Brief Report

Binding of pradimicin A derivative BMY-28864 to neoglycolipids bearing mannose residues at the non-reducing ends

Huanli Xu[1], Fengshan Wang[1], Tsuguo Mizuochi[2], Munehiro Nakata[2,3,*]

[1] *Institute of Biochemical and Biotechnological Drugs, School of Pharmacy, Shandong University, Jinan, Shandong, China;*
[2] *Department of Applied Biochemistry, Tokai University, Hiratsuka, Kanagawa, Japan;*
[3] *Shandong University China-Japan Cooperation Center for Drug Discovery & Screen, Jinan, Shandong, China.*

Summary **BMY-28864 is a derivative of carbohydrate-binding antibiotic pradimicin A. This study aimed to examine the carbohydrate-binding specificity of BMY-28864 by a direct binding assay using neoglycolipids synthesized by conjugation of various oligosaccharides with dipalmitoylphosphatidylethanolamine (DPPE). Neoglycolipids were chromatographed on a thin layer chromatography plate and then subjected to BMY-28864 binding analysis. Binding of BMY-28864 to neoglycolipids such as Manα1-3Man-DPPE, Manα1-6Man-DPPE, Man3-DPPE, and Man5-DPPE and those bearing oligosaccharides derived from ribonuclease B, all of which bear mannose residues at the non-reducing ends, was detected. This study showed that pradimicin A derivative BMY-28864 selectively bound to mannose residues at the non-reducing ends and that neoglycolipids bearing various carbohydrate structures will be helpful as carbohydrate probes to detect carbohydrate-binding low molecular weight compounds.**

Keywords: Carbohydrate, Neoglycolipid, Lectin, Antibiotic

1. Introduction

Pradimicin A is an antifungal antibiotic that Oki *et al*. isolated from the culture filtrate of *Actinomadura hibisca* (*1*). Pradimicin A and its derivative BMY-28864, which has improved water solubility, have a broad antifungal spectrum against a wide variety of pathogenic fungi such as *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus in vitro* and in mice (*2-4*). Notably, several studies have shown that the compounds cause precipitation and alteration of the absorbance spectrum in the presence of mannose in a calcium ion-dependent manner (*3-5*). Therefore, these low molecular weight compounds are suggested to have a lectin-like carbohydrate-binding ability. The carbohydrate-binding specificityof an increasing number of lectins has been characterized by means of a direct binding assay using neoglycolipids prepared by lipidation of carbohydrates as carbohydrate probes (*6,7*). The present study used the direct binding assay using neoglycolipids to examine the interaction of BMY-28864 with carbohydrates.

**Correspondence to:* Dr. Munehiro Nakata, Department of Applied Biochemistry, Tokai University, Hiratsuka, Kanagawa 259-1292, Japan;
e-mail: nak@keyaki.cc.u-tokai.ac.jp

2. Materials and Methods

2.1. *Materials*

BMY-28864 was kindly provided by Dr. Toshikazu Oki, Toyama Prefectural University, Toyama, Japan, and dissolved in water at 2 mg/mL as a stock solution. The following commercially available carbohydrates were purchased from Funakoshi, Tokyo, Japan: Manα1-3Man, Manα1-6Man, mannotriose (Man3) with a structure of Manα1-6(Manα1-3)Man, mannopentaose (Man5) with a structure of Manα1-6(Manα1-3)Manα1-6(Manα1-3)Man, core-pentaose (GlcNAc2Man3) with a structure of GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Man, maltooligomer with a structure of Glcα1-(-4Glcα1-)$_{0\text{-}3}$-4Glc, isomaltooligomer with a structure of Glcα1-(-6Glcα1-)$_{0\text{-}3}$-6Glc, cellooligomer with a structure of Glcβ1-(-4Glcβ1-)$_{1\ or\ 3}$-4Glc, laminarioligomer with a structure of Glcβ1-(-3Glcβ1-)$_{1\ or\ 3}$-3Glc, chitooligomer with a structure of GlcNAcβ1-(-4GlcNAcβ1-)$_{0\text{-}3}$-4GlcNAc, and sialyllactose with a structure of NeuAcα2-3Galβ1-4Glc. Glycoproteins such as bovine pancreas ribonuclease B (RNase B), human

IgG, and human fibrinogen were purchased from Sigma-Aldrich Japan, Tokyo, Japan. *N*-linked oligosaccharides of these glycoproteins were prepared by hydrazinolysis and re-*N*-acetylation followed by cellulose column chromatography (*8,9*). Oligosaccharides obtained from IgG and fibrinogen were then asialylated by sialidase treatment as described previously (*8*).

