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AUGMENTATION OF EFFECTS OF INTERFERON-STIMULATED GENES BY REVERSAL OF EPIGENETIC SILENCING: POTENTIAL APPLICATION TO MELANOMA

Ernest C. Borden*

* *Ernest C. Borden, MD, Taussig Cancer Center and Case Comprehensive Cancer Center, Cleveland Clinic Foundation, 9500 Euclid Ave, Cleveland, OH 44195, Email: bordene@ccf.org, Phone: 216-444-8183, Fax: 216-636-2498*

Abstract

Increased expression of genes, silenced by methylation of their promoters, could have relevance for increasing effects of not only interferons (IFNs) but also APO2L/TRAIL, cytotoxics and immunotherapeutics for melanoma and other malignancies. A resistant melanoma cell line, A375, lacked APO2L/TRAIL or apoptosis induction by either IFN-α2 or IFN-β. However, apoptosis was induced by IFNs in A375 cells by 5-aza, 2′deoxycytidine, evaluated based upon the postulate that promoter methylation might be silencing pro-apopoptotic IFN-stimulated genes (ISGs). RASSF1A, commonly methylated at high frequency in many tumors including melanoma, which we discovered to be also an IFN-regulated gene, was increased by 5-Aza-dC. RASSF1A was important in enhancing apoptotic effects of not only IFNs and APO2L/TRAIL but also cisplatin. Unraveling epigenetic regulatory mechanisms, as yet only partially identified, will result in new biological insights and improved strategies for therapeutic use of IFNs or ISGs such as APO2L/TRAIL.

Keywords

APO2L/TRAIL; azacytidine; apoptosis

Introduction

Despite use over the past 15 years, interferons (IFNs) have yet to reach their full therapeutic potential in oncology. To do so, 1) augmentation of function and regulation of induced genes and proteins must be attained, 2) interventions to overcome cellular resistance developed, and 3) integration with other therapeutic modalities improved. From the initial introduction of IFNs into clinical trials, melanoma has been a prototype for applications in oncology [1–10]. Approximately 75,000 individuals in the United States will be newly diagnosed with melanoma this year; 60% of those who die will be individuals under 60 years of age who would otherwise have had a life expectancy of 25 years or more. Unfortunately, since the advances marked by identification of IFNs and IL-2 as human proteins therapeutically active in melanoma, the past 15 years has been notable more for negative Phase III trials than for clinically significant reduction in mortality from systemic metastatic disease [11–13]. While sorafenib and

antibodies to the T cell ligand, CTLA-4, hold new promise, improved therapies for the high risk primary and metastatic patient are much needed.

One approach to potentially enhance effects of IFNs is to reverse epigenetic silencing of gene expression, since methylation of gene promoters occurs commonly in malignancies. Thus, in the spirit of the Milstein awards of encouraging research to improve clinical outcomes, this article will describe rationale and results of one specific ongoing investigational approach. It however grows out of research on gene regulation and melanoma that has involved ideas and collaborations with many individuals including friends and other Milstein award winners, Paula Pitha-Rowe, Jordan Gutterman, Susan Krown, John Kirkwood, Bryan Williams, Robert Silverman, Ganes Sen, and George Stark.

Interferons and stimulated genes in melanoma

Although no systemic modality has substantial activity, IFN-α2 has resulted in response rates in metastatic melanoma equivalent to the best of any single agent. It results in metastatic disease regression in 15% of patients in summarized data from clinical studies [2,3,5,9]. Approximately 3% of patients will have a complete response of significant duration. Prolongation of disease-free survival and a strong trend toward prolonged overall survival have emerged from use of IFN-α2 as an adjuvant to surgery for high-risk patients with primary melanoma [6–8,13]. Though randomized studies, meta-analyses and cost benefit analyses all support usage in these patients with Stage IIc and III disease, clinical adoption has been slowed by perceptions of a marginally beneficial therapeutic index. Thus ways to enhance gene expression, particularly with improved tolerance, could be of substantial clinical benefit.

Protein products, encoded by interferon-stimulated genes (ISGs), underlie the changes in cellular function that result in clinical effects. ISG expression translates into alterations of the state of differentiation, rate of proliferation, and functional activity of endothelial cells, immune effector cells, and tumor cells. Changes in ISGs vary both quantitatively and qualitatively depending upon tissue and IFN type. Building upon the identification of transcriptional induction of genes by IFNs [10,14,15], our prior studies have focused on the functional significance of over a dozen different ISGs ([6–24]. In clinical studies, this work has identified differences in IFN types and helped guide considerations of schedule, route, and dose.

After receptor binding by IFNs-α or IFN-β, the tyrosine kinases, tyk2 and JAK-1, are phosphorylated; these subsequently phosphorylate STAT (signal transducers and activators of transcription) 1 and/or STAT 2 leading to activated dimers [14,15]. Receptor signaling is suppressed by the phosphatases SHP-1 and SHP-2 and by the induced SOCS and PIAS proteins [1,14,25]. The phosphorylated ISGF-3 complex is translocated to the nucleus and forms (with the addition of a third subunit, p48 or IRF-9), a DNA binding complex specific for the IFN-stimulated response element.

Suppression of the IFN response system of the host in and/or by malignant cells has emerged as a potentially significant contributor to development of clinical disease. Mutation in an ISG, RNase L, increased prostate cancer risk [26,27]. Gene expression profiling and cytogenetic analyses have identified homozygous and heterozygous deletion or decreased expression of ISGs in melanoma, colon, breast, hematologic and other malignancies [28–60]..

Another important mechanism of ISG suppression may be epigenetic silencing with potential influence on tumor development [45,51–60]. Decreases in ISGs has been identified in transformed compared to diploid cells of varying histogenesis [45,54,58,61]. Inhibition of normal ISG expression could be the basis for effectiveness of IFNs and/or inducers in suppressing tumor emergence in carcinogen-induced murine tumors [61,62]. Activation of the

IFN system, as has been used chronic hepatitis to decrease risk of hepatocellular carcinoma [63–67], suggests IFNs or inducers could eventually play a role in chemoprevention.

Postulating that suppressed cellular responses to IFNs in tumors could in part result from aberrant methylation of the promoters of ISGs, we have examined inhibitors of epigenetic alteration in gene expression. 5-aza,2′deoxycytidine (5-Aza-dC) is a nucleoside analogue, which after incorporation into DNA, inhibits DNA methyltransferase 1 (DNMT1) by covalent binding. DNMT1 is thus trapped and not available at the DNA replication fork to copy methylation patterns from mother to daughter strand. Similar but more specific inhibition of DNMT1 can result from antisense oligonucleotides to DNMT1. Both have been utilized in our initial studies to identify changes in expression of pro-apoptotic genes, RASSF1A and XAF1 [57,58], two which are decreased in expression in patients with melanoma.

