Necrosis Binding of Ac-Lys⁰(IRDye800CW)-Tyr³-octreotate: Pros and Cons of Using Cyanine-labeled Small Molecules

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Research Article

Keywords: 800CW-TATE, molecular fluorescence guided surgery, somatostatin receptor subtype 2, fluorescent molecular probes, necrosis-avidity

Posted Date: March 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-297703/v1>

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Abstract

Background

There is a growing body of nuclear contrast agents that are repurposed for fluorescence guided surgery. New contrast agents are obtained by substituting the radioactive tag with, or adding a fluorescent cyanine to the molecular structure. This enables intra-operative fluorescent detection of cancerous tissue, leading to more complete tumor resection. However, these fluorescent cyanines can have a remarkable influence on pharmacokinetics and tumor uptake. Here we demonstrate the effect of cyanine-mediated dead cell binding of Ac-Lys⁰(IRDye800CW)-Tyr³-octreotate (800CW-TATE) and how this can be used as an advantage for the purpose of fluorescence guided surgery. We exposed cultured U2OS cells (alive or dead, with or without SSTR₂-expression) and frozen human tumor tissue sections of NCI-H69 (SSTR₂ positive) and CH-157MN (SSTR₂ negative) to 800CW-TATE. We then injected 800CW-TATE into NCI-H69-tumor-bearing mice. Blocking experiment were included with DOTA⁰-Tyr³-octreotate (DOTA-TATE). Paraffin sections of the resected tumors were imaged for near infrared fluorescence and cell death staining was performed.

Results

Binding of 800CW-TATE could be blocked with DOTA-TATE and was absent in SSTR₂ negative cells. However, strong binding was observed to dead cells, which could not be blocked with DOTA-TATE and was also present in dead SSTR₂ negative cells. No SSTR₂-mediated binding was observed in frozen sections, possibly due to disruption of the cells in the process of sectioning the tissue before exposure to the contrast agent. DOTA-TATE blocking resulted in incomplete reduction of $61.5 \pm 5.8\%$ fluorescence uptake by the tumors in the mice. Paraffin sections revealed that fluorescence uptake persisted in necrotic regions upon blocking.

Conclusion

This study shows that labeling peptides with cyanines can result in dead cell binding. This does not hamper the ultimate purpose of fluorescence guided surgery, as necrotic tissue appears in most solid tumors. Hence, the necrosis binding can increase the overall tumor uptake. Moreover, necrotic tissue should be removed as much as possible: it cannot be salvaged, causes inflammation, and is tumorigenic. However, when performing binding experiments to cells with disrupted membrane integrity, which is routinely done with nuclear probes, this dead cell binding can resemble non-specific binding. This study will benefit development fluorescent contrast agents.

Background

There is a growing interest in development of optical molecular contrast agents for the purpose of fluorescence-guided surgery [1, 2]. In most cases, successful targeting vectors from nuclear imaging agents are repurposed, either by replacing the radiolabeling part by a fluorescent tag for single modal imaging, or by adding both a radioactive and a fluorescent chemical moiety for bi-modal imaging. These approaches have already yielded ample probes, with some explored in clinical trials [3, 4]. Most of these tracers are based on antibodies, which are relatively big molecules and therefore can carry the molecular burden of both tags without affecting the epitope affinity [1, 5]. Currently, a growing body of peptides and small molecules are advanced as targeting vectors for optical imaging. However, fluorescent tags can have a drastic influence on the pharmacokinetic properties of smaller vectors due to their relatively big size, electrostatic properties, solubility, and binding to serum proteins. However, these effects are difficult to generalize [6–8].

Here, we describe the dead cell binding properties of Ac-Lys⁰(IRDye800CW)-Tyr³-octreotate (800CW-TATE, Fig. 1), a contrast agent for molecular fluorescence guided surgery of meningiomas [9]. IRDye800CW is a widely applied fluorescent cyanine dye, which has fluorescent properties in the near infrared region (NIR). Tyr³-octreotate [10] serves as a targeting vector, able to bind to the somatostatin receptor subtype 2 (SSTR₂), which is commonly upregulated in neuroendocrine tumors (NET) [11], such as meningioma's [12]. Tyr³-octreotate conjugated to DOTA and labelled with gallium-68 or lutetium-177 is routinely used for diagnosis and radionuclide therapy of NET patients, respectively [13–15]. In our previous work we found that cyanine dyes such as IRDye800CW specifically bind to cells that lost membrane integrity and thus, to necrotic regions in tumors [16–19]. The presence of necrotic regions is common in fast growing tumors and necrosis binding of the dye can lead to unexpected results when studying optical probes.

