

Short interfering RNA directed against the SLUG gene increases cell death induction in human melanoma cell lines exposed to cisplatin and fotemustine

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Abstract. *Background:* Melanoma remains largely resistant to currently available chemotherapy, and new strategies have been proposed to flank standardized therapeutic protocols in an effort to improve efficacy. Such an approach requires good knowledge of the mechanisms involved in the resistance and survival of melanoma cells. In this context, the SLUG gene has recently been characterized as a major regulator of melanocytes and melanoma cell survival. *Methods:* We tested the hypothesis that an oligonucleotide-based short interfering RNA (siRNA) directed against the SLUG gene increases the susceptibility of melanoma cells to drugs such as cisplatin and fotemustine, which are frequently used to treat this cancer. *Results:* It was found that SLUG siRNA increased cisplatin-induced cell death and rendered the drug active *in vitro* at half its plasmatic peak concentration. Such activity was correlated with an upregulation of the pro-apoptotic gene, PUMA. Furthermore, SLUG siRNA increased the capacity of fotemustine to elicit cell death and induced p21WAF1 upregulation, resulting in cell cycle arrest. Interestingly, this pathway did not require functional p53. *Conclusion:* These findings suggest that SLUG siRNA enhances the efficacy of two of the most widely used drugs to treat melanoma.

Keywords: Slug gene, melanoma, short interfering RNA, cisplatin, fotemustine

1. Introduction

Melanoma is the most aggressive form of skin cancer and is notoriously resistant to most current modalities of cancer therapy [18]. For several years the attempt to treat metastatic melanoma has been based on new cytotoxic agents tested alone or in combination, or on other biologically active molecules, such as INF- γ [16]. Recently, the identification of molecules involved in the regulation and induction of apoptosis in melanoma has generated new biological

treatments aimed at enhancing chemotherapy-induced cell death [1,2,11]. One of the most widely investigated molecules is the antisense oblimersen, a single stranded phosphorothioate oligodeoxynucleotide targeted against Bcl-2 [1,11].

The human SLUG gene belongs to the highly conserved Slug/Snail family of transcription repressors, which are master regulators of neural crest cell specification and melanocyte migration during development in vertebrates [5,9,13,15,17]. Interestingly, the SLUG gene is required for the metastatic progression of melanoma cells [8], and the anti-melanoma effect of IFN- γ is associated with the downregulation of SLUG [7,16]. In addition, SLUG promotes survival and hinders cell death by directly repressing PUMA, a key BH3-only antagonist of the anti-apoptotic Bcl-2 protein [19]. SLUG, therefore, has two basic functions that

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render it a target for melanoma gene therapy – it is involved in melanocytic differentiation and ontogeny, and it antagonizes cell death.

The aim of the present study was to investigate and define the ability of SLUG siRNA to increase the susceptibility of melanoma cell lines to currently used cytotoxic drugs.

2. Methods

2.1. Cell lines

The study was performed on three melanoma cell lines, M14, M66, and M79. M14, a commercial cell line, was obtained from the American Type Culture Collection (Rockville, MD, USA), while M66 and M79 cell lines were isolated in our laboratory from primary and metastatic lesions of two melanoma patients (W. Zoli, unpublished observation). Cell lines were maintained as a monolayer at 37°C and sub-cultured weekly. Culture medium was composed of DMEM/HAM'S F12 (1:1) supplemented with fetal calf serum (10%), glutamine (2 mM), non-essential aminoacids (1%) (Mascia Brunelli s.p.a., Milan, Italy), and insulin (10 mg/ml) (Sigma-Aldrich, St. Louis, MO, USA). Cells were used in the exponential growth phase in all the experiments.

2.2. siRNA transfections

Three different double strand siRNA oligonucleotides (Invitrogen, Carlsbad, CA, USA) were utilized alone or pooled together. A validated medium GC scramble (SCR) double strand siRNA oligonucleotide (Invitrogen) was used as control for transfection. The siRNA oligonucleotide showing the highest efficiency of SLUG mRNA knocking-down in the three cell lines was utilized for the experiments reported in the manuscript. To perform transfection, cells were seeded the day before the experiment in 25-cm² flasks at a density of 18×10^4 cells (60% confluence). Transfections were carried out using Lipofectamine 2000 (Invitrogen) and Opti-MEM GlutaMax medium (Invitrogen) medium without antibiotics. The incubation time for oligonucleotide/Lipofectamine 2000 complexes was 5 h. The total incubation time before drug treatment was 72 h at 37°C.