Pro-apoptotic genes and cellular effects of IFNs

The pathway that led to a focus on epigenetic silencing of gene expression emerged from studies of genes and pathways that regulate IFN-induced apoptosis. We and others had consistently identified greater antiproliferative effects for IFN-β when compared to IFNs-α [68–78]. Despite their binding to the same heterodimeric receptor and a signal transduction pathway with common elements, this occurs presumably as a result of differing three-dimensional ligand conformations [79,80]. We undertook studies which were among the first to identify IFNs through TNF-related apoptosis-inducing ligand or Apo2L (APO2L/TRAIL) induction as activating the extrinsic apoptotic cascade in melanoma and other cell lines [72,73,81].

By both TUNEL and Annexin V assays, apoptosis was identified with up to 50% apoptotic cells. Although IFN-α2 and IFN-β have equivalent antiviral activity per mg of protein, IFN-β was >5x more potent for apoptosis induction [72]. Antiviral specific activity remains the WHO-recommended standard for determining potency of an IFN. Thus to confirm labeled biological activity of the pharmaceutical grade IFNs, the two were confirmed as equivalent in the same cell line [72]. This work has suggested that antiviral specific activity may not be the best standard for IFNs for clinical antitumor applications [82].

To identify ISGs that might be involved, RNA samples from WM9 cells and WM35 melanoma cell lines were treated with IFN-α2 or IFN-β and assessed on a 10k Affymetrix oligonucleotide array. For 95% of genes assessed, IFN-β was more potent than IFN-α2 in inducing ISG expression; 910 genes were identified as induced by IFN-β at 500 units and 260 ISGs as significantly induced by IFN-β at both 50 and 500 units. Of these 260 ISGs, 209 were new ISGs based upon the array analysis [83]. Increased expression of 28 genes was further confirmed by Northern blot or semi-quantitative or quantitative RT-PCR analysis.

Nearly half of the 260 genes were functionally categorized as encoding growth regulatory proteins. Of the 104 with described growth regulatory function, 71 were induced more than 3x by 500 units and 2x by 50 units of IFN-β; 48 were new or not previously considered as ISGs. Included in this latter category were APO2L/TRAIL, XIAP-Associated Factor 1 (XAF1), galectin 9, a cyclin E binding protein, amphiphysin 1, MyD88, and ubiquitin pathway genes. Direct comparison of the gene modulatory potential of IFN-α2 and IFN-β was possible for 73 of the new and previously identified ISGs. For these, comparisons in both WM9 and WM35 melanoma cell lines, IFN-β potency was >IFN-α2 for induction of gene expression in 137/146 instances (95%).

Most known and novel ISGs were induced by both IFNs to a similar extent in the apoptosis-sensitive WM9 and apoptosis-resistant WM35 cells. However, some of the potentially apoptosis-related genes were specific to one or the other cell line. For example, APO2L/TRAIL was higher in sensitive WM9 as compared to resistant WM35 melanoma cells. Kinetic studies

of induction by IFN-α and IFN-β with specific RNA probes confirmed APO2L/APO2L/TRAIL, XAF-1, K12, MYD88, SP100, AIPC, UBEL6, TSG6, Cyclin E associated, USP18, RIG-G, BST-2, and ISG27; these studies identified genes that peaked in expression early (8h) and late (36h) [83]. APO2L/TRAIL however was sustained at high levels throughout in the sensitive WM9 melanomas (83). The anti-apoptotic XIAP was present at higher levels in resistant cells; XAF1 had been defined as an XIAP interacting protein and thus potentially a tumor suppressor [84,85]. As assessed by caspase inhibitors, IFN-β-induced apoptosis was dependent on activation of the caspase cascade with cleavage of caspases 3, 8, and 9 and of the caspase 3 substrate, poly (ADP-ribose) polymerase. These changes together with the release of cytochrome *c* from mitochondria to cytoplasm were also identified in response to Apo2L/APO2L/TRAIL (but not for FAS or FASL). Other sensitive melanoma cell lines had a similar induction by IFN-β of APO2L/TRAIL. Antibody to APO2L/TRAIL inhibited IFN-β-induced apoptosis in the WM9 cells sensitive to IFN-α2 and IFN-β (Fig 1). In resistant A375 cells, IFN-β did not induce APO2L/TRAIL expression. Thus, induction of APO2L/TRAIL was critical for IFNs initiated apoptotic cascade.

To further probe the mechanism of cellular refractoriness to apoptosis, resistant melanoma cell lines were analyzed for their sensitivity to recombinant APO2L/TRAIL protein [73,86]. As assessed by Annexin V and TUNEL assays, all were also resistant to apoptosis induction by APO2L/TRAIL protein (the Zn based trimer kindly provided by A. Ashkenzi (Genentech). No correlation existed between expression of apoptosis regulators (Apaf1, FLIP, caspase-8, caspase-9, caspase-3, cIAP, Bcl-2 or Bax) and resistance to APO2L/TRAIL induced apoptosis. APO2L/TRAIL activated caspase-8 and caspase-3, but subsequent apoptotic events, such as PARP cleavage and DNA fragmentation, were not observed, suggesting a possible role of inhibitors of apoptosis downstream of caspase-3. Postulating that in addition to APO2L/TRAIL, induction of one or more IFN stimulated genes might sensitize cells to APO2L/TRAIL, melanoma cell lines were treated with IFN-β for 16–24 h prior to APO2L/TRAIL; more than 30% of cells underwent apoptosis. Induction of apoptosis by IFN-β and APO2L/TRAIL correlated with synergistic activation of caspase-9, decrease in mitochondrial potential and cleavage of PARP. Cleavage a p19 fragment from XIAP to an inactive p29 and other fragments, following IFN-β or APO2L/TRAIL occurred in WM9 (Fig 2), FEMX, A375 and WM3211 cells and correlated with apoptosis [86]. Treatment with IFN-β after APO2L/TRAIL did not result in potentiated apoptosis, suggesting induction of a gene(s) by IFN-β that potentiated apoptosis. Thus *in vitro* IFN-β and APO2L/TRAIL in combination had more potent apoptotic and anti-growth effects compared to either cytokine alone in melanoma cells lines, an effect postulated to result from modulation by IFNs of anti-apoptotic action of IAPs.

