Cell Death via DR5, but not DR4, Is Regulated by p53 in Myeloma Cells

Sylvanie Surget1,2,3, David Chiron1,2,3, Patricia Gomez-Bougie1,2,3,4, Géraldine Descamps1,2,3, Emmanuelle Ménot1,2, Régis Bataille1,2,3,5, Philippe Moreau1,2,3,4, Steven Le Guoil1,2,3,4, Martine Amiot1,2,3, and Catherine Pellet-Deceunynck1,2,3

Abstract

Myeloma cells are sensitive to TRAIL through the two death receptors DR4 and DR5. Because p53 directly modulates expression of death receptors, we investigated here whether p53 can modulate myeloma sensitivity to TRAIL. We found that p53 affects the sensitivity of myeloma cells to the DR5 agonistic human antibody lexatumumab but not the DR4 antibody mapatumumab. TP53 wild-type myeloma cells overexpressed DR5 in correlation with sensitivity to lexatumumab. Both nongenotoxic (nutlin-3a) and genotoxic (melphalan) p53-inducing stresses increased DR5 expression only in TP53 wild-type cells and synergistically increased lexatumumab efficiency yet did not increase DR4 expression, nor sensitivity to mapatumumab. Silencing of p53 strongly decreased DR5 expression and induced resistance to nutlin-3a and lexatumumab but did not modulate DR4 expression or sensitivity to mapatumumab. Increase of lexatumumab efficiency induced by nutlin-3a was related to a p53-dependent increase of DR5 expression. In primary myeloma cells, nutlin-3a increased DR5 expression and lexatumumab efficiency but did not increase mapatumumab efficiency. Taken together, our findings indicate that p53 controls the sensitivity of myeloma through DR5 but not DR4 and suggest that a subset of patients with multiple myeloma may benefit from DR5 therapy.

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Introduction

The receptors DR4/TRAILR1/TNFRSF10A, DR5/TRAILR2/TNFRSF10B, Fas/TNFRSF6, and TNFR2/TNFRSF1B are members of the death receptor family (1). Cancer cells, but not normal cells, are sensitive to TRAIL, making it an attractive target for cancer therapy (1). However, the mechanisms responsible for this selective tumor sensitivity remain unclear. TRAIL binds to 2 different receptors, and it remains undefined whether the tumor sensitivity to TRAIL is mediated by both receptors. Both DR4 and DR5 genes have been shown to be a transcriptional target of p53 (2–4). TP53 is frequently mutated and/or deleted in tumor cells (5). Although the loss of TP53 or mutations of TP53 are both associated with cancer, mutated cells have a gain-of-function compared with cells with a loss of expression (5, 6). p53 controls many of the pathways of cell homeostasis (proliferation, autophagy, apoptosis), and its transient activation via numerous stresses allows cells either to survive the stress or to enter apoptosis (7–9). p53 has numerous target genes, such as MDM2, CDKN1A, BAX, and DR5, which are involved in p53 regulation, cell-cycle arrest, and apoptosis, respectively. It is well known that mutations of (7–9) p53 prevent its transactivation ability of numerous genes including DR5 (2, 10).

In multiple myeloma, as in lymphomas, the presence of a deletion of the TP53 locus (17p13) or TP53 mutation at diagnosis has a negative effect on the overall survival of patients, regardless of the treatment regimen followed (11–15). Recently, Lode and colleagues reported that 17p13 monallelic deletion in myeloma cells at diagnosis was associated with the frequent presence of TP53 mutations (16). Weak or absent TP53 expression in myeloma cells affects the expression of numerous genes including DR5 (17).

We previously reported that the human agonist anti-DR4, mapatumumab, and anti-DR5, lexatumumab, monoclonal antibodies induced massive cell death of myeloma cells through the activation of both the extrinsic and intrinsic pathways of apoptosis (18). This previous study also showed that mapatumumab was more efficient than lexatumumab, suggesting that myeloma cells were not equally sensitive to TRAIL through the 2 receptors. In the present study, we show that p53 directly affects the expression of DR5 but not of DR4 as well as myeloma sensitivity to lexatumumab.
Regulation of TRAIL-R–Mediated Cell Death by p53

Materials and Methods

**Human myeloma cell lines and primary myeloma cells**
All human myeloma cell lines (HMCL) used in this article have been previously extensively characterized and authenticated (19). HMCLs BCN, MDN, NAN-1, -3, -6, -7, SBN, and XG-1, -2, -3, -5, -6, -7, -11 were derived in the Nantes or Montpellier laboratories in the presence of interleukin (IL)-6. ANBL-6 was kindly provided by Dr. D.F. Jelinek (Mayo Clinic, Rochester, MN), KMS-11, KMS12-BM, KMS12-PE, and KMM1 were kindly provided by Dr. T. Otsuki (Kawasaki Medical School, Kurashiki, Japan). JNJ3, JMM3, Karpass620, and MM15 were, respectively, kindly provided by Drs. I. Van Biet (Vrije Universiteit Brussel, Brussels, Belgium), L.C. MacLennan (University of Birmingham, Birmingham, UK), A. Karpas (University of Cambridge, Cambridge, UK), and S. Rosen (Northwestern University, Chicago, IL). AMO1, LP1, L363, NC-I-H929, SKMM2, U266, and OPM2 were from the DSMZ, and RPMI8226 was from American Type Culture Collection. HMCLs were cultured in RPMI-1640 containing 5% fetal calf serum (FCS) and in the presence of 3 ng/mL IL-6. ANBL-6 was kindly provided by Dr. D.F. Jelinek (Mayo Clinic, Rochester, MN). NCI-H929, SKMM2, U266, and OPM2 were from the DSMZ, and RPMI8226 was from American Type Culture Collection.

