A Receptor-Targeted Near-Infrared Fluorescence Probe for In Vivo Tumor Imaging

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Small-molecular targeted probes for biological imaging can be effective for overcoming in vivo delivery barriers often encountered by large affinity molecules such as monoclonal antibodies, antibody fragments, and proteins. Small molecule agents often show preferable pharmacokinetic properties and are also often less immunogenic. As a result, recent developments have focused on the creation of small-molecule targeted compounds for fluorescent biological imaging, in particular peptide–fluorochrome conjugates.[1–3] Recently developed near-infrared-fluorescent (NIRF; 700–900 nm), rather than visible-fluorescent, cyanines allow biological tissues to be interrogated more efficiently since light propagation improves in the near infrared.[4] Indeed, recent studies have shown that penetration depths of 10–20 cm are feasible,[5] while certain photon detection techniques are also quantitative.[6]

We previously developed a number of biologically compatible NIR fluorochromes with improved pharmacologic, toxicologic, and physicochemical properties.[7] Since our primary goal is to utilize these reporters in targeted and/or enzyme-activated imaging probes,[8–10] we functionalized the fluorochromes for the attachment of small affinity molecules. In this study we demonstrate this approach to in vivo optical imaging by targeting the folate receptor, which is overexpressed by many tumor types, particularly ovarian cancer.[11] This receptor is used as a model system to show the feasibility of conjugation and the biological efficacy of small-molecule modification to attain target specificity. We hypothesized that the derivatized NIR fluorochromes would have significant advantages over nonspecific fluorochromes, which are often used for nontargeted image enhancement.[12, 13] The concept of tagging NIR fluorochromes can potentially be extended to a myriad of other small-molecule receptor systems and may also be useful for in vivo screening of limited libraries and/or for identification of structure-activity relationships.

To prepare the near infrared fluorescence labeled folate receptor agent (Scheme 1), we first reacted folic acid with a hydrophilic spacer, 2,2′-(ethylenedioxy)-bis(ethylamine) (EDBEA) in anhydrous dimethyl sulfoxide with diisopropylcarbodiimide/*N*-hydroxysuccinimide (DCC/NHS) as the coupling agent. This enabled us to convert the carboxy group into a functional amino group. The folate–EDBEA conjugate was purified by HPLC and characterized by mass spectroscopic analysis. A biocompatible, highly stable, asymmetric near-infrared-fluorescent compound (NIR2) was synthesized de novo as an NHS ester as previously described.[7] The NHS ester of NIR2 was then coupled to the amino-derivatized folic acid in a mixed solvent of 0.1 M $NaHCO_3$/dimethylformamide. The reaction product was purified by reversed-phase HPLC and the successful conjugation of NIR2 to the folate–EDBEA was confirmed by mass spectroscopic analysis ($[M]^+$ calcd: 1401, found: 1402). The absorption and fluorescence spectra of the final product showed both the characteristics of folic acid (excitation at 358 nm and emission at 430 nm) and those of NIR2 (excitation at 665 nm and emission at 686 nm; Figure 1).

High expression levels of folate receptors have been shown to occur in many ovarian cancers.[14–16] We therefore chose the OVCAR3 cell line as a putative receptor and the human lung carcinoma A549[17] as a putative negative control cell line. In order to measure receptor expression levels, we first determined cellular binding/internalization by using ^{3}H-folate. OVCAR3 or A549 (10^6 cells) grown in 12-well plates were incubated for different times with 50 nM ^{3}H-folate. Cells were then washed and counted. Figure 2A shows the temporal binding/uptake of folate in the two cell lines. Folate was internalized in significant amounts by OVCAR3 cells and this process reached a plateau in 60 minutes (1.5 pmol/10^6 cells). In contrast, the A549 cell line showed essentially no uptake of folate (Figure 2A). We subsequently performed competitive inhibition studies in which OVCAR3 cells were incubated with different amounts of NIR2–folate or folic acid. Results from these studies confirmed that fluorochrome attachment did not interfere with folate receptor binding and that NIR2–folate uptake could be inhibited by excess folate. In subsequent experiments we used near infrared fluorescence microscopy to determine cellular distribution of the compound. OVCAR3 and A549 were incubated for 30 minutes with 0.1 μM NIR2–folate conjugate added to the cell culture medium. Cells were then washed and subjected to fluorescence microscopy (Figure 2B). An extensive, bright punctate fluorescence signal was observed for the OVCAR3 cells whereas there was essentially no binding/uptake in the negative control A549 cells (Figure 2B). These results confirm those of the quantitative uptake studies and show that fluorescence is associated with punctuate clusters that occur during endosome formation and internalization.