The aim of this study is to investigate the effect of the IRDye800CW-mediated dead cell binding on physiological properties of 800CW-TATE. These dead cell binding properties should be kept in mind when developing novel cyanine-labeled contrast agents because these can interfere with results from routine experiments.

Materials And Methods

In vitro dead cell binding

Human osteosarcoma cells (U2OS), either transfected with SSTR₂ [20] or wild-type (without SSTR₂ expression) were cultured in DMEM (Gibco Life Technologies, Waltham, MA, USA) with 10% fetal calf serum and 1% penicillin. The cells were seeded in 24 well plates (10⁵ cells per well) and grown until confluent. The cells were kept alive or killed with 50 μ L EtOH (70%) and incubated with 800CW-TATE (10 nM, piCHEM, Grambach, Austria) in culturing medium for 30 min. A blocking experiment was included by co-incubation of 800CW-TATE (10 nM) with DOTA-TATE (10 μ M, Bachem, Bubendorf, Switzerland). Control experiments were included by incubation of the cells with a NIR-fluorescent dye that does not bear a cyanine motive: Rhodamine 800 (10 nM, Sigma-Aldrich). Additionally, wells without seeded cells were

exposed to PBS or EtOH and then incubated with 800CW-TATE, this to investigate non-specific binding to plastic. After gentle washing with PBS, NIR-fluorescence imaging of the whole plate was performed on an Odyssey flatbed scanner (Li-Cor, Lincoln, USA). 800CW-TATE binding was based on fluorescent signal $\pm$ SD and each experiment was performed in $n = 12$.

In vitro dead cell binding [^{111}In]In-DOTA-TATE

U2OS cells, either transfected with SSTR₂ [20] or wild-type were seeded in 24 well plates (10^5 cells per well) and grown until confluent. The cells were kept alive or killed with 50 μL EtOH (70%) and incubated with [^{111}In]In-DOTA-TATE (10 nM, 50 MBq/nmol) in culturing medium for 30 min. A blocking experiment was included by co-incubation with DOTA-TATE (10 μM). After gentle washing with PBS, autoradiography of the whole plate was performed using a super resolution phosphor screen and a Cyclone® Plus system (Perkin Elmer, Waltham, MA, USA). Then, the cells were detached with NaOH (1 M, 1 mL) and collected in tubes for γ -counting (1480 γ -counter Perkin Elmer, Waltham, MA, USA). Each experiment was performed in $n = 6$.

Microscopy

U2OS cells, either transfected with SSTR₂ [20] or wild-type were seeded on 10 mm coverslips in 24 well plates (10^5 cells per well). The cells were kept alive or killed with 50 μL EtOH (70%) and incubated in culturing medium with or without 800CW-TATE (100 nM) for 30 min. After PBS washing, the cells were incubated at 37°C with Calcein AM (1 μM) and Propidium iodide (1 μM) in PBS for 15 minutes to stain live and dead cells, respectively. The cells were washed with PBS, fixated with PFA (4% in PBS, 20 min) and nuclei were stained with DAPI (Sigma-Aldrich, 1:1000 in PBS, 5 min). After final washing with PBS, the coverslips were removed from the well plate and mounted on a microscope slide for fluorescence microscope imaging using a magnification of 20x.

Ex vivo experiments

Frozen NCI-H69 and CH-157MN xenografts were sectioned in 10 μm thick slices, which were mounted on Starfrost glass slides (Thermo Fisher). To prevent non-specific binding, fresh sections were incubated for 10 min at room temperature (RT) with washing buffer (167 mM Tris-HCl, 5 mM MgCl_2) with bovine serum albumin (BSA, 0.25 gram/L). Slides were drained and incubated for 1 hour at RT with a fixed volume of 100 μL washing buffer, containing 800CW-TATE (100 nM or 1 μM) and BSA (1 gram/L). To determine specificity, additional sections were co-incubated with increasing concentrations of DOTA-TATE block (0x, 1x, 5x, 10x, 50x, 100x, 250x, 500x, or 1000x the concentration of 800CW-TATE). The sections were drained, washed and after drying, placed in an Odyssey CLx (Li-cor Biosciences) for NIR-fluorescence detection. Subsequently, H&E staining was performed on the sections, based on which regions of interests (ROIs) were drawn on the viable sections of the tumors for quantification of the NIR-fluorescence signal using ImageJ.

