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Cancer is a major public health problem. Although progress has been made in reducing incidence and mortality rates, cancer still causes numerous deaths worldwide.\textsuperscript{1-2} Therefore, the early diagnosis and treatment of cancer are particularly important. At present, cancer diagnostic methods include magnetic resonance imaging (MRI), ultrasound, positron emission tomography (PET) imaging, X-ray imaging and single-photon emission computed tomography (SPECT).\textsuperscript{3,5} However, each of these methods has its drawbacks, such as limited spatial resolution of ultrasound, high instrument cost and radiological hazards\textsuperscript{4} resulting from PET, SPECT and X-ray imaging. Besides, these methods are often not effective until the middle and last-stage cancer.\textsuperscript{7-8} The main treatment protocols are extensive surgical resection, along with chemotherapy and radiotherapy.\textsuperscript{9-10} When performing surgery, the accurate localization and adequate visualization of tumors are vital in optimizing surgical resection.\textsuperscript{11} Failure to adequately recognize tumor margins and an incomplete resection may increase tumor recurrence and decrease survival rate.\textsuperscript{12-13} Fluorescence imaging, serving as an alternative low-cost approach with relatively low toxicity, high sensitivity and resolution, noninvasive real-time capabilities, has become a powerful technique for the localization and dynamic monitoring of biomolecules in living systems.\textsuperscript{14-17} Because the overexpressed enzymes are promising candidates of cancer-imaging target, the development of enzyme-activatable probes that report the species associated with cancer cells will allow for the discrimination of diseased and healthy tissue.\textsuperscript{8, 18-19}

Cyclooxygenases (COXs) regulate the synthesis of prostaglandins and play an important role in tumor development and progression.\textsuperscript{5, 20} Among the COXs, COX-1 is widely distributed and constitutively expressed in all tissues, whereas COX-2 is absent or expressed at very low levels in most normal cells, but is found at high levels in inflammatory lesions and many types of tumors.\textsuperscript{20-24} Clinical data suggest that the overexpression of COX-2 promotes tumor growth, angiogenesis, and metastasis of cancer cells.\textsuperscript{25-26} Recently, Uddin \textit{et al.}\textsuperscript{27} studied the selective inhibition of COX-2 using diverse fluorescent conjugates of COX-2 inhibitors. They\textsuperscript{22} also reported a promising fluorescence method using COX-2-specific molecular probes. Zhang \textit{et al.}\textsuperscript{24, 25} discovered two fluorescent probes based on COX-2 as the cancer-imaging target. One of the probes specially targeted the Golgi apparatus, and the other discriminated tumors from inflammatory lesions. However, the absorption and emission wavelengths of these probes were short, thus limiting their application to a certain extent. Near-infrared (NIR) light (650–900 nm) has several advantages such as minimum photodamage to biological samples, minimal interference from background autofluorescence, and acceptable penetration of the fluorescent light through biological tissues.\textsuperscript{29-30} NIR optical imaging offers great potential for the noninvasive detection of cancer sites \textit{in vivo}; therefore, it is suitable for the wide exploration of possible clinical utility warranted.\textsuperscript{11-12} Consequently, innovative strategies for the development of NIR fluorescent probes for bioimaging are actively sought after.

In this study, we report a novel COX-2-specific fluorescent probe, \textit{Niblue-C6-IMC}, in which indomethacin (IMC) is linked to Nile Blue dye using a hexanediamine linker (Scheme 1). The probe has advantages of optical properties with NIR absorption (630 nm) and emission (670 nm) and can detect cancer cells by fluorescence imaging method. Instant and complete activation of the probe make it possible to use in the study of cancer progression and surgical resection procedures.

Nile Blue dye was selected as the fluorophore because of its distinguished properties such as NIR excitation and emission wavelengths, a high fluorescent quantum yields and a large absorption coefficient.\textsuperscript{33-35} COX-2 was selected as the cancer-imaging target because it is overexpressed in most cancer cell lines. Uddin \textit{et al.}\textsuperscript{27} confirmed that IMC conjugates bound most tightly and selectively to COX-2. When selecting a linear hexanediamine as the linker, on the one hand, the long alky side-chain allows the IMC functionality to fully insert into the binding pocket of COX-2; on the other hand, the side-effect of the fluorophore on IMC is avoided. The spectroscopic properties of \textit{Niblue-C6-IMC} in different solutions are summarized in Figure S1 and Table S1. The detailed synthetic route is described in Scheme 1. Both \textit{Niblue-C6-IMC} and the intermediates were well characterized by \textit{1H} NMR, \textit{13C} NMR, and time-of-flight mass spectrometry (TOF-MS) (See the Supporting Information and Fig. S9–S17).

