Published in final edited form as:

J Cell Physiol. 2013 April ; 228(4): 764–772. doi:10.1002/jcp.24224.

Inhibition of PRC2 histone methyltransferase activity increases TRAIL-mediated apoptosis sensitivity in human colon cancer cells

Yannick D. Benoit¹, Kristian B. Laursen¹, Mavee S. Witherspoon², Steven M. Lipkin^{2,3}, and Lorraine J. Gudas^{1,2,*}

¹Pharmacology Department, Weill Cornell Medical College, New York, New York 10065, USA

²Department of Medicine, Weill Cornell Medical College, New York, New York 10065, USA

³Department of Genetic Medicine, Weill Cornell Medical College, New York, New York 10065, USA

Abstract

Colorectal cancer is ranked among the top leading causes of cancer death in industrialized populations. Polycomb group proteins (PcGs), including Suz12 and Ezh2, are epigenetic regulatory proteins that act as transcriptional repressors of many differentiation-associated genes and are overexpressed in a large subset of colorectal cancers. Retinoic acid (RA) acts as a negative regulator of PcG actions in stem cells, but has shown limited therapeutic potential in some solid tumors, including colorectal cancer, in part because of RAR β silencing. Through treatment with RA, Suz12 shRNA knockdown, or Ezh2 pharmacological inhibition with 3-deazaneplanocin A (DZNep), we increased TRAIL-mediated apoptosis in human colorectal cancer cell lines. This increased apoptosis in human colon cancer cells after RA or DZNep treatment was associated with a ~2.5-fold increase in TNFRSF10B (DR5) transcript levels and a 42% reduction in the H3K27me3 epigenetic mark at the TNFRSF10B promoter after DZNep addition. Taken together, our findings indicate that pharmacological inhibition of PRC2 histone methyltransferase activity may constitute a new epigenetic therapeutic strategy to overcome RA non-responsiveness in a subset of colorectal tumors by increasing TRAIL-mediated apoptosis sensitivity.

Keywords

Polycomb; Retinoic acid; Epigenetics; Apoptosis; Colon cancer

Introduction

Colorectal cancer is among the leading causes of cancer death in the USA (Jemal et al., 2011). Following chemotherapy, more than 50% of patients will relapse, and this is associated with metastasis (Rodriguez-Moranta et al., 2006). Colorectal tumors of higher stages are notoriously resistant to chemotherapy and show a poor, 10% 5-year survival (Jemal et al., 2011). Consequently, it is crucial to develop new strategies to improve treatments, particularly for advanced disease.

*To whom correspondence should be addressed: Dr. Lorraine J. Gudas, Department of Pharmacology, Weill Cornell Medical College, New York, New York 10065, USA, Phone: (212) 746-6262, Fax: (212) 746-8835, ljgudas@med.cornell.edu.

Retinoids are biologically active metabolites of vitamin A (retinol) that play essential roles in vertebrate embryonic development via the regulation of aspects of cell growth, differentiation, and apoptosis (Gudas and Wagner, 2011; Mongan and Gudas, 2007; Niederreither and Dolle, 2008). Specifically, the physiological effects of retinoids are mediated by heterodimers of retinoic acid receptors (RARs α , β , γ) and retinoid X receptors (RXRs α , β , γ) of the steroid/nuclear receptor family that regulate chromatin structure and transcriptional activity (Bastien and Rochette-Egly, 2004). All-trans retinoic acid (RA) has been extensively used in many different protocols to initiate the differentiation of embryonic stem cells (Gudas and Wagner, 2011). Additionally, RA signalling is frequently compromised early in carcinogenesis and a reduction in retinoid signalling may be required for tumor development (Tang and Gudas, 2011). Significant, anti-tumorigenic effects of RA and other retinoids have been reported in promyelocytic leukemia, as well as in different types of carcinomas, such as prostate, breast, and colon (de The and Chen, 2010; Tang and Gudas, 2011; Zhang et al., 2010). For instance, retinyl acetate was recently shown to increase TNF-related apoptosis-inducing ligand (TRAIL) dependent cell death in mouse *adenomatous polyposis coli* (Apc)-mutant intestinal cells, without affecting normal cells, by up-regulating TRAIL-activated death receptors TNFRSF10A and B (DR4 and DR5) transcripts and by reducing decoy receptor TNFRSF10C and D (DcR1 and DcR2) transcripts (Zhang et al., 2010). APC-null cells activate caspase-8 in response to death receptor stimuli through the repressive effect of c-myc on the cellular FLICE-like inhibitory protein (c-FLIP) (Dhandapani et al., 2011; Zhang et al., 2010). In addition, Dhandapani *et al.* recently identified retinoic acid response elements (RAREs) in the promoter of human death receptor-4 gene (TNFRSF10A), confirming the direct effects of retinoids on TRAIL-mediated apoptosis in head and neck carcinoma cells (Dhandapani et al., 2011). Conversely, epigenetic silencing of both RAR β promoters has been reported in a variety of human cancers (Mongan and Gudas, 2007; Sirchia et al., 2000; Youssef et al., 2004). Such epigenetic silencing can render tumor cells refractory to the growth inhibitory, pro-differentiation, and pro-apoptotic effects of RA, resulting in poor outcomes in the treatment of advanced stage colorectal cancer.

