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 Ménoret1,2, Régis Bataille1,2,3,5, Philippe Moreau1,2,3,4, Steven Le Gouill1,2,3,4, Martine Amiot1,2,3, and Catherine Pellat-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 over-expressed 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/TRAIRL1/TNFRSF10A, DR5/TRAIRL2/ 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.
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 MM1S were, respectively, kindly provided by Drs. I. Van Biet (Vrije Universiteit Brussel, Brussels, Belgium), I.C. MacLennan (University of Birmingham, Birmingham, UK), A. Karpas (University of Cambridge, Cambridge, UK), and S. Rosen (Northwestern University, Chicago, IL). AMO1, LP1, I363, NCI-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 (Novartis Pharmaceuticals) for ANBL-6, BCN, MDN, NaNs, SBN, and XGs. Blood or bone marrow samples from patients with multiple myeloma at diagnosis were collected after informed consent at the Department of Hematology at University Hospital of Nantes (Nantes, France) or at the Intergroupe Francophone du Myélome. Plasma cells were obtained after density gradient centrifugation using Ficoll Hypaque and purification with CD138 immunomagnetic beads (Miltenyi Biotech). In all cases, the purity of the plasma cells was higher than 90%, as assessed by morphology. Purified cells were cultured for 24 hours in RPMI-1640 containing 10% FCS and 3 ng/mL IL-6.

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'-ATGGAGGACGCAGCTCA-3', 5'-GGCTCGCGTCCAATGCGG-3' for amplification codon 1–250 and 5'-GGCCCTCCCTCTCAGCATCTC-3', 5'-TCCCCATCTCCTCCACA-3' for amplification codon 187–370. cDNA sequencing was conducted using the Plate-Forme de Séquençage-Génomptype 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 (TP53-), 5 have a structurally abnormal TP53 (TP53abnor), 15 have a missence point mutation (TP53mut). Only 2 HMCLs with a TP53mut express a wild-type allele (TP53Wt TP53mut, 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-TABM-937 and E-TABM-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 Biosciences, and anti-DR4-PE and anti-DR5-PE were purchased from ebiosis.

Cell death assays

The cytométric 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-Abcyx), DR5 (Millipore Bioscience Research Reagents), MDM2 (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 lysed 40 for 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 mg of lystate proteins were immunoprecipitated in each assay).

TP53 silencing

A stably modified myeloma cell line was obtained via lentiviral cell transduction. The TP53mut cell line NCI-H929 was transduced with a lentivirus carrying short hairpin RNA (shRNA) control or an shRNA designed to knockdown p53 (CCGGTGCCATGAGCTCCTCCAGAATCGAGTTCGGA-GCTTCATCTGGACTTTTT, 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...
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 performed 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'-AGACCTTGGCTGTTG-3' and reverse 5'-CGGGAATTTACACCAAGTGG-3'), which amplify a sequence of 271bp 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 of p53 and melphalan were analyzed using the combination index (CI) method with the CalcuSyn software program (Bio-soft). A CI of less than 0.9 indicates synergism, 0.9 to 1.1 additive, and greater than 1.1 antagonism.

**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 (TP53*Wt*) 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 TP53*Abn* HMCLs (see Materials and Methods).

Transcriptomic Affymetrix analysis of HMCLs showed that several p53 target genes such as MDM2, CDKN1A, BAX, and DR5 but not DR4 were overexpressed in TP53*Wt* compared with TP53*Abn* 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 TP53*Wt* HMCLs (Fig. 1B), whereas DR4 was more expressed by TP53*Abn* HMCLs (Fig. 1F), P = 0.1 and P = 0.03, Mann–Whitney test, respectively. Lexatumumab significantly killed TP53*Wt* HMCLs but not TP53*Abn* 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). TP53*Abn* HMCLs were more sensitive to mapatumumab than TP53*Wt* 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, TP53*Wt* HMCLs expressed DR5 and were killed by lexatumumab in contrast to TP53*Abn* HMCLs, which expressed DR4 and were killed by mapatumumab.