The NIR2–folate conjugate was subsequently tested in a murine ovarian xenograft tumor model to determine whether this targeting probe can be used for identification of folate-receptor-positive tumors. OVCAR3 tumors were implanted in the lower abdomen of nude mice (number of mice, $n = 3$) and the

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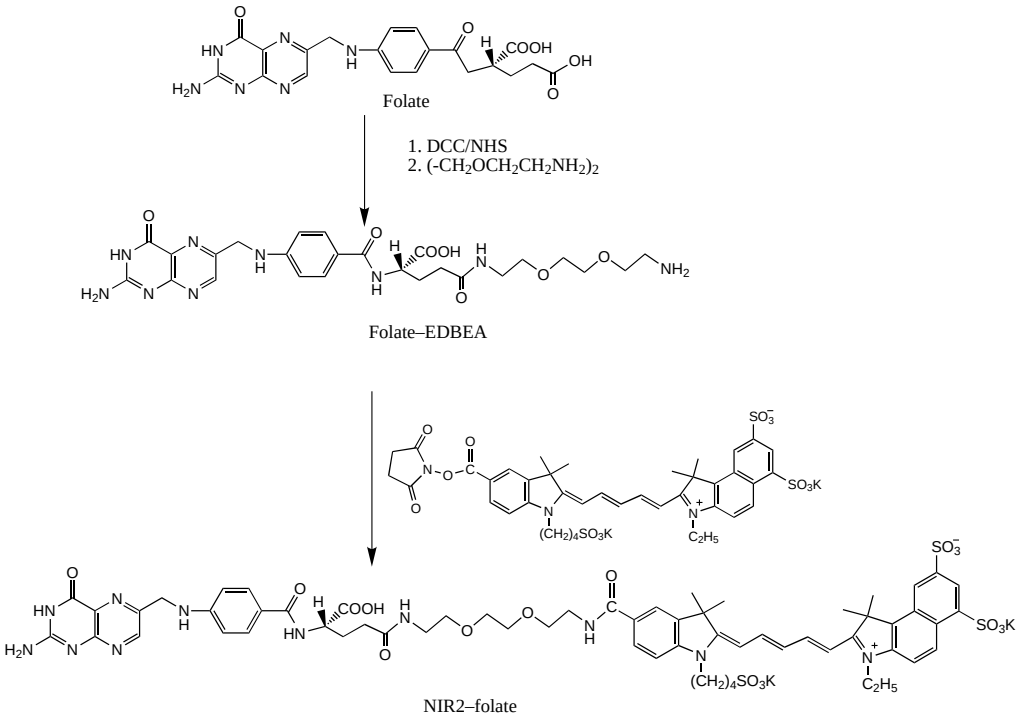

Scheme 1. *Synthesis of the NIR2 – folate conjugate.*

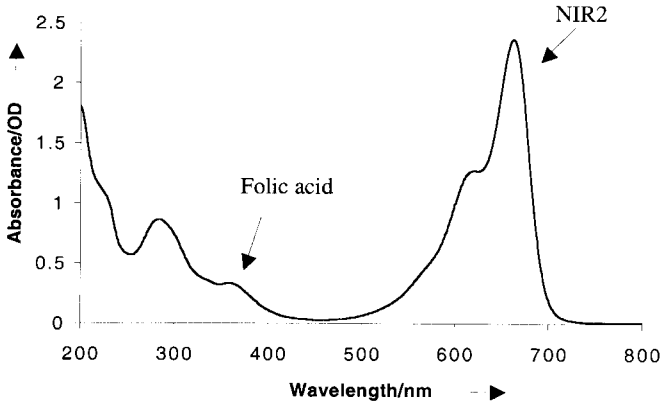

Figure 1. *Absorption spectrum of the NIR2 – folate conjugate. The peaks at 358 nm and 665 nm represent absorption by folic acid and NIR2, respectively. OD = optical density.*