To further probe the role of IAPs in conferring resistance to APO2L/TRAIL-induced apoptosis, melanoma cells were transfected with siRNAs to XIAP or survivin [86]. Since higher expression of inhibitors of apoptosis such as Bcl-2 or FLIP could also play a role, siRNAs to them were also assessed. Compared to both scrambled and mismatch controls, target specific siRNAs (si-Bcl-2, si-XIAP, si-FLIP or si-Surv), followed by APO2L/TRAIL resulted in marked increase in apoptosis. Compared to si-Bcl-2 or si-FLIP, siRNAs against XIAP and survivin were most potent in sensitizing melanoma cells. A substantial increase in apoptosis also occurred in renal carcinoma cells (SKRC-45, Caki-2), following inhibition of either XIAP or survivin by siRNAs. Thus, APO2L/TRAIL resistance could be overcome by interfering with expression of XIAP and survivin and to a lesser extent by inhibitors of the intrinsic (mitochondrial) pathway of apoptosis.

IFNs induced high expression of XAF1 predominantly in cell lines sensitive to the apoptotic effects of IFN-β [87]. In apoptosis-resistant cells, including WM164 and WM35 melanoma, U937 lymphoma and HT1080 fibrosarcoma cells, XAF-1 mRNA was strongly upregulated but XAF1 protein was expressed only weakly or not at all. APO2L/TRAIL is a critical mediator

of IFN-β induced apoptosis, but most melanoma cell lines were resistant to exogenous recombinant APO2L/TRAIL protein. A375 melanoma cells, for example, were defective in APO2L/TRAIL induction by IFN-β and were resistant to APO2L/TRAIL-induced apoptosis. Thus, APO2L/TRAIL was necessary for apoptosis induction but only effective in the presence of XAF-1.

Examining the converse interaction, evidence that XAF-1 was a component of IFN-β sensitization for APO2L/TRAIL was provided by constitutively overexpressing XAF1 in A375 cells. A375 cells expressing XAF1 constitutively were more sensitive to APO2L/TRAIL-induced apoptosis as compared to empty vector transfected cells; sensitization correlated with the level of XAF1 expressed. Furthermore, overexpression of only the zinc-finger portion of XAF1 blocked IFN sensitization of A375 melanoma cells to the proapoptotic effects of APO2L/TRAIL [87]. These results suggested that induction of the ISG, XAF1, possibly in part due to its interaction with XIAP, determined directly cellular sensitivity to the proapoptotic actions of APO2L/TRAIL and indirectly IFNs. In melanoma, renal carcinoma and myeloma cells, if both were not induced by IFNs, no apoptosis resulted, an effect confirmed by use of MAb to APO2L/TRAIL and a dominant negative construct of XAF1 [72,87]

Epigenetic silencing of gene expression

Expression profiling of melanoma cell lines when compared to melanocytes identified many ISGs that were constitutively suppressed [45]. Although direct consequences for malignant cell function or survival was not evaluated, gene profiling and expression studies had identified genes in IFN pathways that were epigenetically silenced by hypermethylation of their 5′ regulatory regions [51–56,109,110]. Maintenance of DNA methylation in promoters can result in heritable silencing of genes that control DNA stability, cell proliferation, and apoptosis, and like mutation, are integral to the neoplastic process [90–94]. The relative degree of importance of the maintenance DNA methyltransferase 1 (DNMT1) and *de novo* DNMTs such as DNMT 3b [95] is still an unresolved issue; however, at least in colon, breast, and lung cancer cell lines, inhibition of DNMT1 by oligonucleotide antisense or siRNA was sufficient for re-expression of silenced genes [96,97].

Sensitization to apoptosis in cell lines resistant to IFN-β occurred at 5-Aza-dC concentrations that alone did not result in apoptosis and resulted in an augmented apoptotic response to IFNs (Fig 3) [57]. 5-Aza-dC and also an antisense to DNMT1 (DNMT1 AS) depleted available DNMT1 protein (no similar effect with control oligonucleotide for DNMT1 AS); both DNMT1 AS and 5-Aza-dC resulted in augmented apoptotic response to IFNs in SK-RC-45 renal carcinoma (up to 80% apoptotic cells with IFN-β) and A375 cells, which were otherwise resistant to IFN-induced apoptosis (Fig 3). Confirming these results was an almost 10x reduction with 5-azacytidine in IC50 of two melanoma cell lines resistant to apoptosis induction by IFNs, FEMX and Minors (data not shown). In contrast, 5-Aza-dC caused only slight apoptosis in normal kidney epithelial cells or melanocytes (<10% TUNEL+ cells) [57]. Neither IFN-β, APO2L/TRAIL, 5-AZA-dC, nor the combinations resulted any substantial degree of apoptosis in normal melanocytes; representative data for IFN-β are presented (Fig 4). Thus for induction of apoptosis, treatments were relatively specific for malignant cells [58].

DNMT1 inhibitors reactivated the proapoptotic tumor suppressor gene, RASSF1A, in SKRC45 and in ACHN renal carcinoma cells and A375 melanoma cells by demethylating its promoter [57]. A375 cells treated with IFNs had further augmented RASSF1A protein expression [57]. In IFN sensitive WM9 cells, in which RASSF1A was basally expressed, IFN-β increased RASSF1A protein without 5AZA-dC pretreatment. RASSF1A siRNA reduced IFN-induced apoptosis in WM9 cells and in DNMT1 depleted ACHN RCC cells [57] (Fig 5). Conversely lentiviral overexpression of RASSF1A in the absence of reversal by inhibitors of DNMT1

resulted in IFN-β or APO2L/TRAIL-induced apoptosis [57]. These results defined RASSF1A, commonly silenced by DNA methylation in melanoma and renal carcinoma, as an IFN regulated gene that participates in IFN-induced apoptosis [57]. Primary melanomas and nephrectomy specimens have a high frequency of silencing of RASSF1A by promoter hypermethylation [98]. RASSF1A interacts with the proapoptotic kinase MST1 and scaffolding protein CNK1 to induce apoptosis and has sensitized cells to apoptosis induction and mitosis regulation [98–105].

In addition to reduced RASSF1A, XAF1 was also decrease in expression in malignant cells [106]. Treatment of A375 melanoma identified increase in expression by 5-Aza-dC [58]. Methylation specific PCR confirmed demethylation of the 5′regulatory region of XAF1 and DNMT1 depletion and augmented XAF1 protein expression by IFN in A375 melanoma and ACHN cells [58]. XAF1 siRNA reduced IFN-induced apoptosis in 5-Aza-dC treated ACHN cells and in IFN sensitive melanoma cells (A375.S2). Conversely, lentiviral overexpression of XAF1 overcame resistance to apoptosis by IFN-β (Fig 6). In 6/11 melanoma cell lines, greater than 10-fold increase in XAF-1 expression resulted from DNMT1 inhibition (Table 1). Thus expression of XAF1, first identified as an ISG in our array studies, was also epigenetically silenced in melanoma and in renal carcinoma cells. Its re-expression may well contribute to augmentation of apoptosis by interactions with XIAP or by other mechanisms.

