Drugs that modify cholesterol metabolism alter the p38/JNK-mediated targeted and non-targeted response to alpha and Auger radioimmunotherapy

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Abstract

Purpose: For the development of new anti-cancer therapeutic radiopharmaceuticals, including alpha particle emitters, it is important to determine the contribution of targeted effects in irradiated cells, and also of non-targeted effects in non-irradiated neighboring cells because they may affect the therapeutic efficacy and contribute to side effects.

Experimental Design: Here, we investigated the contribution of non-targeted cytotoxic and genotoxic effects in vitro and in vivo in (xenografted mice) during alpha (\(^{212}\)Pb/\(^{212}\)Bi, \(^{213}\)Bi) and Auger (\(^{125}\)I) radioimmunotherapy (RIT).

Results: Between 67 and 94% (alpha RIT) and 8 and 15% (Auger RIT) of cancer cells were killed by targeted effects, whereas 7-36% (alpha RIT) and 27-29% (Auger RIT) of cells were killed by non-targeted effects. We then demonstrated that the non-targeted cell response to alpha and Auger RIT was partly driven by lipid raft-mediated activation of p38 kinase and c-JUN N-terminal kinases (JNK). Reactive oxygen species also played a significant role in these non-targeted effects, as demonstrated by NF-kB activation and the inhibitory effects of antioxidant enzymes and radical scavengers. Compared with RIT alone, the use of RIT with ASMase inhibitor (imipramine) or with a lipid raft disruptor (e.g., methyl-beta-cyclodextrin or filipin) led to an increase in clonogenic cell survival in vitro and to larger tumors and less tissue DNA damage in vivo. These results were supported by an inhibitory effect of pravastatin on Auger RIT.

Conclusions: Cell membrane-mediated non-targeted effects play a significant role during Auger and alpha RIT, and drugs that modulate cholesterol level, such as statins, could interfere with RIT efficacy.
STATEMENT OF TRANSLATIONAL RELEVANCE

This study shows that alpha radioimmunotherapy activity is mediated by targeted and non-targeted effects. Non-targeted effects and to a lower extent, targeted effects are modulated by the formation of ceramide-enriched large platforms and the subsequent activation of p38- and JNK-mediated signaling pathways. Non-targeted effects contribute to the cytotoxic and genotoxic effects beyond the particle range, and may also counterbalance the heterogeneity in vector distribution. As targeted effects are dose-related but not the non-targeted effects, these results have consequences on the planning and prediction of the therapeutic efficacy and side effects of targeted radionuclide therapy (TRT) using alpha particles. Moreover, patients undergoing treatment to modify lipid metabolism could respond differently to TRT.
Introduction

During the last decade, several new radiopharmaceuticals were approved for targeted radionuclide therapy (TRT) of solid tumors or evaluated in clinical trials (1-6). These radiopharmaceuticals are obtained by coupling a radionuclide to a peptide or a monoclonal antibody (for radioimmunotherapy; RIT). Once administered to patients, they specifically recognize tumor cells and produce targeted irradiation to eradicate them. While conventional external beam radiotherapy (EBRT) is mostly dedicated to localized tumors, TRT can be used for treating diffuse, metastatic disease or tumors close to organs at risk (7). DNA has been for long considered as the main, if not the only target of radiation; however, it is today admitted that other subcellular targets, including mitochondria and cell membrane, should be considered during radiotherapy (8,9). The cell membrane contains proteins, alcohols, such as sterols, and lipids (e.g., sphingolipids, glycolipids, phospholipids) that are structured in a bilayer to provide variable fluidity. We know from EBRT studies that radiation-induced reactive oxygen species (ROS) react with polyunsaturated fatty acids (10), leading to the generation of breakdown molecules, such as malondialdehyde, acrolein and 4-hydroxy-2-nonenal, that can react with cellular biomolecules (e.g., DNA, RNA and also amino acids) to generate adducts. Irradiation also induces rapid formation of ceramide through the activation of acidic sphingomyelinases (ASMases) and the subsequent sphingomyelin hydrolysis at the plasma membrane (11,12). Ceramide could be considered as a mediator of radiation that has an effect on the plasma membrane via the generation of ceramide-enriched microdomains termed rafts, but also on intracellular signaling molecules (8,13,14).

Moreover, besides its direct response to radiation (defined as a targeted effect), cell membrane can also be involved in secondary intercellular communications that will further alter the biological functions of neighboring cells (15-18). When observed in cells that were not directly irradiated, these effects are defined as non-targeted effects of radiotherapy. Non-
targeted effects have been predominantly described after low dose (<0.5Gy) of EBRT, when the probability of cells to be hit by particles (targeted effect) is low (19-27). They may contribute to tumor eradication; however, they could also affect treatment planning because they are not dose-related and lead to unexpected side effects. As most of the vectors used in TRT binds to cell surface receptors, the probability for the cell membrane to be hit is much higher than during EBRT. This is even more marked when radionuclides used in clinical trials emit high linear energy transfer (high-LET) particles, namely alpha particles or Auger electrons (3,5,6,28). This radiation type produces dense ionization in tissues over a short range (<100µm), leading to unrepairable complex lesions in biological constituents and possibly generating deleterious signals towards neighboring cells. Therefore, in association with strong dose heterogeneity and protracted exposure, non-targeted effects involving the cell membrane might play a significant role in the final therapeutic outcome of TRT.

Here, we investigated in vitro and in vivo the role of the cell membrane and of drugs that modulate cholesterol metabolism in targeted and non-targeted effects of RIT using alpha particle emitters, namely $^{212}$Pb/$^{212}$Bi and $^{213}$Bi (alpha RIT), and the Auger emitter $^{125}$I (Auger RIT).

Materials and Methods

Cell lines and antibodies

The A-431 vulvar squamous carcinoma, SK-OV-3 ovarian carcinoma, AN3CA endometrial carcinoma, and HCT116 colon adenocarcinoma human cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HER2-positive A-431 cells were transfected with constructs encoding carcinoembryonic antigen (CEA, expressed by colorectal carcinoma cancer) and luciferase (29) to obtain the A-431$^{\text{CEA}}$ cell line. AN3CA cells naturally express Müllerian inhibiting substance receptor II (MISRII), while SK-OV-3
cells were transfected with a construct to express MISRII (SK-OV-3MISRII cells). HCT116 cells naturally express CEA. Cell line identities were confirmed using Powerplex 21 Kit (Promega). Mycoplasma test was routinely performed using the MycoAlert Mycoplasma Detection Kit (Lonza).

Then, SK-OV-3MISRII and AN3CA cells were chosen because they mimic ovarian peritoneal carcinomatosis when grafted intraperitoneally in mice. Similarly, HCT116 and 431CEA cells were chosen because they mimic colorectal peritoneal carcinomatosis. Small volume peritoneal carcinomatosis originating from ovarian or colorectal cancer are suitable candidates for TRT using radionuclides that emit short-range particles (Auger, alpha) (2,30).

Parental and A-431CEA and SK-OV-3MISRII cells were grown in Dulbecco’s Modified Eagle Medium, and HCT116 cells in RPMI 1640 medium. Media were supplemented with 10% (v/v) fetal calf serum (FCS), 2 mmol/L L-glutamine, 0.1 U/ml penicillin and 100 µg/ml streptomycin, and 1% geneticin (only for A-431CEA and SK-OV-3MISRII cells). AN3CA cells were cultured in MEM medium with 10% FCS, 1% penicillin, 0.1% streptomycin, 1% sodium pyruvate and 1% non-essential amino acids. Cells were kept at 37°C in a humidified atmosphere containing 5% CO2. The murine IgG1k monoclonal antibody (mAb) 35A7 against the CEA Gold 2 epitope, and the anti-HER2 mAb trastuzumab (Herceptin®, Genentech, San Francisco, CA) were used to target CEA and HER2 expressing cells, respectively. The murine IgG1 anti-MISRII mAb 16F12 produced by our team was used to target MISRII (31). The non-specific PX IgG1 mAb from the mouse myeloma MOPC 21 (32) was used for control in vivo experiments.