In vivo experiments

Tumor tissue from mice described in another publication were used [9]. Briefly, BALB/c-nu mice were inoculated with 5×10^6 human small cell lung cancer cells (NCI-H69) in 1:1 medium:matrigel [9, 10, 21]. When the tumors reached a size of 400 mm³, 800CW-TATE (3 µg, 1.36 nmol, 50 µL saline) was injected retro-orbitally. A blocking study was included, for which a mouse received two injections of DOTA-TATE (3 mg, 2.2 µmol, 2x50 µL saline) followed by one injection with 800CW-TATE (3 µg, 1.36 nmol, 50 µL saline), with 5 min intervals. Four-hour post injection, the mice were sacrificed, after which tumors were collected and embedded in paraffin for histological analysis. Adjacent paraffin sections of 4 µm were prepared of the tumors. The sections were imaged for NIR-fluorescence on an Odyssey. Immunofluorescence (IF) staining was performed for SSTR₂ (SSTR₂ primary antibody, Abcam; 1:100; secondary antibody goat anti-rabbit Alexa 596, Abcam, 1:1000). Histochemical TUNEL dead cell staining was performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany). Stitched fluorescence microscope images of the stained sections were acquired on a fluorescence microscope (TriPath-Imaging, Burlington, USA) equipped with an Aziocam MRm (Zeiss, Oberkochen, Germany) and an Axiocam 208 color (Zeiss) using a magnification of 40x.

The Institutional Review Board of the University of Groningen approved the study and animal care complied with the Guide for the Care and Use of Laboratory Animals.

Results

In vitro binding of 800CW-TATE on plated cells was demonstrated (Fig. 2). The four wells on the left contain U2OS cells with stable expression of SSTR₂ whereas the four wells on the right contain the wild-type U2OS cells which do not express SSTR₂. The cells were either killed or kept alive before incubation with either 800CW-TATE or 800CW-TATE in presence of an excess of DOTA-TATE. It is apparent that there was SSTR₂-mediated binding of 800CW-TATE, as it was effectively blocked by DOTA-TATE. Striking was the strength of the signal observed from the dead cells. It was not mediated by SSTR₂ since the signal was not blocked by the excess DOTA-TATE. Similarly, fluorescence was observed from the dead cells without SSTR₂-expression. The dead cell binding was significant as it was double as high as SSTR₂-mediated binding (206%, $P < 0.001$). In contrast, [¹¹¹In]In-DOTA-TATE did not showcase dead cell mediated binding (Supplementary data 1.1). No 800CW-TATE binding was observed to ethanol exposed wells without cells (Supplementary data 1.2), therefore excluding binding to the ethanol-exposed plastic of the wells. Moreover, dead and alive cells with and without SSTR₂-expression were exposed to Rhodamine 800, a NIR-fluorescent dye which does not bear a cyanine motive. There was no binding observed of Rhodamine 800, indicating that this dead cell binding is mediated via the cyanine motive of IRDye800CW (Supplementary data 1.3). Further confirmation of 800CW-TATE binding to dead cells was obtained from microscopy images. Cells incubated in culturing medium without 800CW-TATE showcased no fluorescence, therefore indicating that the NIR-fluorescent signal is not caused by autofluorescence from dead cells (Supplementary data 2).

The NIR-fluorescence uptake in the frozen tumor sections exposed to 800CW-TATE was heterogeneous. On the basis of H&E staining viable regions of the tumors were selected to draw ROIs (Supplementary data 3.1). It was observed that fluorescent signal was highest in disrupted regions of the tissue sections. Nonetheless, the results from *ex vivo* experiments on NCI-H69 and CH-157MN cryosections were not conclusive. NCI-H69 tumors have well established expression of SST₂ whereas CH-157MN are SST₂-negative. However, co-incubation with DOTA-TATE did not block the fluorescence signal from 800CW-TATE in the NCI-H69 sections. Moreover, the CH-157MN sections showcased similar uptake intensities, thereby further confirming that the fluorescence binding to the sections is not SST₂-mediated (Fig. 3). This finding is not in line with the results from the *in vitro* experiment in Fig. 2, where SST₂-mediated binding is effectively blocked from live, SST₂-expressing cells with DOTA-TATE. Quantification of the mean fluorescent uptake is shown in Supplementary data 3.