2.2. *Synthesis of neoglycolipids*

Neoglycolipids were synthesized by conjugation of oligosaccharides with dipalmitoylphosphatidylethanolamine (DPPE, Sigma-Aldrich Japan) by reductive amination (*10,11*). Briefly, oligosaccharides (0.1-10 mg) were dissolved in water and mixed with 15.7 vol. of DPPE solution (10 mg/mL in chloroform/methanol, 1:1, v/v) and 3.3 vol. of sodium cyanogenborohydrate solution (20 mg/mL in methanol). The reaction mixture was incubated at 80°C for 5 h with occasional sonication. Synthesis of neoglycolipids was confirmed by a thin layer chromatography (TLC) with detection of carbohydrate moiety by orcinol staining and lipid moiety by primulin staining (*12*). Neoglycolipids derived from commercially available oligosaccharides were then purified by HPLC with a silica gel column (Shim-Pack PREP SIL, 2 × 25 cm, Shimadzu, Kyoto, Japan) and subsequently subjected to chromatography with a Bond Elut C18-cartridge column (Varian, Palo Alto, CA, USA) (*13*).

2.3. *Assay of BMY-28864 binding to neoglycolipids*

Neoglycolipids were spotted on a TLC plate (Merck, Darmstadt, Germany) and developed using chloroform/methanol/water (60:35:8, v/v) or chloroform/methanol/water (105:100:28, v/v) as a solvent system (*12*). After drying, the plate was plasticized by soaking it in 0.1% polyisobutyl methacrylate in *n*-hexane for 30 s and then it was blocked by 1% (w/v) gelatin for 2 h at room temperature. The plate was overlaid with 50 μg/mL of BMY-28864 in 10 mM Tris-HCl, pH 7.3, containing 0.15 M NaCl and 1 mM $CaCl_2$ (Ca-TBS) and incubated for 1 h at room temperature with gentle shaking. After incubation, the plate was rinsed with Ca-TBS and air-dried.

3. Results and Discussion

First, purified neoglycolipids derived from commercially available carbohydrates (1 μg each per lane) were chromatographed on a TLC plate and then incubated with 50 μg/mL of BMY-28864 in Ca-TBS for 1 h at room temperature (Figure 1). As shown in Figure 1B, binding of BMY-28864, which was observed as red-colored spots, to neoglycolipids such as Manα1-3Man-DPPE (lane h), Manα1-6Man-DPPE (lane i), Man3-DPPE (lane j), and Man5-DPPE (lane k), all of which bear mannose residue(s) at the non-reducing end(s), was detected. In contrast, binding to Man-DPPE (lane g), in which mannose moiety does not form a ring, GlcNAc2Man3 (lane f), in which mannose residues are not at the non-reducing ends, and neoglycolipids that did not contain mannose residue was not detected. The binding of BMY-28864 to neoglycolipids bearing mannose residue(s) at non-reducing end(s) was completely inhibited in the presence of 100 mM mannose or 2 mM EGTA, while binding was not inhibited by 100 mM of glucose and galactose (data not shown).

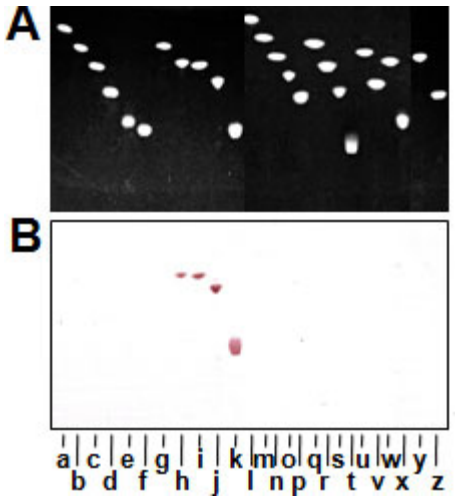

Figure 1. A direct binding assay of BMY-38864 with purified neoglycolipids prepared from commercially available carbohydrates. Neoglycolipids were developed on a TLC plate using chloroform/methanol/water (60:35:8, v/v) as a solvent system. (*A*) The neoglycolipids on the plate were visualized by primulin staining. (*B*) 50 μg/mL of BMY-28864 in Ca-TBS was incubated on the plate for 1 h at room temperature. *Lane a*, GlcNAc-DPPE; *lanes b-e*, chitooligomer-DPPE bearing 2-5 GlcNAc residues, respectively; *lane f*, GlcNAc2Man3-DPPE, *lane g*, Man-DPPE, *lane h*, Manα1-3Man-DPPE; *lane i*, Manα1-6Man-DPPE; *lane j*, Man3-DPPE; *lane k*, Man5-DPPE; *lane l*, Glc-DPPE; *lanes m-p*, maltooligomer-DPPE bearing 2-5 Glc residues, respectively; *lanes q-t*, isomaltooligomer-DPPE bearing 2-5 Glc residues, respectively; *lanes u and* v, cellooligomer-DPPE bearing 3 and 5 Glc residues, respectively; *lanes w and x*, laminarioligomer-DPPE bearing 3 and 5 Glc residues, respectively; *lane y*, lactose-DPPE; *lane z*, sialyllactose-DPPE.

