IGF-1R Targeting Increases the Antitumor Effects of DNA-Damaging Agents in SCLC Model: An Opportunity to Increase the Efficacy of Standard Therapy

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Abstract
Insulin-like growth factor receptor-1 (IGF-1R) inhibition could be a relevant therapeutic approach in small cell lung cancer (SCLC) given the importance of an IGF-1R autocrine loop and its role in DNA damage repair processes. We assessed IGF-1R and pAkt protein expression in 83 SCLC human specimens. The efficacy of R1507 (a monoclonal antibody directed against IGF-1R) alone or combined with cisplatin or ionizing radiation (IR) was evaluated in H69, H146, and H526 cells in vitro and in vivo. Innovative genomic and functional approaches were conducted to analyze the molecular behavior under the different treatment conditions. A total of 53% and 37% of human specimens expressed IGF-1R and pAkt, respectively. R1507 showed single-agent activity in H146 and H526 cells but not in H69 cells. R1507 exhibited synergistic effects with both cisplatin and IR in vitro. The triple combination R1507-cisplatin-IR led to a dramatic delay in tumor growth compared with cisplatin-IR in H526 cells. Analyzing the apparent absence of antitumoral effect of R1507 alone in vivo, we observed a transient reduction of IGF-1R staining intensity in vivo, concomitant to the activation of multiple cell surface receptors and intracellular proteins involved in proliferation, angiogenesis, and survival. Finally, we identified that the nucleotide excision repair pathway was mediated after exposure to R1507-CDDDP and R1507-IR in vitro and in vivo. In conclusion, adding R1507 to the current standard cisplatin-IR doublet reveals remarkable chemo- and radiosensitizing effects in selected SCLC models and warrants to be investigated in the clinical setting. Mol Cancer Ther; 12(7): 1213–22. ©2013 AACR.

Introduction
Small cell lung cancer (SCLC) accounts for 15% of all lung cancers and is the cause of death in 90% to 95% of affected individuals within 5 years (1). Over the past 20 years, progress in treatment has been limited and the standard of care is still therapy combining cisplatin plus ionizing radiation (IR) and cisplatin-based polychemotherapy for locally advanced and extended disease, respectively (2). New strategies based on a better understanding of SCLC biology are urgently needed.

The presence of an insulin-like growth factor-1 (IGF1)/IGF-1 receptor (IGF1R) autocrine loop is thought to be instrumental in driving the aggressive course of SCLCs (3). This receptor is a well-recognized promoter of malignant transformation, proliferation, and survival through the transduction of phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathways (4). Furthermore, the IGF-1R pathway has been implicated in resistance to DNA-damaging agents, suggesting its role in DNA damage repair (5–8). Previous studies on in vitro models of SCLCs suggest that inhibition of the IGF-1R pathway might enhance the sensitivity of SCLCs to chemotherapy through the inhibition of PI3K/Akt activation (9, 10). However, these studies did not include in vivo validation and comprehensive molecular analyses on the interaction between chemotherapy and IGF-IR inhibitors. As a result, there is little rationale to support the 4 phase I/II studies that are currently investigating agents targeting the IGF-1R/IGF axis in patients with SCLCs: OSI-906 versus topotecan (NCT01387386 and NCT01533181, phase II trials), cisplatin etoposide and cixutumumab (NCT00887159, phase II trial), pasireotide and topotecan (NCT01417806, phase II trial).

The aim of the present study was to investigate the efficacy of R1507 (an IgG1 monoclonal antibody directed...
against the IGF-1R both in vitro and in vivo in a context mimicking the clinical course of patients treated for SCLCs (IR combined with chemotherapy). We first evaluated the expression of the phosphorylated forms of IGF-1R and of Akt in 83 human SCLC specimens. We then assessed the efficacy of R1507 against selected SCLC cell lines and examined the consequences on IGF-1R downstream signaling. We also investigated whether R1507 could potentiate the effects of cisplatin and IR in vitro and in vivo. Finally, we conducted an integrated genomic and functional analysis to explore the adaptive mechanisms occurring (i) after long-term versus short-term R1507 exposure and (ii) with cisplatin- and IR-R1507 combinations.