**TP53 cDNA sequencing**
Sequencing of TP53 cDNAs was conducted on 2 overlapping reverse transcription and polymerase chain reaction (RT-PCR) products. The extraction of RNAs and reverse transcription were conducted as described previously (20). The primers used for PCR assays contained the following sequences: 5'-ATGGAGGAGCGCGAGCTA3'; 5'-GGCCTCGGTTATCGGC3' for amplification codon 1–250 and 5'-GGCCCTCCTTCAGACAGTCTT3' and 5'-TCCCCCACTCCTCGA3' for amplification codon 187–370. cDNA sequencing was conducted using the Ready-Forme de Séquençage-Génotypage OUEST Génopole. Nine HMCLs express a wild-type TP53 (TP53wt) and 22 HMCLs express an abnormal TP53 (TP53mut). Of these, 2 HMCLs have no TP53 expression (TP53del), 5 have a structurally abnormal TP53 (TP53mut;exon deletion, intron insertion, stop codon), and 15 have a missense point mutation (TP53mut). Only 2 HMCLs with a TP53mut express a wild-type allele (TP53wt;mut KMM1, and NAN3).

**Gene expression profiling**
Gene expression profiling of HMCLs has been reported previously (19) and deposited in the ArrayExpress database (accession numbers E-TAB-937 and E-TAB-1088). Gene expression data of HMCLs and primary myeloma cells were analyzed with RAGE (http://rage.montp.inserm.fr/) and Amazonia (http://amazonia.montp.inserm.fr/) bioinformatics platforms (21).

**Reagents and antibodies**
Human mapatumumab and lexatumumab mAbs were provided by Human Genome Sciences. Apo2.7-PE, CD138-PE, and IgG1-PE mAbs were purchased from BD Bioscience, and anti-DR4-PE and anti-DR5-PE were purchased from eBioscience.

**Cell death assays**
The cytometric evaluation of cell death in HMCLs was conducted using the combined analysis of APO2.7 staining and the altered cellular morphology characteristic of apoptosis [lower forward scatter (FSC)-H and higher side scatter (SSC)-H] as described previously (18). Cell death of primary myeloma cells was measured via the loss of CD138 staining, which is characteristic of myeloma apoptosis (22).

**Western blotting and immunoprecipitation assays**
Protein expression was conducted by Western blotting as described previously (18). The following antibodies were used: p53 (Oncogene Science), DR4 (Eurobio-Abcys), DR5 (Millipore Bioscience Research Reagents), MM2 (R&D Systems), NOXA (Alexis Biotech), caspase-3 (Santa Cruz Biotechnology), caspase-8 (Cell Signaling), caspase-9 (Santa Cruz Biotechnology), PUMA (Calbiochem, Merck), Bax (Immunotech, Beckman Coulter), and actin (Millipore Bioscience Research Reagents).

**Immunoprecipitation assays** were conducted in cells treated up to 90 minutes with 10 μg/mL mapatumumab or lexatumumab or in untreated cells (control). Cells were lyzed for 40 minutes on ice in 10 mmol/L Tris-HCL, pH 7.6, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonylfluoride, 2 mg/mL aprotinin, 1% digitonin. For control assays, mapatumumab or lexatumumab (10 μg) was added to the lysates. The lysates were immunoprecipitated with a mix of proteins A and G (2 μg of lysate proteins were immunoprecipitated in each assay).

**TP53 silencing**
A stably modified myeloma cell line was obtained via lentiviral cell transduction. The TP53wt cell line NCI-H929 was transduced with a lentivirus carrying short hairpin RNA (shRNA) control or an shRNA designed to knockdown p53 (CCGGGTCCAGATGAGCTCCAGAACTCGAGTTCTGGAGCCTCATCTGGACTTTTT, RefSeq NM_000546; Sigma-Aldrich). Cells were plated in a 24-well plate (20,000 cells per mL) and infected with lentivirus (multiplicity of infection = 2). Selection with puromycin (4 μg/mL) was started 4 days after infection. Silenced HMCLs were stable and cultured under the continuous pressure of puromycin.

**Chromatin immunoprecipitation assays**
Chromatin immunoprecipitation (ChIP) assay was conducted using the ChIP-It Express Kit purchased from Active Motif following manufacturer’s instruction. Briefly, NCI-H929 cells (15 × 10⁶ cells) were treated for 3 hours with or without 2.5-μmol/L nutlin-3a; then genomic DNA and proteins were cross-linked by addition of formaldehyde (1% final concentration) directly into the culture medium. After cell lysis, nuclei

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were sonicated to generate DNA fragments. p53 immunoprecipitation assays were conducted by using a mix of 2 antibodies directed against p53, mouse Pab 240 mAb (Abcam) and rabbit p53 Ab (Active Motif). Control ChIP assays were conducted with a mix of mouse and rabbit IgG control Abs (Jackson ImmunoResearch Laboratories).