2.3. Drugs

Cisplatin (Mayne Pharma Pty Ltd, Mulgrave, Australia) and fotemustine (Laboratories Thissen, Braine L'Alleud, Belgium) were diluted in 0.9% saline solution. Cisplatin was stored at 25°C and fotemustine at 4°C, and both were freshly diluted in culture medium before each experiment.

2.4. Drug exposure

Cells were exposed to cisplatin for 6 h or to fotemustine for 45 min to reproduce the clinical conditions of melanoma treatment. Taking into account that the peak plasma level is 3 µg/ml for cisplatin and 2.8 µg/ml for fotemustine, we tested 1.5- and 3-µg/ml concentrations for cisplatin and 1.4- and 2.8-µg/ml concentrations for fotemustine. Evaluation of the cytotoxic effect was performed 48 h after the end of drug exposure.

2.5. RNA extraction and amplification

Total RNA was extracted from cell lines by RNA easy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. One microgram of RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA) in a final volume of 20 µl. The reaction was carried out at 42°C for 30 min and was stopped by heating to 85°C for 5 min. Real Time PCR was performed with MyiQ Single Color Real-Time PCR Detection System (BioRad) using SYBR Green I dye chemistry. *β2-microglobulin* mRNA was amplified as control for to verify the quality and quantity of input RNA (similar results were obtained when *18S ribosomal subunit* RNA and *glyceraldehyde-3-phosphate dehydrogenase genes* were used as controls [data not shown]). Primers used for *β2-microglobulin* and *Slug* amplification were designed using Beacon Designer Software (version 4, BioRad). Primer sequences were forward 5'-CGC TAC TCT CTC TTT CTG GC-3' and reverse 5'-AGA CAC ATA GCA ATT CAG GAA AT-3' for *β2-microglobulin*, and forward 5'-AGA TGC ATA TTC GGA CCC ACA-3' and reverse 5'-CCT CAT GTT TGT GCA GGA GAG-3' for *Slug*. Real Time PCR reactions were carried out in triplicate in a volume of 25 µl containing 50 ng of cDNA template, 1 × SYBR Green Mix, and 200 nM or 400 nM of forward and reverse primers (for *β2-microglobulin* and *Slug*, respectively). Samples were heated to 95°C for one and a half min, followed by 30 amplification

cycles for 15 sec at 95°C and for 30 sec at 60°C (for $\beta 2$ -microglobulin) or 60.5°C (for *Slug*) for annealing and amplification. Product purity was controlled by melting point analysis. The amount of *Slug* mRNA was normalized to the endogenous reference $\beta 2$ -microglobulin and expressed as n-fold *Slug* mRNA levels relative to *Slug* mRNA SCR of each experiment or as n-fold SLUG mRNA levels relative to SLUG mRNA of M79 cell line using Gene Expression Macro Software (Version 1.1, BioRad).

Semiquantitative RT-PCR analysis was carried out to evaluate PUMA, Bcl-2, p21WAF1 and $\beta 2$ -microglobulin mRNA expression. One microgram of RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (BioRad) as previously described. To verify the quality and quantity of the cDNA obtained, amplification of the endogenous $\beta 2$ -microglobulin gene was performed. The following primers were used for PCR reactions: forward 5'-TGG GGA TGT CCG TCA GAA CC-3' and reverse 5'-TGG AGT GGT AGA AAT CTG TCA TGC T-3' for *p21WAF1*; forward 5'-TGT GGC CTT CTT TGA GTT CG-3' and reverse 5'-GAC ACG CCG GGA AGC AAC AA-3' for *Bcl-2*; forward 5'-CAG ACT GTG AAT CCT GTG CT-3' and reverse 5'-ACA GTA TCT TAC AGG CTG CC-3' for *PUMA*; forward 5'-ACC CCC ACT GAA AAA GAT GA-3' and reverse 5'-ATC TTC AAA CCT CCA TGA TG-3' for $\beta 2$ -microglobulin.

PCR reactions were carried out in a volume of 25 μ l containing 50 ng of cDNA template, 2.5 units of recombinant Taq DNA polymerase (Invitrogen), 10 \times reaction buffer, 20 μ M of dNTPs and 200 nM of forward and reverse primers. Samples were pre-heated at 94°C for 2 min, followed by 29 amplification cycles for 1 min at 94°C, 1 min at 60°C for *p21* and *Bcl-2*, 62°C for *PUMA* and 58°C for $\beta 2$ -microglobulin, 1 min at 72°C, and a final extension of 7 min at 72°C. Twenty microliters of the amplified DNA fragments were separated on an ethidium bromide-stained 2% agarose gels in 1 \times TBE and visualized under UV light.

