Molecular Radiotherapy using Cleavable Radioimmunoconjugates that Target EGFR and γH2AX

Bart Cornelissen, Andrew Waller, Sarah Able, Katherine A Vallis

CR-UK/MRC Gray Institute for Radiation Oncology and Biology, Department of Oncology, University of Oxford, Oxford, United Kingdom

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To whom correspondence should be addressed:

Professor Katherine A Vallis
Gray Institute for Radiation Oncology and Biology,
Department of Oncology,
University of Oxford,
Old Road Campus Research Building,
Off Roosevelt Drive,
Oxford,
OX3 7DQ
Tel: +44 (0)1865 225850
Fax: +44 (0)1865 857127
Email: katherine.vallis@oncology.ox.ac.uk
ABSTRACT

Many anticancer therapies, including ionizing radiation (IR), cause cytotoxicity through generation of DNA double-strand breaks (DNA DSB). Delivery of therapeutic radionuclides to DNA DSB sites can amplify this DNA damage, for additional therapeutic gain. Herein, we report on two radiopharmaceuticals, radiolabeled with the Auger electron emitter, $^{111}$In, with dual specificity for both the intranuclear, DNA damage repair signaling protein, γH2AX, and the epidermal growth factor receptor (EGFR). The EGFR ligand, EGF, was conjugated to a fluorophore- or $^{111}$In-labeled anti-γH2AX antibody, linked via a nuclear localization sequence (NLS) to ensure nuclear translocation. EGF conjugation was achieved either through a non-cleavable PEG linker (PEO₆), or a cleavable disulphide bond. Both conjugates selectively bound EGFR on fixed cells and γH2AX in cell extracts. Both compounds enter EGFR-expressing cells in an EGF/EGFR-dependent manner. However, only the cleavable compound was seen to associate with γH2AX foci in the nucleus of irradiated cells. Intracellular retention of the cleavable compound was prolonged in γH2AX-expressing cells. Clonogenic survival was significantly reduced when cells were exposed to IR (to induce γH2AX) plus $^{111}$In-labeled cleavable compound compared to either alone, and compared to non-specific controls. In vivo, uptake of $^{111}$In-labeled cleavable compound in MDA-MB-468 xenografts in athymic mice was 2.57 ± 0.47 percent injected dose/g (%ID/g), but increased significantly to 6.30 ± 1.47 %ID/g in xenografts where γH2AX was induced by IR ($P<0.01$). This uptake was dependent on EGF/EGFR and anti-γH2AX/γH2AX interactions. We conclude that tumor-specific delivery of radiolabeled antibodies directed against intranuclear epitopes is possible using cleavable antibody-peptide conjugates.
INTRODUCTION

DNA double-strand breaks (DNA DSB) are highly deleterious and their formation is a major determinant of the cytotoxicity of radiation therapy and several widely used anticancer chemotherapeutic agents (1). An early event following the formation of DSBs is the phosphorylation of the X isoform of histone H2A at serine-139 by the PI3-like kinases (DNA-PKcs, ATM and ATR), resulting in $\gamma$H2AX (1, 2). Many hundreds of copies of $\gamma$H2AX form foci at DSB sites. We previously identified $\gamma$H2AX as an attractive target for molecular imaging and quantification of DSBs in vivo (3). This exclusively nuclear protein can be targeted using anti-$\gamma$H2AX antibodies, conjugated to the cell-penetrating peptide (CPP), TAT, which also harbours a nuclear localization sequence (NLS), to allow cellular internalization and nuclear translocation. Anti-$\gamma$H2AX-TAT was then conjugated to fluorophores or the radionuclide $^{111}$In, to allow fluorescence microscopy or SPECT imaging, respectively.

Apart from being a target for imaging DSBs, we have shown that $\gamma$H2AX is also a good target for Auger electron radiotherapy (4). Auger electrons are low energy electrons (1-10 eV) with very limited track length (in the nm-μm range) that, when emitted in close proximity of DNA, are highly damaging (5). When anti-$\gamma$H2AX-TAT was radiolabeled with the Auger electron-emitter, $^{111}$In, at high specific activity (SA), it caused amplification of DNA damage induced by chemotherapy and IR, reduction of clonogenic survival in vitro, and inhibition of tumor growth in vivo. However, none of the constituent moieties of anti-$\gamma$H2AX-TAT ensure the conjugate’s tumor-specific uptake, and any non-specific internalization in normal cells would be undesirable as it may cause toxicity.

Here, we report on two $^{111}$In-labeled radioimmunoconjugates that target not only $\gamma$H2AX but also, through the incorporation of the epidermal growth factor (EGF) peptide, the
EGF receptor (EGFR) which is frequently overexpressed in cancer e.g. MDA-MB-468 human breast cancer cells express $1.3 \times 10^6$ EGFR/cell (6), but is expressed to a much lower extent in normal tissues e.g. normal hepatocytes express $8 \times 10^4$ to $3 \times 10^5$ EGFR/cell (7). These dual-specific immunconjugates incorporate the nuclear localization sequence (NLS) of SV-40 large T-antigen, to promote nuclear accumulation of the $\gamma H2AX$-seeking moiety.

Two different synthetic strategies were pursued. First, EGF conjugated to NLS was covalently bound to anti-$\gamma H2AX$ antibody via a non-cleavable poly-ethylene glycol (PEG$_6$) linker. Additionally, EGF was linked to NLS-conjugated anti-$\gamma H2AX$ antibody via a cleavable disulphide bond (8). We report on the in vitro and in vivo characteristics of both compounds, and demonstrate the potential of the $^{111}$In-labeled cleavable compound as an Auger electron therapeutic radiopharmaceutical.