animals were injected intravenously with 2 nmol conjugate when the tumors had grown to 3 – 5 mm in diameter. Animals were imaged by using a laboratory-built fluorescence reflectance imaging system with appropriate excitation (630 ± 15 nm) and emission (700 ± 20 nm) filters.[18] Image analysis confirmed that tumor visibility was significantly enhanced after administration of the probe (Figure 3). Interestingly, tumoral fluorescence could be detected as early as 1 hour after administration but reached a plateau at 24 hours, which suggests that active accumulation does occur, as one would expect with a receptor-targeted agent. Conversely, when the NIR2 compound (not conjugated to folate) was used as an

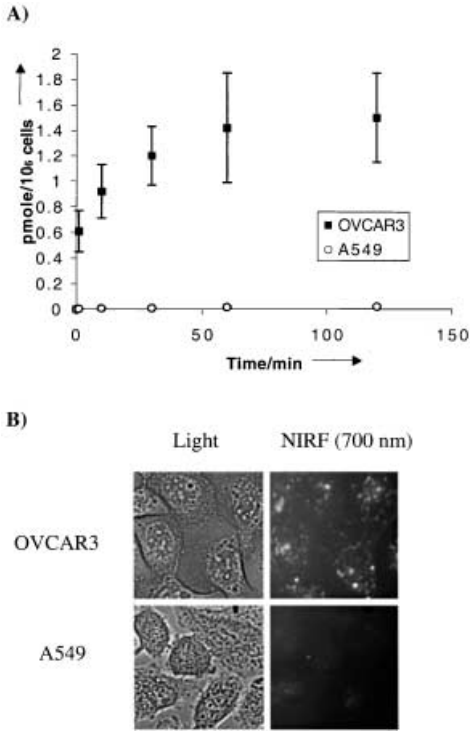

Figure 2. *A) Determination of the number of folate receptors on experimental cells. OVCAR3 and A549 tumor cells were incubated with ^{3}H-folic acid (50 nM) and cellular binding/uptake was quantified by scintillation counting. Note the receptor-positive nature of the OVCAR3 cells and the absence of receptors in the negative control cell line. B) NIR fluorescence microscopy shows cellular uptake of the NIR2 – folate agent into receptor-positive OVCAR3 but not into A549 cells (incubation with NIR2 – folate (0.1 μM) for 30 min; magnfication: 20 $\times$; identical window and level settings were used for all images).*

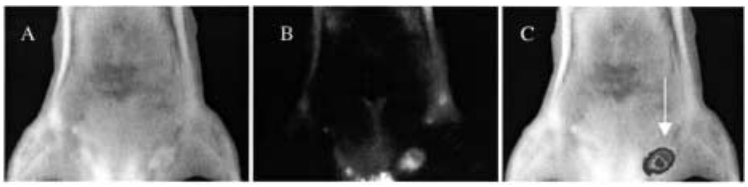

Figure 3. *In vivo image of an OVCAR3 tumor in a nude mouse. The tumor was grown for 9 weeks to reach a size of approximately 3 – 5 mm and groups of animals were injected intravenously with 2 nmole NIR2 – folate conjugate. The images show white light (A) and NIR (B) views and a superimposed false-color image of A and B (C). NIR-fluorescent tumor enhancement was acquired with narrow bandpass filters (excitation: 630 ± 15 nm; emission: 700 ± 20 nm). Note the enhancement of the receptor-positive tumor in the lower right abdomen (arrow).*

imaging agent, no appreciable enhancement could be observed at the 1- or 24-hour time points after injection.

In conclusion, we have shown that near-infrared fluorochromes can be modified by small molecules other than peptides and can be used for targeting receptor systems. These probes, and the modifications thereof, open the door to more extensive biological and medical applications. Apart from use of the conjugates in cell-based assays, it should be feasible to conjugate libraries of small molecules for rapid identification of targeting ligands. The developed compounds may also be used for the detection of small tumors by endoscopy,[1, 19] noninvasive measurement of receptors in vivo,[6] and determination of the efficacy of receptor-targeted therapeutic agents.

