J. Microbiol. Biotechnol. (2002), **12**(5), 834–837

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Effect of 2-NBDG, a Fluorescent Derivative of Glucose, on Microbial Cell Growth

SHIN, DONG-SUN[1] AND KI-BONG OH[1,2]*

[1]*Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 110-460, Korea*
[2]*Graduate School of Agricultural Biotechnology, Seoul National University, Suwon 441-744, Korea*

Received: February 14, 2002
Accepted: June 21, 2002

Abstract A fluorescent glucose analogue, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), which had previously been developed for the analysis of glucose uptake in living cells, was investigated to determine its biological activity on microorganisms. 2-NBDG did not show any inhibitory effect on growth of yeast cells and bacteria. In contrast, 2-NBDG exhibited strong inhibitory effects on filamentous fungal growth. The growth of filamentous fungi was completely inhibited, when 2-NBDG was supplemented as sole carbon source. The inhibitory effect was decreased by the addition of glucose in the test medium. Furthermore, 2-NBDG inhibited chitinase activity of *Trichoderma* sp. These results suggested that the inhibitory effects of 2-NBDG on filamentous fungi might be partially due to the inhibition of chitinase.

Key words: Fluorescent glucose probe, fungi, bacteria, growth inhibitory activity

The measurement of growth inhibition and/or viability are often considered to be desirable features of antimicrobial susceptibility tests [5, 10]. Because of diversity of the methods employed and the constitutive microbiology involved in a suspension, antimicrobial agents might not be evenly distributed [4]. Various fluorescent dyes have been developed and used to indicate a cell's physiological state [2, 8, 9, 11, 12, 14, 15]. Such fluorescence techniques are uniquely suitable for probing living or dead cells, because of their sensitivity and specificity.

Glucose uptake is a crucial phenomenon closely related to metabolic activity [3, 16], and thus considered to be a more accurate indicator of cell viability than indicators of membrane permeability or of a particular enzyme activity.

*Corresponding author
Phone: 82-2-740-8915; Fax: 82-2-745-1015;
E-mail: ohkibong@snu.ac.kr

We have recently developed a fluorescent D-glucose derivative, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG), that allows a more sensitive measurement of glucose uptake in real time in single living cells [19]. 2-NBDG is a fluorescent derivative of D-glucose that has been modified with a 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino group at the C-2 position. Previous studies have shown that 2-NBDG is taken up into the cytoplasm of both *Escherichia coli* cells and yeast *Candida albicans* cells and that this process is useful for assaying viability of cells [20, 21]. Uptake of 2-NBDG into these cells is inhibited by D-glucose but not L-glucose, suggesting that 2-NBDG is transported into the microbial cells through the glucose transporter system. In living mammalian cells, 2-NBDG is incorporated into the cells through glucose transporters GLUTs [18]. In addition, 2-NBDG is metabolized to a phosphorylated fluorescent derivative at the C-6 position, *i.e.* 2-NBDG 6-phosphate, after incorporation, and then to a nonfluorescent derivative in *E. coli* [20]. In this work, in order to determine the scope of the biological activity of 2-NBDG toward various bacteria, yeast, and filamentous fungi, the effect of this fluorescent probe on the microbial cell growth was investigated.

2-NBDG (Fig. 1) was synthesized as described previously with some modifications [19]. In brief, 0.5 g of D-glucosamine dissolved in 10 ml of 0.1 M $NaHCO_3$ was added to a

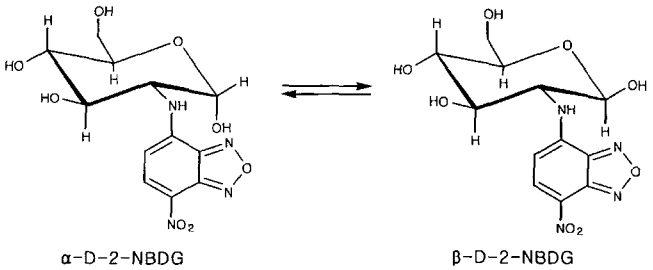

Fig. 1. Molecular structure of 2-NBDG.

