

Evaluation of DNA damage in a *Her2*+ cell line induced by an Auger-emitting immunoconjugate

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ABSTRACT

Introduction: Auger electron based radioimmunotherapy (RIT) using ^{111}In -DOTA-trastuzumab (^{111}In -DOTA-antiHer2) feasibility was studied *in vitro* on a HER2/neu positive cell line, SkBr3.

Methods: ^{111}In -DOTA-antiHer2 was prepared according to the optimized conditions followed by quality control tests including radiochemical purity; immunoreactivity). SkBr3 as a HER2/neu positive cell line was used to determine the degree of internalization and cell viability of ^{111}In -DOTA-antiHer2. For comet assay freshly cultivated SkBr3 cells incubated with ^{111}In -DOTA-antiHer2 in 3.7 and 7.4 MBq doses at 37°C for 24h.

Results: ^{111}In -DOTA-antiHer2 (>95% radiochemical purity; >79% immunoreactivity) demonstrated significant internalization in SKBr3 cells in 24 h. no significant cell viability difference observed for ^{111}In -DOTA-antiHer2 and ^{111}In cation treatments. Comet assay at 3.7 MBq demonstrated no significant DNA damage, while at 7.4 MBq dose DNA damage observed at least 16% more than control In-111 chloride after 24 h.

Conclusion: Although the internalization of ^{111}In -DOTA-antiHer2 was approved in this study, however, lack of cell death and slight DNA breakage for ^{111}In -DOTA-antiHer2 treatment suggests absence of nucleus entry which is essential for demonstration of DNA damage according to Auger electron range at cellular level.

Key words: Auger therapy; ^{111}In -DOTA-antiHer2; SkBr3; Immunoreactivity; Internalization; Cell viability; Comet assay

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INTRODUCTION

In targeted therapy, the shorter the range of the particle emission, the better targetry and lower imposed dose to the surrounding cells, on condition that the radionuclide is close enough to target DNA molecules in nucleus and/or mitochondria in case of mammalian cells. β -emitters now are the frontier radionuclides used in the development of therapeutic radiopharmaceuticals, however their relative particle long range often impose adverse effects to the surrounding cells [1]. On the other hand α -emitters offer better opportunities for targeted therapy applications in case they are internalized into target cells or even closer to the DNA molecules. Their low availability and high costs in production and distribution has limited application of these radionuclides for vast therapeutic applications [2]. Auger electrons are produced in cascades during electronic shell transitions followed by electronic capture and/or conversion in some radionuclides with a very low energy (from a few eV to a few keV). They are considered as high LET particles (from 4 to 26 keV/ m; energy <1 keV). Thus, their path length in biological matter is very short, and efforts have been done to bring Auger electrons in the cell nucleus to obtain the highest cytotoxicity. The clinical radioimmunotherapy (RIT) studies using Auger electrons such as I-125 labeled to monoclonal antibodies (mAbs) have shown no solid results [3, 4]. The possibility of iodinated antibody instability and long half-life limited the application I-125 agents for human use. In-111 is an interesting radionuclide used in the development various radiolabeled antibodies

for imaging studies during last 3 decades. This cyclotron-produced radionuclide produced usually via $^{112}\text{Cd}(p,2n)^{111}\text{In}$, is available in no carrier added form and its 67 h half- life matches the biological half-life of full size mAbs (1-3 d) for ultimate use in radioimmunosciintigraphy and also allows enough shelf life for transport to rather distant centers from the production site. Interestingly In-111 is a source of Auger electrons demonstrating potentials for targeted therapy in case of reaching close to nucleus while carried by appropriate internalizing agents such as antibiotics [5], peptides [6] and antibodies [7] (Table 1).

Not many reports on evaluation and application of In-111 therapeutic agents are available in the literature, however most of the studies have been focused on the development of mAbs and peptides as some have been presented in Table 2.

Recently some In-111 labeled mAbs have been developed for human studies [9, 10] based on interesting chelating agent moiety DOTA, having potentials for radiolabeling of various theranostic radionuclides such as Y-90, Lu-177, Ga-68, etc., in one case ^{111}In -DOTA-trastuzumab (^{111}In -DOTA-antiHer2) production has been reported and in continuation of current Auger electron therapy we were interested in evaluation of its DNA breakage effect *in vitro* based on the comet assay (single-cell gel electrophoresis), a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells as well as cell death.

