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Multifactorial diagnostic NIR imaging of CCK2R expressing tumors

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ABSTRACT

Optical imaging-based diagnostics identify malignancies based on molecular changes instead of morphological criteria in a non-invasive, irradiation free process. The aim of this study was to improve imaging efficiency by the development of a new Cholecystokinin-2-receptor targeted fluorescent peptide that matches the clinical needs regarding biodistribution and pharmacokinetics while displaying superior target specificity. Furthermore we performed multifactorial imaging of Cholecystokinin-2-receptor and tumor metabolism, since simultaneous targeting of various tumor biomarkers could intensely increase tumor identification and characterization. Affinity and specificity of the fluorescent Cholecystokinin-2-receptor targeted minigastrin (dQ-MG-754) were tested *in vitro*. We conducted *in vivo* imaging of the dQ-MG-754 probe alone and in a multifactorial approach with a GLUT-1 targeted probe (IR800 2-DG) on subcutaneous xenograft bearing athymic nude mice up to 24 h after intravenous injection ($n = 5$/group), followed by *ex vivo* biodistribution analysis and histological examination. We found specific, high affinity binding (Kd = 1.77 nM ± 0.6 nM) of dQ-MG-754 to Cholecystokinin-2-receptor expressing cells and xenografts as well as favorable pharmacokinetics for fluorescence-guided endoscopy. We successfully performed multifactorial imaging for the simultaneous detection of the Cholecystokinin-2-receptor and GLUT-1 targeted probe. Prominent differences in uptake patterns of the two contrast agents could be detected. The results were validated by histological examinations. The multifactorial imaging approach presented in this study could facilitate cancer detection in diagnostic imaging and intraoperative and endoscopic applications. Especially the dQ-MG-754 probe bears great potential for translation to clinical endoscopy imaging, because it combines specific high affinity binding with renal elimination and a favorable biodistribution.

1. Introduction

Optical imaging is rapidly advancing toward clinical application for early cancer detection and intraoperative imaging [1]. Beyond the recognition of only visible morphological changes, it enables the non-invasive detection of molecular changes in malignant cells. In this context, fluorescent probes have been developed for a wide range of tumor specific targets, mostly addressing only one specific marker in each approach. Considering that pathobiological mechanisms are very complex, the visualization of several tumor markers at a time could fundamentally improve tumor detection and characterization. The implementation of such multifactorial approaches, for example in fluorescence-guided endoscopy, could foster early identification of malignant cells [2] and prognosis by assessment of their developmental stage.

Particularly in relation to the identification of colon cancers, the second leading cause of death in men and women in developed countries [3,4], small or flat lesions are often missed in conventional endoscopic screening [5]. In these cancers the cholecystokinin-2-receptor (CCK2R), is one of the receptors which is overexpressed early in the transition from adenoma-carcinoma, allowing for early discovery of malignant lesions [6]. The receptor

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has been shown to be involved in tumorigenesis, progression and invasiveness of various tumor types [7–10] by CCK2R dependent activation of several tumorigenic pathways [11–14]. The expression of the receptor in normal tissue is primarily located in the central nervous system and in the stomach, whereas it has been reported that in the normal colon the CCK2R is not expressed [6].

In 1999, a short 7 amino acid sequence called "minigastrin" has been selected from a large screening for CCK2R binding peptides [15]. Since then several chelate based minigastrin analogs with high target affinity, fast kinetics and renal elimination have been developed to improve tumor targeting properties [16,17]. Replacement of the radioactive moiety with a near infrared fluorophore makes these substances available for fluorescence endoscopy or intraoperative imaging [18]. However, improved fluorescent minigastrins with suitable pharmacokinetics and superior target affinity remain to be identified [19].