PCR amplifications of p53-BS2 in DR5 gene were conducted before (input) and after ChIP assays with specific primers (forward 5’-AAGACCCCTTGCTGTTGTG-3’ and reverse 5’-CGGGAATTTACACCAAGTGG-3’), which amplify a sequence of 271pb including p53-BS2 located within intron 1 (2, 23). p53-BS2 amplification assays were conducted at 65°C with 35 cycles for input samples (after a 50-fold dilution) or with 40 cycles for ChIP samples. PCR products were run in 2% agarose gel and analyzed with Gel Smart software (Clara Vision).

Statistical analysis
Statistical analyses were conducted using the Mann–Whitney, Fisher exact, Spearman, ANOVA, or paired Student t tests. The combination effects between the anti-TRAIL-R mAbs and

Results

Cell death via death receptors depends on TP53 status
We previously showed that mapatumumab and lexatumumab, 2 human agonistic mAb directed against DR4 and DR5, respectively, induced myeloma cell death (18). Because p53 modulates expression of death receptor genes, we wondered whether sensitivity of myeloma cells to mapatumumab and lexatumumab could be related to p53 status. Nine HMCLs express a wild-type TP53 (TP53Wt) and 22 HMCLs express an abnormal TP53 (Supplementary Table S1; ref. 19). All HMCLs expressing an abnormal TP53 or lacking TP53 expression were gathered and designated as TP53Abn HMCLs (see Materials and Methods).

Transcriptomic Affymetrix analysis of HMCLs showed that several p53 target genes such as MDM2, CDK11A, BAX, and DR5 but not DR4 were overexpressed in TP53Abn compared with TP53Wt HMCLs (P < 0.01, Mann–Whitney test; Fig. 1A and E; Supplementary Fig. S1 and Supplementary Table S2). Flow cytometric analysis of DR5 expression confirmed that DR5 had a trend to be more expressed on TP53Abn HMCLs (Fig. 1B), whereas DR4 was more expressed by TP53Abn HMCLs (Fig. 1F), P = 0.1 and P = 0.03, Mann–Whitney test, respectively. Lexatumumab significantly killed TP53Abn HMCLs but not TP53Wt HMCLs (median cell death 40% vs. 6%; P = 0.001, Mann–Whitney test; Fig. 1C) in correlation with the level of DR5 expression (P = 0.001, Spearman test; Fig. 1D). TP53Abn HMCLs were more sensitive to mapatumumab than TP53Wt HMCLs (Fig. 1G; median cell death 57.5% vs. 7%, P = 0.005, Mann–Whitney test) in correlation with the level of DR4 expression (P = 0.014, Spearman test; Fig. 1H). Not surprisingly, no correlations were found between DR5 expression and mapatumumab efficiency or between DR4 expression and lexatumumab efficiency (data not shown, P > 0.2).

In summary, TP53Abn HMCLs expressed DR5 and were killed by lexatumumab in contrast to TP53Wt HMCLs, which expressed DR4 and were killed by mapatumumab.

Nongenotoxic and genotoxic stresses increased DR5 and lexatumumab-induced cell death in TP53Abn HMCLs
To further show whether p53 could modulate killing of myeloma via DR4, DR5, or both, we next pharmacologically increased p53 and reciprocally silenced it in myeloma cells. We used nutlin-3a, which increases half-life of p53 (Supplementary Fig. S2) by disrupting p53/MDM2 interactions and induces cell death only in TP53Abn cells (24). As shown in Fig. 2A, all TP53Abn HMCLs were killed by nutlin-3a, whereas all TP53Wt HMCLs were resistant (P = 0.0004, ANOVA test). As shown in Fig. 2B, nutlin-3a (6 hours, 10 μmol/L) increased the expression of DR5 in all 3 TP53Abn HMCLs but in none of 3 TP53Wt HMCLs. The median value of the DR5 expression level in treated cells was 230% (range, 137–300) and 111% (range, 89–118) of untreated cells for TP53Abn and TP53Wt HMCLs, respectively (P = 0.001, Mann–Whitney test). Pretreatment of myeloma cells with a low dose of nutlin-3a (6 hours, 2.5 μmol/L) significantly increased the cell death induced by lexatumumab in the 3 TP53Abn HMCLs (median value of 38%) but in none of the 3 TP53Wt (0.6%, P < 0.001, Mann–Whitney test; Fig. 2C).

Melphalan increases p53 and induces myeloma cell death (Supplementary Fig. S2) through the intrinsic pathway of apoptosis (24, 25). TP53Abn HMCLs were more sensitive to melphalan than TP53Wt HMCLs (Fig. 2D; P = 0.0019, ANOVA test). Similar to nutlin-3a, melphalan (5 μmol/L) increased DR5 expression only in TP53Abn HMCLs (Fig. 2E). The median value of DR5 expression in treated cells was 139% (range, 119–170) and 106% (range, 90–114) of untreated TP53Abn and TP53Wt cells, respectively (P = 0.0002, Mann–Whitney test). Pretreatment of myeloma cells with a low dose of melphalan (24 hours, 1.25 μmol/L) significantly and synergistically increased the cell death induced by lexatumumab in all TP53Abn HMCLs but in none of the TP53Wt (P < 0.001, Mann–Whitney test). These data showed that a nongenotoxic (nutlin-3a) or genotoxic (melphalan) stress of TP53Abn HMCLs enhanced DR5 expression and sensitivity of myeloma cells to lexatumumab.