Materials and Methods

Human established SCLC cell lines NCI-H146, NCI-H69, and NCI-H526 were obtained from the American Type Culture Collection (ATCC-LGC Standards, authentication by ATCC using short tandem repeat profiling). No further authentication was conducted on these cell lines: they were passaged within the 6 months of the purchase. All cell lines cultured in the following medium: RPMI-1640 (GIBCO-Invitrogen) supplemented with 1% Glutamax, 10% FBS, and 1% penicillin/streptomycin. Cells growing in floating aggregates were maintained in plastic flasks and incubated at 37°C in 5% CO2 humidified atmosphere.

Reagents

R1507 (RO4858696; Robatumumab, Roche), is a fully humanized IgG1 monoclonal antibody directed against the extracellular portion of IGF-1R. R1507 was provided by Roche Diagnostics and stored at −80°C. It binds with high selectivity to the extracellular domain of IGF-1R (and not to insulin receptor), leading to displacement of IGF-1 binding and loss of protein at the cell surface due to receptor internalization and degradation. Its chemical name is immunoglobulin G1-kappa, anti-(Homo sapiens IGF1R, IGF-1R, IGF-1 receptor, CD221), Homo sapiens monoclonal antibody; gamma1 heavy chain (1-448) [Homo sapiens VH (IGHV3-33’01 (91.80%) -IGHD)-IGHJ2’01] [8.8.11] (1-118) –IGHGI1’01 (119-448)], (221-215’)-disulfide with kappa light chain (1’-215’) [Homo sapiens V-KAPPA (IGKV3-11’01 (97.90%) –IGKJ1’01) [6.3.10] (1’-108’) –IGKC’01 (109’-215’)]; (227-227’-230-230’)-disulfide dimer. The molecular formula of R1507 is C6476H10012N1748O2000S40 and its molecular weight is 145.6 kDa. Its 3-dimensional structure has been described elsewhere (11). R1507 does not cross react with mouse IGF1R (11). Toxicity in the clinical setting has already been described elsewhere (12). Cisplatin was purchased from Mylan Pharmaceuticals and stored at room temperature. Its chemical formula is Cis-PtCl2(NH3)2 and its molecular weight is 230”-bisdisulfide dimer. The molecular formula of R1507 (RO4858696) is C6476H10012N1748O2000S40 and its molecular weight is 145.6 kDa. Its 3-dimensional structure has been reported elsewhere (10). The antibody ERCC1 mouse mAb (6F1) (1:500 dilution), XPA mouse mAb (1:1,000 dilution), and β-actin mouse mAb (1:5000 dilution) were purchased from Abcam Inc., Thermo Scientific Pierce Products, and Sigma Aldrich, respectively. All primary antibodies were purchased from Cell Signaling Technology. The following secondary antibodies were used: goat anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Invitrogen; 1:3,000 dilution), and goat anti-mouse antibodies (Alexa Fluor 488) at 1:1,000 dilution.

Immunohistochemical analysis

The SCLC human specimens were handled according to standardized methods in the Department of Pathology at Institut Guysvaste Roussy using the Discovery XT automated platform (Ventana Medical Systems). After deparaffinization, slides were incubated in pIGF-1R rabbit monoclonal antibody [CONFIRM anti-IGF-1R (G11) antibody, Ventana Medical Systems] directed against the cytoplasmic domain of the phosphorylated form of the pIGF-1R, pAKT expression was also assessed with a rabbit monoclonal antibody (1:50 dilution, Cell Signaling). A section of normal human breast was used as a negative control, whereas an invasive ductal carcinoma that was positive for IGF1R protein expression was included as a positive control. The scoring system used to interpret immunohistochemical staining was based on the distribution and the intensity of staining: no staining, slight staining or staining in <10% cells (0), slight staining in >10% and <50% cells (1), marked staining in 10%–50% cells (2+), and strong staining in >50% cells (3+) as previously described (14). All the IHC analyses were centrally conducted in a blinded manner by a senior pathologist (J. Calderaro).