In terms of multifactorial imaging, an important functional change in cancer development is acceleration of glucose metabolism [20]. The observations of prevalent GLUT-1 overexpression in tumors and correlations to poor prognosis and chemotherapy resistance are already being widely exploited in ^{18}F-FDG nuclear imaging of tumors [21]. Translation of GLUT-1 imaging to optical imaging led to the development of a commercially available 2-deoxy-D-glucose (2-DG), where the radioligand was replaced by the near infrared dye IRDye 800CW [22]. So far, reports on the reliability of the probe have been somewhat contradictory. Whereas some research reports proposed a specific binding mechanism [22,23], another research proposed an EPR effect dependent accumulation [24].

Here, we explored the feasibility of multifactorial NIR optical imaging with a CCK2R targeted peptide and a fluorescent 2-DG probe. We aimed at synthesizing a CCK2R targeted probe by linking a NIRF dye via a hydrophilic linker to a high-affinity peptide to obtain pharmacokinetic properties fitted to prospective clinical endoscopic imaging. Our hypothesis was that probe properties could be selectively modulated to increase affinity, specificity, and renal elimination. To show the feasibility of the multispectral approach, we explored and characterized differences in uptake patterns of the two contrast agents in subcutaneous xenografts *in vivo*. We propose that the approach described here could foster the development of multifactorial approaches in optical imaging.

2. Material and methods

2.1. Cell lines

A431/CCK2R and A431/WT, human epidermoid carcinoma cells were a gift from Dr. Luigi Aloj (Department of Nuclear Medicine, Instituto Nazionale Tumouri, Fondazione "G. Pascale", Naples, Italy). The cells were stably transfected with either the CCK2-Receptor (A431/CCK2R) or with empty vector (A431/WT) [25]. Authenticity of the cells was confirmed by DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen) in 2012. HT-29 and LS174T colorectal adenocarcinoma cell lines were obtained from DSMZ and CLS. All cell lines were grown in a monolayer culture at 37 °C in a 5% CO_2 humidified atmosphere. They were maintained in D-MEM medium with 10% (v/v) FBS (both Gibco® (Invitrogen)) and passaged regularly at 70–80% confluency.

2.2. Animals

Female athymic nude mice (Hsd:Athymic Nude-Foxn1^{nu} nu/nu; Harlan Laboratories) were housed under standard conditions with water and food ad libitum. Mice were maintained under a low-pheophorbide diet (C 1039; Altromin) to reduce tissue autofluorescence. All procedures were approved by the regional animal committee and were in accordance with international guidelines on the ethical use of animals. Throughout all procedures, animals were anesthetized with 2% isoflurane. To implement subcutaneous xenografts, 5×10^5 cells (A431/CCK2R and A431/WT) or 2×10^6 cell (HT-29 and LS174T) were dispensed in Matrigel™ (BD Biosciences) and injected into right (A431/CCK2R cells) and left (A431/WT cells) flank or the middle of the back (HT-29 and LS174T) of 8–12 weeks old animals 10–15 days before *in vivo* imaging.

2.3. Target expression in A431/CCK2R und A431/WT cells

RNA isolation from 5×10^6 cells with TRIzol® (Invitrogen) was performed according to standard protocol [26]. After cDNA synthesis with the QuantiTect RT Kit (Qiagen), SYBR® green qRT-PCR was performed on a RotorGene Q (Qiagen). Reactions consisted of RotorGene™ SYBR® Green Mastermix (Qiagen), forward/reverse Primer (see Fig. S1) DEPC water and cDNA. Samples were denaturized at 95 °C and amplification was carried out over 40 cycles of 5 s 95 °C and 10 s 60 °C. CCK2R gene expression was relatively quantified using the ΔΔCt method.

2.4. Synthesis of CCK2R targeted probe "dQ-MG-754" with improved pharmacokinetics

We designed a NIRF peptide probe for CCK2R imaging. The hemicyanine fluorescent dye DY-754 (Dyomics) was used as signaling moiety of the probe construct. This dye was selected out of six different hemicyanine NIR dyes (DY-676, DY-677, DY-678, DY-751, DY-752 and DY-754) due to its low nonspecific binding to the gastrointestinal tract and renal elimination pattern (see Supplementary Figs. 1 and 2). Then, a *N*-Hydroxysuccinimide-ester of DY-754 was N-terminally coupled to the minigastrin peptide analog with the sequence H_2N-$(DGln)_6$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-amid [27]. The probe construct was named "dQ-MG-754". Peptide synthesis, dye coupling and HPLC purification were carried out by Peptide Specialty Laboratories (PSL).

