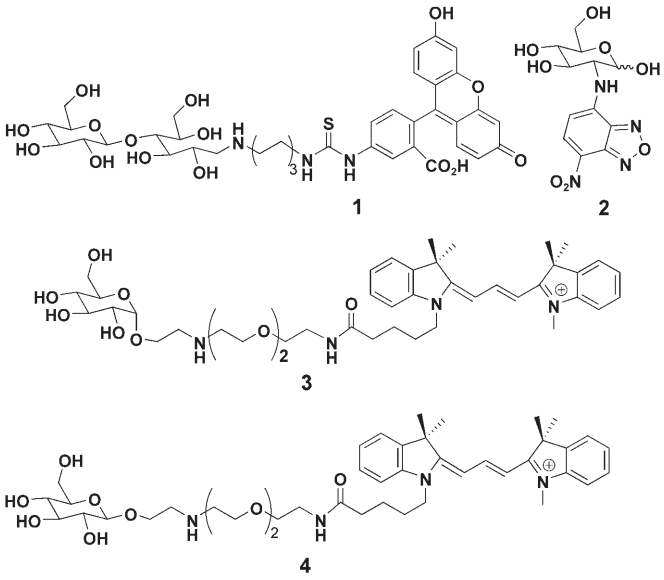
Fluorescent Bioprobes

DOI: 10.1002/anie.200604364

Development of a Cy3-Labeled Glucose Bioprobe and Its Application in Bioimaging and Screening for Anticancer Agents**

*Jongmin Park, Hyang Yeon Lee, Myung-Haing Cho, and Seung Bum Park**

Glucose is the most important energy source for cell growth; therefore, a fast-growing cancer cell requires more glucose than a normal cell. One of the biochemical markers in tumor malignancy is enhanced tumor glycolysis, primarily due to the overexpression of glucose transporters (GLUTs) and the increased activity of mitochondria-bound hexokinases in tumors.[1] The in vitro and in vivo assessment of glucose utilization has been of considerable interest. One of the applications of this assessment is tumor diagnosis by using [^{18}F]-2-fluoro-2-deoxyglucose (^{18}FDG) based positron-emission tomography (PET).[2a,b] PET with ^{18}FDG is a molecular-imaging modality that monitors metabolic perturbation in tumor cells and allows imaging of the exact positions of tumors in the human body; therefore, it is widely applied in the diagnosis of various tumors.[2c,d] A fluorescent 2-deoxyglucose analogue, 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]-2-deoxy-D-glucose (2-NBDG), was developed and extensively studied, primarily by Yoshioka et al.[3] 2-NBDG has been widely applied in various studies, especially for tumor imaging and the examination of GLUT-related cell metabolism.[3–8] In addition, some other 2-deoxyglucose analogues have been reported.[9,10] However, these analogues have several limitations; for instance, 2-NBDG is applicable only in a non-physiological sugar-depleted environment. T; therefore, we designed and synthesized a novel fluorescence-labeled glucose analogue, a Cy3-linked *O*-1-glycosylated glucose, which was not *N*-2 glycosylated like the previous analogues (Scheme 1).



Scheme 1. Structure of fluorescence-labeled glucose bioprobes: FITC-labeled cellobiose (**1**); 2-NBDG (**2**); Cy3-labeled α-glucose (**3**); Cy3-labeled β-glucose (**4**).

In our initial approach, the natural disaccharide cellobiose was directly labeled with fluorescein isothiocyanate (FITC) by reductive amination[11] with sodium borohydride (see the Supporting Information). However, this approach has several disadvantages: first, the yield is low and the purification process is tedious; second, only the β-anomer form of the bioprobe was prepared by using this procedure. In addition, FITC was not the appropriate fluorescent dye for this study due to its photochemical properties, especially fast photobleaching during experimentation with a confocal laser scanning microscope (CLSM) or fluorescence microscope. Therefore, a second approach was pursued to improve the reaction yields by using simple purification steps and to synthesize both anomers (α and β) of D-glucose labeled with photoresistant dye.