The anti-CEA, anti-MISRII and PX mAbs were purified from mouse hybridoma ascitic fluids by ammonium sulfate precipitation followed by ion exchange chromatography on DE52 cellulose (Whatman, Balston, UK).

**Antibody conjugation and radiolabeling with** $^{212}\text{Pb}$, $^{213}\text{Bi}$ or $^{125}\text{I}$
The anti-HER2, 35A7 and PX mAbs were conjugated with TCMC (Macroyclics, Dallas, TX, USA) prior to radiolabeling at the specific activity of 37MBq/mg with $^{212}$Pb (Orano Med, Bessines, France), as described in (33) (Supplementary Fig. S1). The anti-MISRII mAb was conjugated with 2-(P-SCN-benzyl)-cyclohexyl acid A-diethylenetriaminepentaacetic (CHX-A"- DTPA) and radiolabeled with $^{213}$Bi (JRC, Karlsruhe, Germany) at the specific activity of 37MBq/mg, as described in (31). The anti-CEA mAb was radiolabeled also with $^{125}$I (Perkin Elmer, Boston) at the specific activity of 370MBq/mg, as described in (34).

In vivo radioimmunotherapy using $^{212}$Pb-, $^{125}$I- and $^{213}$Bi-labeled antibodies

Female athymic nude Foxn1$^{nu}$ mice (6–8 week-old) (Envigo RMS Laboratories, Gannat, France) were acclimated for 1 week. They were housed at 22°C and 55% humidity with a light-dark cycle of 12h, and food and water ad libitum. Body weight was determined weekly, and mice were clinically examined throughout the study. All animal experiments were performed in compliance with the French government guidelines and the INSERM standards for experimental animal studies (agreement B34-172-27). They were approved by the ethics committees of the Institut de Recherche en Cancérologie de Montpellier (IRCM/INSERM) and the Languedoc Roussillon region (CEEA LR France No. 36) for animal experiments (reference number: 1056).

To establish intraperitoneal tumor xenografts, $1\times10^6$ A-431$_{\text{CEA}}$ or $4.5\times10^6$ AN3CA cells in 0.3 mL DMEM or 0.3 mL DMEM/Matrigel™ (1:1), respectively were intraperitoneally (i.p.) grafted in mice. Six days post-A-431$_{\text{CEA}}$ graft, mice received i) one single i.p. injection of 1.48 MBq $^{212}$Pb-anti-CEA, $^{212}$Pb-anti-HER2, or $^{212}$Pb-PX mAbs (n=10, 7 and 6 mice) (alpha RIT). The survival of these mice was previously reported (33); or ii) $^{125}$I-anti-CEA mAb [Auger RIT: two i.p. injections of 37 MBq at day 8 and 11]; or methyl-$\beta$-cyclodextrin (MBCD, lipid raft disruptor) (300 mg/kg; daily, from day 6 to 15); or both
125I-anti-CEA mAb and MBCD (n=10 mice). Tumor growth was followed by bioluminescence (Xenogen, Perkin Elmer) measurement.

Mice xenografted with AN3CA cells (7 mice/group) were treated with: i) NaCl; or ii) daily i.p. injections of 300 mg/kg MBCD from day 7 to 13 post-graft; or iii) one injection of 213Bi-anti-MISRII mAb (37MBq) at day 11; or (iv) both 213Bi-anti-MISRII mAb and MBCD. At day 30 post-graft, mice were sacrificed, tumors collected, and the tumor mass was determined.

To investigate the role of pravastatin in Auger RIT, mice (8 mice/group) were subcutaneously xenografted with 1×10^6 A-431 CEA cells in Matrigel™ and treated with: i) NaCl; or ii) daily i.p. injections of 40 mg/kg pravastatin from day 10 pre-graft until day 18 post-graft; or iii) one injection of 125I-anti-CEA mAb (37MBq) at day 8 and one at day 11; or (iv) both 125I-anti-CEA mAb and pravastatin. Tumor growth was followed by caliper measurement.

**Ex-vivo autoradiography and DNA damage assessment after RIT**

At various times (4h, 17h and 22h) following alpha (212Pb/212Bi) or 24h after Auger RIT, mice bearing i.p. A-431 CEA tumor cell xenografts were anesthetized, bled, and dissected. Tumors were collected and frozen after inclusion in OCT embedding matrix. Two consecutive 10µm-thick frozen sections were analyzed by digital autoradiography (DAR) or by immunodetection of 53BP1, as described in (34).

**In vitro determination of targeted and non-targeted cytotoxic effects of alpha RIT**

SK-OV-3MISRII (100 to 1500 cells/well) and A-431 CEA (100 to 5000 cells/well) cells were plated in 6-well plates with 2 ml of medium. The following day, cells were incubated with 213Bi-anti-MISRII mAb (SK-OV-3MISRII cells) or 212Pb-anti-CEA mAb and 212Pb-anti-HER2 mAb (A-431 CEA cells) (0-0.5MBq/mL), or the corresponding unlabeled mAbs (0-27µg/mL) for 90min (donor cells). Culture medium was then removed and cells were washed.
twice with 4 mL PBS. For investigating the non-targeted cytotoxic response, fresh medium (2 mL) was added to donor cells for 2h and then transferred to recipient cells that had been plated the day before (100 to 300 cells/well in 6-well plates).

For standard clonogenic assays, donor and recipient cells were then grown for 12 days, and colonies stained with crystal violet (2.5 g/l in 45:5 30% (v/v) methanol/paraformaldehyde). Colonies containing 50 or more cells were scored and the surviving fraction was calculated.

**In vitro measurement of DNA damage**

A-431_{CEA} and SK-OV-3_{MISRII} donor cells were seeded on coverslips in 6-well plates. The following day, they were incubated with 0.5 MBq/mL of $^{212}$Pb-mAbs (A-431_{CEA} cells) or 0.5MBq/mL of $^{213}$Bi-mAbs (SK-OV-3_{MISRII} cells) for 90min. After radiolabeled antibody removal, fresh medium (2 ml) was added to donor cells for 2h, and then transferred to recipient cells. Both donor and recipient cells were fixed with paraformaldehyde (PFA) and permeabilized, as described in (34) (and Supplementary methodology) for γ-H2AX/53BP1 foci detection.

The formation of micronuclei was determined in A-431_{CEA} donor cells exposed to 0.5 MBq/mL $^{212}$Pb-mAbs for 90min and in recipient cells, as described in (35).

