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Inhibition of the Redox Function of APE1/Ref-1 in Myeloid Leukemia Cell Lines Results in a Hypersensitive Response to Retinoic Acid-induced Differentiation and Apoptosis

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Abstract

Objective—The standard of care for promyelocytic leukemia includes use of the differentiating agent all-*trans* retinoic acid (RA) and chemotherapy. RA induces cell differentiation through retinoic acid receptor (RAR) transcription factors. Because redox mechanisms influence how readily transcription factors bind to DNA response elements (RARE), the impact of small molecule (E3330) inhibition of the redox regulatory protein, apurinic-apyrimidinic endonuclease/redox effector factor (APE1/Ref-1) on RAR DNA binding and function in RA-induced myeloid leukemia cell differentiation and apoptosis was investigated.

Materials and Methods—The redox function of APE1 was studied using the small molecule inhibitor E3330 in HL-60 and PLB acute myeloid leukemia cells. Electrophoretic mobility shift assays (EMSA) were employed to determine effect of inhibitor on APE1/Ref-1 redox signaling function. Trypan blue assays, Annexin-V/PI and CD11b staining, and real time PCR analyses were employed to determine survival, apoptosis and differentiation status of cells in culture.

Results—RAR α binds to its RARE in a redox-dependent manner mediated by APE1/Ref-1 redox regulation. Redox-dependent RAR-RARE binding is blocked by E3330, a small molecule redox inhibitor of APE1/Ref-1. Combination treatment of RA + E3330 results in a profound hypersensitivity of myeloid leukemia cells to RA-induced differentiation and apoptosis. Additionally, redox inhibition by E3330 results in enhanced RAR target gene, BLR-1, expression in myeloid leukemia cells.

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Conclusion—The redox function of APE1/Ref-1 regulates RAR binding to its DNA RAREs influencing the response of myeloid leukemia cells to RA-induced differentiation. Targeting of APE1/Ref-1 redox function may allow manipulation of the retinoid response with therapeutic implications.

Keywords

APE1/Ref-1; redox; differentiation; myeloid leukemia; retinoic acid

INTRODUCTION

Acute promyelocytic leukemia (APL) represents about 10% of the cases of acute myelogenous leukemia in children and adults, and is most often characterized by the t(15,17) chromosomal translocation involving the RAR- α and PML genes. Standard treatment for APL combines all-*trans* retinoic acid (ATRA, hereafter referred to as “RA”) with chemotherapy to induce terminal granulocytic differentiation of APL cells, representing one of the first uses of truly targeted therapy for a malignancy. Disease-free outcomes of greater than 70% have been achieved with this combination therapy [1]. However, approximately 25% of APL patients treated with RA develop differentiation syndrome, a toxicity caused by rapid expansion of maturing myeloid cells, accompanied by release of cytokines (IL-1 β , INF- γ) [2]. In addition, 5 to 30% of APL patients require additional, more intense therapy to achieve disease control [3]. Higher doses of RA may not achieve a therapeutic response in such cases. Finding a therapeutic approach that enables RA to work at lower doses would potentially decrease the risk of toxicities and increase the effectiveness of RA over a broader range of patients. Being one of the first targeted therapies for a malignancy, considerable work has been done to characterize the molecular basis for the RA response. RA ligand binds retinoic acid receptors (RARs) which form heterodimers (RXR:RAR) that bind specific retinoic acid response elements within target gene promoter elements to regulate gene expression [4–5].

An intricate network of co-transcriptional elements complex to provide inhibitory (non-liganded) or activating (liganded) configurations that influence target gene expression [5–6]. One factor that mediates transcription factor binding to DNA response elements through its redox signaling activity is APE1/Ref-1. While the importance of APE1/Ref-1’s functions in DNA repair has been well established, its redox function is still being interrogated, especially in leukemia. We previously demonstrated that in HL-60 cells treated with RA, the protein and mRNA levels of APE1/Ref-1 decrease [7]. The basis of APE1/Ref-1 redox mediated transcription factor binding to DNA response elements has been characterized in multiple transcription factors including Fos/Jun, NF κ B and others [8–9]. Recent investigations by others suggest that RAR activity is also influenced by redox activity [5].

