing to the lack of the sialic acid terminal sugar on several kidney glycoproteins.

Recombinant proteins as therapeutic drugs

Recombinant human glycoproteins are finding increasing applications in therapy and the trend is likely to escalate as new discoveries are harnessed through cell culture technologies. Furthermore, therapeutic applications are evolving for diseases in which there has never been a prior form of therapy. Consequently, there is a great deal of interest in developing new products for the healthcare sector. The compound annual growth rate of these recombinant human glycoproteins is 16%; double the average growth rate of the pharmaceutical sector.[6]

There is normally some level of sialylation of specific glycans within a glycoprotein, but with the observation that incomplete sialylation leads to reduced biological activity and/or increased immunogenicity, it becomes relevant to assess the cell-lines and culture media to improve performance and yield, and reduce manufacturing costs. ManNAc is a potential non-nutrient culture medium ingredient that can increase sialylation to therapeutically effective levels that is receiving attention. This report assesses the benefits of using ManNAc in Chinese Hamster Ovary (or CHO cells, the mammalian "work-horse" cell type for the production of therapeutic recombinant human glycoproteins) cultures to produce sialylated glycoproteins.

Issues around ManNAc and glycoprotein sialylation

The benefits of using ManNAc as an ingredient in a model CHO cell line to produce recombinant human interferon-γ was investigated in some detail.[2] ManNAc was chosen over sialic acid as the preferred additive to the culture medium because it is a specific precursor for intracellular synthesis of sialic acid and it has greater cell membrane

permeability than sialic acid itself and CMP-sialic acid, at physiological pH. The authors (Gu and Wang[2]) were able to measure a nearly 30-fold increase in intracellular concentration of CMP-sialic acid upon ManNAc feeding, and increased incorporation of the precursor into the sialylated product. While sialylation was significant, it was always incomplete on the pre-sialylated biantennary glycan structures at specific asparagine glycosylation sites. The incomplete sialylation might have been because a sialidase removed the sialic acid once formed on the glycan, or perhaps there was limited access of the CMP-sialic acid to the Golgi apparatus[7] in the cell nucleus, or perhaps there was limited steric accessibility for the sialylation to occur. The authors concluded that more dramatic sialylation could occur for proteins with low sialylation profiles and more easily accessible sialylation sites.

Another group published their work soon after the Gu and Wang paper[2] and noted that while the CMP-sialic acid pool increased, they did not identify increased sialylation of the human tissue inhibitor of metalloproteinase (TIMP-1) glycoprotein either in CHO cells or in the murine myeloma NS0 cell lines.[6] It was suggested that there could be three basic variables that could affect sialylation: (i) availability of sialyltransferases; (ii) abundance of competing acceptor sites; (iii) availability of nucleotide-sugar substrate in the Golgi. These three issues continue to be the key problems today.

A significant secondary outcome from the addition of ManNAc was the increase in the ratio of NeuAc versus N-glycolylneuraminic acid (NeuGc) moieties on the therapeutic protein product. The increase in the proportion of NeuAc over NeuGc helps to "humanise" the glycoprotein because NeuGc is not produced in the human body.[8]

Research on the benefits of adding ManNAc to various glycoprotein production systems continues. It is clear that the structural heterogeneity of glycans is sensitive to fed batch (initial single large dose) to interval feeding (periodic smaller doses through the production), culture type and environment, nutrient balance, waste accumulation, oxygen levels, pH and temperature. Therefore, process control is critical to improving the production of the glycoproteins.

Erythropoietin (EPO) is a heavily sialylated glycoprotein hormone, and recombinant human EPO is used therapeutically to enhance the production of red blood cells. There are up to 14 sialic acids per EPO molecule with 4 sialic acid molecules on each tetra-antennary *N*-glycan and 2 sialic acid molecules on the *O*-glycans. By use of a novel CHO-EPO cell line, it was demonstrated[9] that the addition of ManNAc increased the sialylation of the EPO, although a genetic modification of the GNE enzyme was claimed as best of all. However, genetic modification of cell lines is a competitive R&D area and a commercially very sensitive and secretive operation, and scaling up to commercial production introduces technical and regulatory complications. ManNAc supplementation is likely to enhance a range of culture types without breach of specific patents, and furthermore, supplementation does not affect the regulatory environment because it is not changing the way the cell synthesises the glycoprotein. This is particularly important when a company files an Investigational New Drug (IND) application with the FDA in the USA. INDs need to be accepted by the FDA prior to any organisation starting any first in human, or clinical, trials in the USA.

Baker[10] noted the availability of CMP-sialic acid could be limited in the Golgi. However, she also demonstrated that the addition of ManNAc beneficially increased the ratio of NeuAc versus NeuGc in the glycan structure.

Bork[9] reported that ManNAc "could influence" cell proliferation and differentiation, which are indicative signs of toxicity. Bork's concern was proved not to be a problem in mammals themselves because high dose cell culture and oral toxicity studies in two animal species have been undertaken and there was no observable toxicity.[11] Whether this is important in cell cultures is a moot point.

