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# A multifunctional heptamethine near-infrared dye for cancer theranosis

Shenglin Luo [a], Xu Tan [a], Qingrong Qi [b], Qinyuan Guo [a], Xinze Ran [a], Lilong Zhang [a], Erlong Zhang [a], Yufeng Liang [b], Lingling Weng [b], Hu Zheng [b], Tianmin Cheng [a], Yongping Su [a], Chunmeng Shi [a,*]

[a] Institute of Combined Injury, State Key Laboratory of Trauma, Burns and Combined Injury, Chongqing Engineering Research Center for Nanomedicine, College of Preventive Medicine, Third Military Medical University, 30, Gaotanyan Road, Chongqing 400038, China
[b] Key Laboratory of Drug-Targeting and Drug-Delivery Systems of the Ministry of Education, Department of Medicinal Chemistry, West China School of Pharmacy, Sichuan University, Chengdu 610041, China

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ABSTRACT

Personalized oncology significantly relies on the development of cancer theranostic agents to integrate cancer therapeutics and diagnostics. Current most common strategy for development of such multifunctional agents requires multistep chemical conjugation with cancer targeted ligands, contrast agents and therapeutic agents. Here we report the chemical synthesis and biological characterization of a new heptamethine dye, termed as IR-808DB, natively with multifunctional characteristics of cancer targeting, near-infrared fluorescence imaging, and efficient anticancer activity. The tumor inhibition effect of IR-808DB is higher than that of cyclophosphamide (CTX) toward a broad spectrum of tumor xenograft models. These findings provide IR-808DB a promising prospect as a new cancer theranostic agent that would enable integration of cancer targeted therapeutics and diagnostics without requirement of multi-component chemical conjugation.



## 1. Introduction

Despite significant advances that have been achieved in understanding cancer biology in recent years, cancer therapies remain daunting owing to inefficient early diagnosis, poor drug specificity and lack of a sensitive modality to monitor therapeutic responses in real-time [1–3]. Cancer theranosis, an integration of therapeutics and diagnostics for optimizing efficacy and safety of cancer treatment, has been considered recently as a significant alternativeness to overcome these challenges [4–6]. Therefore, theranostic agents have received a great deal of recent research interest in cancer diagnosis and treatment [7–11]. Generally, the development of such multifunctional agents are explored through advanced nanotechnology or multi-step chemical conjugation of different functional agents which may respectively serve as a tumor-specific ligand, contrast agent, anticancer drug or simultaneously perform two or more of the above duties [5,7,12–16]. Despite the above strategies have demonstrated some efficacy, it remains considerable challenges to apply the theranostic nanomaterials in humans, such as low specificity for passive tumor targeting [17,18], adverse immunogenic reaction with antibody-based active targeting [19], difficulties in large-scale preparation with high reproducibility and safety concern about potential toxicity [20,21]. Moreover, the efficiency of drug delivery and treatment remains limited since the conjugation may alter the functional activities of contrast agents, tumor-targeted ligands or therapeutic agents.

In addition to current chemical conjugation strategy to develop cancer targeted theranostic agents, we have recently described an alternative strategy to develop such multifunctional agents without chemical conjugation [22]. A couple of cancer-selective near-infrared (NIR) fluorescent heptamethine indocyanine dyes have been identified for cancer targeting and imaging [23–26]. Among them, a protypical compound termed as IR-808, is shown to preferentially accumulate in the mitochondria of tumor cells and function in the wavelength range of 700–900 nm in which the intrinsic chromophores in native tissue are extremely low to visualize tumors in vivo sensitively and noninvasively [26]. This dye also shows photodependent cytotoxic activity, suggesting a potential theranostic agent for tumor targeting, imaging and photodynamic therapy (PDT). Nevertheless, the relatively low quantum yield of singlet oxygen produced by IR-808, limited tissue penetration of light irradiation, requirement of strict procedure and special equipments would preclude IR-808 for efficient cancer treatment and widespread application for clinical purpose. In order to overcome these drawbacks, the development of derivatives of IR-808 as cancer targeted theranostic agents without requirement of multi-component chemical conjugation is warranted.

* Corresponding author. Tel.: +86 23 65462263.
*E-mail address:* shicm1010@yahoo.com.cn (C. Shi).