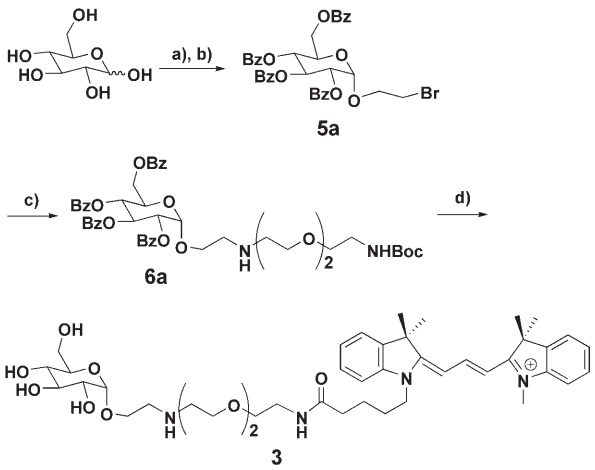
The cyclic form of D-glucose is recognized to be a hemiacetal ring. Owing to the special reactivity of the anomeric hydroxy group, 2-bromoethanol can be regioselectively introduced into the anomeric position by acid-catalyzed Fischer glycosylation with a 2:1 α:β ratio, as confirmed by NMR spectroscopy.[12] The resulting reaction mixture was benzoylated for isolation of the α and β anomers (**5a** and **5b**, respectively; only the synthesis with the α anomer is shown in Scheme 2).[13] After the coupling of the poly(ethylene glycol)-amine linker *N*-Boc-3,6-dioxaoctane-1,8-diamine[14] with both anomers (**5a** and **5b**) separately, the obtained sugars (**6a** and

[*] J. Park,[+] H. Y. Lee,[+] Prof. S. B. Park
Department of Chemistry
College of Natural Science
Seoul National University
Seoul 151-747 (Korea)
Fax: (+82) 2-889-1568
E-mail: sbpark@snu.ac.kr
Prof. M.-H. Cho
Laboratory of Toxicology
College of Veterinary Toxicology
Seoul National University
Seoul, 151-747 (Korea)

[+] These authors contributed equally to this work.

[**] This work is supported by the Korea Science and Engineering Foundation (KOSEF), the Molecular and Cellular BioDiscovery Research Program from the Ministry of Science and Technology (MOST), and MarineBio21, Ministry of Maritime Affairs and Fisheries, Korea (MOMAF). M.-H.C. is supported by NSI-NCRC through KOSEF. H.Y.L. is grateful for the award of a Seoul Science Fellowship award. J.P. and H.Y.L. are grateful for the award of a BK21 fellowship award.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 2. Synthetic scheme for Cy3-labeled glucose analogues: a) 2-bromoethanol, Dowex 50WX8-400 ion-exchange resin, 70 °C reflux; b) benzoyl chloride, pyridine, DMAP; c) *N*-Boc-3,6-dioxaoctane-1,8-diamine, triethylamine, DMF, 50 °C; d) 1. NaOMe, MeOH; 2. 50% TFA/DCM; 3. Cy3-COOH, EDC, DIPEA, DMF. Bz: benzoyl, Boc: *tert*-butoxycarbonyl, DMAP: 4-dimethylaminopyridine, DMF: *N*,*N*-dimethylformamide, TFA: trifluoroacetic acid, DCM: dichloromethane, EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, DIPEA: diisopropylethylamine.

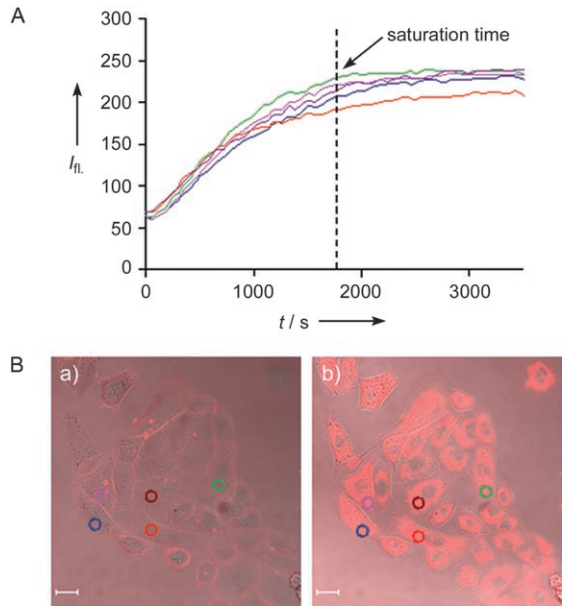


Figure 1. A) Cy3-Glc-α uptake by A549 cells. Fluorescence intensities $I_{fl.}$ were determined by continuous measurement from regions of interest in five independent cells marked in (B) and based on an unbiased selection. B) Merged phase-contrast and fluorescence images in A549 cells captured by live-cell imaging with a CLSM: a) after 0 min; b) after 60 min. The scale bar represents 20 μm.