**ASMase and ceramide measurement, detection of lipid rafts**

Cells were incubated with $^{213}$Bi-anti-MISRII mAb (0.5 MBq/mL) for 90min, or H$_2$O$_2$ (25mM) at 37°C for 30min. After three PBS washes, they were fixed in 3.7% (v/v) PFA for 15min and then washed three times in PBS. Cells were then incubated with a polyclonal rabbit anti-ASMase antibody (2µg/mL Santa Cruz Biotechnology, Inc. Heidelberg, Germany) and the anti-ceramide 15B4 mAb (1:50; Alexis Biochemicals Heidelberg, Germany) at 37°C for 1h. After three washes in PBS/2% FCS, cells were incubated with Alexa Fluor 555-conjugated anti-rabbit (1:500; Invitrogen; Saint Aubin, France) or with Alexa Fluor 488-
conjugated anti-mouse IgM (1:200; Invitrogen; Saint Aubin, France) in the dark for 1h. After three washes and resuspension in PBS, cells were analyzed by flow cytometry (Muse®, Merck Millipore, Molsheim, France) and the G mean value (arbitrary units) was determined. To confirm ASMase role in RIT cytotoxicity, SK-OV-3MISRII cells were pre-incubated with 50 µM imipramine (ASMase inhibitor) for 30min following addition of 213Bi-anti-MISRII mAb (0.5 MBq/mL) at 37°C for 90min.

To visualize lipid rafts, SK-OV-3MISRII cells were plated on 12-mm glass coverslips in culture dishes. After treatment, they were fixed in 3.7% (v/v) PFA, and incubated with Alexa-488-conjugated cholera toxin B (Molecular Probe, Illkirch, France) at 37°C for 45 min. After three washes in PBS, cells were fixed again in PFA for 10min and coverslips were then mounted in Mowiol and analyzed using a 63×NA objective and a Leica (Leica Microsystems, Wetzlar, Germany) inverted microscope.

**In vitro role of lipid rafts in alpha RIT cytotoxic effects**

The role of lipid raft integrity in 213Bi-mAb-induced non-targeted cytotoxic effects was investigated by pre-incubating SK-OV-3MISRII cells with 4mM MBCD (Sigma-Aldrich, St Louis, MO) or 2.5 µg/mL filipin (Sigma-Aldrich, St Louis, MO) for 30min before adding 213Bi-anti-MISRII mAb for another 90min in the presence of these lipid raft inhibitors.

MBCD effect on cholesterol concentration in SK-OV-3MISRII cells incubated with MBCD was determined using a Cholesterol Quantification Kit (Sigma-Aldrich, St Louis, MO).

**Proteome kinase analysis and western blotting**

A Human Phospho-Kinase Array (Proteome Profiler Array, R&D Systems, Minneapolis, MN) was used to detect the relative phosphorylation levels of 46 kinases in cell extracts of SK-OV-3MISRII donor (incubated with 0.5MBq/ml 213Bi-anti-MISRII mAb, or with 0.5MBq/ml 213Bi-anti-MISRII mAb and 4mM MBCD or 2.5 µg/ml filipin) and recipient cells, according to the manufacturer’s protocol.
For western blotting, HCT116, AN3CA and SK-OV-3MISRII cell membrane, cytosolic protein fractions and total lysates (40 µg of each) were separated by SDS–PAGE and electrotransferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). After incubation with anti-phosphorylated (p) ERK, -p-SAP/JNK, -p-p38, and -p-NFκB (p65) primary antibodies (Cell Signaling Technology, Danvers, MA), immune reactions were detected with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (Sigma-Aldrich, St Louis, MO) secondary antibodies and the ECL detection system (Amersham Biosciences, Saclay, France).

**In vitro analysis of p38 and JNK role**

SK-OV-3MISRII donor cells were pre-incubated with 10 µM SP600125 (JNK inhibitor) or 10 µM SB203580 (p38 inhibitor) for 30min before addition of 0-0.5MBq/mL $^{213}$Bi-anti-MISRII mAb in the presence of the inhibitors for another 90min. Then, medium was removed and the standard medium transfer protocol to donor cells described before was used.

**Analysis of oxidative stress**

SK-OV-3MISRII donor cells were pre-incubated with 20µg/mL catalase (Sigma-Aldrich St Louis, MO) or 0.5% DMSO (Sigma-Aldrich St Louis, MO) for 20min before RIT with $^{213}$Bi-anti-MISRII mAb in the presence of catalase or DMSO for 90min. Next, cells were washed twice with PBS and new medium was added, and cells cultured until the appearance of colonies.

**Apoptosis induction and proliferation**

Apoptosis was measured in SK-OV-3MISRII donor cells pre-incubated with SP600125 (JNK inhibitor), SPB203580 (p38 inhibitor), imipramine (ASMase inhibitor) or MBCD (lipid raft disruptor) for 30min before addition of 0-0.5MBq/mL $^{213}$Bi-anti-MISRII mAb for 90min. Then, radioactivity was removed, cells were washed with PBS, and apoptosis was measured.
at 48h post-treatment using the TUNEL Detection Kit (Promega, France) according to the manufacturer’s instructions.

Cell proliferation was measured in SK-OV-3\textsubscript{MISRII} cells using the EdU Cell Proliferation Assay (Merck Millipore, St Quentin-en-Yvelines, France). Briefly, EdU was added to the culture medium at a final concentration of 10 μM immediately after RIT and left for 24h. Then, cells were fixed with 3.7% PFA and permeabilized with 0.1% Triton X-100. Fluorescent EdU was detected according to the manufacturer’s instructions, and cells analyzed using a 63×NA objective and a Leica (Leica Microsystems, Wetzlar, Germany) inverted microscope.

**Statistical Analysis**

All in vitro data were obtained from four independent experiments in triplicate. Data were analyzed using the Stata software v.13 (StataCorp, College Station, TX) and described using mean, standard deviation (SD), median and range. Comparisons between radiolabeled antibodies and control were performed using the non-parametric Kruskal-Wallis test (significance level set at 0.05). Pairwise comparisons were performed using the non-parametric Mann-Whitney test (considering a significance threshold at 0.013 to account for multiple testing). For in vivo experiments, radioactivity (cpm)-related DNA damage (number of foci) was modeled by linear regression.

**Results**

**Non-targeted effects could contribute to the $^{212}\text{Pb}$-anti CEA mAb efficacy**

The Kaplan Meyer analysis in our previous study (33) showed a significantly higher survival rate ($p < 0.05$) in mice bearing intraperitoneal A-431\textsubscript{CEA} tumors and treated with $^{212}\text{Pb}$-anti-HER2 mAb compared with $^{212}\text{Pb}$-anti-CEA mAb (Fig. 1A). The absence of anti-tumor effect of the non-specific $^{212}\text{Pb}$-PX mAb ($p=0.85$ vs NaCl) demonstrated the absence of
In vivo cytotoxic effects when $^{212}$Pb is not targeted to tumor cells. The higher therapeutic efficacy of the $^{212}$Pb-anti-HER2 mAb could not be explained by a higher mean tumor uptake, because the mean doses were 28.1 ± 2.1 Gy and 36.1 ±2.7 Gy for the $^{212}$Pb-anti-HER2 and $^{212}$Pb-anti-CEA mAb, respectively (Supplementary Methodology). Conversely, DAR analysis (Fig. 1B) showed that compared with tumors treated with $^{212}$Pb-anti-HER2 mAb, some tumor regions incorporated very low or no $^{212}$Pb-anti-CEA mAb. Voxel dosimetry (Supplementary Methodology) indicated that about 30% of the tumor volume from $^{212}$Pb-anti-CEA mAb-treated mice received 0 Gy (Fig. 1C). Conversely, in the $^{212}$Pb-anti-HER2 mAb group, all regions received some radioactivity, although the maximal voxel dose was about two times lower (77 for $^{212}$Pb-anti-HER2 mAb vs 140 Gy for $^{212}$Pb-anti-CEA mAb).

