Agents Described in the Molecular Imaging and Contrast Agent Database for Imaging Carbonic Anhydrase IX Expression

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Abstract

Carbonic anhydrase IX (CA IX) is selectively expressed in a range of hypoxic tumours and is a validated endogenous hypoxia marker with prognostic significance, hence CA IX is of great interest as a molecular imaging target in oncology. In this review we present an overview of the different imaging agents and imaging modalities that have been applied for the in vivo detection of CA IX. The imaging agents reviewed are all entries in the Molecular Imaging and Contrast Agent Database (MICAD) and comprise antibody, antibody fragments and small molecule imaging agents. The effectiveness of these agents for imaging CA IX in vivo gave variable performance, however a number of agents proved very capable. As molecular imaging has become indispensable in current medical practice we anticipate that the clinical significance of CA IX will see continued development and improvements in imaging agents for targeting this enzyme.
1. Molecular Imaging and Contrast Agent Database (MICAD)
The Molecular Imaging and Contrast Agent Database (MICAD) is a collection of data comprising molecular imaging and contrast agents that have in vivo animal or human data published in peer-reviewed scientific journals\(^1\), \(^2\). MICAD is part of the National Institute of Health (NIH) Common Fund and aims to promote research using molecular imaging probes and to facilitate the translation of preclinical findings to clinical practice\(^1\)-\(^3\). As of March 2013 there were approximately 1 400 agents listed in MICAD. Of this, the majority were for positron emission tomography (PET) (41\%), followed by single photon emission computed tomography (SPECT) (29\%), optical imaging (14\%), magnetic resonance imaging (MRI) (11\%), ultrasound (2\%), multimodal imaging (1\%), X-ray/computer tomography (X-ray/CT) (1\%) and photoacoustic imaging (1\%).

Cancers are traditionally staged and monitored using invasive methods, for example biopsies or exploratory surgery, characterizing treatment response by a change in tumour volume\(^4\). These methods are limited however as biochemical changes happen much faster than morphological ones. Molecular imaging allows a repeated in vivo measurement of several critical molecular features such that molecular imaging has become indispensable in current medical practice\(^4\). Of the MICAD entries, ten agents target carbonic anhydrase IX (CA IX), Table 1. CA IX is overexpressed in several solid cancer types in hypoxic conditions, while expression in normal tissues is minimal. CA IX is thus considered a marker for tumour hypoxia as it provides differentiation between normal and hypoxic tissue, allowing the opportunity for selective imaging of solid tumours. This paper presents the ten CA IX imaging agents in MICAD and outlines their effectiveness for imaging CA IX in vivo.

Insert Table 1 here

2. Carbonic Anhydrase IX as a tumour marker
Carbonic anhydrase IX (CA IX) is a dimeric zinc metalloenzyme that catalyzes the reversible hydration of CO\(_2\) to HCO\(_3^-\) and H\(^+\) to play an important role in pH homeostasis\(^24\). Each CA IX monomer is comprised of an intracellular domain, a single transmembrane helix domain, an extracellular proteoglycan domain and an extracellular catalytic domain\(^25\),\(^26\). CA IX is
overexpressed in several solid cancer types, including cervical, clear cell renal cell, pancreatic, breast, bladder, head and neck, soft tissue and non-small cell lung carcinoma. In solid tumours, hypoxia inducible factor 1 (HIF-1) regulates a signalling cascade involving ~100 genes, including CA IX, to enable adaptive cellular functions to support tumour cell survival, proliferation and metastasis in hypoxia. The extracellular pH (pH_e) of tumour cells is lower than in normal tissue owing to elevated metabolism leading to increased production and export of acidic metabolites such as lactic acid. At a molecular level, CA IX overexpression leads to dramatically increased production of HCO_3^- and H^+ at the extracellular surface of hypoxic tumour cells. The HCO_3^- is actively transported into the cell to buffer pH_i while the H^+ contributes to acidosis of the extracellular space to support invasion and metastasis. The proteoglycan domain makes CA IX unique among CAs, and is proposed to assist CA IX to function efficiently at a more acidic pH.

Solid tumour masses are heterogeneous and show significant variation in oxygen concentrations. These heterogeneously distributed areas of hypoxia are referred to as the “hypoxic fraction” and arise from the insufficient organization of blood vessels within the solid tumours. Hypoxic tumours have a high metastatic potential associated with a more aggressive phenotype, this results in a resistance to radio- and chemotherapy and therefore a poor outcome for the patient. Gray and co-workers reported that tumour cells lacking oxygen were resistant to death via radiation by 2.5 to 3-fold compared to fully oxygenated cells. These results were obtained separately by other groups. Hypoxia has been established as a key feature that can significantly influence tumour behaviour and predict a response to therapy. There are several invasive and non-invasive methods available for the detection of tumour hypoxia, but these are not fully reliable. These techniques either yield a variable diagnosis or have functional limitations due to the incomplete penetration of the tumours or resistance to some tumour types. For example, hypoxia can be determined post-mortem in animal models given pimonidazole by i.v. administration 30 minutes prior to sacrifice. Pimonidazole is a 2-nitroimidazole compound that is reduced in hypoxia, the reduced form can react with and stain intracellular macromolecules in hypoxic regions to provide generic, but selective hypoxia detection. Co-localization analysis between pimonidazole staining and ex vivo CA IX expression detected by monoclonal antibodies (mAbs) to CA IX has yielded conflicting results,
this has been attributed to different levels of oxygen at which pimonidazole is reduced or CA IX overexpressed, respectively; local temporal fluctuations in hypoxia level; or non-hypoxia-related factors that affect CA IX expression\textsuperscript{16}. For in vivo hypoxia measurements, an invasive oxygen electrode can be used but this is not practical for all cancer types and causes significant patient discomfort. CA IX offers several features that make it a promising clinically relevant candidate for diagnostic imaging\textsuperscript{29,31}. Firstly, the active site domain is extracellular and can be targeted by imaging agents that do not have to transverse the cell membrane\textsuperscript{29}, and secondly it is expressed in few normal tissues but is overexpressed to high concentrations in hypoxic conditions with a long half-life\textsuperscript{7,31}. It has been postulated that while some cell lines may subvert known hypoxia mechanisms and that CA IX is not overexpressed in all cancer cell types, a vast array of human solid tumours typically overexpress CA IX\textsuperscript{7,31}.