6b) were treated for debenzoylation and Boc deprotection (Scheme 2).

After basification of the deprotected products with DIPEA in order to remove any residual TFA, the resulting chiral glucoside with a primary amine group can be coupled with any probe, such as fluorescent dyes or biotin. Our choice for the final modification was Cy3 fluorescent dye instead of FITC because the former is tolerant to intense light sources and compatible with various bioassay systems. We used in-house Cy3 carboxylic acid synthesized with high yields by well-documented procedures.[15] EDC coupling of Cy3 carboxylic acid with the resulting glucosides yielded the desired Cy3-labeled D-glucoses after preparative HPLC purification. Both anomers of Cy3-labeled D-glucose were prepared by using identical procedures with complete control over the stereochemistry on the glucosides, and the final products were completely characterized by ^{1}H and ^{13}C NMR spectroscopy, MALDI-TOF MS, and HRMS (see the Supporting Information for detailed procedures).

After completion of chiral Cy3-labeled D-glucose synthesis, we proceeded to evaluate the applicability of our bioprobes in a bioassay system. First, we measured the efficiency of Cy3-Glc-α (**3**) uptake into cells by using a CLSM. To decide the optimum concentration of Cy3-Glc-α, we allowed A549 cells (human lung carcinoma cell line) to incorporate our bioprobe at Cy3-Glc-α concentrations of 100, 50, 25, 12.5, and 6 μM. Based on the repeated tests, we selected 12.5 μM as the optimum concentration for cellular uptake experiments (see the Supporting Information). With an optimized concentration for live-cell imaging, we used a CLSM to measure the optimum incubation time required to achieve the maximum uptake of our bioprobe. As shown in Figure 1, the uptake of our bioprobe by A549 cells reaches the maximum within 35 min. Previous work with 2-NBDG (**2**) reported that an apparent maximum uptake of 2-NBDG was reached within 30 min, which is consistent with our observation.[16] Under these optimized conditions, we tested whether or not our bioprobe acts as a glucose analogue.

Unlike 2-NBDG, our bioprobe is a glucose analogue that is *O*-glycosylated at the C-1 position; therefore, we asymmetrically synthesized both anomers simultaneously under the assumption that the behavior of these anomers could be different, because the molecular conformation of Cy3-Glc-α and Cy3-Glc-β would be quite distinct. To confirm our hypothesis, we performed real-time uptake measurements with Cy3-Glc-α by live imaging of A549 cells with an inverted fluorescence microscope and we compared the findings with those for Cy3-Glc-β. As shown in Figure 2 A, the uptake of Cy3-Glc-α was 40% greater than that of Cy3-Glc-β. This led us to the conclusion that the configuration at the C-1 anomeric position definitely influences the efficiency in mimicking glucose; this might be due to the binding orientation of D-glucose in GLUTs.[17] Based on this observation, further studies in bioimaging and bioapplication were performed only with the α anomer of the Cy3-labeled glucose analogue as our choice of the bioprobe. We also measured the efficiency of Cy3-Glc-α uptake; in particular, we focused our attention on the differentiation of GLUT-overexpressing cancer cells (A549: lung carcinoma cell line; HeLa: cervical carcinoma cell line) from normal cells (WI-38: lung normal cell line; NIH/3T3: murine fibroblast cell line). Cy3-Glc-α uptake in NIH/3T3 cells was only 30% of that in A549 cells. Interestingly, Cy3-Glc-α uptake in HeLa cells was almost identical to that in A549 cells, which confirms the selective

