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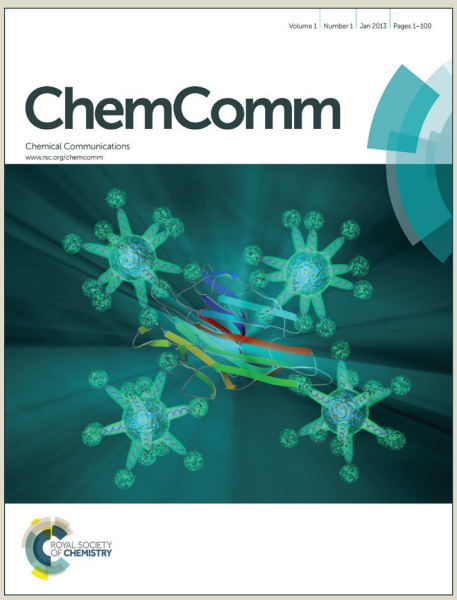

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COMMUNICATION

Development of an Enzymatic Pretargeting Strategy for Dual-Modality Imaging

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A pretargeted imaging strategy based on the HaloTag dehalogenase enzyme is described. Here, a HaloTag-Trastuzumab conjugate has been used as the primary agent targeting HER2 expression, and three new radiolabelled HaloTag ligands have been used as secondary agents, two of which offer dual-modality (SPECT/optical) imaging capability.

Antibodies labelled with radioisotopes can facilitate the *in vivo* detection and/or characterisation of cancers by positron emission tomography (PET) or by single-photon emission computed tomography (SPECT) imaging.[1-4] While antibodies can offer extremely high binding affinities and specificities towards their target antigens, they suffer from prolonged residence times within the circulation. Consequently, high tumour-to-blood (T/B) contrast ratios are only observed several days after administration of the radiolabelled antibody.[5] This is unfavourable as the patient is exposed to an increasing radiation burden over this timeframe.

To circumvent this problem, a variety of pretargeting strategies have been developed which involve the sequential administration of an antibody and a radioactive secondary agent which rapidly binds to the antibody yet has a short biological half-life.[6-8] The main attraction of this approach is that the radioactive agent is administered only after the antibody has reached an optimal T/B ratio. This results in a significantly reduced radiation dose to the patient and leads to an improvement of imaging contrast at earlier time points after administration of the radioactive agent.

Crucially, both the antibody and the radiolabelled secondary agent must be suitably designed so that they bind rapidly upon contact at the tumour. The first examples of pretargeting involved bispecific antibodies which exhibit high affinity for both a target antigen and a radiolabelled hapten molecule.[9] While this approach has yielded promising results in the clinic[10, 11], it is reliant on entirely non-covalent interactions which can lead to dissociation of the radiolabelled hapten from the antibody. Furthermore, the high cost and practical complexity of developing bispecific antibodies has stimulated the search for alternative pretargeting strategies.

Several clinically relevant antibodies and radiolabelled secondary agents have since been reported containing complementary reactive groups which rapidly form strong chemical interactions under physiological conditions. However, the number of appropriate chemical pairings investigated for this purpose has so far been limited to a few distinct classes: including biotin/(strept)avidin[12, 13], complementary oligomers, particularly those containing a morpholino-backbone[14, 15], and components for *trans*-cyclooctene/tetrazine bioorthogonal click chemistry[16-19]. These approaches offer their own benefits and limitations which are discussed in recent review articles.[6, 7]

HaloTag is a dehalogenase enzyme (33 kDa) which contains an engineered cavity designed to accommodate the reactive chloroalkane group of a HaloTag ligand (HTL).[20] Upon entering the enzyme cavity, the terminal chlorine atom rapidly undergoes nucleophilic displacement and a covalent adduct is formed, effectively anchoring the HaloTag ligand in a precise location. Importantly, this reaction has been shown by Cai *et al.* to be highly specific, even under physiological conditions[21, 22] and proceeds with a second order rate constant of up to 2.7×10^6 $M^{-1}s^{-1}$ which is comparable to the biotin/(strept)avidin interaction[20] and faster than bioorthogonal chemistry-based approaches.[6] Based on these attractive properties, we realised that this reaction could form the basis of a novel pretargeted imaging strategy as depicted in Figure 1. It is important to highlight that in a similar manner to the biotin/(strept)avidin pretargeting approach, the administration of the HaloTag foreign protein may provoke an immunogenic response in vivo.