Table 1: List of some important Auger-emitting radionuclides properties [8].

Radioisotope	Half-life	Average number of Auger and Coster-Kronig electrons	Energies (keV)	Dose from electrons $\Delta(\text{ie-rad.g.}\mu\text{-l.h-1}$
$^{99\text{m}}\text{Tc}$	6.05 h	3	12	n.a.
^{111}In	2.1 d	8	16	0.074
^{125}I	60.5 d	20	24	0.041
^{123}I	13.3 h	11	21	0.032

Table 2: Some selected studies on the therapeutic effects of In-111 radiolabeled biomolecules.

Agent	Malignancy	Study model	Reference
^{111}In -antiCD123	Acute myeloma	Mice engrafted with primary human AML	[11]
^{111}In -dual-antiHER2/EGFR	Human Breast Cancer	Xenografts	[12]
^{111}In -[Lys40(Ahx-DTPA-111In)NH2]-exendin-4	Pancreatic neuroendocrine tumors	Mouse model	[13]
^{111}In -DTPA-hEGF	Human Breast Cancer	231-H2N xenografts	[14]
^{111}In -NLS-antiHER2	Human Breast Cancer	MDA-MB-231 tumors	[15]
^{111}In -HuM195 anti-CD33	Acute myeloma	HL-60 and HL-60-MX-1 cells	[16]
^{111}In -DTPA-hEGF	Human breast cancer	MDA-MB-468	[17]
^{111}In -labeled N-myc antisense oligonucleotides.	Human neuroblastoma cells	Human neuroblastoma cells	[18]
^{111}In -labeled antibodies to HER-2	Human Carcinoma	SK-OV-3.ip.1 cells	[19]
^{111}In -DTPA-octreotide	Thyroid cancer	Human subject	[20, 21]

In comet assay method, cells embedded in agarose on a microscope slide are lysed to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix followed by electrophoresis at high pH. The tail of structures resembling comets, observed by fluorescence microscopy shows the intensity and the number of DNA breaks [22]. In this work we report brief preparation, cell binding, cell internalization, cell viability and comet assay results of ¹¹¹In-DOTA-trastuzumab (¹¹¹In-DOTA-antiHer2) on breast cancer SkBr3 cells as a Her2 positive line.

METHODS

NHS-DOTA was purchased from Macrocycles (NJ, USA). Trastuzumab (Herceptin) was a pharmaceutical sample purchased from Roche Co. Radiochromatography was performed by using a Bioscan AR-2000 radio thin layer chromatography (TLC) scanner instrument (Bioscan, Paris, France). A high purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL) multichannel analyzer and a dose calibrator ISOMED 1010 (Dresden, Germany) were used for radioactive measurements. Calculations were based on the 171 keV peak for ¹¹¹In. All values were expressed as mean ± standard deviation (mean ± SD) and the data were compared using Student's t-test. Statistical significance was defined as P < 0.05.

Production and quality control of ¹¹¹In-InCl₃ sterile solution for *in vitro* studies

Indium-111 chloride was prepared by 22 MeV proton bombardment of the enriched cadmium-112 target at a 30 MeV cyclotron, with a current of 100 μA for 48 min (80 μAh) according to the reported method [23]. Briefly, after the dissolution of the irradiated target by conc. HBr, the solution was passed through a cation exchange Dowex 50 × 8 resin, followed by washing with HBr conc. solution and DDH₂O. Indium-111 was eluted with 1 N HCl as ¹¹¹InCl₃ for labeling use and quality control experiments. For cell studies the appropriate activity was evaporated under reduced pressure followed by the addition of pH.5.5 acetate buffer.

Production and quality control of ¹¹¹In-DOTA-antiHer2

Detailed production and extensive quality control of ¹¹¹In-DOTA-antiHer2 has been reported [24]. Briefly, Trastuzumab (Roche) as a lyophilized powder was purified by Vivaspin-2 filters using ultrafiltration in 0.2 M Na₂CO₃ (pH 9.2) buffer solution followed by antibody concentration measurement and conjugated with DOTA-NHS in bicarbonate buffer and