To elucidate the mechanism of the fluorescence “off–on” process, the Gaussian 09 (DFT at the B3LYP/6-31G level) was used to calculate the frontier molecular orbital...
energies. The oscillator strength of HOMO to LUMO transition was only 0.033, indicating that the electron transition from HOMO to LUMO is prohibited (Fig. S2). The calculated results show photoinduced electron transfer (PET) between Nile Blue and IMC; therefore, the fluorescence is quenched. In the presence of COX-2, Nileblue-C6-IMC binds to three amino acids of COX-2, namely, Arg120, Tyr355, and Glu522, and the IMC moiety can be held by the large hydrophobic cavity of COX-2’s homodimer.24 28 We assume that the probe can adopt the unfolded conformation, resulting in the suppression of PET; thus, the fluorescence can be restored.

Scheme 1. Synthesis route of Nileblue-C6-IMC. Reagents and conditions: a) 2-methoxyethanol, CuI, CsCO3, 125 °C, 24 h; b) ethanol, 90 °C, 2.5 h; c) DMF, EDCI, HOBr, H2O, DMAP, rt, 24 h.

First, we evaluated whether Nileblue-C6-IMC can specifically target COX-2 by native polyacrylamide gel electrophoresis (Native-PAGE) analysis. MCF-7 cancer cells, with high endogenous COX-2 levels, were treated with Nileblue-C6-IMC (0–10 µM) in situ for 30 min, followed by in-gel fluorescence analysis. The analysis (Fig. 1) demonstrated that a fluorescent band appeared in the gel, consistent with purified COX-2; moreover, the intensity was concentration-dependent. In contrast, the pretreatment of cells with celecoxib, one of the potent reagents to control the active site of COX-2 in cancer cells, resulted in much weaker fluorescence. These data clearly indicate that the specific conjugation of Nileblue-C6-IMC to the COX-2 structure significantly turned on the fluorescence, which inspired us to use this probe for potential applications in biological samples containing multiple COX-2.

Fig. 1 Native-PAGE analysis of Nileblue-C6-IMC labeling. (a) Coomassie Brilliant Blue staining and (b) fluorescence image with excitation λ = 630 nm. Lane 1: purelified Cytochrome c; Lane 2-6: protein extracts of MCF-7 cells incubated with various concentrations of Nileblue-C6-IMC for 30 min; Lane 7: protein extracts of MCF-7 cells preincubated with 13.0 µM celecoxib for 3 h, and then the addition of 5 µM Nileblue-C6-IMC for another 30 min.

Next, enzyme-linked immunosorbent assay (ELISA) was used to determine the amount of COX-2 in different cell lines. The results (Fig. 2C) indicate that COX-2 is highly expressed in cancer cell lines (MCF-7 cell, 3.759 µg/mL; HepG2 cell, 3.590 µg/mL; Hela cell, 2.131 µg/mL), while minimally expressed in normal cell lines (COS-7 cell, 0.0075 µg/mL; LO-2 cell, 0.0145 µg/mL; OB cell, 0.0175 µg/mL). Then, these cell lines were incubated with 2.5 µM Nileblue-C6-IMC for 30 min and imaged using a confocal fluorescence microscope. Both the cancer cell lines showed strong fluorescence; in contrast, all the COX-2-negative normal cell lines showed negligible fluorescence upon excitation at 635 nm (Fig. 2A). The fluorescence intensity (Fig. 2B) correlated with the concentration of COX-2 in these cell lines, which was measured by ELISA (Fig. 2C). These results indicate that Nileblue-C6-IMC was cell membrane permeable and capable of distinguishing cancer cells from normal cells by labeling overexpressed COX-2 in cancer cells. Moreover, Nileblue-C6-IMC strongly stained cancer cells within 30 min, and the fluorescence intensity remained almost unchanged after prolonged incubation time (120 min). However, the normal cells still maintained the minimal signal during the long incubation time (Fig. S3). The time scan results indicate that Nileblue-C6-IMC served as a NIR marker for long-time observations in living cancer cells.

Fig. 2 (A) Living cells staining with Nileblue-C6-IMC (2.5 µM). a, c, e, g, i and k are white light images, b, d, f, h, j and l are fluorescence images. Excitation wavelength = 635 nm, scan range = 640-700 nm; (B) Quantitative analysis of fluorescence responds of Nileblue-C6-IMC; (C) Content of COX-2 in different cells by ELISA.