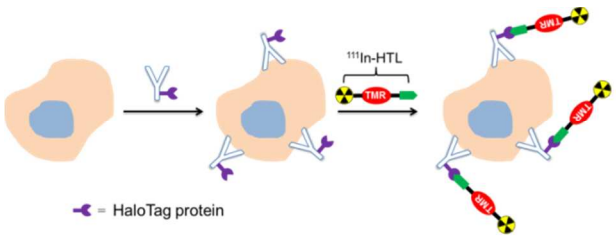

Fig. 1 A schematic representation of a two-step pretargeting strategy based on HaloTag Technology

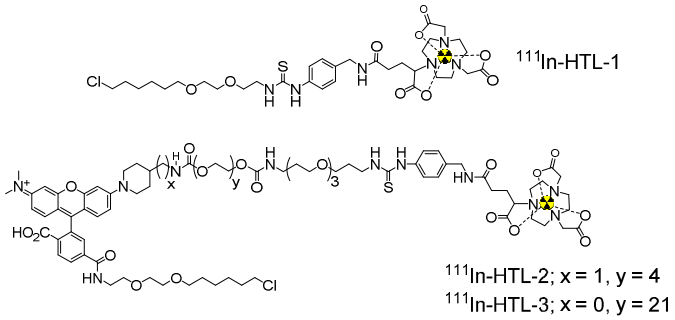

Fig. 2 The mono-modal HaloTag ligand, ^{111}In-HTL-1 (top), and the dual-modality HaloTag ligands, ^{111}In-HTL-2 and ^{111}In-HTL-3 (bottom)

However, the proposed HaloTag system has a specific advantage in that it has no competing endogenous species, whilst the biotin/(strept)avidin pretargeting strategy is hampered by the presence of endogenous biotin (10^{-7}-10^{-8} M).

To test this hypothesis, three new HaloTag ligands were synthesised and each labelled with the SPECT radionuclide indium-111 (^{111}In-HTL-1, 2, and 3; Fig. 2: described in greater detail in the supplementary information). The chelating agent NODAGA was used to facilitate radiolabelling with indium-111 which resulted in moderate radiochemical yields (46±12%, 23±4%, and 22±3% for ^{111}In-HTL-1, 2, and 3, respectively). While the use of a larger macrocycle such as DOTA may have resulted in higher radiochemical yields, several studies investigating ^{111}In-NODAGA-containing compounds have shown highly comparable *in vivo* biodistribution, pharmacokinetics, and stability to analogous ^{111}In-DOTA-containing compounds.[23-25] ^{111}In-HTL-2 and ^{111}In-HTL-3 also contain a tetramethylrhodamine fluorophore (TMR; $\lambda_{ex/em}$=552/578 nm) which allows the acquisition of complementary optical imaging data, thereby creating a dual-modality approach.

The ability of these ^{111}In-labelled HaloTag ligands to bind HaloTag protein was assessed using U2OS human osteosarcoma and 4T1 murine breast cancer cell lines which had been stably transfected with HaloTag on the extracellular surface (U2OS-ECS and 4T1-ECS). The degree of binding was determined by assaying cell-bound radioactivity after increasing incubation times up to 24 h (Fig. S3). These preliminary experiments revealed a substantial degree of specific binding on both U2OS-ECS and 4T1-ECS cells lines that increased over 24 h, yielding maximum uptake values of 37±17%, 53±7%, and 26±10% on the U2OS-ECS cells, and 7±1%, 21±4%, and 8±2% on the 4T1-ECS cells for ^{111}In-HTL-1, 2, and 3, respectively. Furthermore, we were able to detect significantly enhanced binding on the U2OS-ECS cell line after just 15 minutes incubation compared to the wild-type U2OS cells and also U2OS-ECS cells which had been pre-incubated with a blocking concentration of non-radiolabelled HaloTag ligand (^{111}In-HTL-1: P<0.05; ^{111}In-HTL-2 and ^{111}In-HTL-3: P<0.001). Confocal microscopy experiments involving the two fluorescent HaloTag ligands HTL-2 and HTL-3 confirmed HaloTag-mediated binding to U2OS-ECS and 4T1-ECS cell lines which was not observed for the corresponding wild-type cell lines (Fig. S4).