**MATERIALS AND METHODS**

**Cell lines**

MDA-MB-468 and SQ20b human breast cancer cells were obtained from Cell Services, CR-UK London Research Institute. MDA-MB-231 human breast cancer cells, stably transfected with the HER2-gene, yielding MDA-MB-231/H2N cells (hereafter referred to as 231-H2N) were a gift from Robert Kerbel (Sunnybrook Health Sciences Centre, Toronto, ON) (9). These cells were tested and authenticated by the provider, using short tandem repeat profiling. The length of time in culture of these cells was less than 6 months after retrieval from liquid nitrogen storage. The number of EGFR/cell for MDA-MB-468 and 231-H2N cells is $1.3 \times 10^6$ and $2 \times 10^5$, respectively (10). Cells were cultured in 5% CO$_2$ in DMEM cell culture medium (Sigma-Aldrich, Dorset, UK) supplemented with 10% fetal calf...
serum (Invitrogen, Paisley, UK), and penicillin/streptomycin, 100 units/mL (Invitrogen, Paisley, UK).

**Synthesis of Non-Cleavable Immunoconjugates**

A schematic overview of the synthesis of non-cleavable immunoconjugates is shown in Supplementary Figure 1A. A full description of the synthesis is available in the Supplementary Methods. Anti-γH2AX antibody or non-specific IgGs from rabbit serum was conjugated to benzyl-DTPA (BnDTPA) to allow $^{111}$In labeling, or to Cy3 or AlexaFluor555 (AF555) to allow fluorescence microscopy. This was reacted with (SM$(\text{PEO}_6$), yielding maleimide-activated conjugates, BnDTPA-anti-γH2AX-(PEO)$_6$-mal or BnDTPA-rIgG-(PEO)$_6$-mal. Epidermal growth factor (EGF) was conjugated to the NLS-peptide, GGPKKKRKVGYGCG, using EDC/NHS chemistry, yielding EGF-NLS. EGF-NLS was conjugated to BnDTPA-anti-γH2AX-(PEO)$_6$-mal or BnDTPA-rIgG-(PEO)$_6$-mal. The size of BnDTPA-anti-γH2AX-PEO$_6$-NLS-EGF was determined using polyacrylamide gel electrophoresis (PAGE) electrophoresis followed by Coomassie staining. $^{111}$In-labeling was achieved by addition of $^{111}$In-chloride ($^{111}$InCl$_3$; Perkin Elmer, Boston, MA). For brevity and because BnDTPA was used as the radiometal chelator for all radioimmunoconjugates reported here, BnDTPA-anti-γH2AX-PEO$_6$-NLS-EGF and BnDTPA-rIgG-PEO$_6$-NLS-EGF are referred to hereafter as anti-γH2AX-PNE and rIgG-PNE, respectively.

**Synthesis of Cleavable Immunoconjugates**

A schematic overview of the synthesis of cleavable immunoconjugates is shown in Supplementary Figure 1B. A full description of the synthesis is available in the Supplementary Methods. Anti-γH2AX antibody or non-specific IgGs from rabbit serum was conjugated to BnDTPA to allow $^{111}$In labeling, or to Cy3 or AlexaFluor555 (AF555) to allow
fluorescence microscopy. This was reacted with SANH for addition of a hydrazine moiety, and subsequently with an N-terminal serine-containing NLS-peptide (SGPKKKRKVGYGCG) and sodium periodate. EGF was modified with a disulphide function using SMPT. EGF-SMPT was added to BnDTPA- or fluorophore-tagged IgG-NLS, yielding BnDTPA-anti-γH2AX-NLS-SS-EGF and BnDTPA-rIgG-NLS-SS-EGF (referred to hereafter as anti-γH2AX-N-SS-E and rIgG-N-SS-E). 111In-labeling and determination of radiolabeling yield was achieved as described above. The size of BnDTPA-anti-γH2AX-NLS-SS-EGF was determined using PAGE electrophoresis followed by Coomassie staining. To study the influence of the NLS sequence, the same conjugate was prepared using a disrupted NLS sequence (dNLS, SGGPGGKRKVGYGCG), denoted as 111In-anti-γH2AX-dN-SS-EGF.

**Competition Binding Assay**

EGFR- and γH2AX-binding by anti-γH2AX-PNE and anti-γH2AX-N-SS-E were compared to that of unmodified EGF or anti-γH2AX antibody in competition assays against 125I-EGF or 125I-labeled anti-γH2AX antibody as previously described (10). 125I-labeling of EGF (Peprotech, London, UK) and anti-γH2AX antibody (Merck, Nottingham, UK) was achieved using the Iodogen method (11). This method is available in the Supplementary information.

**Cleavage of Disulphide Linker**

To determine whether the disulphide linker was cleavable under reducing conditions, a cell-free assay was performed using glutathione as the reducing agent. rIgG-N-SS-E was synthesized, as described above, using 125I-EGF in place of EGF, to yield rIgG-N-SS-*E. SEC was performed on IgG-N-SS-*E using a G50 sephadex mini-column, and the amount of
\(^{123}\)I in serial fractions was measured in an automated gammarounter. IgG-N-SS-*E was exposed to 1 mM glutathione for 2 h at room temperature, and SEC performed using a G50 sephadex minicolumn.

