Role of Apollon in human melanoma resistance to anti-tumor agents that activate the intrinsic or the extrinsic apoptosis pathways

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Running title: Targeting Apollon to rescue melanoma apoptosis

Keywords: Melanoma, Apoptosis, IAP, Apollon, BRUCE

Financial support: this investigation was supported by grants from Associazione Italiana per la Ricerca sul Cancro (A.I.R.C., Milan), the European Community Integrated Project 037665 “CHEMORES, Fondazione Cariplo (Grant #2008-2411), Alleanza contro il Cancro, Ministero della Salute, Rome (to A.A.) and Grant #9998 of the “Special Program Molecular Clinical Oncology - 5 per mille” of A.I.R.C. (to A.M.G. and A.A.), and by the PHS grants R01 CA113369 and R01 CA138188 awarded by the National Cancer Institute (S.F.).

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Conflicts of interest: the authors declare no conflict of interest.

Word count: 4898. 6 Figures.
Statement of translational relevance.

The identification of key regulators of apoptosis in advanced melanoma may provide new therapeutic targets to improve efficacy of anti-tumor 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 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$^{V600E}$ 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 IAP may improve the efficacy of currently available treatments.
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, MEK-, BRAFV600E- and mTOR-specific inhibitors, TRAIL and anti-HLA class II mAbs. Mitochondrial depolarization, caspase activation, apoptosis assays and gene expression profiling were used to test effects of Apollon silencing, by siRNA, on melanoma response to anti-tumor 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 anti-tumor 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, -8, -9 and -3 activation in response to different anti-tumor 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 anti-tumor agents that trigger the intrinsic or the extrinsic apoptosis pathways.
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 (MAPK) and phosphoinositide 3-kinase (PI3K)-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 demonstrated 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 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 anti-tumor 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 pro-apoptotic molecule Smac/DIABLO and of caspase-9 (14, 15). In addition, Apollon depletion promotes p53 stabilization leading to caspase-2- and -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 anti-tumor 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 pharmacological treatments of melanoma.
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 biological 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, San Diego, CA); goat anti-c-IAP1 (R&D Systems, Minneapolis, MN, USA); mouse anti-α-tubulin (Calbiochem, Merck KGaA, Darmstadt, Germany); anti-β-actin (Abcam Inc., Cambridge, MA). The HLA class II specific 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 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 FACS-Calibur cyt fluorimeter (Becton Dickinson, Franklin Lakes, NJ). Values were expressed as mean fluorescence intensity (MFI) after subtracting the
MFI of cells stained only with the secondary Ab. Mitochondrial membrane depolarization was assessed by using the fluorescent probe tetramethylrhodamine ethyl ester (TMRE, Invitrogen Life Technologies, Camarillo, CA). 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 cytofluorimeter (BD).

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

**Immunohistochemistry.** Immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissues as described (21), by staining with mAbs to Apollon (Abcam) or to gp100 (HMB45, DakoCytomation, Denmark). Sections were counterstained with haematoxylin followed by dehydration and mounting. Images were acquired with an Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany) 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 h with: camptothecin (Aventis Pharma, Milan, Italy) at 50 µmol/L, celecoxib (Pfizer, NY) at 50 µmol/L, temozolomide (Sigma-Aldrich, St.Louis, MO) at 20 µmol/L, fotemustine (Muphoran, Italfarmaco, Milan, Italy) at 150-300 µmol/L, mTOR inhibitor Rapamycin (Sigma-Aldrich) at 10 nmol/L, MEK inhibitor PD0325901 (Cayman Chemicals, Ann Arbor, MI) at 5-10 nmol/L, or BRAFV600E-specific inhibitor PLX4720 (Selleck Chemicals, Houston, TX) at 500 nmol/L. Melanoma cells were treated for 24 h with 10 ng/mL of recombinant sTRAIL (KillerTRAIL, Alexis Biochemicals, Enzo Life Sciences, Exeter, UK). Cells were treated with HLA class II antigen-specific or HLA class I antigen-specific mAbs (25 µg/ml). Quantification of apoptotic
cells was performed by flow cytometry by staining with 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 anti-tumor agents for 48 h, were also treated with lactacystin (Sigma-Aldrich) at 10 \( \mu \text{M} \) during the last 24 h. Samples were acquired by a FACS-Calibur cytofluorimeter (BD).

