Role of Apollon in Human Melanoma Resistance to Antitumor Agents That Activate the Intrinsic or the Extrinsic Apoptosis Pathways

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Abstract

Purpose: To assess the role of Apollon in melanoma resistance to intrinsic and extrinsic pathways of apoptosis and to identify strategies to reduce its expression.

Experimental Design: Apollon expression was assessed in melanoma cells in vitro and in vivo. Apollon modulation and melanoma apoptosis were evaluated by Western blot and/or flow cytometry in response to cytotoxic drugs, mitogen-activated protein/extracellular signal–regulated kinase (MEK)-, BRAFV600E-, and mTOR-specific inhibitors, TRAIL and anti-HLA class II monoclonal antibodies (mAb). Mitochondrial depolarization, caspase activation, apoptosis assays, and gene expression profiling were used to test effects of Apollon silencing, by siRNA, on melanoma response to antitumor agents.

Results: Apollon was constitutively expressed by melanoma cells, in vitro and in vivo, and at higher levels than in benign melanocytic lesions. Melanoma apoptosis correlated significantly with Apollon protein downmodulation in response to cytotoxic drugs, MEK, or BRAFV600E-specific inhibitors. Combinatorial treatment with MEK and mTOR inhibitors and HLA class II ligation, by a specific mAb, promoted Apollon downmodulation and enhanced melanoma apoptosis. Apollon downmodulation induced by antitumor agents was caspase independent, but proteasome dependent. Knockdown of Apollon, by siRNA, triggered apoptosis and/or significantly enhanced melanoma cell death in response to cytotoxic drugs, MEK- and BRAFV600E-specific inhibitors, and soluble or membrane-bound TRAIL. Apollon silencing promoted mitochondrial depolarization and caspase-2, caspase-8, caspase-9, and caspase-3 activation in response to different antitumor agents and altered the profile of genes modulated by MEK or BRAFV600E-specific inhibitors.

Conclusions: Targeting of Apollon may significantly improve melanoma cell death in response to antitumor agents that trigger the intrinsic or the extrinsic apoptosis pathways. Clin Cancer Res; 18(12); 3316–27. ©2012 AACR.

Introduction

Advanced melanoma is an aggressive disease hardly curable by currently available treatments (1). Different genetic alterations in this tumor fuel the constitutive activation of the mitogen-activated protein kinases and phosphoinositide 3-kinase–AKT–mTOR pathways (2), which, in turn, promote resistance to cell death regulated by the intrinsic and extrinsic pathways of apoptosis (3, 4). Targeted therapy aimed at inhibiting one of these intracellular signaling pathways (5) has indeed shown highly significant clinical results in metastatic patients, as shown by a phase III trial with a BRAFV600E-specific inhibitor (6). Interestingly BRAFV600E-selective inhibitors, as PLX4720, may contribute to promote/rescue melanoma susceptibility to apoptosis (7, 8).

However, melanomas can show primary (9, 10) or acquired resistance to BRAF inhibitors (see ref. 11 for review), suggesting that targeting of a single signaling pathway may not be sufficient in all instances for blocking...
Translational Relevance

The identification of key regulators of apoptosis in advanced melanoma may provide new therapeutic targets to improve efficacy of antitumor agents that activate the intrinsic or the extrinsic apoptosis pathways. We found that melanoma susceptibility to apoptosis by cytotoxic drugs and target-specific inhibitors correlated with downmodulation of Apollon protein. Combinatorial treatment with mitogen-activated protein/extracellular signal–regulated kinase (MEK) + mTOR inhibitors, or cell surface HLA class II ligation promoted Apollon downmodulation and enhanced melanoma apoptosis. Targeting of Apollon, by siRNA, enhanced significantly caspase-dependent melanoma apoptosis in response to cytotoxic drugs, MEK, and BRAF<sup>V600E</sup> inhibitors and soluble or membrane-bound TRAIL. The results suggest that Apollon is a potentially relevant therapeutic target in melanoma and that effective strategies aimed at downmodulating this inhibitor of apoptosis protein may improve the efficacy of currently available treatments.

