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Drugs That Modify Cholesterol Metabolism Alter the p38/JNK-Mediated Targeted and Nontargeted Response to Alpha and Auger Radioimmunotherapy

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Abstract

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

Experimental Design: Here, we investigated the contribution of nontargeted cytotoxic and genotoxic effects in vitro and in vivo (in xenografted mice) during alpha (212Pb/212Bi, 213Bi) and Auger (125I) 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% to 36% (alpha RIT) and 27% to 29% (Auger RIT) of cells were killed by nontargeted effects. We then demonstrated that the nontargeted cell response to alpha and Auger RIT was partly driven by lipid raft–mediated activation of p38 kinase and JNK. Reactive oxygen species also played a significant role in these nontargeted effects, as demonstrated by NF-κB 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 nontargeted effects play a significant role during Auger and alpha RIT, and drugs that modulate cholesterol level, such as statins, could interfere with RIT efficacy.

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 an mAb (for radioimmunotherapy, RIT). Once administered to patients, they specifically recognize tumor cells and produce targeted irradiation to eradicate them. Although 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
Antigen (CEA, expressed by colorectal carcinoma cancer) and were transfected with constructs encoding carcinoembryonic antigen Type Culture Collection (ATCC). HER2-positive A-431 cells adenocarcinoma human cell lines were obtained from the American Type Culture Collection (ATCC). AN3CA endometrial carcinoma, and HCT116 colon carcinoma, AN3CA cells were chosen because they mimic ovarian peritoneal carcinomatosis when grafted i.p. 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; refs. 2, 30).

Parental and A-431CEA and SK-OV-3MISRII cells were grown in DMEM, and HCT116 cells in RPMI 1640 medium. Media were supplemented with 10% (v/v) FCS, 2 mmol/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% nonessential amino acids. Cells were kept at 37°C in a humidified atmosphere containing 5% CO2. The murine IgG1k mAb 35A7 against the CEA Gold 2 epitope and the anti-HER2 mAb trastuzumab (Herceptin, Genentech) 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).

**Antibody conjugation and radiolabeling with 212Pb, 213Bi, or 125I**

The anti-HER2, 35A7 and PX mAbs were conjugated with TCMC (Macroyclics) prior to radiolabeling at the specific activity of 37MBq/mg with 212Pb (Orano Med), as described in ref. 33 (Supplementary Fig. S1). The anti-MISRII mAb was conjugated with 2-(P-SCN-benzyl)-cyclohexyl acid A-diethylenetriamine-pentaacetic (CHX-A”-DTPA) and radiolabeled with 213Bi (JRC) at the specific activity of 37 MBq/mg, as described in ref. 31. The anti-CEA mAb was radiolabeled also with 125I (Perkin Elmer) at the specific activity of 370 MBq/mg, as described in ref. 34.

**In vivo RIT using 212Pb, 125I, and 213Bi-labeled antibodies**

Female athymic nude Foxn1nu mice (6–8 weeks old; Envigo RMS Laboratories) were acclimated for 1 week. They were housed at 22°C and 55% humidity with a light-dark cycle of 12 hours, 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 × 106 A-431CEA or 4.5 × 106 AN3CA cells in 0.3 mL DMEM or 0.3 mL DMEM/Matrigel (1:1), respectively, were i.p. grafted in mice. Six days after A-431CEA 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 days 8 and 11); or methyl-β-cyclodextrin (MBCD, lipid raft disruptor; 300 mg/kg; daily, from days 6 to 15); or both $^{125}$I-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 days 7 to 13 after graft; or (iii) one injection of $^{213}$Bi-anti-MISRII mAb (37 MBq) at day 11; or (iv) both $^{213}$Bi-anti-MISRII mAb and MBCD. At day 30 after 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 s.c. xenografted with $1 \times 10^6$ A-431CEA cells in Matrigel and treated with (i) NaCl; or (ii) daily i.p. injections of 40 mg/kg pravastatin from day 10 before graft until day 18 after graft; or (iii) one injection of $^{125}$I-anti-CEA mAb (37 MBq) at day 8 and one at day 11; or (iv) both $^{125}$I-anti-CEA mAb and pravastatin. Tumor growth was followed by caliper measurement.