IGF1R gene copy number in the 3 selected cell lines was analyzed by FISH according to the Vysis protocol (unmodified; Abbott Molecular). BlueFISH probes (Blue-gnome) were used. The BAC clone RP11-14C10 (labeling with SpectrumOrange) is located on 15q26.3 and contains

Clonogenic survival assay

Clonogenic survival assays were conducted as previously described (13). A total irradiation dose of 2 Gy was delivered using a linear accelerator (200-kV X-ray irradiator, dose rate = 0.96 Gy/min) within the hour after seeding. Colonies consisting of at least 50 viable cells were estimated 21 days posttreatment. The surviving fraction was normalized to the corresponding untreated control. Each experiment was repeated 3 times.

Western blot

Western blotting were conducted as previously described (13). Signals were detected using the SuperSignal West Pico chemoluminescent system (Pierce Biotechnology). IGF-1R β rabbit antibody (1:1,000 dilution), pan-Akt rabbit mAb (1:1,000 dilution), phospho-Akt (Ser473) rabbit mAb (1:1,000 dilution), p44/42 MAPK rabbit mAb (1:1,000 dilution), phospho-p44/42 MAPK (Thr202/Thr204) rabbit mAb (1:1,000 dilution), phospho-MEK 1/2 (Ser217/221) rabbit mAb (1:1,000 dilution), and cleaved Caspase-9 (Asp330) rabbit mAb (1:1000 dilution) were purchased from Cell Signaling Technology. The antibodies ERCC1 mouse mAb (6F1) (1:500 dilution), XPA mouse mAb (1:1,000 dilution), and β-actin mouse mAb (1:5000 dilution) were purchased from Abcam Inc., Thermo Scientific Pierce Products, and Sigma Aldrich, respectively. All primary antibodies were purchased from Cell Signaling Technology. The following secondary antibodies were used: goat anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Invitrogen; 1:3,000 dilution), and goat anti-mouse antibodies (Alexa Fluor 488) at 1:1,000 dilution.
IGF-1R. The BAC clone RP-263I19 (labeling with SpectrumGreen) located on 15q15.3 was used as a control probe. At least 50 nuclei were examined to score the number of signals from each probe per cell and the IGF1R/control ratio.

Gene expression analysis

All samples were obtained from H526 xenografts in nude mice. Total RNA was isolated from tissues using the RNAlater protocol. Affymetrix human genome U133 Plus 2.0 arrays were used for expression profiling, and the information concerning each probe on the array was extracted from the image data according to the manufacturer’s instructions. The raw intensity values from the gene expression files were imported into the R statistical Software, which is used for all data input, diagnostic plots, normalization (RMA), and quality checking steps of the analysis process. Generalized linear models were computed (Limma, Bioconductor) to determine the differentially expressed probe sets (≥2-fold change, adjusted P values were computed for multiple testing (Benjamini and Hochberg method). Fisher exact tests were computed to test the overlap with gene sets from selected molecular signature databases [Gene Ontology gene sets and Canonical Pathways (KEGG, Biocarta, and Reactome)]. Only overlaps with P < 0.05 significance were retained for further analysis. The data reported in this article have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE45626). The RMA normalized gene expression data were uploaded to the Synapse public portal (https://synapse.sagebase.org) for convenience under the following entity: "IGF1R targeting in SCLC (R1507)" (synapse ID syn1588571). Extensive information on Synapse can be found at: https://sagebionetworks.jira.com/wiki/display/SYND/. Further information about the use of the Synapse R Client is available on the synapse wiki: https://sagebionetworks.jira.com/wiki/display/SYNR/R+Synapse+Client+Vignette.

