Targeting the Nucleus: An Overview of Auger-Electron Radionuclide Therapy

Bart Cornelissen* and Katherine A Vallis

MRC/CRUK Gray Institute for Radiation Oncology and Biology, University of Oxford, Oxford, United Kingdom

Abstract: The review presented here lays out the present state of the art in the field of radionuclide therapies specifically targeted against the nucleus of cancer cells, focussing on the use of Auger-electron-emitters. Nuclear localisation of radionuclides increases DNA damage and cell kill, and, in the case of Auger-electron therapy, is deemed necessary for therapeutic effect. Several strategies will be discussed to direct radionuclides to the nucleoplasm, even to specific protein targets within the nucleus. An overview is given of the applications of Auger-electron-emitting radionuclide therapy targeting the nucleus. Finally, a few suggestions are made as how radioimmunotherapy with nuclear targets can be improved, and the challenges that might be met, such as how to perform accurate dosimetry measurements, are examined.

Keywords: Auger-electron, radionuclide, radioimmunotherapy, nucleus, targeted radiotherapy, molecular radiotherapy, PRRT.

1. INTRODUCTION

The management of solid cancers relies on a combination of surgery, systemic chemotherapy and locally delivered radiotherapy. External beam X-ray radiotherapy (XRT) is used in approximately 50% of all cancer patients as it is able to cause DNA damage to tumour cells, thereby initiating apoptosis, senescence, necrosis, genetic instability, mitotic catastrophe and eventually tumour regression [1, 2]. XRT, as a stand-alone modality or in combination with chemotherapy, has a good record in the management of locally confined disease, but is not effective for metastatic lesions or disseminated disease. Radionuclide therapy uses radioisotopes that emit charged particles such as electrons or alpha particles to cause DNA damage and kill the tumour. These radionuclides are sometimes used as such, as is the case for ^{131}I for thyroid ablation therapy [3], or ^{89}Sr for the management of pain due to bone metastases [4]. More often than not, the radionuclides are incorporated in a "carrier" molecule, which delivers the radionuclide to the tumour. This carrier can be (1) a small molecule (e.g. $^{125/131}$I-labelled metaiodobenzylguanidine (MIBG), ^{211}At-labelled metaastatobenzylguanidine (MABG), ^{32}P-orthophosphate, ^{153}Sm-ethylenediamine tetra(methylene phosphonic acid) (EDTMP) or ^{186}Re-1-Hydroxy Ethylidene-1,1-Diphosphonic Acid (HEDP)); (2) a peptide (^{111}In-octreotide, ^{177}Lu-octreotide); (3) a protein (^{111}In-labelled epidermal growth factor (EGF)), (4) an oligonucleotide, (5) an affibody or antibody or its fragments (^{211}At-anti-Her2 affibodies, Bexxar, Zevalin), or (6) a nanoparticle into which one of the above is incorporated. This complex of radionuclide and carrier molecule is referred to as a radiopharmaceutical. Once a radiopharmaceutical is localised in a cancerous lesion, it can irradiate the tumour from within. Because of tumour-specific delivery, radionuclide therapy is also called targeted or molecular radiotherapy (tRT). Where antibodies or peptides are used to guide the radionuclide to epitopes or receptors on malignant cells, the terms radioimmunotherapy (RIT) or peptide receptor radionuclide therapy (PRRT) are used, respectively. Especially for non-resectable or metastatic disease, tRT can be a valuable addition to the therapeutic repertoire.

The types of radionuclides used for tRT emit either $^{4}He^{2+}$ particles (alpha-particles), high energy electrons originating from the isotope's nucleus (beta-particles), or low energy electrons from the electron mantle (Auger-electrons (AE), Coster-Kroning (CK) and super-Coster-Kroning electrons (sCK), and internal conversion electrons (CE)) [5-7]. All of these particles can result in DNA damage, and all do so by causing direct damage to DNA, as well as ionisation of water molecules, creating reactive oxygen species (ROS), which in turn cause secondary ionisations and damage of DNA. The contribution of direct DNA ionisation is believed to be much higher for high linear energy transfer (LET) particles like alpha-particles and Auger-electrons, compared to low-LET radiation like XRT and beta-particles [5-17]. The range or track-length of alpha and beta particles depends on their kinetic energy, but ranges from 50 to 100 μm for alpha particles and is in the centimetre range for beta particles. Therefore, alpha- or beta-emitting isotopes are able to deposit their energy into cells, and cause DNA damage, even if their decay takes place outside those cells. The case for AEs is very different as their track length is much shorter, 1 nm to 30 μm in water, depending on their kinetic energy. In the case of ^{125}I, most of the AE have a low energy, and most of the energy is deposited in a very small sphere around the decaying radionuclide - up to 10^7 Gy in a 5 nm sphere [18] - causing a large number of ionisations, although the more energetic AE, CE, and diffusion of long-living ROS can induce ionisations micrometres away from the decay site [19]. When emitted in the nucleus, Auger-electrons exhibit the characteristics of

*Address correspondence to this author at the MRC/CRUK Gray Institute for Radiation Oncology and Biology, University of Oxford, Oxford, United Kingdom; Tel: ++44 1865 225874; Fax: ++44 1865 857127;
E-mail: bart.cornelissen@rob.ox.ac.uk

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high linear energy transfer (LET) particles [18]. CK and sCK electrons have even lower kinetic energies and cause less ionisations than AE. CEs have higher energies, and have a much larger range compared to Auger-electrons, some as large as 250 μm (for ^{125}I, at least [20, 21]), and are also less densely ionising [22, 23]. An overview of a selection of Auger-electron-emitters and their characteristics is given in Table **1**.

The review presented here, will mainly focus on the use of Auger-electron-emitting radiopharmaceuticals for tRT.

2. AUGER-ELECTRON THERAPY: WHY IS NUCLEAR TARGETING NECESSARY?

Auger-electrons, discovered in 1922 by Lise Meitner [24] and in 1923 by Pierre Auger [25], are formed when after electron capture, the vacancy created in an inner shell is filled with an electron from a higher energy level. Most of the excess energy is emitted as X-ray energy, but some is released as kinetic energy given to another electron, then called Auger electron, which is emitted. [5, 8, 26-29]. A summary of the characteristics of Auger-electron-emitters and their emissions is given in Table **1**.

The Auger-electron-emitters mainly used for *in vitro* or *in vivo* therapy are ^{125}I, ^{111}In, and ^{123}I; and to a lesser extent ^{67}Ga, ^{99m}Tc, and ^{201}Tl [9, 29]. ^{125}I is the most studied Auger-electron-emitter, and has been used for many *in vitro* experiments, studying the different effects of low-energy electrons on DNA [9, 18, 30-32]. Its long half-life, 60 days, however, makes it somewhat less practical for clinical applications. The physical half-life of the radionuclides should preferably be in the same order of magnitude as the biological half-life. A too long physical half-life increases the necessary amount of radionuclides to be delivered to the tumour cells to allow for reasonable amounts of decays before excretion. A shorter physical half-life, on the other hand, will not give enough time for the targeting process to take place. It seems reasonable to assume that the most suitable physical half-lives range from a few hours up to some days when targeting of disseminated cells is considered. Longer physical half-lives (up to one or a few weeks) might be desirable if high uptakes in solid tumour masses are needed.