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

At various times (4, 17, and 22 hours) following alpha ($^{211}$Pb/$^{211}$Bi) or 24 hours after Auger RIT, mice bearing i.p. A-431CEA tumor cell xenografts were anesthetized, bled, and dissected. Tumors were collected and frozen after inclusion in OCT embedding matrix. Two consecutive 10-μm-thick frozen sections were analyzed by digital autoradiography (DAR) or by immunodetection of 53BP1, as described in ref. 34.

**In vitro determination of targeted and nontargeted cytotoxic effects of alpha RIT**

SK-OV-3MISRII (100 to 1,500 cells/well) and A-431CEA (100 to 5,000 cells/well) cells were plated in 6-well plates with 2 mL of medium. The following day, cells were incubated with $^{211}$Bi-anti-MISRII mAb (SK-OV-3MISRII cells) or $^{212}$Pb-anti-CEA mAb and $^{212}$Pb-anti-HER2 mAb (A-431CEA cells; 0.5 MBq/mL), or the corresponding unlabeled mAbs (0–27 μg/mL) for 90 minutes (donor cells). Culture medium was then removed, and cells were washed twice with 4 mL PBS. For investigating the nontargeted cytotoxic response, fresh medium (2 mL) was added to donor cells for 2 hours 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 45:50% (v/v) methanol/paraformaldehyde (PFA)]. Colonies containing 50 or more cells were scored, and the surviving fraction was calculated.

**In vitro measurement of DNA damage**

A-431CEA and SK-OV-3MISRII 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-431CEA cells) or 0.5 MBq/mL of $^{211}$Bi-mAbs (SK-OV-3MISRII cells) for 90 minutes. After radiolabeled antibody removal, fresh medium (2 mL) was added to donor cells for 2 hours and then transferred to recipient cells. Both donor and recipient cells were fixed with PFA and permeabilized, as described in ref. 34 (and Supplementary Methodology) for γ-H2AX/53BP1 foci detection.

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

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

Cells were incubated with $^{211}$Bi-anti-MISRII mAb (0.5 MBq/mL) for 90 minutes or H$_2$O$_2$ (25 mmol/L) at 37°C for 30 minutes. After three PBS washes, they were fixed in 3.7% (v/v) PFA for 15 minutes and then washed 3 times in PBS. Cells were then incubated with a polyclonal rabbit anti-ASMase antibody (2 μg/mL; Santa Cruz Biotechnology, Inc.) and the anti-ceramide 15B4 mAb (1:50; Alexis Biochemicals) at 37°C for 1 hour. After three washes in PBS/2% FCS, cells were incubated with Alexa Fluor 555–conjugated anti-rabbit (1:500; Invitrogen) or with Alexa Fluor 488–conjugated anti-mouse IgM (1:200; Invitrogen) in the dark for 1 hour. After three washes and resuspension in PBS, cells were analyzed by flow cytometry (Muse, Merck Millipore), and the G mean value (arbitrary units) was determined. To confirm ASMase role in RIT cytotoxicity, SK-OV-3MISRII cells were preincubated with 50 μmol/L imipramine (ASMase inhibitor) for 30 minutes following addition of $^{211}$Bi-anti-MISRII mAb (0.5 MBq/mL) at 37°C for 90 minutes.

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) at 37°C for 45 minutes. After three washes in PBS, cells were fixed again in PFA for 10 minutes, and coverslips were then mounted in Mowiol and analyzed using a 63 × NA objective and a Leica (Leica Microsystems) inverted microscope.

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

The role of lipid raft integrity in $^{211}$Bi-mAb–induced nontargeted cytotoxic effects was investigated by preincubating SK-OV-3MISRII cells with 4 mmol/L MBCD (Sigma-Aldrich) or 2.5 μg/mL filipin (Sigma-Aldrich) for 30 minutes before adding $^{211}$Bi-anti-MISRII mAb for another 90 minutes 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).

**Proteome kinase analysis and Western blotting**

A Human Phospho-Kinase Array (Proteome Profiler Array, R&D Systems) was used to detect the relative phosphorylation levels of 46 kinases in cell extracts of SK-OV-3MISRII donor (incubated with 0.5 MBq/mL $^{211}$Bi-anti-MISRII mAb, or with 0.5 MBq/mL $^{213}$Bi-anti-MISRII mAb and 4 mmol/L 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). After incubation with anti-phosphorylated (p) ERK, p-SAP/JNK, p-p38, and p-NF-κB (p65) primary antibodies (Cell Signaling Technology), immune reactions were detected with horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit (Sigma-Aldrich) secondary antibodies and the ECL detection system (Amersham Biosciences).
**In vitro analysis of p38 and JNK role**