Nongenotoxic and genotoxic stresses neither increased DR4 nor mapatumumab-induced cell death in HMCLs
DR4 expression was not increased by nutlin-3a in TP53Abn but very slightly decreased in TP53Wt HMCLs (Fig. 3A). The median fold change of DR4 expression level in treated cells was 100% (range, 89–109) and 87% (range, 83–96; P = 0.001, Mann–Whitney test) in TP53Abn and TP53Wt HMCLs, respectively. The cell death induced by mapatumumab was not modified by nutlin-3a (Fig. 3B). Similarly, DR4 expression was not increased by melphalan in TP53Abn but very slightly decreased in TP53Wt HMCLs (P = 0.006, Mann–Whitney test; Fig. 3C). Pretreatment of myeloma cells with 1.25 μmol/L of melphalan either did not modify or slightly decreased cell death induced by mapatumumab in TP53Abn or TP53Wt HMCLs (Fig. 3D).

Thus, in contrast to DR5, DR4 expression was not increased by nutlin-3a or melphalan in TP53Abn HMCLs and neither nutlin-3a nor melphalan increased mapatumumab-induced cell death.
Silencing TP53 decreases DR5 expression and cell death induced by lexatumumab or nutlin-3a

To confirm the involvement of p53 in TRAIL killing of myeloma cells, we silenced p53 in the TP53\textsuperscript{WT} cell line NCI-H929. The decrease of p53 expression was associated with a marked decrease of constitutive expression of several p53 target genes, that is, p21, MDM2, Puma, Noxa, and DR5 (Fig. 4A and B). The level of DR5 expression in shp53 NCI-H929 cells was significantly reduced by 25\% ± 8\% compared with the shcontrol NCI-H929 cells (P = 0.01 paired t test) whereas that of DR4 remained unchanged (Fig. 4B). Compared with the shcontrol, shp53 NCI-H929 cells were significantly more resistant to nutlin-3a, melphalan, and lexatumumab (P < 0.05) but remained resistant to mapatumumab (P > 0.09); the decrease of death was of 50\% ± 7\% for nutlin-3a (10 \(\mu\)mol/L), 34\% ± 10\% for melphalan (10 \(\mu\)mol/L), 39\% ± 5\% for lexatumumab (6 \(\mu\)g/mL), and 8\% ± 4\% for mapatumumab (Fig. 4C). Moreover, as illustrated in Fig. 4D, the increase of DR5 expression induced by nutlin-3a was significantly reduced by 43\% ± 6\% in shp53 NCI-H929 cells compared with shcontrol NCI-H929 cells (P = 0.008).

Sensitivity of myeloma cells to lexatumumab or mapatumumab is related to recruitment and activation of caspase-8

In all cell lines treated with either lexatumumab or mapatumumab, the activation of caspase-8 correlated with cell death, suggesting that resistance was mainly associated with the lack of its activation (18). We showed that sensitivity of myeloma cells to lexatumumab or mapatumumab correlated with the level of DR5 or DR4 expressed at the cell surface, suggesting that the amount of complexes between DR5 or DR4 and caspase-8 should govern the cell response. Thus, we
analyzed caspase-8/TRAIL-R complexes by conducting immunoprecipitation assays of DR4 or DR5 in HMCLs killed by both mAbs (MDN), or by lexatumumab (LP1) or by mapatumumab (LP1). The cells were treated with mapatumumab or lexatumumab for 45 to 90 minutes at 37°C, and immunoprecipitation assays were conducted by adding a mix of protein A and G to the lysates to immunoprecipitate DR4 or DR5 engaged by the antibodies. As shown in Fig. 5, cleaved forms of caspase-8, 41 and/or 43 kDa (26), were found within the immunoprecipitates of DR4 or DR5 in treated cells in correlation with the sensitivity to either mapatumumab (LP1, MDN) or lexatumumab (MDN, NCI-H929). In contrast, no significant amounts of the caspase-8 cleaved forms were associated with DR4 or DR5 in resistant cells (LP1/lexatumumab, NCI-H929/mapatumumab) or in untreated cells (control IP). Of note, despite cleavage of both 53- and 55-kDa isoforms in the whole lysates, only the 41-kDa was associated with lexatumumab-induced cell death of TP53WT HMCLs. TP53WT and TP53Abn HMCLs were incubated for 6 hours with or without 2.5 μmol/L nutlin-3a (N) before addition of 6 μmol/L melphalan, and cell death was determined by Apo2.7-PE staining. The data were expressed as the mean ± SD of 3 experiments. The cell death was increased by 18% ± 10% for NCI-H929 (P = 0.019, CI < 0.9), 11% ± 3% for XG-6 (P = 0.005, CI < 0.9), and 9% ± 1% for MM1S (P = 0.008, CI < 0.9), whereas increased cell death was not observed in the TP53Abn cell lines (0% ± 2% for KMS12-PE, 0% ± 1% for LP1, and 0% ± 2% for OPM2).