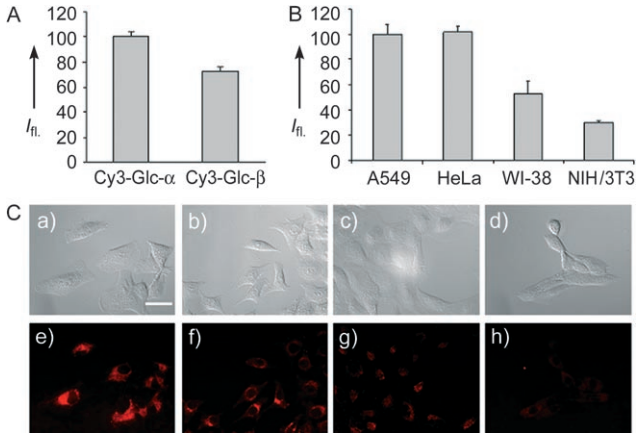


Figure 2. A) Cy3-Glc-α and Cy3-Glc-β uptake by A549 cells. Fluorescence intensities were determined by fluorometry, and data are the mean measurements from 35–50 cells in an experimental representative of at least two independent experiments. B) Efficiency of Cy3-Glc-α uptake in cancer cells (A549, HeLa) versus normal cells (WI-38, NIH/3T3). C) Comparison of Cy3-Glc-α uptake by A549 cells (a, e), HeLa cells (b, f), WI-38 cells (c, g), and NIH/3T3 cells (d, h); a–d) phase-contrast images; e–h) fluorescence images. The scale bar represents 40 μm.

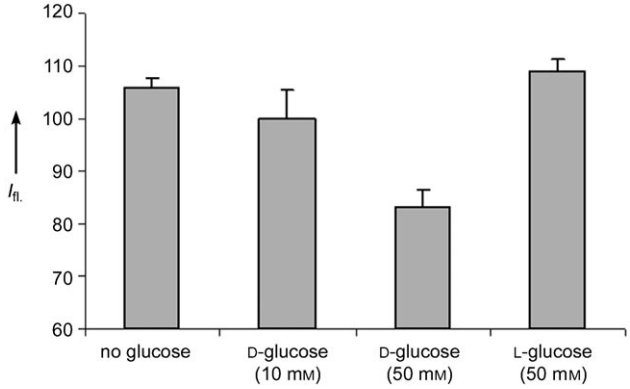


Figure 3. Dose-dependent inhibition of Cy3-Glc-α uptake in A549 cells in the absence or presence of 10 or 50 mM D-glucose. To demonstrate specific inhibition by D-glucose, an identical experiment was performed in the presence of 50 mM L-glucose, and this resulted in no uptake inhibition. Fluorescence intensities were determined by fluorometry, and the data are the mean measurements from 35–50 cells in an experimental representative of at least two independent experiments.

uptake of Cy3-Glc-α in cancer cells with enhanced glucose metabolism (Figure 2B). These data demonstrated that the cellular uptake of Cy3-Glc-α is affected by the higher glucose metabolism in cancer cells, which in turn relies on the adenosine triphosphate (ATP) generated from glycolysis in order to meet the energy requirements of rapidly replicating tissue. Therefore, glucose metabolism is strongly correlated with the GLUT/hexokinase expression levels. These data led us to the application of Cy3-Glc-α in bioimaging and bioassay in cancer studies.

To confirm whether the intracellular uptake pathway of glucose analogues is relevant to that of D-glucose, the direct competition experiment has been utilized in many studies.[1,8,9,16] If the cellular uptake of a certain glucose analogue depends on the concentration of D-glucose but not on that of L-glucose, then that particular glucose analogue would enter the cell through a GLUT-mediated glucose-uptake system. Based on prior experiments, we tested the process of the cellular uptake of Cy3-Glc-α by measuring the efficiency of Cy3-Glc-α uptake by A549 cells at 37 °C in RPMI1640 medium lacking glucose or containing 10 mM D-glucose, 50 mM D-glucose, or 50 mM L-glucose. As shown in Figure 3, the uptake of Cy3-Glc-α decreased as the concentration of D-glucose in the medium increased. However, the uptake of Cy3-Glc-α was not influenced by the L-glucose in the medium. In addition, we measured the uptake of Cy3-Glc-α by A549 cells in a medium containing 55 mM alanine, in order to ensure that the osmotic pressure in the medium does not affect Cy3-Glc-α uptake; we observed no difference in the uptake efficiency in the medium with or without 55 mM alanine (see the Supporting Information). The uptake of Cy3-Glc-α by A549 cells is inhibited by D-glucose but not by L-glucose; this suggests that Cy3-Glc-α is taken up by the cell through a glucose-specific transport system, not by passive diffusion. Therefore, Cy3-Glc-α can function as a D-glucose mimic and can be applied as a research tool in the study of glucose metabolism.