To evaluate the ability of ^{111}In-labelled HaloTag ligands to bind HaloTag protein in an *in vivo* environment, each was administered intravenously to BALB/c mice bearing either 4T1-ECS or wild-type 4T1 tumours. By performing small animal SPECT/CT imaging at 3 h and 24 h post-injection and *ex vivo* biodistribution experiments at 24 h p.i., we observed that each ligand had a unique pharmacokinetic profile and revealed varying degrees of tumour uptake (Figs. S6 and S7). The ligand which performed best in the preceding *in vitro* experiments, ^{111}In-HTL-2, also exhibited the most optimal pharmacokinetic profile and HaloTag binding properties *in vivo*, yielding significantly higher uptake in 4T1-ECS *versus* wild-type 4T1 tumours after 24 h (3.49±0.58 and 2.64±0.34 %ID/g, respectively; P<0.05).

For the validation of a novel pretargeting strategy based on these HaloTag ligands, we opted to target human epidermal growth factor receptor 2 (HER2) expression in a series of *in vitro* experiments. HER2 is amplified in 30% of early-stage breast cancers and its overexpression is strongly correlated to reduced survival, making it a target of high clinical significance.[26] The humanized anti-HER2 monoclonal antibody Trastuzumab (Herceptin) was selected as the primary targeting agent and was modified with HaloTag protein *via* the *trans*-cyclooctene/tetrazine ligation. Whilst Trastuzumab internalises upon binding the HER2 receptor which potentially renders the primary agent inaccessible, the rate of internalisation is slow ($t_{1/2}$ ~11 h)[27] and therefore it does not present a meaningful obstruction to pretargeting within the timescales of the reported experiments. All three ^{111}In-labelled HaloTag ligands were evaluated as secondary agents.

A proof-of-concept model was adapted from a previous study by Devaraj *et al.,* who reported an *in vitro* pretargeting strategy based on norbornene/tetrazine bioorthogonal chemistry.[28] This involved first incubating either MDA-MB-231/H2N (high HER2 expressing; 6.1×10^5 receptors/cell)[29] or MDA-MB-231 (HER2 negative; 0.4×10^5 receptors/cell)[29] cells with the Trastuzumab-HaloTag primary agent (200 nM) at room temperature for 30 minutes. The primary agent was removed and the cells were then incubated with the relevant ^{111}In-labelled HaloTag ligand for a further 30 minutes. The cell culture medium was collected and the remaining monolayer of cells was then lysed with 0.1 M NaOH. The radioactivity present in both the cell culture medium (unbound fraction) and in the cell lysates (bound fraction) was then measured using a gamma counter. Pretargeting experiments were also conducted using unmodified Trastuzumab, unmodified HaloTag, and a non-specific rabbit IgG-HaloTag conjugate, as primary targeting agents.

All three ^{111}In-labelled HaloTag ligands exhibited significantly higher binding to the HER2 expressing MDA-MB-231/H2N cells which had been pretargeted with the Trastuzumab-HaloTag conjugate protein compared with the negative control experiments (Fig. 3). Furthermore, experiments involving the HER2 negative cell line (MDA-MB-231) did not show appreciably higher signal when pretargeted with the Trastuzumab-HaloTag agent. The overall background uptake varied for each HaloTag ligand according to their relative propensity to non-specifically bind proteins (Fig. S5). Consequently, the ligand with the lowest non-specific protein binding, ^{111}In-HTL-3, resulted in the highest fold increase compared with background signal (as defined by the mean average of all negative control experiments).