Figure 2. Nutlin-3a (N) or melphalan (M) increased the expression of DR5 and the cell death induced by lexatumumab (Lexa) in TP53WT HMCLs. A, nutlin-3a only killed TP53WT HMCLs. Cells (500,000 cells/mL) were treated for 48 hours with 10 μmol/L nutlin-3a, and the percentage of cell death was determined by Apo2.7-PE staining. Each symbol represents one HMCL (the value is the mean of 3 experiments). B, nutlin-3a increased DR5 expression in TP53WT HMCLs. TP53WT (NCI-H929, MM1S, XG-6) and TP53Abn (LP1, OPM2, KMS12-PE) HMCLs were incubated for 6 hours with or without 10 μmol/L nutlin-3a before determination of DR5 expression as described in Fig. 1. Representative experiments out of 3. Overlay histograms represent the DR5-specific fluorescence of nutlin-3a-treated cells (thick lines) over that of control cells (thin lines). Histograms of control staining of control and treated cells were identical and not represented. C, nutlin-3a synergistically increased lexatumumab-induced cell death of TP53WT HMCLs. TP53WT and TP53Abn HMCLs were incubated for 6 hours with or without 2.5 μmol/L nutlin-3a (N) before addition of 6 μmol/L of lexatumumab (Lexa) and incubated for an additional 24 hours. Cell death was determined by Apo2.7-PE staining. The data were expressed as the mean ± SD of 3 experiments. The combination increased the cell death by 38% ± 10% for NCI-H929 (P = 0.012, CI < 0.9), 18% ± 7% for XG-6 (P = 0.053, CI < 0.9), and 58% ± 10% for MM1S (P = 0.009, CI < 0.9). D, TP53WT HMCLs were more sensitive to melphalan than TP53Abn HMCLs. Cells were incubated for 48 hours with or without 10 μmol/L melphalan, and cell death was determined by Apo2.7-PE staining. The data were expressed as the mean ± SD of 3 experiments. The median values of cell death were 68% (range, 35–100) and 23.5% (range, 1–68) for TP53WT and TP53Abn, respectively (P = 0.0005, Mann–Whitney test). E, melphalan increased DR5 expression in TP53WT HMCLs. TP53WT (NCI-H929, MM1S, XG-6) and TP53Abn (LP1, OPM2, KMS12-PE) HMCLs were incubated for 24 hours with or without 5 μmol/L melphalan before determination of expression of DR5 as described in Fig. 1. Representative experiments out of 3. Overlay histograms represent the DR5-specific fluorescence of melphalan-treated cells (thick lines) over that of control cells (thin lines). Histograms of control staining of control and treated cells were identical and not represented. F, melphalan synergistically increased cell death induced by lexatumumab of TP53WT HMCLs. HMCLs were incubated for 24 hours with or without 1.25 μmol/L melphalan before the addition of 6 μmol/L of lexatumumab and incubated for an additional 24 hours. Cell death was determined by Apo2.7-PE staining. The data were expressed as the mean ± SD of 3 experiments. The cell death was increased by 18% ± 10% for NCI-H929 (P = 0.019, CI < 0.9), 11% ± 3% for XG-6 (P = 0.005, CI < 0.9), and 9% ± 1% for MM1S (P = 0.008, CI < 0.9), whereas increased cell death was not observed in the TP53Abn cell lines (0% ± 2% for KMS12-PE, 0% ± 1% for LP1, and 0% ± 2% for OPM2).
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Nutlin-3a (N) or melphalan (M) did not increase the expression of DR4 and the cell death induced by mapatumumab (Mapa) in HMCLs. A, nutlin-3a did not modulate the expression of DR4. TP53WT and TP53Abn HMCLs were incubated for 6 hours with or without 10 μM nutlin-3a before the determination of DR4 expression by flow cytometry. A representative experiment of 3 is shown. Histograms represent the DR4-specific fluorescence of nutlin-3a-treated cells (thick line) over that of control cells (thin line). Histograms of control staining of control and treated cells were identical and not represented. B, nutlin-3a did not increase the cell death induced by mapatumumab. TP53WT and TP53Abn HMCLs were incubated for 6 hours with or without 2.5 μM nutlin-3a before the addition of 6 μg/mL mapatumumab and incubated for an additional 24 hours. Cell death was determined by Apo2.7–PE staining. The data were expressed as the mean ± SD of 3 experiments. The drug combination increased the cell death by 7% ± 4% for NCI-H929 (P = NS) and 1% ± 1.5% for LP1 (P = NS) and decreased it by -2% ± 3% for LP1 (P = NS), and -3% ± 1% for MM1S (P = NS), -3 ± 5% for KMS12-PE (P = NS), and -2% ± 3.5% for OPM2 (P = NS). C, melphalan did not modulate the expression of DR4. TP53WT and TP53Abn HMCLs were incubated for 24 hours with or without 5 μM melphalan before the determination of the expression of DR4 by flow cytometry. A representative experiment of 3 is shown. Histograms represent the DR4-specific fluorescence of melphalan-treated cells (thick line) over that of control cells (thin line). Histograms of control staining of control and treated cells were identical and not represented. D, melphalan did not increase the cell death induced by mapatumumab. TP53WT and TP53Abn HMCLs were incubated for 24 hours with or without 1.25 μM melphalan before the addition of 6 μg/mL mapatumumab and incubated for an additional 24 hours. Cell death was determined by Apo2.7–PE staining. The data were expressed as the mean ± SD of 3 experiments. The drug combination increased the cell death by 2% ± 1% for NCI-H929 (P = NS) and decreased it by -1% ± 1% for LP1 (P = NS), -6% ± 1% for MM1S (P = NS), -3 ± 2% for KMS12-PE (P = NS), and -17% ± 3% for OPM2 (P = 0.001, CI > 1.1).