3. Antibody and antibody fragment imaging agents that target CA IX

3.1 G250 and cG250 monoclonal antibodies and antibody fragments, A3 and CC7 monoclonal antibodies

Monoclonal antibodies (mAb) are a homogenous population of immunoglobulins (eg. IgG) directed against a single epitope\textsuperscript{36}. G250 is a mAb that was isolated more than 20 years ago and the antigen identified much later as an epitope of CA IX\textsuperscript{6,37,38}. cG250 is a chimeric form of G250 that was developed to be less immunogenic than G250 whilst retaining the CA IX antigen specificity ($K_a = 4 \times 10^9$ M\textsuperscript{-1})\textsuperscript{6,7,10}. Antibody fragments have several advantages over parent antibodies in the IgG format for molecular imaging owing to altered physicochemical properties. This includes more rapid clearance from the blood and normal tissues, reduced residence time, a reduction in the radiation absorbed by the bone marrow, improved tumour penetration - particularly into hypoxic tumour regions, and potentially reduced immunogenicity\textsuperscript{39}. The increased clearance of fragments has several caveats as the absolute tumour uptake may be lower while nonspecific uptake by other organs may be higher\textsuperscript{12,39}. In 2009, Neri and coworkers\textsuperscript{18} reported the generation and characterization of two high-affinity human mAbs specific to extracellular CA IX, named A3 and CC7\textsuperscript{18}. These are the first fully human mAbs with high affinity (nM) for CA IX, importantly they do not inhibit CA IX activity and specificity to CA IX over other forms of CA was also confirmed\textsuperscript{18}. Due to limitations in antibody diffusion within solid tumour masses\textsuperscript{40,41,42}, A3 and CC7 antibodies in the small immunoprotein (SIP) format were
prepared with a lower molecular weight (~25 kDa) cf. A3 and CC7 with a molecular weight of 76 kDa when in the IgG format. Researchers have undertaken work using these mAbs and mAb fragments conjugated to a range of clinically used radionuclides for imaging CA IX using PET and SPECT, Table 2.

3.2 Directly radiolabelled CA IX antibodies

With direct radiolabelling the mAb structure remains virtually unchanged and retains high specificity for the target antigen. Direct iodination of mAbs with $^{131}$I, $^{124}$I and $^{125}$I is relatively easy to perform and provides high specific activities$^{46}$. It is typically achieved via the Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril) method, although other methods are available$^{7,10,45,47-51}$. The radioactive iodine is provided via an aqueous solution of NaI$^{48}$. Iodogen, a strong oxidising agent, is used to convert the I$^-$ anion to an electrophilic I$^+$ cation, this electrophile then reacts to insert an iodine ortho to the hydroxyl groups of tyrosine residues of the antibody, Figure 1$^{49}$. The iodogen reagent also limits oxidative damage to the protein during radiolabelling$^{8,48}$.

131$I has a higher intensity gamma ray when compared to $^{124}$I and $^{125}$I, it is also widely available, relatively inexpensive and was the first to be established as a radiolabelling agent for cG250$^{45,46,50}$. $^{131}$I was however readily released from the tumour cells following internalization of $^{131}$I-cG250 and the abundant high energy gamma photons could affect normal tissues such as the bone marrow, limiting the dose of $^{131}$I-cG250 that could be used therapeutically$^{50,52}$. Additionally, the high energy single-photon emission compromises image quality$^{6}$. With these drawbacks, research moved to the direct iodination of cG250 using $^{124}$I (Table 1, entry 1) and $^{125}$I (Table 1, entry 2)$^{6}$. $^{124}$I has a half-life of ~4 days, this allows both long term pharmacokinetics to be determined and for centralized production and wide distribution of $^{124}$I radiolabelled antibody$^{6}$. $^{125}$I is considered a pure gamma emitter with a low radiation energy and medium range half-life of ~60 days, whereas $^{131}$I is both a beta and gamma emitter with medium range energy.
and a short range half-life of ~8 days\textsuperscript{53}. \textsuperscript{125}I gives a higher counting efficiency than \textsuperscript{131}I such that about half the amount of \textsuperscript{125}I is needed for tracer purposes\textsuperscript{53}.

RCC is the most common malignancy in the kidney and each year in the United States ~39 000 people are diagnosed with and ~13 000 people die from RCC\textsuperscript{45}. Approximately 80\% of RCCs are of the more aggressive clear cell type (ccRCC), of these >95\% express the G250/CA IX antigen on the cell surface\textsuperscript{6, 7, 10, 37, 39}. In RCC it has been shown that production of CA IX begins after the loss of the tumour suppressor gene VHL (Von Hippel-Landau protein), whose product is integral to the HIF-1\alpha pathway\textsuperscript{7, 12, 18}. This upregulation of CA IX has been determined to be one of the most prominent accessible markers of RCC\textsuperscript{18}. There is debate within the literature regarding the mechanism leading to CA IX overexpression in RCC, irrespective of mechanism however the high cell surface production of CA IX renders RCC an excellent model for investigating the properties of CA IX imaging agents.

\textsuperscript{124}I-cG250 (Table 1, entry 1)\textsuperscript{5}. Divgi et al carried out the first phase I trial with patients with ccRCC using \textsuperscript{124}I-cG250 pre-operatively to predict ccRCC, the only human clinical trial in this review\textsuperscript{6}. The aim of this research was to determine if PET with \textsuperscript{124}I-cG250 was able to discern ccRCC from other renal masses. For this clinical trial, 25 patients who were surgical candidates for kidney tumour resection, accrued via one surgeon, were administered with 185 MBq/10 mg of \textsuperscript{124}I-cG250 over 20 min. PET and CT images were taken 3 h later with positive PET images being indicative of ccRCC. Both PET and CT modalities were used to unequivocally determine tumour location and structure. Out of the 25 patients imaged pre-surgery, 15 gave positive PET images. Following imaging, surgery determined that these 15 patients all had ccRCC. Of the 10 patients with negative PET images there was only one patient surgically determined to have ccRCC.

Animal model studies with \textsuperscript{124}I-cG250 have been undertaken by Lawrentshuck et al\textsuperscript{7}. The purpose of this investigation was to identify if there was direct data that linked hypoxia and CA IX expression in RCC. They provided two hypotheses based on the loss of the VHL: (i) that CA IX will reflect hypoxia, or (ii) that tumours subvert this pathway with CA IX expression unrelated to hypoxia. SK-RC-52 human RCC xenograft models were established in nude mice. At day 0, 1, 3, 7 and 10 invasive oxygen electrodes were used to investigate intratumoural
hypoxia directly before injection with 5 MBq of $^{124}$I-cG250 (25 μg) in 0.2 mL PBS followed by PET imaging. The PET results showed localization of $^{124}$I-cG250 in the tumour as well as the cardiac blood pool. After imaging, the animals were sacrificed and their tumours and organs removed, weighed and biodistribution determined. To investigate CA IX expression, one mouse was taken at 1, 2, 3 and 5 day time points and autoradiography, histology and immunohistochemistry performed. They found no significant correlation between any oxygen parameter and the CA IX expression and concluded that in this model, the level of CA IX expression could not be clearly related to hypoxia with only a weak correlation between hypoxia and the cG250 uptake.