2.6. Western blot

Cells were lysated and cellular proteins were denatured, separated on 10% SDS-polyacrylamide gel and then electroblotted onto Hybond-C extra membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was stained with Ponceau S (Sigma-Aldrich) to verify equal amounts of sample loading and then incubated for 2 h at room temperature with T-PBS 5% non fat dry milk. The membrane was

probed overnight at 4°C with the primary antibody and then with a horseradish peroxidase-conjugated secondary antibody diluted 1:1000 (Dako Corporation, Glostrup, Denmark). The bound antibodies were detected by enhanced chemiluminescence (ECL) using an ECL kit (Amersham Biosciences). The following primary antibodies were used: anti Bcl-2, mouse monoclonal antibody, dilution 1:100 (Dako Corporation); anti α *Slug*, rabbit monoclonal antibody, dilution 1:250 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti PUMA, rabbit polyclonal antibody, dilution 1:500 (Upstate Biotechnology, Charlottesville, VA, USA); anti p21, mouse monoclonal antibody, dilution 1:100 (NeoMarkers, Fremont, CA, USA), anti actin, rabbit polyclonal antibody, dilution 1:5000 (Sigma-Aldrich),

2.7. Cell cycle distribution

After exposure to fotemustine, cells were harvested, fixed in ethanol (70%) and stained in a solution containing 10 μ g/ml of propidium iodide (Sigma Aldrich), 10 Kunits/ml of RNase (Sigma Aldrich) and 0.01% of NP40 (Sigma Aldrich). After 30–60 min, samples were analyzed by flow cytometry using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA, USA). Data acquisition (10,000 events were collected for each sample) was performed using CELLQuest software (Becton Dickinson). Data were elaborated using Modfit (DNA Modelling System) software (Verity Software House Inc., Topsham, ME, USA) and expressed as fractions of cells in the different cycle phases.

2.8. Apoptosis

TUNEL assay

After exposure to cisplatin or fotemustine, cells were trypsinized, fixed in 1% paraformaldehyde in PBS on ice for 15 min, suspended in ice cold ethanol (70%) and stored overnight at –20°C. Cells were then washed twice in PBS and incubated with 50 μ l of solution containing TdT and FITC-conjugated dUTP deoxynucleotides 1:1 (Roche Diagnostic GmbH, Mannheim, Germany) in a humidified atmosphere for 60 min at 37°C in the dark. Samples were then washed in PBS containing 0.1% Triton X-100, counterstained with 3 μ g/ml of propidium iodide (Sigma Aldrich) and 10 Kunits/ml of RNase (Sigma-Aldrich) for 30 min at 4°C in the dark, and finally analyzed by flow cytometry using a FACS Vantage flow cytometer (Becton Dickinson). Data acquisition and analysis were performed using CELLQuest software (Becton Dickinson). For each sample, 10,000 events were recorded.

2.9. Statistical analysis

Data were analyzed by Anova. $p < 0.05$ was considered significant.

3. Results

3.1. SLUG expression

SLUG mRNA and protein levels were evaluated in M79, M66, M14 melanoma cell lines. All 3 cell lines expressed detectable levels of SLUG mRNA and protein (Fig. 1a). When the cell lines were exposed to three RNA double strand SLUG-specific short interfering oligonucleotides (SLUG siRNA), a downregulation of the SLUG mRNA and protein was observed with respect to exposure to an appropriate oligonucleotide control (scramble, SCR siRNA) (Fig. 1b).

3.2. SLUG modulation of cisplatin-induced cell death

The 3 cell lines were then exposed to cisplatin, a drug currently used in melanoma therapy. Drug concentrations were based around *in vivo* peak plasma levels, and the 6-h exposure time was chosen on the basis of the drug's plasmatic half-life. Cell death analysis performed by TUNEL assay showed, at both concentrations (1.5 $\mu\text{g/ml}$ and 3 $\mu\text{g/ml}$), a higher cell death in SLUG siRNA-treated cells compared to SCR siRNA-

treated cells (Fig. 2a,b). Moreover, we observed that treatment with SLUG siRNA elicited an upregulation of PUMA mRNA and protein levels with respect to SCR siRNA in all 3 cell lines exposed to cisplatin (Fig. 2c). No upregulation of PUMA mRNA or protein expression was present in any of the 3 cell lines that were exposed to SLUG siRNA and not treated with cisplatin (data not shown). Conversely, the SLUG siRNA-induced downregulation of another recently identified SLUG-regulated gene, *Bcl-2*, was not observed (Fig. 2d).