Having confirmed that Cy3-Glc-α acts as a D-glucose analogue, we compared Cy3-Glc-α with a fluorescent analogue of 2-deoxyglucose, namely, 2-NBDG.[1,3–8] The cellular uptake of 2-NBDG cannot be detected under identical experimental conditions to those used for Cy3-Glc-α. To achieve a fluorescence intensity with 2-NBDG that is up to 80% that of Cy3-Glc-α, a 10-fold increase in 2-NBDG concentration and a 20-fold increase in lens exposure time in D-glucose-depleted medium were needed with identical experimental setups. In addition, the cellular uptake of 2-NBDG in normal media (containing 10 mM D-glucose) is extremely low (>60% uptake reduction in normal media) and can hardly be detected by using fluorescence-based imaging methods.[16] It is detectable only in glucose-depleted media, and this is a critical limitation on its application in biologically significant environments. In contrast to the results with 2-NBDG, the reduction in Cy3-Glc-α uptake in glucose-containing media is 5% compared to that in glucose-depleted media (Figure 4). Therefore, Cy3-Glc-α can be applied in a bioassay system without glucose starvation.

Based on the fact that Cy3-Glc-α could be taken up by cells as a D-glucose analogue through a D-glucose-specific transport mechanism in normal glucose-containing medium, we applied Cy3-Glc-α to the screening of small-molecule modulators involved in cellular metabolism. We postulated that the depression of cellular metabolism in cancer cells, which is closely related to the reduction in glucose uptake, can be caused by anticancer agents. We intended to monitor this phenomenon with our fluorescent bioprobe Cy3-Glc-α. As a proof-of-principle experiment, we treated A549 cancer cells with taxol, an anticancer agent, and we measured the uptake of Cy3-Glc-α at $t = 3$, 6, 12, and 24 h after treatment. When we treated the cells with 9.8 μM taxol, the cellular uptake of our bioprobe was reduced as the incubation time increased (Figure 5); this clearly demonstrates the potential of Cy3-

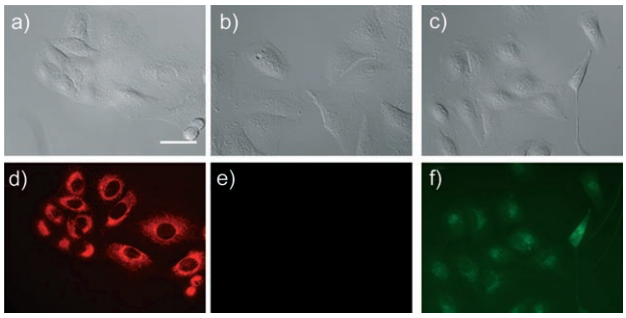


Figure 4. Uptake images of 2-NBDG and Cy3-Glc-α by A549 cells under different conditions: a, d) 12.5 μM Cy3-Glc-α with lens exposure of the CCD camera for 500 ms; b, e) 12.5 μM 2-NBDG with lens exposure of the CCD camera for 500 ms; c, f) 125 μM 2-NBDG with lens exposure of the CCD camera for 11 000 ms; a–c) phase-contrast images; d–f) fluorescence images. Fluorescence of 2-NBDG cannot be detected under the same conditions as those used for Cy3-Glc-α. Even 125 μM 2-NBDG cannot be detected with a lens exposure of the CCD camera for 500 ms. A 10-fold increase in concentration and 20-fold increase in lens exposure time was needed to get measurable fluorescence intensity with 2-NBDG. The scale bar represents 40 μm.