SK-OV-3MISRII donor cells were preincubated with 10 μmol/L SP600125 (JNK inhibitor) or 10 μmol/L SB203580 (p38 inhibitor) for 30 minutes before addition of 0 to 0.5 MBq/mL 213Bi-anti-MISRII mAb in the presence of the inhibitors for another 90 minutes. 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 preincubated with 20 μg/mL catalase (Sigma-Aldrich) or 0.5% DMSO (Sigma-Aldrich) for 20 minutes before RIT with 212Bi-anti-MISRII mAb in the presence of catalase or DMSO for 90 minutes. 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 preincubated with SP600125 (JNK inhibitor), SPB203580 (p38 inhibitor), imipramine (ASMase inhibitor), or MBCD (lipid raft disruptor) for 30 minutes before addition of 0 to 0.5 MBq/mL 213Bi-anti-MISRII mAb for 90 minutes. Then, radioactivity was removed, cells were washed with PBS, and apoptosis was measured at 48 hours after treatment using the TUNEL Detection Kit (Promega) according to the manufacturer’s instructions.

Cell proliferation was measured in SK-OV-3MISRII cells using the EdU Cell Proliferation Assay (Merk Millipore). Briefly, EdU was added to the medium at a final concentration of 10 μmol/L immediately after RIT and left for 24 hours. 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 were analyzed using a 63 × NA objective and a Leica (Leica Microsystems) 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) and described using mean, SD, median, and range. Comparisons between radiolabeled antibodies and control were performed using the nonparametric Kruskal–Wallis test (significance level set at 0.05). Pairwise comparisons were performed using the nonparametric 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**

Nontargeted effects could contribute to the 212Pb-anti-CEA mAb efficacy

The Kaplan–Meier analysis in our previous study (33) showed a significantly higher survival rate (P < 0.05) in mice bearing intraperitoneal A-431CEA tumors and treated with 212Pb-anti-HER2 mAb compared with 212Pb-anti-CEA mAb (Fig. 1A). The absence of antitumor effect of the nonspecific 212Pb-PX mAb (P = 0.85 vs. NaCl) demonstrated the absence of in vivo cytotoxic effects when 212Pb is not targeted to tumor cells. The higher therapeutic efficacy of the 212Pb-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 212Pb-anti-HER2 and 212Pb-anti-CEA mAb, respectively (Supplementary Methodology). Conversely, DAR analysis (Fig. 1B) showed that compared with tumors treated with 212Pb-anti-HER2 mAb, some tumor regions incorporated very low or no 212Pb-anti-CEA mAb. Voxel dosimetry (Supplementary Methodology) indicated that about 30% of the tumor volume from 212Pb-anti-CEA mAb–treated mice received 0 Gy (Fig. 1C). Conversely, in the 212Pb-anti-HER2 mAb group, all regions received some radioactivity, although the maximal voxel dose was about 2 times lower (77 for 212Pb-anti-HER2 mAb vs. 140 Gy for 212Pb-anti-CEA mAb).

To confirm in vitro the contribution of targeted and nontargeted cytotoxic effects during alpha RIT, we determined the clonogenic cell survival of A-431CEA and SK-OV-3MISRII donor cells exposed to radiolabeled antibodies and of the corresponding recipient cells. In donor A-431CEA cells, 212Pb-mAbs strongly reduced clonogenic cell survival at test activities as low as 0.03 MBq/mL (Fig. 2A). Beyond 0.06 MBq/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 (nontargeted effects). Targeted and nontargeted cytotoxic effects...
**Figure 1.**

*In vivo* evidence for nontargeted effects. **A,** Kaplan–Meier survival analysis of mice bearing intraperitoneal A-431 tumor cell xenografts that received a single i.p. injection of NaCl, or 1.48 MBq of $^{212}$Pb-anti-CEA mAb, $^{212}$Pb-anti-HER2 mAb, or $^{212}$Pb-PX (nonspecific mAb) at day 5 after graft (data from ref. 33). **B,** Representative DAR images of tumor sections at 17 hours after RIT. Gray 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-431CEA 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,** IHC analysis of 53BP1 foci in the frozen section adjacent to the one used for DAR. DAR images (as reported in **B**) 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 IHC 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/mm²) determined by DAR in the corresponding tumor area.
Figure 2.