2.5. In vitro dQ-MG-754 probe uptake into A431/CCK2R and A431/WT cells

For determination of probe uptake, cells were seeded into chamber slides and incubated with 5 nM dQ-MG-754 in culture medium at 4 °C and 37 °C for 30 min. Cell membranes were stained with Alexa-555 conjugated wheat germ agglutinin (WGA-555; Invitrogen) and subsequently fixated with 4% paraformaldehyde (Roth). Cells were mounted with PermaFluor® mounting medium (ThermoFischer) containing Hoechst 33258 DNA stain (Applichem). Using a laser scanning microscope (LSM 510, Zeiss), cell nuclei were visualized with a 405 nm laser diode and a 420–480 nm bandpass filter. For cell membranes, a 543 nm HeNe laser and a 550–615 nm bandpass filter were applied, fluorescence of dQ-MG-754 was excited with a 633 Argon Laser and emission was detected with a 650 nm longpass filter. Binding specificity was controlled by incubation of cells with either the probe together with 10-fold excess unlabeled peptide or an equimolar concentration of DY-754.

Furthermore interactions of dQ-MG-754 probe with tumor (A431/CCK2R and A431/WT), endothelial (HMEC-1) and kidney cells (HEK293) were investigated by the means of real time impedance measurement (xCELLigence, Roche). Therefore, cells were incubated with 50 μM probe over a period of 96 h. Change of impedance by proliferation activities of cells was measured as "Cell Index" [28] and normalized to untreated cells.

2.6. Fluorescence based determination of binding affinity

To obtain a saturation binding curve, 2×10^6 A431/CCK2R cells were incubated with serial dilutions from 0.1 to 50 nM of dQ-MG-754 for 5 min at room temperature to measure total probe binding. To determine nonspecific binding, the same probe concentrations were used in presence of 100-fold excess unlabeled peptide (D-Leu-minigastrin, Bachem). After centrifugation cell pellets were immediately imaged with the small animal imaging system Maestro™ (CRi). A region of interest (ROI) was assigned to each cell pellet in order to measure fluorescence intensity semi-quantitatively. Fluorescence intensities were described as average signal (scaled counts/s). This value represents count levels after scaling for exposure time, camera gain, binning and bit depth, hence measurements are comparable among each other. Binding curves and dissociation constants were determined using Graphpad Prism 5. Specific binding was assessed by subtracting nonspecific binding signal from total binding signal.

Determination of the Ki value was carried out by preincubating cells with increasing concentrations of unlabeled peptide from 0.1 to 2000 nM directly before adding a fixed probe concentration of 2 nM to all samples. Thereafter, the same procedure as described above was followed.

2.7. In vivo imaging of CCK2R expression and elevated rate of glycolysis

When subcutaneous xenografts reached a diameter of 5–7 mm, *in vivo* imaging was initiated by tail vain injection of the respective contrast agent. For solitary imaging of dQ-MG-754, we injected 108 nmol/kg. To control signal specificity, animals received probe together with a 10-fold excess of unlabeled D-Leu-minigastrin or the native fluorophore (108 nmol/kg of DY-754). For multifactorial imaging of CCK2R expression and elevated rate of glycolysis we injected 108 nmol/kg dQ-MG-754 and 608 nmol/kg IRDye 800CW 2-DG (short: IR800 2-DG; LI-COR) simultaneously.

At defined time points after injection, animals were imaged in the Maestro® using filters for excitation (670–710 nm) and emission (longpass > 750 nm). Semiquantitative analysis of fluorescence intensities was carried out as described above. Each contrast agent was analyzed separately for its distinct fluorescence spectrum after subtraction of autofluorescence. A tumor to background ratio (TBR)