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Certain indocarbocyanine, thiacarbocyanine, and oxacarbocyanine dyes (wavelength below 600 nm) in high concentration have been revealed with mitochondrial toxicity to preferentially inhibit the NADH-ubiquinone reductase [27]. The increased mitochondrial cytotoxicity of these dyes is shown at least partially in correlation to the increased length of alkyl chains [28]. Because mitochondria are encircled with two specialized (outer and inner) lipophilic membranes and lipophilic agents are more readily transported across bistratal membranes to reach a higher concentration. Therefore, increasing lipophilicity of a mitochondrial toxic compound with suitable lipid/water partition coefficient may also improve its cytotoxicity [29,30]. Basing on these investigations, we hypothesized that the chemical modification of IR-808 by increasing the alkyl side-chains length and the lipophilicity could enhance its cytotoxic activity. To testify this hypothesis, we screened a series of IR-808 analogs and IR-808DB, a butyl ester derivative of IR-808, was identified with simultaneous cancer targeted NIR imaging and potent anticancer activities. Here, we describe the chemical synthesis and biological characterization of this newly developed multifunctional dye.

## 2. Materials and methods

### 2.1. Materials

Except special mention, all chemicals and solvents were purchased from Aladdin Chemistry Reagent Company (Beijing, China) and straightforward used without further purification or dryness. All reactions were performed under argon-protected dark and anhydrous atmosphere, monitored by TLC on GF254 plates (Qingdao Haiyang Chemical Group Co., Qingdao, China). Flash chromatography was carried out using silica gel (300–400 mesh). $^1$H NMR spectra were recorded with a Varian 400 MHz NMR in $CDCl_3$ with trimethylchlorosilane (TMS) as an internal standard. High-resolution mass spectra (HRMS) were performed on a Bruker BioTOF IIIQ system. Photoluminescence was determined using a Cary Eclipse Fluorometer (Varian Instruments, Palo Alto, CA) with 740 nm excitation, continuous wavelength from 750 to 900 nm emission. UV–VIS NIR spectroscopy was recorded using a Shimadzu UV-3600 Scanning Spectrophotometer. NIR optical imaging was performed by using a Kodak In-Vivo FX Professional Imaging System (New Haven, CT).

### 2.2. Synthesis and structural characterization

A 500 ml round-bottom flask equipped with water separator and condenser, was added 2-chloro-1-formyl-3-hydroxymethylene cyclohex-1-ene **1** (0.645 g, 3.728 mmol), quaternary ammonium salt **2** (2.780 g, 7.809 mmol), and n-butanol/toluene [7:3(v/v), 250 ml]. The mixture was heated under reflux for 16 h at 120 °C. After completion of the reaction, toluene and n-butanol were evaporated under reduced pressure to yield yellow green solid (3.395 g). The crude was dissolved in chloroform (50 ml) and precipitated with excess ether (150 ml). Further purification was performed with column chromatography, by using dichloromethane/methanol (30:1) as the eluent. Eventually green powder of IR-808DB was afforded with a yield of 84% (2.753 g). With the same method, compound **1** and **2** was mixed in a refluxed solution of toluene and corresponding alcohol (n-hexyl alcohol or cyclohexanol) to afford IR-808DH, and IR-808DCH, respectively. The structures of synthesized compounds were confirmed by nuclear magnetic resonance spectra ($^1$H NMR and $^{13}$C NMR) and HRMS.

### 2.3. Determination of optical properties

The ester derivatives of IR-808DB were dispersed in methanol (MeOH), 100% fetal bovine serum (FBS) and dimethylsulfoxide (DMSO) with a final concentration of 2 μM. Photoluminescence and absorbance spectra were determined respectively. The molar absorption conefficient ($\varepsilon$, $mol^{-1}$ $cm^{-1}$ L) in different solvents was calculated by using the Beer's law. The relative fluorescence quantum yield was determined using the model: $\Phi_{F(x)} = [A_s/A_x] \times [F_x/F_s] \times \Phi_{F(s)}$, where $\Phi_F$ is the fluorescence quantum yield, $A$ is the absorbance, $F$ is the area under the emission curve. The subscripts $s$ and $x$ represent the standard and the unknown, respectively. ICG (sigma-aldrich Co. LLC.) was used as a reference standard, which has the value of 0.040 in MeOH.

### 2.4. Cell culture

The aggressive rat dermal stem cell-derived sarcoma cells (rTDMCs) were established in our lab [26] and was cultured in IMDM medium supplemented with 10% FBS. All the other cells: human lung cancer cells (A549, NCIH-460), human breast cancer cells (MDA231 and MCF-7), human hepatoma cells (SMMC-7721, HepG2) used in our study were purchased from ATCC (Manassas, VA) and cultured in ATCC recommended media with 10% FBS. All the cells were added with 1% penicillin/streptomycin and incubated at 37 °C with 5% $CO_2$. At 80% confluence, cells were split 1:3 and cultured for one passage.