In vitro evidence for nontargeted effects. Clonogenic cell survival was assessed in donor and recipient cells 12 days after exposure to increasing activities (0–0.5 MBq/mL) of radiolabeled mAbs for 90 minutes (donor cells), or incubation in culture medium in which donor cells were cultured for 2 hours (recipient cells). 

A, A-31CEA donor cells were exposed to $^{212}$Pb-anti-CEA (gray) or $^{212}$Pb-anti-HER2 mAbs (black).

B, SK-OV-3MISRII donor cells were exposed to $^{213}$Bi-anti-MISRII mAb.

C, The mean number ± SD of 53BP1 and γH2AX foci per cell was determined by immunofluorescence in SK-OV-3MISRII donor cells exposed to 0.5 MBq/mL $^{213}$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. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with untreated cells.
were also observed in SKOV3-MISRII donor cells exposed to the \(^{213}\)Bi-anti-MISRII mAb and in recipient cells (Fig. 2B). In both cell models, neither targeted nor nontargeted cytotoxic effects were detected after exposure to unlabeled antibodies (Supplementary Fig. S2).

DNA double-strand breaks induced by targeted and nontargeted effects of alpha RIT are associated with different types of 53BP1 and \(\gamma\)H2AX foci

We then evaluated the expression of 53BP1 and \(\gamma\)H2AX, two DNA double-strand break (DSB) markers, in SK-OV-3-MISRII donor cells exposed to \(^{212}\)Bi-anti-MISRII mAbs for 90 minutes and in recipient cells (Fig. 2C and D; Supplementary Fig. S3A). Quantification of the mean number of 53BP1 and \(\gamma\)H2AX 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 nontargeted 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 ± 0.1, 3.7 ± 0.2, and 5.4 ± 0.3, respectively (Fig. 2D). Conversely, the mean number of 53BP1 small foci was 1.8 ± 0.3 in recipient cells, and 0.7 ± 0.1 in untreated cells. We obtained similar results for \(\gamma\)H2AX foci (Fig. 2D, right plot), and in A-431CEA cells exposed to \(^{213}\)Bi-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 \(\gamma\)H2AX and 53BP1 proteins compared with medium and small foci, and that consequently, they corresponded to more complex lesions. As a consequence of mis repaired DNA DSBs, the number of micronuclei was significantly increased in both donor and recipient A-431CEA cells after exposure to \(^{212}\)Pb-mAbs (Supplementary Fig. S3C).

Lipid rafts participate in the targeted and nontargeted 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 nontargeted effects. Using cholera toxin B, we first showed the formation of lipid raft domains in SK-OV-3-MISRII cells exposed to \(^{213}\)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, top) and ceramide production (Fig. 3B, bottom) were increased in SK-OV-3-MISRII cells exposed to \(^{213}\)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 \(^{213}\)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).

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 \(^{213}\)Bi-anti-MISRII mAbs in the presence of MBCD (Fig. 5A; Supplementary Fig. S5B), which decreased the mean cholesterol levels (Fig. 3E), and filipin (Supplementary Fig. S6). Phosphorylated p38 and JNK1/2/3 expression levels were strongly reduced in both donor and recipient cells exposed to \(^{213}\)Bi-anti-MISRII mAb + MBCD or filipin compared with cells incubated only with \(^{213}\)Bi-anti-MISRII mAb. Western blot analysis of tumors collected from mice treated with 37 MBq \(^{213}\)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 nontargeted response to alpha RIT

As ROS can activate ASMase, we assessed the role of oxidative stress in the targeted and nontargeted 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,
Figure 3.