3.3. SLUG modulation of fotemustine-induced cell death and cell cycle arrest

We then assessed whether SLUG siRNA modulates the effects of fotemustine, a cytostatic drug used in melanoma therapy. The modality of drug administration was once again based on the *in vivo* peak plasma level, and the 45-min exposure time was chosen on the basis of the drug's plasmatic half-life. SLUG siRNA-treated cells displayed a higher degree of cell death than SCR siRNA-treated cells (Fig. 3a,b). Moreover, at least in M79 cells, SLUG siRNA induced an increase in G2/M phase with respect to SCR siRNA (Fig. 3a,b). SLUG siRNA cells treated with fotemustine showed an upregulation of the cyclin inhibitor p21WAF1 mRNA and protein levels with respect to SCR siRNA-treated cells (Fig. 3c), whereas no important changes were observed in PUMA or *Bcl-2* mRNA and protein lev-

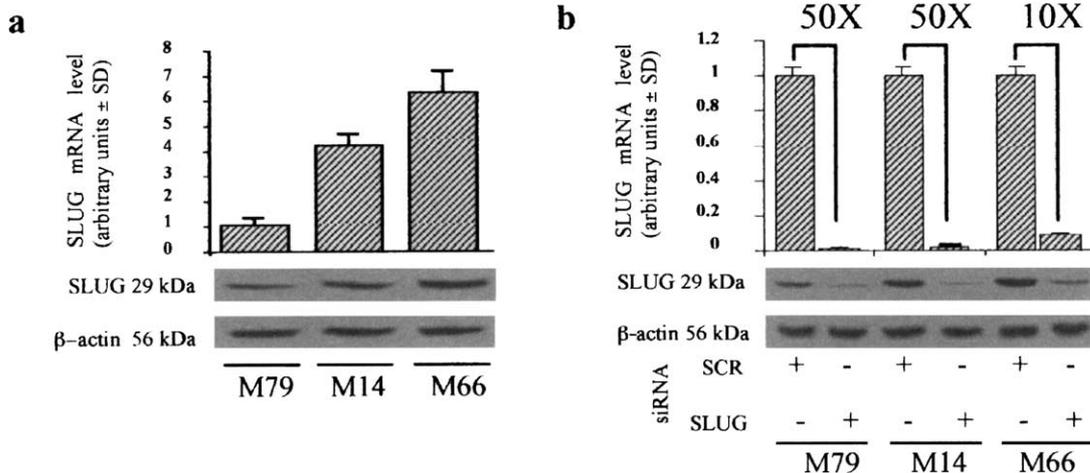


Fig. 1. SLUG expressed in melanoma cell lines. (a) M79, M14, and M66 melanoma cells. Real time PCR analysis of SLUG mRNA level (arbitrary units; \pm SD, standard deviation of 3 replicates, upper part) and Western blot (WB) analysis of SLUG and β -actin (lower part). (b) M79, M14, M66 cells administered with either SLUG-specific (SLUG) or control (scramble, SCR) short interfering RNA (siRNA). Real time PCR analysis of mRNA levels, normalized over SCR mRNA level (arbitrary units; \pm SD, standard deviation of 3 replicates, upper part). WB analysis of SLUG and β -actin (lower part) (+ administered, - not administered SLUG siRNA or SCR siRNA).