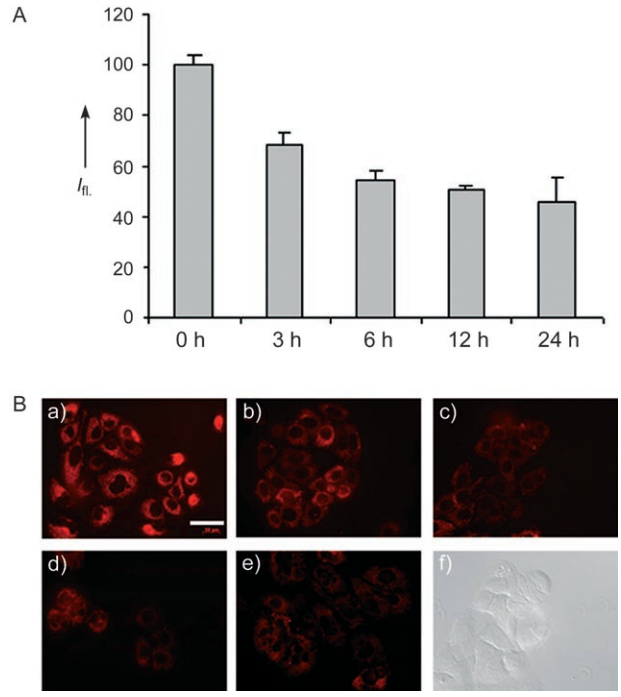


Figure 5. A) Cy3-Glc-α uptake by A549 cells is measured after 0, 3, 6, 12, and 24 h of treatment with taxol (9.8 μM) at 37°C. Fluorescence intensities were determined by fluorometry, and the data are the mean measurements from 35–50 cells from an experimental representative of at least two independent experiments. B) Fluorescence images of Cy3-Glc-α uptake by A549 cells after treatment with taxol (9.8 μM) at 37°C for a) 0, b) 3, c) 6, d) 12, and e) 24 h. After incubation with taxol, each image was captured with a fluorescence microscope after 30 min of Cy3-Glc-α treatment as described in the Supporting Information. The fluorescence intensity decreased as the incubation time with taxol was increased. The scale bar represents 40 μm. f) Phase-contrast image after 6 h incubation.

Glc-α for evaluation of the metabolic perturbation caused by bioactive small molecules in live cells under physiological conditions. Therefore, Cy3-Glc-α should not affect cell viability. By measuring the viability of cells treated with Cy3-Glc-α (12.5 μM) by using a CCK-8 kit (Dojindo, Japan), we concluded that Cy3-Glc-α treatment does not affect cell viability under our incubation conditions (see the Supporting Information).

We also observed the dose dependency of Cy3-Glc-α uptake by changing the taxol concentration from 490 to 49 nM (Table 1). Another anticancer agent, combretastatin, also

Table 1: Dose dependence of Cy3-Glc-α uptake by A549 cells in the presence of various concentrations of anticancer agents.

	After 6 h	After 12 h
taxol (9.8 μM)	54.8%	50.2%
taxol (490 nM)	88.4%	61.8%
taxol (49 nM)	96.5%	86.9%
combretastatin (2 μM)	57.4%	45.0%

prohibited cellular proliferation by disturbing cellular metabolism, as indicated by a reduction in the cellular uptake of Cy3-Glc-α. Based on these proof-of-principle experiments, we are confident that Cy3-Glc-α can be used to evaluate the behavior of bioactive small molecules in cells in a similar manner to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which measures the mitochondrial function.[18] The advantages of a screening system with Cy3-Glc-α over an MTT assay are as follows: first, the measurement time is short. MTT assays usually take 24 h and may take up to 72 h in many cases, that is, when taxol and combretastatin are used; however, the screening system with Cy3-Glc-α showed significant differences in 6 h and the maximum difference in 12 h. Second, the observation channel of a screening system with Cy3-Glc-α is quite different from that of a cell-viability assay; the former involves measuring glucose-uptake efficiency, while the latter involves measuring mitochondria function. Therefore, we envision that the two assay systems will compensate for each others limitations.

In conclusion, we designed and completed the asymmetric synthesis of novel fluorescent glucose analogues and we demonstrated the importance of stereochemistry at the anomeric C-1 position of glucose for efficiency of cellular uptake in A549 lung carcinoma cells. The chiral bioprobe of our choice, namely Cy3-Glc-α, showed superior properties as a glucose-uptake tracer compared to the previously reported 2-NBDG. We also developed a novel system for the screening of anticancer agents through the detection of metabolic perturbation by measuring glucose uptake in cancer cells with our bioprobe Cy3-Glc-α. The development of a high-throughput screening (HTS) system with Cy3-Glc-α is currently underway, not only for the discovery of novel anticancer agents but also for that of glucose-uptake regulating agents for the treatment of obesity or diabetes.[16]

Received: October 25, 2006
Published online: February 7, 2007

Keywords: antitumor agents · bioassays · bioimaging · fluorescent probes · glucose

[1] M. Zhang, Z. Zhang, D. Blessington, H. Li, T. M. Busch, V. Madrak, J. Miles, B. Chance, J. D. Glickson, G. Zheng, *Bioconjugate Chem.* **2003**, *14*, 709 – 714.