melanoma growth and rescuing susceptibility to apoptosis. To achieve such result, potentially effective strategies include the combinatorial usage of inhibitors of different signaling pathways (12) or the identification and targeting of key regulators of melanoma resistance to apoptosis (13). The latter strategy may improve the antitumor efficacy of currently available treatments, including targeted therapy or even conventional chemotherapy (see ref. 1 for review). To this end, in this study we investigated the expression in melanoma cells, and role in apoptosis, of the inhibitor of apoptosis protein (IAP) Apollon/BIRC6 (14–16). Apollon, a 528-kDa membrane-associated IAP, inhibits the mitochondrial pathway of apoptosis by promoting ubiquitination and degradation of the proapoptotic molecule Smac/DIABLO and of caspase-9 (14, 15). In addition, Apollon depletion promotes p53 stabilization leading to caspase-2- and caspase-3–dependent apoptosis (16, 17). Although the expression of several IAPs in melanoma and their role in chemoresistance have been previously investigated (18–20), the potential role of Apollon in regulating melanoma cell apoptosis activated by the intrinsic and/or extrinsic pathways of programmed cell death needs to be clarified.

The results indicated that Apollon plays a relevant role in melanoma resistance to apoptosis by antitumor agents that trigger the intrinsic or the extrinsic apoptosis pathways. Furthermore, the results suggest that this IAP may be evaluated as a potentially relevant therapeutic target, to improve the efficacy of currently available pharmacologic treatments of melanoma.

Materials and Methods

Melanoma cell lines and CD34<sup>+</sup> cells

Melanoma cell lines were established as described (21) from surgical specimens of patients admitted to Fondazione IRCCS Istituto Nazionale dei Tumori, Milan. All the lesions were histologically confirmed to be cutaneous malignant melanomas. Molecular and biologic characterization of the cell lines and methods for identification of mutations in BRAF, NRAS, and p53 genes have been reported previously (21–24). All cell lines were maintained as described (21). CD34<sup>+</sup> cells were enriched as described (25) from leukaphereses of consenting patients undergoing peripheral blood stem cell mobilization and harvest, in view of autologous transplantation. Written informed consent was obtained from patients.

Antibodies

The following antibodies were used for flow cytometry and/or Western blot analysis: mouse anti-Apollon, anti-c-IAP2, anti-XIAP, and anti-Bax (BD Biosciences); goat anti-c-IAP1 (R&D Systems); mouse anti-α-tubulin (Calbiochem, Merck KGaA); anti-β-actin (Abcam Inc.). The HLA class II–specific monoclonal antibody (mAb) LGII-612.14 has been described elsewhere (26). The mAb H2-27.F5 was generated from a BALB/c mouse immunized with human B lymphoid cells WIL2. The HLA class II antigen specificity of mAb H2-27.F5 is indicated by its selective reactivity with HLA class II antigen bearing cells and by the characteristic electrophoretic profile of the molecules immunoprecipitated from cultured B lymphoid cells. H2-27.F5 mAb and the HLA class I antigen-specific mAb EP15.25.39 (27, 28) were purified as described (28).

Flow cytometry assays

Expression of Apollon, c-IAP1, c-IAP2, and XIAP was determined by intracytoplasmic flow cytometry in saponin-permeabilized cells as described (21). Samples were acquired by a fluorescence-activated cell sorting (FACS)-Calibur cytometer (Becton Dickinson). Values were expressed as mean fluorescence intensity (MFI) after subtracting the MFI of cells stained only with the secondary antibody. Mitochondrial membrane depolarization was assessed by using the fluorescent probe tetramethylrhodamine ethyl ester (TMRE; Invitrogen Life Technologies). Cells were washed, incubated with 50 nmol/L TMRE at 37°C for 15 minutes in the dark and then analyzed by a FACS-Calibur cytometer (Becton Dickinson).

Western blot analysis

SDS-PAGE was carried out using 30 μg of protein samples on 3% to 8% NuPAGE Tris-Acetate (for Apollon) or 4% to 12% NuPAGE Bis-Tris (for c-IAP1, c-IAP2, XIAP, and Bax) polyacrylamide gels (Invitrogen). Development was carried out by the chemiluminescence method with ECL Plus Western Blotting Detection System (GE Healthcare).

Immunohistochemistry

Immunohistochemistry (IHC) was carried out with formalin-fixed, paraffin-embedded tissues as described (21), by staining with mAbs to Apollon (Abcam) or to gp100 (HMB45; DakoCytomation). Sections were counterstained with hematoxylin followed by dehydration and mounting.
Images were acquired with an Axiovert 100 microscope (Carl Zeiss) equipped with a digital camera (AxioCam MrC5; Zeiss).