**Silencing of Apollon by small interfering RNA (siRNA).** Cells were transfected with two different Apollon-specific siRNAs: siRNA#2 (sequence AGAAAUUGACCUGAGUUA, Eurofins MWG Operon, Ebersberg, Germany, ref. 17) or with siRNA#4 (Stealth RNAi siRNA, sequence GGGCAUGCUUGGAAUGUUGACGUAA, 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 co-culture 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 (BD) and TRAIL-PE mAb (CD253, BD Pharmingen). Melanoma cells were stained with 2 \( \mu \text{mol/L} \) 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Invitrogen) as described (29). TRAIL-expressing CD34\(^+\) cells (or untransduced CD34\(^+\) cells as control) were co-cultured for 24 hours at 1:1 ratio with melanoma cells labelled with CFSE and transfected with Apollon- or control-siRNA. After co-culture, cells were stained with the far-red fluorescent DNA dye DRAQ7 (Biostatus Limited, UK) that stains nuclei only in dead cells. By flow cytometry analysis of melanoma-CD34\(^+\) co-culture 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, -3, -8 and -9 on 50 μg/well of cell lysate was performed by using Caspase-2/ICH-1, Apopcyto/Caspase-3, Apopcyto/Caspase-8 and Apopcyto/Caspase-9 Fluorometric Assay Kits (Medical and Biological Laboratories, Naka-ku Nagoya, Japan) according to manufacturer’s instructions by TECAN Infinite M1000 (Tecan Group Ltd., Mannedorf, Switzerland). Results were expressed as relative fluorescence units (RFU). Melanoma cells were treated with general caspase inhibitor z-VAD-fmk or control z-FA-fmk (BD Pharmingen) at 5 μmol/L, 5 h 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., Hercules, CA). 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, s.c. 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 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 four IAPs by ANOVA followed by Bonferroni post-test), nor with the p53 status (data not shown). By immunohistochemistry 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. 1), or even within the same lesion (see insets Fig. 1D and Supplementary Fig. 1). 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 anti-tumor agents.** Time course experiments in two BRAFV600E mutant melanoma cell lines (Me14464 and Me23682) showed that apoptosis was associated with Apollon downmodulation in response to the MEK inhibitor PD0325901, the BRAFV600E-specific inhibitor PLX4720 or the cytotoxic drug fotemustine (Fig. 2A). In a larger panel of cell lines (Fig. 2B for correlation analysis and 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, BRAFV600E mutant tumors). In the same tumors, no correlation was found between apoptosis by each drug and modulation of c-IAP2 or XIAP, while c-IAP1 modulation showed a significant correlation.
with apoptosis only by PLX4720 and fotemustine, but not by PD0325901 (Supplementary Table 1).

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, as well as in tumor subsets defined by BRAF or NRAS mutational status (Supplementary Fig. 2).

Overall, these results indicated that melanoma susceptibility to apoptosis by different anti-tumor 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 to 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 h, compared to 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, C and D). In a panel of 11 melanoma cell lines enhanced Apollon downmodulation correlated significantly with enhanced cell death by combined PD0325901+rapamycin treatment compared to 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 hematological malignancies (30, 31), could impact on expression of Apollon and whether this promoted melanoma apoptosis by anti-tumor agents. Pre-incubation of HLA class II⁺ melanoma cells (Me32669, BRAF<sup>V600E</sup> p53<sup>wt</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. 3A). This rescued apoptosis in response to PD0325901 and to fotemustine (Supplementary Fig. 3B).

Melanoma treatment with the HLA class I antigen-specific mAb TP25.99 (27) did not affect apoptosis by these drugs (Supplementary Fig. 3B). Similar results were obtained by treating HLA class II⁺ melanoma cells with a different anti-HLA class II-specific mAb LGII-612.14 (26, data not shown). Taken together, these results suggest 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 anti-tumor agents is not caspase-dependent.** We asked whether Apollon downmodulation, in melanoma cells treated with anti-tumor agents, was a consequence of caspase activation, as caspases can degrade Apollon (15). In cell lines, as Me23682, susceptible to 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 anti-tumor 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, C). These results suggested that Apollon downmodulation, promoted by different anti-tumor agents, is caspase-independent and proteasome-dependent.

**Apollon silencing enhances melanoma cell death in response to cytotoxic drugs, to MEK and BRAF^{V600E} 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 24-96 hours by the specific siRNA, as detected by Western blot and flow cytometry (Fig. 5A and 5B), while 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. 4), and to inhibitors as PD0325901 (Fig. 5C).

