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Two-photon fluorescent probe for detection of nitroreductase and hypoxia-specific microenvironment of cancer stem cell

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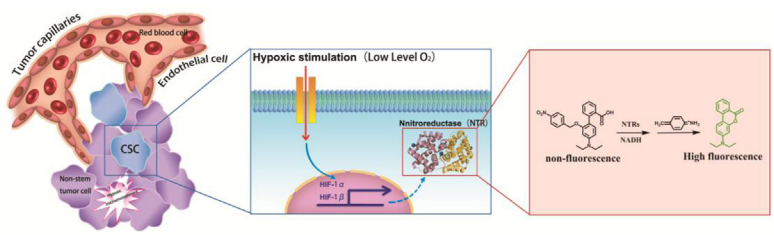
HIGHLIGHTS

- A two-photon fluorescence probe is developed.
- The two-photon fluorescence probe detected the hypoxic microenvironment of cancer stem cells (CSCs) *in vivo*.
- The two-photon absorption cross section extends fluorescent excitation spectra to the NIR region, allowing light penetration for deep-tissue imaging, which will greatly promote the application in biological systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Hypoxia plays a crucial role in cancer progression, and it has great significance for monitoring hypoxic level in biosystems. Cancer stem cells (CSCs) represent a small population of tumour cells that regard as the key to seed tumours. The survival of CSCs depend on the tumour microenvironment, which is distinct region has the hypoxic property. Therefore, the detection of the hypoxic CSC niche plays a pivotal role in the destructing the 'soil' of CSCs, and eliminating CSCs population. Numerous one-photon excited fluorescent probes have been developed to indicate the hypoxic status in tumours through the detection of nitroreductase (NTR) level. However, the biomedical application of one-photon fluorescent probes is limited due to the poor tissue penetration. In the present work, we reported a two-photon fluorescent probe to detect the NTR in CSCs and monitor the hypoxic microenvironment *in vivo*. The two-photon fluorescent molecular probe with a hypoxic specific response group can be reduced by NTR under hypoxic conditions. We used the two-photon probe to detect the hypoxia status of 3D cultured-CSCs *in vitro* and *in vivo* CSCs' microenvironment in tumour. The two-photon absorption cross section extends fluorescent excitation spectra to the near infrared region, which dramatically promotes the tissue penetration for hypoxic microenvironment detection of CSC *in vivo*.

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1. Introduction

Cancer is a common disease and the first or second major cause of death in the world. Recently, the theory of the cancer stem cell (CSC) has both challenged and disrupted the previous understandings of cancer biology. CSCs defined the existence of a small group of tumour cells have a stem cell-like property, which may drive tumor formation, progression, metastasis, recurrence, and current anticancer drug resistance [1,4,19,22]. CSCs live in niches, which have the distinct hypoxic feature in the tumour microenvironment. This hypoxic microenvironment activates the expression of stemness genes, which maintain the stem-like features of CSCs, prevent attackers from the immune system, facilitate their metastasis and DNA damage repair, and protect them phenotype [9,12,33]. Therefore, the early detection and assessment of hypoxia microenvironment plays a key role in the destructing the 'soil' of CSCs, eliminating CSCs population, and developing CSCs-targeted therapies for their eventual adoption into routine clinical practice. In addition, the hypoxia status of microenvironment in tumour would hint the exits of CSCs, impact early diagnosis of tumour, and report cancer metastasis and recurrence after surgical treatment. However, despite current research into the early stages of tumour microenvironment imaging, measurement of tumour hypoxia within lesions still remains a tough challenge because of the limitations on the water solubility and sensitivity of the existing molecular fluorescence probes, and intrinsic background interference [32,36]. Therefore, there is an acute need to develop sensitive fluorescent probes to monitor the tumour formation, which spans from the proliferation and differentiation of CSCs to growth of a solid tumour.