The use of low-energy electrons has some advantages over the use of high-energy electron beta-emitters. Because of their long range, beta-particles will overshoot single disseminated cells and small metastases [19, 33-37]. Instead, most of the damage will be done in the surrounding healthy tissues. Auger-electron-emitters therefore cause much less off-target effects than beta-emitters. On the other hand, for larger tumours, this cross-firing from beta-emitters will result in a more homogeneous deposition of energy in the tumour mass even if the radiopharmaceutical has an uneven distribution inside the tumour [38-40]. Also, for larger tumours, a much larger number of Auger-electron-emitting radionuclides is needed to cause the same cytotoxic effects, unless the Auger-electrons are emitted inside the cell's nucleus. In a study by Capello *et al.*, the influence of tumour size on the effectiveness of ^{111}In-octreotide peptide receptor radionuclide therapy (PRRT) was evaluated [38]. In rats bearing small (<1 cm^3) tumours, complete responses were observed, but only partial tumour regressions were observed in the rats bearing larger (> 8 cm^3) lesions. Auger-electron therapy can thus be compared conceptually with alpha- and beta-therapy based largely on their pathlength and their LET. Direct comparison of the various Auger electron emitting isotopes with each other, however, is complicated by the diverse electron emission spectra of these isotopes. For example, ^{125}I emits on average 12.2 Auger-electrons per decay, compared to 6.8 Auger-electrons emitted per decay by ^{111}In, but for ^{111}In the average energy of the AE is higher, and it emits more than three-fold more higher-energy conversion electrons, and is accompanied by two gamma-photon emissions which are considerably higher in energy than the 35 keV photon from ^{125}I (Table **1**) [19, 41].

The relatively short range of most Auger-electrons necessitates the proximity of the radionuclide to the DNA. Microdosimetric modelling shows that, when placed inside the nucleus of a cell, the amount of energy deposited by Auger-electron-emitters on the DNA is approximately 30-fold higher than when it is placed on the membrane [42-44], highlighting the need for nuclear localisation [19, 45, 46]. Therefore, the carrier molecule transporting the radionuclide must direct it there. This can be achieved by (1) allowing the carrier to be built into DNA, as for example is the case with ^{125}I-labelled 5-iododeoxyuridine (^{125}I-IUdR) [47-49]; (2) translocation of the radioisotope to the nucleus, as for ^{111}In-octreotide [50], ^{111}In-labelled epidermal growth factor (EGF) [51-54], or radiolabelled steroids such as ^{125}I-tamoxifen [55-58], by using the normal nuclear translocation of the targeted receptor (somatostatin (sst), EGF receptor (EGFR), or estrogen receptor (ER) respectively); (3) using peptides or proteins that have been modified to include a nuclear localisation sequence (NLS) [59, 60]; (4) making use of the ability of radiolabelled oligonucleotides or peptide nucleic acids (PNAs) to bind to nuclear DNA [7, 19, 61-63]. Examples from these categories will be highlighted in section 3 of this review.

Internalisation and nuclear localisation is not necessary for successful targeted radiotherapy using alpha or beta-emitters. This is due to their longer path length, causing ionisations more than one cell diameter away from their site of decay. However, if a radiopharmaceutical is internalised into cancer cells, it is retained longer within the tumour. This will result in increased dose deposition within the tumour, and consequently increased cell kill. Moreover, when translocated to the nucleus, this effect is even more pronounced, especially for internalising proteins directly iodinated on a tyrosine residue, as nuclear localisation decreases the possibility of lysosomal degradation and excretion of radioiodide [64]. Also, the possibility of cellular internalisation and nuclear translocation unlocks all intracellular and intranuclear epitopes as potential targets, rather than being limited to extracellular or membrane-embedded epitopes.

For Auger-electron-emitters, nuclear localisation leads to a 30-fold increase in absorbed dose to the nucleus, and the DNA contained within it [43]. Therefore, nuclear localisation is the norm for Auger-emitting radiopharmaceuticals for therapy. Auger-emitters that are bound to or associated with DNA, such as ^{125}I-IUdR [65], ^{111}In-labelled oligonucleotides [66], or ^{111}In-EGF [51], will be more effective than radio-

Table 1. The Properties of Some Auger-Electron-Emitters (Adapted from Buchegger *et al.* [19, 41, 259]

	^{125}I	^{111}In	^{123}I	^{99m}Tc	^{67}Ga	^{201}Tl
Half-life (days)	59.4	2.80	0.55	0.25	3.26	3.04
Number of Auger electrons/decay	24.9	14.7	14.9	4.0	4.7	36.9
Auger electron Energy per decay (keV)	12.2	6.8	7.4	0.9	6.3	15.3
AE energy range (keV)	0.02 - 30.3	0.04 -25.6	0.02 -30.35	0.2 - 17.8	0.9 - 9.4	0.07-66.9
Range of Auger electrons in water †	1.5nm - 14.0μm	0.25nm - 13.6μm	0.5 nm - 13.5 μm	13nm - 6.5 μm	0.1 - 2.7 μm	3 nm - 40μm
CE electrons/decay	0.9	0.2	0.2	1.1	0.3	1.1
CE Energy (keV/decay)	7.2	25.9	20.2	15.4	28.1	30.2
CE energy range (keV)	3.7-35	145-245	127-159	100-140	82-291	1.6-153
Range of CE in water (μm) †	0.7-16	205-622	100-130	70 – 112	50-300	0.2-126
Associated gamma emissions (keV)	3535	171.3, 245.4	159.0	140.5	9.1, 9.3, 184, 209, 300, 393	135.3, 167.4
Total energy/decay (keV)	61.4	419.2	200.4	142.6	201.6	138.5
Total energy deposited per decay ($\times 10^{-14}$ Gy kg /Bq /s)	1.0	7.0	3.2	2.3	3.14	2.2

†estimated using data from Emfietzoglou *et al*. [259].

pharmaceuticals merely taken up in the nucleoplasm. However, there is an increasing body of evidence to suggest that nuclear uptake is not strictly required for Auger-electron-emitters to exhibit cytotoxic effects. Pouget *et al.* describe successful tRT in A431 and SKOV cells using ^{125}I-labelled non-internalising antibodies [67, 68]. Using these non-internalising (or better, sparingly internalising) antibodies, *in vivo* tumour growth delay was observed. More recently, our group has studied the cytotoxicity of the ^{111}In-labelled, non-internalising peptide, F3 [1]. In our hands, the F3 peptide was shown to have very limited internalisation into a range of cell lines, and to exhibit no significant nuclear localisation. Nonetheless, ^{111}In-F3, like ^{213}Bi-F3 [69], but not unlabelled F3 peptide, was shown to induce a marked drop in clonogenic survival *in vitro*, as well as tumour growth delay *in vivo*. The mechanism by which membrane-associated Auger-electron-emitters cause cytotoxicity has not yet been elucidated.

Even if nuclear localisation is accomplished, it will be almost impossible to target every single cell in a tumour. Given the limited physical cross-fire and short path-length of Auger-electrons, this would mean that only a limited number of cells get damaged directly by the Auger-electrons. The radiation-induced bystander effect (RIBE), however, might be able to induce cytotoxicity in neighbouring cells that have not been directly irradiated themselves [6, 70-73]. Reviewed extensively elsewhere [49, 74-77], the XRT-induced bystander effect has an extensive contribution to tumour growth delay, and bystander effects resulting from Auger-electron therapy have been described [49, 71, 72, 76, 78]. Xue *et al.* demonstrated RIBE *in vivo* with donor-cells treated with milliBequerel amounts of ^{125}I-IUdR per cell, which are typical amounts for Auger-electron therapy [78]. Although its mechanisms are still poorly understood, it is thought that the irradiated cell (donor) signals to the non-irradiated cell (acceptor) though either cell-cell gap junctions, or in a paracrine way *via* excreted signalling factors. The identity of the signalling factors involved in AE-mediated RIBE remains to be elucidated. A review on XRT-mediated bystander effects mentions lipid peroxide products, inosine nucleotides and cytokines such as tumour necrosis factor-α (TNFα), but underlying their actions is the involvement of ROS such as superoxide radicals [79].