To evaluate whether Nileblue-C6-IMC specifically targeted COX-2, HepG2 cells were preincubated with 0, 6.5, or 13.0 µM celecoxib. The fluorescence intensity (670 ± 10 nm) decreased gradually with increasing amount of celecoxib (Fig. 3), because celecoxib prevented the labeling of HepG2 cells by Nileblue-C6-IMC. Similar results (see Fig. S4) were also obtained in Hela and MCF-7 cells. And as shown in Fig. 4, the activity of COX-2 gradually decreased with increasing probe concentration, and a good linearity relationship was obtained. The IC50 value of Nileblue-C6-IMC for COX-2 was 0.71 µM, which is very close to the IC50 value of IMC for COX-2,28 (0.75 µM), indicating that the binding affinity of Nileblue-C6-IMC for COX-2 is as strong as that of IMC. Collectively, these data confirmed that Nileblue-C6-IMC serves as a potent and selective fluorescent inhibitor for COX-2 in cancer cells.

Fig. 3 Labeling of COX-2-expressing cells by Nileblue-C6-IMC. (A) HepG2 cells preincubated with 0, 6.5, or 13.0 µM celecoxib for 3 h prior to Nileblue-C6-IMC treatment. Excitation wavelength = 635 nm, scan range = 640-700 nm; (B) Quantitative image analysis of the average total fluorescence of HepG2 cells, determined from analysis of 10 cells in each sample image.
The cells survived, thus making Niblue-C6-IMC suitable for microscopy imaging applications in living specimens under the experimental conditions.

To further assess Niblue-C6-IMC as a NIR fluorescent probe for bioimaging applications, we primarily investigated the applicability of this probe for tissue imaging. After the incubation of cancerous and normal liver tissue slices with 30 µM Niblue-C6-IMC for 30 min at 37 °C, the fluorescence images were obtained with 635 nm excitation. Significantly, the cancerous tissues revealed a strong fluorescence signal; in contrast, only weak or negligible signals were detected in normal liver tissue slices (Fig. S8). Therefore, the probe could distinguish cancerous tissues from normal tissues by fluorescence imaging.

Finally, the ability of Niblue-C6-IMC in COX-2-targeted cancer imaging was studied using a mouse tumor model. The tumor-bearing nude mice were dosed by subcutaneous injection with 100 µM Niblue-C6-IMC. When a Small Animals Living Imaging System was used with an excitation wavelength of 630 nm and an emission wavelength of 700 nm, strong fluorescence signal was detected in the COX-2 expressing MDA-MB-231 tumor after 1 h (Fig. 6b). We normalized Niblue-C6-IMC signals in the tumor area (T, right flank) with that in the normal area (N, same ROI in left flank) to generate tumor-to-normal (T/N) ratios as high as 9.4. These results support the hypothesis that Niblue-C6-IMC has potential for applications in living systems by selective accumulation in tumor lesions. Further, a COX-2 blocking experiment was performed, in which the nude mice with MDA-MB-231 xenografts were pretreated with celecoxib in PBS by subcutaneous injection prior to the dosing of Niblue-C6-IMC. After 1 h post injection, the celecoxib-pretreated mice showed weak fluorescence signals in tumors (Fig. 6d) and a significantly lower T/N ratio of roughly 4.1. Thereby, celecoxib partially inhibited the activity of COX-2, thus reducing the tumor uptake of Niblue-C6-IMC in vivo. In other words, this indicated that Niblue-C6-IMC labelled the tumor site by specifically binding with COX-2. These results confirm that Niblue-C6-IMC is an important NIR probe for cancer imaging.
Conclusions

In summary, we report the first COX-2-specific NIR fluorescent probe, Niblue-C6-IMC, for visualizing tumor sites in cancer cells and in vivo. In COX-2 negative normal cells, the fluorescence of Niblue-C6-IMC was weak, while its fluorescent signal was selectively and quickly generated by interacting with COX-2 accumulated in the Golgi apparatus of cancer cells. The “off–on” fluorescence enhancement results from the inhibition of PET following the Niblue-C6-IMC binding to COX-2. Simultaneously, the probe could be used to screen cancer cells in a rapid, sensitive, and quantitative manner using flow cytometry. Because NIR optical imaging offers great potential for the noninvasive detection of cancer sites in vivo, the probe may have applications ranging from an accurate cancer diagnosis to guiding tumor resection during surgery.

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