**Treatment of melanoma cells with drugs, TRAIL, or HLA class II mAbs and apoptosis assays**

Cells in log phase of growth were treated for 24 to 72 hours with the following: camptothecin (Aventis Pharma) at 50 μmol/L, celecoxib (Pfizer) at 50 μmol/L, temozolomide (Sigma-Aldrich) at 20 μmol/L, fotemustine (Muphoran, ItalFarmaco) at 150 to 300 μmol/L, mTOR inhibitor rapamycin (Sigma-Aldrich) at 10 nmol/L, mitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibitor PD0325901 (Cayman Chemicals) at 5 to 10 nmol/L, or BRAFV600E-specific inhibitor PLX4720 (Selleck Chemicals) at 500 nmol/L. Melanoma cells were treated for 24 hours with 10 ng/mL of recombinant sTRAIL (KillerTRAIL; Alexis Biochemicals, Enzo Life Sciences). Cells were treated with HLA class II antigen-specific or HLA class I antigen-specific mAbs (25 μg/mL). Quantification of apoptotic cells was carried out by flow cytometry by staining with fluorescein isothiocyanate (FITC)- or APC-conjugated Annexin V (BD Pharmingen) and with propidium iodide (PI; BD Biosciences) as described (22). In some experiments melanoma cells, treated with antisense agents for 48 hours, were also treated with lactacystin (Sigma-Aldrich) at 10 μmol/L during the last 24 hours. Samples were acquired by a FACS-Calibur cytofluorimeter (Becton Dickinson).

**Silencing of Apollon by siRNA**

Cells were transfected with 2 different Apollon-specific siRNAs: siRNA#2 (sequence AGAAAUIGACClUIGA-GUUA; Eurofins MWG Operon, Ebersberg, Germany; ref. 17) or with siRNA#4 (Stealth RNAi siRNA, sequence GGCCAUIGClUIGAAILUIGGCUIUA; Invitrogen) and corresponding negative control siRNAs (siRNA#1 and siRNA#3, respectively Eurofins MWG and Invitrogen) according to Lipofectamine RNAiMAX guidelines (Invitrogen) to reach a final siRNA concentration of 75 nmol/L.

**Adenoviral transduction of CD34+ cells and coculture with melanoma cells**

CD34+ cells were transduced with a replication-deficient adenovirus encoding the human TRAIL gene (Ad-TRAIL; Center for Cell & Gene Therapy, Houston, TX) as described (25). Transduction efficiency was evaluated by flow cytometry by staining cells with CD34-FITC mAb, CD45-PerCP mAb (Becton Dickinson), and TRAIL-PE mAb (CD253; BD Pharmingen). Melanoma cells were stained with 2 μmol/L 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Invitrogen) as described (29). TRAIL-expressing CD34+ cells (or untransduced CD34+ cells as control) were cocultured for 24 hours at 1:1 ratio with melanoma cells labeled with CFSE and transected with Apollon- or control-siRNA. After coculture, cells were stained with the far-red fluorescent DNA dye DRAQ7 (Biosattus Limited) that stains nuclei only in dead cells. By flow cytometry analysis of melanoma-CD34+ coculture experiments, live (DRAQ7−) and dead (DRAQ7+) melanoma cells were identified by gating on CFSE+ melanoma cells.

**Detection of caspase enzymatic activity and caspase inhibitors**

Enzymatic activity of caspase-2, caspase-3, caspase-8, and caspase-9 on 50 μg per well of cell lysate was carried out by using Caspase-2/ICH-1, Apopcyto/Caspase-3, Apopcyto/Caspase-8, and Apopcyto/Caspase-9 Fluorometric Assay Kits (Medical and Biological Laboratories) according to manufacturer’s instructions by TECAN Infinite M1000 (Tecan Group Ltd.). Results were expressed as relative fluorescence units. Melanoma cells were treated with general caspase inhibitor z-VAD-fmk or control z-FA-fmk (BD Pharmingen) at 5 μmol/L, 5 hours after transfection with Apollon siRNA and 1 hour before treatment with drugs. Caspase inhibitor or control at 5 μmol/L were added to cultures every 24 hours.

**Apoptosis antibody array**

The Human Apoptosis Array Kit (R&D Systems) was used according to manufacturer’s instructions. Signals on membranes were detected by chemiluminescence and quantitated by densitometric analysis with Quantity One software (BioRad Laboratories Inc.). After background subtraction, protein expression values were expressed as percentage of the mean of the relative positive controls.