Polycomb group proteins (PcGs) are epigenetic, transcriptional repressors for differentiation-associated genes and play key roles in embryonic stem cell self-renewal and pluripotency (Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010). Briefly, PcGs form multiprotein complexes that possess histone methyltransferase (HMTase) activity responsible for the trimethylation of lysine 27 of histone H3 (H3K27me3) (Sauvageau and Sauvageau, 2010). PcGs are divided into two main transcriptional repressive complexes, PRC1 and PRC2. EED, EZH1/2, and SUZ12 proteins constitute the core components of the PRC2 complex (Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010). The PRC2 complex plays an initiating role in PcG-mediated epigenetic repression by generating specific H3K27me3 marks associated with subsequent DNA methyltransferase (DNMT) recruitment (Margueron and Reinberg, 2011). In contrast, the PRC1 complex is recruited to the epigenetic marks established by the PRC2 complex and is responsible for the sustained, PcG-mediated repression of target genes through histone ubiquitination, chromatin condensation, and RNA polymerase II inhibition (Luis et al., 2012; Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010). Dysregulated expression of several PcG proteins, including Suz12 and Ezh2, was recently documented in different types of human tumors, including colon cancer, and is potentially associated with a poor prognosis (Benoit et al., 2012; Crea et al., 2012; Iliopoulos et al., 2010; Margueron and Reinberg, 2011).

Our laboratory demonstrated that RA signalling is crucial for PcG displacement from promoters of development-associated genes, such as *Hoxa1* and *Cyp26a1*, in F9 teratocarcinoma cells and mouse embryonic stem (ES) cells (Gillespie and Gudas, 2007; Kashyap et al., 2011). SUZ12, a key protein for the assembly/association of the Polycomb

repressive complex 2 (PRC2), was rapidly removed from all of the RA-responsive target genes we tested following RA addition (Amat and Gudas, 2011; Gillespie and Gudas, 2007; Kashyap et al., 2011). Since the TRAIL-activated death receptors were recently identified as retinoid target genes in Apc-mutant intestinal cells (Zhang et al., 2010), we hypothesized that reducing the activity of the PRC2 complex could increase TRAIL-mediated apoptosis in human colorectal cancer cells. Since the PcGs act downstream of RA in the RA signalling pathway, inhibiting PRC2 activity could potentially overcome the resistance to RA associated with the RAR promoter methylation observed in many human colorectal cancers (Tang and Gudas, 2011). Here we report that inhibition of the PRC2 HMTase activity of EZH2 leads to increased TRAIL-mediated apoptosis in human colorectal cancer cells, which is associated with increased TNFRSF10B death receptor expression.

Materials and Methods

Tissue culture and Reagents

Human colon cancer cell lines (HT29, SW480) and human breast cancer cells (MCF-7) were authenticated and obtained from ATCC, and cultured as previously described (Mongan and Gudas, 2005; Sikandar et al., 2010). SW480 cells stably expressing human RAR β and RAR γ receptors and empty vector controls (EV) were established by retroviral infection, as described (Benoit et al., 2010). pQCXIH retroviral vectors containing mouse RAR β 2, RAR γ 2 and eGFP coding DNA sequences were used to generate viral particles in 293T cells (Benoit et al., 2010). Cells were selected using 500 μ g/ml of hygromycin B (Roche) for 15 days. Stable Suz12 knockdown (shRNA sequences TRCN0000038726 and TRCN0000038728) and control shLuciferase (shLuc) HT29 and SW480 cell populations were established as described (Benoit et al., 2012). *All-trans* retinoic acid (RA) (Sigma) and human recombinant TRAIL (R&D Systems) were used at 1 μ M and 50 ng/ml, respectively. 3-Deazaneplanocin A (DZNep) from the National Cancer Institute (Bethesda, MD), was used at 5 μ M in water. For growth curve experiments, 1.0×10^5 cells were plated in 60 mm dishes by day 0, and DZNep was added (5 μ M) 6 hours after plating. Total cell number was counted by day 2, 3, and 4 post-seeding using a Beckman Coulter Z2 Particle Counter.

RNA isolation and PCR reactions

Total RNA was extracted using TriPure isolation reagent (Ambion). mRNA was reverse-transcribed using the qScript cDNA SuperMix (Quanta Biosciences). Semi-quantitative and quantitative PCR assays were performed as described (Laursen et al., 2012). Sequences of primers used for PCR reactions are in Table 1.

Western Blot analysis

Proteins were extracted in SDS final sample buffer, separated by SDS-PAGE, and transferred onto nitrocellulose membranes as previously described (Benoit et al., 2010). Membranes were blocked in PBS containing 5% skim milk and 0.1% TWEEN 20 (BioRad). Primary antibodies used are described (Table 2). For quantitative optical densitometry analysis, Western blot bands were scanned and analyzed using Image J software (National Institutes of Health).

Indirect Immunofluorescence staining and TUNEL assays

Immunofluorescence staining of cells was performed as described (Benoit et al., 2009). Briefly, cells were seeded on serum-pretreated glass coverslips (Fisher) 24 hours prior to treatment. Cell monolayers were fixed using 4% (w/v) paraformaldehyde (Sigma) and membrane permeabilization was performed with 0.3% (w/v) Triton-X 100 (Sigma). 2% BSA (Sigma) was used for blocking for 30 minutes at room temperature prior to incubation

with primary antibodies (Table 2). Phalloidin-TRITC (Millipore, FAK100, 1:1000) was used to stain actin stress fibers (F-actin). Nuclei were stained using DAPI contained in Vectashield® mounting medium for fluorescence (Vector labs). For terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays, permeabilized cells were incubated with *In Situ* Cell Death Detection Kit Fluorescein® enzymatic reaction mixture (Roche) for 1 hour at 37°C according to the manufacturer's instructions (www.roche-applied-science.com). TUNEL positive and DAPI stained cells were counted using NIS-Elements Advanced Research software (Nikon).

Chromatin immunoprecipitation (ChIP) assays

A one-step ChIP protocol involving formaldehyde cross-linking was used for histone chromatin immunoprecipitation assays, as previously described (Gillespie and Gudas, 2007; Kashyap et al., 2011; Laursen et al., 2012). Briefly, immunoprecipitations (IP) of sonicated chromatin were performed using 2 µg of specific antibodies (Table 2). qPCR amplifications of TNFRSF10A, B and D promoter fragments, as well as of a control intergenic region (Jia et al., 2008) were carried out using the primers described in Table 1. The accurate size of each PCR product was confirmed by electrophoresis on 1.5% agarose gel.