Lipid raft-mediated activation of signaling pathways. A, Lipid rafts were detected by immuno-fluorescence analysis using Alexa-488–conjugated cholera toxin B (green) in untreated and treated (213Bi-mAb or 213Bi-mAb + MBCD) SK-OV-3MISRII cells. Nuclei were stained with Hoechst (blue). B, ASMase and ceramide levels were measured in SK-OV-3MISRII cells by flow cytometry analysis after incubation with 25 mmol/L H2O2 (positive control) or with 213Bi-anti-MISRII mAbs. C, Clonogenic survival of SK-OV-3MISRII donor cells incubated with 213Bi-mAb or 213Bi-mAb and imipramine (an ASMase inhibitor). D, Clonogenic cell survival of SK-OV-3MISRII donor cells coincubated or not with 213Bi-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-3MISRII cells. Results are the mean ± SD of three (four for the clonogenic assays) experiments performed in triplicate. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 compared with untreated cells. F, The phosphorylation level of 46 kinases activated in SK-OV-3MISRII donor cells exposed or not (untreated) to unlabeled or 213Bi-labeled anti-MISRII mAbs was assessed using the Human Phospho-Kinase Array (Proteome Profiler Array; R&D Systems). For each kinase, Image J software was used to determine the pixel intensity.
Figure 4.
Pharmacologic 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 NF-κB (p-NF-κB) was determined by Western blotting using extracts of SK-OV-3MISRII cells exposed to 213Bi-mAbs and/or catalase or DMSO. B, EdU and TUNEL (48 hours after RIT) staining of SK-OV-3MISRII donor cells preincubated or not with 10 μmol/L of SP600125 (JNK inhibitor) or 10 μmol/L of SB203580 (p38 inhibitor) for 30 minutes and then exposed to 0–0.5 MBq/mL 213Bi-anti-MISRII mAb in the presence or not of the inhibitors for 90 minutes. C, Quantification of EdU-positive (at 24 and 48 hours after treatment) and TUNEL-positive (at 48 hours) SK-OV-3MISRII cells relative to all cells. D, SK-OV-3MISRII donor cells were preincubated or not with 10 μmol/L of SP600125 or 10 μmol/L of SB203580 for 30 minutes and then exposed to 0–0.5 MBq/mL 213Bi-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; and ***, P < 0.001 compared with untreated cells.
Figure 5.
MBCD effect on protein kinase expression and antioxidant defenses during alpha RIT in SK-OV-3MISRII cells. A, The phosphorylation level of 46 kinases activated in SK-OV-3MISRII 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). For each kinase, ImageJ software was used to determine the pixel intensity. B, The expression of phosphorylated (p)-p38, p-SAPK/JNK, and p-NF-$k$B was determined by Western blotting using extracts of AN3CA tumors of mice exposed to low (3.7 MBq and 7.4 MBq) and high activities (37 MBq) of $^{213}$Bi-anti-MISRII mAb combined or not with MBCD. C, SK-OV-3MISRII donor cells were preincubated with catalase or 0.5% DMSO for 20 minutes 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 mAb. Clonogenic cell survival of donor (left) and recipient cells (middle) was measured. Results are the mean ± SD of four experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ compared with cells exposed only to $^{213}$Bi-anti-MISRII mAb.
coincubation 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 0.25 and 0.5 MBq/mL (P < 0.001) and in recipient cells incubated with medium from donor cells exposed to 0.25 and 0.5 MBq/mL of 211Bi-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-NF-κB 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 over-expressed in donor cells exposed to 211Bi-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-pyrole 2, 3-dioxygenase (IDO), NFκB, p21, paraoxonase 2 and 3 (PON2, PON3), and SOD2. Conversely, in HCT116 cells, the same proteins were induced in donor cells exposed to 125I-anti-CEA mAb (Auger RIT) and in recipient cells (nontargeted effect; Supplementary Fig. S7B). Western blot analysis confirmed the role of oxidative stress, as demonstrated by the increase in phosphorylated NF-κB expression after in vivo Auger and alpha RIT (Fig. 4A; Supplementary Fig. S7C). We found that alpha RIT–targeted cytotoxicity was responsible for 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 125I-UdR (34), Auger and alpha RIT–targeted effects were similar (i.e., 69.6% of cell death).

Using the present experimental data in donor and recipient cells (Figs. 2 and 4C) and previous data we described for Auger RIT and 125I-UdR targeting of the nucleus (34), we could determine the relative contribution of targeted and nontargeted cytotoxicity (Supplementary Methodology and Fig. 7). We found that alpha RIT–targeted cytotoxicity was responsible for 67% to 94% of cell death (Fig. 7), and was much higher than the cell death induced by Auger RIT (<18%). Using 125I-anti-CEA mAb (28.8%), indicating that Auger RIT nontargeted 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 nontargeted cytotoxic effects (Fig. 7), which represents a contribution of about 25% to 33% (i.e., 21%–24% of 67%–94%) of targeted effects and about 50% of nontargeted 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 nontargeted effects (18.9%–21.6% of 27%–29%).