3. HOW TO TARGET THE NUCLEUS: EXAMPLES

Below is an overview of the molecules and molecular targets that have been used in the past, without the goal of giving an exhaustive listing of the applications of Auger-electron therapy. Here, we have broken down this list by the carrier molecule used to deliver the Auger-electron-emitting radionuclide, into (1) small molecules, (2) oligonucleotides and PNAs, (3) peptides, (4) proteins, and (5) antibodies. A schematic overview of the trafficking and nuclear localisation of Auger-electron-emitters is shown in Fig. (**1**).

3.1. Small Molecules

3.1.1. $^{123/125}$I-deoxynucleotides

The first and most extensively studied Auger-electron-emitting radiopharmaceutical is ^{125}I-labelled iododeoxyuridine, or ^{125}I-IUdR [80]. Its applications have been reviewed

[1] Cornelissen B, Target C, Waller A, Kersemans V, Smart S, Vallis K. Molecularly-Targeted Auger Electron Radiotherapy Using Nucleolin-Targeted 111In-F3 Peptide. Proceedings of the 2010 World Molecular Imaging Conference 2010.

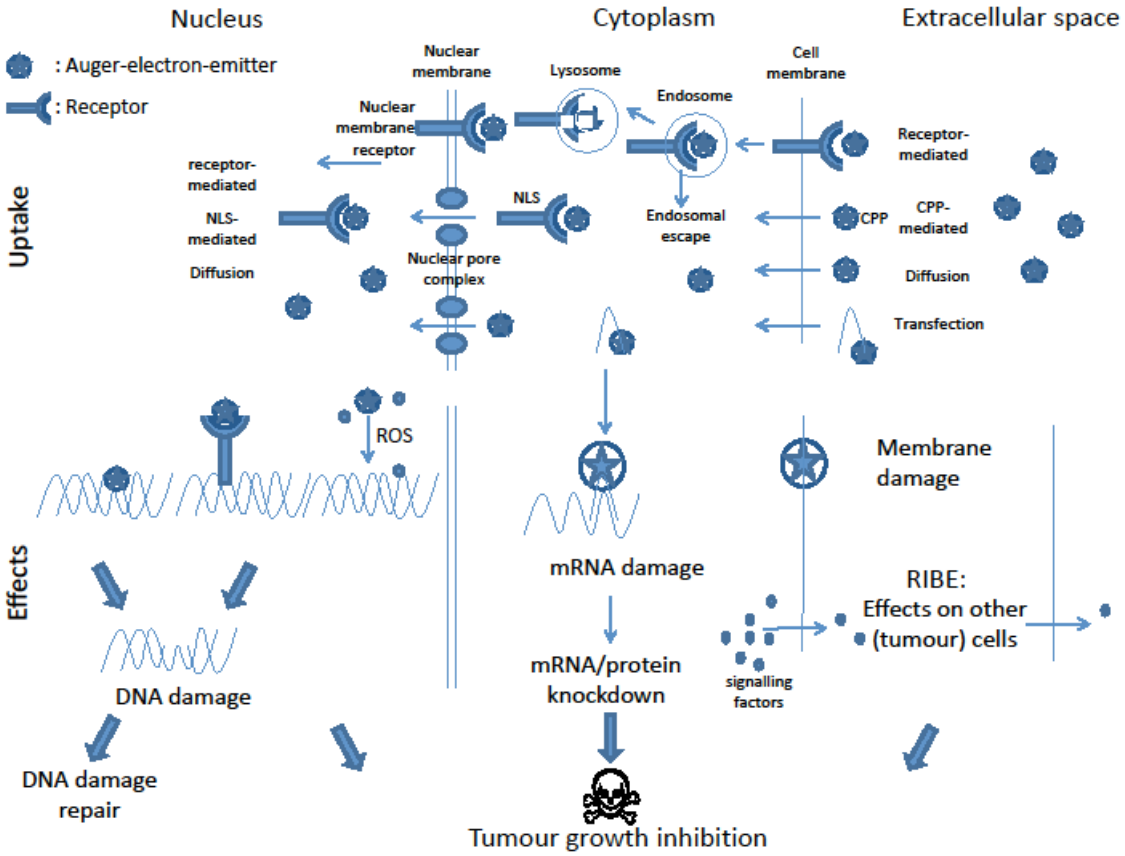

Fig. (1). A schematic overview of the trafficking and nuclear localisation of Auger-electron-emitting radiopharmaceuticals (AER). AER can damage and kill cells by inducing membrane damage, or by internalising into the cell. Internalisation can occur *via* receptor- or cell-penetrating-peptide- mediated endocytosis, or happen *via* diffusion or transfection. Once in the cell, AER internalised by endocytosis escape from the endosome, or are degraded by lysosomes. Nuclear localisation takes place *via* receptor-mediated nuclear transport, or *via* the nuclear pore complex, which can happen *via* diffusion for smaller molecules, or *via* nuclear localisation sequence (NLS)-mediated active transport. AER can bind covalently to DNA, intercalate in the DNA helix, interact with chromatin *via* receptors and scaffolding proteins, or cause ROS species. All of the above results in DNA damage, which is either repaired, or leads to cytotoxicity. Even in cells which have not been affected directly by AER, the radiation-induced bystander effect (RIBE) can induce cytotoxicity *via* excreted signalling factors.

in more detail elsewhere [12]. Since the first studies in the 1970s [81-83], it has advanced to phase I clinical trials [84, 85]. ^{125}I-IUdR or indeed, ^{123}I-IUdR is taken up by tumour cells, where it is phosphorylated by thymidine kinase and thereby trapped in the cell, and is incorporated into DNA during the S-phase of the cell cycle instead of thymidine. *Ex vivo*, nuclear localisation in tumour tissue was shown using micro-autoradiography [12, 86]. Although ^{125}I-IUdR exhibited rapid washout in humans and low tumour uptake (0.005-0.234) [12], tumour to non-tumour ratios were excellent, and tumour control and cure have been observed in the clinic. The main disadvantages of using labelled deoxyribonucleotides are their rapid washout and the limitation to only target cells in the S-phase of the mitotic cycle. Although the percentage of cells in S-phase in the tumour is higher than in normal tissues, only a limited number of cells can be targeted. RIBE is thought to be responsible for the remaining cytotoxicity [49, 72, 78]. Alternatively, it has been suggested that multiple administrations, effectively dose fractionation, of ^{125}I-IUdR could improve cell kill and cure rates. A second way to overcome the short biological half-life of ^{125}I-IUdR after systemic administration is to deliver the radiopharmaceutical locally. This technique has been applied successfully in mouse tumour models of bladder, brain and spinal cord. Other methods have been described by Buchegger *et al.* [19].

Based on the same concept of radiolabelled deoxyribonucleotides, a number of other compounds have been developed. [^{123}I]-5-I-4'-thio-2'-deoxyuridine (^{123}I-IUTdU) was synthesised by Reske *et al.* and shown to cause cell kill in beta-radiation-, gamma-radiation- and doxorubicin-resistant leukaemia cells [87]. Several other groups have utilised thymidine kinase (TK) to selectively trap more radiolabelled thymidine or uridine into cells, by using virally-induced gene expression of this enzyme. ^{123}I-FIAU has been reported to internalise into TK-transfected cells, and to be taken up into the nucleus with cytotoxic effects (Al-Derwish, Mairs, Boyd *et al.*, unpublished results; [88]).