Statistical analysis

All experiments were performed at least 3 times using independent, biological triplicates. Results are presented as means ± SEM. All statistical tests were performed using GraphPad InStat software version 3.10. For TUNEL data analysis, one-way ANOVA followed by Bonferroni post test correction was performed. For all other data, the two-tailed *t*-test was applied. P values of 0.05 were considered significant.

Results

Retinoic acid and TRAIL-mediated apoptosis induction in human colorectal cancer cells

Because of their anti-proliferative, pro-differentiation, and pro-apoptotic roles in many types of mammalian cells, retinoids have great potential for cancer prevention and treatment (de The and Chen, 2010; Tang and Gudas, 2011; Zhang et al., 2010). Retinoids re-sensitize pre-neoplastic APC-mutant, intestinal epithelial cells to TRAIL-mediated apoptosis, suggesting a useful cancer prevention strategy (Zhang et al., 2010). However, in several types of cancer, including colon cancer, epigenetic silencing of RARβ expression leads to retinoid resistance (Tang and Gudas, 2011). We defined the RAR (retinoic acid receptor) mRNA expression profiles in HT29 and SW480, two well-characterized, human colorectal cancer cell lines. Both of these lines are APC-deficient, have elevated tumorigenic potential, and show a poorly differentiated phenotype (Trainer et al., 1988). Semi-quantitative RT-PCR analyses were performed to assess RXRα, RARα, RARβ, and RARγ transcript levels. As previously described, all four receptor transcripts were expressed by HT29 cells (Figure 1A) (Lee et al., 2000; Nicke et al., 1999b). In contrast, SW480 cells exhibited very low RARβ mRNA levels (88% less than the level in HT29) and ~2-fold lower levels of RARγ mRNA as compared to HT29 cells, while the RXRα and RARα transcript levels were similar in both cell lines (Figure 1A).

We then assessed the ability of RA to increase TRAIL-associated apoptosis in these two tumor lines. We treated HT29 and SW480 cells with RA or TRAIL alone, as well as with both drugs together, and compared the number of apoptotic cells in each treated group with the number in untreated controls. The combined RA and TRAIL treatment resulted in a dramatic, 8.3-fold increase (±1.19-fold, *p*<0.0001) in the number of apoptotic HT29 cells compared to the RA-alone, TRAIL-alone, and control groups. However, we observed no

significant changes in the numbers of apoptotic SW480 cells in different treatment conditions (Figure 1B, upper panel).

The immunodetection of Poly-(ADP-ribose) polymerase (PARP), which constitutes a specific substrate for activated caspase-3, confirmed the TUNEL analyses. We observed a stronger band corresponding to PARP (89 kDa cleaved form) only in HT29 cells treated with RA and TRAIL. No significant changes were observed in PARP cleavage in SW480 cells under each of the tested conditions (Figure 1B, lower panel).

As mentioned above, RA signaling is crucial for death receptor transcriptional regulation (Dhandapani et al., 2011; Zhang et al., 2010). Quantitative PCR analyses revealed a 2.2-fold increase in TNFRSF10B transcript levels, and a 7.7-fold reduction in decoy receptor TNFRSF10D levels following RA treatment in HT29 cells (Figure 1C). TNFRSF10A mRNA was unaffected by RA treatment of HT29 cells (Figure 1C). In contrast, the transcript levels of all 3 death receptors tested in SW480 cells decreased in RA treated cells compared to untreated controls (Figure 1C).

The aforementioned results suggest that the decreased levels of RAR β and RAR γ receptor transcripts in SW480 cells could impair TRAIL mediated apoptosis. In order to test this hypothesis, mouse full-length RAR β 2 and RAR γ 2 clones, driven by a CMV promoter, were ectopically re-expressed in SW480 cells by retroviral stable infection. An eGFP vector was used as a control. Ectopic expression of both RAR β 2 and RAR γ 2 was confirmed at the transcript level (Figure 2A). TUNEL assays performed with SW480 eGFP, RAR β 2, and RAR γ 2 stable cell populations revealed significantly increased levels of apoptosis in RA plus TRAIL treated RAR β (~35-fold) and RAR γ (~12-fold) expressing SW480 cells compared to the other experimental conditions (Figure 2B). Thus, active RA signaling greatly enhances TRAIL-mediated apoptosis in colon cancer cells that express high levels of RAR β and RAR γ receptors.

Polycomb group proteins act as downstream effectors of retinoid signaling

Polycomb group proteins (PcG) were previously identified as downstream targets of RA signaling in F9 and ES cells (Amat and Gudas, 2011; Gillespie and Gudas, 2007; Gudas and Wagner, 2011). In these cells, RA treatment results in a decrease of histone H3 lysine 27 trimethylation (H3K27me₃), a marker of active PRC2 transcriptional repression (Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010). Thus, one potential way to circumvent RAR silencing in colon cancer and re-sensitize malignant cells to TRAIL mediated apoptosis could be through the inhibition of RA signaling downstream targets, *i.e.* PcG proteins. In order to test whether PcGs influence colorectal cancer cell survival, we established shRNA-based stable Suz12 knockdowns in HT29 and SW480 cells. Targeting Suz12 has already been shown to disrupt PRC2 complex functionality, reflected by a decrease in PRC2-associated epigenetic histone marks (Benoit et al., 2012). In addition to previous validation experiments for the shRNA constructs used herein (Benoit et al., 2012), indirect immunofluorescence and Western blot analyses confirmed the lower Suz12 protein levels in both HT29 and SW480 Suz12 shRNA stable knockdown lines (Figure 3A, B) as compared to the luciferase shRNA lines. We observed no Suz12 knockdown when we compared the parental cell lines with control, non-silencing (shLuc) shRNA infected stable cell lines (Figure 2A, B).