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 Figure 5C, Apollon silencing induced apoptosis, without any additional treatment with anti-tumor agents (compare bars labelled “control siRNA” and “Apollon siRNA” in Fig. 5C at 72 h). Apoptosis induction upon Apollon silencing was observed in 3 additional cell lines out of 12 tested (data not shown).

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. 5). Taken together, these results indicate that inhibition of Apollon rescues apoptosis in melanoma cells in response to anti-tumor agents that activate the intrinsic or extrinsic pathways of apoptosis.

**Apollon silencing promotes mitochondrial depolarization and caspase activation in response to anti-tumor agents.** To gain insight into the mechanism of enhanced melanoma apoptosis upon Apollon silencing we initially used apoptosis protein arrays. Six out of 35 apoptosis-specific proteins (Fig. 6A, black symbols) showed a significantly different modulation in melanoma cells by “PD0325901+Apollon siRNA”, vs. “PD0325901 + control siRNA” (Fig. 6A). “PD0325901+Apollon siRNA” downmodulated the anti-apoptotic protein Bcl-x and upregulated the pro-apoptotic proteins Bax and Bad (Fig. 6A and Supplementary Fig. 6 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 48-72h), and association with PD0325901 treatment further enhanced this effect (Fig. 6B).

Apollon knockdown increased activation of initiator caspases of the intrinsic (caspase-2 and -9) and of the extrinsic (caspase-8) pathways, and of the effector caspase-3, in response to PD0325901 in cell lines bearing wt p53 (Me23682, Fig. 6C) or even mutant p53 (Supplementary Fig. 7), as well as in response to cytotoxic drugs as camptothecin or celecoxib (Supplementary Fig. 8). As expected, based on apoptosis assays (already shown in Fig. 5C) in tumors as Me23682 Apollon silencing also induced significant activation of caspase-2, -8 and -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 indicate that Apollon silencing increases expression of pro-apoptotic Bcl-2 family members, promotes mitochondria depolarization and activation of caspase-dependent apoptosis.

**Apollon silencing alters the profile of genes modulated by anti-tumor agents.** To test the effects of Apollon silencing on genes modulated by anti-tumor agents we used whole genome microarray analysis of melanoma cells (Me23682) bearing expressed vs. 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. 9A). 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. 9).

However, pairwise significance analysis of modulation of each of these genes showed a few notable exceptions (gene symbols in bold, color in Supplementary Fig. 9B): the CCND1 gene, encoding the cell cycle regulator cyclin D1, had a fold change (FC) of -5.0 by PD0325901, but -7.0 by PD0325901 plus Apollon silencing (results confirmed by RT-PCR, data not shown), suggesting that Apollon knockdown may enhance the anti-proliferative 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), IL-8 and JUN (Supplementary Fig. 9B).

We then classified, by Venn diagrams, all genes with significant modulation by any of the treatments, but irrespective of FC (Supplementary Fig. 10A and Supplementary Fig. 11A for PD0325901 and PLX4720, respectively). By Ingenuity Pathway Analysis 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. 10B and 11B). This was also shown by IPA analysis of genes modulated only by the combination of drug treatment and Apollon silencing (Supplementary Fig. 10 C,D and 11 C,D). Detailed analysis of the latter set of genes showed that the majority had a FC <1.5, but a minority showed a FC between 1.5 and 2 (Supplementary Fig. 12). Many of the genes with a FC >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. 12).

Taken together, these results suggest that Apollon knockdown may contribute to the anti-tumor effects of inhibitors as PD0325901 and PLX4720 even by altering the overall profile of genes modulated by such anti-tumor agents.
Discussion

The present study indicates that Apollon is constitutively expressed in human melanoma cells in-vitro and in-vivo, and 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 anti-tumor agents is critically dependent on downmodulation of this IAP. Furthermore, Apollon downmodulation, observed in melanomas that are susceptible to the anti-tumor 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 pro-apoptotic 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 even 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, since it was significantly inhibited by z-VAD-fmk. However, the same pan-caspase inhibitor did not prevent Apollon downmodulation promoted by anti-tumor 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 anti-tumor agents (such as cytotoxic drugs, MEK and BRAF\textsuperscript{V600E}-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 et al. (16), where 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 small molecule inhibitors. Such Apollon antagonists could rescue/improve melanoma susceptibility to apoptosis by different anti-tumor agents. In agreement, depletion of Apollon, by oncolytic adenovirus-mediated shRNA, promotes tumor apoptosis in-vitro, inhibits tumor growth and enhances effects of chemotherapy in-vivo in nude mice (43).
References


19. Chawla-Sarkar M, Bae SI, Reu FJ, Jacobs BS, Lindner DJ, Borden EC. Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes