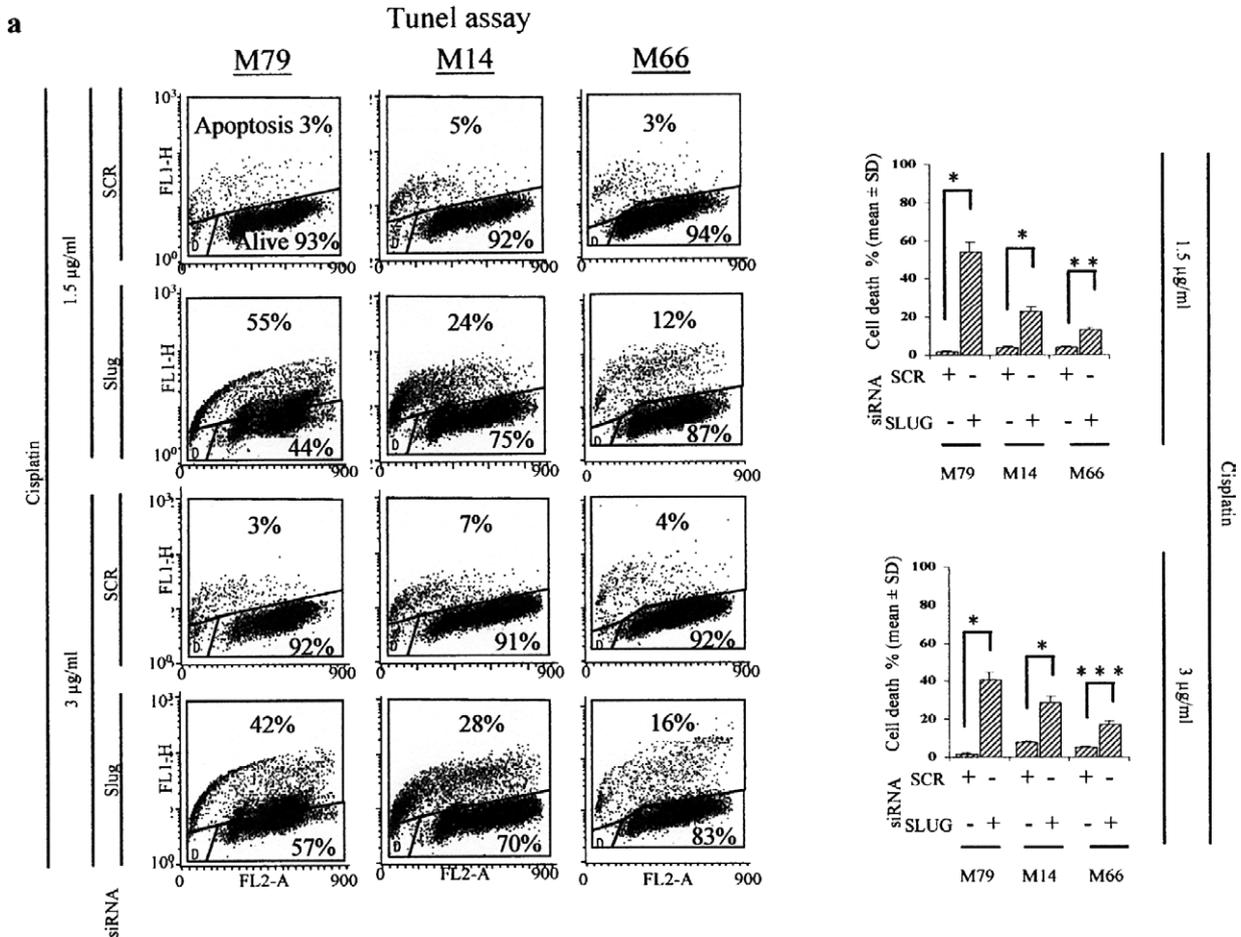


Fig. 2. SLUG modulation of cisplatin-induced cell death in melanoma cells. M79, M14, and M66 exposed to cisplatin (1.5 and 3 $\mu\text{g/ml}$) and treated with SLUG or SCR siRNA. (a) Tunel assay (representative experiments), with percentage of dead cells ('D' refers to debris, which never exceeded 5%) (left part) and median values of each experiment, which was repeated 3 times (right part, * $p < 0.001$, ** $p < 0.01$, *** $p = 0.004$).

els (data not shown). No upregulation of p21 WAF1 mRNA or protein expression was present in any of the 3 cell lines that were exposed to SLUG siRNA and not treated with fotemustine (data not shown).

3.4. p53 mutation analysis

Finally, as PUMA and p21WAF-1 are known to be p53-regulated, we sequenced the full p53 cDNA of the 3 cell lines and observed that M14 and M79 cell lines carry a mutation at codon 266 of p53, and that M66 cells carry a mutation at codons 258 and 259 of the same gene (data not shown). p53 mutations in the 3 cell lines resulted in an amino acid substitution in the protein, which functionally inactivated the protein (IARC TP53 Mutation Database, www.iarc.fr) [4].

4. Discussion

Melanoma has proven resistant to most available chemotherapies and immunotherapies [1,18]. In the present study we showed that SLUG siRNA increases the susceptibility of three melanoma cell lines to the cytotoxic action of cisplatin and the cytostatic effect of fotemustine, which are currently used for the treatment of melanoma [18]. Drug concentrations were based on *in vivo* peak plasma levels, and the exposure time was chosen on the basis of each drug's plasmatic half-life [10,14,16]. We found that, in the presence of SLUG siRNA, cisplatin induced cell death at a concentration corresponding to half of its peak plasma level, which is not active in SCR siRNA cells. The mechanism by which SLUG siRNA induced an increase in cisplatin-induced apoptosis involves the upregulation of PUMA,