Based on these observations, we hypothesized that in the absence of non-targeted effects, tumor cells that received 0 Gy in the $^{212}$Pb-anti-CEA mAb-treated group should grow as fast as those in the NaCl group. Then, we established a theoretical growth curve using exponential tumor growth parameters of the NaCl-treated group and considering an initial tumor volume corresponding to 30% of the volume of NaCl-treated tumors measured at day 5 (Fig. 1D). According to this theoretical curve, the 30% of tumor volume that received 0 Gy in the $^{212}$Pb-anti-CEA mAb-treated group should have grown much faster than what experimentally observed (Fig. 1D). The slower experimental growth rate of $^{212}$Pb-anti-CEA mAb-treated tumors could be explained by the presence of molecular signals (i.e., non-targeted cytotoxic effects) between irradiated and non-irradiated tumor regions.

$^{212}$Pb-anti-CEA mAbs produce similar DNA damage levels in irradiated and non-irradiated tumor regions

To test the in vivo occurrence of non-targeted effects associated with the $^{212}$Pb-anti-CEA mAb treatment, we asked whether genotoxic effects were present also in non-irradiated tumor regions. To this aim, we investigated in tumor sections from mice treated with $^{212}$Pb-
mAbs the relationship between the number of 53BP1 foci, a protein that rapidly relocalizes to nuclear foci upon DNA damage, and radioactivity level in the tumors collected at 17h post-RIT, when the mean dose rate was still about 50% of the initial dose rate (1.1 vs 2.1 Gy.h⁻¹ at 4h post-RIT, data not shown) (Fig. 1E). The number of 53BP1 foci per mm² tumor was relatively constant in ²¹²Pb-anti-CEA mAb-treated tumors, independently of the radioactivity level calculated by DAR (between 5.9±0.2 and 7.6±0.3 foci/cpm/mm²), and the slope of the corresponding regression curve was not different from zero (p= 0.072). Conversely, 53BP1 foci were proportional to the radioactivity level in ²¹²Pb-anti-HER2 mAb and ²¹²Pb-PX-treated tumors (Fig. 1F), and the slope was significantly different from zero (p = 0.012 and p = 0.023, respectively). The number of 53BP1 foci per cell ranged from 4.0±0.3 to 8.6±0.4 for ²¹²Pb-anti-HER2 mAb, from 1.9±0.1 to 3.5±0.2 for ²¹²Pb-PX mAb, and was above the background level of 0.8±0.1 foci per cell in NaCl-treated tumors.

Non-targeted cytotoxic effects are observed after alpha RIT

To confirm in vitro the contribution of targeted and non-targeted cytotoxic effects during alpha RIT, we determined the clonogenic cell survival of A-431cia and SK-OV-3MISRII donor cells exposed to radiolabeled antibodies and of the corresponding recipient cells. In donor A-431cia cells, ²¹²Pb-mAbs strongly reduced clonogenic cell survival at test activities as low as 0.03MBq/mL (Fig. 2A). Beyond 0.06MBq/mL, clonogenic cell survival was lower than 1%. Although less pronounced than in donor cells (targeted effect), clonogenic cell survival was reduced also in recipient cells (non-targeted effects). Targeted and non-targeted cytotoxic effects were also observed in SKOV3MISRII donor cells exposed to the ²¹³Bi-anti-MISRII mAb and in recipient cells (Fig. 2B). In both cell models, neither targeted nor non-targeted cytotoxic effects were detected after exposure to unlabeled antibodies (Supplementary Fig. S2).
DNA double-strand breaks (DSB) induced by targeted and non-targeted effects of alpha RIT are associated with different types of 53BP1 and γ-H2AX foci

We then evaluated the expression of 53BP1 and γH2AX, two DNA DSB markers, in SK-OV-3\textsubscript{MISRII} donor cells exposed to $^{213}\text{Bi}$-anti-MISRII mAbs for 90min and in recipient cells (Fig. 2C-D and Supplementary Fig. S3A). Quantification of the mean number of 53BP1 and γH2AX foci per cell showed that upon irradiation, DNA DSBs were produced in both donor and recipient cells compared with untreated control (Fig. 2C), confirming the occurrence of non-targeted genotoxic effects. Moreover, we could classify foci in three subgroups according to their size (arbitrarily defined as large, medium, and small) in donor cells, but we only detected small foci in recipient and control cells (Fig. 2D). Foci sizes are reported in Supplementary Fig. S3A. The mean number of large, medium, and small 53BP1 foci per donor cell was $0.8 \pm 0.1$, $3.7 \pm 0.2$, and $5.4 \pm 0.3$, respectively (Fig. 2D). Conversely, the mean number of 53BP1 small foci was $1.8 \pm 0.3$ in recipient cells, and $0.7 \pm 0.1$ in untreated cells. We obtained similar results for γH2AX foci (Fig. 2D, right panel), and in A-431\textsubscript{CEA} cells exposed to $^{212}\text{Pb}$-mAbs (Supplementary Fig. S3B; only for 53BP1 foci for technical reasons). We hypothesized that large foci were caused by the recruitment of a higher number of γ-H2AX and 53BP1 proteins compared with medium and small foci, and that consequently, they corresponded to more complex lesions. As a consequence of misrepaired DNA DSBs, the number of micronuclei was significantly increased in both donor and recipient A-431\textsubscript{CEA} cells after exposure to $^{212}\text{Pb}$-mAbs (Supplementary Fig. S3C).

Lipid rafts participate in the targeted and non-targeted cytotoxic effects

As radiolabeled mAbs bind to cell surface receptors, the cell membrane is the first target of irradiation. We investigated whether cell membrane modifications could be involved in alpha RIT targeted and non-targeted effects. Using cholera toxin B, we first showed the formation of lipid raft domains in SK-OV-3\textsubscript{MISRII} cells exposed to $^{213}\text{Bi}$-mAbs (Fig. 3A).
Ceramide, a class of cell membrane sphingolipids, can be formed by sphingomyelin hydrolysis catalyzed by ASMase. With cholesterol and proteins, ceramide can contribute to the formation of ceramide-enriched large domains that can be identified as lipid rafts. We found that compared with untreated cells, ASMase activation (Fig. 3B upper panel) and ceramide production (Fig. 3B, lower panel) were increased in SK-OV-3_{MISRII} cells exposed to $^{213}\text{Bi}$-mAbs. To test the role of ASMase and of lipid rafts in alpha RIT cytotoxic effects, we incubated SK-OV-3_{MISRII} cells with $^{213}\text{Bi}$-anti-MISRII mAb alone or with imipramine, an ASMase inhibitor (Fig. 3C), or with lipid raft disruptors (Fig. 3D): filipin that sequesters cholesterol, or MBCD that depletes cholesterol. Clonogenic cell survival of both donor and recipient cells was increased in the presence of imipramine or of lipid raft disruptors. We confirmed that the cholesterol level of cells treated with MBCD was lower than in untreated cells (Fig. 3E).

**The MAP kinases p38 and JNK1/2 are activated during alpha RIT and contribute to cell death**

As lipid raft are known to activate intracellular signaling pathways, we analyzed SK-OV-3_{MISRII} donor (i.e. exposed to the $^{213}\text{Bi}$-anti-MISRII mAb) and recipient cells extracts using a phosphoprotein kinase array (Fig. 3F and Supplementary Fig. S4A). Compared with untreated cells or cells exposed to unlabeled anti-MISRII mAb, several kinases were activated in cells exposed to $^{213}\text{Bi}$-anti-MISRII mAb. p38-α kinase, c-JUN N terminal kinases 1/2/3 (JNK 1/2/3), and the downstream c-JUN transcription factor were among the most significantly activated phosphorylation pathways. Some growth factors also were activated, such as ERK1/2, AKT1/2, CREB, GSK3, and MSK1/2. Yes, Fyn and Fgr of the SrcA family of protein tyrosine kinases, LCK, WNK, STAT 3 and 6, HSP60 and HSP27 also were activated (Fig. 3F).
We obtained similar results in recipient cells. Among the most notable differences was the activation of proline-rich AKT1 substrate 1 (PRAS40) and 5’-AMP-activated protein kinase (AMPK) subunit α1 (Supplementary Fig. S4A).