Figure Legends

**Figure 1. Apollon expression in melanoma.** (A) Expression of Apollon, c-IAP2, c-IAP1 and XIAP in 34 melanoma cell lines by flow cytometry. Horizontal bars: median normalized MFI. (B) Western blot for Apollon, c-IAP2, c-IAP1 and XIAP in 7 melanoma cell lines. (C) IAPs expression according to BRAF or NRAS mutations of tumors shown in A. (D) Immunohistochemistry for Apollon expression in tissue sections from a s.c. or a lymph node (LN) metastasis of two melanoma patients. 1: staining with secondary Ab only of the same area shown in 2. 2: Apollon expression (20x magnification). 3-4: details of the indicated areas. (E). Staining of a compound nevus with gp100-specific HMB45 mAb or with Apollon mAb (magnification 20x). Right hand panels: details of the indicated area.

**Figure 2. Melanoma apoptosis correlates with Apollon downmodulation.** (A) Time course analysis of Apollon expression by flow cytometry and apoptosis in Me14464 (upper panels) and Me23682 (lower panels) after treatment with PD0325901 (10 nmol/L), PLX4720 (500 nmol/L) or fotemustine (300 μmol/L). Results expressed as ratio of values (MFI and Annexin-V/PI live cells) in treated cells to untreated cells. (B) Correlation between Apollon modulation (48 h) and apoptosis (72 h) 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, D) Apoptosis by Annexin-V/PI assay (upper graphs, 72 h) and Apollon expression (lower graphs, 48 h) in 6 BRAF^{V600E} p53^{wt} melanoma cell lines treated with PD0325901, fotemustine, or PLX4720 as in A. Bars are mean of three independent experiments ± SD. Significant differences are expressed as follows: *, p < 0.05; **, p < 0.01.

**Figure 3. Co-targeting of MEK and mTOR pathways promotes Apollon downmodulation and improves the apoptotic response of melanoma cells.** (A) Time course analysis of apoptosis (upper graphs) and Apollon downmodulation (lower graphs) in a
BRAF^{V600E} p53^{wt} 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 two BRAF^{V600E} p53^{wt} melanoma cell lines treated for 48 h as in panel A. (C) Modulation of c-IAP1, c-IAP2 and Apollon expression in three BRAF^{V600E} p53^{wt} 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 h. (E) Correlation between extent of apoptosis and Apollon modulation by co-targeting MEK and mTOR pathways vs. MEK-targeting alone, in 12 cell lines treated for 72 h as in panel A. MEKi= PD0325901; mTORi= rapamycin. Statistical analysis in A, C and D by ANOVA followed by Bonferroni post-test. *:p<0.05; **:p<0.01; ***:p<0.001. Data in A, C and D are means of three independent experiments.

**Figure 4.** Apollon downmodulation, by anti-tumor agents or combinatorial treatments with signaling pathway inhibitors, is not caspase-dependent. (A) Apoptosis (% of live cells, upper graphs) and Apollon expression (MFI, lower graphs) of a BRAF^{V600E}, p53^{wt} melanoma cell line (Me23682) treated for 48 and 72 h 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, C) Apollon expression (MFI) and apoptosis (% of live cells) at 48 h in Me23682 cells treated with PD03205901 or fotemustine (B), or with PD0325901 plus rapamycin (C) in the presence or not of lactacystin (10 μM/L, last 24 h). Statistical analysis and annotation of P values as in Fig. 3. Bars in A, B, C: means of three independent experiments.

**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 expression 48 and 72 h after transfection of Me23682 cells with two different pairs of control siRNA (siRNA#1 and #3) and Apollon-specific siRNA (siRNA#2 and #4). (B) Apollon protein expression, in Me23682 cells by flow cytometry at
24, 48, 72 and 96 h after transfection with control siRNA#3 (continuous line) or Apollon-specific siRNA#4 (dotted line). Filled histogram, cells stained with secondary antibody only. (C) 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 (5 nmol/L). (D) Melanoma apoptosis in cell lines transfected with control- or Apollon-siRNA and then treated with fotemustine or PD0325901 (as in panel C) or PLX4720 (500 nmol/L) for 72 h. Empty symbols: tumors with wt p53. Black symbols: tumors with the following p53 mutations: Me2211, ▼p53S127F; Me14362, □p53E258K; Me20842M2, ●p53G187S; Me15479, ♦p53W146X; Me4405 ▲p53null (see refs. 23 and 36 for these tumors). The values of % Annexin-V+PI+ in cells transfected with control siRNA were subtracted from all data points. Statistical analysis by Student’s paired T test. (E) Extent of cell death (% DRAQ7+ cells) in CFSE-labelled Me23682 cells transfected with control- or Apollon-siRNA and then treated for 24 h with 10 ng/ml of soluble TRAIL (sTRAIL), untransduced CD34+ cells (CD34), or TRAIL-expressing CD34+ cells (CD34-TRAIL). Bars in C and E: means of three independent experiments ± SD. Statistical analysis and annotation of P values as in Fig. 3.