solution of 0.5 g NBD-Cl (7-chloro-4-nitrobenz-2-oxa-1, 3-diazole) in 20 ml of methanol. The reaction mixture was stirred at 35°C for 15 h during which it became very dark. The reaction mixture was partially concentrated by evaporation under vacuum to remove the methanol and then passed through a DEAE Sephadex A-50 (PharmaciaBiotech) column (15×200 mm) with water as eluant. The orange-colored eluate was further purified by the Sephadex LH-20 (PharmaciaBiotech) column (22× 350 mm). The fractions that contained the bright yellow fluorescent product were loaded onto an amide column (TSKgel Amide-80, Tosoh Co., Tokyo, Japan) and fractionated with acetonitrile-water (17:3, v/v) using HPLC. The column was eluted at the flow rate of 1 ml/min, and the eluate was detected at 475 nm.

In order to confirm the effect of 2-NBDG on yeast cell growth, the minimum inhibitory concentration (MIC) was determined by a macrobroth dilution method M27-T, proposed by the National Committee on Clinical Laboratory Standards (NCCLS) [13]. Briefly, 2-NBDG was dissolved in sterile distilled water to prepare a stock solution of 10 mg/ml. The stock solution was diluted with yeast nitrogen base (YNB; Difco, Detroit, U.S.A.) supplemented with or without 5 g/l of glucose to prepare serial 2-fold dilutions in the range of 0.01–100 μg/ml. The yeast cells were harvested and washed twice with sterile distilled water. Ten μl of cell suspension was added to each well containing 990 μl of the above-mentioned medium (10^3 cells/ml) and incubated for 3 days at 28°C. The MIC was taken as the concentration at which no growth was observed. The effect of 2-NBDG on the growth of filamentous fungi was determined by the macrobroth dilution method of Association of Official Analytical Chemists (AOAC) [1]. Thus, a spore suspension was collected with 0.1% Tween-80 solution from potato dextrose agar (Difco) plates that had been incubated at 28°C for 2 weeks. Standardization adjustments of the spore suspension were made to obtain initial inoculum size of 10^5 spores/ml. The MIC values were determined as described above. The effect of 2-NBDG on the bacterial growth was determined by a 2-fold microtiter broth dilution method [17]. Thus, bacteria were grown overnight into standard methods broth (SM broth, Difco), harvested, and then washed twice with sterile distilled water. The stock solution of 2-NBDG was diluted with SM broth supplemented with or without 2 g/l of glucose to prepare serial 2-fold dilutions as described above. One-hundred μl of the broth containing about 10^4–10^5 CFU/ml of test bacteria was added to each well of a 96-well microtiter plate and the MIC was determined after incubation for 24 h at 37°C.

We also evaluated the effect of 2-NBDG on the fungal chitinase as follows. Chitinase from *Trichoderma* sp. AF6-T8 (Takara Shuzu Co., Shiga, Japan) was assayed by mixing a 0.5-ml aliquot of appropriately diluted enzyme suspension with 1.0 ml of 0.2% (w/v) glycol chitin (Seikagaku Co., Tokyo, Japan) in sodium acetate buffer (100 mM, pH 5.2). After incubation for 10 min at 37°C, 2 ml of the color reagent (0.5 g of potassium fericyanide in 1 liter of 0.5 M sodium carbonate solution) were mixed with the suspension and incubated in boiling water for 15 min. The amount of liberated reducing sugar was measured by the Schales procedure [7].

Table 1. *In vitro* inhibitory activity of 2-NBDG on fungal growth.*

Organism	MIC (μg/ml)**	
	Glucose (+)	Glucose (-)
Aspergillus fumigatus HIC 6094	6.25	<0.01
Aspergillus niger ATCC 9642	12.5	<0.01
Cladosporium cladosporioides IFO 6348	12.5	<0.01
Fusarium solani HIC 5670	6.25	<0.01
Fusarium oxysporium HIC 5651	6.25	<0.01
Geotrichum candidum HIC 5700	6.25	<0.01
Monilia sp. HIC 5745	3.12	<0.01
Penicillium citrinum HIC 7043	6.25	<0.01
Penicillium expansum IFO 5453	12.5	<0.01
Rhizopus stolonifer IFO 30816	25	<0.01
Trichoderma sp. 101	25	<0.01
Trichophyton mentagrophytes HIC 8185	1.56	<0.01
Trichophyton mentagrophytes IFO 40996	1.56	<0.01
Trichophyton rubrum IFO 9185	1.56	<0.01
Trichophyton rubrum IFO 6204	1.56	<0.01
Candida albicans ATCC 10231	>100	>100
Candida tropicalis ATCC 750	>100	>100
Saccharomyces cerevisiae IFO 1234	>100	>100

*Growth inhibitory activity of 2-NBDG upon fungi was tested with yeast nitrogen base medium supplemented with 5 g/l of glucose (+) or without glucose (-).