Phospho-protein arrays

Human Phospho-RTK arrays were processed according to the manufacturer’s instructions (2010 Cell Signaling Technology, Inc., Pathscan RTK signaling antibody array kit #7982). Briefly, 50 mg of protein lysates was incubated with blocked membranes that were subsequently washed and exposed to a chemiluminescent reagent and to an X-ray film. Dot images were captured by an ImageQuant 350 CCD camera controlled by ImageQuant 350 capture software (GE Healthcare), at 3-, 5-, and 6-minute exposure time. The antibody directed against pIGF-R1 in this kit is selective and does not cross-react with pINSR (the phosphorylated form of the insulin receptor).

In vivo experiments (including ethical requirements)

In vivo experiments were carried out at the Institut Gustave Roussy (Animal Care license N° 94-076-11). Seven-week-old female athymic nude mice were purchased from Janvier (CERT), maintained in laminar flow cabinets under pathogen-free conditions. Two different cell lines (H146 and H526) were selected for the in vivo experiments, according to recent guidelines (15). Mice were injected with 5 × 10^6 H526 cells into the flank. Animals were randomized to different treatment groups of 5 to 7 mice each when xenograft diameters attained 5 mm. R1507 was administered via an intraperitoneal (i.p.) injection once weekly at a dose of 18 mg/kg and continued until the end of the experiment. Cisplatin was administered at a dose of 3 mg/kg i.p. at days 1, 8, and 15. IR was delivered in 4 fractions of 2 Gy on days 1 to 4 (A 200-kV X-ray irradiator, dose rate = 0.45 Gy/min), using mouse jigs to exclusively expose the tumor beds. Bidimensional tumor measurement on each mouse was conducted twice weekly to measure the tumor volume using the following formula: (length × width^2)/2. Animals were sacrificed when the main tumor volume per group had attained 2,200 to 2,800 mm^3.

Statistical analysis

The description of the results is based on classic statistical methods: median, range, and 95% confidence intervals. For clonogenic assays and WST-1 proliferation assays, data are presented as the mean ± SEM from a minimum of 3 independent experiments and were compared using the Student t test. The synergy effect between different conditions was assessed using the 2-way ANOVA interaction test. Tumor sizes were compared using Student t tests. Tumor growth delay (days) was analyzed using the Kaplan–Meier method, considering any event as the moment when the tumor volume had attained 5-fold the baseline volume (RTV5). For all the tests conducted, P < 0.05 was considered statistically significant, and all tests were 2-tailed. The statistical analyses were conducted using the R statistical software and Bioconductor packages (affy, limma).

Results

IGF-1R and pAkt were frequently expressed in human SCLC tissue specimens

We assessed IGF-1R expression and Akt phosphorylation in one of the largest cohort of SCLC human specimens (83 patients). Positive IGF-1R immunohistochemical staining (Fig. 1A) was observed in 44 patients (53%), whereas pAkt (Fig. 1B) was detected in 31 patients (37%). A total of 17 (21%) and 22 (26%) of all tumors exhibited a minimum of 2+ staining intensity for IGF-1R and pAkt, respectively. Concomitant expression of both proteins was present in 24 samples (29%) suggesting that IGF pathway could be a relevant target in SCLCs.

R1507 downregulated IGF-1R protein expression and disrupted its downstream signaling in selected SCLC cell lines

Three SCLC cell lines were selected according to their high basal IGF1R protein expression and their specific mutation profiles: H146 cells are mutated for MAP2K4
(c.814_891del78), whereas H69 cells are mutated for both PIK3CA (c.317_325del9) and c-Met (c.2962C>T) (Cosmic database). Western blot analyses showed a downregulation of IGF-1R protein expression in the presence of R1507 (200 nmol/L) in all the selected cell lines (Fig. 1C).

R1507 decreased IGF-1-induced downstream effector pAKT in H526 and H146 cells but not in H69 cells (Fig. 1D). R1507 also inhibited ligand-induced phosphorylation of MEK in H526 cells. However, the MAPK pathway (pMAPK, pMEK) appeared to be stimulated by R1507 in a dose-dependent manner in H146 cells.