**Genome-wide expression profiling**

Gene expression profiles of melanoma cells transfected with control-siRNA or Apollon siRNA and treated with PD0325901 (5 nmol/L) or PLX4720 (500 nmol/L) were assessed and analyzed as described in detail in Supplementary Methods. Expression profiles were deposited in NCBI’s Gene Expression Omnibus (GEO) with accession number GSE34686.

**Results**

**Constitutive Apollon expression in human melanoma cells**

By flow cytometry, Apollon (as well as c-IAP1, c-IAP2, and XIAP) was found expressed in 34 human melanoma cell lines (vertical growth phase primary tumors: n = 8; lymph node metastases: n = 25, subcutaneous metastases n = 1), although with a wide range of fluorescence intensity (Fig. 1A). Western blot analysis in cell lines representative of different genetic backgrounds (in terms of BRAF or NRAS oncogenic mutations) confirmed expression of Apollon and of other IAPs (Fig. 1B).

The level of expression of Apollon and other IAPs was not significantly associated with the presence of mutated BRAF or NRAS oncogenes in the cell lines (Fig. 1C, P > 0.05 for all 4 IAPs by ANOVA followed by Bonferroni posttest), nor with the p53 status (data not shown). By IHC in surgical samples, Apollon was found expressed in melanoma cells in vivo, although with variable levels of staining intensity in different tumors (Fig. 1D and Supplementary Fig. S1), or even within the same lesion (see insets Fig. 1D and Supplementary Fig. S1).
Supplementary Fig. S1). Apollon expression in benign melanocytic lesions was weaker than in melanomas (Fig. 1E).

**Melanoma apoptosis correlates with downmodulation of Apollon protein expression in response to different antitumor agents**

Time course experiments in 2 BRAF<sup>V600E</sup>-mutant melanoma cell lines (Me14464 and Me23682) showed that apoptosis was associated with Apollon downmodulation in response to the MEK inhibitor PD0325901, the BRAF<sup>V600E</sup>-specific inhibitor PLX4720, or the cytotoxic drug fotemustine (Fig. 2A). In a larger panel of cell lines (Fig. 2B for correlation analysis and Fig. 2C for representative results), we found a significant correlation between extent of Apollon protein modulation and extent of cell death by PD0325901 (n = 10), fotemustine (n = 10), and PLX4720 (n = 7, BRAF<sup>V600E</sup>-mutant tumors). In the same tumors, no correlation was found between apoptosis by each drug and modulation of c-IAP2 or XIAP, whereas c-IAP1 modulation showed a significant correlation with apoptosis only by PLX4720 and fotemustine, but not by PD0325901 (Supplementary Table S1).

In contrast, other BRAF<sup>V600E</sup>-mutant melanoma cell lines that were resistant to apoptosis by these agents did not show Apollon downmodulation (Fig. 2D for representative results). Apollon baseline levels of expression and susceptibility to PD0325901, PLX4720, or fotemustine showed no significant correlation, either in a panel of 34 cell lines or in tumor subsets defined by BRAF or NRAS mutational status (Supplementary Fig. S2).

Overall, these results indicated that melanoma susceptibility to apoptosis by different antitumor agents correlates with downmodulation of Apollon protein.

**Treatment with MEK plus mTOR inhibitors, and cell surface HLA class II ligation promote Apollon downmodulation and enhance melanoma apoptosis**

We tested whether combinatorial treatment with inhibitors targeting different signaling pathways was associated with Apollon downmodulation and enhanced melanoma cell death, compared with single treatments. In time course
experiments, in Me15392 cells, the combination of a MEK inhibitor (PD0325901) and an mTOR inhibitor (rapamycin) promoted Apollon downmodulation (Fig. 3A and B, left hand blots), and this was associated with enhanced cell death at 48 and 72 hours, compared with single drugs (Fig. 3A).

Enhanced Apollon downmodulation (Fig. 3C) was specifically promoted by PD0325901 + rapamycin in cell lines (Me15392 and Me14464) that also showed enhanced apoptosis by this combinatorial treatment (Fig. 3D), but not in other tumors (Me4023) that were resistant to apoptosis by such association of inhibitors (Fig. 3B–D). In a panel of 11 melanoma cell lines, enhanced Apollon downmodulation correlated significantly with enhanced cell death by combined PD0325901 + rapamycin treatment compared with treatment with PD0325901 only (Fig. 3E).