We confirmed the increased phosphorylation of p38, JNK1/2/3 in vitro by western blotting using protein lysates of untreated and donor and recipient SK-OV-3MISRII and AN3CA cells (Fig.4A and Supplementary Fig. S4B).

We showed using EdU that cell proliferation was reduced in SK-OV-3MISRII cells exposed to 213Bi mAbs and that apoptosis (using TUNEL assay) was induced (Fig. 4B and 4C, and Supplementary Fig. S5A). Conversely, cell proliferation was restored at 48h and apoptosis reduced when pharmacological inhibitors of p38 (SB203580) and of JNK1/2/3 (SP600125) (and also MBCD or imipramine) were used (Fig. 4B and 4C).

Finally, compared with exposure to 213Bi-mAbs alone, clonogenic cell survival was increased in both donor and recipient cells when SK-OV-3MISRII donor cells were incubated with 213Bi-mAbs in the presence of SB203580 or SP600125. These results indicate that the p38 and JNK1/2/3 signaling pathways contribute to the targeted and non-targeted cytotoxicity of 213Bi-mAbs (Fig. 4D).

Lipid raft disruption is accompanied by loss of alpha RIT-induced p38 and JNK1/2/3 phosphorylation

To investigate the role of lipid raft integrity on the RIT-induced expression of the previously identified signaling pathways, we performed a phosphoprotein kinase array analysis using donor and recipient cells exposed to 213Bi-anti-MISRII mAb in the presence of MBCD (Fig. 5A and Supplementary Fig. S5B), which decreased the mean cholesterol levels (Fig. 3E), or filipin (Supplementary Fig. S6). Phosphorylated p38 and JNK1/2/3 expression levels were strongly reduced in both donor and recipient cells exposed to 213Bi-anti-MISRII mAb + MBCD or filipin compared with cells incubated only with 213Bi-anti-MISRII mAb.
Western blot analysis of tumors collected from mice treated with 37MBq $^{213}$Bi-anti-MISRII mAb confirmed the decreased phosphorylation of p38 and SAPK/JNK in the presence of MBCD treatment (Fig. 5B) in vivo.

**Oxidative stress is involved in the non-targeted response to alpha RIT**

As ROS can activate ASMase, we assessed the role of oxidative stress in the targeted and non-targeted effects of alpha RIT by incubating donor cells with catalase or DMSO (two radical scavengers) during exposure to $^{213}$Bi-anti-MISRII mAb (Fig. 5C). Compared with cells exposed to $^{213}$Bi-anti-MISRII mAb alone, co-incubation with catalase led to a significant increase in clonogenic cell survival of donor (p<0.001) and recipient cells (p<0.05). For DMSO, survival was only statistically increased in donor cells exposed to 0.25MBq/mL (p<0.001) and in recipient cells incubated with medium from donor cells exposed to 0.25 and 0.5 MBq/mL of $^{213}$Bi-anti-MISRII mAb (p<0.05 and p<0.01, respectively). Catalase and DMSO also reduced the level of p-p38, p-SAPK/JNK and p-NFкB p65 (Fig. 4A). However, comparison of the catalase and DMSO effects in donor and recipient cells indicated that the relative contribution of ROS to the cytotoxic effect was much lower in donor than in recipient cells (Fig. 5C).

Then, we used an oxidative stress membrane array to analyze the expression of proteins involved in redox mechanisms (Supplementary Methodology). In SK-OV-3$_{MISRII}$ cells, few proteins (Cited-2, HIF1α, HSP60, and SIRT2) were overexpressed in donor cells exposed to $^{213}$Bi-anti-MISRII mAb compared with untreated cells (Supplementary Fig. S7A). Conversely, in recipient cells, additional proteins were induced: ADAMTS1, BCL2, carbonic anhydrase IX, COX-2, DKK-4, FAB-P1, HIF2α, phosphorylated HSP27, indoleamine-pyrrole 2, 3-dioxygenase (IDO), NFK-B, p21, paraoxonase 2 and 3 (PON2, PON3), and SOD2. Conversely, in HCT116 cells, the same proteins were induced in donor cells exposed to $^{125}$I-anti-CEA mAb (Auger RIT) and in recipient cells (non-targeted effect) (Fig. S7B).
Western blot analysis confirmed the role of oxidative stress, as demonstrated by the increase in phosphorylated NF-kB expression after \textit{in vitro} Auger and alpha RIT (Fig. 4A and Supplementary Fig. S7C). However, this increase was not confirmed \textit{in vivo} in tumors collected at 6h after alpha-RIT (Fig. 5B).

\textbf{MBCD and pravastatin reduce RIT efficacy \textit{in vivo}}

Then, to assess the role of lipid rafts in $^{213}$Bi-mAb therapeutic efficacy \textit{in vivo}, we treated mice harboring intraperitoneal AN3CA cell tumor xenografts with NaCl, MBCD alone, $^{213}$Bi-anti-MISRII mAb alone, or $^{213}$Bi-anti-MISRII mAb + MBCD. At day 30 post-RIT, tumor mass was significantly reduced in the $^{213}$Bi-anti-MISRII mAb group (RIT) compared with the NaCl group ($p = 0.0321$, RIT-treated to untreated tumor mass ratio=0.02) (Fig. 6A, left panel). Although not statistically significant, cholesterol levels tended to be lower in MBCD–treated than in NaCl-treated tumors (Fig. 6A, middle panel). Although MBCD alone was effective in reducing tumor mass and $^{213}$Bi-anti-MISRII mAb + MBCD showed a therapeutic efficacy, the combination was less effective than RIT ($p = 0.0284$, RIT + MBCD to MBCD tumor mass ratio=0.05) (Fig. 6A), confirming \textit{in vivo} the involvement of lipid rafts in alpha RIT therapeutic efficacy. We also observed a similar trend, although not significant, when we used lower activities (12.5 MBq and 7.4 MBq) of $^{213}$Bi-anti-MISRII mAb (Supplementary Fig S8), at earlier time point (day 14) post-RIT.

Moreover, the number of 53BP1 foci per cell was lower in tumors from mice harboring A-431\textsubscript{CEA} tumor cell xenografts after combined treatment with $^{125}$I-anti-CEA mAb and MBCD compared with $^{125}$I-anti-CEA mAb alone (Fig. 6A, right panel).

Similarly, pravastatin modulated Auger RIT efficacy in mice harboring subcutaneous A-431\textsubscript{CEA} tumor cell xenografts (Fig. 6B). Compared with the NaCl-treated group, pravastatin delayed tumor growth and increased survival (median survival: 49 vs 39 days for control; $p=0.17$) (Fig. 6B). Survival was comparable in mice treated with $^{125}$I-anti CEA mAb alone or
\(^{125}\)I-anti CEA mAb + pravastatin (median survival = 55 vs 57 days, respectively; \(p = 0.92\)) (Fig. 6B). However, at day 39 (at a time where most of the mice were still monitored) or at day 58 (end of the follow-up period), the highest tumor mass reduction (NaCl to RIT tumor mass ratio= 0.52 and 0.71, respectively) was observed with RIT alone, whereas it was comparable for the pravastatin and pravastatin + RIT groups (pravastatin to pravastatin + RIT tumor mass ratio=0.5 and 0.99, respectively) (Fig. 6C).