**Nongenotoxic and genotoxic stresses increased DR5 and lexatumumab-induced cell death in TP53*Wt* 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 TP53*Wt* cells (24). As shown in Fig. 2A, all TP53*Wt* HMCLs were killed by nutlin-3a, whereas all TP53*Abn* 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 TP53*Wt* HMCLs but in none of 3 TP53*Abn* 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 TP53*Wt* and TP53*Abn* 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 TP53*Wt* HMCLs (median value of 38% but in none of the 3 TP53*Abn* (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). TP53*Wt* HMCLs were more sensitive to melphalan than TP53*Abn* HMCLs (Fig. 2D; P = 0.0019, ANOVA test). Similar to nutlin-3a, melphalan (5 μmol/L) increased DR5 expression only in TP53*Wt* HMCLs (Fig. 2E). The median value of DR5 expression in treated cells was 139% (range, 119–170) and 106% (range, 90–114) of untreated TP53*Wt* and TP53*Abn* 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 TP53*Wt* HMCLs but in none of the TP53*Abn* (P < 0.001, Mann–Whitney test). These data showed that a nongenotoxic (nutlin-3a) or genotoxic (melphalan) stress of TP53*Wt* 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 TP53*Wt* but very slightly decreased in TP53*Abn* 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 TP53*Wt* and TP53*Abn* 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 TP53*Wt* but very slightly decreased in TP53*Abn* 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 TP53*Wt* or TP53*Abn* HMCLs (Fig. 3D).

Thus, in contrast to DR5, DR4 expression was not increased by nutlin-3a or melphalan in TP53*Wt* 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 TP53Wt 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 μmol/L), 34% ± 10% for melphalan (10 μmol/L), 39% ± 5% for lexatumumab (6 μg/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, MDN) 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 caspase-8 isoforms in the whole lysates, only the 41-kDa was associated with DR4 or DR5 in the 2 TP53WT HMCLs MDN and NCI-H929. Immunoprecipitation of DR5 was hardly detectable in NCI-H929 unless the cells were preincubated for 2 hours with 2.5 μm/L nutlin-3a before adding lexatumumab or mapatumumab. Nutlin-3a increased activation of caspase-8 induced by lexatumumab.
Nutlin-3a did not modulate the expression of DR4. TP53WT and TP53Abn HMCLs were incubated for 6 hours with or without 10 μmol/L 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 μmol/L 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 11% ± 1.5% for LP1 (P < NS) and decreased it by −2% ± 3% for XG-6 (P = NS), −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 μmol/L 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 μmol/L 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 XG-6 (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).

Nutmol-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 μmol/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.

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 upexpressed the p53 target genes CDKN1A, MDM2, BAX, and DR5 but not DRA (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 μmol/L), lexatumumab (6 μg/mL), or mapatumumab (6 μg/mL), as indicated in Fig. 6. Cell death and DR5 expression were measured using flow...
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 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/- 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 b2-microglobulin levels to chromosomal abnormalities (27). Among the latter, the deletion of the short arm of chromosome 17 containing the 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, del17p 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 TP53 at diagnosis are found in patients with advanced or aggressive disease, especially in those with 17p

**Figure 4.** Silencing p53 in the TP53wt HMCL NCI-H929 inhibited cell death induced by nutlin-3a, melphalan, and lexatumumab and decreased expression of DR5. A, Western blot analysis of p53, DR5, MDM2, p21, Bax, Noxa, and Puma expression in shcontrol (shCt) and shp53 NCI-H929 cells. Western blotting was conducted as described in Materials and Methods. B, expression of DR5 or DR4 in shCt and shp53 NCI-H929 cells. Cells were stained with control-PE or anti-DR4-PE or anti-DR5-PE mAbs before analysis by flow cytometry. Overlay histograms represent the specific fluorescence of shp53 cells (thick line) over that of shCt cells (thin line). C, cell death of shCt and shp53 cells induced by nutlin-3a, melphalan, lexatumumab, or mapatumumab. ShCt and shp53 NCI-H929 cells were incubated for 48 hours with nutlin-3a (10 \( \mu \)mol/L) or melphalan (10 \( \mu \)mol/L) or lexatumumab or mapatumumab (6 \( \mu \)g/mL) before the determination of cell death by Apo2.7-PE staining. The data represent the mean \( \pm \) SD of 3 independent experiments. *, \( P < 0.05 \). D, DR5 expression of shCt and shp53 induced by nutlin-3a. ShCt and shp53 NCI-H929 cells were incubated for 6 hours with or without 10 \( \mu \)mol/L nutlin-3a before staining with control-PE or anti-DR5-PE mAbs.
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 transcription 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 TP53$^{Abn}$ HMCLs are significantly more resistant to melphalan, which triggers the intrinsic pathway of apoptosis (25). All TP53$^{Abn}$ HMCLs were sensitive to nutlin-3a, whereas all TP53$^{Wt}$ HMCLs were resistant. These results confirm a previous study that showed that 2 TP53$^{Abn}$ HMCLs were sensitive to nutlin-3a whereas 4 TP53$^{Wt}$ 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.