We then tested whether ligation of cell surface HLA class II molecules, a treatment that can promote apoptosis in hematologic malignancies (30, 31), could impact on expression of Apollon and whether this promoted melanoma apoptosis by antitumor agents. Preincubation of HLA class II+ melanoma cells (Me32669, BRAF<sup>Wild</sup> p53<sup>Wild</sup> tumor) with the HLA class II–specific mAb H2–27. F5 mAb decreased levels of Apollon and, to a lesser extent, of c-IAP1 (Supplementary Fig. S3A). This rescued apoptosis in response to PD0325901 and to fotemustine (Supplementary Fig. S3B).

Melanoma treatment with the HLA class I antigen-specific mAb TP25.99 (27) did not affect apoptosis by these drugs (Supplementary Fig. S3B). Similar results were obtained by treating HLA class II− melanoma cells with a different anti-HLA class II–specific mAb LGII–612.14 (ref. 26, data not shown). Taken together, these results suggested that combinatorial treatment with target-specific inhibitors and HLA class II ligation promote downmodulation of Apollon and enhance melanoma apoptosis.

**Apollon downmodulation, after melanoma treatment with antitumor agents is not caspase dependent**

We asked whether Apollon downmodulation, in melanoma cells treated with antitumor agents, was a consequence of caspase activation, as caspases can degrade Apollon (15). In cell lines, as Me23682, susceptible to apoptosis...
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by drugs as PLX4720 and fotemustine, the pan-caspase inhibitor z-VAD-fmk could significantly inhibit apoptosis, but did not prevent Apollon downregulation (Fig. 4A).

Apollon can be degraded even by the ubiquitin–proteasome pathway (15, 32), therefore, we tested whether the proteasome inhibitor lactacystin could inhibit Apollon downmodulation. Moreover, melanoma cells can be resistant to cell death by proteasome inhibitors (33). This allowed us to test the effect of lactacystin on both Apollon downmodulation and apoptosis by different antitumor agents. In response to PD0325901 or fotemustine (Fig. 4B), or PD0325901 + rapamycin (Fig. 4C), Apollon downmodulation was significantly inhibited by lactacystin treatment, associated with reduced apoptosis (Fig. 4B and C). These results suggested that Apollon downmodulation, promoted by different antitumor agents, is caspase independent and proteasome dependent.

**Apollon silencing enhances melanoma cell death in response to cytotoxic drugs, to MEK and BRAFV600E inhibitors and to soluble or membrane-bound TRAIL.**

To directly assess the role of Apollon in melanoma apoptosis, we carried out Apollon silencing experiments by siRNA. Two siRNAs (#2 and #4) were evaluated in the initial studies (Fig. 5A), and siRNA#4 was selected for subsequent studies. Apollon protein expression was inhibited at 24 to 96 hours by the specific siRNA, as detected by Western blot and flow cytometry (Fig. 5A and B), whereas c-IAP1 and c-IAP2 were not affected (Fig. 5A). Apollon silencing in melanoma cells markedly increased the extent of apoptosis in response to drugs that activate the intrinsic pathway of apoptosis (34, 35), including temozolomide and fotemustine (Fig. 5C), camptothecin and celecoxib (Supplementary Fig. S4), and to inhibitors as PD0325901, mTOR = rapamycin. Statistical analysis in A, C, and D by ANOVA followed by Bonferroni posttest. **P < 0.05; ***P < 0.01; ****P < 0.001. Data in A, C, and D are means of 3 independent experiments.

