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Photoacoustic imaging of integrin-overexpressing tumors using a novel ICG-based contrast agent in mice

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ABSTRACT

PhotoAcoustic Imaging (PAI) is a biomedical imaging modality currently under evaluation in preclinical and clinical settings. In this work, ICG is coupled to an integrin binding vector (ICG-RGD) to combine the good photoacoustic properties of ICG and the favourable $\alpha_v\beta_3$-binding capabilities of a small RGD cyclic peptidomimetic. ICG-RGD is characterized in terms of physicochemical properties, biodistribution and imaging performance. Tumor uptake was assessed in subcutaneous xenograft mouse models of human glioblastoma (U-87MG, high $\alpha_v\beta_3$ expression) and epidermoid carcinoma (A431, low $\alpha_v\beta_3$ expression). ICG and ICG-RGD showed high PA signal in tumors already after 15 min post-injection. At later time points the signal of ICG rapidly decreased, while ICG-RGD showed sustained uptake in U-87MG but not in A431 tumors, likely due to the integrin-mediated retention of the probe. In conclusion, ICG-RGD is a novel targeted contrast agents for PAI with superior biodistribution, tumor uptake properties and diagnostic value compared to ICG.

1. Introduction

Photoacoustic imaging (PAI) is a biomedical imaging modality based on laser-generated ultrasounds that has gained attention particularly over the last decade [1,2]. Of relevance, the most recent advancement in the PAI filed has been the development of Multispectral Optoacoustic Tomography (MSOT), in which the sample is irradiated (in a tomographic setup) with multiple wavelengths, allowing it to detect ultrasound waves emitted by different photoabsorbing molecules in the tissue, whether endogenous (oxygenated and deoxygenated hemoglobin, melanin) or exogenous (imaging probes, nanoparticles). Computational techniques such as spectral unmixing deconvolute the ultrasound waves emitted by these different absorbers, allowing each emitter to be visualized separately in the target tissue [3,4]. While the detection of endogenous chromophores has already been exploited in clinical settings (*i.e.*, Hemoglobin), the development and bench-to-bedside translation of exogenous agents is still lacking [5]. Fluorescent dyes developed for Optical Imaging (OI) applications can be exploited for PAI, particularly those with a high molar extinction coefficient and low fluorescence efficiency, as the non-radiative conversion of light energy to heat is maximized [6]. Indeed, the heptamethine cyanine dye Indocyanine green (ICG), clinically available for fluorescence angiography and liver function assessment by OI, is currently being evaluated off-label for photoacoustic imaging applications [7]. In recent clinical trials on melanoma patients [8,9], ICG was evaluated non-invasively using fluorescence imaging and multispectral optoacoustic tomography (MSOT) to detected non-invasively sentinel lymph nodes after intradermal injection in proximity of the primary tumor. While fluorescence imaging revealed only superficial nodes, PAI picked up ICG-positive lymph nodes up to a depth of 5 cm [8]. *In vivo* PAI of sentinel lymph node using ICG revealed comparable results with the gold standard ^{99m}Tc lymphoscintigraphy. The detection of metastatic melanoma lymphnode mapping using the photoacoustic signal of endogenous chromophore melanin granted 100% sensitivity compared to histopathology, but only 48% and 62% specificity *in vivo* and *ex vivo*, respectively [8]. This study showed that a non-invasive approach using photoacoustic imaging could reduce the number of patients with negative sentinel lymph nodes subjected to surgical excision. However, the specificity is still limited, probably due to the lack of a cancer-specific tracer able to discriminate between tumor and healthy tissues, as already reported in studies based on fluorescence imaging [10,11]. Integrins are adhesion proteins present at basal levels in normal tissues but are overexpressed in several types of cancer, particularly during tumor neoangiogenesis, [12,13]. Molecular imaging of integrins (*i.e.*,

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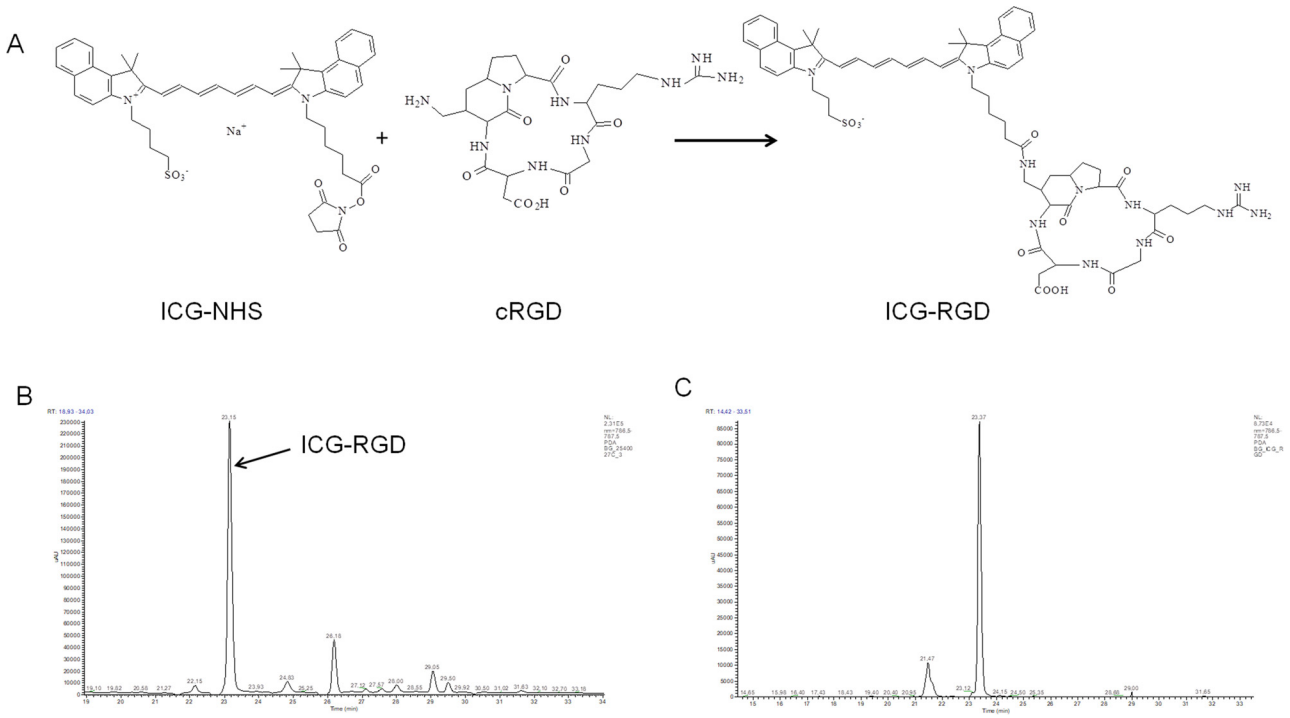