Primary myeloma cells without del17p were sensitive to nutlin-3a and lexatumumab

To study whether p53 could modulate lexatumumab killing of primary cells, we assessed whether DR5 expression was linked to TP53 abnormalities. Indeed, primary myeloma cells with a TP53 deletion constitutively upregulated the p53 target genes CDKN1A, MDM2, BAX, and DR5 but not DR4 (Supplementary Table S2). Thus, we assessed the sensitivity of primary myeloma cells to nutlin-3a, lexatumumab, and mapatumumab in 13 samples in relation to chromosome 17p deletion (Table 1). Myeloma cells were incubated for 24 hours with or without nutlin-3a (10 μM/L), lexatumumab (6 μg/mL), or mapatumumab (6 μg/mL), as indicated in Fig. 6. Cell death and DR5 expression were measured using flow (input) as well as the amount of 41 kDa caspase-8 in lexatumumab immunoprecipitation. In contrast, nutlin-3a increased neither activation of caspase-8 by mapatumumab nor the amount of activated caspase-8 within the DISC via DR5 but not via DR4.

Nutlin-3a directly increased DR5 gene expression via p53

To determine whether p53 directly increased expression of DR5 gene, we conducted quantitative real-time RT-PCR and ChIP assays. Indeed, quantitative real-time RT-PCR assays showed that DR5 mRNA quickly increased after addition of 2.5 μM/L nutlin-3a. In 3 hours, the amount of DR5 mRNA was increased by 373% ± 49% (P < 0.01, n = 3) in good agreement with an increase of 180% ± 37% (P < 0.05, n = 3) of DR5 expression at the cell surface (Supplementary Fig. S4A). To further confirm that p53 was directly involved, p53 ChIP assays were conducted in untreated and nutlin-3a-treated NCI-H929 cells. As shown in Supplementary Fig. S4B, nutlin-3a strongly increased the amount of p53 bound to p53-BS2 in DR5 gene.
cytometry via the loss of CD138 expression (as shown in Fig. 6A) and by direct staining, respectively (Fig. 6B). In contrast to HMCLs, abnormalities of \textit{TP53} are not found within 100% of myeloma cells but in a minority or majority of them. Samples with less than 20% of myeloma cells with a del17p were considered as del17p$^{-}\text{C0}$ whereas those with more than 75% were considered as del17p$^{+}$. Myeloma cells were heterogeneously sensitive to nutlin-3a, lexatumumab, or mapatumumab with no significant differences (Table 1). In del17p$^{-}$ samples (patients 1–10; Fig. 6C), the combination of nutlin-3a and lexatumumab significantly induced a cell death superior to the cell death induced by nutlin-3a plus that induced by lexatumumab (1.7-fold mean increase; Fig. 6C, left; \(P < 0.001\), paired \(t\) test). Nutlin-3a significantly increased DR5 expression (median value, 170%; \(n = 9\); \(P < 0.001\); Fig. 6C, middle). The expression of DR4 was not investigated in the primary samples, but as observed in HMCLs, the combination of nutlin-3a with mapatumumab induced a cell death inferior or equal to that induced by nutlin-3a plus mapatumumab (\(P < 0.001\); Fig. 6C, right). In the 3 del17p$^{+}$ samples, nutlin-3a neither increased lexatumumab-induced cell death (\(P > 0.4\); Fig. 4D, left) nor DR5 expression (\(P > 0.4\), middle) nor mapatumumab-induced cell death (\(P > 0.4\), right).

**Discussion**

Multiple myeloma remains an incurable but treatable disease, despite the successful introduction of new therapies with bortezomib and thalidomide/lenalidomide. Multiple myeloma is characterized by a wide number of prognostic values ranging from the sera $\beta$-2-microglobulin levels to chromosomal abnormalities (27). Among the latter, the deletion of the short arm of chromosome 17 containing the \textit{TP53} locus (17p13) has a high adverse prognosis (11–14). In primary cells, monoallelic deletion of chromosome 17p is not frequent at diagnosis, ranging from 7% to 25% depending on reports, but it increases with the successive relapses (28–32). In contrast, del17p13 is frequent in extramedullary diseases such as plasma cell leukemia (83% in secondary plasma cell leukemia), multiple myeloma involving central nervous system (89%), or plasmacytoma (28, 33–35). Mutations of \textit{TP53} at diagnosis are found in patients with advanced or aggressive disease, especially in those with 17p deletions.
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deletion (36). This finding is in good agreement with the strong expression of p53 in tumors of patients with a hemizygous del17p13, because mutated p53 proteins are strongly expressed (37). In addition, it appears that the p53 pathway is also altered in myeloma cells because of the MDM2 amplification in cells with a TP53 wild-type sequence (38). Taken together, these reports show that the p53 pathway is altered in myeloma cells as it is in most, if not all, cancers. HMCLs are usually derived from extramedullary disease with a high labeling index occurring mainly at relapse, although some HMCLs were derived at the time of diagnosis (19). Most of the HMCLs have a monoallelic deletion associated with a mutated sequence of TP53 (67%). However, 30% of HMCLs have a wild-type TP53 arguing against a full association between extramedullary disease and TP53 deletion and/or mutation.