**Relative contribution of targeted and non-targeted cytotoxicity in alpha and Auger RIT**

Using the present experimental data in donor and recipient cells (Fig. 2 and 4C) and previous data we described for Auger RIT and \(^{125}\)I-UdR targeting of the nucleus (34), we could determine the relative contribution of targeted and non-targeted cytotoxicity (Supplementary Methodology and Fig.7). We found that alpha RIT targeted cytotoxicity was responsible of 67% to 94% of cell death (Fig. 7), and was much higher than the cell death induced by Auger RIT (<18%). However, when the emitter was located in the DNA, for instance when using \(^{125}\)I-UdR (34), Auger and alpha RIT targeted effects were similar (i.e., 69.6% of cell death). Although the non-targeted cytotoxic effects of the \(^{212}\)Pb-anti-HER2 mAb killed only about 7% of cells, those of the \(^{212}\)Pb-anti-CEA and \(^{213}\)Bi-anti-MISRII mAbs caused 30% to 36% of cell death (Fig. 7), which means that about 50% of killing by alpha particles is due to non-targeted effects. We obtained similar non-targeted effect values for Auger RIT using the \(^{125}\)I-anti CEA mAb (28.8%), indicating that Auger RIT non-targeted effects kill about twice more cells than the corresponding targeted effects.

Moreover, for alpha RIT, lipid rafts contributed significantly to killing and in a similar proportion for targeted and non-targeted cytotoxic effects (Fig. 7), which represents a contribution of about 25-33% (i.e., 21-24% of 67-94%) of targeted effects and about 50% of non-targeted effects (i.e., 17.6-20.9% of 7-36%). For Auger RIT, the contribution of lipid
rafts was higher: about 50% for targeted effects (9.2-9.4 of 18%) and higher (65%-72%) for non-targeted effects (18.9-21.6% of 27-29%).

Discussion

In this study, we demonstrated that cell membrane-mediated non-targeted effects play a significant role (up to 50% depending on situations) during Auger and alpha RIT, and that drugs regulating cholesterol level, such as MBCD, filipin and statins, could interfere with RIT efficacy.

We first showed in mice bearing small-volume peritoneal carcinomatosis and treated with $^{212}$Pb-anti-CEA mAbs that non-targeted effects occur, and could limit the negative effect of the antibody distribution heterogeneity by producing similar levels of damage in both irradiated and non-irradiated tumor areas, contributing to the therapeutic efficacy. It must be noted that we showed previously that unlabeled used here antibodies had no therapeutic efficacy under RIT conditions (33). As DNA damage (DNA DSBs) increases proportionally with the irradiation dose (a feature of targeted effects) (36,37), in the absence of non-targeted signals the level of DNA lesions in non-irradiated areas should be similar to that measured in NaCl-treated tumors (Fig. 1D).

Conversely, for the $^{212}$Pb-anti-HER2 mAb, the relative contribution of targeted effects was higher than that of non-targeted effects, and the yield of DNA damage ex vivo was proportional to the dose (36). We then confirmed the non-targeted cytotoxic and genotoxic effects of alpha RIT in vitro using conditioned medium transfer experiments (Fig. 2).

As during RIT the cell membrane is subject to high energy deposition, we hypothesized that it could actively participate in targeted and non-targeted effects. Relatively few studies have investigated the effects of irradiation on the cell membrane (38,39). We found that ceramide production was increased during alpha RIT through ASMase activation.
(Fig. 3B and C). Ceramide is known to be a 2nd messenger of apoptosis, a cell death process observed during alpha-RIT (Fig. 4C). Moreover, sphingolipids, such as ceramide, interact with each other via hydrophilic interactions between the sphingolipid head groups, and form complexes that are stabilized by cholesterol that fills the gaps between large sphingolipid molecules. These complexes constitute very small floating domains in the membrane (called lipid rafts) that are in a liquid ordered phase and are insoluble in cold non-ionic detergents (40,41). We confirmed the formation of lipid raft during alpha-RIT (Fig. 3A) which corroborated a previous study showing also their formation during Auger-RIT (34). Upon irradiation, ceramide production leads to aggregation of lipid raft domains that are converted into larger membrane platforms. These platforms represent lateral sub-compartments that participate in the segregation of molecules and the re-organization of receptor molecules, membrane signaling and trafficking. Ceramide also interacts and activates the serine/threonine phosphatases PP2A and PP1 that in turn act on different signaling proteins, including MAPKs (AKT, c-JUN), PKC isoforms (PKCα and ζ), kinase suppressor of Ras (KSR), pRB, and BCL-2 (8,42-44). Here, we found that p38 and JNK 1/2/3 are activated during alpha RIT and play a significant role in targeted and non-targeted effects in vitro (Fig. 3F, 4 and 5A) and in vivo (Fig. 5B). However, phosphorylation of p38 and JNK1/2/3 was lost and clonogenic cell survival was increased when cell cultures and mice bearing tumor cell xenografts were treated with the combination of alpha RIT and MBCD (and also filipin in vitro), a drug that disrupt lipid rafts by cholesterol depletion (Fig. 5A and B).

We showed that MBCD and filipin reduced the efficacy of alpha-RIT in vitro (Fig. 3D) and also in vivo (Fig. 6A and Supplementary Fig. S8). In vivo, DNA damage level (53BP1 foci) was reduced in tumors of mice treated with RIT + MBCD. Similarly, Auger RIT efficacy was decreased when performed in the presence of pravastatin, an inhibitor of hydroxymethylglutaryl-CoA reductase (involved in cholesterol synthesis) and also of low-
density lipoprotein synthesis. This result is important because older patients undergoing alpha RIT are often also treated for hypercholesterolemia.

Moreover, our results suggest a role for ROS during alpha-RIT (Fig. 4A and Supplementary Fig. S7). Besides producing DNA lesions (45), HO° could participate in the oxidation of cysteine 629 in ASMase C-terminus, leading to ASMase activation (46-48).

We showed that catalase and DMSO limited the non-targeted and also targeted (to a lower extent) cytotoxic effects of alpha RIT (Fig. 5C) and also reduced the level of p-p38, p-JNK1/2/3 and p-NFkB in vitro (Fig. 4A). We did not observe any change in p-NFkB level in tumors (Fig 5B). However, overall, p-NFkB expression was very low and was not correlated with any treatment. It would be of interest in further experiments to use NFkB knock out mice to better delineate the role of the latter transcription factor in vivo. In recipient cells, ROS production could lead to simple DNA DSBs that recruit a lower number of DNA repair proteins, as shown by the presence of small 53BP1 and γH2AX foci only. Conversely, ROS seems to be less implicated in alpha RIT targeted effects that generate dense DNA ionization, leading to more complex lesions and the recruitment of a higher number of DNA repair proteins, as suggested by the larger 53BP1 and γH2AX foci (Fig. 2C and 2D).

Therefore, these results indicate that Auger and alpha RIT, which have been described as potential therapeutic tools mainly dedicated to the treatment of microscopic diseases, can also be efficient for small volume (several mm in size) tumors, far beyond the expected range of Auger and alpha particles. This is also relevant for radionuclide therapy in which radiolabeled vectors cannot gain access to all tumor cells. Non-targeted effects could thus contribute to the observed therapeutic efficacy of the new radiopharmaceuticals currently under evaluation (49,50).