3.1.2. ^{123}I-MIBG

The well characterised radiopharmaceutical $^{123/131}$I meta-iodo-benzyl-guanidine (MIBG) is applied for the detection

and therapy of various tumours such as phaeochromocytomas, neuroblastomas and carcinoids. Radioiodinated MIBG is selectively concentrated in neuroendocrine tumors *via* the noradrenaline transporter (NAT), resulting in selective irradiation of the target tumor cells with relative sparing of normal tissues not expressing NAT. At least in phaeochromocytomas, MIBG uptake is based on a specific catecholamine type I active uptake mechanism and stored in adrenergic storage vesicles [89]. The Auger-electron emission of ^{123}I-MIBG was used by Reingard, Zalutsky and Reilly for the therapy of neuroblastoma cells and spheroids [90-97]. Some studies on the bystander effect have been performed using ^{123}I-MIBG [72], and are reviewed by Mairs [71].

3.1.3. ^{125}I-Steroids

The use of radiolabelled steroids to target steroid-receptor-rich tumour cells has been suggested as early as 1980. Radiolabelled estrogens and ER antagonists bind to the ER, a nuclear membrane receptor, which then translocates to the nucleus. This strategy has been explored using estrogens and androgens labelled with such radioisotopes as ^{123}I, ^{125}I, or ^{80m}Br [98-100]. The use of ^{125}I-labelled tamoxifen, an oestrogen receptor (ER) antagonist, was described by Bloomer *et al.* [101]. Although ^{123}I-tamoxifen has been used as a diagnostic tool to determine ER status in patients with breast or head-and-neck cancer [102], and its potential use as a therapeutic tool has been suggested since the 80's, there are no reports of the use of radiolabelled steroids as a clinical therapeutic agent. *In vitro*, ^{123}I-estrogen (^{123}I-IE) therapy has proven successful in ER-transfected CHO cells and in ER-positive MCF-7 cells, both in monolayer and spheroid cultures reported by Kearney *et al.* [37, 99, 103-106] and Schwartz *et al.* [107]. The latter showed for example that an increasing amount of ^{123}I-IE in cells caused increased amounts of DNA single and double strand breaks (ssb and dsb), as well as chromosomal breaks, and that this correlated with survival. Beckmann *et al.* proved the nuclear localisation and ER-specific uptake of ^{125}I-IE, and also studied chromosomal breaks caused by ^{125}I-IE, but did not quantify this [108]. Neto *et al.* reported the influence of various chemical modifications on the binding affinity of ^{125}I-labelled estrogens [109], which has a great impact on therapeutic effectiveness, as shown by Desombre in a mathematical modelling paper [105]. More recently, Fisher *et al.* have used a radioiodinated (^{123}I, ^{123}I and ^{131}I) version of the ER antagonist diethylstilbestrol (DES), which caused a decrease in cell proliferation, and showed an increase in cell apoptosis [110]. In conclusion, the study of radiolabelled estrogens for tRT has produced some promising findings in terms of tumour specificity, nuclear localisation and tumour cell survival, but these results have yet to be translated to the clinic.

3.1.4. ^{111}In-bleomycin

^{111}In easily forms a complex with bleomycin (^{111}In-BLMC) [111, 112], and it has been observed to be a tumour-targeting agent in head-and-neck squamous cell carcinoma and glioma patients, thus being a useful diagnostic tracer [113-116]. In an autoradiography study by Hou and Maruyama, 78% of ^{111}In-BLMC was localised in the nucleus and nuclear membrane of human small cell lung cancer cells [117, 118]. ^{111}In-BLMC induced more chromosome aberrations than BLMC [118, 119]. The cytotoxicity of ^{111}In-BLMC has been demonstrated in human small cell lung cancer and head-and-neck carcinoma cell lines [120-122]. Moreover, in glioma- or head-and-neck-tumour xenograft bearing nude mice, ^{111}In-BLMC diminished tumour size better than unlabelled BLMC [123-125]. ^{111}In-BLMC is stable *in vitro* and *in vivo* and because it does not bind to transferrin, it does not cause toxicity to the bone marrow [117, 126-128]. ^{111}In-BLMC was used in phase I clinical studies, and the biodistribution, pharmacokinetic behaviour and dosimetry was determined [113], but therapeutic studies were not performed.

3.1.5. $^{191/193m}$Pt-Cisplatin

The platinum core of the chemotherapy agent, cisplatin, can be replaced by Auger-emitting Pt isotopes, such as ^{191}Pt, ^{193m}Pt, or ^{195m}Pt. Howell *et al.* reported that ^{193m}Pt-cisplatin increased the survival of mice injected with B16 melanoma cells, compared to cisplatin [129]. Also, ^{191}Pt-cisplatin was more effective than non-radiolabelled cisplatin in controlling the growth of human squamous-cell carcinoma xenograft tumours in a mouse model [130, 131]. Indeed, the cytotoxicity of 5 mg/kg of ^{191}Pt-cisplatin was estimated to be equivalent to that of 9 mg/kg of unlabelled cisplatin. Further evaluation of ^{191}Pt-cisplatin in Wistar rats revealed no increase in toxicity to the liver, kidneys, or bone marrow, compared to cisplatin [131].

3.1.6. Others

Other small-molecule Auger-emitting radiopharmaceuticals include ^{125}I-daunorubicin, ^{111}In-folate, and ^{125}I-plumbagin. ^{125}I-daunorubicin was synthesized by Ickenstein *et al.* [132]. Cell growth of SK-BR-3 cells was inhibited by ^{125}I-daunorubicin, but not by unlabelled daunorubicin, or by ^{127}I-daunorubicin. Nuclear targeting was demonstrated by fluorescence microscopy. Fondell *et al.* packaged a ^{125}I-labelled doxorubicin analogue in a PEG-stabilised EGF-conjugated liposome nanoparticle dubbed nuclisome [133]. Nuclisomes targeted EGFR-positive cells, and ^{125}I-doxorubicin was internalised. It was previously shown that it was then transported to the nucleus, intercalated into DNA and caused DNA double strand breaks [133]. Doubling time of EGFR-positive U-343 cells was decreased approximately 3-fold by ^{125}I-doxorubicin nuclisome treatment.

3.1.7. Oligonucleotides/PNAs

The natural ability of oligonucleotides and the oligonucleotide mimetic peptide nucleic acids (PNAs) and phosphorodiamidate morpholinos (MORF), to anneal with RNA and DNA makes them an appealing vehicle to bring radionuclides in close proximity to the RNA/DNA. Both ^{125}I and ^{111}In have been used to radiolabel oligonucleotides and have been applied successfully to target over-expression of certain genes involved with cancer. The biggest disadvantage is that predominantly mRNA is targeted, which is mainly localised in the cytoplasm, and not in the nucleus where the Auger-electron emissions would be more effective. One way to circumvent this, is to increase nuclear targeting of the radio-oligonucleotides, which can be achieved by forming a complex of the oligonucleotide with the cell penetrating peptide

(CPP) TAT, which contains a nuclear localisation sequence (NLS) [60].

Zhang *et al.* synthesised ^{125}I-labelled triple-helix forming oligonucleotides (TFO) against the mRNA and DNA encoding the androgen receptor, which is overexpressed in prostate cancer [134]. In a mouse model, subcutaneous tumour growth was inhibited and androgen receptor mRNA and protein expression decreased by ^{125}I-TFO more effectively than by unlabelled TFO. Remarkably, the ^{125}I-TFO was injected without the aid of any transfection agents or other vehicles. Cheng *et al.*, by contrast, packaged ^{125}I-oligonucleotides directed against the α-fetoprotein gene in a chitosan nanoparticle [135]. Inhibition of the growth of the hepatic cancer cell line HepG2 by chitosan-packaged ^{125}I-oligonucleotides was two-fold better compared to non-packaged oligonucleotides. Watanabe at al. used ^{111}In-labelled antisense (AS) oligonucleotides against c-myc mRNA for Auger-electron therapy of neuroblastoma [63]. Packaged within a cationic reverse phase evaporation nanoparticle, ^{111}In-AS exposure reduced c-myc mRNA expression and tumour cell proliferation *in vitro*, compared to unlabelled AS control. *In vivo*, ^{111}In-AS was marginally better in controlling tumour xenograft growth than unlabelled AS and vehicle controls.