Despite induction of APO2L/TRAIL by IFN-β in SK-MEL28 and SK-MEL3 human melanoma cells, neither underwent apoptosis in response to IFN-β or IFN-α2b (1500 U/ml). However pretreatment with 5-Aza-dC (0.1 μM) over 4d sensitized for IFN-induced apoptosis (>70% TUNEL + cells after 100 U/ml of IFN-β (data not shown). As in prior studies, Westerns confirmed decrease in DNMT1. APO2L/TRAIL R1 RNA was increased by 0.1 μM of 5-Aza-dC in SK MEL 28 and SK MEL 3 when assayed by qRT-PCR (Table 1) with a concomitant increase in protein expression on the cell surface of APO2L/TRAIL R1(unpublished, S. Bae, V. Cheriyath, 2006). Thus resistance to apoptosis from IFNs or APO2L/TRAIL could also be overcome by 5-Aza-dC in these cells through an increase in APO2L/TRAIL R1 expression

To demonstrate the potential applicability of findings to other histologies, we have extended studies of methylation inhibition to hepatocellular carcinoma (HCC) with IFN-β and leiomyosarcomas (LMS) with APO2L/TRAIL in cells completely resistant to the cytokines For example, in two LMS cell lines fewer than 5% apoptotic cells were identified after APO2L/TRAIL alone but almost 100% apoptotic cells resulted from pretreatment with 5-Aza-dC (Fig 7). In the LMS cells, the role of RASSF1A was suggested by identification of >1000x augmentation of RASSF1A by qRT-PCR after 5-Aza-dC. Similar results occurred with IFN-β in HCC cell lines (Y Ren, E Borden, unpublished, 2006).

Overall summary and perspectives

The focus of our research has been and is to define cellular effects of IFNs and how to thus enable IFNs and their induced genes, such as APO2L/TRAIL, to reach their full clinical antitumor potential. Expression of APO2L/TRAIL proved necessary but not sufficient for apoptosis induction. To identify new therapeutic strategies for IFNs in melanoma, we have sought ways to overcome resistance. The putative tumor suppressor genes, XAF1 and RASSF1A, which are ISGs augmenting apoptosis, have been identified. The low clinical levels in melanomas of XAF1 and RASSF1A (and also possibly APO2L/TRAIL-R1) may reflect epigenetic silencing; our studies of reversal of hypermethylation by 5-Aza-dC have identified markedly augmented gene expression and functional effects. These results have led to design of a Phase I combination trial to establish gene modulation of the combination of 5-Aza-dC with IFNA. In addition to the in vitro studies, we have also established effects of 5-Aza-dC in combination with IFN-β in human tumor xenografts in nude mice [58]. Further rationale for

such a trial was increase in global DNA demethylation in peripheral blood mononuclear cells (PBMCs) of patients treated with 5-Aza-dC [107].

These latter findings on influence of 5-Aza-dC on gene expression in PBMCs are in accord with our findings on influence of 5-Aza-dC on dendritic cell function. Recent papers suggest methylation silencing of genes could have a critical role in host tumor interactions: changes in antigen presentation, recognition, T cell function, and immunosurveillance [45,107,108,109–114]. We have defined an additional effect of methylation silencing of gene expression in dendritic cells (DC). Human myeloid DCs were matured from peripheral blood with CSF-GM and IL-4, treated with 5-Aza-dC (0.1 uM) for 4d, and then with poly I:poly C, as a representative ligand for TLR3. 5-Aza-dC resulted in a 20x augmentation in IL-12 production when compared to cultures not treated with the methylation inhibitor (Fig 8). Furthermore no increase resulted in IL-10 suggesting a potential influence on enhancement of Th1 cell function and suppression of Th2 cell activity (M Whitmore, E Borden, unpublished).

Increase in expression of other methylation-silenced genes, such as other apoptosis related genes (PTEN, p16), ISGs (IRF7, ISG15, or HLA-DR) or melanoma-associated antigens (MAGEs or membrane proteoglycans) could all also potentiate effects of IFNs, APO2L/TRAIL, immunoaugmenting agents, or cytotoxics such as cisplatin for melanoma and other tumors (Fig 7, Fig 9). Furthermore, clinical findings on reversal of methylation could further stimulate studies of epigenetic silencing in other signaling pathways, such as wnt, hedgehog, steroid hormones, or those influencing angiogenesis, which may be in part each regulated by promoter methylation [115–118].

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Biography

Ernest C. Borden, M.D. Ernest C. Borden obtained his medical degree from Duke University and joined Cleveland Clinic in 1998 to direct the Center for Cancer Drug Discovery and Development. In 2005 the 5 laboratories in the Case Western Reserve Cleveland Clinic College of Medicine were integrated to form the Center for Hematology and Oncology Molecular Therapeutics (CHOMT) with Dr. Borden named as Director. He is also a staff member in the Department of Solid Tumor Oncology and a Professor in Cancer Biology in the Lerner Research Institute. In the 1980s, he was amongst the first to initiate clinical trials of interferons, the first human protein effective in stimulating immune mechanisms to fight cancer. In addition to developing improved approaches to clinical assessment of interferons and its inducers, Dr.

Borden's laboratory has focused on function and action of genes stimulated by interferons and anti-tumor effects of other biological therapeutics such as monoclonal antibiodies. In addition to targeted biological therapies, he has an international reputation for research and treatment of melanomas and sarcomas. Dr. Borden has also been listed in *Best Doctors of America* for the past 10 years and honored by the Milstein Award from the International Society of Interferon and Cytokine Research (ISICR) in 2004 and as an American Cancer Society Professor of Clinical Oncology. Dr. Borden has also served as consultant to several biotechnology companies, including CIBA GEIGY (now Novartis AG in Basel, Switzerland), Ares-Serono (Geneva, Switzerland), IDEC Pharmaceuticals (LaJolla, CA), Ribozyme Pharmaceuticals Inc. (Boulder, CG) and Igeneon Inc. (Vienna, Austria). He holds 4 patents.