**Confocal Microscopy**

231-H2N, SQ20b, or MDA-MB-468 cells, expressing $2 \times 10^5$, $1.04 \times 10^6$, or $1.3 \times 10^6$ EGFR receptors per cell, respectively, were seeded on coverslips and allowed to adhere overnight. To determine the intracellular distribution of conjugates, cells were exposed to 16 nM AF555-labeled immunoconjugate, with or without 16 nM AF488-EGF (Invitrogen, Paisley, UK), for up to 4 h at 37°C. In some cases, cells were irradiated (4 Gy) 1 h after addition of fluorophore-labeled immunoconjugates, after which the growth medium was replaced with fresh medium. Cells were washed twice with PBS, fixed for 10 min at room temperature with 4% paraformaldehyde (Sigma), washed again with PBS, permeabilized at room temperature using 1% Triton X-100 in PBS (Sigma) and blocked (1 h at 37°C; 2% BSA in PBS). For γH2AX immunostaining, fixed cells were incubated with anti-γH2AX primary antibody raised in mouse (JBW301; Millipore; 1:800 dilution) for 1 h at 37°C. Following 3 washes, cells were exposed to goat anti-mouse antibody (Invitrogen; 1:250 dilution) labeled with AF488 or AF555 for 1 h at 37°C. After 3 washes coverslips were mounted on slides using Vectashield plus DAPI (Vector Laboratories, Burlingame, USA). Confocal microscopy was performed on a Zeiss 530 confocal microscope (Zeiss, Welwyn Garden City, UK). To determine the distribution of EGFR, cells were fixed and permeabilized as above, and stained for EGFR using anti-EGFR antibodies (Invitrogen; 1:250 dilution) and AF555-labeled goat anti-mouse antibodies (Invitrogen; 1:250 dilution).
Intracellular Distribution and Retention

To investigate the intracellular distribution of immunoconjugates, aliquots of 2 x 10^5 MDA-MB-468 or 231-H2N cell in 500 µL of growth medium were exposed to 16 nM ^111^In-anti-γH2AX-N-SS-E or ^111^In-anti-γH2AX-dN-SS-E in 200 µL DMEM (0-6 MBq/µg). At selected times, supernatant was removed from the cells, cells were washed with 0.1 M glycine.HCl pH 2.5 to remove cell-surface bound radioactivity, the cytoplasmic membrane was lysed (25 mM KCl, 5 mM MgCl2, 10 mM Tris–HCl and 0.5% NP- 40), and cell nuclei were pelleted and lysed using 0.1 M NaOH, as previously described (12). Radioactivity in cytoplasmic and nuclear fractions was counted in a gammarcounter. Retention of ^111^In-labeled compounds in MDA-MB-468 and 231-H2N cells was determined using a load-chase assay as previously described (12). Briefly, cells were seeded in a 24 well plate (2 x 10^5 cells/well) and left to adhere overnight. Cells were exposed to 0.25 µg/mL of ^111^In-labeled compound for 1 h, irradiated (4 Gy) or sham-irradiated and then washed twice with PBS and supplied with fresh culture medium. At selected time points, the amount of ^111^In remaining in cells was determined as previously described (12).

Clonogenic Survival Assay

Aliquots of MDA-MB-468 (1.3 x 10^6 EGFR/cell) or 231-H2N cells (2 x 10^5 EGFR/cell) were exposed to various concentrations of unlabeled anti-γH2AX-PNE, ^111^In-anti-γH2AX-PNE (6 MBq/µg), ^111^In-anti-γH2AX-N-SS-E (0-6 MBq/µg), ^111^In-rIgG-N-SS-E (0-6 MBq/µg) either alone or in combination with IR. The highest concentration used, 0.25 µg/mL, can be easily achieved in vivo by injection of a 20 g mouse with 10 µg of conjugate. After incubation at 37°C for 24 h, 2 x 10^3 cells were seeded per well in triplicate on a 6-well plate, and incubated with fresh growth medium (2 mL). After 7-14 days, plates were washed with PBS and cell colonies were stained with methylene blue (2% methylene blue in
water:methanol 1:1), and counted using a Gelcount automated plate reader (Oxford Optronics, Oxford, UK).

**Biodistribution**

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with local ethical committee approval. MDA-MB-468 xenografts were established in female BALB/c nu/nu mice (Harlan, UK). $^{111}$In-anti-$\gamma$H2AX-N-SS-E or IgG-N-SS-E (10 $\mu$g; 5 MBq) was administered intravenously (i.v.). $\gamma$H2AX was induced by X-irradiation of the tumor (10 Gy) 1 h post injection (p.i.) using a Gulmay 320 kV X-irradiator; 2.0 Gy/min. Apart from the tumor, the body of the mouse was shielded from radiation using 1 cm thick brass. The extent of $\gamma$H2AX expression was demonstrated by immunohistochemistry on sections from excised xenografts, 24 h after irradiation, processed as previously reported (3). Images were acquired using a confocal microscope as above. Enumeration of foci was performed manually, on a single 1 $\mu$m optical section through the tumor, in at least 100 cells. To demonstrate targeting of $\gamma$H2AX by $^{111}$In-anti-$\gamma$H2AX-N-SS-E, tumor sections were stained for $\gamma$H2AX as before (3), using an antibody raised in mouse and an AlexaFluor488-conjugated goat anti-mouse secondary antibody. An AlexaFluor555-conjugated goat anti-rabbit antibody was used to stain the IgG moiety of $^{111}$In-anti-$\gamma$H2AX-N-SS-E. In some cases, a 100-fold excess of EGF was co-injected to block EGFR. For SPECT imaging, mice were anesthetized using isoflurane at 24 h p.i. and SPECT-CT images were acquired using a nanoSPECT-CT scanner (Bioscan, Washington DC, USA). Volume of interest (VOI) analysis on SPECT images was performed using the Inveon Research Workplace software package (Siemens, Camberley, UK).
**Statistical Analysis**

All statistical analyses and non-linear regression was performed using Graphpad Prism (Graphpad Software Inc). 1-way or 2-way ANOVA was used for multiple comparisons. The F-test was used to compare parameters between curves.