R1507 inhibited colony formation of selected SCLC cell lines in vitro

Exploring the sensitivity of selected SCLC cell lines to R1507, we observed a 35% and 45% relative reduction in colony formation of H526 cells (P < 0.0001) and H146 cells (P < 0.0001), respectively (Fig. 2A). No significant reduction in the colony-forming capacity was observed in H69 cells (data not shown). Although we did not study any SCLC cell lines without IGF-1R basal expression, such feature does not appear to be correlated with sensitivity to R1507. Indeed, R1507 treatment had no effect on H69 cells, whereas these cells present IGF1R basal expression (Fig. 1D). Interestingly, only the cell lines (H526 and H146) with IGF-1R gene amplification exhibited sensitivity to R1507, whereas those devoid of IGF1R gene amplification (H69) did not respond to R1507 (Fig. 2B).

R1507 sensitized H146 and H526 cells to cisplatin and IR effects

Because cisplatin (CDDP)-based chemotherapy and IR are the standard treatments for SCLC, we investigated whether R1507 could enhance their efficacy in SCLC cell lines. To formally assess the synergy relationship, 2-way ANOVA were computed. We observed that the combination of R1507 and CDDP decreased their colony-forming capacity by 90% and 87% in H526 and H146 cells, respectively (P < 0.001; Fig. 2C). We observed a synergistic inhibitory effect of the CDDP-R1507 combination on the...
The colony-forming capacity of H146 cells (2-way ANOVA interaction test; $P = 0.008$) and an additive inhibitory effect in H526 cells (2-way ANOVA interaction test; $P = 0.691$). The R1507-IR combination decreased the colony-forming capacity by 87% and 88% in H526 and H146 cells, respectively ($P < 0.001$; Fig. 2D). We also noted a synergistic effect of R1507-IR on the colony-forming capacity of H146 cells (2-way ANOVA interaction test; $P = 0.0001$) and an additive inhibitory effect in H526 cells (2-way ANOVA interaction test; $P = 0.101$).

The triple combination of R1507-cisplatin-IR potentiated the antitumor effect of cisplatin-IR (current standard treatment) in H146 and H526 cells in vitro

Cisplatin (CDDP) combined with IR remains the standard of care for the treatment of limited-stage SCLCs. We herein observe that the addition of R1507 to CDDP-IR resulted in a 32% and 45% relative reduction in the colony-forming capacity compared with CDDP-IR in H146 and H526 cells, respectively ($P < 0.001$ and $<0.00001$; Fig. 3).
The addition of R1507 to CDDP or IR induced nonsignificant delays in tumor growth in vivo

To evaluate the delay in tumor growth according to the various treatment conditions in vivo, we assessed the RTV5 in H526 and H146 xenografts (Fig. 4A and B and Supplementary Figs. S1 and S2). Single-agent R1507 induced a nonsignificant delay in tumor growth in H526 and in H146 cells (Supplementary Table S1). Similarly, the R1507-CDDP and R1507-IR combination produced a nonsignificant delay in tumor growth in both H146 cells and H526 cells (Fig. 4B).

The triple combination CDDP-IR-R1507 caused a dramatic delay in tumor growth in H526 xenografts as compared with the CDDP-IR combination

The triple combination R1507-CDDP-IR produced a significant delay in tumor growth compared with the standard CDDP-IR regimen in H526 cells (median RTV5: R1507—cisplatin—IR = 50 days vs. cisplatin—IR = 34 days, \( P = 0.006 \); Fig. 4A and B). Notably, the experimental regimen yielded an 84% (\( P = 0.01 \)) reduction of the mean tumor volume compared with the CDDP-IR regimen (tumor assessment at day 42; Supplementary Table S1).

Integrated genomic and functional analysis revealed adaptive mechanisms under R1507

Because R1507 as a single-agent showed clear antitumor activity in vitro but not in vivo, we hypothesized that (i) R1507 as a single agent would not be sufficient to inhibit IGF-1R in vivo and/or (ii) molecular adaptive mechanisms would occur under anti-IGF-1R treatment in vivo.