Bork also suggested that ManNAc was too expensive for commercial applications, so NZP undertook calculations to assess the cost of its cell culture grade ManNAc[12] versus the value of the glycoprotein in question. ManNAc becomes a minimal cost at just a few dollars per gram compared to some therapeutic glycoproteins valued at up to US$1000 per gram. The issue raised by Bork becomes inconsequential and ManNAc addition to the culture medium is rightfully the first-choice approach to increase intracellular sialic acid concentrations in a large-scale production process.[1]

In another review,[13] published concurrently with the review by Bork,[9] it was noted that in all technologies to produce therapeutic glycoproteins – be they by CHO, NS0, HEK, BHK or PERC.6 cell lines – there are issues with expressing the complete human glycoprotein with constraints in glycosylation. There was also an emphasis on glycoform profiling because of the growing issues surrounding the NeuGc glycan variant.

In a study on the impact of feeding nucleoside sugar precursors to CHO cells,[14] it was demonstrated that ManNAc plus cytidine increased the CMP-sialic acid pool in line with published results from Gu and Wang,[2] but it did not lead to a synergistic increase in the glycoprotein sialylation. Along with the range of results found in this study, it provided further evidence that mechanisms exist in the Golgi that inhibit glycoprotein sialylation.

In order to overcome the problems associated with effecting utilisation of raised levels of the CMP-sialic acid for enhanced glycoprotein sialylation, there have been attempts to insert improved transporters and sialyltransferases in the CHO cell.[15] At the same time the investigators improved the function of the GNE enzyme, although this seems a little pointless considering ManNAc feeding has already been proven to increase the CMP-sialic acid pool. The authors argue that ManNAc is too expensive, but it is now recognised that the current cost of the chemical at scale is insignificant compared to the value of the glycoprotein and so this argument can be dismissed.

In the most recent relevant publication,[16] a "high throughput method" for the quantification of sialylation on gly-

coproteins was developed and the authors' assessment confirmed earlier observations that there are different mechanisms operated by different mammalian cells to produce glycoproteins. This is unsurprising and within their own work they detected significant interclonal variability in the sialylation of the interferon-γ glycoprotein model. A key result of this thesis once again demonstrated that the addition of ManNAc at concentrations as low as 2mM (plus a specific metal ion cofactor) enhances the sialylation and production of interferon-γ. It will be interesting to note the breadth of the relevance of the metal ion co-factor in future work.

Conclusion

The addition of ManNAc to the culture medium can make a special and essential contribution to maximising the sialylation of certain therapeutic glycoproteins. Some mammalian cell cultures are more efficient with the sialylation mechanism than other types of mammalian cell lines, although there are many factors that can influence the sialylation yield. It is likely that the best utility of ManNAc will be in the production of proteins that do not have sterically hindered sites that prevent the sialyltransferase from operating efficiently in the final step of the glycosylation process. The addition of ManNAc to the medium might also gain extra value where the NeuGc content in a glycoprotein should be minimised. Increased sialylation reduces the risk of immunogenicity and extends the half-life of therapeutic glycoproteins.

Owing to the recent availability of ManNAc in large volumes at modest cost, it has become an essential culture medium ingredient for research with the possibility that it could provide an important function in large scale recombinant human therapeutic glycoprotein production.

Acknowledgement

Comments from Dr Barry Old (NZP) and Dr Richard Furneaux (IRL) are gratefully acknowledged.

References:

1. Bork, K.; Horstkorte, R.; Weidermann, W. *J. Pharm. Sci*. **2009**, *98*(10), 3499.
2. Gu, X.; Wang, D.I.C. *Biotech and Bioeng*. **1998**, *58*(6), 642.
3. Weiss, P.; Ashwell, G. *Prog. Clin. Biol. Res*. **1989**, *300*, 169.
4. Huizing, M.; Krasnewich, D.M. *Biochim. Biophys. Acta*. **2009**, *1972*(9), 881.
5. Galeano, B.; Klootwijk, R.; Manoli, I.; Sun, M.; Ciccone, C.; Darvish, D.; Starost, M.F.; Zerfas, P.M.; Hoffmann, V.J.; Hoogstraten-Miller, S.; Krasnewich, D.M.; Gahl, W.A.; Huizing, M. *J. Clin. Invest.* **2007**,*117*(6), 1585.
6. Werner, R.G.; Kopp, K.; Schlueter, M. *Acta Paediatrica*. **2007**, *96*(s455), 17.
7. The Golgi apparatus, also known as the Golgi complex or Golgi body, is an organelle found in most eukaryotic cells.
8. Varki, N.M.; Varki, A. *Lab. Invest*. **2007**, *87*(Sept), 851
9. Bork, K.; Reutter, W.; Weidemanna, W.; Horstkorte, R. *FEBS Lett*. **2007**, *581*(22), 4195.
10. Baker, K.N.; Rendall, M.H.; Hills, A.E.; Hoare, M.; Freedman, R.B.; James, D.C. *Biotech. Bioeng.* **2001**, *73*(3), 188.
11. Shen, J.; NCATS, TRND.; private communication.
12. www.nzp.co.nz/index.php/products/microbiological-media-and-fermentation-ingredients/n-acetyl-d-mannosamine-mannac.html.
13. Hossler, P.; Khattak, S.F.; Zheng, J.L. *Glycobiol*. **2009**, *19*(9), 936
14. Wong, N.S.C.; Wati, L.; Nissom, P.M.; Feng, H.T.; Lee, M.M.; Yap, M.G.S. *Biotech. Bioeng*. **2010**, *107***(**2), 321
15. Son, Y.D.; Jeong, Y.T.; Park, S.Y.; Kim, J.H. *Glycobiol*. **2011**, *21*(8), 1019.
16. Markley, L.R.A.; Wang D.I.C. *DSpace@MIT*. **2011**, http://dspace.mit.edu/handle/1721.1/65762.