**RESULTS**

**Synthesis, Affinity and Cleavage**

Two radioimmunoconjugates were synthesized, based on anti-γH2AX antibodies and EGF. The linkage between the antibody and EGF moieties was made either using a non-cleavable PEG linkage, or using a cleavable disulfide bond. All compounds were synthesized so that they contained, on average, a 1:1 ratio of the various moieties. Radiolabeling yield for $^{111}$In labeling was >95%. The affinity for EGFR of EGF-containing conjugates was compared to that of $^{123}$I-EGF. Similarly, the affinity of anti-γH2AX-containing conjugates was compared to $^{123}$I-anti-γH2AX. $IC_{50}$ values were not significantly different between the conjugates and native EGF or anti-γH2AX (Supplementary Figure S2). To demonstrate reductive cleavage of the disulphide, rIgG-N-SS-*E was exposed to glutathione (Supplementary Figure S3). After addition of glutathione, $^{123}$I became associated with low MW fractions, compared to high MW species before addition. This was ascribed to cleavage of the disulphide bond. These results are described in more detail in the Supplementary information.
Confocal Microscopy: Co-localization of Immunoconjugates with EGF and γH2AX

MDA-MB-468 cells were exposed to the fluorophore-labeled, non-cleavable, anti-γH2AX and EGF-containing immunoconjugate, AF555-rIgG-PNE and to AF488-EGF. A focal pattern of uptake of both fluorophores was observed in the cytoplasm, mainly in the perinuclear region, but not in the nucleus at 1 and 4 h time points (Figure 1A). AF555-rIgG-PNE colocalized closely with AF488-EGF, suggesting that the EGF moiety of AF555-rIgG-PNE is a major determinant of its intracellular distribution. γH2AX foci were observed in irradiated, but not sham irradiated MDA-MB-468 cells, at 4 and 24 h after irradiation (Figure 1B, green). The number of foci, counted in a single focal plane, in MDA-MB-468 cells was 14.80 ± 0.76 vs. 9.14 ± 1.03 γH2AX foci/cell at 4 versus 24 h after irradiation. In MDA-MB-231/H2N cells, 14.45 ± 0.70 versus 6.12 ± 0.45 γH2AX foci/cells were counted, respectively. In SQ20b cells, 22.13 ± 0.36 versus 8.54 ± 0.60 γH2AX foci/cells were counted, respectively. Neither AF555-anti-γH2AX-PNE nor AF555-rIgG-PNE were detected in the nuclei of irradiated or unirradiated cells (Figure 1B, red), and therefore failed to colocalize with γH2AX foci present in irradiated cells. AF555-anti-γH2AX-PNE was not taken up into MDA-MB-231/H2N cells, consistent with their low EGFR density, and did therefore not colocalize with γH2AX foci (Supplementary Figure S4A).

In contrast to the non-cleavable immunoconjugate, AF555-anti-γH2AX-N-SS-E did colocalize with γH2AX foci in the nuclei of irradiated EGFR-positive MDA-MB-468 and SQ20b cells, 4 and 24 h after IR (Figure 2A, Supplementary Figure S5). At the 4 h post-IR time point, AF555-anti-γH2AX-N-SS-E was visible predominantly in the cytoplasm of MDA-MB-468 cells, but also in the nucleus where it colocalized with γH2AX foci. By 24 h, cytoplasmic staining was no longer present, and all AF555 signal colocalized closely with γH2AX foci in the nuclei. AF555-anti-γH2AX-N-SS-E colocalized with every observed γH2AX focus. This is in contrast to AF555-anti-γH2AX-PNE which showed no
colocalization with γH2AX foci. This suggests that in the case of AF555-anti-γH2AX-N-SS-E, intracellular cleavage at the S-S bond does occur as intended, releasing AF555-anti-γH2AX-NLS which is able to bind γH2AX in the nucleus where it is retained. There was no accumulation of AF555-anti-γH2AX-N-SS-E in the nuclei of sham-irradiated cells which lacked γH2AX foci, and the non-specific conjugate, AF555-rIgG-N-SS-E, did not localize in nuclei, whether cells were irradiated or sham-irradiated. To investigate the specificity of EGFR-binding by immunoconjugates, MDA-MB-468 cells were exposed to an excess of EGF. This effectively blocked binding of AF555-rIgG-N-SS-E and AF488-EGF to the membrane (Figure 2B) and prevented internalization, confirming that cellular uptake of AF555-rIgG-N-SS-E is EGF-dependent. Additionally, AF555-anti-rIgG-N-SS-E was not taken up into 231-H2N cells, consistent with their low EGFR density (Supplementary Figure S4B, Supplementary Figure S6).

**Intracellular Distribution and Retention of Immunoconjugates**

The intracellular distribution of $^{111}$In-anti-γH2AX-N-SS-E, $^{111}$In-anti-γH2AX-dN-SS-E and $^{111}$In-rIgG-N-SS-E is shown (Figure 3A). The results are displayed to show the proportion of the total internalized radioactivity that accumulated in the cytoplasm and nucleus for the two cell lines, MDA-MB-468 and 231-H2N. EGFR expression on MDA-MB-468 cells or 231-H2N cells was not altered after exposure to ionizing radiation (data not shown). In each cell line, the uptake of $^{111}$In-anti-γH2AX-N-SS-E, $^{111}$In-anti-γH2AX-dN-SS-E, or $^{111}$In-rIgG-N-SS-E after incubation for 4 h was not significantly different ($P>0.05$). The absolute amount of total internalized radioactivity was 7-fold less in 231-H2N (0.15 ± 0.008% of added radioactivity was found to be cell-associated after a 1h exposure) compared to MDA-MB-468 cells (1.11 ± 0.04%), in line with the > 6-fold lower expression of EGFR in 231-H2N cells (Supplementary Figure S6A). The initial distribution of EGFR in MDA-MB-
468 cells is shown in Supplementary Figure S6B. In irradiated MDA-MB-468 cells, the proportion of the total internalized radioactivity that accumulated in the nucleus, was greater for $^{111}$In-anti-$\gamma$H2AX-N-SS-E than for $^{111}$In-rIgG-N-SS-E ($P=0.0003$). The amount of intranuclear radioactivity following exposure of cells to $^{111}$In-anti-$\gamma$H2AX-N-SS-E was significantly higher in irradiated cells compared to sham irradiated cells ($P<0.05$). $^{111}$In-anti-$\gamma$H2AX-dN-SS-E, containing a disrupted NLS sequence, showed limited nuclear uptake (10% of the internalized amount of $^{111}$In was found in the nucleus; $P<0.05$). In 231-H2N cells, nuclear localization of $^{111}$In was 50% higher in irradiated cells exposed to $^{111}$In-anti-$\gamma$H2AX-N-SS-E compared to $^{111}$In-rIgG-N-SS-E ($P < 0.05$). In both cell lines the amount of non-specific immunoconjugate, anti-rIgG-N-SS-E, that accumulated in the nucleus was lower in irradiated compared to non-irradiated cells but this difference did not reach statistical significance ($P>0.05$).