To validate the specificity of the Suz12 knockdown on the PRC2 associated epigenetic marks in these cell lines, immunodetection of EZH2-associated H3K27me₃ (Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010), the trithorax group-mediated H3K4me₃ mark, and the H3K36me₂ elongation mark (Schmitges et al., 2011) was performed. In both of the Suz12 knockdown cell lines only the H3K27me₃ mark was decreased compared to

the parental HT29 and SW480 lines (not shown) and the shLuc stable lines, and other epigenetic marks were unchanged (Figure 2B). For each case, off-target effects from the Suz12 lentiviral shRNA system were assessed using a second shSuz12 sequence (Figure 2A + data not shown). TRAIL-mediated apoptosis was then measured in the Suz12 knockdown and control HT29 and SW480 cells by TUNEL (Figure 3C) and PARP cleavage (Figure 3D) analyses. In contrast to the experiments involving RA-treated cells in which only HT29 cells exhibited TRAIL-associated apoptosis (Figure 1B), both HT29 and SW480 cells demonstrated an 8 to 10-fold increased apoptosis induction when Suz12 knockdown was combined with TRAIL treatment (Figure 3C). Our results suggest that PRC2 disruption induces TRAIL-associated apoptosis in human colorectal cancer cells, irrespective of their RA receptor mRNA levels.

Pharmacological inhibition of PRC2 in human colorectal cancer cells enhances apoptosis

As lentiviral-based therapeutic strategies have limitations in patients, we focused our attention on a pharmacological approach in order to inhibit PRC2 activity. 3-Deazaneplanocin A (DZNep) was recently identified as a potent EZH2 inhibitor (Tan et al., 2007). Initially developed as an inhibitor of S-adenosylhomocysteine hydrolase (Glazer et al., 1986), DZNep was also shown to reduce H3K27 trimethylation in various cancer cell types and in ES cells (Crea et al., 2012). In addition, the effectiveness of DZNep in reducing PRC2-associated epigenetic marks in HT29 and SW480 cells was demonstrated previously (Glazer et al., 1986; Jiang et al., 2008). As expected, we saw a marked decrease in H3K27me3 levels in DZNep treated HT29 and SW480 cells compared to untreated controls (Figure 4A). While the effect of DZNep was relatively specific for H3K27me3 inhibition in HT29 cells, DZNep also reduced H3K4me3 and H3K36me2 marks in SW480 cells (Figure 4A). As reported by others, DZNep treatment caused a decrease in Suz12 protein levels in SW480 cells, whereas Suz12 protein level was unaffected in HT29 cells (Figure 4A). No changes were observed in EZH2 protein levels following DZNep treatment in both tested cell lines (Figure 4A).

Growth inhibition caused by RA was previously documented in human colon cancer cells, including HT29 and SW480 lines (Nicke et al., 1999b; Paulsen and Lutzow-Holm, 2000; Shah et al., 2002), and such a process was proposed to be mediated through either RAR α or RAR β receptors (Nicke et al., 1999a; Nicke et al., 1999b). Consistently, we observed large reduction in cell counts in the DZNep treated HT29 and SW480 cultures as compared to untreated controls (Figure 4B). Such a decrease in cell counts is, by deduction, caused by growth inhibition since by TUNEL assays (Figure 4C) and PARP cleavage immunodetection (Figure 4D) DZNep treated cells, compared to controls, exhibited no changes in apoptosis. However, we detected greater than 10-fold increases in apoptosis in HT29 and SW480 cells when these cells were subjected to combined DZNep and TRAIL treatment (Figure 4C, D).

Pharmacological inhibition of PRC2 increases specific TRAIL death receptor transcripts

As mentioned above, TRAIL induces apoptosis through its association with two closely related transmembrane receptors, TNFRSF10 A and B (DR4 and DR5) (Takeda et al., 2007). We investigated the effect of DZNep on promoter epigenetic regulation and on transcript levels for the death receptors TNFRSF10A and TNFRSF10B, as well as the decoy receptor TNFRSF10D, in HT29 and SW480 cells. Because of the specific, inhibitory effects of DZNep on the placement of the H3K27me3 marks in HT29 cells (Figure 4A), these cells were used for investigations of promoter regulation and transcript expression. From chromatin immunoprecipitation (ChIP) assays we observed a decrease in the PRC2-associated H3K27me3 mark at the TNFRSF10A and TNFRSF10B promoters (56%, $\pm 5.9\%$, $p=0.015$ and 42%, $\pm 10.1\%$, $p=0.023$ respectively) in DZNep-treated, as compared to

untreated HT29 cells (Figure 5A). We also detected a similar decrease in H3K27me3 at TNFRSF10D but this was statistically non-significant. We detected a ~56% ($\pm 5\%$, $p=0.041$) decrease in the level of the PRC1-associated epigenetic mark H2K119ub at the TNFRSF10B promoter following DZNep treatment (Figure 5A). A similar, but non-significant, trend was observed for H2K119 ubiquitylation at the TNFRSF10A and TNFRSF10D promoters (Figure 5A). No changes in PcG-associated marks (H3K27me3 and H2K119ub) were observed at a random, intergenic region in DZNep-treated compared to untreated HT29 cells (Figure 5A).

In addition to the different histone modifications observed at the TNFRSF10A, TNFRSF10B, and TNFRSF10D promoter sequences, we detected a 2.6-fold increase (± 0.38 , $p=0.0005$) in TNFRSF10B transcript levels in DZNep-treated compared to untreated HT29 cells by qPCR (Figure 5B). No changes were observed in TNFRSF10A and TNFRSF10D transcript levels in DZNep treated HT29 cells (Figure 5B).