$^{125}$I-mAb (Table 1, entry 2)$^9$. Zhang et al$^8$ have described a $^{125}$I-labelled monoclonal antibody ($^{125}$I-mAb) that recognized CA IX without cross-reactivity to other human CAs. A HT-29 xenograft model was established in athymic male mice and an in vivo binding study, biodistribution study and SPECT planar imaging were carried out with $^{125}$I-mAb. To determine in vivo binding, the mice were injected with 3.70 MBq of $^{125}$I-mAb and sacrificed 24 h later. Their blood was collected, the unbound $^{125}$I-mAb removed and the activity quantified using a gamma counter. Radioactivity in the blood was high: the radioactivity was 5.5% that of the blood activity at 24 h p.i. which the authors attributed to a small amount of the tracer diffusing into blood cells. For the biodistribution study, 1.295 MBq of $^{125}$I-mAb was injected intravenously into mice and the radioactivity of both normal tissues and tumour measured using a gamma counter at a range of time points$^8$. Uptake of $^{125}$I-mAb by the tumour was significantly higher than for normal tissues, $4.9 \pm 1.2\%$ID/g (% of the injected dose per gram of tissue) at 24 h p.i.$^8$. For SPECT imaging, mice were injected with 5.55 MBq of $^{125}$I-mAb and after 48 h planar static images (duration 30 s) were acquired using a gamma camera. In parallel a control study was also undertaken using a 100-fold molar excess of unlabelled mAb injected into the mouse 24 h prior to injection with $^{125}$I-mAb. The SPECT results showed that the $^{125}$I-mAb preferentially concentrated in the HT-29 tumour at 48 h p.i. however it also accumulated in the thyroid, while in the control study unlabelled mAb blocked accumulation of $^{125}$I-mAb in both the tumour and the thyroid. Although the signal to noise ratio was promising, the high levels of radioactivity in the blood make $^{125}$I-mAb currently unsuitable for as a diagnostic tool$^8$. 
3.3 Indirectly radiolabelled CA IX antibodies and antibody fragments

Direct radiolabelling of antibodies with most other radionuclides is not possible and instead an indirect approach with a host-linker moiety is needed. In this indirect labelling method the host coordinates to the radionuclide while the linker allows covalent attachment to the antibody or antibody fragment with minimal disruption to structure. A summary of host-linkers and their method of attachment to the CA IX antibody component are shown in Figure 2. Brouwers et al\textsuperscript{10} (Table 1, entry 7) and Hoeben et al\textsuperscript{16} (Table 1, entry 3) used a desferrioxamine (Df) backbone as host-linker for \(^{89}\text{Zr}\) incorporation. Carlin et al\textsuperscript{12} employed an amide linkage to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) for \(^{111}\text{In}\) incorporation (Table 1, entries 4, 5 and 6). Finally, Neri and coworkers\textsuperscript{18} conjugate diethylenetriaminepentaacetic acid (DTPA) via a thiourea linker for radiolabelling with \(^{177}\text{Lu}\) (Table 1, entry 8).

Insert Figure 2 here

\(^{89}\text{Zr-Df-cG250}\) (Table 1, entry 3)\textsuperscript{11}. To synthesize \(^{89}\text{Zr-Df-cG250}\) Brouwers et al\textsuperscript{10} conjugated the desferrioxamine (as the mesylate salt) host to cG250 via amide bond formation followed by radiolabelling with 165 MBq of \(^{89}\text{Zr}\), Figure 3. \(^{89}\text{Zr}\) was selected as radionuclide because its half-life (3.26 days) is similar to the clearance time of the cG250\textsuperscript{10}. PET imaging with \(^{89}\text{Zr-Df-cG250}\) was used in combination with radioimmunoscintigraphy (RIS) in a SK-RC-52 xenograft rat model\textsuperscript{10}. Results were compared both to \(^{18}\text{F-FDG}\) (\(^{18}\text{F}-\)fluorodeoxyglucose), the gold standard imaging agent for oncology with PET, and to \(^{111}\text{In-DTPA-cG250}\) for RIS\textsuperscript{10}. Eight rats were injected with 20 MBq of \(^{89}\text{Zr-Df-cG250}\) and PET imaging undertaken at 5 min, 24, 48 and 72 h post-injection. After imaging the animals were sacrificed and their organs removed, weighed and the activity of the samples calculated. The PET images showed that \(^{18}\text{F-FDG}\) did not accumulate in the tumours, while at 24 h p.i. \(^{89}\text{Zr-Df-cG250}\) successfully labelled even small (~100 mg) tumours. The \(^{89}\text{Zr-Df-cG250}\) PET images improved over time with optimal images taken at 72 h p.i..

The comparison of the RIS images acquired with \(^{89}\text{Zr-Df-cG250}\) to those acquired with \(^{111}\text{In-DTPA-cG250}\), allowed researchers to conclude that the tumour uptake of \(^{89}\text{Zr-Df-cG250}\) was identical to \(^{111}\text{In-DTPA-cG250}\) (5.0 ± 2.4 and 4.9 ± 2.9 %ID/g, respectively) as was the blood
levels at the 72 h timepoint (1.4 ± 0.4 and 1.7 ± 0.7 %ID/g, respectively). The uptake of both antibodies was similar in other organs assessed, however the authors state that in some cases, the PET images produced using $^{89}\text{Zr}$-DF-cG250 were highly superior to the $^{111}\text{In}$-DF-cG250 images and therefore this agent may be of value in the planning and monitoring of RCC patients\textsuperscript{10}. $^{111}\text{In}$-DTPA-cG250 is not listed as a CA IX targeting agent in MICAD however the synthesis and imaging properties in patients with RCC metastases has been reported and showed improved visualization and a higher activity compared with $^{131}\text{I}$-cG250\textsuperscript{46}. Unlike $^{131}\text{I}$, $^{111}\text{In}$ is a residualizing radionuclide, that is, one that is retained inside a cell as the radioactive catabolites are trapped in the lysosomes\textsuperscript{46,54}. Based on increasing evidence that cG250 can be internalized by CA IX expressing RCC the use of residualizing radionuclides was suggested as beneficial. Previous studies had shown that labelling cG250 with the residualizing nuclides $^{88}\text{Y}$ and $^{177}\text{Lu}$ had approximately 3-4 times the activity of $^{125}\text{I}$-cG250 at 7 days p.i.\textsuperscript{46}. Due to the similarity between the RIS results for $^{111}\text{In}$-DTPA-cG250 and $^{89}\text{Zr}$-Df-cG250 in tumour uptake, blood level and also a similarity amongst other tissues, the authors suggest that $^{89}\text{Zr}$ could be re-classed as a residualizing radionuclide\textsuperscript{54}.