Retention of $^{111}$In-anti-$\gamma$H2AX-N-SS-E was prolonged in irradiated MDA-MB-468 cells, compared to sham-irradiated cells (retention half-life: 2.9 h, 95% CI = 1.787 - 7.729 versus 0.4 h, 95% CI = 0.3670 - 0.4687, respectively; $P<0.0001$) (Figure 3B). It was confirmed that this observation was not due to an increase in the number of EGFR per cell following radiation (see Supporting Information). Retention of $^{111}$In-rIgG-N-SS-E was modest in both sham-irradiated and irradiated cells (retention half-life: 0.4 h (0.3718 - 0.4004) versus 0.5 h (0.4314 - 0.6541); $P > 0.05$). In 231-H2N cells, uptake of both immunoconjugates was low and this was reflected in modest retention of $^{111}$In-anti-$\gamma$H2AX-N-SS-E and $^{111}$In-rIgG-N-SS-E which was not significantly influenced by IR ($P>0.05$) (Figure 3C).
Cytotoxicity of Radioimmunoconjugates

In clonogenic survival assays the surviving fraction (SF) of MDA-MB-468 cells was reduced to 52 ± 1.3% following exposure to IR (4 Gy) alone (P < 0.0001) (Figure 4). Unlabeled, non-cleavable immunoconjugate, anti-γH2AX-PNE, was not itself cytotoxic and did not significantly enhance the cytotoxic effect of IR (P > 0.05). Radiolabeled non-cleavable immunoconjugate, 111In-anti-γH2AX-PNE, reduced SF to 62 ± 4% and showed an additive effect in combination with IR when SF was reduced to 41 ± 3% (P < 0.05).

Increasing the concentration of 111In-rIgG-N-SS-E or 111In-anti-γH2AX-N-SS-E had no effect on the SF of sham-irradiated 231-H2N cells (Figure 4A). Similarly, 111In-rIgG-N-SS-E had no effect on SF in irradiated cells. However, the addition of 111In-anti-γH2AX-N-SS-E did enhance the cytotoxicity of IR, although only at the highest concentration tested (50% at 0 μg/mL versus 31% at 0.5 μg/mL; P < 0.01). As for 231-H2N cells, 111In-rIgG-N-SS-E did not affect survival in MDA-MB-468 cells (Figure 4A). In contrast, in irradiated MDA-MB-468 cells, 111In-anti-γH2AX-N-SS-E reduced the clonogenic survival in a dose-dependent manner across the range of concentrations tested (75% at 0 μg/mL versus 19% at 0.25 μg/mL; Spearman P-value = 0.0028; P<0.001), whereas 111In-anti-γH2AX-N-SS-E caused a less marked but statistically significant reduction in SF in sham-irradiated cells (100% at 0 μg/mL vs. 67% at 0.25 μg/mL; P < 0.001) (Figure 4B).

Increasing the specific activity (SA) of 111In-anti-γH2AX-N-SS-E resulted in a SA-dependent reduction in clonogenic survival in irradiated cells (Figure 4C,D). This effect was more profound in MDA-MB-468 cells that have high EGFR-overexpression, but was also observed in 231-H2N cells. Also, at high SA 111In-anti-γH2AX-N-SS-E caused a modest fall in SF in sham-irradiated MDA-MB-468 but not 231-H2N cells (Figure 4C,D). One possible explanation is that MDA-MB-468 cells have a higher background number of γH2AX foci compared to 231-H2N cells (13), which would be expected to lead to some retention of the
anti-\(\gamma\)H2AX antibody containing immunoconjugate. \(^{111}\)In-rIgG-N-SS-E had no significant effect on SF in irradiated or sham-irradiated EGFR positive or negative cells. As a control, \(^{111}\)InCl\(_3\) was added to cells in equivalent amounts to the immunoconjugates, but it did not influence clonogenic survival in irradiated or sham irradiated cells (\(P>0.05\)).