3.2. Peptides

Radiolabelled peptides have been very extensively studied for use in radionuclide therapy. This is due to their excellent binding efficiencies, selectivity, and favourable pharmacokinetic behaviour. PRRT is discussed more extensively elsewhere, and we will focus here only on the Auger-electron emitting radionuclide labelled versions. Radiolabelled somatostatin analogues have been studied extensively – more than 200 papers have been published on the subject since 1990. Mainly ^{111}In-octreotide has been studied for Auger-electron PRRT. Also the peptides minigastrin and exendin have been used. Their disadvantage is dose-limiting toxicity to the kidneys, but methods have been developed to overcome this [136-140].

3.2.1. Somatostatin Analogues

The success of somatostatin analogues, such as octreotide, rests on the ability of these peptides to bind to the somatostatin receptor type 2 (sst2), overexpressed on the cellular membrane of neuroendocrine tumours. Upon binding of the peptide to the receptor, the complex is internalised in the cell and then partly translocated to the nucleus [141-144]. In this nuclear localisation lies the opportunity for Auger-electron-emitters coupled to somatostatin analogues. In the early clinical studies, however, ^{111}In-octreotide was not effective, and only minor responses in terms of tumour regression were obtained. A more recent study by the same group [38] suggests that the size of the tumour plays a major role in the responsiveness to ^{111}In-octreotide PRRT.

Ginj *et al.* recently reported on the use of an octreotide analogue (TOC) conjugated to the SV40 large-T antigen nuclear localisation sequence (NLS), labelled with ^{111}In using 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) [145]. ^{111}In-NLS-DOTA-TOC showed enhanced cellular uptake and a 6-fold increase in the cellular retention in SSTR-positive rat AR4-2J pancreatic tumour cells, compared to its parent compound without the NLS sequence. Moreover, nuclear uptake was 45-fold higher when NLS was incorporated in ^{111}In-DOTA-TOC.

3.2.2. NLS-Peptides

Haeflinger *et al.* synthesized a ^{99m}Tc-labelled NLS-peptide derived from the SV-40 large T antigen, conjugated to a DNA-intercalating pyrene [146]. The complex was taken up into B16F1 mouse melanoma cells and translocated to the nucleus, where the pyrene ring brings the ^{99m}Tc in close contact with the DNA [146]. Ginj *et al.* reported the inability of an ^{111}In-labelled version of the same NLS peptide to bind and/or internalise into a panel of cancer cells lines [145], but ^{99m}Tc-NLS-pyrene reduced clonogenic survival of B16F1 cells ten-fold at concentrations as low as 5 nM, ten-fold better than unconjugated $^{99m}TcO_4^-$. The *in vivo* biodistribution or therapeutic applicability of this compound has not yet been reported, but is bound to be limited by the non-specificity of the CPP/NLS, and non-target organ toxicity could be high.

3.2.3. Exendin

Exendin binds the glucagon-like peptide-1 (GLP-1) receptor, overexpressed in tumours such as insulinomas. Exendin-4 analogues, radiolabelled with ^{111}In, have been used with much success for imaging as well as radioimmunotherapy [147-151]. In a Rip1Tag2 transgenic mouse model of multistage insulinoma the therapeutic efficacy of [Lys40(Ahx-DTPA-^{111}In)NH2]-Exendin-4 was investigated. Rip1Tag2 transgenic mice express large-T antigen of simian virus 40 under the control of the rat insulin promoter and subsequently develop tumours of the pancreatic ß-cells in a highly reproducible multistage tumour progression pathway. After intravenous administration of [Lys40(Ahx-DTPA-^{111}In)NH_2]-Exendin-4, the tumour mass in the pancreas was reduced about 10-fold, within 7 days [149]. This reduction lasted for a minimum of 10 days. Moreover, H&E staining showed that haemorrhagic tissue was formed in tumour tissue, but normal islets were unaffected, proving the highly selective nature of this tRT agent.

3.3. Proteins

Several cancer cell signalling proteins, such as epidermal growth factor (EGF), insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF), bind to cell-surface receptors, and are internalised upon receptor binding. Their respective receptors are overexpressed on a large range of tumours. The EGF receptor (EGFR), overexpressed on breast, head-and-neck, and non-small cell lung carcinoma (NSCLC), includes a nuclear localisation sequence, and 10% of the EGF/EGFR complex is translocated to the nucleus [51, 54, 152, 153]. This nuclear translocation was exploited by Reilly *et al.* to produce an ^{111}In-labelled EGF construct, which is selectively radiotoxic to EGFR-overexpressing tumour cells [51, 54]. Nuclear localisation of ^{111}In-EGF was measured to be more than 10% of the internalised protein. A fraction of the ^{111}In-EGF in the nucleus became associated with chromatin. DNA dsb formation in EGFR-overexpressing cells was increased 7-fold. Proliferation, as well as clonogenic survival and *in vivo* tumour growth were inhibited by ^{111}In-EGF in EGFR overexpressing cells and

tumours [73, 154]. Because ^{111}In-EGF uses the EGFR as a carrier to transport it into the nucleus of the target cell, it was possible to increase the cytotoxicity of ^{111}In-EGF by increasing the nuclear translocation of the EGFR by interfering with the intracellular trafficking by using a selective tyrosine kinase inhibitor [54]. A phase I clinical trial has been undertaken to test the safety of ^{111}In-EGF administration, but its results remain to be reported (personal communication).

VEGF, and mainly the alternative splicing forms $VEGF_{121}$ and $VEGF_{165}$, have been used for radionuclide imaging of VEGF receptor subtypes 1 and 2 (VEGFR-1, VEGFR-2), which are correlated to neoangiogenesis [155-159]. The VEGFR-2 receptor possesses a native nuclear localisation sequence, as does the EGFR [160]. Chan *et al.* have attempted to improve the nuclear localisation of radiolabelled $VEGF_{165}$ by adding an NLS-peptide to it [157]. Conjugating the NLS sequence from SV-40 large T antigen improved radiometal nuclear localisation of ^{111}In labelled VEGF by only 50%, but it increased DNA dsb formation approximately 10-fold, and reduced clonogenic survival of VEGFR expressing cells 4-fold. In the case of small target volumes like the vascular endothelial wall, the use of Auger-electrons for therapy is ideal, because the use of large-range high-energy beta emitters like ^{90}Y or ^{177}Lu would result in the deposition of most of the energy outside of the vessel wall. However, where neoangiogenesis is the basis of tumour growth, it might not necessarily be a disadvantage to irradiate the tumour tissue as well as its vasculature.