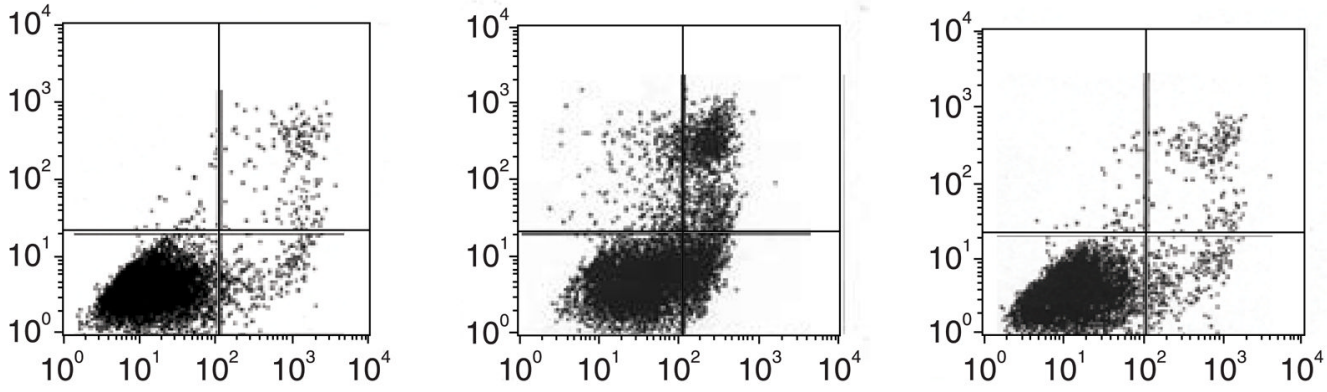

Fig 1. Neutralization of IFN-β mediated apoptosis by APO2L/TRAIL MAb

WM9 cells were untreated (left panel), 6% apoptotic cells by Annexin V assay; treated with IFN-β for 72 h (center), 31% apoptotic cells; or IFN-β with a MAb to APO2L/TRAIL (right), 7% apoptotic cells. Controls (MAb alone, isotype control) for each sample gave expected result. Reproduced with permission.

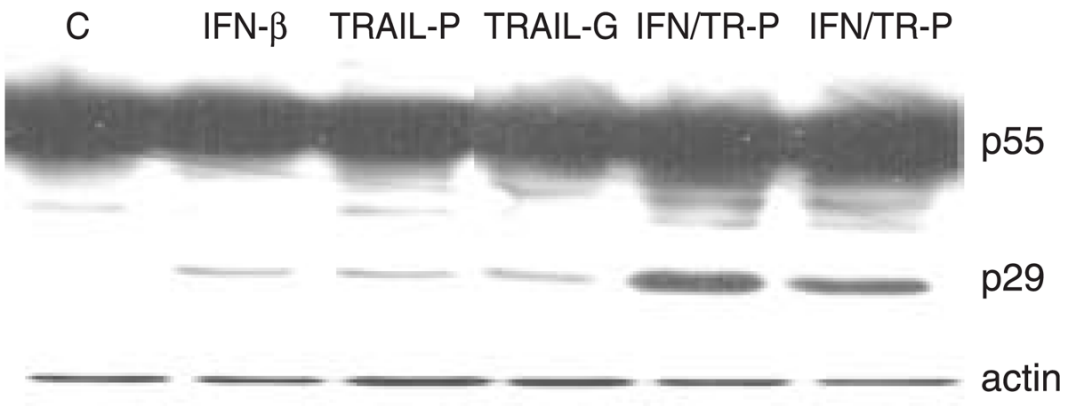

Fig 2.

IFN-β and APO2L/TRAIL synergistically induce cleavage of inactivating p29 fragment from intact XIAP following combination treatment of WM9 cells, detected by Western blot; similar results in A375 (after exogenous APO2L/TRAIL) and WM3211 melanoma cells. No cleavage in cells resistant to apoptosis from IFN-β, APO2L/TRAIL or the combination (WM164, WM35 melanoma) (data not shown). Two different APO2L/TRAIL preparations (Preprotech-APO2L/TRAIL-P and Genentech-APO2L/TRAIL-G). Reproduced with permission.