incubated at room temperature for 12 h. The mixture was then transferred on a Vivaspin 2 cut-off filter (30 kDa) and centrifuged and the conjugation step terminated using ammonium acetate buffer and the antibody concentration was re-measured for determination of the average chelate: antibody ratio the spectrophotometric method was used in presence of arsenazo yttrium(III) complex (2:1, 1 ml) [25]. For radiolabeling, 37 MBq of ¹¹¹In-InCl₃ (in 0.2M HCl) was added to a conical vial and dried under a flow of nitrogen followed by the addition of acetate buffer and vortexing for 10 min and incubating at 40°C for 90 min followed by radiochemical purity determination by RTLC and HPLC methods. Ethylene di-amine tetra acetic acid (ETDA) solution was added to the labeling mixture to scavenge the unlabeled In-111 cation followed by passing the mixture through the disposable PD10 De-salting column. The radioimmunoconjugate was analyzed for integrity by SDS-PAGE according to the method of Laemmli [26].

Cell culture

A HER2/neu positive cell line, SkBr3, was obtained from Iranian Biological Resources Center (Tehran, Iran) and was grown in RPMI-1640 medium supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM glutamine, 100 Iu/ml streptomycin and 100 IU/ml penicillin.

Immunoreactivity of ¹¹¹In-DOTA-antiHer2 towards SkBr3 cell line

Immunoreactivity of ¹¹¹In-DOTA-antiHer2 towards SkBr3 cell line was determined using a double-inverse of the binding data which may be considered a modification of the Lineweaver-Burk plot as follows. Different numbers (5×10^6 , 2.5×10^6 , 1.25×10^6 , 0.62×10^6 , 0.31×10^6 and 0.15×10^6) of SkBr3 cell were located in tubes; 30000 cpm of ¹¹¹In-DOTA-antiHer2 was added and incubated for 3 h at 4°C. After incubation, the radioactivity of tubes was read by γ-counter. Then tubes were centrifuged in 3000 g and the radioactivity of cells (the pellet, not the supernatant) were read by γ-counter. The immunoreactivity was determined using a Lineweaver-Burk plot and data were analyzed by the Lindmo method [27] as known:

$$V = \frac{V_{\max} [S]}{K_m + [S]}$$

Internalization assays

Internalization studies were performed using acid dissociation of ¹¹¹In-DOTA-antiHer2 [28] SkBr3 cells were harvested and re-suspended at 1×10^5 cells/200 μl in medium and cultured in 24 well plates.

After 24 h, 30000 cpm of ¹¹¹In-DOTA-antiHer2 were added to each well and incubated at 37°C. At various times (4, 8, 12, 16, 24, 30 and 36 h), RPMI medium in wells were removed and the cells, half of wells, were then exposed to 1 ml solution of 0.1 M NaOH and half of wells were exposed to 1 ml solution of 0.1 M acetic acid pH 4.0. After 15 min, the radioactivity content of the supernatant was measured by γ -counter. NaOH solution destroyed the whole SKBr3 cells and this solution was considered as total activity (internalized, bound and dissociated ¹¹¹In-DOTA-antiHer2). Acetic acid was used to remove the interaction between ¹¹¹In-DOTA-antiHer2 and HER2/neu at the SKBr3 cell surface and the radioactivity of the solution was considered as bound and dissociated ¹¹¹In-DOTA-antiHer2. Differences between the two activities were considered as internalized ¹¹¹In-DOTA-antiHer2 fraction.

Cell Treatment for comet assay

SKBR-3 cell line was propagated by DMEM media and 10% fetal calf serum (FCS) as monolayer in 105 cells per well into 12-wells plate, after 48 h treated using of ¹¹¹In-DOTA-antiHer2. Then 1 ml of ¹¹¹In-DOTA-antiHer2 in two concentrations (3.7 and 7.4 MBq) was added to the wells and was incubated at 37 °C, after 1.5 h, the cells were washed by Phosphate buffered saline (PBS) for removing the free ¹¹¹In, then added 1 ml of DMEM medium and incubated at 37 °C for 24 hours. The treated cells were harvested using a Teflon scraper, suspended in PBS and counted by a Neobar slide. The viability percent was tested by trypan blue dye and counted white and blue cells. Finally 50 μ l of the treated SKBR-3 cells (with concentration: 10 \times 10⁶ cells per 1 ml PBS) was mixed with 50 μ l of Low Melting Point agarose 1% and coated on a pre-coated slide with agarose 1%. Calculations were based on Cell Viability (%) = total viable cells (unstained) \div total cells (stained and unstained) \times 100 (n=5).