3.4. Antibodies

Antibodies have been used for radioimmunotherapy since the early 1980's. Bexxar and Zevalin are both FDA-approved beta-emitter radionuclide labelled anti-CD20 antibodies for the therapy of lymphomas [161-164]. However, these antibodies do not internalise upon binding to their target epitope, making them less suitable for Auger-electron tRT. In 1989, Woo *et al* reported the use of the internalising antibody anti-17-1a labelled with ^{125}I, targeted to a tumour-specific antigen associated with gastrointestinal adenocarcinomas [165]. The authors showed that, compared to non-internalising antibodies, ^{125}I-17-1a was selectively radiotoxic, and it caused more chromosomal damage and micronuclei than the non-internalising ^{125}I-R11D10 antibody. In contrast, Pouget *et al.* reported in 2008 that ^{125}I-labelled non-internalising antibodies are more cytotoxic (normalised for the number of accumulated decays per cell, or the deposited dose to the nucleus) [67]. It is hypothesized by the authors that when ^{125}I decays inside an endosome or lysosome, there is a lack of target molecules, whereas on the cell membrane, the high-density energy deposition might generate ceramide within membrane sphingolipid-enriched microdomains, which induces apoptosis.

A well-developed example of clinical application of Auger-electron therapy using antibodies is that of ^{125}I-labelled mAb-425 [17, 166-169]. The internalising anti-EGFR antibody mAb-425 was originally studied in the 90's as a new potential therapy for glioblastoma multiforme (GBM), an EGFR-positive aggressive brain tumour with limited therapeutic options and high morbidity. mAb-425, proved successful in *in vitro* assays, as it reduced clonogenic survival of EGFR-overexpressing cells, in contrast to cells with lower EGFR membrane expression [168]. ^{125}I-mAb425 as well as ^{131}I-mAb-425 reduced tumour growth in mouse xenograft models. In a large phase II clinical trial, a total of 192 patients with GBM were treated with ^{125}I-mAb-425 over a course of 3 weekly intravenous injections of 1.8 GBq following surgery and radiation therapy [169]. Treatment with ^{125}I-mAb-425 alone resulted in a median survival of 14.5 months, compared to the control arm of patients receiving standard care, but not ^{125}I-mAb-425, which had a median survival of just 10.2 months. Combination of ^{125}I-mAb-425 and temozolomide provided the greatest survival benefit with a median survival of 20.4 months. This combination was safe and well tolerated with little added toxicity. Nuclear localisation of the antibody was not reported.

All previous examples make use of an unmodified antibody, whether internalising or not. However, introduction of a NLS-sequence on the antibody would result in more radionuclide to localise to the cell nucleus, closer to the DNA, which could increase its cytotoxicity, and at the same time protect the radioimmunoconstruct from lysosomal degradation. This strategy was explored in a few studies by the Reilly group [60, 170-176]. In a paper by Chen *et al.* conjugation of the SV-40 large T antigen NLS to an ^{111}In-labelled anti-CD33 antibody was reported for the first time [170]. Conjugation of 8 NLS peptides to the antibody increased nuclear localisation 8-fold and decreased clonogenic survival of CD33 positive cells. In 7 out of 9 cases, ^{111}In-anti-CD33-NLS reduced clonogenic survival of cells obtained from plasma of AML patients. Kersemans *et al.* demonstrated that ^{111}In-anti-CD33-NLS could reduce clonogenic survival of multidrug resistant AML cell lines [172]. Similarly, Costantini *et al.* showed that addition of up to 6 NLS peptides to the ^{111}In-labelled anti-HER2 antibody trastuzumab (Herceptin) increased internalisation and nuclear localisation of the antibody and decreased clonogenic survival of HER-2 positive cells [174]. ^{111}In-trastuzumab-NLS was shown to overcome IGF-receptor-induced trastuzumab resistance in breast cancer cells *in vitro* and in a mouse xenograft model [176].

The Hnatowich group used the TAT-peptide to create a streptavidin based ^{111}In-labelled radioimmunoconstruct, containing the HER2-targeting antibody trastuzumab, the CPP/NLS-peptide TAT, and an antisense MORF oligonucleotide against RIα messenger RNA, encoding for the RIα regulatory subunit of cAMP-dependent protein kinase [177, 178]. After only 3 hours of incubation with HER2- and RIα-positive SK-BR-3 cells, 90% of the internalised ^{99m}Tc-MAG3-labelled MORF version of the complex was found in the nucleus. An ^{111}In-DTPA-labelled MORF complex caused decreased clonogenic survival, but significantly more so using the antisense MORF compared to the sense MORF complex. The antisense MORF complex also showed higher nuclear localisation than the sense MORF complex. Biodistribution studies in SUM190 xenograft bearing mice showed excellent concentration of the ^{99m}Tc-labelled streptavidin-MORF-trastuzumab complex in the tumour, and uptake in the liver, kidneys and spleen. Tumour uptake was no different than that of ^{99m}Tc-labelled trastuzumab, although liver and kidney uptake were much higher. Nuclear uptake of a Cy3-labelled complex was demonstrated by immunohisto-

chemistry. These very promising results suggest that *in vivo* tumour growth inhibition could be very successful with this three-component radioimmunoconstruct, labelled with ^{99m}Tc, ^{111}In, or $^{123/125}$I.

A study by our own group used the same NLS-containing TAT-peptides conjugated to ^{111}In-labelled antibodies in a pre-amplification approach [2]. Irradiation with X-rays (XRT) induced DNA dsbs. These tend to be repaired more quickly in normal tissue than in cancer cells. The histone H2AX is rapidly phosphorylated in distinct foci around dsbs, forming γH2AX. We have used ^{111}In-labelled monoclonal antibodies directed against γH2AX, conjugated to the CPP/NLS-peptide TAT, to amplify the DNA damage done by XRT. The number of DNA dsbs was significantly and super-additively increased in a panel of breast cancer cell lines treated with the combination of XRT and ^{111}In-anti-γH2AX-TAT. Clonogenic survival was decreased for the combination treatment, and in a mouse xenograft tumour model, the combination of a single dose of XRT and ^{111}In-anti-γH2AX-TAT produced complete responses in 3 out of 6 mice, compared to none with either treatment alone.

4. IMPROVING NUCLEAR TARGETED AUGER-ELECTRON TRT

All of the radiopharmaceuticals mentioned above are capable to reduce tumour growth *in vitro* as well as *in vivo* in animal models and in the clinic. However, many improvements can still be made to the design of existing pharmaceuticals, or new strategies could be employed. The two main physical barriers to deliver Auger-electrons to the tumour cell nucleus are the cellular and the nuclear membrane. An overview of the possible strategies to overcome these physical barriers and other ways to improve Auger-electron tRT are given in Table **2**. These other strategies include reduction of heterogeneity, increase of Auger-electron emission, combination treatments, dual targeting, pretargeting, pre-activation, and meta-activation.

Increase of the amount of radionuclide delivered to the tumour compared to non-target normal tissues can be achieved for example by the use of bispecific agents, targeting more than one epitope on the tumour cell membrane, such as the recently reported RGD-^{111}In-DTPA-octreotate, which binds the $\alpha_v\beta_3$ integrin as well as the somatostatin receptor [179]. RGD-^{111}In-DTPA-octreotate showed increased tumour apoptosis, compared to ^{111}In-DTPA-octreotate. Karragiannis suggests the use of antibodies to target extracellular epitopes, conjugated *via* an intracellularly cleavable linker to an Auger-electron-conjugated DNA-ligand [180]. Upon internalisation into an endosome, the linker is cleaved, and the DNA-binding ligand is released. Therefore, the intracellular trafficking of this DNA-ligand would not be affected by the antibody used for tumour delivery. Alternatively, the DNA-binding ligand could be contained within a nanoparticle, and be released upon antibody-mediated internalisation.