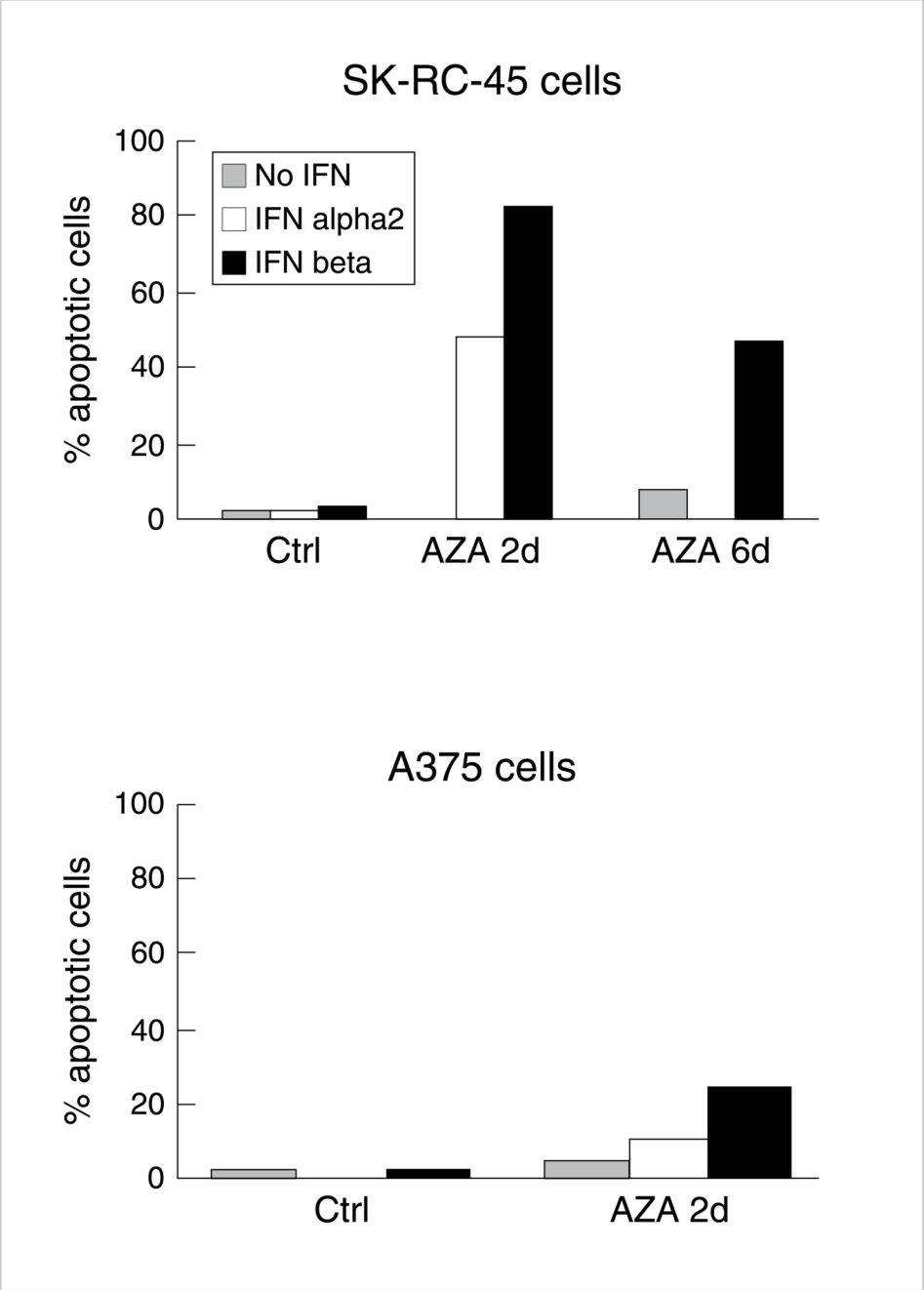

Fig 3. 5-Aza-dC effects on resistance of SK-RC-45 RCC and A375 melanoma cells to IFN-induced apoptosis

TUNEL assay: (FITC) positive DNA was used to assess apoptosis of cells that had been treated with IFNs over 4 d 16h after plating. Cell lines were resistant to apoptosis induction by up to 1500 U/ml of IFN-α2 or IFN-β. Pretreatment with 200 nM 5-Aza-dC, daily over 2–6 d before IFN overcame resistance to apoptosis induction by 50 – 100 U/ml IFN-α2 or IFN-β, while causing little to moderate apoptosis alone (5–20% TUNEL + cells). Marked reduction in DNMT1 protein was confirmed d after 4d of 5-Aza-dC at 200 nM on western blot in SK-RC-45, and A375 cells. Reproduced with permission (57).

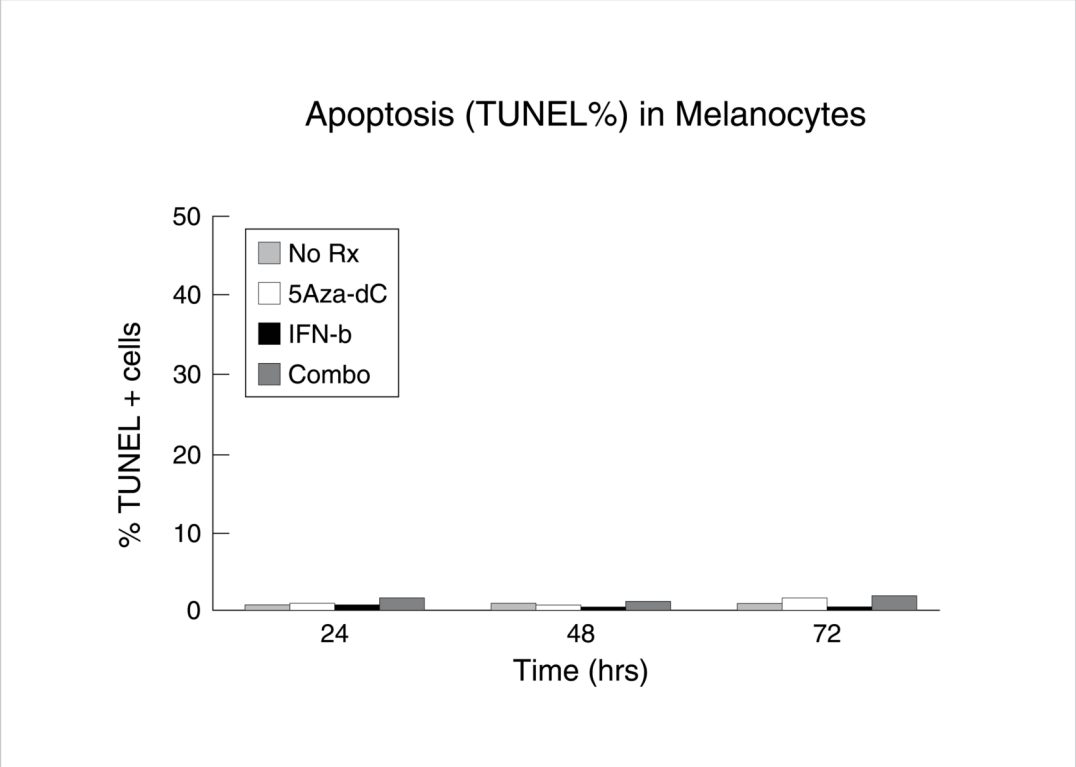

Fig 4. Lack of effect of 5-aza-dC on apoptosis induction for melanocytes or in combination with IFN-β

Normal human melanocytes were obtained from Cambrex Bioscience, Walkersville MD. Melanocytes were cultured from neonatal foreskins, confirmed for gp75/TRP-1 and L-DOPA activity, maintained in the Clonetics Melanocyte culture system according to manufacturer instructions, and then treated with 0.2 μM 5-aza-dC, 250 units of IFN-β, or the combination for the indicated times and apoptosis quantified by TUNEL assay (contrast to quantitative effects on apoptosis induction in tumor cells in Figs 3, 4–7, 9).

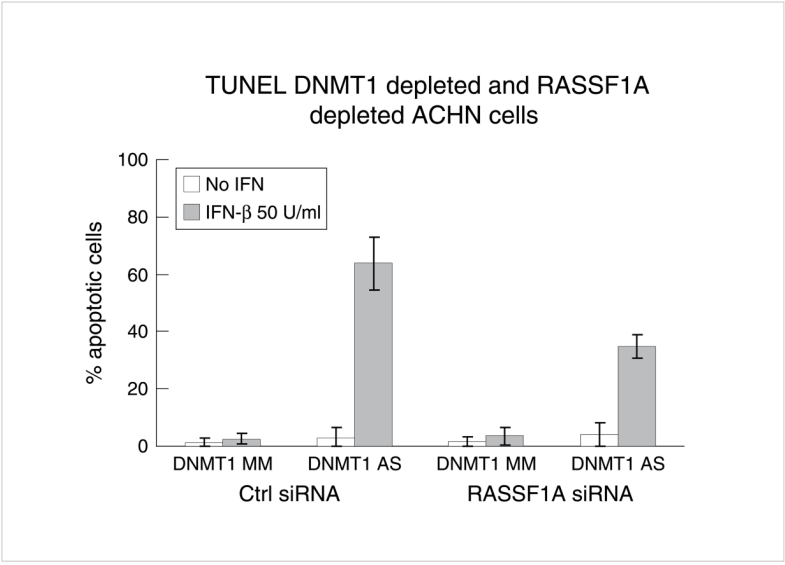

Fig 5. siRNA inhibition of apoptosis resulting from RASSF1A reactivation

ACHN RCC were transfected first with an antisense to DNMT1 or mismatch (8d), then treated with siRNA to RASSF1A for 4 hrs, then IFN-β for 16hrs later. Apoptosis by TUNEL assay after 4 additional days Reproduced with permission (57)