**MICs were determined after 3 days of incubation at 28°C.

ATCC: American Type Culture Collection; IFO: Institute for Fermentation, Osaka, Japan; HIC: National Institute of Health Sciences, Tokyo, Japan.

The effects of 2-NBDG on fungal and bacterial growth are summarized in Tables 1 and 2, respectively. As shown in Table 1, 2-NBDG did not display any inhibitory effect on the growth of yeast cells such as *C. albicans*, *C. tropicalis*, and *S. cerevisiae* at the highest concentration tested (100 μg/ml) with or without glucose. Similar results were also observed with bacterial growth (Table 2). These results indicate that 2-NBDG, a fluorescent glucose probe, is incorporated into yeast and bacterial cells and is metabolizable in the glycolytic pathway. In the previous works, we showed that 2-NBDG was taken up into the cytoplasm of both *E. coli* cells and yeast *C. albicans* cells via glucose transporter in the cell membrane, was metabolized to a phosphorylated fluorescent derivative at the C-6 position, *i.e.* 2-NBDG 6-phosphate, and then decomposed to a nonfluorescent derivative in *E. coli* [20, 21]. Thus, the fluorescent intensity should reflect a dynamic balance between the level of generation and decomposition

Table 2. *In vitro* inhibitory activity of 2-NBDG upon bacterial growth.*

Organism	MIC (μg/ml)**	
	Glucose (+)	Glucose (−)
Escherichia coli ATCC 8739	>100	50
Salmonella typhimurium ATCC 14028	>100	>100
Proteus vulgaris ATCC 6380	>100	>100
Bacillus subtilis ATCC 6633	>100	50
Klebsiella pneumoniae IFO 13541	>100	>100
Staphylococcus aureus ATCC 6538p	>100	50

*Growth inhibitory activity of 2-NBDG upon bacteria was tested with standard methods broth medium supplemented with 2 g/l of glucose (+) or without glucose (−).

** MICs were determined after 24 h incubation at 37°C.

ATCC: American Type Culture Collection; IFO: Institute for Fermentation, Osaka, Japan.

of the fluorescent derivative, thus indicating a promising property of 2-NBDG for the measurement of glucose uptake in living yeast and bacterial cells. In contrast, 2-NBDG exhibited strong inhibitory effects on the filamentous fungal growth. The growth of filamentous fungi was completely inhibited, when 2-NBDG was supplemented as the sole carbon source in the YNB medium (MICs <0.01 μg/ml), as shown in Table 1. The addition of glucose to the test medium resulted in a dramatic decrease in inhibitory activity of 2-NBDG (MICs: 1.56–25 μg/ml). In addition, exposure of fungi to 2-NBDG induced morphological changes in hyphae. For example, when *Trichoderma* sp. 101 was exposed to YNB (supplemented with 5 g/l of glucose) containing 30 μg/ml of 2-NBDG, the linear growth of hyphae stopped, which was accompanied by the swelling of hyphal tips, the burst of the swollen

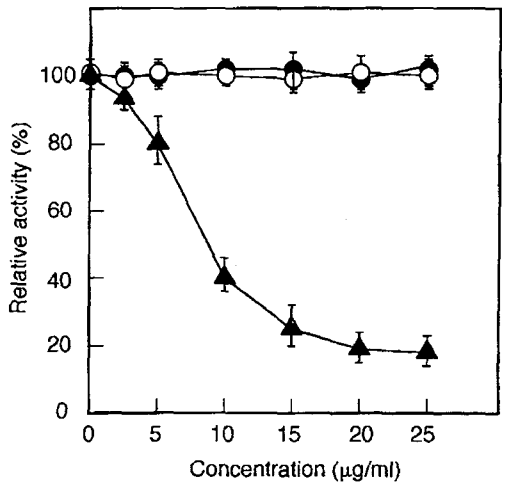

Fig. 2. Effect of 2-NBDG on the activity of chitinase from *Trichoderma* sp. AF6-T8.
●, NBD-Cl; ○, D-glucosamine; ▲, 2-NBDG.