[2] a) P. Som, H. L. Atkins, D. Bandoypadhyay, J. S. Fowler, R. R. MacGregor, K. Matsui, Z. H. Oster, D. F. Sacker, C. Y. Shiue, H. Turner, C. N. Wan, A. P. Wolf, S. V. Zabinski, *J. Nucl. Med.* **1980**, *21*, 670 – 675; b) H. Yorimitsu, Y. Murakami, H. Takamatsu, S. Nishimura, E. Nakamura, *Angew. Chem.* **2005**, *117*, 2768 – 2771; *Angew. Chem. Int. Ed.* **2005**, *44*, 2708 – 2711; c) P. S. Conti, D. L. Lilien, K. Hawley, J. Keppler, S. T. Grafton, J. R. Bading, *Nucl. Med. Biol.* **1996**, *23*, 717 – 735; d) J. Czernin, M. E. Phelps, *Annu. Rev. Med.* **2002**, *53*, 89 – 112.

[3] K. Yoshioka, H. Takahashi, T. Homma, M. Saito, K. B. Oh, Y. Nemoto, H. Matsuoka, *Biochim. Biophys. Acta* **1996**, *1289*, 5 – 9.

[4] K. Yoshioka, M. Saito, K. B. Oh, Y. Nemoto, H. Matsuoka, M. Natsume, H. Abe, *Biosci. Biotechnol. Biochem.* **1996**, *60*, 1899 – 1901.

[5] A. Natarajan, F. Srienc, *J. Microbiol. Methods* **2000**, *42*, 87 – 96.

[6] K. B. Oh, H. Matsuoka, *Int. J. Food Microbiol.* **2002**, *76*, 47 – 53.

[7] P. G. Lloyd, C. D. Hardin, M. Sturek, *Physiol. Res.* **1999**, *48*, 401 – 410.

[8] K. Yamada, M. Nakata, N. Horimoto, M. Saito, H. Matsuoka, N. Inagaki, *J. Biol. Chem.* **2000**, *275*, 22278 – 22283.

[9] Z. Cheng, J. Levi, Z. Xiong, O. Gheysens, S. Keren, X. Chen, S. S. Gambhir, *Bioconjugate Chem.* **2006**, *17*, 662 – 669.

[10] a) Z. Zhang, H. Li, Q. Liu, L. Zhou, M. Zhang, Q. Luo, J. Glickson, B. Chance, G. Zheng, *Biosens. Bioelectron.* **2004**, *20*, 643 – 650; b) Y. Ye, S. Bloch, S. Achilefu, *J. Am. Chem. Soc.* **2004**, *126*, 7740 – 7741.

[11] K. D. McReynolds, A. Bhat, J. C. Conboy, S. S. Saavedra, J. Gervay-Hague, *Bioorg. Med. Chem.* **2002**, *10*, 625 – 637.

[12] F. Fazio, M. C. Bryan, O. Blixt, J. C. Paulson, C.-H. Wong, *J. Am. Chem. Soc.* **2002**, *124*, 14397 – 14402.

[13] a) M. A. Maier, C. G. Yannopoulos, N. Mohamed, A. Roland, H. Fritz, V. Mohan, G. Just, M. Manoharan, *Bioconjugate Chem.* **2003**, *14*, 18 – 29; b) R. E. Campbell, M. E. Tanner, *J. Org. Chem.* **1999**, *64*, 9487 – 9492.

[14] a) M. Trester-Zedlitz, K. Kamada, S. K. Burley, D. Fenyo, B. T. Chait, T. W. Muir, *J. Am. Chem. Soc.* **2003**, *125*, 2416 – 2425; b) Y. Li, Y.-M. Zhu, H.-J. Jiang, J.-P. Pan, G.-S. Wu, J.-M. Wu, Y.-L. Shi, J.-D. Yang, B.-A. Wu, *J. Med. Chem.* **2000**, *43*, 1635 – 1640.

[15] G. A. Korbel, G. Lalic, M. D. Shair, *J. Am. Chem. Soc.* **2001**, *123*, 361 – 362.

[16] R. G. O'Neil, L. Wu, N. Mullani, *Mol. Imaging Biol.* **2005**, *7*, 388 – 392.

[17] P. W. Hruz, M. M. Mueckler, *Mol. Membr. Biol.* **2001**, *18*, 183 – 193.

[18] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55 – 63.