To evaluate whether R1507 could efficiently inhibit IGF-1R in tumor tissue, we examined IGF-1R expression in H526-xenografted mice at different treatment times. We observed an expected decrease in IGF-1R expression on day 2, but a secondary increase on day 7 (Fig. 5A). This did not appear to be attributable to a lack of R1507 availability on day 7, as the product half-life in mice is estimated at 10 days (11).

We thus hypothesized that some adaptive mechanisms occurred upon R1507 exposure and resulted in the secondary increase in IGF1R expression on day 7. The overall regulation of gene expression induced by R1507 in H526 xenografts at the following time points: baseline (vehicle), R1507 day 1, and R1507 day 7. A total of 2,396 probe sets were differentially expressed at R1507 day 1, whereas only 254 probe sets were differentially expressed at R1507 day 7. Interestingly, only 6.7% (\( n = 156 \)) of the initially deregulated probe sets (R1507 day 1) remained differentially expressed at R1507 day 7 (Fig. 5B, Supplementary Fig. S3). To investigate the biologic basis of the gene expression alterations, we computed the overlap between the differentially expressed probes and gene set databases. The following pathways were enriched in the R1507-day 1 condition: cell-to-cell adhesion signaling, MAPK pathway, β-arrestin, and vitamin D receptor (Supplementary Table S2). Interestingly, the downregulation of the MAPK pathway for R1507 day 1 was consistent with our previous results in vitro (Fig. 1D). However, some pathways like MAPK signaling appeared to be inversely deregulated between R1507-day 1 and R1507-day 7 (Supplementary Table S2), suggesting a role in the escape to R1507 alone in vivo.