Comet assay

The double strand breaks of DNAs were determined using the neutral comet assay method with slight modifications [29, 30]. The treated SKBR-3 cells were diluted to a concentration of 10 \times 10⁶ cells/ml in PBS and 50 μ l of the cell dilution was mixed at 37°C with 50 μ l of freshly prepared 1% low melting point agarose (LMP, Merck, Cat No: K39061202-835) in distilled water. An aliquot of 50 μ l of the mixture was placed on a pre-coated slide with agarose 1% (Cinnagen, Cat No: MR7740), then covered with a coverslip and transferred to an ice-cold plate to promote fast gelling. As soon as the gel solidified, coverslips were smoothly removed and the slides were submerged in lysing solution (lysing solution; 2.5M NaCl, 100 mM EDTA, 1% Triton X-100, 10

mMTris, pH:10), overnight at 4 °C to lysis the cells. The slides were rinsed in TBE buffer (0.09 mol/l Tris–borate, 0.002 mol/l EDTA, pH 7.5) for 20 min to allow DNA unfolding, then transferred to an electrophoresis tank and immersed in fresh TBE electrophoresis buffer. Electrophoresis was performed at 20 V (6 V/cm), 12 mA for 40 min. The slides were washed in a neutralization buffer (0.4 mol/l Tris-HCl, pH 7.5) for 15 min and fixed with 10 min washes in absolute ethanol, air-dry for at least 2 h at room temperature. Finally, DNA was stained in two methods with SYBR Green I (Cinnagen, DNA safe stain, Cat No: PR381603) at a 1:10000 dilution in PBS and ethidium bromide (2 g/ml in water) (Cinnagen, Cat No: MR7721C). Samples could be immediately analyzed or stored at room temperature in the dark until needed. The Comets could be assessed by visual scoring or digitalization and image processing. At least 10 cells were measured in each slid, the tail moment of DNA (%tail DNA \times tail length) was evaluated by the fluorescence microscopy and comet-Score software.

RESULTS

Figure 1 shows the step-wise approach of ¹¹¹In-DOTA-antiHer2 preparation. It has been reported that moderate increase of chelator to mAb ratio leads to a slight decrease in immunoreactivity which is likely due to non-specific conjugation of chelators to mAb, which possibly attaches a chelator in the region of the antigen-binding site [31].

In radiochemical purity determination, for ¹¹¹In³⁺ detection, the best solvent mixture was 1 mM DTPA pH. 5 solution (data not shown), while for ¹¹¹In-DOTA detection, mixture of 10% ammonium acetate: Methanol (1:1) can be used (Rf 0.3).

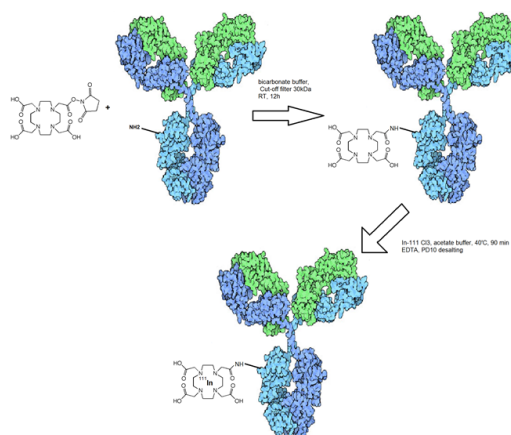


Fig 1. Detailed conjugation and radiolabeling steps of ¹¹¹In-DOTA-antiHer2 at optimized conditions.

¹¹¹In-DOTA-antiHer2 remains at the origin in all systems showing at least 95 ± 0.5% radiochemical purity (Figure 2).

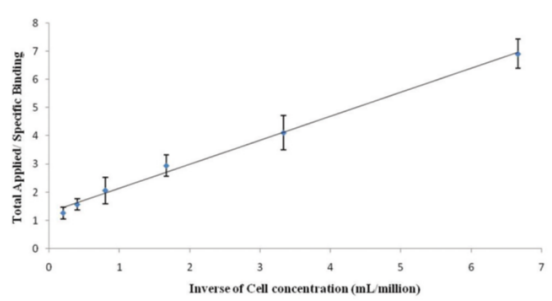


Fig 2. Immunoreactivity of ¹¹¹In-DOTA-antiHer2 using SkBr3 cell-binding assay (n = 5).