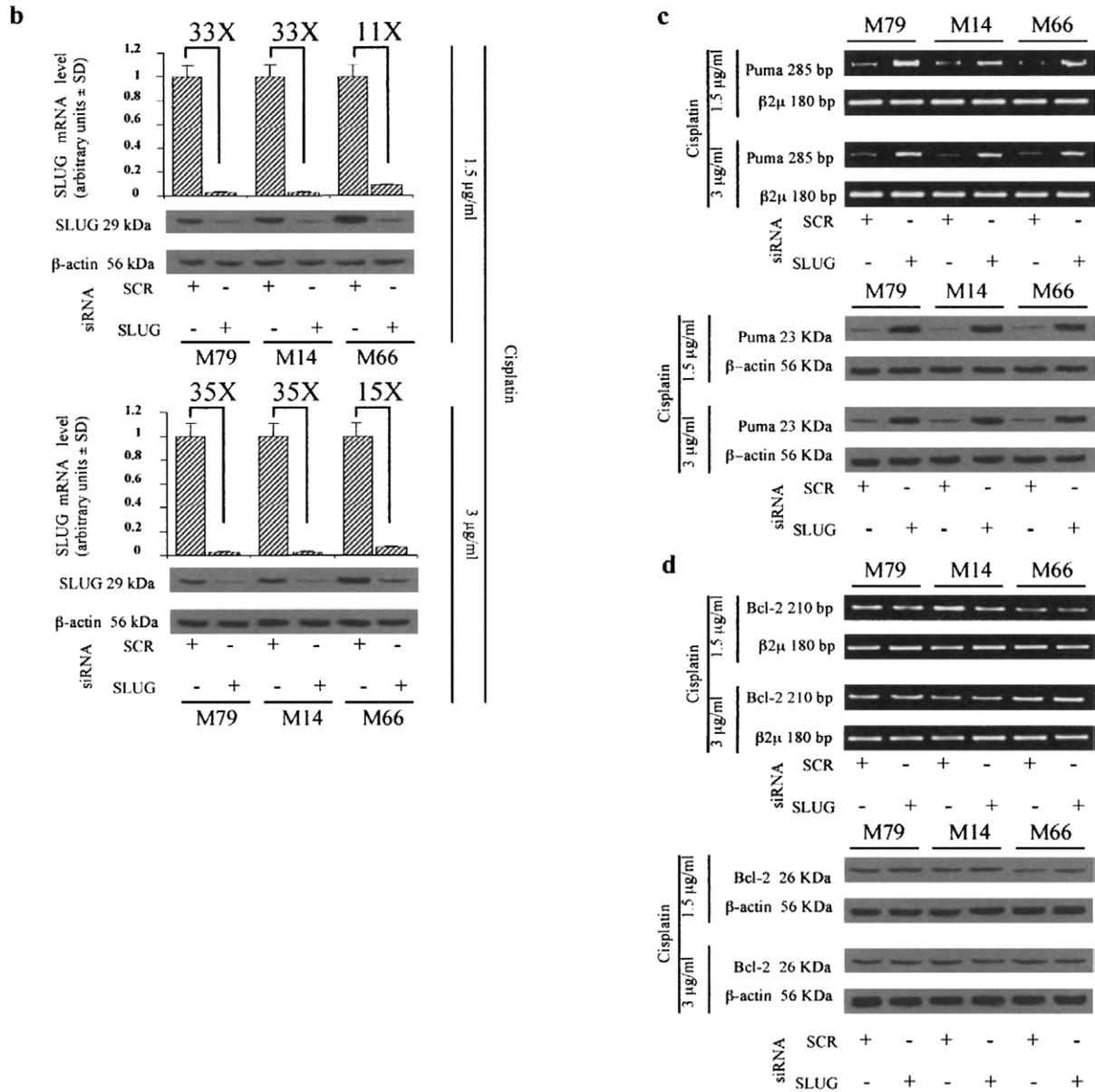


Fig. 2. (b) Real time PCR analysis of SLUG mRNA (arbitrary units; \pm SD, standard deviation of 3 replicates) and WB analysis of SLUG and β -actin. (c) RT-PCR analysis of PUMA and β 2-microglobulin (β 2 μ) mRNA (upper part); WB analysis of PUMA and β -actin (lower part). (d) RT-PCR analysis of Bcl-2 and β 2 μ mRNA (upper part) and WB analysis of Bcl-2 and β -actin (lower part) (+ administered, - not administered SLUG siRNA or SCR siRNA).