To confirm the microarray results at the protein level, we conducted low-scale protein arrays (Fig. 5C, Supplementary Fig. S4). H526 cells were previously cultured with continuous 4-week R1507 exposure (200 nmol/L) as follows: (i) vehicle, (ii) short-term R1507 exposure (ST-R1507: R1507 200 nmol/L—3-hour exposure), (iii) long-term R1507 exposure (LT-R1507: R1507 200 nmol/L—4-week exposure). We observed several striking results: (i) a transient inhibitory effect on the IGF-1R axis after ST-R1507 exposure, with reduced expression of the phosphorylated forms of IGF-1R (insulin receptor; INSR) and Akt (Ser 473), respectively. After LT-R1507 exposure, we observed a return to the baseline expression of the activated forms of IGF-1R and INSR. This is consistent with our previous results in vivo (Fig. 5A). (ii) After LT-R1507 exposure, we observed a return to the baseline expression of the activated forms of IGF-1R and INSR. This is consistent with our previous results in vivo (Fig. 5A). (ii) After LT-R1507 day 1 condition: cell-to-cell adhesion signaling, MAPK pathway, β-arrestin, and vitamin D receptor (Supplementary Table S2). Interestingly, the downregulation of the MAPK pathway for R1507 day 1 was consistent with our previous results in vitro (Fig. 1D). However, some pathways like MAPK signaling appeared to be inversely deregulated between R1507-day 1 and R1507-day 7 (Supplementary Table S2), suggesting a role in the escape to R1507 alone in vivo. To confirm the microarray results at the protein level, we conducted low-scale protein arrays (Fig. 5C, Supplementary Fig. S4). H526 cells were previously cultured with continuous 4-week R1507 exposure (200 nmol/L) as follows: (i) vehicle, (ii) short-term R1507 exposure (ST-R1507: R1507 200 nmol/L—3-hour exposure), (iii) long-term R1507 exposure (LT-R1507: R1507 200 nmol/L—4-week exposure). We observed several striking results: (i) a transient inhibitory effect on the IGF-1R axis after ST-R1507 exposure, with reduced expression of the phosphorylated forms of IGF-1R (insulin receptor; INSR) and Akt (Ser 473), respectively. After LT-R1507 exposure, we observed a return to the baseline expression of the activated forms of IGF-1R and INSR. This is consistent with our previous results in vivo (Fig. 5A). (ii) After LT-R1507 day 1 condition: cell-to-cell adhesion signaling, MAPK pathway, β-arrestin, and vitamin D receptor (Supplementary Table S2). Interestingly, the downregulation of the MAPK pathway for R1507 day 1 was consistent with our previous results in vitro (Fig. 1D). However, some pathways like MAPK signaling appeared to be inversely deregulated between R1507-day 1 and R1507-day 7 (Supplementary Table S2), suggesting a role in the escape to R1507 alone in vivo. To confirm the microarray results at the protein level, we conducted low-scale protein arrays (Fig. 5C, Supplementary Fig. S4). H526 cells were previously cultured with continuous 4-week R1507 exposure (200 nmol/L) as follows: (i) vehicle, (ii) short-term R1507 exposure (ST-R1507: R1507 200 nmol/L—3-hour exposure), (iii) long-term R1507 exposure (LT-R1507: R1507 200 nmol/L—4-week exposure). We observed several striking results: (i) a transient inhibitory effect on the IGF-1R axis after ST-R1507 exposure, with reduced expression of the phosphorylated forms of IGF-1R (insulin receptor; INSR) and Akt (Ser 473), respectively. After LT-R1507 exposure, we observed a return to the baseline expression of the activated forms of IGF-1R and INSR. This is consistent with our previous results in vivo (Fig. 5A). (ii) After LT-R1507 day 1 condition: cell-to-cell adhesion signaling, MAPK pathway, β-arrestin, and vitamin D receptor (Supplementary Table S2). Interestingly, the downregulation of the MAPK pathway for R1507 day 1 was consistent with our previous results in vitro (Fig. 1D). However, some pathways like MAPK signaling appeared to be inversely deregulated between R1507-day 1 and R1507-day 7 (Supplementary Table S2), suggesting a role in the escape to R1507 alone in vivo. To confirm the microarray results at the protein level, we conducted low-scale protein arrays (Fig. 5C, Supplementary Fig. S4). H526 cells were previously cultured with continuous 4-week R1507 exposure (200 nmol/L) as follows: (i) vehicle, (ii) short-term R1507 exposure (ST-R1507: R1507 200 nmol/L—3-hour exposure), (iii) long-term R1507 exposure (LT-R1507: R1507 200 nmol/L—4-week exposure). We observed several striking results: (i) a transient inhibitory effect on the IGF-1R axis after ST-R1507 exposure, with reduced expression of the phosphorylated forms of IGF-1R (insulin receptor; INSR) and Akt (Ser 473), respectively. After LT-R1507 exposure, we observed a return to the baseline expression of the activated forms of IGF-1R and INSR. This is consistent with our previous results in vivo (Fig. 5A). (ii) After LT-R1507
exposure, we observed a major increase in the expression of receptors and signaling proteins involved in angiogenesis (PDGFR, VEGFR2, EphA2, EphA3, EphB3, EphB4), in proliferation (Akt, MAPK, S6 ribosomal protein, STAT3, ALK), and in survival (Akt, ALK). In this respect, the low-scale tyrosine kinase array results are also consistent with our previous genomic analyses (Supplementary Table S2).

Integrated genomic and functional analysis revealed the involvement of nucleotide excision repair and mitochondrial apoptotic pathways in the response to CDDP- and IR-R1507 combinations

To identify the molecular mechanisms involved in the response to CDDP- and IR-R1507 combinations, we conducted global gene expression profiling on mice bearing H526 xenografts treated with the following treatment conditions: vehicle, R1507 CDDP, IR, CDDP-R1507, and IR-R1507 (Fig. 6A and B, Supplementary Figs. S5 and S6, Supplementary Table S2). To investigate the biologic basis of the gene expression variation, we computed the overlap between the differentially expressed probes and gene set databases.