Insert Figure 3 here

$^{111}\text{In}$-DOTA-cG250, $^{111}\text{In}$-DOTA-cG250-Fab and $^{111}\text{In}$-DOTA-cG250-F(ab')\textsubscript{2} (Table 1, entries 4, 5 and 6)\textsuperscript{13-15}. To evaluate the potential of cG250 fragments as imaging agents Carlin and co-workers compared the binding affinity for the CA IX antigen of two low molecular weight fragments of cG250, cG250-Fab and cG250-F(ab')\textsubscript{2}, with the parent cG250\textsuperscript{12}. The fragments were prepared via enzymatic degradation of the intact cG250 using the hydrolytic enzymes pepsin and papain to give cG250-F(ab')\textsubscript{2} and cG250-Fab, respectively\textsuperscript{12,39}. The F(ab')\textsubscript{2} fragment is bivalent (two antigen binding sites), whereas the Fab fragment is monovalent (one antigen binding site)\textsuperscript{39}. cG250, cG250-Fab and cG250-F(ab')\textsubscript{2} were each conjugated to DOTA and then complexed to the residualizing radionuclide $^{111}\text{In}$, Figure 4\textsuperscript{56}. The conjugation occurred in two steps. Firstly, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) are used to synthesize the active monoester of DOTA. Secondly, the DOTA active ester is linked to the mAb or fragment via amide bond formation through a side chain lysine residue on the mAb\textsuperscript{56}. The stoichiometry of incorporated DOTA moieties, binding affinity ($K_d$) for CA IX
expressed by SK-RC-38 cells and the saturation binding data ($B_{max}$) of the mAb and fragments varied, Table 3.

**Insert Figure 4 here**

**Insert Table 3 here**

Athymic male mice with HT-29 tumours were injected with 1.6 MBq of each $^{111}$In-DOTA-cG250 $^{111}$In-DOTA-cG250-Fab and $^{111}$In-DOTA-cG250-F(ab’)$_2$ for in vivo biodistribution studies. $^{111}$In-DOTA-cG250 (tested at 2, 4 and 7 days p.i.) showed a high uptake of radioactivity into the HT-29 tumour. At 2 days p.i. uptake (20.1 ± 4.8% ID/g) was similar to that obtained with two nonspecific $^{111}$In labelled control antibodies, the specificity of the $^{111}$In-DOTA-cG250 was however confirmed over the next 5 days as the tumour uptake increased, while uptake of the control antibodies decreased. Compared to $^{111}$In-DOTA-cG250, the radiolabelled fragments were tested at 6 h and 24 h p.i due to faster clearance and showed a lower absolute tumour uptake. For F(ab’)$_2$ uptake was 7.6 ± 1.4 and 9.3 ± 2.1% ID/g at 6 h and 24 h p.i., respectively, while for Fab uptake was lower again (3.6 ± 1.3 and 3.5 ± 1.7% ID/g at 6 h and 24 h p.i., respectively) due to a much faster clearance from circulation. Biodistribution was similar for all three $^{111}$In-DOTA-antibodies across other organs. The tumour/blood (T/B) and tumour/muscle (T/M) values were compared for all three agents, Table 4. Both the F(ab’)$_2$ and the Fab fragments showed an improvement in the T/B and T/M ratios between 6 h and 24 h p.i. with the T/B ratio for the Fab fragment increasing dramatically from 2.8 to 16.6, this ratio is higher than for the $^{111}$In-DOTA-cG250 at 7 days p.i., Table 4.

**Insert Table 4 here**

To correlate $^{111}$In-DOTA-cG250 to regions of perfusion, hypoxia and CA IX expression, PET images were compared to fluorescent images of the same tumour section stained with Hoechst 33342, pimonidazole and a CA IX fluorescent antibody at 2 days p.i. of $^{111}$In-DOTA-cG250. The Hoescht stain revealed high perfusion around the rim of the tumour and disorganised perfusion at
the centre, the anti-correlation of the PET image. The regions of CA IX staining colocalized with pimidazole, showing that the CA IX expression was limited to the hypoxic regions but more importantly for this study, it was shown that although there was some targeting of CA IX with $^{111}$In-DOTA-cG250 close to the blood vessels, the uptake of $^{111}$In was not directly proportional to the endogenous CA IX. At 4 days and 7 days p.i., the correlation of $^{111}$In-DOTA-cG250 to CA IX improved.

Similarly, the $^{111}$In-DOTA-fragment PET images were compared to the Hoescht, pimidazole and endogenous CA IX fluorescent images at 6 and 24 h p.i. At 6 h p.i. the correlation of $^{111}$In-F(ab$'$)$_2$-cG250 to CA IX is comparable to that of the intact cG250 after 2 days p.i. At 24 h, the $^{111}$In matches almost perfectly to the endogenous CA IX image, demonstrating excellent targeting. Additionally, except for the later data points, there is almost an inverse correlation with Hoechst staining$^{12}$. Due to the antibody fragments clearing faster into the blood, likewise, diffusion into the tumour is faster, allowing more rapid correlation with CA IX expression. The immunohistochemistry results for the F(ab$'$)$_2$ and Fab fragments showed a diffuse uptake in the core of the tumour, where the CA IX production is high. The Fab fragment had the fastest blood clearance and from digital analysis of the images, the authors concluded that the Fab fragment was the most rapid and reliable indicator for CA IX expression in hypoxic tumour regions. However, despite this conclusion, due to the lower absolute tumour uptake for both the fragments, and additional problems posed by the nonspecific tracer uptake and poor perfusion, the authors recommend using the intact $^{111}$In-DOTA-G250 at 7 days p.i. as this gave the most sensitive and accurate detection of CA IX$^{12}$. The late images of the $^{111}$In-DOTA-cG250 were comparable to the early images of the fragments but the higher tumour/non-tumour contrast of $^{111}$In-DOTA-cG250 would reduce imaging times.

$^{89}$Zr-Df-cG250-F(ab$'$)$_2$ (Table 1, entry 7)$^{17}$. Hoeben et al$^{16}$ investigated Df-cG250-F(ab$'$)$_2$ fragments using $^{89}$Zr as the radionuclide. They selected $^{89}$Zr as its half-life (3.26 days) matched the pharmacokinetics of the fragment. The labelling chemistry for Df-cG250-F(ab$'$)$_2$ with $^{89}$Zr to generate $^{89}$Zr-Df-cG250-F(ab$'$)$_2$ is similar to that presented in Figure 3$^{16}$. A cohort of BALB nu/nu mice subcutaneously implanted with SCCNij3 cell xenograft tumours, a head and neck tumour model, were used to qualitatively determine the intratumoural distribution of radiolabeled
cG250 via autoradiography and compared to $^{111}$In-DTPA-cG250. The preparation of $^{111}$In-DTPA-cG250 is described by Troost et al. It was found that the autoradiography uptake signal of $^{89}$Zr-Df-cG250-F(ab')$_2$ was very similar to that of $^{111}$In-DTPA-cG250 with all correlations over two timepoints $P<0.0001$. The uptake of $^{111}$In-DTPA-cG250 was much higher than $^{89}$Zr-Df-cG250-F(ab')$_2$ ($25.2 \pm 6.2 \% \text{ID/g versus } 2.68 \pm 1.30 \% \text{ID/g}$) however this was expected due to the longer circulatory half-life of IgG. That being said the $^{89}$Zr-Df-cG250-F(ab')$_2$ showed a faster accumulation in CA IX rich regions at 4 h p.i.