800CW-TATE was studied as a novel contrast agent for molecular fluorescence-guided surgery (MFGS) on NCI-H69 tumor xenograft bearing mice. In this model the tracer was able to detect tumor tissue for MFGS [9]. To verify SSTR₂ specific binding to the tumor, a blocking experiment was performed by pre-administration of DOTA-TATE, thereby outcompeting 800CW-TATE for binding to SSTR₂. It was observed, however, that fluorescence was only partially reduced by DOTA-TATE blocking (reduction of $57.8 \pm 5.2\%$). Moreover, after dissection of the tumors, a heterogeneous uptake of the fluorescent compound was observed in paraffin embedded sections (Fig. 4, red). Adjacent sections were subjected to H&E staining (Fig. 4), fluorescently stained for cell death using TUNEL staining (Fig. 4, green), or for SSTR₂-expression (Supplementary data 4). The top and bottom sections in Fig. 4 represent a tumor from a mouse injected with 800CW-TATE and from the blocking experiment, respectively. A strong overlap of the NIR-fluorescence uptake and the necrotic regions was observed in these tumors. Similar to what we observed with cultured cells in Fig. 2, DOTA-TATE blocked the signal from viable cells, as the NIR-fluorescence signal in the viable regions of the tumors was reduced by DOTA-TATE blocking.

Discussion

In the current paper we have shown that the necrosis avidity of cyanine-dyes such as IRDye800CW, can drastically contribute to the binding behavior of targeting vectors conjugated to cyanines, intended for MFGS. This has some disadvantages and advantages. One disadvantage is that these probes cannot be used in routine protocols frequently used for radionuclide probes to determine specific binding of the probe directly on tissue sections. We showed in this study that these experiments resulted in high amounts of unspecific binding, which can be explained by the fact that in the process of cutting the tissue sections, the cellular membranes in the tissue are compromised. We showed in an earlier publication that cyanine dyes, such as IRDye800CW, bind to endoplasmic proteins that become available when cells loose membrane integrity [16].

When 800CW-TATE is injected systemically in SSTR₂-expressing tumor bearing mice, it will bind to SSTR₂ via the targeting octreotate peptide, but also to necrotic tissue via IRDye800CW. This was also observed

by us in an earlier study using 800CW-2DG and 800CW-EGF, where the targeting molecules bind to their specific target and IRDye800CW to necrosis [16]. 800CW-EGF has a molecular weight of around 7 kD and could still bind to necrosis. IRDye800CW conjugated to a 25 kD polyethyleneglycol (PEG)-chain on the other hand, could not bind to necrosis, most probably due to steric hindering. This suggests that small molecules or peptides conjugated to IRDye800CW with a molecular weight < 7 kD still retain necrosis avidity [16]. This also explains *in vitro* the presence of specific 800CW-TATE signal in live SSTR₂ expressing U2OS cells and a non-specific signal in dead cells (Fig. 2). The latter was confirmed by the fact that the signal could not be blocked by excess DOTA-TATE and was present in both SSTR₂-expressing and non-expressing dead cells. Moreover, in tissue sections of SSTR₂-positive NCI-H69 tumor-bearing mice treated with 800CW-TATE (Fig. 4) the NIR-fluorescence signal (red) co-localized (yellow-orange) in a great part with the TUNEL signal (green) indicating dead cells. When an excess of DOTA-TATE was co-administered, the specific signal disappeared but the necrosis specific signal remained.

These findings could also explain retrospectively our previous unexpected results *in vivo* using

a dual labeled octreotate peptide. This peptide was attached to both DTPA as a chelator for indium-111 labeling and the fluorescent cyanine dye Cy5 [21]. We observed that the *in vitro* affinity of the dual labeled octreotate dropped by more than a logarithmic unit compared with the peptide only labeled with indium-111. Unexpectedly though, there was no significant difference in overall tumor-uptake *in vivo* between the dual labeled peptide and the radioactive peptide. This might be due to necrosis binding of the cyanine moiety, which added up for the uptake in the tumor and therefore compensated for the reduced affinity for the target protein.

Most solid tumors develop necrotic tissue, especially in the center of the tumor appearing as a necrotic core [22, 23]. This is due to improper functioning of immature tumor blood vessels and rapid tumor growth leading to insufficient oxygen supply in the center followed by tissue necrosis [24]. This is furthermore correlated with poor prognosis of the disease [25]. Moreover, we have shown that imaging of necrosis can be used for early determination of therapy efficacy of irradiation therapy [17] or chemotherapy [18]. For this reason, we recently developed and validated [¹¹¹In]In-DOTA-PEG₄-800CW, as a specific probe to image tumor necrosis [19].