In this report, we show that the HMCLs with abnormal TP53 sequence underexpressed transcriptional target genes of p53, that is, MDM2, CDKN1A, BAX and DR5. These genes were also underexpressed in primary myeloma cells with a low TP53 expression corresponding to a TP53 deletion (17). DR5 and Bax are involved in the extrinsic and intrinsic pathways of apoptosis, respectively, and their underexpression suggests that myeloma cells expressing an abnormal p53 should be more resistant to apoptosis through both pathways. Indeed, we show that TP53Abn HMCLs are significantly more resistant to melphalan, which triggers the intrinsic pathway of apoptosis (25). All TP53Wt HMCLs were sensitive to nutlin-3a, whereas all TP53Abn HMCLs were resistant. These results confirm a previous study that showed that 2 TP53Abn HMCLs were sensitive to nutlin-3a whereas 4 TP53Abn HMCLs were resistant (24). Of interest, we show that HMCLs expressing both a wild-type and an abnormal TP53 (KMM1 and NAN3) were resistant to nutlin-3a too, showing that the mutations exert a dominant-negative effect. The silencing of p53 significantly decreased the cell death induced by nutlin-3a or melphalan, showing that p53 plays a role in cell response. The p53-dependent molecular mechanisms of cell death induced by nutlin-3a and melphalan are under investigation.

TP53Abn HMCLs were resistant to DR5-mediated cell death, in agreement with the underexpression of DR5. This result is in agreement with that of Xiong and colleagues who identified that DR5 gene was modulated upon reintroduction of a wild-type TP53 in a TP53−/− cell line (17). Interestingly, the DR4 gene is not regulated by p53 in myeloma cells, because DR4 gene expression level is not underexpressed in TP53Abn HMCLs and because DR4 gene expression was not found modulated upon reintroduction of a wild-type TP53 in a TP53−/− cell line (17). However, DR4 expression was notably higher on LP1 compared with TP53Wt HMCLs (Fig. 1B). The molecular mechanism supporting this higher DR4 expression on TP53Abn HMCLs remains to be elucidated. In correlation with the expression of DR4 and DR5, we show that death through DR5 was rather restricted to TP53Wt HMCLs whereas that through DR4 was rather restricted to TP53Abn HMCLs. The involvement of decoy receptors in the response to mapatumumab or lexatumumab should be excluded, because these mAbs do not bind to the decoy receptors (39). Exclusively in TP53Abn HMCLs, an increase of p53 expression induced by genotoxic (melphalan) or nongenotoxic (nutlin-3a) stress enhanced both the expression of DR5 and cell death induced by lexatumumab. nutlin-3a or melphalan never increased DR4 expression, nor mapatumumab killing in TP53Wt or TP53Abn. Reciprocally, the decrease of p53 expression by shRNA reduced DR5 expression and cell death induced by lexatumumab but did not overcome the resistance of NCI-H929 to mapatumumab and did not modulate the expression of DR4. This result argues once again against a direct involvement of p53 in the regulation of DR4. In a limited panel of primary myeloma cells without del17p, we show that nutlin-3a significantly increased the expression of DR5 and the cell death induced by lexatumumab. In contrast, nutlin-3a never increased the efficiency of mapatumumab.
Our data show that TRAIL receptors are differentially regulated. In contrast to Fas-L, but as TNF-α, TRAIL has 2 receptors. However, in contrast to TNF-α receptors, both TRAIL receptors induce cell death. TRAIL differentially binds to TRAIL-R as illustrated by the phosphorothioate-modified CpG nucleotides, which block the binding of TRAIL to DR5 but not to DR4 and similarly bind to lexatumumab and not to mapatumumab (40). TRAIL mutants with selective binding to DR4 or DR5 also showed that binding of TRAIL to DR4 and DR5 involves different amino acids (41). Most tumor cells, unlike nonmalignant cells, are sensitive to TRAIL, but the involvement of one or the other receptor has not been systematically investigated. In myeloma cells, resistance to lexatumumab or mapatumumab is associated with a lack of caspase 8 activation (18). The caspase-8/Flip ratio has been proposed to control the sensitivity of myeloma cells (42). However, we did not find any relationship between this ratio and the sensitivity to TRAIL via DR4 or DR5 (data not shown). Moreover, quite all myelomas are sensitive through one or the other receptor, excluding a mechanism of resistance involving the lack of caspase-8 activation because of a high expression of Flip. Recently, Reis and colleagues showed that the increased sensitivity to a DR4-selective TRAIL mutant was associated with an increase in the activation of caspase-8 (43). Moreover, Szegedi and colleagues showed that receptor-selective TRAIL variants enhanced the kinetics of receptor activation and that apoptosis largely depends on the relative amounts of its receptors (44). In melanoma, Kurbanov and colleagues showed that the resistance of cells to DR4-mediated apoptosis was related to the low level of caspase-8 or -10 and DR4 (45). Fan and colleagues also reported that the p53-independent increased expression of DR5 induced by the cap translation inhibitor 4EGI-1 supported the enhanced response of human lung cancer cells to TRAIL (46). These reports suggested that sensitivity or resistance of cancer cells to TRAIL is mainly dependent on the amount of caspase-8/death receptor complexes, regardless of the cancer model. In myeloma, we show that the cleaved forms of caspase-8, 41 and/or 43 kda were associated with DR4 or DR5 in correlation with the sensitivity of cells to mapatumumab or lexatumumab. Furthermore, the pretreatment of NCI-H929 with a low dose of nutlin-3a highly increased DR5 as well as the amount of cleaved caspase-8 within the DISC, explaining the cell death increase. We showed that DR5 increase induced by nutlin-3a was directly related to the increased amount of p53 bound to p53-BS2 in DR5 gene, arguing again for a direct relationship between DR5 expression and myeloma sensitivity to lexatumumab. In contrast, no p53 binding on DR4 gene could be evidenced by ChIP (data not shown). In myeloma cells at least, DR5, but not DR4, is directly regulated by p53. Altogether, these data showed that cell death through DR4 or DR5 correlated with the amount of each receptor and the amount of caspase-8 recruited within the DISC. The strong activation of caspase-8 induced by lexatumumab or mapatumumab in myeloma cells argues against a requirement of the intrinsic pathway, despite its activation. Indeed, silencing of caspase-9 or bax did not inhibit cell death induced by lexatumumab or mapatumumab (data not shown).