Next, neoglycolipids derived from *N*-linked oligosaccharides of glycoproteins (5 μg of carbohydrate equivalent per lane) were developed on a TLC plate and then BMY-28864 binding was analyzed (Figure 2). As shown in Figure 2B, BMY-28864 apparently bound to neoglycolipids bearing high mannose type oligosaccharides derived from RNase B (*14*) but not to those bearing complex type oligosaccharides derived from IgG (*15*) and fibrinogen (*16*).

The present direct binding assay using neoglycolipids showed that pradimicin A derivative BMY-28864 selectively bound to neoglycolipids bearing mannose residues at the non-reducing ends (Figure 1B and 2B). This binding was inhibited by mannose but not by glucose and galactose. Therefore, BMY-28864, a low molecular weight organic compound, can be considered to fall under the new category of "carbohydrate

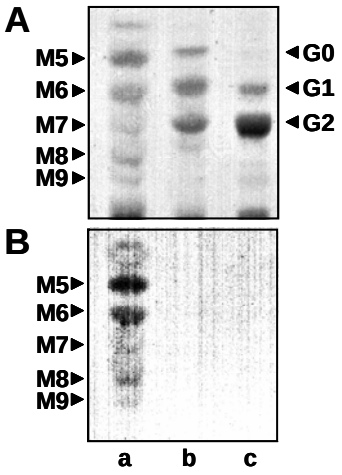

Figure 2. A direct binding assay of BMY-28864 with neoglycolipids prepared from *N*-linked oligosaccharides of various glycoproteins. Neoglycolipids were prepared from high mannose type oligosaccharides of bovine RNase B (*lane a*) and asialylated complex type oligosaccharides of human IgG (*lane b*) and human fibrinogen (*lane c*) and then developed on a TLC plate using chloroform/methanol/water (105:100:28, v/v) as a solvent system. (*A*) The neoglycolipids on the plate were visualized by orcinol staining. (*B*) 50 μg/mL of BMY-28864 in Ca-TBS was incubated on the plate for 1 h at room temperature. M5-M9 denote neoglycolipids bearing high mannose type oligosaccharides containing 5-9 mannose residues, respectively. G0-G2 denote neoglycolipids bearing complex type oligosaccharides containing 0-2 galactose residues, respectively.

binding compounds" that do not fall under any of the conventional categories.

Mannose-binding compounds will prove useful not only in detecting glycoconjugates bearing mannose resides but also in preventing infection by pathogens with surfaces covered in mannose residues. In fact, the antifungal ability of pradimicin A is thought to be due to its binding to mannan on the fungal surface. Furthermore, research has suggested that pradimicin may prevent human immunodeficiency virus infection (*17,18*) possibly because of the binding of the antibiotic to virus envelope glycoprotein gp120, which possesses a number of high mannose type oligosaccharides (*19,20*). Therefore, carbohydrate-binding compounds such as pradimicin A and BMY-28854 will be effective tools for carbohydrate targeting in biochemical and medical studies (*21*). In addition, other types of carbohydrate-binding antibiotics or organic compounds may exist in nature. Neoglycolipids bearing various carbohydrate structures will be helpful as carbohydrate probes to screen and discover novel carbohydrate-binding compounds.

Acknowledgements

The authors are thankful to Mrs. Jing Gao for her excellent secretarial assistance.