TP53$^{Abn}$ 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 TP53$^{Abn}$ 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 TP53$^{Abn}$ compared with TP53$^{Wt}$ HMCLs (Fig. 1B). The molecular mechanism supporting this higher DR4 expression on TP53$^{Abn}$ HMCLs remains to be elucidated. In correlation with the expression of DR4 and DR5, we show that death through DR5 was rather restricted to TP53$^{Wt}$ HMCLs whereas that through DR4 was rather restricted to TP53$^{Abn}$ HMCLs. The involvement of decoy receptors in the response to matatumumab or lexatumumab should be excluded, because these mAbs do not bind to the decoy receptors (39). Exclusively in TP53$^{Abn}$ 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 matatumumab killing in TP53$^{Wt}$ or TP53$^{Abn}$. 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 matatumumab 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 matatumumab.

Figure 5. Cleaved caspase-8 was associated with DR4 or DR5 in relation to mapatumumab or lexatumumab sensitivity. Immunoprecipitation (IP) assays of mapatumumab (Mapa) or lexatumumab (Lexa) were conducted in LP1, MDN, and NCI-H929 cells. Cells ($20 \times 10^6$) were incubated with or without (control) 10 $\mu$g/mL of mapatumumab (45 minutes) or lexatumumab (90 minutes) at 37°C in RPMI-1640 containing 5% FCS. For NCI-H929, cells were pretreated for 2 hours with 2.5mM/L nutlin-3a (N) before addition of lexatumumab. The lysates were immunoprecipitated with a mix of proteins A and G (2 mg of lysate proteins were immunoprecipitated in each assay). For untreated cells, IP was conducted after addition of mapatumumab or lexatumumab to the lysates. The immunoprecipitates were analyzed for the presence of the indicated proteins by immunoblot (IB) analysis. Input, lysates of untreated or treated cells; Ct IP, IP of control lysates (untreated cells); IP, IP of lysates obtained from cells treated with mapatumumab or lexatumumab. * heavy chain of mapatumumab or lexatumumab.

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 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 TP53$^{Abn}$ 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 TP53$^{Abn}$ compared with TP53$^{Wt}$ HMCLs (Fig. 1B). The molecular mechanism supporting this higher DR4 expression on TP53$^{Abn}$ HMCLs remains to be elucidated. In correlation with the expression of DR4 and DR5, we show that death through DR5 was rather restricted to TP53$^{Wt}$ HMCLs whereas that through DR4 was rather restricted to TP53$^{Abn}$ HMCLs. The involvement of decoy receptors in the response to matatumumab or lexatumumab should be excluded, because these mAbs do not bind to the decoy receptors (39). Exclusively in TP53$^{Abn}$ 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 matatumumab killing in TP53$^{Wt}$ or TP53$^{Abn}$. 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 matatumumab 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 matatumumab.
Surget et al.

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

<table>
<thead>
<tr>
<th>Patient Status</th>
<th>Origin</th>
<th>del17p</th>
<th>Lexa</th>
<th>Mapa</th>
<th>Nl</th>
<th>Nm</th>
<th>Untreated</th>
<th>Nutlin-3a</th>
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<tr>
<td>D BM</td>
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<td>66</td>
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<td>1</td>
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<td>99</td>
<td>49</td>
<td>1.4</td>
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<tr>
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<td>20</td>
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<td>37</td>
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<tr>
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<td>5</td>
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<td>12</td>
<td>3</td>
<td>12</td>
<td>13</td>
<td>0.9</td>
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</table>

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.

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/Flp ratio has been proposed to control the TRAIL 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 receptors, excluding a mechanism of resistance involving the lack of caspase-8 activation because of a high expression of Flp. 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, Kurbakan 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).

Recently, phase I/II clinical trials have reported that DR5 and DR4 agonistic antibodies (PRO95780 and mapatumumab) are well tolerated in patients with advanced cancers and have...
shown some antitumor activity (47, 48). As observed in cell lines, combination of nutlin-3a with lexatumumab 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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References


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Sylvanie Surget, David Chiron, Patricia Gomez-Bougie, et al.


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