These data suggest that DZNep treatment causes a decrease in polycomb repressive complex-related epigenetic repressive marks at the promoters of TNFRSF10A and TNFRSF10B. However, in the HT29 cells only the TNFRSF10B transcript level increased in response to DZNep, matching the observations we made in RA treated cells (Figure 1C). These results suggest that an increase in TNFRSF10B expression is sufficient to sensitize human colon cancer cells to TRAIL-mediated apoptosis.

Discussion

By using human colorectal cancer cell lines we demonstrated that indirect inhibition of EZH2 by DZNep increases TRAIL-mediated apoptosis sensitivity. Treating cells with the PRC2 HMTase inhibitor 3-deazaneplanocin A (DZNep) led to increased expression of the death receptor TNFRSF10B in TRAIL-resistant HT29 and SW480 human cell lines. We also propose that PRC2 inhibition represents a new alternative to overcome RA resistance, associated with epigenetically silenced RAR, in the treatment of colon cancer.

Therapeutic application of retinoic acid in colorectal cancer: Relationship to receptor expression

Retinoids in cancer treatment have shown promise, especially when they are combined with other anti-cancer drugs (de The and Chen, 2010; Dhandapani et al., 2011; Lee et al., 2000; Mongan and Gudas, 2005; Tang and Gudas, 2011; Zhang et al., 2010). Recently, a link was established between RA and TRAIL responsiveness in both colon cancer and head and neck carcinoma cells (Dhandapani et al., 2011; Zhang et al., 2010). Briefly, TRAIL targets only neoplastically transformed cells based on their expression of the pro-oncogene c-myc, which leads to c-FLIP repression and enables subsequent caspase-8 activation (Zhang et al., 2010). However, the development of TRAIL resistance is a major obstacle to effective TRAIL-based therapy (Zhang and Fang, 2005). TRAIL resistance can be caused by dysfunction in various steps of the signaling cascade, and one mechanism to overcome such resistance is to increase expression of death receptors (Sung et al., 2010).

We addressed the fact that aberrant expression of RARs, as observed in SW480 cells, compromises the pro-apoptotic effects of RA (Figure 1). RA treatment induced an increase in TNFRSF10B mRNA in RA-responsive HT29 cells, in accord with previous studies in other neoplastic models (Dhandapani/Zhang 2010), while such an increase in TNFRSF10B mRNA was not observed in RA treated SW480 cells (Figure 1). Surprisingly, SW480 cells exhibited decreased levels of all death receptor transcripts tested following RA treatment. In addition, we demonstrate that increased ectopic expression of RAR β or RAR γ in SW480 cells, cells that express these endogenous receptor transcripts at low levels, leads to an

increase in TRAIL-mediated apoptosis following RA treatment (Figure 2). These data confirmed our hypothesis that the effects of RA plus TRAIL on death receptor-mediated apoptosis depend on the RAR expression profile in colorectal cancer cells.

PRC2 inhibition enhances TRAIL signaling in colon cancer cells

In an attempt to recreate such an effect without any ectopic gene expression, we decided to inhibit the activity of PRC2, an epigenetic silencing complex that is displaced from its target gene promoters by RA signaling in some cell types (Amat and Gudas, 2011; Gillespie and Gudas, 2007; Gudas and Wagner, 2011). Both SUZ12 knockdown and EZH2 pharmacological inhibition led to increased TRAIL-mediated apoptosis in both colon cancer cell lines tested, irrespective of the RA responsiveness of the cells (Figures 3, 4). Moreover, combining RA and DZNep treatment did not cause an additional increase in apoptosis in HT29 and SW480 cells, either in the presence or absence of TRAIL (not shown), suggesting that RA and DZNep act through the same signal transduction pathway.

In accordance with previous observations made in SUZ12 knockdown intestinal progenitor cells (Benoit et al., 2012), inhibition of PRC2 HMTase activity by DZNep inhibited the proliferation of both tested colon cancer cell lines (Figure 4). These data further support the anti-tumorigenic potential of DZNep in human colorectal cancers.

Further investigation of histone tail epigenetic modifications showed that DZNep treatment causes a decrease in several epigenetic marks, including H3K27me3 and H2K119ub, which are associated with active PRC2 and PRC1, respectively (Figure 5A). The statistically significant decreases in H3K27me3 and H2K119ub at the TNFRSF10B promoter suggest a role for PcGs in the negative regulation of transcription of this death receptor (Figure 5A). However, a similar, but not statistically significant trend was also observed at the TNFRSF10A and D promoters. While DZNep affects the levels H3K27me3 by causing EZH2 inhibition, the observed decrease in H2K119ub is assumed to be a direct consequence of H3K27me3 depletion, since PRC1 proteins are generally recruited to target genes by the recognition of the H3K27me3 mark (Margueron and Reinberg, 2011; Sauvageau and Sauvageau, 2010). Of the three tested death receptors, only TNFRSF10B transcripts increased in response to DZNep treatment of colon cancer cells (Figure 5B). Thus, both RA treatment and Ezh2 inhibition were associated with increased TNFRSF10B transcript levels, while TNFRSF10A levels were unaffected. These data further support other studies of the importance of TNFRSF10B in TRAIL responsiveness in colorectal cancer cells (Stolfi et al., 2011; Sung et al., 2010; Zhang et al., 2010). In an attempt to provide an explanation for the specific increase in TNFRSF10B transcript, we performed computational transcription factor binding sites mapping on death receptor promoters using MatInspector software (Genomatix Software GmbH, www.genomatix.de). By analyzing the first 1kb upstream of the transcription start sites of TNFRSF10A, B and D, we observed the presence of RXR heterodimer binding sites in all three proximal promoters (Suppl. Fig 1). We did not detect differences in transcription factor binding sites that could explain why TNFRSF10B, but not TNFRSF10A and D, transcript levels are increased by both RA (Figure 1C) and DZNep (Figure 5B). It would be of interest to knockdown TNFRSF10B to determine if this knockdown prevents the increased apoptosis in response to RA plus TRAIL (Figure 1B) and DZNep plus TRAIL (Figure 4C) in these cells.