References

1. Oki T, Konishi M, Tomatsu K, Tomita K, Saitoh K, Tsunakawa M, Nishio M, Miyaki T, Kawaguchi H. Pradimicin, a novel class of potent antifungal antibiotics. J Antibiot (Tokyo) 1988; 41:1701-1704.
2. Oki T, Tenmyo O, Hirano M, Tomatsu K, Kamei H. Pradimicins A, B, and C: New antifungal antibiotics II. *in vitro* and *in vivo* biological activities. J Antibiot (Tokyo) 1990; 43:763-770.
3. Sawada Y, Murakami T, Ueki T, Fukagawa Y, Oki T, Nozawa Y. Mannan-mediated anticandidal activity of BMY-28864, a new water-soluble pradimicin derivative. J Antibiot (Tokyo) 1991; 44:119-121.
4. Oki T, Kakushima M, Nishio M, Kamei H, Hirano M, Sawada Y, Konishi M. Water-soluble pradimicin derivatives, synthesis and antifungal evaluation of *N*,*N*-dimethyl pradimicins. J Antibiot (Tokyo) 1990; 43:1230-1235.
5. Fujikawa K, Tsukamoto Y, Oki T, Lee YC. Spectroscopic studies on the interaction of pradimicin BMY-28864 with mannose derivatives. Glycobiology 1998; 8:407-414.
6. Nakao M, Kajiya T, Sato Y, Somamoto T, Kato-Unoki Y, Matsushita M, Nakata M, Fujita T, Yano T. Lectin pathway of bony fish complement: Identification of two homologs of the mannose-binding lectin associated with MASP2 in the common carp (*Cyprinus carpio*). J Immunol 2006; 177:5471-5479.
7. Takahashi M, Iwaki D, Matsushita A, Nakata M, Matsushita M, Endo Y, Fujita T. Cloning and characterization of mannose-binding lectin from lamprey (Agnathans). J Immunol 2006; 176:4861-4868.
8. Mizuochi T. Microscale sequencing of *N*-linked oligosaccharides of glycoproteins using hydrazinolysis, Bio-Gel P-4, and sequential exoglycosidase digestion. In: Methods in Molecular Biology: Vol. 14 Glycoprotein analysis in biomedicine (Hounsell T, ed.). Humana Press, Totowa, NJ, USA, 1993; pp. 55-68.
9. Shimizu Y, Nakata M, Kuroda Y, Tsutsumi F, Kojima N, Mizuochi T. Rapid and simple preparation of *N*-linked oligosaccharides by cellulose-column chromatography. Carbohydrate Res 2001; 332:381-388.
10. Sugimoto M, Ohishi K, Fukasawa M, Shikata K, Kawai H, Itakura H, Hatanaka M, Sakakibara R, Ishiguro M, Nakata M, Mizuochi T. Oligomannose-coated liposomes as an adjuvant for the induction of cell-mediated immunity. FEBS Lett 1995; 363:53-56.
11. Sakai K, Shimizu Y, Chiba T, Matsumoto-Takasaki A, Kusada Y, Zhang W, Nakata M, Kojima N, Toma K, Takayanagi A, Shimizu N, Fujita-Yamaguchi Y. Isolation and characterization of phage-displayed single antibodies. 1. A new strategies for generation of anti-carbohydrate antibodies. Biochemistry 2007; 46:253-262.
12. Feizi T, Stoll MS, Yuen CT, Chai W, Lawson AM. Neoglycolipids: probes of oligosaccharide structure, antigenicity, and function. Methods Enzymol 1994; 230:484-519.
13. Shimizu Y, Nakata M, Matsunuma J, Mizuochi T. Simultaneous quantification of components of neoglycolipid-coated liposomes using high-performance liquid chromatography with evaporative light scattering detection. J Chromatogr B 2001; 745:127-133.
14. Liang C-J, Yamashita K, Kobata A. Structural study of the carbohydrate moiety of bovine pancreatic ribonuclease B. J Biochem (Tokyo) 1980; 88:51-58.
15. Mizuochi T, Taniguchi T, Shimizu A, Kobata A. Structural and numerical variations of the carbohydrate moiety of immunoglobulin G. J Immunol 1982; 129:2016-2020.

16. Mizuochi T, Taniguchi T, Asami Y, Takamatsu J, Okude M, Iwanaga S, Kobata A. Comparative studies on the structures of the carbohydrate moieties of human fibrinogen and abnormal fibrinogen. J Biochem (Tokyo) 1982; 92:283-293.
17. Tanabe-Tochikura A, Tochikura TS, Yoshida O, Oki T, Ymamoto N. Pradimicin A inhibition of human immunodeficiency virus: attenuation by mannan. Virology 1990; 176:467-473.
18. Balzarini J, Van Laethem K, Daelemans D, Hatse S, Bugatti A, Rusnati M, Igarashi Y, Oki T, Schols D. Pradimicin A, a carbohydrate-binding nonpeptidic lead compound for treatment of infections with viruses with highly glycosylated envelopes, such as human immunodeficiency virus. J Virol 2007; 81:362-373.
19. Mizuochi T, Spellman MW, Larkin M, Solomon J, Basa LJ, Feizi T. Carbohydrate structures of the human-immunodeficiency-virus (HIV) recombinant envelope glycoprotein gp120 produced in Chinese-hamster ovary cells. Biochem J 1988; 254:599-603.
20. Mizuochi T, Nakata M. HIV infection and oligosaccharides: a novel approach to preventing HIV infection and the onset of AIDS. J Infect Chemother 1999; 5:190-195.
21. Balzarini J. Carbohydrate-binding agents: a potential future cornerstone for the chemotherapy of enveloped viruses? Antivir Chem Chemother 2007; 18:1-11.

(*Received April 17, 2008; Revised June 11, 2008; Accepted June 12, 2008*)