Fig. 1. Synthesis of ICG-RGD. A) Scheme of synthesis; B) UV–vis trace of HPLC chromatogram of ICG-am-cRGD in DMF before purification extracted at 787 nm; C) UV–vis trace of HPLC chromatogram of ICG-am-cRGD extracted at 787 nm in MilliQ/DMF 2:1 after purification.

$\alpha_v\beta_3$, $\alpha_v\beta_5$) has been extensively exploited for cancer detection, and several targeted contrast agents have been proposed for PET [14], MRI [15], ultrasound [16,17] and optical/photoacoustics imaging [18,19]. Small integrin-targeting near-infrared fluorescent probes carrying the detection moiety Arg-Gly-Asp (RGD) were proposed in the last years for *in vivo* cancer detection, but mainly for fluorescent imaging application [18–21]. Given the greater penetration depth capabilities of PAI compared to optical imaging, PAI may allow to image deeper tumor lesions, to detect non-invasively deep metastatic lymph node, and to facilitate the identification of residual disease underneath the tissue surface during surgery [22]. Therefore, targeted photoacoustic imaging could be a valid approach to increase specificity of detection particularly in those applications where near-infrared fluorescence imaging is sub-optimal. To this purpose, a novel integrin-targeting photoacoustic probe was synthesized to combine the good photoacoustic properties of ICG and the favourable $\alpha_v\beta_3$-binding capabilities of a cyclic RGD peptidomimetic moiety previously used for fluorescence imaging [23,24]. Here, the physicochemical properties (including albumin-binding properties), biodistribution and imaging performance of this novel contrast agent for photoacoustic imaging of cancer were evaluated and directly compared to ICG both *in vitro* and *in vivo*.

2. Material and methods

2.1. Synthesis and characterization of ICG-RGD

ICG-NHS ester (10 mg, 0.012 mmol, Intrace Medical) was first dissolved in dry N,*N*-Dimethylformamide, and then mixed with the peptidomimetic azabicycloalkane integrin-binding vector am-cRGD [23] (10 mg, 0.0186 mmol). After 24 h stirring in the dark, HPLC analytical control (YMC-Triart Phenyl column 250 × 4.6 mm 5 µm, 50–100% of acetonitrile in ammonium acetate buffer) was performed. The solution was then evaporated to obtain a green powder. A further purification was performed on reverse phase HPLC (Lichrosorb RP-8 250 × 25 mm 7 µm, 30 mL/min, with a gradient of ammonium acetate – acetonitrile, UV detection at λ = 780 nm). The solution was then evaporated and lyophilized three times. To characterize the product an HPLC-MS analysis was performed (YMC-Triart Phenyl 250 × 4.6 mm 5 µm, column temperature 40 °C, flow 1 mL/min, ammonium acetate and acetic acid and ACN). Data were processed using Xalibur software for qualitative and qualitative analysis. Purity was calculated from the area under the curve of the peaks detected at λ = 787 nm.

2.2. Cell line and animal models

Human glioblastoma cells (U-87MG), human epidermoid carcinoma (A431) and melanoma (WM-266) cells were supplied by ATCC. U-87MG and WM-266 cells were cultured in EMEM medium, A431 cells were cultured in DMEM high glucose medium; both media were supplemented with 10% FBS, 2 mM glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. BALB/c nu/nu mice were provided by Charles River Italia Laboratories. Under isoflurane anaesthesia, mice were subcutaneously implanted in the right flank with either five million of A431 cells or two million of U-87MG cells. Imaging experiments were performed approximately 14 days after cells implantation. All the procedures involving the animals were conducted according to the national and international laws on experimental animals (L.D. 26/2014; Directive 2010/63/EU).

2.3. Imaging systems

The photoacoustic instrument used for this work was the VevoLAZR 2100 (Visualsonics), equipped with a transducer (Vevo LAZR LZ250) with a broadband ultrasound frequency of 13 MHz–24 MHz, producing an axial resolution of 75 µm. The optical imaging experiments were performed on IVIS Instrument SPECTRUM (Perkin Elmer), equipped with a CCD camera and a series of excitation (ex) and emission (em) filters. To image ICG and ICG-RGD, the optimal filter pair were 745 nm (ex) and 820 nm (em).