**Biodistribution**

SPECT was performed 24 h following i.v. administration of \(^{111}\)In-anti-\(\gamma\)H2AX-N-SS-E or \(^{111}\)In-rIgG-N-SS-E to MDA-MB-468 xenograft-bearing mice treated with IR (10 Gy) delivered to the xenograft. The tumor uptake of \(^{111}\)In-anti-\(\gamma\)H2AX-N-SS-E appears modest on SPECT images because of the proximity of tumors to the kidneys, however VOI analyses were indicative of a detectable signal-to-background signal (Figure 5A). At 24 h p.i, the tumor uptake of \(^{111}\)In-anti-\(\gamma\)H2AX-N-SS-E was greater in irradiated xenografts than in unirradiated control animals (6.30 ± 1.47 %ID/g versus 2.57 ± 0.47 %ID/g, respectively; \(P<0.01\)). Tumor uptake of the non-specific control agent \(^{111}\)In-IgG-N-SS-E was significantly lower in irradiated animals, compared to \(^{111}\)In-anti-\(\gamma\)H2AX-N-SS-E (2.01 ± 0.01 %ID/g versus 6.30 ± 1.47 %ID/g, respectively; \(P<0.01\)). When an excess of EGF was co-injected with \(^{111}\)In-anti-\(\gamma\)H2AX-N-SS-E to block EGFR, uptake in irradiated xenografts decreased to 1.07 ± 0.14 %ID/g (\(P<0.001\)). \(\gamma\)H2AX staining of sections of tumor, taken from mice after 10 Gy irradiation, showed marked induction of \(\gamma\)H2AX foci (9.8 ± 0.4 foci/cell), even at 24 h after irradiation, compared to sham-irradiated tumors (2.2 ± 0.3 foci/cell) (Figure 5B). To demonstrate \(\gamma\)H2AX targeting, tumor sections were stained for \(\gamma\)H2AX and anti-\(\gamma\)H2AX-N-SS-E (Figure 5C). Some degree of non-specific background staining of anti-\(\gamma\)H2AX-N-SS-E was observed, but anti-\(\gamma\)H2AX-N-SS-E colocalized with every \(\gamma\)H2AX focus.

Taken together, these results demonstrate that \(^{111}\)In-anti-\(\gamma\)H2AX-N-SS-E uptake in tumors is dependent on \(\gamma\)H2AX expression, anti-\(\gamma\)H2AX/\(\gamma\)H2AX interaction and EGF/EGFR
binding. Liver and kidney uptake of $^{111}$In-anti-$\gamma$H2AX-N-SS-E was relatively low (4.67 ± 1.29 %ID/g and 9.00 ± 1.02 %ID/g at 24 h p.i., respectively). There was no significant difference in liver and kidney uptake whether $^{111}$In-anti-$\gamma$H2AX-N-SS-E or $^{111}$In-rIgG-N-SS-E was administered. Liver and kidney uptake was similar in irradiated and control mice for both tracers ($P > 0.05$).

**DISCUSSION**

Radionuclides that emit Auger electrons are of interest as therapeutic agents, due to the high linear energy transfer (LET) and short range in tissue of these particles. Since ionizations are clustered within several cubic nanometres around the point of decay of Auger electron-emitting radionuclides, leading to local absorbed radiation doses in excess of 100 Gy, their targeted delivery to the DNA of cancer cells holds promise as a therapeutic strategy in cancer (14, 15). We have previously shown that the combination of IR and $\gamma$H2AX targeted Auger electron exposure is a very effective tumor control measure *in vitro* as well as *in vivo* (3, 4, 15). Here, our aim was to increase tumor selectivity of a $\gamma$H2AX-targeting Auger electron-emitting compound by adding an EGF moiety.

Using a synthetic strategy similar to one we have employed in the past to target the cyclin-dependent kinase inhibitor protein, p27$^{\text{kip1}}$ (10), EGF was tagged with NLS and covalently linked to an anti-$\gamma$H2AX antibody via a non-cleavable PEO6 linker to generate $^{111}$In-anti-$\gamma$H2AX-PNE (Supplementary Figure S1A). $^{111}$In-anti-$\gamma$H2AX-PNE bound to EGFR on the surface of breast cancer cells, and was internalized and distributed within the cell, mirroring the subcellular distribution of native EGF (Figure 1A). Although anti-$\gamma$H2AX-PNE retained affinity for the $\gamma$H2AX protein (Supplementary Figure S2B), a fluorophore-labeled
conjugate did not co-localize with IR-induced γH2AX foci in cells (Figure 2B). The most likely explanation for this observation is that the concentration of AF555-anti-γH2AX-PNE in the nucleus was too low to generate a detectable signal.

Therefore, the immunoconjugate was re-designed to allow improved DNA-targeting after internalization. A conjugate was synthesized with the EGF and γH2AX-NLS sections separated by a disulphide bond (Supplementary Figure 1B). Disulphide bonds have been used extensively in antibody-drug conjugate (ADC) design, to enable delivery of drugs to cells through linkage to antibody vectors (16). Upon internalization of this conjugate, the disulphide bridge is cleaved through the action of reductive intracellular thiols such as glutathione (8). We have shown that this approach can be used for the release of the DNA-targeting, Auger electron-emitting portion of the radiotherapeutic agent, $^{111}$In-anti-γH2AX-NLS from EGF. Reductive cleavage of disulfide bonds is a general property of all cells, and takes place after acidification of the early endosome. This explains the recent success of antibody-drug conjugates, where tumor-selective antibodies are linked to cytotoxic drugs that, upon cellular internalization, are cleaved from their antibody carriers (17). Peptide-drug conjugates have also been investigated. For example camptothecin linked through disulfide conjugation to substance P, was cytotoxic in a range of cell lines (U87, U251, MCF-7, and MDA-MB-231) that express NK1R, the receptor for substance P (18).

Several endogenous compounds and enzymes play a role in the cleavage of disulfide bonds in endosomes and lysosomes (19). Of these, glutathione and cysteine constitute the major physiological thiols responsible for the intracellular reducing environment, with glutathione being the most prevalent cellular thiol accounting for >90% of the total non-protein sulfur (20). Another mechanism of endosomal disulfide bond cleavage is the activity of the enzyme, GILT (γ-interferon-inducible lysosomal thiol reductase). One study used folate conjugated to a BODIPY fluorescent probe, conjugated to a rhodamine fluorescent
probe through a reducible disulfide linkage. It was shown that the disulfide bond was reduced very efficiently throughout the entire intracellular folate trafficking process (21). Here, we chose to demonstrate disulfide bond cleavage using glutathione since it is the most prevalent intracellular reductive agent. Taken together, reductive cleavage of disulfide linkers is a process that has been observed in the endosomes and cytosol of many different cells, and, as here, provides an excellent mechanism to deliver nuclear targeting radiopharmaceuticals to cancer cells.