At the optimized conditions ¹¹¹In-DOTA-antiHer2 was prepared (RCP >95 ± 0.5%, S.A. 5.3 Ci/ g) with the average number of chelators per antibody of 6:1 showing significant immune-reactivity retention using ELISA. As shown in Figure 3, internalized activity for ¹¹¹In-DOTA-antiHer2 reached to the maximum significantly in 8-12 h incubation at 37°C. After 36 h the internalized activity decreased to 3% in 36 hours as observed in other reports [32] possibly caused by proteolytic degradation in lysosomes, leading to metal trans-chelation to the internal metal containing biomolecules (Figure 3).

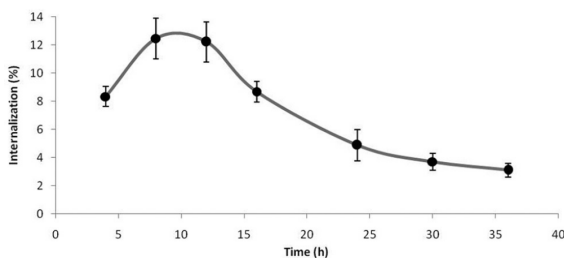


Fig 3. Internalization assay of ¹¹¹In-DOTA-antiHer2 in different time on SkBr3 cell line (n = 5).

Cell viability

The trypan blue exclusion test was used to determine the number of viable cells present in a cell suspension since live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, Eosin, or propidium, whereas dead cells do not [33]. Figure 4 shows the viability of SKBr3 cells based on the percentage of undyed cells compared with dyed cells.

Comet assay

Figure 5 shows the comet assay of samples used in this study using ethidium bromide dye.

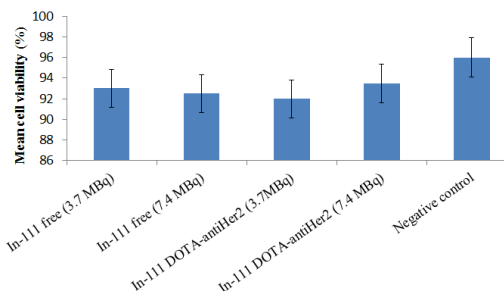


Fig 4. SKBr3 cell viability of In-111 and ¹¹¹In-DOTA-antiHer2 species compared with the negative control (n=5).

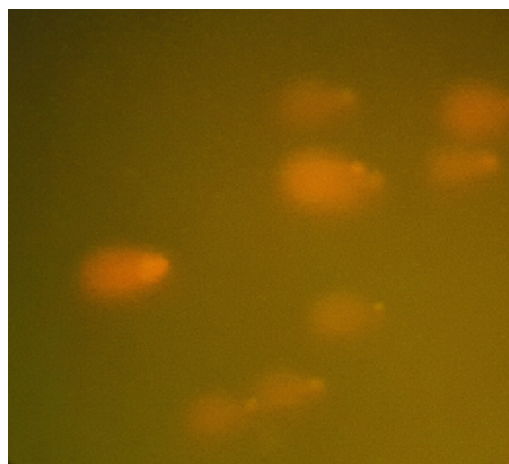


Fig 5. Comet assay of ¹¹¹In-DOTA-antiHer2 on SkBr3 cell line using ethidium bromide dye at low dose.

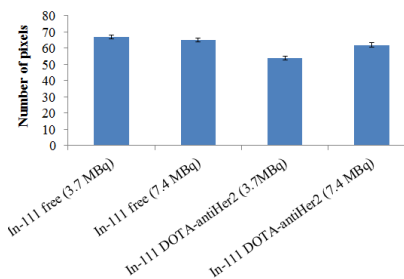


Fig 6. The lengths of comet tails (pixels) in presence of various ¹¹¹InCl₃ and ¹¹¹In-DOTA-antiHer2 activities (3.7 and 7.4 MBq) activities in ethidium bromide dye (n=11).

As shown in Figure 6. no meaningful results has been obtained in low dose and high dose In-111 cation and ¹¹¹In-DOTA-antiHer2 treatment using ethidium bromide, however while using Syb Green, more meaningful results are observed for low dose and high dose in ¹¹¹In-DOTA-antiHer2 showing an increase of comet size of 14-16% in case of high dose

treatment (Figure 6) it seems that based on this experience the use of Syb Green dye is more favored (Figure 7).