Ten mice were injected with $6.85 \pm 0.55 \text{ MBq of } ^{89}\text{Zr-Df-cG250-F(ab')$_2$}$, eight mice (two groups of $n = 4$) were examined via a small animal PET imaging scanner at 4 and 24 h p.i. At 30 min prior to PET imaging, mice were injected with 80 mg/kg of pimonidazole (hypoxia marker). One minute before sacrifice, mice were injected with Hoechst 33342 (perfusion marker). The tumours were clearly visible in the PET images at both 4 and 24 h p.i.. The radioactive uptake of the $^{89}$Zr-Df-cG250-F(ab')$_2$ was compared to the positive area of the tumour stained for CA IX and pimonidazole in tumour sections. The standardized uptake value maximum (SUV$_{\text{max}}$) of $^{89}$Zr-Df-cG250-F(ab')$_2$ for tumour, muscle and tissue was determined, Table 5. The kidneys and liver displayed high uptake of $^{89}$Zr-Df-cG250-F(ab')$_2$ however this is a common side effect of using F(ab')$_2$ fragments: it is probable that the fragment can be reabsorbed, which may cause problems for imaging in the future.

A significant correlation ($P<0.0001, r = 0.57-0.74$) between the CA IX staining and radioactivity uptake was observed at both time points. Similarly, there was a significant correlation ($P<0.0001, r = 0.46-0.68$) between pimonidazole (hypoxia) staining and tracer uptake. A strong positive correlation ($P=0.0067, r = 0.93$) was also observed between SUV$_{\text{max}}$ on the PET images and the measured tumour uptake found from the analysis of tissue (%ID/g), this correlation is similar to that found by Lawrentschuk et al for $^{124}$I-cG250 in a SK-RC-52 model. This study concluded that $^{89}$Zr-Df-cG250-F(ab')$_2$ could be useful in determining CA IX-positive hypoxic areas for head and neck carcinomas via demonstrating a significant spatial correlation of $^{89}$Zr-Df-cG250-F(ab')$_2$ and CA IX expression however, the authors state that further research is required.

Insert Table 5 here
**177Lu-DTPA-A3-SIP, 177Lu-DTPA-CC7-SIP** (Table 1, entry 8)\(^{19}\). Radiolabelled A3-SIP and CC7-SIP antibodies were prepared by covalent modification of a side chain lysine residue of the SIP by reaction with \(p\)-isothiocyanate benzyl DTPA (\(p\)-SCN-Bn-DTPA) to form a thiourea link, followed by coordination of \(^{177}\)Lu radionuclide to the DTPA host, Figure 5\(^{18, 58}\). Mass spectrometry analysis showed a stoichiometry of \(~1.8\) DTPA molecules per antibody molecule\(^{18}\). The biodistribution of \(^{177}\)Lu-DTPA-A3-SIP and \(^{177}\)Lu-DTPA-CC7-SIP (0.259-0.407 MBq) was evaluated following injection into LS174T colorectal xenograft models (\(n = 4\)) and compared to \(^{177}\)Lu-DTPA-SIP-L19 (a clinical stage antibody that targets neo-vasculature structures). The mice were sacrificed 24 h after SIP administration, the organs removed, weighed and the radioactivity counted. Very high kidney and high liver uptake values was observed for both \(^{177}\)Lu-DTPA-A3-SIP and \(^{177}\)Lu-DTPA-CC7-SIP. \(^{177}\)Lu-DTPA-A3-SIP showed a \(~4\)-fold lower tumour accumulation compared to \(^{177}\)Lu-DTPA-SIP-L19 accumulation (2.4 %ID/g versus 9.3 %ID/g, respectively) however had an improved T/B ratio (16.7 versus 5.8, respectively). Biodistribution data for \(^{177}\)Lu-DTPA-CC7-SIP was not presented. This study concluded that simultaneous use of vascular-targeting and hypoxia-targeting antibodies may be desirable to achieve a more homogenous distribution and detection of tumours.

**Insert Figure 5 here**

4. **Small molecule imaging agents that target CA IX**

Two of the ten agents in MICAD that target CA IX are small molecules.

**\(^{99m}\)Tc-PS** (Table 1, entry 9)\(^{21}\). Almost all reported CA small molecule ligands comprise a zinc binding group in their structure of which the primary sulfonamide moiety (-SO\(_2\)NH\(_2\)) is the premier example. The primary sulfonamide anion coordinates to the active site Zn\(^{2+}\) of CAs, acting as an ‘anchor’ on to which variable ‘tail’ groups, selected to incorporate desirable properties, can be tethered\(^{55}\). An important finding by Dubois et al was that the binding of sulfonamide based ligands to cell surface CA IX occurs only under hypoxic conditions\(^{30}\). Akurathi et al\(^{20}\) synthesized a \(^{99m}\)Tc-labelled small molecule sulfonamide compound, \(^{99m}\)Tc-PS, with the aim of imaging CA IX expression to determine regional tumour hypoxia using SPECT.
99mTc is the most widely used radionuclide for diagnostic nuclear medicine (> 90%)\textsuperscript{59}. It can be generated at low cost, it has a suitable half-life (6 h) and its gamma ray emission energy is not accompanied by beta emissions, allowing a more precise alignment of imaging detectors\textsuperscript{59, 60}. Additionally, its emission energy is 141 eV, enough to be detected by gamma cameras but low enough to not carry out significant damage to the body\textsuperscript{20, 59}. 4-(2-Aminoethyl)benzenesulfonamide) (AEBS) was chosen as the core structure of 99mTc-PS as it has a high affinity for CA IX ($K_i = 33$ nM) and the other cancer associated CA (CA XII, $K_i = 3.2$ nM) but low affinity for off-target cytoplasmic CA I and CA II and membrane-bound CA IV ($K_i = 21 \mu$M, 160 nM and 2.4 \mu M, respectively). 99mTc requires a chelating agent and $[^{99m}\text{Tc}(\text{H}_2\text{O})_3\text{(CO)}_3]^+$ (prepared using an Isolink™ kit) together with N-2-picolyl-N-acetic acid were used in the synthesis of 99mTc-PS as the $[^{99m}\text{Tc}(\text{H}_2\text{O})_3\text{(CO)}_3]^+$ can exchange the three water molecules with the amine, aromatic N-heterocycle and carboxylate donor of N-2-picolyl-N-acetic acid, while the latter is also able to covalently attach to the AEBS small molecule through amide bond formation, Figure 6. A rhenium analogue of this sulfonamide ligand, Re-PS, was synthesized as a ‘cold’ control compound for 99mTc-PS to enable the indirect study of the affinity of 99mTc-PS for CA I, II, IX and XII under in vitro conditions\textsuperscript{20}. The in vitro inhibition data for Re-PS is comparable to AEBS ($K_i$s of 3440, 50, 58 and 45 nM at CA I, CA II, CA IX and CA XII, respectively. It is worthwhile to note that the $K_i$ of Re-PS for CA IX is similar to that for AEBS while it is more potent than AEBS at CA XII.