Additional pretargeting experiments using the same experimental conditions were also conducted using the non-radiolabelled, fluorescent HaloTag ligands HTL-2 and HTL-3. Following the labelling procedure, the cells were imaged by confocal microscopy. MDA-MB-231/H2N cells pretargeted with the Trastuzumab-HaloTag conjugate protein showed higher fluorescence signal at the cell membrane compared to

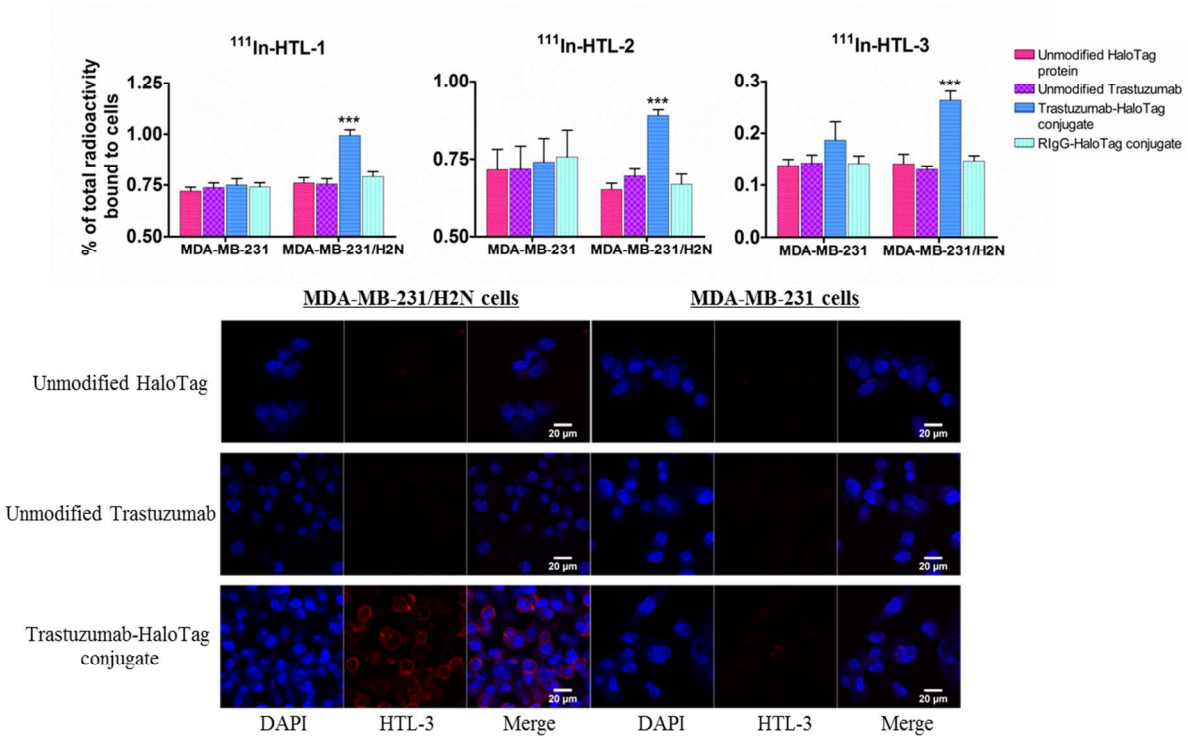

Fig. 3 Top: *In vitro* pretargeting experiments involving ^{111}In-HTL-1,2, and 3 as secondary agents revealed an ability to detect HER2 expression when pretargeted with the Trastuzumab-HaloTag conjugate protein. Bottom: Confocal microscopy experiments involving the non-radiolabelled fluorescent secondary agent HTL-3 further confirmed the effectiveness of this pretargeting strategy.

negative control experiments (Fig. 3).

In summary, these experiments demonstrate the feasibility of using this novel enzymatic pretargeting strategy to detect a clinically relevant cancer biomarker *in vitro* using both gamma emission and fluorescence imaging. This approach is highly modular as HaloTag protein can be readily conjugated to any antibody using standard bioconjugation chemistry. Given the rapid rate of this reaction and its ability to proceed selectively *in vivo*, it is clear that this dual modality pretargeting strategy has strong potential to be translated into an *in vivo* setting for molecular imaging and nuclear medicine applications.

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Notes and references

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Electronic Supplementary Information (ESI) available: Detailed experimental methods, full characterisation data for all compounds, cell labelling data, SPECT/CT images and *ex vivo* biodistribution data. See DOI: 10.1039/c000000x/

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