To study whether the redox function of APE1/Ref-1 is a major controlling factor for RAR activity, we used the highly characterized and specific small molecule inhibitor of the redox function of APE1/Ref-1, E3330 [10–12]. E3330 effectively blocks specific APE1/Ref-1 redox-mediated NF κ B binding to its DNA response elements thus blocking NF κ B transcriptional activity [11,13–14]. E3330 inhibition of APE1/Ref-1 redox activity was utilized in these studies to determine the impact of redox activity on RA-induced myeloid differentiation. Initial electrophoretic mobility shift assays (EMSA) demonstrated loss of binding of RARs to their DNA response elements in the absence of APE1/Ref-1 redox activity (similar to the redox sensitivity seen with NF κ B in other studies [13–14]). E3330 alone induced a reversible growth inhibition of HL-60 and PLB cells, and by itself did not induce myeloid differentiation in either cell line. However combined with RA, E3330 produced a profound hypersensitive response to RA-induced myeloid differentiation and

apoptosis. These studies suggest a working model in which redox regulation may control the balance of DNA and non-DNA RAR binding partners to impact the RA-response. Given our recent characterization of non-DNA targets for RARs [15] that mediate a differentiation response and the significant enhancement of differentiation observed in the present studies, we postulate that E3330 represents a novel therapeutic agent by specifically interfering with APE1/Ref-1 redox activity, inducing RARs to come off their DNA binding sites (RAREs) and allowing interaction with alternate binding partners, to enhance the differentiation pathway. We have recently identified cytoplasmic c-SRC as one such RAR target capable of inducing RA-mediated neuronal differentiation in neuroblastoma cells [15].

RA treatment of APL cells activates NFκB activity and this activity, although not essential for differentiation, results in an accumulation of differentiated cells. The authors conclude that this accumulation could contribute to the differentiation syndrome observed in patients [16]. The inhibition of NFκB activity by E3330 [13] may prohibit the expansion of mature cells and the transcription of anti-apoptotic genes by NFκB. [17][18] Redox reagents like E3330 may be useful in manipulating the redox status of retinoid receptors as well as NFκB through APE1/Ref-1 and have therapeutic implications.

MATERIALS AND METHODS

Cell culture

Both cell lines were obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 media (Gibco; Carlsbad, CA) and supplemented with Cosmic Calf Serum (Hyclone; Logan, UT) and Penicillin (100 units/mL)-Streptomycin (100μg/mL): HL-60 acute myeloid leukemia cells; 5% Cosmic Calf Serum and PLB-985 (myeloblastic) cells; 10% Cosmic Calf Serum.

Materials

HL-60 and PLB cells were differentiated with the addition of all-*trans* retinoic acid (RA), (Sigma; St. Louis, MO) that was reconstituted in 100% absolute ethanol (EtOH). The redox function of APE1/Ref-1 was blocked pharmacologically using E3330 that was synthesized as previously described [10] and suspended in EtOH. EtOH was utilized as vehicle control in all experiments. The purified APE1/Ref-1 utilized in the EMSA assays was purified as previously reported [19].

Electrophoretic mobility shift assay (EMSA)

EMSA's were performed as previously described [19] with the following modifications. Briefly, 10 μg/μl purified APE1/Ref-1 protein was reduced with 1.0 mM DTT for 10 min and diluted in PBS buffer to yield final concentrations of 2 μg/μl protein and 0.2 mM DTT. Two μl of reduced APE1/Ref-1 protein was incubated with increasing amount of E3330 in EMSA reaction buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 5% [vol/vol] glycerol) in a total volume of 12 μl for 30 min. Previously characterized K562 cells that overexpress retroviral (LXSN) constructs containing human RARα, RARγ, or RXRα [20] were used as the source of RAR proteins for shift assays. 6 μg of nuclear extract (treated with 0.01mM diamide for 10 min) was added for 30 min, followed by 1 μl poly(dI-dC) · poly(dI-dC) (1 μg/ul) (Amersham Biosciences, Piscataway, NJ) for 5 min and 1 μl of 5' hexachlorofluorescein phosphoramidite (HEX)-labeled double-stranded oligonucleotide DNA (The Midland Certified Reagent Company, Midland, TX) containing the RXRα-RARα DR5 direct repeat consensus sequence 5'-AGG GTA GGG TTC ACC GAA AGT TCA CTC-3' (0.1 pmol) for 30 min at room temperature [21]. The final concentration of DTT in the redox reactions was 0.02 mM. Then the mixture was further incubated for 30 min at room temperature. Samples were loaded on a 5% non-denaturing polyacrylamide gel and

subjected to electrophoresis in 0.5× TBE buffer (200 V for 1 h at 4°C) with detection performed using the Hitachi FMBio II Fluorescence Imaging System (Hitachi Genetic Systems; South San Francisco, CA). The HEX fluorophore was excited by a solid-state laser at 532 nm (Perkin-Elmer, Waltham, MA) and emitted a fluorescent light signal at 560 nm, which was then measured using a 585 nm filter.