**Discussion**

In this study, we demonstrated that cell membrane–mediated nontargeted 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 212Pb-anti-CEA mAbs that nontargeted effects occur and could limit the negative effect of the antibody distribution heterogeneity produced by similar levels of damage in both irradiated and nonirradiated 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; refs. 36, 37), in the absence of nontargeted signals, the level of DNA lesions in nonirradiated areas should be similar to that measured in NaCl-treated tumors (Fig. 1D). Conversely, for the 212Pb-anti-HER2 mAb, the relative contribution of targeted effects was higher than that of nontargeted
Figure 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 days 7 to 13 after graft, (iii) one injection of 37 MBq of $^{213}$Bi-anti-MISRII mAb at day 11, or (iv) both $^{213}$Bi-anti-MISRII mAb and MBCD. At day 30 after graft, mice were sacrificed, tumors were collected, and the total tumor mass was determined (left). Tumors were also collected in mice ($n = 3$ mice/group) for assessing MBCD effect on the tumor cholesterol level (middle). IHC detection of 53BP1 foci in tumor sections from mice bearing i.p. A-431CEA cell tumor xenografts and killed 24 hours after (i) two injections of 37 MBq of $^{125}$I-anti-CEA mAb at days 8 and 11 after graft, or (ii) daily i.p. injections of MBCD from days 6 to 15 after graft (MBCD) combined with two injections of 37 MBq of $^{125}$I-anti-CEA mAb at days 8 and 11 after graft (right). B, Mice bearing subcutaneous A-431CEA tumor cell xenografts were treated with (i) NaCl, or (ii) daily i.p. injections of 40 mg/kg pravastatin from day 10 before graft until day 18 after graft, or (iii) two injections of 37 MBq of $^{125}$I-anti-CEA mAb at days 8 and 11 after graft, or (iv) $^{125}$I-anti-CEA mAb and MBCD. The average tumor volume in each treatment group ($n = 8$) was determined, and Kaplan-Meier survival curves were established. Mice were sacrificed when tumor volume reached 2,000 mm$^3$. C, The ratio between the tumor masses of the indicated groups was calculated. *, $P < 0.05$ compared with control animals.
Figure 7.

Contribution of targeted and nontargeted effects during Auger RIT using $^{125}$I-mAbs or alpha RIT using $^{212}$Pb- or $^{213}$Bi-mAbs. The contribution of targeted and nontargeted effects of RIT with $^{212}$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-κB and downstream signaling pathways. Alpha RIT–targeted effects produced large 53BP1 and γH2AX foci (assimilated to complex DNA lesions), as indicated by immunofluorescence analysis (see Fig. 1). Conversely, Auger RIT–targeted effects and Auger and alpha RIT–nontargeted effects produced small foci that could represent single DNA lesions. Antioxidant agents reduce the nontargeted 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. iNOS, inducible nitric oxide synthase; NO, nitric oxide; SM, sphingomyelin.
effects, and the yield of DNA damage \textit{ex vivo} was proportional to the dose (36). We then confirmed the nontargeted cytotoxic and genotoxic effects of alpha RIT \textit{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 nontargeted 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 second 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 nonionic 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 subcompartments that participate in the segregation of molecules and the reorganization 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-RUN), PKC isoforms (PKCa and \(\zeta\)), kinase suppressor of Ras (KSR), pRB, and BCL-2 (8, 42–44). Here, we found that p38 and JNK 1/2/3 are activated in p-NF-\(\kappa\)B expression was very low and was not correlated with any treatment. It would be of interest in further experiments to use NF-\(\kappa\)B knockout mice to better delineate the role of the latter transcription factor \textit{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 \(\gamma\)H2AX 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 \(\gamma\)H2AX foci (Fig. 2C and D).

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. Nontargeted effects could thus contribute to the observed therapeutic efficacy of the new radiopharmaceuticals currently under evaluation (49, 50).

Finally, it must be noted that nontargeted 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 immune-competent preclinical models (53). This study showed that alpha RIT is mediated by targeted and nontargeted effects. Depending on the radiopharmaceuticals, nontargeted 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 INK-mediated signaling pathways are partly responsible for the nontargeted effects and, to a lower extent, for targeted effects. As targeted effects are dose-related but not nontargeted 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.

\textbf{Disclosure of Potential Conflicts of Interest}

No potential conflicts of interest were disclosed.

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References


Drugs That Modify Cholesterol Metabolism Alter the p38/JNK-Mediated Targeted and Nontargeted Response to Alpha and Auger Radioimmunotherapy

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