When targeted to and accumulated into the tumour tissue, internalisation and consecutive nuclear localisation has proven to be the best way to ensure cytotoxicity of Auger-electron-emitting agents. To increase nuclear localisation of oligonucleotides, peptides, proteins and antibodies, the conjugation of NLS-containing peptides will increase nuclear localisation. The strategy has been successfully applied for tRT by the Reilly, Vallis and Hnatowich groups [62, 66, 157, 170-172, 174, 178]. Before the NLS-sequence can bind to the importins in the cytoplasm needed for transportation through the nuclear pore [60], it needs to escape from the endosome in which it was encapsulated after receptor-binding induced endocytosis. The use of endosomal escape sequences (EE) could possibly increase the efficiency of this process, and increase nuclear translocation. Also, it will decrease lysis of the radiopharmaceutical by lysosomal enzymes [60]. Interference with signalling pathways by using e.g. kinase inhibitors can influence the intracellular trafficking of radiopharmaceuticals. In the case of ^{111}In-EGF, the use of gefitinib, a EGFR tyrosine kinase inhibitor, increases EGFR nuclear internalisation and therefore ^{111}In-EGF nuclear uptake [54].

As mentioned elsewhere in this review, heterogenic uptake of the Auger-electron-emitting radiopharmaceutical in tumour tissue is an important issue that can be optimised. Because not every cell is being targeted, there will only be limited cross-fire to non-targeted tumour cells, and this is the main argument to choose for long-range beta and alpha particle therapy – ignoring the RIBE. However, there are strategies that result in more homogeneous delivery of the radiopharmaceutical. Inclusion of a cell penetrating protein in the design of the carrier molecule, for example, increases the penetration of the radionuclide to all parts of the tumour [59, 60, 181]. Also, delivery of drugs and radiopharmaceuticals is highly regulated by the tumour microvasculature [182]. Vascular normalisation techniques could be applied ahead of radiopharmaceutical administration, thereby reducing the poorly vascularised areas in the tumour tissue for a short period of time, and increasing radiopharmaceutical delivery during that time [183].

Auger-electron-emitters that have been used so far for targeted radiotherapy are mostly limited to ^{99m}Tc, $^{123/125}$I, and ^{111}In. $^{191/193m}$Pt has only been used in a few cisplatin-based studies. ^{195m}Pt has the highest Auger-electron yield with 33 electrons emitted per decay versus 24.9 electrons for ^{125}I, 14.9 electrons for ^{123}I, 14.7 electrons for ^{111}In, and between 4 and 7 electrons for ^{77m}Br, ^{67}Ga, ^{55}Fe, and ^{99m}Tc [12]. Therefore, the amount of energy deposited per decay in a 5 nm sphere is much greater for ^{195m}Pt (2000 eV) than for ^{125}I (1000 eV), ^{123}I (550 eV), and ^{111}In (450eV). Given the increasing interest in the use of tRT in general and the use of Auger-electron-emitters in particular, more and more centres are now developing novel radiopharmaceuticals labelled with more potent Auger-electron-emitters, with more low-energy electrons being emitted per decay. Some of the promising radioisotopes include ^{67}Ga [180], ^{119}Sb [184], ^{201}Tl, ^{165}Ho, ^{103m}Rh [185, 186], ^{195m}Hg [187], and ^{195m}Pt [187]. The main challenge here is the implementation of new chemistries, to make the conjugation of these radionuclides possible. Iodine, gallium and platinum complexes have been de-

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Table 2. An Overview of the Various Strategies to Improve Tumour Selectivity, Cellular Uptake and Nuclear Localisation of Auger-electron-Emitting Radiopharmceuticals

Opportunity for Improvement	Solution	References
Radiopharmaceutical not able to enter cell	Use of cell penetrating peptides	[60]
Target intracellular	Use of cell penetrating peptides	[60]
Tumour selectivity low	Bispecificity	[179]
Intracellular trafficking unfavourable	Cleavable linker systems	[180]
Intracellular trafficking unfavourable	Pharmacological interference with trafficking	[54]
Nuclear localisation low	Use of nuclear localisation sequences	[62, 66, 157, 170-172, 174, 178, 260]
Lysosomal degradation	Use of endosomal escape sequences	[60]
Auger-electron yield low	Alternative isotopes	[20, 180, 184-187]
Poor tumour delivery	Vascular normalisation	[182]
Tumour uptake low, and/or heterogenic	Locoregional administration	[191, 192]
Heterogeneity	Fractionation	[191-193, 196-203]
Heterogeneity	Use of cell penetrating peptides	[59, 60, 181]
Normal tissue toxicity	Fractionation	[191-193, 196-203]
Renal toxicity	Nephroprotection	[136-140]
Therapeutic index low	Chemoradiation	[176, 204, 205, 207]
Therapeutic index low	Combination XRT/tRT	[208, 210-212]
Radiopharmaceutical has poor pharmacokinetic behaviour	Pre-targeting	[28, 214-216]
Low/no target expression	Pre-activation	[28]
Use of non-radioactive materials	Meta-activation	[28, 226, 227, 229-231]

scribed before, but as far as the other proposed nuclides are concerned, no satisfactory radiolabelling strategies exist to date. Radiolabelling strategy, the choice of radionuclide, and the choice of chelator are of importance when designing radiopharmaceuticals for therapy, since they can have great impact on the behaviour and efficacy of the compound. A common example is the difference between iodination of peptides, proteins and antibodies *via* the Iodogen method versus the Bolton-Hunter method [188]. Iodogen iodination will result in an unstable bond between radioiodine and tyrosine, which is readily broken *in vivo*. In the case of metal ion radionuclides, a host of chelators is available, but the choice should be driven by suitability for the application. Octreotate, radiolabelled with ^{90}Y, ^{177}Lu or ^{111}In, has a different biodistribution pattern [189, 190], so the choice of the radioisotope itself is also important.

The administration of Auger-electron-emitting radiopharmaceuticals can also be optimised. Not only could locoregional or intratumoural injection be advantageous over systemic administration [191, 192], also the question of fractionation is important in light of the therapeutic index. In low-LET XRT and high-LET external beam charged particle therapy (e.g. protons, ^{12}C ions), the dose deposited in a certain mass (up to 80 Gy), is divided up into many 1-2 Gy subdoses, administered over many days to limit toxicity to off-target tissues. Could this also be the case for radionuclide therapy? Certainly this is so for low-LET beta-emitters. Baum *et al.* [193] showed that, using ^{90}Y-DOTA-TATE or ^{177}Lu-DOTA-TATE at multiple cycles, it is possible to retain tumour regression, but without nephrotoxicity and only minor haematological toxicity. In contrast, Bodei *et al.* found no clear correlation between fractionation of ^{90}Y-DOTA-TOC and ^{177}Lu-DOTA-TATE and toxicity [194]. DeNardo *et al.* state in a 2002 review [195] that preclinical and clinical data have shown that toxicity can be better controlled, the total dose extended, and efficacy increased by multiple dosing at or near the maximum tolerated dose MTD [196-198]. If radionuclide dose is selected at the MTD, then an interval must be chosen between doses that permits adequate normal tissue recovery. DeNardo *et al.* [199, 200] have shown good results for fractionated RIT in patients with non-Hodgkin lymphoma (NHL), using low doses of the beta-emitter ^{131}I-labeled mAbs in 4 or more cycles. Recently, is has been suggested that, especially for high-LET particle therapy, hypofractionation, i.e. a lower number of fractions, or even administration of the entire dose in one fraction might be advantageous [201-203]. This follows from the higher α/β

ratio at higher LET values of normal, healthy, tissues compared to the DNA-repair deficient tumour tissues - α and β being the coefficients of the linear quadratic dose-response curve. This concept might also be valid in tRT with high-LET alpha and Auger-electron-emitters, even though dose rates are lower compared to proton- or carbon-ion therapy. Although fractionation reduces the effects of non-uniform dose-distribution within a tumour, it might be advantageous to administer high-LET tRT in one large dose, or in just a few, hypofractionated doses, compared to many fractions.