Anti-γH2AX-N-SS-E functions as intended, as it was shown to bind to EGFR (Supplementary Figure 2A) and to internalize into cells in an EGF/EGFR dependent manner (Figure 2). In confocal microscopy experiments, AF555 signal was clearly seen in the nuclei of irradiated cells that expressed abundant γH2AX, suggesting that cleavage at the disulfide bond of AF555-anti-γH2AX-N-SS-E had occurred, with release and translocation to the nucleus of AF555-anti-γH2AX-NLS (Figure 2B, Figure 3A). This process, which would be expected to be similar for 111In-labeled anti-γH2AX-N-SS-E, would result in the localization of the Auger electron-emitting radioisotope, 111In, near the DNA, leading to reduced clonogenic survival, as shown in Figure 4. The proposed mechanism of action of 111In-anti-γH2AX-N-SS-E is summarized in Supplementary Figure S7. First, 111In-anti-γH2AX-N-SS-E binds to EGFR, expressed on the cell surface and is internalized by ligand-induced endocytosis into clathrin-coated pits. The disulfide bond is reductively cleaved, with release of 111In-anti-γH2AX-NLS, which escapes from the endosome into the cytoplasm. The mechanism of this endosomal escape remains to be elucidated, but has been observed before by Costantini et al, who showed increased cytotoxicity of 111In-labeled trastuzumab when conjugated to NLS (22). The escape mechanism may be similar to that of the TAT-peptide, which permeabilizes membranes by generating topologically active saddle-splay ("negative Gaussian") membrane curvature through multidentate hydrogen bonding of lipid head groups.
(23, 24). Then, the NLS tag interacts with importins so that anti-γH2AX-NLS is transported into the nucleus, where it binds to IR-induced γH2AX, expressed in foci at the sites of DSBs.

The release of $^{111}$In-anti-γH2AX-NLS from the delivery-moiety, EGF, led to marked nuclear accumulation of $^{111}$In in irradiated cells with induced γH2AX expression (Figure 3A), and localization at DSB sites (Figure 2A). Importantly, it was shown that increased intranuclear accumulation of $^{111}$In was not the consequence of increased cellular uptake of the compound due to EGFR copy number modulation, which remained unchanged after irradiation, under the experimental conditions used (see Supplementary Figure S6). The extensive nuclear localization of $^{111}$In following exposure of cells to $^{111}$In-anti-γH2AX-N-SS-E translated into a marked reduction in clonogenic survival (Figure 4B, 4D). Reduction in survival of EGFR-overexpressing MDA-MB-468 cells that expressed γH2AX through prior exposure to IR, was radiation dose-dependent, since it correlated with the specific activity of the cleavable conjugate, $^{111}$In-anti-γH2AX-N-SS-E (Figure 4D).

Given their very short pathlength, Auger electrons emitted from radionuclides are most radiotoxic when emitted close to DNA. This makes their use distinctly different from previously reported EGFR-targeting radioimmunotherapeutics, which mainly incorporate alpha- or beta-emitting radionuclides. The use of $^{177}$Lu (25), $^{90}$Y (26, 27), and $^{231}$Bi (28) labeled anti-EGFR antibodies has shown great promise for radioimmunotherapy. However, because these agents were investigated using different cell models, and given the unique radiobiological characteristics of Auger electron emitters (29), a direct comparison with the method reported here is not straightforward. Direct comparison between $^{111}$In-anti-γH2AX-N-SS-E and $^{111}$In-DTPA-EGF is possible since they are both labeled with $^{111}$In (6, 13). In MDA-MB-468 cells, a similar concentration and amount of $^{111}$In only reduced clonogenic survival to 81 ± 5%, compared to 100% for untreated cells, 74 ± 13% for 4 Gy IR, or 19 ± 3% for $^{111}$In-anti-γH2AX-N-SS-E (plus 4 Gy IR). The main difference between $^{111}$In-anti-
γH2AX-N-SS-E and ¹¹¹In-DTPA-EGF is that the former has a defined intranuclear DNA-associated target, whereas the latter does not, so relies on nuclear accumulation due to EGFR NLS. A similar mechanism accounts for the radiotoxicity of the ¹²⁵I-labeled anti-EGFR mAb 425 antibody (30) or ¹²⁵I-m225 (31). Although several studies show that even ¹²⁵I-labeled non-internalizing antibodies can have potent cytotoxic effects (31), the recently described ¹¹¹In-labeled anti-EGFR antibody, nimotuzomab, which is in itself a cytotoxic agent, conjugated to a nuclear localization sequence, demonstrates very elegantly that conjugation of a nuclear localization sequence to the anti-EGFR antibody results in increased nuclear uptake, and therefore leads to increased radiotoxicity (32). One could hypothesize that all of these agents could be further improved if a DNA-targeting moiety were to be added.

*In vivo* biodistribution studies confirm that ¹¹¹In-anti-γH2AX-N-SS-E uptake in EGFR-expressing MDA-MB-468 xenograft tumors is dependent on γH2AX expression, anti-γH2AX/γH2AX interaction and EGF/EGFR binding (Figure 5A,B), and that anti-γH2AX-N-SS-E targets γH2AX in irradiated tumor tissue *in vivo* (Figure 5C). Taken together, the results reported in this paper show the EGFR-specific, tumor-specific delivery of a γH2AX-targeting radiopharmaceutical for Auger electron therapy, and encourage further investigation of this type of bispecific conjugate.