Table 1. Sensitivity of myeloma cells to lexatumumab, mapatumumab, and nutlin-3a

<table>
<thead>
<tr>
<th>Patient Status</th>
<th>Cell death (%)</th>
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<tr>
<td>Patient</td>
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NOTE: Primary samples were obtained from bone marrow (BM), pleural effusion (PE), and peripheral blood (PB) from patients at diagnosis (D) or relapse (R). Bone marrow myeloma cells were purified with anti-CD138 immunomagnetic beads. Myeloma cells from both peripheral blood and pleural effusion samples were not purified because of high plasma cell content. Deletion of chromosome 17p was assessed by FISH. Cells were treated for 24 hours with 10 μmol/L nutlin-3a (N), 6 μg/mL lexatumumab (Lexa), or 6 μg/mL mapatumumab (Mapa) alone or in combination as indicated. DR5 expression level was assessed by flow cytometry and expressed as a ratio of specific fluorescence divided by control fluorescence. Cell death was assessed by the loss of CD138 expression (see Fig. 6A). Abbreviation: nd, not done.
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shown some antitumor activity (47, 48). As observed in cell lines, combination of nutlin-3a with lextatumumab is synergistic in del17p– samples, whereas that of nutlin-3a with mapatumumab is at best additive (3 cases of 8). Our results suggest that
treatment of patients with DR5 agonistic mAb should be more efficient in combination with a p53-activating drug, only if the cells have retained a functional p53 pathway. In contrast, treatment of patients without del17p with DR4 agonistic mAb treatment should not be more efficient in combination with a p53-activating drug. Concerning del17p+ samples, we did not observe that mapatumumab was very efficient, but we could obtain only 3 samples. Because the correlation between p53 abnormalities and mapatumumab sensitivity was found when more than 20 HMCls were assessed, more primary samples are required before reaching a first conclusion.

In summary, we have shown that the death of myeloma cells induced by DR5, but not DR4, agonistic antibody depends on p53 and can be synergistically increased by drugs inducing DR5 expression such as those activating the p53 pathway.

Disclosure of Potential Conflicts of Interest
R. Bataille is a consultant/advisory board member for Celgene. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S. Surget, C. Pellat-Deceunynck
Development of methodology: S. Surget, P. Gomez-Bougie, C. Pellat-Deceunynck
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Surget, D. Chiron, G. Descamps, E. Ménoret, P. Moreau, S. Le Gouill, C. Pellat-Deceunynck
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Surget, C. Pellat-Deceunynck
Writing, review, and/or revision of the manuscript: S. Surget, G. Descamps, R. Bataille, S. Le Gouill, M. Amiot, C. Pellat-Deceunynck
Study supervision: C. Pellat-Deceunynck
Carried out experiments: S. Surget, D. Chiron, P. Gomez-Bougie, G. Descamps, E. Ménoret

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Figure 6. Nutlin-3a increases cell death of primary cells induced by lextatumumab. A, purified primary myeloma cells from patient 3 were incubated for 24 hours with or without 10 μM nutlin-3a or 6 μg/mL lextatumumab or 6 μg/mL mapatumumab or the combinations as indicated in the figure before staining with anti-CD138-PE mAb. Cell death was determined by the percentage of CD138+ cells that have lost CD138 expression (22); for example, mapatumumab induced 1% of cell death [100 × (83–82)/83 = 1%]. B, purified primary myeloma cells from patient 3 were incubated for 24 hours with or without 10 μM nutlin-3a before the determination of DR5 expression. Histograms represent the specific fluorescence (thick line) over that of control staining (thin line). C and D, purified primary myeloma cells from 13 patients were incubated for 24 hours with or without 10 μM nutlin-3a or 6 μg/mL lextatumumab or mapatumumab alone or in combination before staining with anti-CD138-PE mAb to assess cell death (left and right, respectively). N = L, the sum of death induced by nutlin-3a and lextatumumab alone; NL, the death induced by the combination; N + M, the sum of death induced by nutlin-3a and mapatumumab alone; NM, the death induced by the combination. Expression of DR5 was determined in cells treated for 24 hours with or without 10 μM nutlin-3a using flow cytometry (middle). G, patients with less than 20% of myeloma cells with a 17p deletion. D, samples with more than 75% of myeloma cells with a 17p deletion.

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Cell Death via DR5, but not DR4, Is Regulated by p53 in Myeloma Cells

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