The stability of 99mTc-PS in whole blood and plasma of mice was analyzed at 1 h p.i. In plasma 65% of the radioactivity was in intact 99mTc-PS while 35% was present as a polar radiometabolite. Biodistribution studies with 99mTc-PS were then carried out in mice bearing HT-29 colorectal tumours at 0.5, 1, 2 or 4 h p.i. Accumulation was initially highest in the liver and it was proposed that this may be due to an active transport mechanism on the liver surface. The tracer is moderately hydrophilic resulting in liver clearance, hence at 2 h and 4 h p.i. ~18% ID/g was found in the intestines, indicating clearance to the hepatobiliary pathway. The tracer was also found initially in a significant quantity in the kidneys (36-42% ID/g at 0.5 h and 1 h p.i.), an indication that the tracer may be binding to CA XII or CA XIV, which are highly expressed on the membrane of the kidneys. Control experiments using AEBS as a blocking agent were used to evaluate this theory and it was concluded that the tracer uptake in the kidneys was not related to
interaction with CA XII or CA XIV. A minimal amount of $^{99m}$Tc-PS was shown to diffuse into red blood cells (0.1 % ID/g at 1 h p.i.) and is assumed to bind to either red blood cell CA I or CA II$^{20}$. Disappointingly, the tumour uptake of $^{99m}$Tc-PS was low at each of the four tested timepoints (0.1 ± 0.1% ID/g at 0.5 h then 0.0 ± 0.0 thereafter).

Insert Figure 6 here

$^{13}$C-labelled bicarbonate (Table 1, entry 10)$^{23}$. Gallagher et al$^{22}$ designed hyperpolarized $^{13}$CO$_3$ as a MRI imaging agent to investigate the redox properties of carbonic anhydrase potentially including CA IX, but not specifically for CA IX. Hyperpolarized $^{13}$CO$_3$ was developed on the basis of dynamic nuclear polarization (DNP), a technique that increases the sensitivity of solution-state magnetic resonance spectroscopy (MRS). By injection hyperpolarized $^{13}$CO$_3$, their aim was to produce a high resolution pH map of a transverse slice through an animal that may offer a new way of detecting disease and monitoring treatment response. pH changes can indicate inflammation, ischemia or renal disease amongst other pathological conditions, however there is currently no clinical tool to measure in vivo pH. Using the Henderson-Hasselbach equation, the tissue pH can be determined from the measurements of the concentration ratio between $^{13}$CO$_3$ and $^{13}$CO$_2$, assuming that the pKa of bicarbonate is constant at 6.17. The replacement of $^{12}$C with $^{13}$C does not alter the biochemistry of the molecule while it provides a >10,000 fold enhancement of the $^{13}$C MRS signals thus allowing changes in pH to be monitored in vivo$^{22}$.

After validation of the MRI method for pH determination, hyperpolarized $^{13}$CO$_3$ (200 μL, 40 mg/kg) was injected into three groups of mice with EL4 murine lymphoma tumours. Group 1 were untreated controls, group 2 were given sodium bicarbonate in the drinking water to increase the tissue pH and group 3 were given an ammonium chloride gavage to decrease the tissue pH$^{22}$. For comparison, several mice were injected with the pH sensitive molecule 3-aminopropylphosphonate (3-APP) so that $^{31}$P spectra could be obtained for comparison. 3-APP is a nontoxic MRS-visible extracellular marker that is relatively unaffected by temperature or ionic strength, the chemical shift frequency of 3-APP is sensitive to pH allowing the determination of
pH in vivo$^{61}$. $^{13}$C and $^{31}$P MRS imaging at 9.4 T were performed$^{22}$. The spatial distribution of $^{13}$CO$_2$ and H$^{13}$CO$_3$ in the transverse slice was determined. It was shown that the highest concentration of $^{13}$CO$_2$ was in the tumour mass compared to H$^{13}$CO$_3$ which was highest in the aorta. Although minimal differences were shown in signal intensity between muscle/tumour tissue, a pH map was successfully calculated and a low pH value for the tumour mass was shown, demonstrating that it was possible to image tissue pH non-invasively in vivo. Comparing the untreated mice (tumour pH 6.71), NaHCO$_3$ treated mice (tumour pH 7.02) with NH$_4$Cl (tumour pH 6.47) suggests that the H$^{13}$CO$_3$ measurement reflects more of the extracellular pH. The authors suggest this could be due to the acquisition of spectra immediately after H$^{13}$CO$_3$ is injected and that normally there is a higher concentration of bicarbonate in the extracellular environment. A limitation of the method was also highlighted, namely small differences in the rate at which H$^{13}$CO$_3$ and $^{13}$CO$_2$ lose polarization will lead to the calculated pH varying over time. Imaging therefore must be acquired with a few of the $T_1$ relaxation times. The $T_1$s were found to be 10.1 ± 2.9s ($n = 9$) for H$^{13}$CO$_3$ and 9.8 ± 2.5 s for $^{13}$CO$_2$ ($n = 7$) with the presence of CA increasing this exchange.

Properties of antibodies and small molecules as imaging agents

Although antibodies provide excellent specificity towards their antigen, there are several factors that make tumour targeting with antibodies difficult. Solid tumours are heterogeneous masses and a common limitation of antibody-based imaging agents is slow or incomplete tissue distribution, especially to areas with poor vasculature$^{31}$ as the drugs cannot completely penetrate the tissue$^{62}$. The contributing factors to this include molecular size (IgG ~150 KDa), the antigen barrier and the high tumour interstitial pressure. Additionally, slow plasma clearance maintains high drug levels which complicate imaging and therapy$^{62}$ and present a high radiation dose risk to the bone marrow$^{13}$. Rudnick and Adams have discussed in depth the interaction between the affinity, avidity and efficacy of antibodies in tumour targeting$^{63}$. Although not the focus of this paper, the critical parameters they specify that affect a binding site are: antigen density, mAb internalization and metabolism and the mAb binding affinity$^{63}$.

Antibody fragments may provide advantages over the intact antibodies. Their reduced molecular size (for example, cG250-F(ab’)$_2$ fragments ~100 kDa compared to intact cG250 ~ 150 kDa) may
aid in tissue penetration and their reduced clearance time from the blood can result in higher tumour uptake compared to normal tissues. However, fragments may have increased renal uptake as they are reabsorbed by the renal tubular cells and may lead to radiation-induced nephrotoxicity. Similarly, scFv fragments of ~25 kDa may have excellent tumour penetration but their rapid clearance from the blood may be advantageous or disadvantageous. Other disadvantages include that they are monovalent and there is a possibility of aggregation. SIPs consist of two scFv fragments held via a hinge, making them bivalent, and their size of ~80 kDa means that they have a slower clearance time. They have an increased total affinity and have reduced immunogenicity. However, they have no effector functions and can be difficult to generate as the synthesis involves several steps.