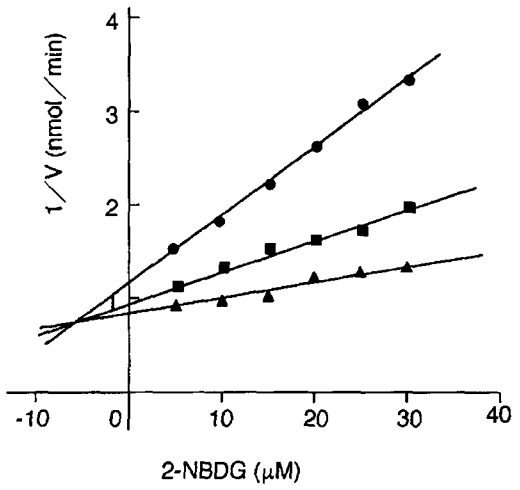

Fig. 3. Kinetics of the *Trichoderma* sp. chitinase inhibited by 2-NBDG.
Inhibition kinetics was determined by Dixon plot analysis after assaying the enzyme activity in the presence of various concentrations of substrate and inhibitor. Glycolchitin concentrations used for the inhibition by 2-NBDG were 0.1 mM (●), 0.25 mM (■), and 1 mM (▲).

tips, and release and scattering of the hyphal cytosol (unpublished data). These results implied that 2-NBDG itself or its metabolite might have inhibitory activity on filamentous fungi. Based on the data obtained, we next focused our attention on the effect of 2-NBDG on the fungal chitinase, because it has long been suggested that chitinase activity is involved in apical extension of fungal hyphae and that fungal cell wall morphogenesis is the result of a delicate balance between synthesis by chitin synthetase and lysis by chitinase [6]. As shown in Fig. 2, 2-NBDG inhibited *Trichoderma* sp. chitinase activity, having about 80% inhibitory activity at 20 μg/ml. On the other hand, D-glucosamine and NBD-Cl, chemicals used for 2-NBDG synthesis, did not display any inhibitory effect on the chitinase activity. Dixon plot analysis indicated that 2-NBDG inhibited the enzyme activity competitively with apparent K_i value of approximately 6 μM (Fig. 3). These results indicate that the inhibitory activity of 2-NBDG on filamentous fungi might have partially been due to inhibition of chitinase. Currently, however, we cannot fully explain the reason for the difference of 2-NBDG activity toward different microorganisms as well as the action mechanism of 2-NBDG toward filamentous fungi. Further studies on the growth inhibition mechanism of 2-NBDG toward filamentous fungi are in need.

Acknowledgment

This study was partially supported by a grant of the Korea Health Ministry of Health & Welfare, Republic of Korea (HMP-00-CD-01-0001).