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[1] A. Becker, C. Hessenius, K. Licha, W. Semmler, B. Wiedenmann, C. Groetzinger, *Nat. Biotechnol.* **2001**, *19*, 327 – 331.
[2] S. Achilefu, R. B. Dorshow, J. E. Bugaj, R. Rajagopalan, *Invest. Radiol.* **2000**, *35*, 479 – 485.
[3] J. E. Bugaj, S. Achilefu, R. B. Dorshow, R. Rajagopalan, *J. Biomed. Opt.* **2001**, *6*, 122 – 133.
[4] B. Chance, *Ann. NY Acad. Sci.* **1998**, *838*, 29 – 45.
[5] V. Ntziachristos, J. Ripoll, R. Weissleder, *Opt. Lett.* **2002**, *27*, 333 – 335.
[6] V. Ntziachristos, C. H. Tung, C. Bremer, R. Weissleder, *Nature Med.* **2002**, in press.
[7] Y. Lin, R. Weissleder, C. H. Tung, *Bioconjugate Chem.* **2002**, *13*, 605 – 610.
[8] R. Weissleder, C. H. Tung, U. Mahmood, A. Bogdanov, *Nat. Biotechnol.* **1999**, *17*, 375 – 378.
[9] C. Tung, U. Mahmood, S. Bredow, R. Weissleder, *Cancer Res.* **2000**, *60*, 4953 – 4958.
[10] C. Bremer, C. H. Tung, R. Weissleder, *Nat. Med. (Tokyo, Jpn.)* **2001**, *7*, 743 – 748.
[11] S. Wang, R. J. Lee, C. J. Mathias, M. A. Green, P. S. Low, *Bioconjugate Chem.* **1996**, *7*, 56 – 62.
[12] V. Ntziachristos, A. G. Yodh, M. Schnall, B. Chance, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 2767 – 2772.
[13] B. Ebert, U. Sukowski, D. Grosenick, H. Wabnitz, K. T. Moesta, K. Licha, A. Becker, W. Semmler, P. M. Schlag, H. Rinneberg, *J. Biomed. Opt.* **2001**, *6*, 134 – 140.
[14] S. D. Konda, M. Aref, S. Wang, M. Brechbiel, E. C. Wiener, *MAGMA* **2001**, *12*, 104 – 113.
[15] G. Toffoli, C. Cernigoi, A. Russo, A. Gallo, M. Bagnoli, M. Boiocchi, *Int. J. Cancer* **1997**, *74*, 193 – 198.
[16] S. Miotti, M. Bagnoli, F. Ottone, A. Tomassetti, M. I. Colnaghi, S. Canevari, *J. Cell Biochem.* **1997**, *65*, 479 – 491.
[17] S. Wang, J. Luo, D. A. Lantrip, D. J. Waters, C. J. Mathias, M. A. Green, P. L. Fuchs, P. S. Low, *Bioconjugate Chem.* **1997**, *8*, 673 – 679.
[18] U. Mahmood, C. Tung, A. Bogdanov, R. Weissleder, *Radiology* **1999**, *213*, 866 – 870.
[19] K. Marten, C. Bremer, K. Khazaie, M. Sameni, B. Sloane, C. H. Tung, R. Weissleder, *Gastroenterology* **2002**, *122*, 406 – 414.

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Photoregulation of the Transcription Reaction of T7 RNA Polymerase by Tethering an Azobenzene to the Promoter

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Regulation of gene expression by external stimuli is one of the current important and attractive themes.[1] If gene expression could be triggered or terminated only by photoirradiation at a specific wavelength, the scope of application of photoregulation should be extended. For this purpose, we have synthesized photoresponsive DNA that carries an azobenzene moiety in the side chain of a residue and successfully photoregulated the formation and dissociation of its duplex and triplex. The planar *trans*-azobenzene moiety intercalated between the base pairs stabilizes the duplex as a result of stacking interactions, whereas nonplanar *cis*-azobenzene destabilizes the duplex.[2] Furthermore, elongation of primer DNA by T7 DNA polymerase has been successfully photoregulated by use of this photoresponsive DNA as a modulator.[3] Photoregulation of gene expression requires either the transcription or translation step to be controlled by light irradiation. In the present paper, an azobenzene moiety is tethered to the promoter sequence and transcription by RNA polymerase (RNAP) is photoregulated. Here, we use T7 RNAP because useful in vitro translation systems

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