![Figure 3. Cotargeting of MEK and mTOR pathways promotes Apollon downmodulation and improves the apoptotic response of melanoma cells. A. time course analysis of apoptosis (top graphs) and Apollon downmodulation (bottom graphs) in a BRAFV600E p53wt melanoma line (Me15392) after treatment with PD0325901 (10 nmol/L), or rapamycin (10 nmol/L), or both drugs. Results expressed as in Fig. 2A. B. Western blot for Apollon expression in 2 BRAFV600E p53wt melanoma cell lines treated for 48 hours as in panel A. C, modulation of c-IAP1, c-IAP2, and Apollon expression in 3 BRAFV600E p53wt melanoma cell lines, after treatment as in panel A. D, apoptosis, of cell lines shown in C, after treatment as in panel A. for 72 hours. E, correlation between extent of apoptosis and Apollon modulation by cotargeting MEK and mTOR pathways versus MEK-targeting alone, in 12 cell lines treated for 72 hours as in panel A. MEKi = PD0325901; mTOR = rapamycin.](https://www.aacjournals.org/article-pdf/10.1158/1078-0432.CCR-11-2232/4012032/1078-0432.CCR-11-2232.pdf)
Figure 4. Apollon downmodulation, by antitumor agents or combinatorial treatments with signaling pathway inhibitors, is not caspase dependent. A, apoptosis (% of live cells, top graphs) and Apollon expression (MFI, bottom graphs) of a BRAF\textsuperscript{V600E}, p53\textsuperscript{wt} melanoma cell line (Me23682) treated for 48 and 72 hours with PLX4720 (500 nmol/L) or fotemustine (300 \textmu mol/L) in the presence or not of the pan-caspase inhibitor z-VAD-fmk or the control z-FA-fmk (both at 5 \mu mol/L). B and C, apollon expression (MFI) and apoptosis (% of live cells) at 48 hours in Me23682 cells treated with PD0325901 or fotemustine (B), or with PD0325901 plus rapamycin (C) in the presence or not of lactacystin (10 \textmu mol/L, last 24 hours). Statistical analysis and annotation of \( P \) values as in Fig. 3. Bars in A, B, C: means of 3 independent experiments.

\* \( P < 0.05; \) ** \( P < 0.01; \)** \( P < 0.001; \)** \( P < 0.0001.\)
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Table and Figure 5. Apollon silencing improves melanoma cell death in response to agents that trigger the intrinsic or extrinsic apoptosis pathways. Western blot analysis of Apollon, c-IAP2, and c-IAP1 expressions 48 and 72 hours after transfection of Me23682 cells with 2 different pairs of control siRNA (siRNA#1 and #3) and Apollon-specific siRNA (siRNA#2 and #4). A, Apollon protein expression in Me23682 cells by flow cytometry at 24, 48, 72, and 96 hours after transfection with control siRNA#4 (continuous line) or Apollon-specific siRNA#4 (dotted line). Filled histogram, cells stained with secondary antibody only. B, melanoma apoptosis in Me23682 cells transfected with control- or Apollon-specific siRNA and then treated with fotemustine (150 μmol/L), or temozolomide (150 μmol/L), or PD0325901 (500 nmol/L). D, melanoma apoptosis in cell lines transfected with control- or Apollon-siRNA and then treated with fotemustine (150 μmol/L), or temozolomide (150 μmol/L), or PD0325901 (500 nmol/L). E, extent of cell death (% DRAQ7+ cells) in CFSE-labeled Me23682 cells transfected with control- or Apollon-siRNA and then treated for 24 hours with 10 ng/mL of soluble TRAIL (sTRAIL), or untransduced CD34-CD34+ cells (CD34), or TRAIL-expressing CD34+ cells (CD34-TRAIL). Bars in C and E: means of 3 independent experiments ± SD. Statistical analysis by Student paired t test. E, extent of cell death (% DRAQ7+ cells in CFSE-labeled Me23682 cells transfected with control- or Apollon-siRNA and then treated for 24 hours with 10 ng/mL of soluble TRAIL (sTRAIL), or untransduced CD34-CD34+ cells (CD34), or TRAIL-expressing CD34+ cells (CD34-TRAIL). Bars in C and E: means of 3 independent experiments ± SD. Statistical analysis by Student paired t test. E, extent of cell death (% DRAQ7+ cells in CFSE-labeled Me23682 cells transfected with control- or Apollon-siRNA and then treated for 24 hours with 10 ng/mL of soluble TRAIL (sTRAIL), or untransduced CD34-CD34+ cells (CD34), or TRAIL-expressing CD34+ cells (CD34-TRAIL). Bars in C and E: means of 3 independent experiments ± SD. Statistical analysis by Student paired t test. E, extent of cell death (% DRAQ7+ cells in CFSE-labeled Me23682 cells transfected with control- or Apollon-siRNA and then treated for 24 hours with 10 ng/mL of soluble TRAIL (sTRAIL), or untransduced CD34-CD34+ cells (CD34), or TRAIL-expressing CD34+ cells (CD34-TRAIL). Bars in C and E: means of 3 independent experiments ± SD. Statistical analysis by Student paired t test.