Figure 6. Apollon silencing in melanoma cells promotes mitochondrial depolarization and caspases activation. (A) Antibody array analysis of 35 apoptosis-related proteins at 48 h 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, -9, -8 and -3 catalytic activity in Me23682 cells 24 h 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 h), after transfection of Me23682 cells with control- or Apollon-siRNA, associated with PD0325901
treatment as in Fig. 5C. Bars in B-E, means of three independent experiments ± SD. Statistical analysis and annotation of P values as in Fig. 3.
Figure 2

A) PD0325901, PLX4720, and Fotemustine were treated with PD0325901, PLX4720, and Fotemustine, respectively. The Apollon MFI ratio and % live cells ratio were measured at 18h, 24h, 48h, and 72h. Pearson r = 0.913, P = 0.0002 for PD0325901. Pearson r = 0.943, P = 0.0014 for PLX4720. Pearson r = 0.786, P = 0.0070 for Fotemustine.

B) Apollon MFI ratio and % live cells ratio were calculated for PD0325901, PLX4720, and Fotemustine. Pearson r = 0.913, P = 0.0002 for PD0325901. Pearson r = 0.943, P = 0.0014 for PLX4720. Pearson r = 0.786, P = 0.0070 for Fotemustine.

C) PD0325901 (Me15392), PLX4720 (Me21158), and Fotemustine (Me13294) were treated with PD0325901, PLX4720, and Fotemustine, respectively. % live cells were measured at 18h, 24h, 48h, and 72h. ** indicates P < 0.01.

D) PD0325901 (Me18732), PLX4720 (Me2934), and Fotemustine (Me32669) were treated with PD0325901, PLX4720, and Fotemustine, respectively. % live cells were measured at 18h, 24h, 48h, and 72h. ** indicates P < 0.01.
Figure 3

A. 

B. 

C. 

D. 

E. 

Figure 3
**Figure 5**

A. Western blot analysis showing protein expression at 48 h and 72 h.

- 528 kDa: Apollon
- 72 kDa: c-IAP2
- 68 kDa: c-IAP1
- 42 kDa: β-actin

B. Flow cytometry analysis showing cell events at 24 h, 48 h, 72 h, and 96 h.

C. Graphs showing the percentage of annexin-V-PI- cells at 48 h and 72 h for different treatments:
- **Fotemustine**
- **Temozolomide**
- **PD0325901**

D. Graphs showing the percentage of annexin-V-PI+ cells at 48 h and 72 h for different treatments:
- **Fotemustine**
- **PD0325901**
- **PLX4720**

E. Graphs showing the percentage of DRAQ7+ cells for different treatments:
- control siRNA
- Apollon siRNA
- control siRNA + drug
- Apollon siRNA + drug
Figure 6

A) Expression levels of Caspase-2, Caspase-9, Bad, Bcl-x, and TRAIL-R1 were investigated using siRNA-mediated knockdown. The expression levels were normalized to Catalase.

B) Cell viability was assessed using TMRE staining. The percentage of TMRE-negative cells was determined at 24, 48, and 72 hours post-treatment. PD0325901 and control siRNA were used as controls, and Caspase-2, Caspase-3, Caspase-8, and Caspase-9 activities were measured.

C) Caspase activities were measured using a fluorometric assay. The activities of Caspase-2, Caspase-3, Caspase-8, and Caspase-9 were determined for PD0325901 and control siRNA.

D) Annexin V/PI staining was used to assess cell apoptosis. The percentage of Annexin V-positive cells was determined for PD0325901 and control siRNA.

E) The percentage of live cells was determined using Annexin V/PI staining. The results show the effectiveness of PD0325901 and control siRNA in inducing cell death.
Clinical Cancer Research

Role of Apollon in human melanoma resistance to anti-tumor agents that activate the intrinsic or the extrinsic apoptosis pathways

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Clin Cancer Res  Published OnlineFirst May 2, 2012.

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

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