### 2.5. Tumor xenograft models

To establish rTDMC tumor xenografts, male Sprague–Dawley (SD) rats (3–4 weeks old and weighing 70-90g), or athymic nude mice (4–6 weeks old and weighting 20–25g) were injected subcutaneously in the right flank with $1 \times 10^7$ rTDMC cultured cells suspended in 200ul PBS. Lewis lung carcinoma (LLC) model was established as described by Myungmi Oh [31]. Approximately $1.0 \times 10^7$ LLC cells suspended in 200ul PBS was subcutaneously implanted into the right flank of each male C57BL/6 mouse (10 weeks old, weighing 20–25 g). To establish HeLa and A549 tumor xenografts, athymic nude mice (4–6 weeks old and weighting 20–25 g) were injected subcutaneously in the right flank with $1.0 \times 10^7$ HeLa or A549 cells suspended in 200ul PBS respectively. All the animals were purchased from the laboratory animal center of the Third Military Medical University and the animal protocols were in accordance with the "Animal Care and Use Committee Guidelines" of the university.

### 2.6. In vivo NIR imaging for biodistribution and tumor targeting of IR-808DB

Normal mice without tumor inoculation were used to assess distribution of IR-808DB. These mice were sacrificed at 0, 24, 48 and 120 h ($n = 3$ each) after tail vein injection of IR-808DB at a dose of 0.4 mg/kg. Dissected organs were subjected to NIR imaging by a Kodak In-Vivo FX Professional Imaging System (New Haven, CT). For assessment of tumor targeting ability, the SD rats or athymic nude mice bearing rTDMC, Male C57BL/6 mice bearing LLC, athymic nude mice bearing HeLa or A549 tumor xenografts were injected IR-808DB at a dose of 0.4 mg/kg by tail vein. To evaluate the dynamic accumulation and retention of the dye in tumors, the contrast index (CI) values were calculated according to the formula CI = [(Ftumor-Fauto)/(Fnorm-Fauto)]. Ftumor and Fnorm are the fluorescence mean intensities of tumor and normal contralateral region, respectively. Fauto is the autofluorescence from the corresponding region measured before dye injection. The fluorescence intensity of different time points was calculated using Kodak MI software 5.0.1. Then, dissected organs and tumors were obtained for NIR fluorescent imaging at the day of sacrifice.

### 2.7. In vitro and in vivo tumor inhibitory effects of IR-808DB

Cytotoxicity of IR-783, Rhodamine 123 (Rho123), IR-808 and its ester derivatives in vitro were performed on A549 human lung cancer cells using a typical Cell Counting Kit-8 (CCK-8) assay. IR-783 and Rho123 were purchased from Sigmae-Aldrich (MO,USA). A number of 5000 cells were seeded in each well of a 96-well plate. Followed a 24 h-period for cells to adhere, IR-783, Rho123, IR-808 and its ester derivatives at different concentrations were added with 6 wells for each concentration. Cells viability was tested to measure the 50% inhibitory concentration ($IC_{50}$). $IC_{50}$ of IR-808DB on the other cancer cells (NCIH-460, MDA231, MCF-7, SMMC-7721 and HepG2) were also investigated. To determine the in vivo tumor inhibitory effect of IR-808DB, rTDMC, LLC, HeLa and A549 tumor xenograft models were used. There were three groups for each tumor xenograft model: control group, IR-808DB group and cyclophosphamide (CTX) group. Animals in the IR-808DB group or CTX group were injected intraperitoneally 5 mg/kg IR-808DB or 20 mg/kg CTX respectively every two days and repeated for 5 times. In the control group, all the animals were just given the same volume of PBS each time. Size of tumor was measured from two dimensions by a caliper. Tumor volumes were calculated as: tumor volume = largest diameter × (smallest diameter)$^2$/2. After drug administration, animals were sacrificed, subsequently tumors and organs were dissected to be fixated by 4% paraformaldehyde. Hematoxylin and eosin staining were carried out for tumors to determine the histologic changes after paraffin-embedded, sectioned.

## 3. Results and discussion

### 3.1. Synthesis and structure characterization of representative ester derivatives

With an aim to increase the length of alkyl side-chains and the lipophilicity of IR-808, four representative ester derivatives of IR-808, namely n-butyl ester (IR-808DB), n-hexyl ester (IR-808DH), cyclohexyl ester (IR-808DCH), phenyl ester (IR-808DP) were designed to synthesize for the first time. For preparation of IR-808DB, IR-808DH and IR-808DCH (Fig. 1), 2-chloro-1-formyl-3-hydroxy -methylene cyclohexene (**1**) and 1-(5-carboxypentyl) -2,3,3-trimethyl-3H indolenium (**2**) were prepared as the key intermediates according to literature methods [32]; then these