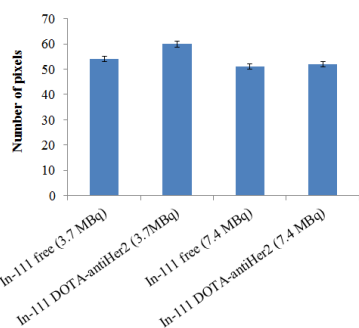


Fig 7. The lengths of comet tails (pixels) in presence of various ¹¹¹InCl₃ and ¹¹¹In-DOTA-antiHer2 activities (3.7 and 7.4 MBq) activities in Syb Green dye (n=11).

DISCUSSION

For removal of excipients from pharmaceutical sample and conjugation step samples were purified by ultra-filtration using the cut-off filters. The conjugation step is performed under alkaline pH, (bicarbonate buffer). The estimation of the number of DOTA prosthetic group on each antibody molecule, the arsenazo yttrium complex (Y[AAlIII] 2) method was used. The absorbance of Y(AAIII) 2 at 652 nm decreases upon the addition of DOTA-conjugate while the corresponding absorbance of AAIII ligand at 538 nm increases. The data demonstrated the DOTA: Antibody ratio of 6:1. The radiolabeling of In-111 with DOTA moiety of the conjugate is usually preformed in 40-90°C, however in case of antibodies the temperatures higher than 40° C is not recommended due to the loss of immunoreactivity.

As an important quality control test the immunoreactivity of ¹¹¹In-DOTA-antiHer2 towards SkBr3 cell line was determined under conditions of antigen excess in HER2/neu antigen-expressing SkBr3 human breast carcinoma cells by using a Lineweaver-Burk plot. Under these conditions, the immunoreactivity of radioimmunoconjugate was found to be 0.79 which is suitable for further studies. In order to make sure that the ¹¹¹In-DOTA-antiHer2 enters the cell and is not just bound to the cell surface internalization study was a mandate. This test is crucial for the most of the therapeutic radiopharmaceuticals *esp.* alpha and Auger electron emitters. Although for Auger emitters, cell entry is necessary but not enough to irradiate the target DNA molecules. The assays were performed with the HER2/neu expressing SkBr3 cell line to determine internalized activity following in vitro incubation at 37°C as a function of time.

The cell viability results shows significant cell death in radioactive groups studied (including In-111 and ¹¹¹In-DOTA-antiHer2 with two doses) compared to negative control. However no significant difference is observed among radioactive groups. Other studies [19] showed that ¹¹¹In-antibody killed SKBr3 target cells compared to nonreactive-labeled. This would be interesting and in accordance with the application of our radiolabeled compound for future studies.

The fluorescent dyes used most frequently are ethidium bromide, propidium iodide, 4,6-diamidino-2-phenylindole (DAPI), SYBR Green I and YOYO-1. For some fluorescent dyes, antifade can be used to greatly reduce the rate of signal quenching allowing the same slide to be scored multiple times. Comet image magnification has generally varied from 200× to 400× being used most commonly. Selection of appropriate magnification mostly depends on the type of cell being evaluated, the range of migration responses to measure, and the constraints of the microscope and/or imaging system. It has been concluded that due to many factors such as lack of nucleus insertion, cellular instability *etc.* at least *in vitro* not significant cell damage based on DNA has been observed using ¹¹¹In-DOTA-antiHer2. This might have been explained that many full size monoclonal antibodies even if integrated into cytoplasm of target cells cannot penetrate the cell nucleus and even get close enough to the nucleus membrane to show any therapeutic effects on the DNA.

CONCLUSION

Preliminary studies based on a recently developed Auger electron emitting radioimmunoconjugate (¹¹¹In-DOTA-antiHer2) using *in vitro* experiments on a HER2/neu positive cell line, SkBr3 using on a targeted. ¹¹¹In-DOTA-antiHer2 (>95% radiochemical purity; >79% immunoreactivity) demonstrated significant internalization in SKBr3 cells in 24 h while no cell death observed using trypan blue assay. Comet assay at 3.7 MBq demonstrated no significant DNA damage, while at 7.4 MBq dose DNA damage observed at least 16% more than control In-111 chloride after 24 h. Due to many factors such as lack of nucleus insertion, cellular instability *etc.* at least *in vitro* no significant cell damage based on DNA target has been observed using ¹¹¹In-DOTA-antiHer2. It seems Auger-emitter therapy based on simple radioimmunoconjugate would not be so effective and application of nucleus transferring moieties as well as application of other smaller targeting vectors such as peptides and oligonucleotides can be favored.

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