REFERENCES

1. Association of Official Analytical Chemists. 1995. *Official Methods of Analysis of AOAC International*, pp. 6:1–6:18. 16th ed. AOAC International, Arlington, U.S.A.
2. Autio, K. and T. Mattila-Sandholm. 1992. Detection of active yeast cells (*Saccharomyces cerevisiae*) in frozen dough sections. *Appl. Environ. Microbiol.* **58:** 2153–2157.
3. Flier, J. S., M. M. Mueckler, P. Usher, and H. F. Lodish. 1987. Elevated levels of glucose transport and transporter messenger RNA are induced by ras or src oncogenes. *Science* **235:** 1492–1495.
4. Graf, B., T. Adam, E. Zill, and U. B. Göbel. 2000. Evaluation of the VITEK 2 system for rapid identification of yeasts and yeast-like organisms. *J. Clin. Microbiol.* **38:** 1782–1785.
5. Hahm, D.-H., M.-J. Yeom, E. H. Lee, I. Shim, H.-J. Lee, and H.-Y. Kim. 2001. Effect of Scutellariae radix as a novel antibacterial herb on the *ppk* (polyphosphste kinase) mutant of *Salmonella typhimurium*. *J. Microbiol. Biotechnol.* **11:** 1061–1065.
6. Heath, I. B. 1990. *Tip Growth in Plant and Fungal Cells*, pp. 31–50. Academic Press Inc., San Diego, U.S.A.
7. Imoto, T. and K. Yagishita. 1971. A simple activity measurement of lysozyme. *Agric. Biol. Chem.* **35:** 1154–1156.
8. Jayapal, V., K. M. Sharmila, G. Selvibai, S. P. Thyagarajan, N. Shanmugasundaram, and S. Subramanian. 1991. Fluorescein diacetate and ethidium bromide staining to determine the viability of *Mycobacterium smegmatis* and *Escherichia coli*. *Lept. Rev.* **62:** 310–314.
9. Jones, K. H. and J. A. Senft. 1985. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. *J. Histochem. Cytochem.* **33:** 77–79.
10. Kim, K.-Y., P. M. Davidson, and H.-J. Chung. 2000. Antimicrobial effectiveness of pine needle extract on foodborne illness bacteria. *J. Microbiol. Biotechnol.* **10:** 227–232.
11. Lopez-Amoros, R., D. J. Mason, and D. Lloyd. 1995. Use of two oxonols and a fluorescent tetrazolium dye to monitor starvation of *Escherichia coli* in seawater by flow cytometry. *J. Microbiol. Methods* **22:** 165–176.
12. McCaig, R. 1990. Evaluation of the fluorescent dye 1-anilino-8-naphthalene sulfonic acid for yeast viability determination. *J. Am. Soc. Brew. Chem.* **48:** 22–25.
13. National Committee for Clinical Laboratory Standards. 1992. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Proposed Standard*, pp. 1–22. NCCLS Document M27-P ed. National Committee for Clinical Laboratory Standards, Villanova, U.S.A.
14. Sano, A., N. Kurita, K. Iabuki, R. Coelho, K. Takeo, K. Nishimura, and M. Miyaji. 1993. A comparative study of four different staining methods for estimation of live yeast from cells of *Paracoccidioides brasiliensis*. *Mycopathologia* **124:** 157–161.
15. Tsuji, T., Y. Kawasaki, S. Takeshima, T. Sekiya, and S. Tanaka. 1995. A new fluorescence staining assay for visualizing living microorganisms in soil. *Appl. Environ. Microbiol.* **61:** 3415–3421.
16. Venama, K. and M. G. Palmgren. 1995. Metabolic modulation of transport coupling ratio in yeast plasma membrane H(+)-ATPase. *J. Biol. Chem.* **270:** 19659–19667.
17. Wu, M. and R. E. W. Hancocks. 1999. Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane. *J. Biol. Chem.* **274:** 29–35.
18. Yamada, K., M. Nakata, N. Horimoto, M. Saito, H. Matsuoka, and N. Inagaki. 2000. Measurement of glucose uptake and intracellular calcium concentration in single, living pancreatic beta-cells. *J. Biol. Chem.* **275:** 22278–22283.
19. Yoshioka, K., H. Takahashi, T. Homma, M. Saito, K.-B. Oh, Y. Nemoto, and H. Matsuoka. 1996. A novel fluorescent derivative of glucose applicable to the assessment of glucose uptake activity of *Escherichia coli*. *Biochim. Biophys. Acta* **1289:** 5–9.
20. Yoshioka, K., M. Saito, K.-B. Oh, Y. Nemoto, H. Matsuoka, M. Natsume, and H. Abe. 1996. Intracellular fate of 2-NBDG, a fluorescence probe for glucose uptake activity, in *Escherichia coli* cells. *Biosci. Biotech. Biochem.* **60:** 1899–1901.
21. Yoshioka, K., K.-B. Oh, M. Saito, Y. Nemoto, and H. Matsuoka. 1996. Evaluation of 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose, a new fluorescent derivative of glucose, for viability assessment of yeast *Candida albicans*. *Appl. Microbiol Biotechnol.* **46:** 400–404.