Apollon knockdown could also enhance melanoma apoptosis activated by the death receptor-dependent extrinsic pathway. In fact, Apollon silencing led to increased melanoma cell death in response to soluble TRAIL (sTRAIL, Fig. 5E) and even to membrane-bound TRAIL (Fig. 5E, CD34-TRAIL, i.e., TRAIL expressed by CD34- cells transduced with a replication-deficient adenovirus encoding the human TRAIL gene, as shown in Supplementary Fig. S5). Taken together, these results indicated that inhibition of Apollon rescues apoptosis in melanoma cells in response to antitu-
Apollon silencing in melanoma cells promotes mitochondrial depolarization and caspases activation. A, antibody array analysis of 35 apoptosis-related proteins at 48 hours in Me23682 cells treated as in Fig. 5C. Black dots, significantly modulated proteins (by Student t test). B, time course analysis of mitochondrial depolarization (by TMRE staining) after Apollon silencing combined or not with PD0325901 treatment in Me23682 cells. C, caspase-2, caspase-9, caspase-8 and caspase-3 catalytic activity in Me23682 cells 24 hours after Apollon silencing combined or not with PD0325901 treatment as in Fig. 5C. D, TMRE assay after Apollon silencing of Me23682 cells treated or not with z-VAD-fmk pan-caspase inhibitor. E, effect of z-VAD-fmk or of the control z-FA-fmk on apoptosis (72 hours), after transfection of Me23682 cells with control- or Apollon-siRNA, associated with PD0325901 treatment as in Fig. 5C. Bars in B-E, means of 3 independent experiments ± SD. Statistical analysis and annotation of P values as in Fig. 3.

These results suggested that Apollon silencing may promote the early steps of the mitochondrial pathway of apoptosis. In agreement with this hypothesis, silencing of Apollon induced a loss of mitochondrial potential in melanoma cells (at 48–72 hours), and association with PD0325901 treatment further enhanced this effect (Fig. 6B).

Apollon knockdown increased activation of initiator caspases of the intrinsic (caspase-2 and caspase-9) and of the extrinsic (caspase-8) pathways, as well as of the effector caspase-3, in response to PD0325901 in cell lines bearing wt p53 (Me23682, Fig. 6C) or even mutant p53 (Supplementary Fig. S7), as well as in response to cytotoxic drugs as camptothecin or celecoxib (Supplementary Fig. S8). As expected, based on apoptosis assays (already shown in Fig. 5C) in tumors as Me23682 Apollon silencing also induced significant activation of caspase-2, caspase-8, and caspase-3, without additional drug treatment (Fig. 6C).

Mitochondria depolarization, an early step in the apoptosis cascade induced by Apollon silencing, was not affected by the pan-caspase inhibitor z-VAD-fmk (Fig. 6D). Instead, the same inhibitor, but not the negative control z-FA-fmk, significantly reduced cell death induced by Apollon silencing and by its combination with PD0325901 (Fig. 6E). Overall, these data indicated that Apollon silencing increases expression of proapoptotic Bcl-2 family members, promotes mitochondrial depolarization, and activation of caspase-dependent apoptosis.

**Apollon silencing alters the profile of genes modulated by antitumor agents**

To test the effects of Apollon silencing on genes modulated by antitumor agents, we used whole genome microarray analysis of melanoma cells (Me23682) bearing...
expressed versus silenced Apollon and treated with PD0325901 or PLX4720. Two major clusters of significantly upregulated and downregulated genes were associated with inhibitor treatment, and not with Apollon silencing (Supplementary Fig. S9A). Almost all of the most modulated genes by either inhibitor (i.e., those with a fold change ≥2) were not significantly affected by concurrent Apollon silencing, as indicated by correlation analysis (Supplementary Fig. S9). However, pairwise significance analysis of modulation of each of these genes showed a few notable exceptions (gene symbols in bold, color in Supplementary Fig. S9B): the CCND1 gene, encoding the cell-cycle regulator cyclin D1, had a fold change of −5.0 by PD0325901, but −7.0 by PD0325901 plus Apollon silencing (results confirmed by reverse transcriptase PCR, data not shown), suggesting that Apollon knockdown may enhance the antiproliferative effects of this MEK inhibitor. Other genes identified by pairwise significance analysis showed less relevant changes when inhibitor treatment was associated to Apollon knockdown: VASN (vasorin, a TGFβ-binding protein), PLA2GT (phospholipase A2), interleukin-8, and JUN (Supplementary Fig. S9B). We then classified, by Venn diagrams, all genes with significant modulation by any of the treatments, irrespective of fold change (Supplementary Fig. S10A and Supplementary Fig. S11A for PD0325901 and PLX4720, respectively). By Ingenuity Pathway Analysis (IPA) on genes defined by these Venn diagrams, we found that Apollon silencing did alter the profiles of genes modulated by PD0325901 and PLX4720 and belonging to several functional classes (listed in Supplementary Fig. S10B and S11B). This was also shown by IPA of genes modulated only by the combination of drug treatment and Apollon silencing (Supplementary Fig. S10 C and D and S11 C and D). Detailed analysis of the latter set of genes showed that the majority had a fold change less than 1.5, but a minority showed a fold change between 1.5 and 2 (Supplementary Fig. S12). Many of the genes with a fold change more than 1.6 had direct or indirect functions related to either apoptosis and/or regulation of proliferation and cell-cycle control (gene symbols in bold, underlined in Supplementary Fig. S12). Taken together, these results suggested that Apollon knockdown may contribute to the antitumor effects of inhibitors as PD0325901 and PLX4720, even by altering the overall profile of genes modulated by such antitumor agents.