Clinical application of DZNep in colorectal cancer treatment

To date, DZNep is one of the most potent inhibitors of Ezh2 described in the literature (Crea et al., 2012; Tan et al., 2007). However, further experiments are required to establish DZNep as an effective Ezh2 inhibitor in different *in vitro* and *in vivo* models (Miranda et al., 2009). In that regard, we observed inhibition of H3K4 and H3K36 methylation in SW480 cells

following DZNep treatment (Figure 4A). These observations point to potentially important effects of DZNep which are unrelated to Ezh2 inhibition and which could differentially affect distinct subset of tumors or cells. It is also noteworthy that the measurements of SUZ12 and EZH2 protein levels following DZNep treatment (Figure 4A) are partially inconsistent with a previous study in which a decrease in the cellular levels of these PRC2 members upon drug treatment was reported (Tan et al., 2007). DZNep inhibits EZH2 activity primarily by perturbing the one-carbon metabolism pathway and indirectly inhibiting histone H3 methylation. Thus, SUZ12 and EZH2 protein levels may not necessarily be affected (Glazer et al., 1986; Miranda et al., 2009). However, the consistency of our results obtained with DZNep and with Suz12 knockdown with respect to TRAIL-mediated apoptosis (Figures 3B, 4C, 4D) supports the involvement of PcGs in death receptor regulation. The development of more specific inhibitors of Ezh2 HMTase activity is a crucial step for the development of clinical, epigenetic therapies targeting PcGs.

Conclusions

Taken together, our findings suggest that pharmacological inhibition of PRC2 HMTase activity constitutes a new epigenetic, therapeutic strategy to surmount RA resistance and increase TRAIL responsiveness in colorectal tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Weill Cornell Medical College for additional funding, and Matthew Bell for a generous donation. The authors also thank Drs. Victor E. Marquez and Joseph J. Barchi at NIH/NCI for providing 3-deazaneplanocin A. Thanks to Dr. Jean-François Beaulieu (Université de Sherbrooke) for the gift of shRNA constructs, and Tamara Weissman for editing the manuscript.

Funding:

Contract grant sponsor: The National Institutes of Health, Contract grant numbers RO1 CA0043796-22 (to LJG), DE010389-17 (to LJG), R21 CA153049 (to SML), R21 CA162483 (to SML).

Contract grant sponsor: Fonds de Recherche en Santé du Québec; Contract grant number: PF1-Benoit-25389 (to YDB).

References

- Amat R, Gudas LJ. RARgamma is required for correct deposition and removal of Suz12 and H2A.Z in embryonic stem cells. *J Cell Physiol.* 2011; 226(2):293–298. [PubMed: 20857416]
- Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene.* 2004; 328:1–16. [PubMed: 15019979]
- Benoit YD, Lepage MB, Khalfaoui T, Tremblay E, Basora N, Carrier JC, Gudas LJ, Beaulieu JF. Polycomb repressive complex 2 impedes intestinal cell terminal differentiation. *J Cell Sci.* 2012
- Benoit YD, Lussier C, Ducharme PA, Sivret S, Schnapp LM, Basora N, Beaulieu JF. Integrin alpha8beta1 regulates adhesion, migration and proliferation of human intestinal crypt cells via a predominant RhoA/ROCK-dependent mechanism. *Biol Cell.* 2009; 101(12):695–708. [PubMed: 19527220]
- Benoit YD, Pare F, Francoeur C, Jean D, Tremblay E, Boudreau F, Escaffit F, Beaulieu JF. Cooperation between HNF-1alpha, Cdx2, and GATA-4 in initiating an enterocytic differentiation program in a normal human intestinal epithelial progenitor cell line. *Am J Physiol Gastrointest Liver Physiol.* 2010; 298(4):G504–517. [PubMed: 20133952]