Finally, it must be noted that non-targeted effects of radiation might also include abscopal and systemic effects that are measured at a distance from the irradiated tumor area.
and that are mediated by the immune system (51,52). Although these effects have been poorly studied during RIT, they might contribute to RIT effectiveness and require the development of dedicated immunocompetent preclinical models (53).

**Conclusion**

This study showed that alpha RIT is mediated by targeted and non-targeted effects. Depending on the radiopharmaceuticals, non-targeted effects contribution can be high. Upon radiolabeled mAb binding to cell surface receptors, the formation of ceramide-enriched large platforms and the subsequent activation of p38- and JNK-mediated signaling pathways are partly responsible for the non-targeted effects and, to a lower extent, to targeted effects. As targeted effects are dose-related but not non-targeted effects, these results have consequences on the planning and prediction of the therapeutic efficacy and side effects of TRT using alpha particles. Moreover, they suggest that patients undergoing treatment to modify lipid metabolism could respond differently to TRT.
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FIGURE LEGENDS

FIG. 1. *In vivo* evidence for non-targeted effects. (A) Kaplan Meyer survival analysis of mice bearing intraperitoneal A-431 tumor cell xenografts that received one single i.p. injection of NaCl, or 1.48MBq of $^{212}$Pb-anti-CEA mAb, $^{212}$Pb-anti-HER2 mAb, or $^{212}$Pb-PX (non-specific mAb) at day 5 post-graft (data from (33)). (B) Representative digital autoradiography (DAR) images of tumor sections at 17h post-RIT. Grey scale bar shows the calculated dose per decay (Gy/Bq.s) (C) Histograms showing the tumor volume in function of the absorbed dose for A-431$_{CEA}$ cell xenografts from mice treated with $^{212}$Pb-anti-HER2 mAb (red), $^{212}$Pb-anti-CEA mAb (blue) and $^{212}$Pb-PX (green). A representative tumor for each group was selected and used as input for the simulation of the voxel absorbed dose. About 30% of the $^{212}$Pb-anti-CEA mAb-treated tumor received 0 Gy of absorbed dose. (D) The experimental tumor growth was determined by measuring the bioluminescence signal over time ($^{212}$Pb-anti-CEA mAb-treated mice). The theoretical curve was established by considering that 30% of the tumor volume of $^{212}$Pb-anti-CEA mAb-treated mice behaved like tumors from NaCl-treated mice (theoretical curve). (E) Immunohistochemical analysis of 53BP1 foci in the frozen section adjacent to the one used for DAR. DAR images (as reported in Fig.1B) are also shown. The distribution of 53BP1 foci was determined in 100 cells for each tumor area (6-7 tumor areas for each mouse). Some of the selected tumor areas are highlighted in yellow in the immunohistochemical analysis panels and the relevant DAR panels. (F) For each radiolabeled $^{212}$Pb-mAb, the average number of 53BP1 foci per cell (counted in each tumor area) was plotted as a function of the activity (cpm/mm$^2$) determined by DAR in the corresponding tumor area.

FIG. 2. *In vitro* evidence for non-targeted effects. Clonogenic cell survival was assessed in donor and recipients cells 12 days after exposure to increasing activities (0-0.5MBq/mL) of...
radiolabeled mAbs for 90 min (donor cells), or incubation in culture medium in which donor cells were cultured for 2 h (recipient cells). (A) A-31<sub>CEA</sub> donor cells were exposed to <sup>212</sup>Pb-anti-CEA (gray) or <sup>212</sup>Pb-anti-HER2 mAbs (black). (B) SK-OV-3<sub>MISRII</sub> donor cells were exposed to <sup>213</sup>Bi-anti-MISRII mAb. (C) The mean number ± SD of 53BP1 and γH2AX foci per cell was determined by immunofluorescence in SK-OV-3<sub>MISRII</sub> donor cells exposed to 0.5 MBq/mL <sup>213</sup>Bi-anti-MISRII mAb and in the corresponding recipient cells (n=100 cells/group). (D) 53BP1 and γH2AX foci were classified as large, medium and small in donor, recipient and untreated cells according to size criteria shown in Supplementary Fig S3. Results are the mean ± SD of three experiments performed in triplicate. *p <0.05, **p <0.01, ***p <0.001 compared with untreated cells.

FIG. 3. Lipid raft-mediated activation of signaling pathways.

(A) Lipid rafts were detected by immunofluorescence analysis using Alexa-488-conjugated cholera toxin B (green) in untreated and treated (<sup>213</sup>Bi-mAb or <sup>213</sup>Bi-mAb + MBCD) SK-OV-3<sub>MISRII</sub> cells. Nuclei were stained with Hoechst (blue). (B) ASMase and ceramide levels were measured in SK-OV-3<sub>MISRII</sub> cells by flow cytometry analysis after incubation with 25 mM H<sub>2</sub>O<sub>2</sub> (positive control) or with <sup>213</sup>Bi-anti-MISRII mAbs. (C) Clonogenic survival of SK-OV-3<sub>MISRII</sub> donor cells incubated with <sup>213</sup>Bi-mAb or <sup>213</sup>Bi-mAb and imipramine (an ASMase inhibitor). (D) Clonogenic cell survival of SK-OV-3<sub>MISRII</sub> donor cells co-incubated or not with <sup>213</sup>Bi-anti-MISRII mAb and filipin or MBCD (two lipid raft disruptors) and of the corresponding recipient cells. (E) Cholesterol level in cell extracts of untreated and MBCD-treated SK-OV-3<sub>MISRII</sub> cells. Results are the mean ± SD of three (four for the clonogenic assays) experiments performed in triplicate. *p <0.05, **p <0.01, ***p <0.001 compared with untreated cells. (F) The phosphorylation level of 46 kinases activated in SK-OV-3<sub>MISRII</sub> donor cells exposed or not (untreated) to unlabeled or <sup>213</sup>Bi-labeled anti-MISRII mAbs was assessed.
using the Human Phospho-Kinase Array (Proteome Profiler Array; R&D Systems, Minneapolis, MN). For each kinase, the Image J software was used to determine the pixel intensity.

FIG. 4. Pharmacological inhibition of MAPK pathways and involvement of lipid rafts in vitro. (A) The expression of phosphorylated p38 (p-p38), SAPK/JNK (p-SAPK/JNK) and NFkB (p-NFkB) was determined by western blotting using extracts of SK-OV-3_{MISRII} cells exposed to $^{213}$Bi-mAbs and/or catalase or DMSO. (B) EdU and TUNEL (48h post-RIT) staining of SK-OV-3_{MISRII} donor cells pre-incubated or not with 10 µM of SP600125 (JNK inhibitor) or 10 µM of SB203580 (p38 inhibitor) for 30min and then exposed to 0-0.5MBq/mL $^{213}$Bi-anti-MISRII mAb in the presence or not of the inhibitors for 90min. (C) Quantification of EdU-positive (at 24h and 48h post-treatment) and TUNEL-positive (at 48h) SK-OV-3_{MISRII} cells relative to all cells. (D) SK-OV-3_{MISRII} donor cells were pre-incubated or not with 10 µM of SP600125 or 10 µM of SB203580 for 30min and then exposed to 0-0.5MBq/mL $^{213}$Bi-anti-MISRII mAb in the presence or not of the JNK and p38 inhibitors. Clonogenic cell survival was determined in donor and recipient cells. Results are the mean ± SD of four experiments performed in triplicate. *p <0.05, **p <0.01, ***p <0.001 compared with untreated cells.

FIG. 5. MBCD effect on protein kinase expression and antioxidant defenses during alpha RIT in SK-OV-3_{MISRII} cells.