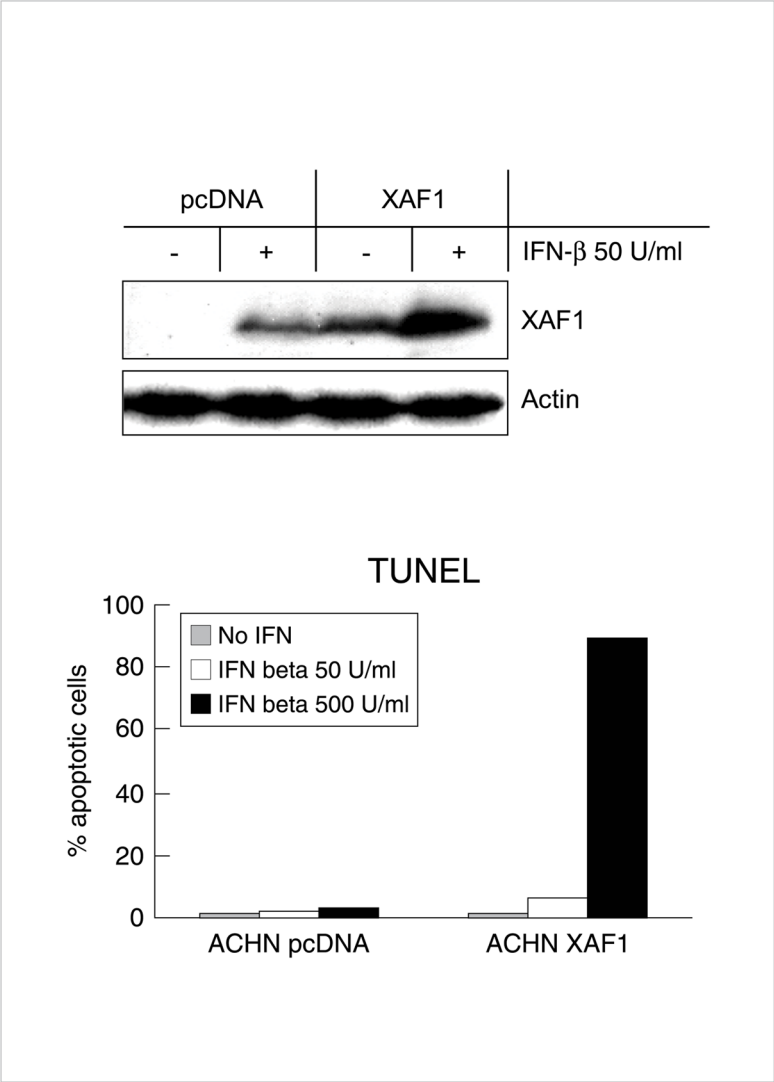

Fig 6.

Reactivation of XAF1 in A375 melanoma cells pretreated with 5-Aza-dC at 200 nM daily over 4d levels achieved by DNA demethylation. Similar effect in ACHN cells. **Resistance of apoptosis induction by IFN-β overcome by overexpression of XAF1.** Overexpression of XAF1plasmid vector in ACHN cells overcame resistance to high doses of IFN-β (500 U/ml, hatched), while lower doses (50 U/ml, solid) had less effect suggesting that reactivation Reproduced with permission. (58)

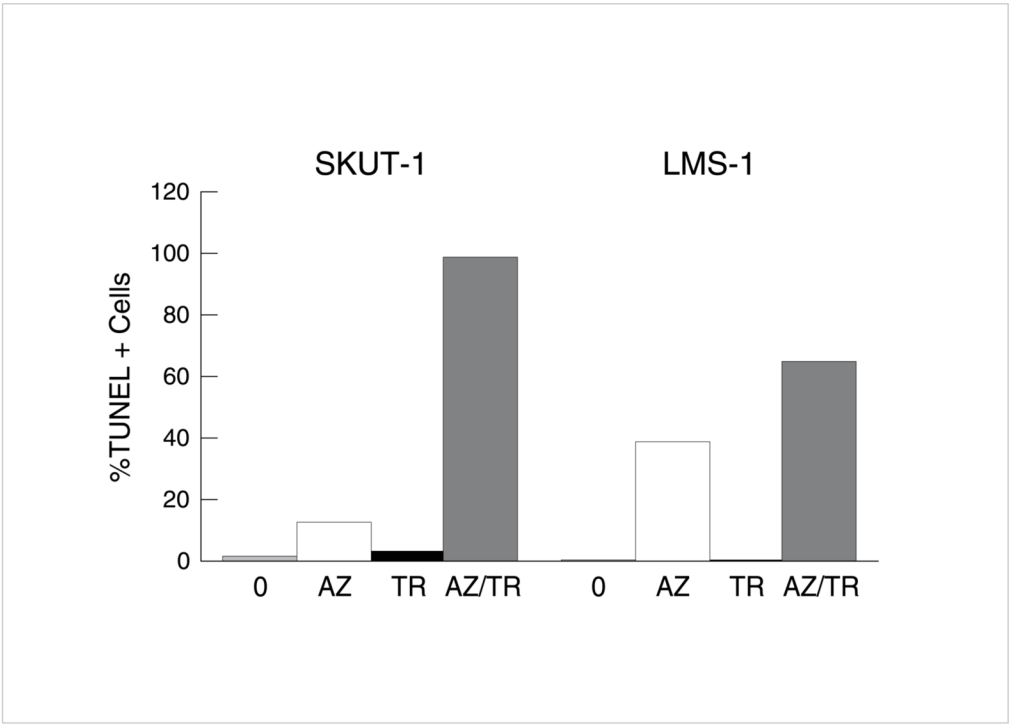

Fig 7. Induction of apoptosis in APO2L/TRAIL-resistant leiomyosarcoma by 5-Aza-dC

SKUT1 and LMS-1 human leiomyosarcoma cell lines, both APO2L/TRAIL resistant, were treated with 5-Aza-dC. Apoptosis was assessed 3d after APO2L/TRAIL (TR) (100ng/ml). Cells were pretreated for 4d with 5-Aza-dC (0.2μM). After wash, APO2L/TRAIL was added. Apoptosis assessed by TUNEL assay 4d later.