Proliferation of HL-60 and PLB cells

The proliferative capacity of HL-60 and PLB cells was assessed using Trypan Blue. Trypan Blue (0.4%; Invitrogen; Carlsbad, CA) was added to aliquots of cell-containing media and the number of viable cells was determined using a hemocytometer. For studies where E3330 was removed from the growth media, HL-60 cells were plated at 1×10^5 cell/mL at Day 0 and E3330 added. Following the addition of E3330, cells were counted at Day 3 and 6. On Day 6, the cells were centrifuged and resuspended in fresh media at the original density (1×10^5 cell/mL), with or without added E3330. The replated cells were then counted using the Trypan Blue assay on Days 3 and 6.

Determination of Differentiation by CD11b Staining

Cell staining for differentiation was performed using PE-conjugated anti-CD11b and a control of PE-conjugated IgG (VWR; West Chester, PA). The cells were centrifuged, washed, and incubated with antibody, diluted 1:10 in PBS, for 30 min at RT in the dark. Following incubation, the cells were washed, spun down, and resuspended in PBS. Cells were then analyzed with a Fluorescence-Activated Cell Sorter (FACS) Calibur (Becton Dickinson; San Jose, CA) using the CELLQuest program (Becton Dickinson; San Jose, CA).

Apoptosis

Annexin-V (BD Pharmingen; San Jose, CA) and Propidium Iodide, or PI (Sigma; St. Louis, MO), were used to stain for programmed cell death as previously described). [22]. Briefly, cells were collected, washed, and resuspended in PBS containing PI (1 μ g/mL), and Annexin-V diluted 1:20. Cells were stained for 30 min at room temperature and then analyzed with a Fluorescence-Activated Cell Sorter (FACS) Calibur (Becton Dickinson; CA, USA) using the CELLQuest program (Becton Dickinson; CA, USA). Quantification of the apoptosis data was performed as follows: cells that stained either Annexin-V positive/PI positive or Annexin-V positive/PI negative were combined to obtain the total percent apoptosis.

Morphology

Aliquots, 5×10^4 cells/mL, of the treated HL-60 cells were cytopspun using a Shandon cytocentrifuge for 3 min at 400 rpm on Superfrost Premium Microscope Slides (Fisher Scientific, Hanover Park, IL). The slides containing cells were fixed with methanol and then stained with Wright-Giemsa (Fisher Scientific; Hanover Park, IL). Morphological evaluation of the differentiated cells was determined using a light microscope at 200–1000× magnification.

Real-time PCR (polymerase chain reaction) analysis

Expression levels of BLR1 and GAPDH mRNA were measured by quantitative PCR. Total RNA was extracted from $3\text{--}8 \times 10^6$ cells treated with drug or vehicle control at the stated time point using a QIAshredder and RNeasy Mini Kit (Qiagen; Valencia, CA) according to manufacturer's protocol. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Foster City, CA) was used to synthesize cDNA, according to the manufacturer's protocol. The reverse transcription step was done using a PCR Sprint Thermocycler (Thermo Scientific; Waltham, MA). BLR1 and GAPDH cDNA were

quantitated by real-time polymerase chain reaction (RT-PCR) using the TaqMan Fluorogenic Detection System (ABI Prism 7500; Applied Biosystems; Foster City, CA). TaqMan gene expression assay (Applied Biosystems; Foster City, CA) primers and probes that span exons for BLR1, Hs00173527_m1, and GAPDH, Hs99999905_m1, were used to exclude the possibility of genomic DNA contamination. For the amplification step, TaqMan Gene Expression Master Mix (Applied Biosystems; Foster City, CA) was used according to manufacturer's protocol. Thermal cycling parameters were as follows: 50°C for 2 min (UDG Incubation) and 95°C for 10 min (Taq DNA activation and reverse transcriptase inactivation). PCR amplification consisted of 40 cycles of 95°C for 15 sec and 60°C for 60 sec. All samples were tested in triplicate, and the quantitative values were normalized to the internal standard, GAPDH.