- Crea F, Paolicchi E, Marquez VE, Danesi R. Polycomb genes and cancer: Time for clinical application? *Crit Rev Oncol Hematol*. 2012; 83(2):184–193. [PubMed: 22112692]
- de The H, Chen Z. Acute promyelocytic leukaemia: novel insights into the mechanisms of cure. *Nat Rev Cancer*. 2010; 10(11):775–783. [PubMed: 20966922]
- Dhandapani L, Yue P, Ramalingam SS, Khuri FR, Sun SY. Retinoic acid enhances TRAIL-induced apoptosis in cancer cells by upregulating TRAIL receptor 1 expression. *Cancer Res*. 2011; 71(15):5245–5254. [PubMed: 21685476]
- Gillespie RF, Gudas LJ. Retinoid regulated association of transcriptional co-regulators and the polycomb group protein SUZ12 with the retinoic acid response elements of Hoxa1, RARbeta(2), and Cyp26A1 in F9 embryonal carcinoma cells. *J Mol Biol*. 2007; 372(2):298–316. [PubMed: 17663992]
- Glazer RI, Knode MC, Tseng CK, Haines DR, Marquez VE. 3-Deazaneplanocin A: a new inhibitor of S-adenosylhomocysteine synthesis and its effects in human colon carcinoma cells. *Biochem Pharmacol*. 1986; 35(24):4523–4527. [PubMed: 3790170]
- Gudas LJ, Wagner JA. Retinoids regulate stem cell differentiation. *J Cell Physiol*. 2011; 226(2):322–330. [PubMed: 20836077]
- Iliopoulos D, Lindahl-Allen M, Polytharchou C, Hirsch HA, Tschlis PN, Struhl K. Loss of miR-200 inhibition of Suz12 leads to polycomb-mediated repression required for the formation and maintenance of cancer stem cells. *Mol Cell*. 2010; 39(5):761–772. [PubMed: 20832727]
- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin*. 2011; 61(2):69–90. [PubMed: 21296855]
- Jia L, Berman BP, Jariwala U, Yan X, Cogan JP, Walters A, Chen T, Buchanan G, Frenkel B, Coetzee GA. Genomic androgen receptor-occupied regions with different functions, defined by histone acetylation, coregulators and transcriptional capacity. *PLoS One*. 2008; 3(11):e3645. [PubMed: 18997859]
- Jiang X, Tan J, Li J, Kivimae S, Yang X, Zhuang L, Lee PL, Chan MT, Stanton LW, Liu ET, Cheyette BN, Yu Q. DACT3 is an epigenetic regulator of Wnt/beta-catenin signaling in colorectal cancer and is a therapeutic target of histone modifications. *Cancer Cell*. 2008; 13(6):529–541. [PubMed: 18538736]
- Kashyap V, Gudas LJ, Brenet F, Funk P, Viale A, Scandura JM. Epigenomic reorganization of the clustered Hox genes in embryonic stem cells induced by retinoic acid. *J Biol Chem*. 2011; 286(5):3250–3260. [PubMed: 21087926]
- Laursen KB, Wong PM, Gudas LJ. Epigenetic regulation by RARalpha maintains ligand-independent transcriptional activity. *Nucleic Acids Res*. 2012; 40(1):102–115. [PubMed: 21911359]
- Lee MO, Han SY, Jiang S, Park JH, Kim SJ. Differential effects of retinoic acid on growth and apoptosis in human colon cancer cell lines associated with the induction of retinoic acid receptor beta. *Biochem Pharmacol*. 2000; 59(5):485–496. [PubMed: 10660115]
- Luis NM, Morey L, Di Croce L, Benitah SA. Polycomb in Stem Cells: PRC1 Branches Out. *Cell Stem Cell*. 2012; 11(1):16–21. [PubMed: 22770239]
- Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. *Nature*. 2011; 469(7330):343–349. [PubMed: 21248841]
- Miranda TB, Cortez CC, Yoo CB, Liang G, Abe M, Kelly TK, Marquez VE, Jones PA. DZNep is a global histone methylation inhibitor that reactivates developmental genes not silenced by DNA methylation. *Mol Cancer Ther*. 2009; 8(6):1579–1588. [PubMed: 19509260]
- Mongan NP, Gudas LJ. Valproic acid, in combination with all-trans retinoic acid and 5-aza-2'-deoxycytidine, restores expression of silenced RARbeta2 in breast cancer cells. *Mol Cancer Ther*. 2005; 4(3):477–486. [PubMed: 15767557]
- Mongan NP, Gudas LJ. Diverse actions of retinoid receptors in cancer prevention and treatment. *Differentiation*. 2007; 75(9):853–870. [PubMed: 17634071]
- Nicke B, Kaiser A, Wiedenmann B, Riecken EO, Rosewicz S. Retinoic acid receptor alpha mediates growth inhibition by retinoids in human colon carcinoma HT29 cells. *Biochem Biophys Res Commun*. 1999a; 261(3):572–577. [PubMed: 10441468]
- Nicke B, Riecken EO, Rosewicz S. Induction of retinoic acid receptor beta mediates growth inhibition in retinoid resistant human colon carcinoma cells. *Gut*. 1999b; 45(1):51–57. [PubMed: 10369704]