The probes differentially expressed exclusively in the CDDP-R1507 combination were associated with downregulation of the nucleotide excision repair (NER) pathway \((P = 0.02)\) through decreases in ERCC1, XAB2, and RAD23A gene expression and upregulation of the apoptotic mitochondrial response to DNA damage pathway \((P = 0.04)\) through gene expression modifications in BCL2L1 and IFI6.
The probes that were exclusively differentially expressed in the R1507-IR combination were associated with a downregulation of the TGF-β pathway (Fig. 6B, Supplementary Table S2, Supplementary Fig. S6), suggesting that the IR-R1507 combination induced the downregulation of this pathway.

We then showed that the R1507-CDDP combination decreased ERCC1 but not XPA protein expression in H526 cells (Supplementary Fig. S7A) and also decreased cleaved caspase-9 expression (Supplementary Fig. S7C). When we examined the effect of R1507-IR on the TGF-β pathway, we did not observe any impact on SMAD4 protein expression (Supplementary Fig. S7D). We next investigated whether the R1507-IR combination could modify ERCC1 (and XPA at the protein expression level. Interestingly, the R1507-IR combination also decreased ERCC1 but not XPA protein expression in H526 cells (Supplementary Fig. S7C).

Discussion

The importance of the IGF1-IGF-1R autocrine axis in SCLC growth and its role in DNA damage repair processes suggest a benefit of combining anti-IGF-1R with cisplatin and ionizing radiation (3–5). To our knowledge, this is the first time that such a dramatic delay in tumor growth is observed after adding an IGF-1R inhibitor to the CDDP-IR standard combination against SCLCs.
We have shown that R1507 efficiently inhibited cell growth in vitro in H146 and H526 SCLC cell lines. Conversely, H69 cells failed to respond to IGF-1R inhibition, despite effective R1507-induced IGF-1R receptor down-regulation. Interestingly, sensitive cell lines (H526 and H146) exhibited both higher IGF-1R gene copy number and lower level of basal phosphorylated Akt expression as compared with resistant cells (H69). Interestingly, these findings are consistent with those of Gong and colleagues (16) and Yeh and colleagues (9), respectively, but warrant further studies to be validated.

We did not observe a significant effect of R107 monotherapy in H526 xenografts. Interestingly, long-lasting responses have been observed with anti-IGF1R agents delivered in monotherapy, in patients diagnosed with Ewing’s sarcoma or with adrenocortical carcinoma (17, 18). However, such responses have not yet been described in the SCLC setting. In this study, we observed that—compared with the short-term setting, the chronic administration of R1507 as a single-agent led to both sustained IGF-1R pathway activity and to the concomitant relief of multiple alternative signaling pathways including the HER family receptors, angiogenesis, STATs, etc. These late effects are likely to promote tumor escape under R1507 exposure. Similar results have already been observed for antibodies targeting tyrosine kinase receptors (19, 20). Although we did not observe clear compensatory effect mediated by the insulin receptor in our model (21). These results require to be validated in human specimens to be confirmed. Because most antineoplastics are delivered over the long term and in a recurrent manner, we believe that greater emphasis should be given to long-term preclinical analyses when evaluating a new drug.

The latter findings also support strategies combining anti-IGF-1R drugs with other antineoplastics. In this regard, the long-lasting R1507-induced sensitizing effect with CDDP and IR is noteworthy. This can be partially explained by the role of IGF-1R in DNA damage repair processes. A large body of literature already exists about the influence of IGF-1R on DNA damage repair through ATM (7, 8). Here, we describe that the R1507-CDDP and R1507-IR combinations were associated with a downregulation of the NER pathway in vitro and in vivo. Given the importance of CDDP and IR as antineoplastic agents across cancer types, the causal implication of NER in the response to combinations of anti-IGF1R antibody with CDDP and IR should be further evaluated.

The triple combination R1507-cisplatin-IR showed major and durable antitumor effects in vitro and in vivo in an SCLC model. Given these encouraging results and the very poor prognosis of SCLCs, it is thus time for clinical testing of this triple combination.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: C. Ferte, Y. Loriot, E. Deutsch
Development of methodology: C. Ferte, Y. Loriot, C. Clemenson, A. Gombos, S. Hamama, E. Deutsch
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