RESULTS

Binding of RAR α to RAREs is mediated by redox conditions through APE1/Ref-1

EMSA assay was employed to examine the effect of redox conditions on RAR α binding to its DNA response element (RARE). Incubation of a labeled RARE with K562-RAR α cell nuclear extract produced the expected retarded band (Figure 1A, lane 1). The specificity of this interaction was demonstrated by a supershift band observed when nuclear extract was pre-incubated with RAR α antibody (Figure 1A, lane 3). EMSA assay was then used to examine the impact of reduced APE1/Ref-1 on RAR-RARE binding (Figure 1B). The presence of reduced APE1/Ref-1 significantly enhanced RAR α binding to RARE compared to unreduced APE1/Ref-1 (Figure 1B). This effect appears to be dependent on reducing conditions as both the presence of reduced APE1/Ref-1 (Figure 2A, lane 3) and DTT (Figure 2A, lane 9) could enhance RAR α -RARE binding compared to negative controls (Figure 2, lanes 1–2). To further define RAR α -RARE binding dependence on the redox activity of APE1/Ref-1, increasing doses of E3330, a specific small molecule redox inhibitor of APE1/Ref-1, were added to the EMSA reaction (Figure 2A, lanes 4–8). The inhibition of the redox function of APE1/Ref-1 by E3330 produced a dose-dependent inhibition of RAR α -RARE binding (Figure 2A, lanes 4–8). As a control in Figure 2A, lane 9 demonstrates with 0.4 mM DTT we can achieve similar binding of RAR α to DNA as with reduced APE1/Ref-1. E3330 treatment does not inhibit the reducing ability of DTT (lanes 10–14) showing E3330 specificity for APE1/Ref-1. E3330 doses that inhibit DNA binding of RAR α also cause a reduction in cell number (Figure 2B). HL-60 cells treated with E3330 for 6 days demonstrate a decrease in cell number when doses are 15 μ M and higher. The ED₅₀ for E3330 is ~ 25 μ M, and this dose was utilized for further experiments in combination with RA. At doses of 40 μ M and higher, cytotoxicity is observed with E3330 treatment alone (Figure 2B).

HL-60 proliferation and morphology following RA and E3330 treatment

To investigate the role of APE1/Ref-1 and redox signaling in RA-induced differentiation of HL-60 cells, we treated cells with 25 μ M E3330 (corresponding to the ED₅₀) in combination with RA. Figures 2C and D clearly demonstrate the expected cell growth arrest and differentiation induced by RA, as well as the 50% reduction in cell number with E3330 treatment alone compared to vehicle control. A further reduction in cell number occurs when RA is used in combination with E3330—with an 8.5-fold decrease in cell number compared to vehicle control and a 3-fold decrease in cell number compared to RA alone (Figure 2C).

Neither E3330 alone nor the vehicle control causes any obvious morphological changes to the HL-60 cells (Figure 2D). Those cells still possess large, round nuclei with high nuclear-to-cytoplasmic ratios. However, E3330 treatment alone does not induce differentiation as is observed with RA. As expected, RA induces cellular differentiation, as indicated by

indented and lobed nuclei, a greater cytoplasmic-to-nuclear ratio, and cytoplasmic granules. Cells treated with RA + E3330 contain more highly segmented nuclei, a greater cytoplasmic-to-nuclear ratio, and more cytoplasmic granules, indicating more differentiation than cells treated with RA alone. In addition, cells treated with RA + E3330 display more apoptotic cells at Day 6 compared to cells treated with RA alone.

E3330's presence in the media inhibits cell growth

To determine if HL-60 cells would proliferate following removal of E3330, we treated HL-60 cells with E3330 for 6 days and then removed the drug from the media. HL-60 cells with continuous exposure to E3330 displayed a 50% reduction in cell number. However, when E3330 was removed from the media the cells began to proliferate again. By Day 6, the cell number does not differ from vehicle-treated cells (Figure 3).

E3330 enhances RA-induced cellular differentiation in HL-60 and PLB cells

In addition to cell morphology, CD11b expression was determined by FACS analysis in the presence and absence of RA and E3330. Neither the vehicle control nor E3330 alone induce differentiation as assayed by CD11b expression (Figure 4A). RA increased the expression of CD11b in HL-60 cells, as expected from previous reports [23]. The histogram depicting the combination of RA plus E3330 illustrates a pronounced shift to the right compared to cells treated with vehicle, E3330 alone, and RA alone (Figure 4A). Quantification of the increase in CD11b expression is shown in Figure 4B. HL-60 cells treated with both RA and E3330 express CD11b 63% more than cells treated with RA alone (Figure 4B). To ensure that the effects of E3330 on RA-induced differentiation and apoptosis were not cell-line dependent, we utilized another promyelocytic leukemia cell line, PLB, to test the combination of RA and E3330. A dose of 45 μ M E3330 results in a 50% reduction in cell number (data not shown); therefore, this dose was used in combination with RA to characterize the effects of CD11b expression as well as apoptosis. Results in PLB cells were similar to those in HL-60 cells: the addition of E3330 to RA induces 66% more differentiation than RA treatment alone (Figure 4). E3330 treatment alone did induce a small population of cells to express higher levels of CD11b, however when RA was added to the E3330 treatment the entire population of cells shifted dramatically in CD11b expression (Figure 4A).