a SLUG-regulated gene in other cell systems [19], but not of Bcl-2, whose expression is SLUG-dependent in mouse [3]. PUMA is a p53-regulated gene and, although an interplay exists between SLUG and functional p53 for PUMA modulation [19], we found that all 3 melanoma cell lines carried a mutated p53 gene that inactivated the protein (IARC TP53 Mutation Database) [4]. These data suggest that SLUG downreg-

ulation leads to PUMA-induced, but not p53-induced, cell death. The apoptotic nature of cell death was confirmed by the fact that the administration of 100 μ M of the caspase-9 inhibitor, Z-LEHD-FMK (BD Pharmingen, San Diego, CA, USA), 1 h before exposure to 1.5 μ g/ml of cisplatin and again at the end of treatment elicited a lower degree of cell death than that induced by cisplatin alone (10% vs 55%, respectively, data not

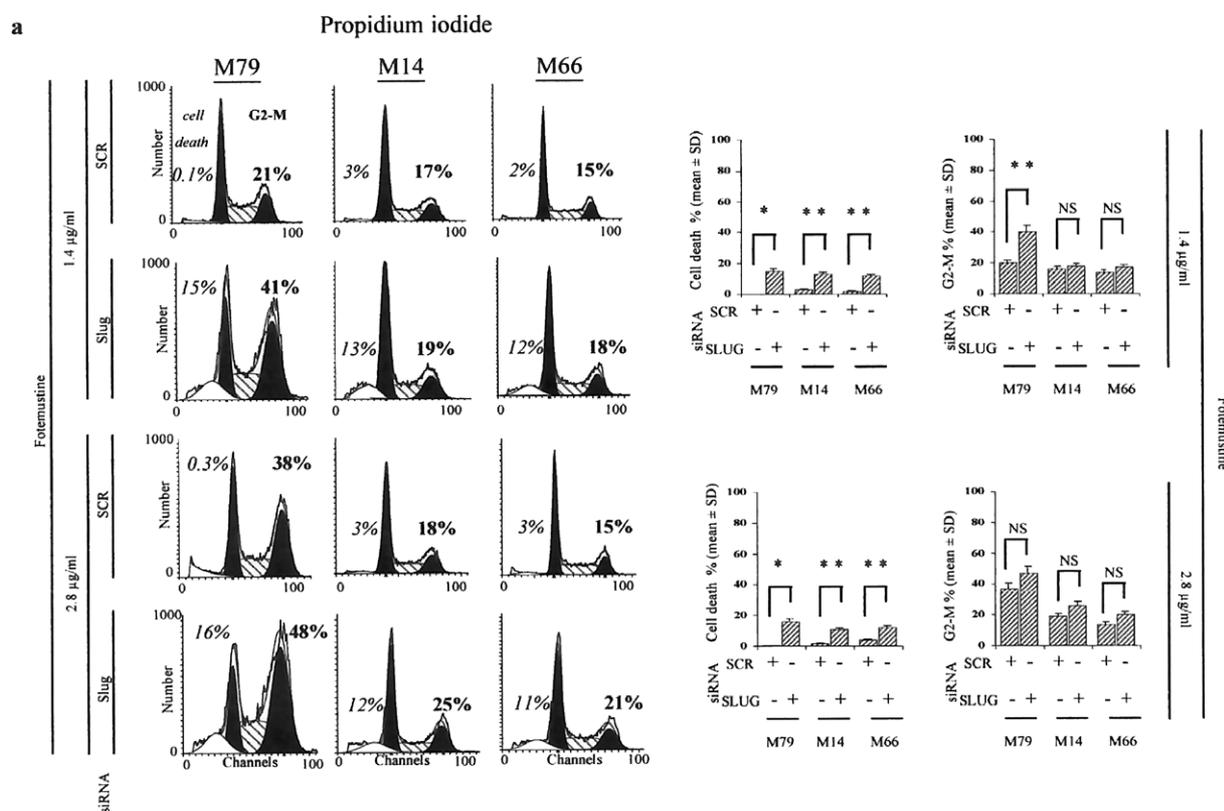


Fig. 3. SLUG modulation of fotemustine-induced cell death and cell cycle arrest in melanoma cells. M14, M66, and M79 exposed to fotemustine (1.4 and 2.8 µg/ml) in the presence of SLUG or SCR siRNA. (a) Cytofluorimetric analysis of hypotonic propidium iodide-stained cells (representative experiments), with percentage of cell death (italics) and G2-M-arrested cells (bold) (left part) and median values for cell death and G2-M arrested cells of each experiment, which was repeated 3 times (right part, * $p < 0.001$, ** $p < 0.01$, NS, not significant, $p > 0.05$).

shown). It was also observed that SLUG siRNA increased the capacity of fotemustine to block cell cycle and induce cell death. Once again, we found that such a phenomenon is accompanied by the upregulation of a p53-dependent gene, p21WAF-1 [6].