- Niederreither K, Dolle P. Retinoic acid in development: towards an integrated view. *Nat Rev Genet.* 2008; 9(7):541–553. [PubMed: 18542081]
- Paulsen JE, Lutzow-Holm C. In vivo growth inhibition of human colon carcinoma cells (HT-29) by all-trans-retinoic acid, difluoromethylornithine, and colon mitosis inhibitor, individually and in combination. *Anticancer Res.* 2000; 20(5B):3485–3489. [PubMed: 11131651]
- Rodriguez-Moranta F, Salo J, Arcusa A, Boadas J, Pinol V, Bessa X, Batiste-Alentorn E, Lacy AM, Delgado S, Maurel J, Pique JM, Castells A. Postoperative surveillance in patients with colorectal cancer who have undergone curative resection: a prospective, multicenter, randomized, controlled trial. *J Clin Oncol.* 2006; 24(3):386–393. [PubMed: 16365182]
- Sauvageau M, Sauvageau G. Polycomb group proteins: multi-faceted regulators of somatic stem cells and cancer. *Cell Stem Cell.* 2010; 7(3):299–313. [PubMed: 20804967]
- Schmitges FW, Prusty AB, Faty M, Stutzer A, Lingaraju GM, Aiwezian J, Sack R, Hess D, Li L, Zhou S, Bunker RD, Wirth U, Bouwmeester T, Bauer A, Ly-Hartig N, Zhao K, Chan H, Gu J, Gut H, Fischle W, Muller J, Thoma NH. Histone methylation by PRC2 is inhibited by active chromatin marks. *Mol Cell.* 2011; 42(3):330–341. [PubMed: 21549310]
- Shah S, Pishvaian MJ, Easwaran V, Brown PH, Byers SW. The role of cadherin, beta-catenin, and AP-1 in retinoid-regulated carcinoma cell differentiation and proliferation. *J Biol Chem.* 2002; 277(28):25313–25322. [PubMed: 12000762]
- Sikandar SS, Pate KT, Anderson S, Dizon D, Edwards RA, Waterman ML, Lipkin SM. NOTCH signaling is required for formation and self-renewal of tumor-initiating cells and for repression of secretory cell differentiation in colon cancer. *Cancer Res.* 2010; 70(4):1469–1478. [PubMed: 20145124]
- Sirchia SM, Ferguson AT, Sironi E, Subramanyan S, Orlandi R, Sukumar S, Sacchi N. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. *Oncogene.* 2000; 19(12):1556–1563. [PubMed: 10734315]
- Stolfi C, Caruso R, Franze E, Rizzo A, Rotondi A, Monteleone I, Fantini MC, Pallone F, Monteleone G. 2-methoxy-5-amino-N-hydroxybenzamide sensitizes colon cancer cells to TRAIL-induced apoptosis by regulating death receptor 5 and survivin expression. *Mol Cancer Ther.* 2011; 10(10):1969–1981. [PubMed: 21817114]
- Sung B, Ravindran J, Prasad S, Pandey MK, Aggarwal BB. Gossypol induces death receptor-5 through activation of the ROS-ERK-CHOP pathway and sensitizes colon cancer cells to TRAIL. *J Biol Chem.* 2010; 285(46):35418–35427. [PubMed: 20837473]
- Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. Targeting death-inducing receptors in cancer therapy. *Oncogene.* 2007; 26(25):3745–3757. [PubMed: 17530027]
- Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, Tan PB, Liu ET, Yu Q. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. *Genes Dev.* 2007; 21(9):1050–1063. [PubMed: 17437993]
- Tang XH, Gudas LJ. Retinoids, retinoic acid receptors, and cancer. *Annu Rev Pathol.* 2011; 6:345–364. [PubMed: 21073338]
- Trainer DL, Kline T, McCabe FL, Faucette LF, Feild J, Chaikin M, Anzano M, Rieman D, Hoffstein S, Li DJ, et al. Biological characterization and oncogene expression in human colorectal carcinoma cell lines. *Int J Cancer.* 1988; 41(2):287–296. [PubMed: 3338874]
- Youssef EM, Estecio MR, Issa JP. Methylation and regulation of expression of different retinoic acid receptor beta isoforms in human colon cancer. *Cancer Biol Ther.* 2004; 3(1):82–86. [PubMed: 14726683]
- Zhang L, Fang B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer Gene Ther.* 2005; 12(3):228–237. [PubMed: 15550937]
- Zhang L, Ren X, Alt E, Bai X, Huang S, Xu Z, Lynch PM, Moyer MP, Wen XF, Wu X. Chemoprevention of colorectal cancer by targeting APC-deficient cells for apoptosis. *Nature.* 2010; 464(7291):1058–1061. [PubMed: 20348907]

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Table 1
Primer sequences used for RT-PCR and ChIP assays

All primers for RT-PCR are designed around introns.

Primer	Application	Forward sequence (5'→3')	Reverse sequence (5'→3')	Product size (bp)
hRXR α	RT-PCR	TTCGCTAAGCTCTTGCTC	ATAAGGAAGGTGTCAATGGG	113
hRAR α	RT-PCR	GTCTGTCAGGACAAGTCCTCAGG	GCTTTGCGCACCTTCTCAATGAG	314
hRAR β	RT-PCR	ATTCCAGTGCTGACCATCGAGTCC	CCTGTTTCTGTGTCATCCATTTC	349
hRAR γ	RT-PCR	TACCACTATGGGGTCAGC	CCGGTCATTTCGCACAGCT	195
mRAR β	RT-PCR	GATCCTGGATTCTACACCG	CACTGACGCCATAGTGGTA	248
mRAR γ	RT-PCR	ATGTACGACTGCATGGAATCGT	GATACAGTTTTTGTACGGTGACAT	366
hTNFRSF10A	qPCR	CATAGCCCTTTGGGAGAGTTGTGT	CGTACAATCCTTGACCTTGACCAT	294
hTNFRSF10B	qPCR	GTGCTCGTTGTCGCCGCGTCCTG	GTGCCTTCTTCGCACTGACACACT	314
hTNFRSF10D	qPCR	GCTGAAGGGTGTGAGAGGAG	CAGGCTGCTTCCCTTTGTAG	261
hpTNFRSF10A	ChIP	TGGTTGAGGAACAGAAGCTGAGA	GGCTCCTGTTGGCTAACCCT	75
hpTNFRSF10B	ChIP	GCGCGGACAGGACCCAGAAA	ATCCTCCGCAAGCGCGTCCAA	110
hpTNFRSF10D	ChIP	GGGAGTACAAGTACCACGCCTT	ACTGCCAGAATAGAACGTGCTCCT	68
hIntergenic	ChIP	GTGAGTGCCAGTTAGAGCATCTA	GGAACCAGTGGGTCTTGAAGTG	142
HPRT1	RT-PCR/qPCR	TGCTCGAGATGTGATGAAGG	TCCCCTGTTGACTGGTCATT	192

Table 2
Antibodies used for Western blot, immunofluorescence, and ChIP assays

Target	Application	Company	Clone/Catalog #	Source
PARP	Wb	Cell Signaling	9542	Rabbit polyclonal
Suz12	Wb, IF,ChIP	Cell Signaling	3737	Rabbit monoclonal
Ezh2	Wb	Millipore	04-1047	Rabbit monoclonal
Actin	Wb	Millipore	MAB1501	Mouse monoclonal
H3K27me3	Wb, ChIP	Millipore	07-449	Rabbit polyclonal
H3K4me3	Wb	Millipore	07-473	Rabbit polyclonal
H3K36me2	Wb	Millipore	07-369	Rabbit polyclonal
H3K9me3	ChIP	Abcam	Ab8898	Rabbit polyclonal
H2K119ub	ChIP	Millipore	AB10029	Rabbit polyclonal
Rabbit IgG	ChIP	Santa Cruz Biotech	Sc 2027	---