Another way of increasing therapy effectiveness is the widely applied method of combination treatment. XRT is commonly used in combination with chemotherapy, and could benefit even more from rationally designed agents that interfere with for example DNA damage repair and cause superadditive effects. The same is true for radioimmunotherapy, but it has not been extensively studied yet. Tijink *at al.* examined the combination of ^{131}I-L19-SIP tRT with cetuximab [204, 205], and Costantini described the combination of the abovementioned ^{111}In-trastuzumab-NLS with the radiation sensitizers methotrexate, paclitaxel, and doxorubicin [176]. Further examples are reviewed by Sharkey [206]. Other combination techniques involve the strategy of pre-activation, where the target of the radiopharmaceutical is induced or upregulated. Examples include Nayak *et al.*, who studied the combination *in vitro* of ^{177}Lu-octreotide with gemcitabine, a DNA-replication inhibitor [207]. They found that pre-treatment with gemcitabine upregulated sst-receptor expression and increased octreotide uptake and apoptosis, and decreased cell viability in a synergistic way. Our group has, as mentioned above, used X-rays or bleomycin to induce the DNA repair protein, γH2AX, as a target for ^{111}In-anti-γH2AX-TAT constructs.

This last work also suggests the strategy of combining low-LET external beam radiotherapy (for control of local disease) with high-LET targeted radioimmunotherapy. This would not only treat disseminated disease, but would also enhance the effect of the XRT [208]. The delivered radiation dose to the tumour will be enhanced, but also the DNA repair response of the tumour tissue to low-LET repair will be altered. Alpha-particle irradiation of tumour cells has been demonstrated to decrease the clonogenic survival following XRT. Combination of the densely ionising alpha particles with XRT inhibits DNA damage repair significantly, because of the complexity of the induced DNA breaks [209]. Finally, XRT may help to improve the uptake and the homogeneity of radiopharmaceutical distribution [208]. A dosimetry approach presented by Bodey *et al.* corroborates some of these suggestions [210-212]. Whenever two therapies are combined, the temporal relationship between the combined agents are very important, and the timing of each agent and each combination will have to be optimised [212, 213].

Pretargeting has been very successfully applied for radioimmunoimaging [28, 214-216]. It involves the administration of a first agent, highly selective to the target, but pharmacokinetically unsuitable for direct radiolabelling. Then, a second, radiolabelled, agent is administered which is highly selective for the first. Pretargeting strategies for tRT have been applied for intraperitoneal as well as systemic tRT [215, 217-224]. When applying pretargeting techniques to Auger-electron-emitters, nuclear localisation becomes an issue for both the primary and the secondary agent.

Another form of pretargeting is dubbed meta-activation [28]. Here, the primary agent is a compound, or is conjugated to a compound, that can be "activated" by external beam irradiation to produce Auger-electrons, and become radiotoxic. This was first demonstrated by Shinohara *et al.* in 1985 using monochromatic synchrotron-produced X-rays, which induce Auger emissions from 5-bromodeoxyuridine in HeLa cells [225]. More examples of meta-activation include the Auger-electron production induced in cold iodine by radio/brachytherapy, as reported by Moiseenko *et al.* [226], induction of low-energy electron emissions from gold nanoparticles when bombarded with high-energy electrons [227], and the activation of Gd-containing compounds by synchrotron produced non-toxic hard X-rays (51 keV) [228], or by neutron capture [229-231].

5. CHALLENGES

Aside from optimising the design and the strategies used in Auger-electron radionuclide therapy, a few challenges remain.

A better understanding of the radiobiology of Auger-electrons, of the types of damage caused by Auger-electrons, and the repair of them, is needed. This could result in increased tumour cell kill and reduced toxicity in normal tissues, but at present the implications for treatment are unclear [32, 232-236]. Actually, the absorbed doses necessary for successful radionuclide therapy are not known, nor are the tolerance doses for normal tissues [37]. Conventional perspectives on the response of tissues to radiation may not adequately describe or predict the effects of targeted radiotherapeutics on tumour and normal tissues [237-239]. tRT is generally characterized by low dose rates, therefore the recently suggested hypersensitivity of mammalian cells to low dose radiation may play a role [240]. Auger-electron-emitters have mixed LET characteristics, as they exhibit low-LET when localised in the cytoplasm, but high-LET characteristics when localised in the nucleus [18]. The bystander effect could also have a profound effect on targeted radionuclide therapy [241]. Moreover, given the proximity needed for the Auger-electron-emitting radionuclides, the structure of the chromatin, i.e. euchromatin vs. heterochromatin, will influence the amount of dsbs formed [242].

Modelling the dose delivered by radionuclide therapy is a topic of much discussion in the literature, not just for Auger, but also for beta and alpha therapies, because of uneven dose distribution within tissues [242]. The widely used whole-body MIRD or OLINDA/EXM algorithms do not take this heterogeneity and nuclear localisation into account [243, 244]. Not only is there non-homogeneous distribution within organs and tissues, but also within substructures, within cells and within the nucleus. Moreover, the distribution of radionuclides in these structures will change over time, due to intracellular trafficking of radiopharmaceutical and its metabolites. All of the aforementioned will profoundly influence tumour regression and normal tissue toxicity, and therefore have to be taken into account when modelling the radionuclide therapy effects. Auger-electron-emitters deposit

most of their energy within a very narrow sphere around their site of decay. As a result, the influence of heterogeneous dose distribution becomes important on a nanometre scale, and modelling should be performed on a nanometre scale as well [245]. However, even the best attempts of modelling have had to make compromises, e.g. in terms of resolution and geometries. The best models, based on Monte-Carlo simulations, can only give S-values on the base of a single cell, or a symmetrically organised monolayer [42, 246]. The early methods used by Charlton and Humm still provide a good estimate of the number of dsbs in single cells [247], but MC methods for Auger microdosimetry [23, 45, 248-251] incorporate all data from Auger, CE and CK electron emissions from radionuclides. Nevertheless, it has proven difficult to take the non-uniformity of intracellular and intranuclear distribution, and the structure of the chromatin itself into account, and the models still only provide dosimetry on a single-cell basis, which can give but an approximation for solid tumours [18, 42, 246]. According to Brans [242], radionuclide dosimetry should be able "(1) to establish individual minimum effective and maximum tolerated absorbed doses; (2) to establish a dose–response relation to predict tumour response and normal organ toxicity on the basis of pre-therapy dosimetry; (3) to objectively compare the dose–response results of different radionuclide therapies, either between different patients or between different radiopharmaceuticals, as well as to perform comparisons with the results routinely obtained with external radiotherapy; (4) to increase the knowledge of clinical radionuclide radiobiology, in part with the aim of developing new approaches and regimens". In order for tRT dosimetry models to do all of the above, they would need to incorporate the repair processes of DNA damage as well. As these processes are very complex and involve a whole host of factors, and the exact role of some of the proteins involved is still unclear [252, 253], this task is a very complex one, and would ultimately have to involve systems biology. Some simulators have already been developed and include DNA damage repair processes in their programs, and have been validated *in vitro* [246, 254-258]. Despite these simulators still using a one cell DNA model, they could provide some insight on potentially useful radionuclides for targeted tumour radiotherapy.

6. CONCLUSION

Molecularly targeted radiotherapy treatments for cancer, based on the low-energy, small-range emission of Auger-electrons, are a promising addition to the anti-cancer armamentarium. Some have been shown to be successful in the clinic, and many more have been evaluated positively in the preclinical setting in animal models. The search continues for more specific cancer-targeting agents, with lower toxicity to non-target organs. Good micro-dosimetry methods are in the process of being developed, but cannot yet predict the dose and toxicity on a DNA-size scale for the whole body.

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