Aromatic sulfonamides are the classical small molecule CA inhibitors that bind to the active site of CA. The CA active sites are extremely tolerant to diverse and variable structures appended to the aromatic sulfonamide moiety. It is possible to vary considerably the size, shape, charge, polarity, hydrophobicity etc. and still have nM CA inhibition. This tolerance allows finetuning of the biopharmaceutical and toxicological properties of the inhibitor via structural manipulations. Additionally, small molecules may be advantageous in their cost and ease of manufacture as well as their improved ADME and DMPK properties when compared to antibodies. However, sensitivity, degradation, toxicity and clearance may all be considerably problematic with these compounds as well as specificity for CA IX over other CAs. To overcome these challenges the small molecule has to be designed very carefully to take the limitations in to account.

Imaging via PET is the most common imaging modality used with the agents described here. PET is advantageous in that it allows the absolute quantitation of tracer uptake unlike determination of uptake via histological sections which is highly subjective. As well as allowing the possibility of serial imaging, it is possible to image the whole tumour volume, instead of biopsy or tissue specimens. PET displays a much higher sensitivity than SPECT (approximately 2-3 times the order of magnitude). Using SPECT however has the potential to increase the observational time window due to the longer half-life of the emitters used. MRI has superior soft tissue contrast resolution, multiplanar image acquisition and functional imaging capabilities. Of note is that only one of these agents has been designed for MRI. With the
growth of MRI in hospitals and clinics, it is likely that more agents will be developed to use this modality which provides high resolution and superior soft tissue contrast. However, the ultimate deciding factor in the choice of imaging modality is the biological considerations of the system. Rahmim and Zaidi warn that in the biological context, generalized comparisons between modalities are not appropriate and instead they need to be performed on a case-by-case basis so that the correct imaging modality is selected.

The development of agents for in vivo imaging of CA IX extends well beyond those agents listed in MICAD. Recent examples include small molecule $^{18}$F labelled PET agents, and an extensive comparison of $^{124}$I-cG250 with $^{89}$Zr-Df-cG250 in murine models with CA IX expressing ccRCC xenografts (SK-RC-52 or NU-12) using PET. The biodistribution results demonstrated very high tumour uptake. In the NU-12 tumour model uptake of the two agents was similar (48.7 ± 15.2%ID/g vs. 32.0 ± 22.9%ID/g, respectively, p = 0.257), while in the SK-RC-52 tumour model the uptake of $^{89}$Zr-Df-cG250 was significantly higher than $^{124}$I-cG250 (114.7 ± 25.2%ID/g vs. 38.2 ± 18.3%ID/g, respectively, p = 0.029). This high uptake gave excellent PET image contrast, for example small intraperitoneal lesions (7 mm$^3$) were visualized with $^{89}$Zr-Df-cG250 and were readily discriminated from the liver and spleen. The results from this study suggest that PET in RCC patients may be more sensitive using $^{89}$Zr-Df-cG250 instead of $^{124}$I-cG250.

Conclusions
Of the MICAD entries, ten agents target CA IX, an antigen overexpressed in several solid cancer types in hypoxic conditions with expression in normal tissues is minimal. CA IX is a challenging diagnostic target for noninvasive molecular imaging, especially given the similarities in the active site for all 12 catalytically active human CAs. It is however an extremely important target as the detection and monitoring of hypoxic tumours can inform clinical decisions surrounding cancer therapy. Continued development of CA IX imaging agents will ideally balance patient safety, good tumour penetration and accurate localization of hypoxia. The ten MICAD agents described herein, highlight the scope and remaining opportunity for development of improved agents targeting CA IX imaging in oncology.

List of Abbreviations
ADME  adsorption, distribution, metabolism, excretion
3-APP  3-aminopropylphosphonate
(M)Bq  (Mega) Becquerels
CA  carbonic anhydrase
ccRCC  clear cell renal cell carcinoma
CT  computed tomography
DMPK  drug metabolism and pharmacokinetics
DNP  dynamic nuclear polarization
DOTA  1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DTPA  diethylenetriaminepentaacetic acid
EDC  1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
FDG  fluorodeoxyglucose
h  hour
HIF  hypoxia inducible factor
%ID/g  % of the injected dose per gram of tissue
IgG  Immunoglobulin G
Iodogen  1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril
i.v.  intravenous
mAb  monoclonal antibody
MICAD  Molecular Imaging and Contrast Agent Database
MRI  magnetic resonance imaging
MRS  magnetic resonance spectroscopy
NIH  National Institute for Health
NHS  N-hydroxysuccinimide
PBS  phosphate buffered saline
PET  positron emission tomography
pH_e  extracellular pH
pH_i  intracellular pH
p.i.  post injection
RCC  renal cell carcinoma
RIS  radioimmunoscintigraphy
ScFv  single chain variable fragment
SIP  small immunoprotein
SPECT  single photon emission computed tomography
$SUV_{\text{max}}$  standardized uptake value maximum
$T/B$  tumour/blood ratio
$T/M$  tumour/muscle ratio
VHL  von Hippel-Lindau protein

**Declaration of Interest**
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Table 1. Agents listed in the Molecular Imaging and Contrast Agent Database (MICAD) that target carbonic anhydrase IX (CA IX).