Nitroimidazole-based compounds, such as metronidazole, misonidazole, pimonidazole, and etanidazole have emerged as the most prevalent type of molecular hypoxia probes for biomedical imaging [11,17]. However, the excitation sources of traditional one-photon fluorescence probes are mostly ultraviolet (UV) light or visible light, which restrict the use of probes in tissue imaging due to the shallow penetration depth less than 80 μm and also damages the healthy cells and organs [10,38,39]. Moreover, phosphorescent transition metal complexes with adjustable excitation wavelength and emission wavelength and high fluorescence quantum yields are sensitive to oxygen and been used in bio-imaging [26,27]. However, these imaging probes have poor specificity that could be uptaken by normal tissues and rapidly cleared by renal, which limit their application to differentiate hypoxic and normoxic status [38,39]. Furthermore, these probes have poor biodistribution because of their water insoluble property. Besides, the probes are excited through UV light and have poor tissue penetration, which prevent their application in biosystem hypoxia imaging [2,14]. Recently, two-photon fluorescence has been accepted as a potential candidate due to its great advantages [5,13,15,21,31]. A two-photon fluorescent probe can be excited by near-infrared (NIR) light, which superiors to UV or visible light, allows deeper tissue penetration, causes little damage to biological samples and photobleaching, and excludes the disturbance of auto-fluorescent from biological tissues. Therefore, it has great significant to develop two-photon probes over traditional techniques in the field of bioimaging.

The ideal molecular probe should have excellent tissue penetration, high sensitivity, and long retention time in hypoxic region. Taking these into consideration, we designed a two-photon fluorescent molecular probe with a hypoxic specific response group, which can be reduced by nitroreductase (NTR) under hypoxic conditions and then undergo intra molecular charge transfer and intra molecular lipid exchange to release 2-carboxy-2′-hydroxybiphenyl (BCM), which emits strong fluorescent. The large two-photon absorption cross section extends fluorescent excitation spectrum to the Near-Infrared region, which allowed the light penetrates into deep-tissue for the efficient imaging, which will greatly promote the tissue penetration and application in biological systems. In addition, CSCs have the ability of three-dimension (3D) tumour spheroid formation, which can mimic *in vivo* microenvironments of tumour tissue and demonstrate a more realistic hypoxic response of molecular probe. In this work, the feasibility of two-photon fluorescent probe in early tumour hypoxic microenvironment visualization and CSCs detection were investigated *in vivo* and *in vitro* using 3D-cultured CSCs tumour spheres and nude mouse CSCs xenograft model. The findings suggest that the probe non-invasively detects the hypoxic status in tumour and early evaluates the hypoxic microvironment of CSCs *in vivo*.

2. Materials and methods

2.1. Materials

Aminoanisole, di-*tert*-butyl dicarbonate, *N*-bromosuccinimide, ethylene glycol dimethyl ether, 1,4-dioxane, *p*-nitrobenzyl bromide, 2-methoxycarbonylphenylboronic acid, 5-fluoro-2′-deoxyuridine nucleoside, and boron tribromide were purchased from Energy Chemical. Anhydrous dimethyl sulfoxide (DMSO) and acetonitrile, dichloromethane, and ethyl acetate were purchased from Alfa-aesar. Malononitrile and *N,N′*-diisopropylcarbodiimide were obtained from Aladdin Reagent. The chemicals used in this work are analytical grade and purchased from Tianjin Analytical Pure Sinopharm Group Chemical Reagent. Newborn bovine serum was purchased from Hangzhou Sijiqing Biotechnology Materials. Low glucose DMEM medium was purchased from Gibco. Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), growth factor N2 and B27, and antibodies were obtained from Thermo Fisher. Nitroreductase (NTR), nicotinamide adenine dinucleotide (NADH), benzyl penicillin and streptomycin, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma. Nude mice used in this wrok were from Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal operations were authorized by the administration office committee of laboratory animal of Hebei University, and complied with the guidance of the care and use of laboratory animals.

2.2. Instrumentation and measurement

NMR spectrum of compounds were record on a Bruker AVANCEIII 600 NMR spectrometer (Bruker, Billerica, MA)) in CD_3Cl or DMSO-d_6. Fluorescence properties of the probe were analyzed by a Hitachi F-7000 luminescence spectrophotometer in 2.5×10 mm quartz cells. Electrospray ionization mass spectrum (ESI-MS) was obtained by using a AB SCIEX 4000 Q TRAP MS system. Cell viability was determined using full-wavelength microplate reader. Two-photon excitated fluorescence imaging of cells were performed on a laser scanning confocal microscope (CLSM, Zeiss LSM-880 microscope, Germany). The animal images were captured by using a PerkinElmer IVIS Lumina II animal imaging system. Optical images of mice were performed on Maestro EX fluorescence imager system (CRI Maestro 2, USA).

2.3. Synthesis and characterization

The detailed synthesis and characterization of the probe were shown in Supporting Information. The UV absorption and fluorescence spectra of probe and BCM were measured by UV-Vis spectrophotometer and Cary Eclipse fluorescence spectrophotometer.

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