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

Elena Tassi1, Marina Zanon1, Claudia Vegetti1, Alessandra Molla1, Ilaria Bersani1, Valentina Perotti1, Marzia Pennati1, Nadia Zaffaroni2, Michele Milella1, Soldano Ferrone2, Carmelo Carlo-Stella3, Alessandro M. Gianni3, Roberta Mortarini1, and Andrea Anichini1

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...
Targeting Apollon to Rescue Melanoma Apoptosis

**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 BRAFV600E 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.

**Materials and Methods**

**Melanoma cell lines and CD34\(^+\) 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\(^+\) 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 WI.2. 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 TP25.99 (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 cytometerfluorimeter (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 cytometerfluorimeter (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.

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.
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 transfected 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 antitumor 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 cytometer (Becton Dickinson).

**Silencing of Apollon by siRNA**

Cells were transfected with 2 different Apollon-specific siRNAs: siRNA#2 (sequence AGAAAAUCACCUUAGAUUGAA; 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-FTTC mAb, CD45-PerCP mAb (Becton Dickinson), and TRAIL-PE mAb (CD253; BD Pharmingen). Melanoma cells were stained with 2 μmol/mL 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 transfected with Apollon- or control-siRNA. After coculture, cells were stained with the far-red fluorescent DNA dye DRAQ7 (Biosstatus 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 were carried out by using Caspase-2/ICH-1, Apocytoc/Caspase-3, Apocytoc/Caspase-8, and Apocytoc/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 oncogene 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
Figure 2. Melanoma apoptosis correlates with Apollon downmodulation. A, time course analysis of Apollon expression by flow cytometry and apoptosis in Me14464 (top) and Me23682 (bottom) after treatment with PD0325901 (10 nmol/L), PLX4720 (500 nmol/L), or fotemustine (500 μmol/L). Results expressed as ratio of values (MFI and Annexin V/PI) in treated cells to untreated cells. B, correlation between Apollon modulation (48 hours) and apoptosis (72 hours) in 10 melanoma cell lines treated with PD0325901 or fotemustine, and in 7 BRAF^{V600E} melanoma cell lines treated with PLX4720 as in A. Results expressed as in A. C and D, apoptosis by Annexin V/PI assay (top graphs, 72 hours) and Apollon expression (bottom graphs, 48 hours) in 6 BRAF^{V600E} p53^{wt} melanoma cell lines treated with PD0325901, fotemustine, or PLX4720 as in A. Bars are mean of 3 independent experiments ± SD. Significant differences are expressed as follows: *, P < 0.05; **, P < 0.01.

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 (Me23669, BRAF^{V600E} p53^{wt} 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.
Targeting Apollon to Rescue Melanoma Apoptosis

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), 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 < 0.01; ***, 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. A, 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 downmodulation.

Enhanced apoptosis by Apollon silencing was corroborated in a panel of melanomas, including cell lines bearing mutant or null p53 (17), in response to fotemustine, PD0325901, and PLX4720 (Fig. 5D). Furthermore, in cell lines as Me23682, shown in Fig. 5C, Apollon silencing induced apoptosis, without any additional treatment with antitumor agents (compare bars labeled "control siRNA" and "Apollon siRNA" in Fig. 5C at 72 hours). Apoptosis induction upon Apollon silencing was observed in 3 additional cell lines of 12 tested (data not shown).
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 BRAFV600E, p53wt melanoma cell line (Me23682) treated for 48 and 72 hours with PLX4720 (500 nmol/L) or fotemustine (300 μmol/L) in the presence or not of the pan-caspase inhibitor z-VAD-fmk or the control z-FA-fmk (both at 5 μg/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 μmol/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.
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+, CD34-TRAIL). Taken together, these results indicated that inhibition of Apollon rescues apoptosis in melanoma cells in response to antitumor agents that activate the intrinsic or extrinsic pathways of apoptosis.

**Apollon silencing promotes mitochondrial depolarization and caspase activation in response to antitumor agents**

To gain insight into the mechanism of enhanced melanoma apoptosis upon Apollon silencing, we initially used apoptosis protein arrays. Six of 35 apoptosis-specific proteins (Fig. 6A, black symbols) showed a significantly
different modulation in melanoma cells by "PD0325901 + Apollon siRNA", versus "PD0325901 + control siRNA" (Fig. 6A). "PD0325901 + Apollon siRNA" downmodulated the antiapoptotic protein Bcl-x and upregulated the proapoptotic proteins Bax and Bad (Fig. 6A and Supplementary Fig. S6 for Western blot analysis of Bax).

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 or mutated Bcl-2 family members (Fig. 6C). 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. S10A and PLX4720, respectively). This was also shown by IPA of genes modulated only by the combination of drug treatment and Apollon silencing (Supplementary Fig. S10A and PLX4720, respectively).

Knockdown of Apollon increased expression of proapoptotic Bcl-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 caspas 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
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conceptualization and design: E. Tassi, C. Carlo-Stella, R. Mortarini, A. Anichini

Development of methodology: E. Tassi, M. Zanon, C. Vegetti, A. Molla, I. Bersani, V. Perotti, R. Mortarini, A. Anichini

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Tassi, M. Zanon, C. Vegetti, A. Molla, I. Bersani, V. Perotti, M. Pennati, N. Zaffaroni, A. Anichini

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Tassi, C. Vegetti, A. Molla, I. Bersani, V. Perotti, M. Milella, R. Mortarini, A. Anichini

References


Clinical Cancer Research

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

Elena Tassi, Marina Zanon, Claudia Vegetti, et al.


Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-2232

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/05/02/1078-0432.CCR-11-2232.DC1

Cited articles  This article cites 43 articles, 14 of which you can access for free at: http://clincancerres.aacrjournals.org/content/18/12/3316.full.html#ref-list-1

Citing articles  This article has been cited by 2 HighWire-hosted articles. Access the articles at: /content/18/12/3316.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.