Discussion

This study indicates that Apollon is constitutively expressed in human melanoma cells in vitro and in vivo, and it is a relevant factor in melanoma cell resistance to cell death activated by agents that trigger the intrinsic and extrinsic pathways of apoptosis. We found a significant correlation between extent of melanoma cell death and extent of Apollon downmodulation induced by cytotoxic agents, signaling pathway inhibitors and HLA class II ligation through a specific mAb. These findings suggest that melanoma apoptosis in response to antitumor agents is critically dependent on downmodulation of this IAP. Furthermore, Apollon downmodulation, observed in melanomas that are susceptible to the antitumor agents used in this study, may be a consequence of apoptosis rather than an initiator of programmed cell death.

Knockdown of Apollon increased expression of proapoptotic Bel-2 family members, enhanced activation of caspase-2, an initiator caspase of the mitochondrial/intrinsic pathway, and even of caspase-8, an initiator caspase of the extrinsic pathway (37). In agreement, Apollon silencing improved melanoma cell death in response not only to cytotoxic drugs and pathway inhibitors that trigger the intrinsic pathway of apoptosis (34–35) but also to soluble or membrane bound TRAIL that activate the extrinsic apoptotic cascade. Melanoma cell death, associated with Apollon downmodulation or enhanced upon Apollon silencing, was caspase dependent, as it was significantly inhibited by z-VAD-fmk. However, the same pan-caspase inhibitor did not prevent Apollon downmodulation promoted by antitumor agents. These results suggested that Apollon degradation, in melanoma cells undergoing apoptosis, was not a consequence of caspase activation. Results of experiments with the proteasome inhibitor lactacystin were consistent with proteasome-dependent degradation of Apollon (15, 30).

Interestingly, we also found that Apollon silencing, without any additional treatment, could activate caspases and trigger apoptosis in some melanoma cell lines. In agreement with findings in mouse embryos (16) and, more recently, in human breast cancer cells (17), these results suggest that Apollon may have a role even as a melanoma survival factor.

Knockdown of Apollon, by siRNA, could rescue apoptosis, by different antitumor agents (such as cytotoxic drugs, MEK-, and BRAFV600E-specific inhibitors) even in melanoma cell lines with mutant or null p53. Interestingly, reduced cell growth after Apollon silencing has been found even in a breast cancer cell line bearing mutant p53 (17). These results are in contrast with the study by Ren and colleagues (16), in which cell death promoted by Apollon ablation was found to require a functional p53 and suggests that Apollon inactivation/depletion may promote apoptosis even by p53-independent mechanisms.

IAP antagonists are currently in preclinical development and early clinical testing (see ref. 38 for review). Several of these are SMAC mimetics developed to target the BIR3 and/or the BIR2 domains of XIAP, c-IAP1, and c-IAP2, and have shown promising preclinical results even in melanoma (39, 40). Although Apollon binds SMAC (14) and its only BIR domain belongs to the type II class, as the BIR2 domain of XIAP, c-IAP1, and c-IAP2 (41), it is not clear whether available SMAC mimetics also inhibit Apollon. However, other recently developed IAP antagonists are antisense oligonucleotides targeting XIAP or survivin (see ref.42 for review), and a similar approach could lead to develop Apollon-specific antagonists.

Taken together, the results of this study suggest that Apollon should be investigated further as a potential new drug target, for example, for the development of specific inhibitors.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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