A) The phosphorylation level of 46 kinases activated in SK-OV-3_{MISRII} donor cells exposed or not (untreated) to $^{213}$Bi-anti-MISRII mAb or $^{213}$Bi-anti-MISRII mAb + MBCD (lipid raft disruptor) was determined using the Human Phospho-Kinase Array (Proteome Profiler Array; R&D Systems, Minneapolis, MN). For each kinase, the ImageJ software was used to
determine the pixel intensity. (B) The expression of phosphorylated (p)-p38, p-SAPK/JNK and p-NFkB was determined by western blotting using extracts of AN3CA tumors of mice exposed to low (3.7MBq and 7.4MBq) and high activities (37MBq) of $^{213}$Bi-anti-MISRII mAb combined or not with MBCD. (C) SK-OV-3$_{MISRII}$ donor cells were pre-incubated with catalase or 0.5% dimethyl sulfoxide (DMSO) for 20min before exposure to $^{213}$Bi-anti-MISRII mAbs in the presence of these ROS scavengers. Control cells were incubated only with $^{213}$Bi-anti-MISRII mAbs. Clonogenic cell survival of donor (left panel) and recipient cells (middle panel) was measured. Results are the mean ± SD of four experiments performed in triplicate. *p <0.05, **p <0.01, ***p <0.001 compared with cells exposed only to $^{213}$Bi-anti-MISRII mAb.

**FIG. 6. In vivo effects of drugs that modify cholesterol metabolism on tumor growth, and DNA damage formation.** (A) Mice bearing i.p. AN3CA tumor cell xenografts were treated with i) NaCl, ii) daily i.p. injections of 300 mg/kg MBCD from day 7 to 13 post-graft, iii) one injection of 37MBq of $^{213}$Bi-anti-MISRII mAb at day 11, or (iv) both $^{213}$Bi-anti-MISRII mAb and MBCD. At day 30 post-graft, mice were sacrificed, tumors collected, and the total tumor mass was determined (left panel). Tumors were also collected in mice (n=3 mice/group) for assessing MBCD effect on the tumor cholesterol level (middle panel). Immunohistochemical detection of 53BP1 foci in tumor sections from mice bearing i.p. A-431$_{CEA}$ cell tumor xenografts and killed 24h after i) two injections of 37MBq of $^{125}$I-anti-CEA mAb at day 8 and 11 post-graft, or (ii) after daily i.p. injections of MBCD from day 6 to 15 post-graft (MBCD) combined with two injections of 37MBq of $^{125}$I-anti-CEA mAb at day 8 and 11 post-graft (right panel). (B) Mice bearing subcutaneous A-431$_{CEA}$ tumor cell xenografts were treated with i) NaCl, or ii) daily i.p. injections of 40 mg/kg pravastatin from day 10 pre-graft until day 18 post-graft, or iii) two injections of 37MBq of $^{125}$I-anti-CEA mAb
at day 8 and 11 post-graft, or iv) $^{125}$I-anti-CEA mAb and MBCD. The average tumor volume in each treatment group (n=8) was determined and Kaplan Meyer survival curves were established. Mice were sacrificed when tumor volume reached 2000 mm$^3$. (C) The ratio between the tumor masses of the indicated groups was calculated. *p <0.05, **p <0.01, ***p <0.001 compared with control animals.

**FIG. 7. Contribution of targeted and non-targeted effects during Auger RIT using $^{125}$I-mAbs or alpha RIT using $^{212}$Pb- or $^{213}$Bi-mAbs.**

The contribution of targeted and non-targeted effects of RIT with $^{213}$Bi-anti-MISRII, $^{212}$Pb-anti-CEA, $^{212}$Pb-anti-HER2, $^{125}$I-anti-CEA mAbs or $^{125}$I-UdR to cell killing in the presence or not of lipid raft disruptors (filipin, MBCD), or antioxidants (catalase, DMSO, or N-acetyl cysteine) was determined using a Bliss independence mathematical model. Both Auger and alpha RIT activate ASMase through ROS production. ASMase catalyzes sphingomyelin hydrolysis to ceramide and phosphorylcholine, and contributes to the formation of ceramide-enriched domains that activate p38/JNK1/2/3, and NF-kB and downstream signaling pathways. Alpha RIT targeted effects produced large 53BP1 and $\gamma$H2AX foci (assimilated to complex DNA lesions), as indicated by immunofluorescence analysis (see Fig. 1). Conversely, Auger RIT targeted effects and Auger and alpha RIT non-targeted effects produced small foci that could represent single DNA lesions. Antioxidant agents reduce the non-targeted effects of both Auger and alpha RIT, but only the targeted effects of Auger RIT. The proportion of cells killed via a lipid raft-mediated process is also indicated.

SM, sphingomyelin; NO, nitric oxide; iNOS, inducible nitric oxide synthase.
Figure 1

A-431<sub>CEA</sub> tumor xenografts

From Boudousq et al. Plos One 2013

![Graph showing survival and average dose](image)

B

$^{212}\text{Pb}$-anti HER2  $^{212}\text{Pb}$-anti CEA  $^{212}\text{Pb}$-irrelevant PX

![Images of xenografts](image)

C

Volume fraction vs. absorbed dose (Gy)

![Graph showing volume fraction](image)

D

Tumor weight (g) vs. time post graft (W)

![Graph showing tumor weight](image)

E

NaCl  $^{212}\text{Pb}$-anti HER2

![Images of xenografts](image)

$^{212}\text{Pb}$-anti CEA  $^{212}\text{Pb}$-irrelevant PX

F

Average number of 53BP1 foci per cell vs. activity (cpm/mm²)

![Graph showing foci per cell](image)
Figure 2

A) A-431<sub>CEA</sub> cells

Donor cells

Recipient cells

Clonogenic Survival (%)

Test activity of donor cells (MBq/mL)

B) SK-OV-3<sub>MISRII</sub> cells

Donor cells

Recipient cells

Clonogenic Survival (%)

Test activity of donor cells (MBq/mL)

C) SK-OV-3<sub>MISRII</sub> cells

Donor cells  □ Recipient cells

Average number of 53BP1 foci/cell

213<sup>Bi</sup>-anti-MISRII  Untreated

D) SK-OV-3<sub>MISRII</sub> cells

Large □ Medium □ Small

Average number of 53BP1 foci/cell

Donor  Recipient  Untreated

Average number of γ-H2AX foci/cell

Donor  Recipient  Untreated
Figure 6

A  **AN3CA** tumor xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor mass (g)</th>
<th>Cholesterol level (µg/µL)</th>
<th>Average number of 53BP1 foci per cell</th>
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<td>NaCl</td>
<td>×0.02</td>
<td>×0.05</td>
<td>3</td>
</tr>
<tr>
<td>MBCD</td>
<td>×0.15</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>219Bi-anti MISRII</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>219Bi-anti MISRII</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B  **A-431**\textsubscript{CEA} tumor xenografts

- NaCl
- Prava
- 125I-anti CEA
- 125I-anti CEA + Prava

C  **A-431**\textsubscript{CEA} tumor xenografts

Day 39 post-graft:
- NaCl
- Prava
- 125I-anti CEA

Day 58 post-graft:
- NaCl
- Prava
- 125I-anti CEA
Clinical Cancer Research

Drugs that modify cholesterol metabolism alter the p38/JNK-mediated targeted and non-targeted response to alpha and Auger radioimmunotherapy

Riad Ladjohounlou, Catherine Lozza, Alexandre Pichard, et al.

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