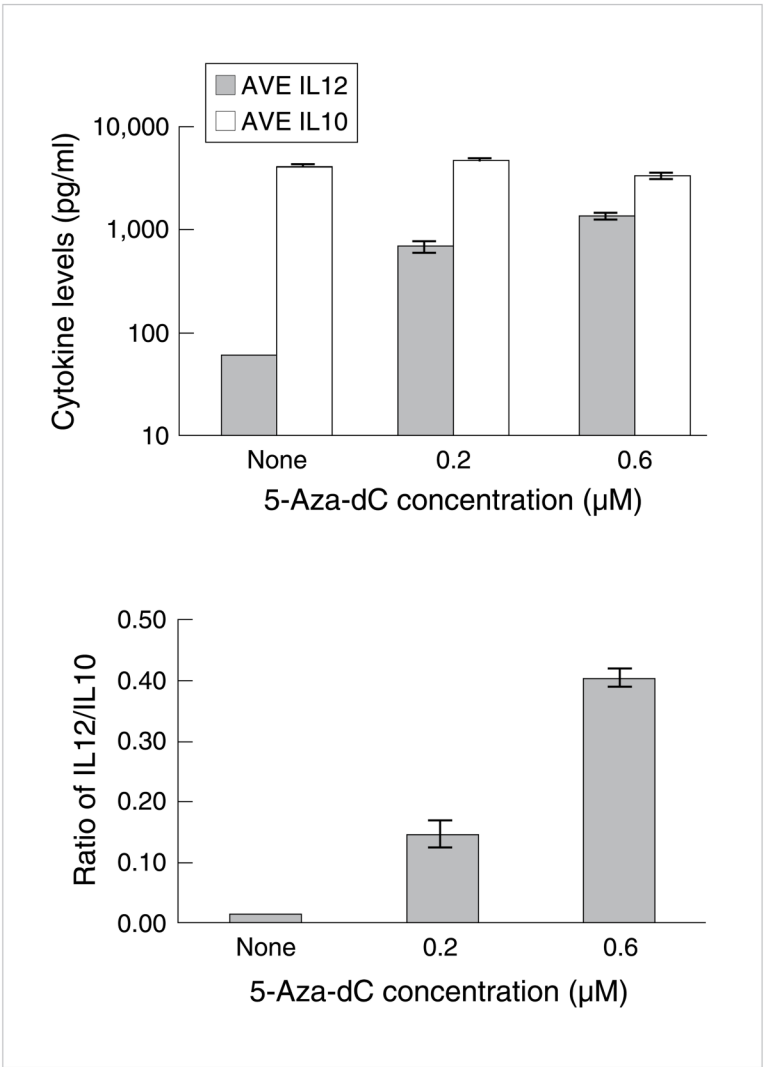

Fig 8. Augmentation of IL-12 production by human dendritic cells by 5-aza-dC

Human myeloid DCs were matured from peripheral blood with CSF-GM and IL-4, treated with 5-Aza-dC (0.1 uM) for 4d, and then poly I:poly C, as a representative ligand for TLR3.

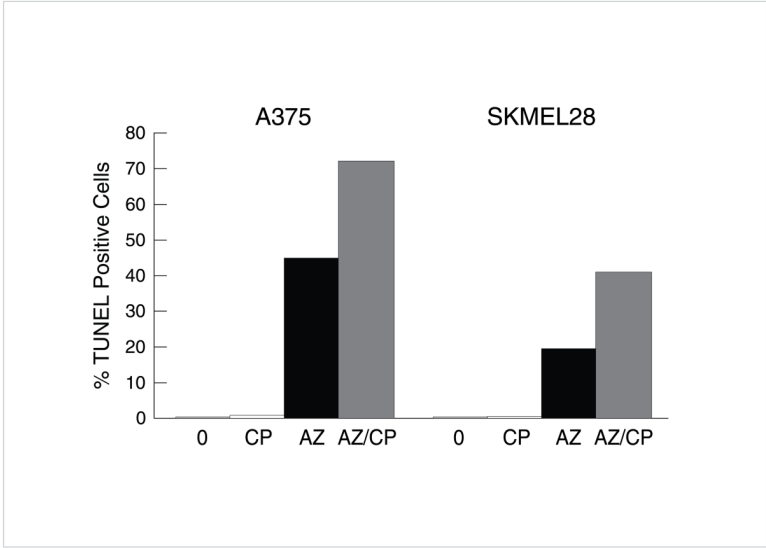

Fig 9. Augmentation of apoptosis by 5-Aza-Dc (AZ) in cisplatin (CP)-resistant melanoma cells (A375 or SKMEL 28)

Cells were first treated with 5-Aza-dC (0.2uM) for 96 hrs, then cisplatin (10uM) added and apoptosis assessed 96 hrs later by TUNEL assay. In both cell lines, RASSF1A was increased >500x by 5-Aza-dC.

Table 1

For qRT-PCR all cell lines were treated with 0.2 μM of 5-AZA-dC for 96 h. Total RNA was extracted using TRIzol Reagent according to the manufacturer's protocol. Two μg of RNA was used for reverse transcription in 20μl of reaction mixture using a Superscript III kit. For quantitative PCR, a reaction mixture of 25 μl of TaqMan Universal PCR Master Mix, 250 nM of Taqman primer, and 60 ng of cDNA were prepared. Primers were purchased from Applied Biosystems for APO2L/TRAIL R1, APO2L/TRAIL R2, and XAF1. GAPDH for gene normalization was measured by TaqMan Human GAPDH control reagents. MUM-2C, MUM-2B, C918, and OCM-1A are ocular melanoma cell lines, while all others are cutaneous melanoma cell lines.

Melanoma Cell	Fold Increase in Gene Expression from 5-Aza-dC		
	XAF-1	APO2L/TRAIL R1	APO2L/TRAIL R2
MUM 2C	150	6	230
MUM 2B	60	<5	<5
C918	20	<5	<5
OCM-1	120	<5	<5
A375	70	<5	<5
SKMEL 1	<5	<5	<5
SKMEL 3	<5	70	<5
SKMEL 28	<5	30	<5
WM164	<5	6	<5
WM3211	<5	<5	<5
MeWo	30	7	<5