**CONCLUSION**

The cleavable radiolabeled immunoconjugate, ¹¹¹In-anti-γH2AX-N-SS-E, binds to EGFR and, following receptor-mediated internalization, associates with γH2AX in the nucleus of cells with DNA DSB. The resulting exposure of DNA to Auger electron radiation causes DNA damage and reduces cell growth.
ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. (A) MDA-MB-468 cells were exposed for 1 or 4 h to AF488-labeled EGF and AF555-labeled anti-γH2AX-PNE, fixed, and mounted with DAPI (blue). (B) MDA-MB-468 cells were exposed for 1 h to AF555-labeled anti-γH2AX-PNE or rIgG-PNE, and irradiated (4 Gy) or sham irradiated. After 4 or 24 h, cells were fixed, stained for γH2AX (green) and mounted with DAPI (blue).

Figure 2. (A) MDA-MB-468 cells were exposed for 1 h to AF555-labeled anti-γH2AX-N-SS-E or rIgG-N-SS-E, and irradiated (4 Gy) or sham irradiated. After 4 or 24 h, cells were fixed, stained for γH2AX (green) and mounted with DAPI (blue). (B) MDA-MB-468 cells were exposed for 2 h, at 4°C to AF488-labeled EGF (green) and AF555-labeled rIgG-N-SS-E (red), with or without an excess of unlabeled EGF. Cells were fixed and mounted with DAPI (blue).

Figure 3. (A) MDA-MB-468 or 231-H2N were exposed to 111In-anti-γH2AX-N-SS-E (indicated by agH), 111In-anti-γH2AX-dNLS-SS-E (with a disrupted NLS sequence; indicated by αγ-H-D) or 111In-rIgG-N-SS-E (incorporating non-specific rabbit IgG; indicated by rIgG) for 4 h, irradiated (4 Gy) or sham irradiated, and the relative amount of 111In in the cytoplasm and the nuclei of cells was determined. Results are shown as the mean internalized amount of 111In, relative to the total added amount, ± SD of three independent measurements; * P < 0.05. In retention assays (B) MDA-MB-468 or (C) 231-H2N cells were exposed for 1 h to 111In-anti-γH2AX-N-SS-E or 111In-rIgG-N-SS-E, irradiated (4 Gy) or sham irradiated, and supplied with fresh growth medium. At selected times, the percentage of 111In remaining in
cells was determined. Results are shown as the mean ± SD of three independent measurements. * $P < 0.05$

Figure 4. (A) 231-H2N or (B) MDA-MB-468 were exposed to increasing amounts (0-0.25 µg in 500 µL; or 0-0.5 µg/mL) of $^{111}$In-anti-γH2AX-N-SS-E or $^{111}$In-rIgG-N-SS-E for 24 h, and irradiated (4 Gy) or sham-irradiated 1 h after addition of immunoconjugates. Clonogenic survival fractions were determined. (C) The effect of increasing specific activity (SA) of immunoconjugates was investigated in 231-H2N or (D) MDA-MB-468 cells which were exposed to PBS (P), unlabeled anti-γH2AX-N-SS-E (0), unlabeled rIgG-N-SS-E (0), $^{111}$In-anti-γH2AX-N-SS-E (1-6 MBq/µg) or $^{111}$In-rIgG-N-SS-E (1-6 MBq/µg) for 24 h, or increasing equivalent amounts of $^{111}$In chloride. Cells were irradiated (4 Gy) or sham-irradiated 1 h after addition of immunoconjugates. Clonogenic survival fractions were determined. All results are shown as the mean ± SD of three independent measurements. * $P < 0.05$

Figure 5. (A) VOI analysis of mice bearing irradiated (10 Gy; 2,3,4) or non-irradiated (1) MDA-MB-468 xenografts 24 h after injection of $^{111}$In-anti-γH2AX-N-SS-E or $^{111}$In-IgG-N-SS-E. In some cases an excess of EGF was co-administered to block EGFR receptors. Quantifications are shown as the average ± standard deviation of three independent measurements. ** $P < 0.01$; *** $P < 0.001$. (B) Sections from irradiated (10 Gy) or sham-irradiated (0 Gy) MDA-MB-468 xenografts, harvested 24 h after irradiation, were stained for γH2AX (scale bar = 10 µm). (C) As in B. Sections from irradiated tumours were also stained using Alexafluor555-conjugated goat anti-rabbit antibodies, to detect rabbit anti-γH2AX antibodies.
Figure 1

A

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B

4 h post IR

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0 Gy

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4 Gy

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Figure 2

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Figure 3

A

[Bar chart showing internalized 111In (%)]

- Cytoplasm
- Nucleus

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B

[Retention (%)]

C

[Retention (%)]
Figure 4

A

Clonogenic survival

Imunoconjugate amount (μg)

B

Clonogenic survival

Imunoconjugate amount (μg)

C

Surviving fraction (%)

P 0 1 2 3 4 6

no IR 4 Gy IR

D

Surviving fraction (%)

P 0 1 2 3 4 6

no IR 4 Gy IR

SA (MBq/μg) or equivalent activity

111In-rIgG-N-SS-E

111In-rIgG-SS-E + IR

111In-anti-γH2AX-N-SS-E

111In-anti-γH2AX-N-SS-E + IR

P 0 1 2 3 4 6

no IR 4 Gy IR

111In-rIgG-N-SS-E

111In-anti-γH2AX-N-SS-E

111In
Figure 5

A

![Bar graph showing %ID/g for different treatments and organs.](image)

B

![Images of DAPI, γH2AX, and merge for 0 Gy and 10 Gy treatments.](image)

C

![Images of DAPI, γH2AX, Cy3-anti-γH2AX-SS-E, and merge for 0 Gy and 10 Gy treatments.](image)
Molecular Cancer Therapeutics

Molecular Radiotherapy using Cleavable Radioimmunoconjugates that Target EGFR and γH2AX

Bart Cornelissen, Andrew Waller, Sarah Able, et al.

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