<table>
<thead>
<tr>
<th>Entry</th>
<th>MICAD Agent Name</th>
<th>Agent Short Name</th>
<th>Category</th>
<th>Imaging Modality</th>
<th>Tumour Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$^{124}$I-chimeric monoclonal antibody G250$^5$</td>
<td>$^{125}$I-cG250</td>
<td>Antibody</td>
<td>PET</td>
<td>1. Human clinical trial ccRCC$^6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>2. SK-RC-52</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Human RCC xenograft$^7$</td>
</tr>
<tr>
<td>2</td>
<td>$^{125}$I-labeled monoclonal antibodies$^{8,9}$</td>
<td>$^{125}$I-mAb</td>
<td>Antibody</td>
<td>SPECT</td>
<td>HT-29</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Human colorectal xenograft</td>
</tr>
<tr>
<td>3</td>
<td>$^{89}$Zr-N-Succinyldesferalchimeric monoclonal antibody G250$^{10,11}$</td>
<td>$^{89}$Zr-Df-cG250</td>
<td>Antibody</td>
<td>PET</td>
<td>SK-RC-52</td>
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<td></td>
<td></td>
<td>Human RCC xenograft</td>
</tr>
<tr>
<td>4</td>
<td>$^{111}$In-DOTA-cG250$^{12,13}$</td>
<td>$^{111}$In-DOTA-cG250</td>
<td>Antibody</td>
<td>SPECT</td>
<td>HT-29</td>
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<td></td>
<td>Human colorectal xenograft</td>
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<td>SK-RC-38</td>
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<td>Human RCC cell line</td>
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<tr>
<td>5</td>
<td>$^{111}$In-DOTA-Fab-cG250$^{12,14}$</td>
<td>$^{111}$In-DOTA-cG250-Fab</td>
<td>Antibody</td>
<td>SPECT</td>
<td>HT-29</td>
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<tr>
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<td></td>
<td>Human colorectal xenograft</td>
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<td>SK-RC-38</td>
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<td></td>
<td></td>
<td></td>
<td>Human RCC cell line</td>
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<tr>
<td>6</td>
<td>$^{111}$In-DOTA-F(ab')$_2$-cG250$^{12,15}$</td>
<td>$^{111}$In-DOTA-cG250-F(ab')$_2$</td>
<td>Antibody</td>
<td>SPECT</td>
<td>HT-29</td>
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<td></td>
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<td></td>
<td>Human colorectal xenograft</td>
</tr>
<tr>
<td></td>
<td>89Zr-labeled N-suc-desferrioxamine-conjugated anti-CA IX chimeric monoclonal antibody cG250-F((\text{ab}'))(_2) fragments(^{16,17})</td>
<td>89Zr-Df-cG250-F((\text{ab}'))(_2)</td>
<td>Antibody</td>
<td>PET</td>
<td>SK-RC-38 Human RCC cell line</td>
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<td>7</td>
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<td></td>
<td></td>
<td></td>
<td>SCCNi3</td>
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<td>177Lu-benzyl-diethylenetriamine pentaacetic acid-anti CA IX small immunoprotein A(^3)(^{18,19})</td>
<td>177Lu-DTPA-A3-SIP</td>
<td>Antibody</td>
<td>SPECT</td>
<td>LS174T Human colorectal xenograft</td>
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<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>[^{99m}\text{Tc}]((\text{CO}_3\text{N-(pyrindin-2-yl-methyl)-N[2-4-sulfamoylphenyl]-ethyl]aminoethylacetate})(^{20,21})</td>
<td>[^{99m}\text{Tc}](\text{PS})</td>
<td>Small molecule</td>
<td>SPECT</td>
<td>HT-29 Human colorectal xenograft</td>
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<td>10</td>
<td>Hyperpolarized (^{13}\text{C})-labeled bicarbonate ((\text{H}^{13}\text{CO}_3))(^{22,23})</td>
<td>(^{13}\text{C})-labelled bicarbonate</td>
<td>Small molecule</td>
<td>MRI</td>
<td>EL4 Murine Lymphoma xenograft</td>
</tr>
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Table 2. PET and SPECT radionuclides combined with antibodies and antibody fragments for imaging CA IX.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half-life (days)</th>
<th>Reference</th>
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<tr>
<td>$^{124}$I</td>
<td>4.16</td>
<td>6, 7, 43</td>
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<tr>
<td>$^{125}$I</td>
<td>59.40</td>
<td>44</td>
</tr>
<tr>
<td>$^{131}$I</td>
<td>8.02</td>
<td>45</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>2.80</td>
<td>5</td>
</tr>
<tr>
<td>$^{89}$Zr</td>
<td>3.26</td>
<td>10, 43</td>
</tr>
<tr>
<td>$^{177}$Lu</td>
<td>6.73</td>
<td>45</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>0.25</td>
<td>20</td>
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</table>
Table 3. Properties of DOTA-CA IX mAb and mAb fragment conjugates\textsuperscript{12}.

<table>
<thead>
<tr>
<th>mAb / fragment</th>
<th>Stoichiometry (molecules of DOTA per mAb)</th>
<th>$B_{\text{max}}$ (binding sites/cell)</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{111}$In-DOTA-cG250</td>
<td>5.2 ± 0.2</td>
<td>120 000 ± 22 000</td>
<td>2.5</td>
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<tr>
<td>$^{111}$In-DOTA- cG250-Fab</td>
<td>1.9 ± 0.1</td>
<td>118 000 ± 21 000</td>
<td>14</td>
</tr>
<tr>
<td>$^{111}$In-DOTA-cG250-F(ab')$_2$</td>
<td>4.1 ± 0.2</td>
<td>114 000 ± 19 000</td>
<td>1.8</td>
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</table>
Table 4. T/B and T/M ratios for $^{111}$In-DOTA-cG250, $^{111}$In-DOTA-cG250-Fab and $^{111}$In-DOTA-cG250-F(ab')$_2$ in mice with HT-29 tumours$^{12}$.

<table>
<thead>
<tr>
<th>mAb/fragment</th>
<th>Time p.i.</th>
<th>T/B ratio</th>
<th>T/M ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{111}$In-DOTA-cG250</td>
<td>7 days</td>
<td>6.6</td>
<td>69</td>
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<td>$^{111}$In-DOTA-cG250-Fab</td>
<td>24 h</td>
<td>16.6</td>
<td>6.7</td>
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<tr>
<td>$^{111}$In-DOTA-cG250-F(ab')$_2$</td>
<td>24 h</td>
<td>4.6</td>
<td>8.9</td>
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</table>
Table 5. The SUV<sub>max</sub> (standardized uptake value) of ⁸⁹Zr-Df-cG250-F(ab')<sub>2</sub> and %ID/g of tissue taken from BALB nu/nu mice subcutaneously implanted with SCCNij3 cell xenograft tumours at 4 h and 24 h p.i.<sup>16</sup>.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>4 h p.i.</th>
<th>24 h p.i.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SUV&lt;sub&gt;max&lt;/sub&gt;</td>
<td>% ID/g</td>
</tr>
<tr>
<td>Tumour</td>
<td>1.65 ± 0.26</td>
<td>3.71 ± 0.97</td>
</tr>
<tr>
<td>Muscles</td>
<td>0.38 ± 0.05</td>
<td>-</td>
</tr>
<tr>
<td>Blood</td>
<td>-</td>
<td>4.34 ± 0.27</td>
</tr>
</tbody>
</table>
Figure Captions

**Figure 1.** Direct iodination of antibodies is typically achieved via the Iodogen method to modify a tyrosine residue\textsuperscript{48,49}.

**Figure 2.** Host-linker moieties that have been employed to indirectly radiolabel antibodies and antibody fragments targeting CA IX.

**Figure 3.** General synthetic methodology for the production of radiolabelled mAbs using desferrioxamine mesylate (desferal, Df) as the host-linker moiety\textsuperscript{55}.

**Figure 4.** Synthetic approach for the production of radiolabelled mAbs and mAb fragments using DOTA as the host-linker moiety.

**Figure 5.** Synthetic approach for the production of radiolabelled antibodies small immunoprotein (SIP) format using $p$-isothiocyanate benzylidiethylenetriamine pentaacetic acid ($p$-SCN-Bn-DTPA) as the host-linker moiety.

**Figure 6.** Synthesis of the small molecule CA IX imaging agent $^{99m}$Tc-PS and control compound Re-PS.