Overall, our data suggest that SLUG siRNA promotes drug-induced cell death and cell cycle arrest by modulating putative p53-regulated genes, also in the absence of functional p53. This is of particular importance as p53 mutations are frequently present in melanoma (IARC TP53 Mutation Database), and a gene that is targeted to improve the efficacy of cytotoxic drugs must therefore not depend on a potentially non functional pathway in such cells. This finding opens up interesting prospects for the use of SLUG siRNA as a support molecule in the chemotherapy of melanoma. Our results also suggest that a feasible translational objective for SLUG siRNA would be to lower the active dose of a chemotherapeutic regimen, thus leading to a decrease in the side-effects of cytotoxic drugs.

There is no doubt that anti-apoptotic molecules are promising targets for cancer therapy, including melanoma [11,12]. G3139, an 18-mer phosphorothioate oligodeoxyribonucleotide targeted to the initiation codon region of the Bcl-2 mRNA, has recently been used in phase III clinical trials for melanoma, showing statistically significant differences in overall survival and time to progression in patients who received the molecule in combination with dacarbazine compared to those treated with dacarbazine alone [2]. Although the mechanisms involved in the antitumor activity of G3139 have not yet been fully understood, recent findings suggest that this oligonucleotide induces Bcl-2 independent cell death [2].

With respect to Bcl-2 regulation, we observed that, although Bcl-2 is a SLUG-regulated gene in mouse [3], no downregulation of Bcl-2 protein or mRNA occurred in cells exposed to SLUG siRNA. This finding indicates that Bcl-2 may not be the only target for gene-based therapy of melanoma, and that other cell death regulatory mechanisms could be targeted.

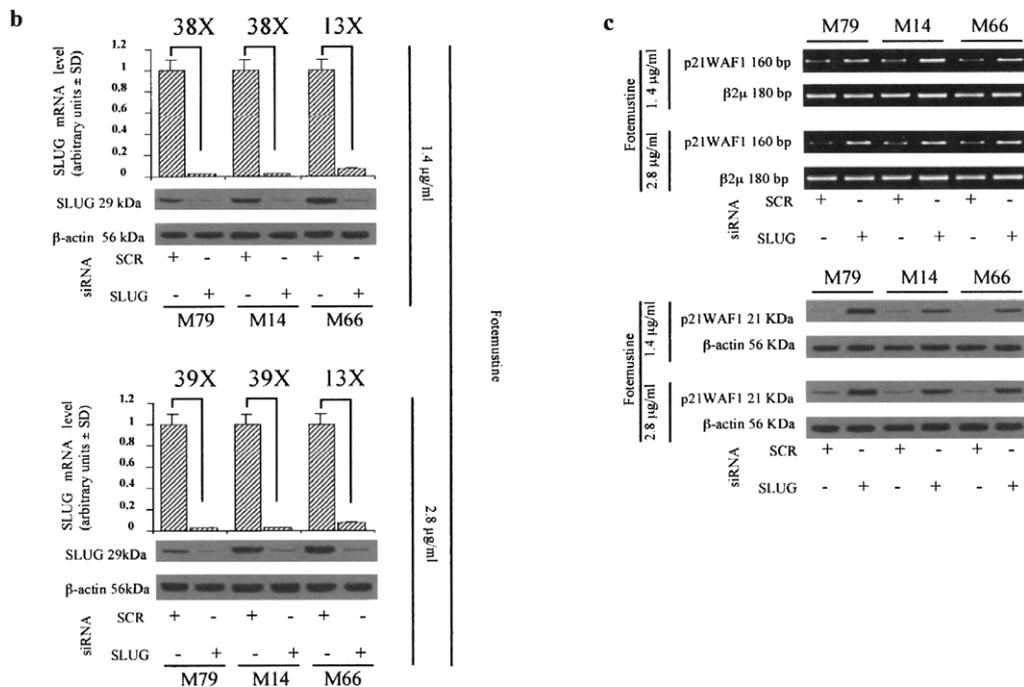


Fig. 3. (b) Real time PCR analysis of SLUG mRNA (arbitrary units; \pm SD standard deviation of 3 replicates) and WB analysis of SLUG and β -actin after fotemustine treatment. (c) RT-PCR analysis of p21WAF1 and β 2 μ mRNA (upper part) and WB analysis of p21WAF1 and β -actin (lower part) (+ administered, - not administered SLUG siRNA or SCR siRNA).

Although SLUG is present in normal melanocytes, it could nonetheless be added to the list of target genes because it is required by cells to acquire a full metastatic phenotype [8]. Further studies would help to clarify the role of SLUG in the regulation of cell death in melanoma and to evaluate its potential use as a molecule to support current therapy regimens.

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