What could be the advantage for MFGS of using a small molecule or peptide probe that is conjugated with a cyanine dye like IRDye800CW that also targets necrosis? As mentioned before, most solid tumors will develop a necrotic core or necrotic regions at a certain time point depending on the growth rate [24]. The ultimate goal of MFGS is to identify tumor tissue and distinguish it from normal healthy tissue during surgery. Apart from binding to a specific tumor target, binding to necrosis which is mostly found in the center of the tumor, will not interfere with the specific binding and will actually strongly contribute to the total tumor to background ratio and the intensity of the signal. Moreover, any necrotic tissue should be removed as much as possible as it cannot be salvaged, causes inflammatory responses, and even releases tumorigenic factors [22, 26].

Conclusion

In conclusion, one has to be aware that tumor targeting molecules or peptides conjugated to a cyanine dye like IRDye800CW will also bind to necrosis, that is, intracellular proteins of necrotic cells that become available when cells have lost membrane integrity. This will prevent its use to determine specific binding of the tumor targeted probe directly on tissue sections because intracellular proteins will be ubiquitously exposed. When using these tumor targeted probes *in vivo* to determine the presence of the specific target, e.g. using NIR-fluorescence imaging or afterwards in histological sections, one needs to be aware of possible binding to necrotic tissue. Despite these disadvantages, a great advantage for MFGS could be that binding to its specific target and necrosis will increase the tumor uptake, contributing to efficiency of MFGS.

Abbreviations

800CW-TATE: Ac- Lys⁰(IRDye800CW)-Tyr³-octreotate; BSA: Bovine serum albumin; DOTA-TATE: DOTA⁰-Tyr³-octreotate; IF: immunofluorescence; MFGS: Molecular fluorescence-guided surgery; NET: Neuroendocrine tumors; NIR: Near infrared; PBS: Phosphate buffered saline; PEG: polyethyleneglycol; SSTR₂: Somatostatin receptor subtype 2.

Declarations

Authors' contributions

All authors contributed to the study design and writing of the manuscript. MCMS, BMD, and SED performed data collection and material preparation. Data analysis was performed by MCMS, BMD, KMP, LM, CWGML, and MdJ. The first draft of the manuscript was written by MCMS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

This research is funded with grant 11089–2017/1 from the Koningin Wilhelmina Foundation (KWF).

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

All animal experiments were supported by research grants of the University of Groningen, the University Medical Center Groningen, and the de Cock-Hadders Stichting.

Acknowledgement

Not applicable.

Consent for publication

Not applicable.

Competing interests

WdD was member of the advisory board for GBM diagnostic testing for AbbVie. MdJ has an unrelated investigator-initiated project contract with Advanced Accelerator Applications, a Novartis company. No potential conflicts of interest relevant to this article exist.

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Figures

Figure 1

Structure of Ac-Lys0(IRDye800CW)-Tyr3-octreotate (800CW-TATE).

Figure 2

NIR-fluorescence imaging of alive or dead U2OS cells with and without SSTR2-expression, exposed to 800CW-TATE (10 nM in culturing medium). Blocking is performed by co-incubation with DOTA-TATE (10 μ M). Fluorescent signal quantified $\pm$ SD, n=12. SSTR2⁺ = U2OS cells transfected with SSTR2; SSTR2⁻ = wild-type U2OS cells without SSTR2-expression; 800CW-TATE = Ac-Lys0(IRDye800CW)-Tyr3-octreotate; DOTA-TATE = DOTA0-Tyr3-octreotate.

a 800CW-TATE (100 nM)

b 800CW-TATE (1 μ M)

Figure 3

Ex vivo NIR-fluorescence uptake in SSTR2-positive NCI-H69 and SSTR2-negative CH-157MN cryosections after incubation of 800CW-TATE (a: 100 nM; b: 1 μ M) in the presence increasing amounts of blocking by DOTA-TATE to block SSTR2-mediated binding. In yellow, ROIs of viable tumor regions. Intensity bar indicates fluorescent signal intensity (arbitrary unit).

Figure 4

Consecutive sections of paraffin-embedded NCI-H69 tumor xenografts from mice, tumors resected 4 hour post injection with either 3 μ g 800CW-TATE (top) or 3 mg DOTA-TATE (which served as a blocking from SSTR2) and 3 μ g 800CW-TATE (bottom). From left to right are depicted H&E staining, fluorescent cell death staining (TUNEL), NIR-fluorescent signal from 800CW-TATE uptake and a merge of dead cell staining and NIR-fluorescence.

Supplementary Files

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