E3330 in combination with RA increases the percentage of cells undergoing apoptosis compared to RA treatment alone

After a granulocyte terminally differentiates, it progresses through its life cycle but dies due to its lack of self-renewal. E3330 treatment alone did not result in an increase in differentiation nor an increase in apoptosis in HL-60 or PLB cells (Figure 4B, C). Although the dose of E3330 utilized decreases the cell number by 50%, E3330 did not increase the percentage of cells undergoing apoptosis—indicating that the cells were not dying, but perhaps undergoing cell cycle arrest. Accompanying the increase in cellular differentiation, Figure 4C shows that RA treatment alone for 6 days, resulted in increased Annexin-V staining in HL-60 and PLB cells. As expected, the combination of RA plus E3330, which resulted in an increase in differentiation, was also accompanied by a statistically significant increase in apoptosis. This increase was statistically significant when compared to E3330 treatment alone as well as RA treatment alone in both cell lines (Figure 4C). The histograms demonstrating CD11b staining of HL-60 and PLB cells indicate that the virtually all of the cells are differentiated with RA and E3330 treatment indicating that the cells that were analyzed for apoptosis were indeed differentiated.

The dose of RA can be reduced 1,000-fold (3 logs) with the addition of APE1/Ref-1 redox inhibitor E3330

In an attempt to lower the dose of RA required to achieve differentiation and apoptosis and thereby reduce the risks of differentiation syndrome in the clinic, we serially reduced the dose of RA while holding the dose of E3330 constant at 25 μ M. HL-60 cells responded in a dose-dependent manner to RA between 10^{-5} M RA to 10^{-8} M RA with or without E3330 present (Table I). As the dose of RA decreased, the expression of CD11b also decreased. Vehicle control (EtOH) or E3330 alone did not induce differentiation (Table I). The addition of E3330 to each dose of RA resulted in significantly greater CD11b expression than the respective RA dose (Table I). We observed a similar degree of differentiation in HL-60 cells treated with 10^{-8} M RA in combination with 25 μ M E3330 versus those treated with 10^{-5} M RA alone (Table I). Combining E3330 with RA, we can reduce the dose of RA 1,000-fold (3 logs) and achieve similar results of cellular differentiation.

Reducing the concentration of RA still induces apoptosis when E3330 is added to the RA treatment

As the dose of RA increased from 10^{-8} to 10^{-5} M RA, the percentage of apoptosis increased in a dose-dependent manner (Table I). At low doses of RA (10^{-8} and 10^{-7} M), there was no difference in apoptosis between RA treatment alone or RA in combination with E3330—even though a difference in the amount of differentiation was observed. At higher doses of RA (10^{-6} M and 10^{-5} M), the addition of E3330 resulted in a statistically significant increase in apoptosis over the respective RA treatment alone (Table I). Although 10^{-8} and 10^{-7} M RA in combination with E3330 could induce CD11b expression to the same degree as 10^{-5} M RA alone, the induction of apoptosis is not as robust as the effects on cellular differentiation at lower doses of RA.

E3330 in combination with RA increases expression of BLR1

BLR1 has been implicated as an important target of RA because it triggers the MAPK (mitogen activated kinase) signaling pathway in HL-60 cells, leading to the desired response of differentiation and growth arrest [24–26]. Therefore, we used real-time PCR to quantitate the expression of BLR1 when E3330 was added to the RA treatment. Eighteen to 72 h after treating HL-60 cells with RA plus E3330, BLR1 was dramatically induced (Figure 5). The difference between RA treatment alone versus RA in combination with E3330 was not significant at 6 or 12 h following treatment (data not shown). Induction of BLR1 with RA treatment alone was observed; however, when E3330 was added to the RA treatment, BLR1 expression increased ~ 2-fold compared to RA treatment, ~ 400-fold over baseline expression, and was sustained longer

DISCUSSION

The observations made in this report indicate that redox regulation is an important factor in the response of myeloid leukemia cells to RA. Others studies have suggested that redox conditions may be important in RA-induced cellular differentiation. Melanoma cells respond to RA under reducing conditions while oxidative treatment with H_2O_2 diminishes the induction of melanoma cell differentiation [5]. More recently, the redox regulatory protein thioredoxin glutathione reductase was found to interact with RARs and enhance their transcriptional activity [27]. A number of the RAR-related transcriptional co-factors are also influenced by redox control [5–6,28]. We have made prior observations that the DNA base excision repair/redox regulatory enzyme, APE1/Ref-1 is modulated during RA-induced granulocytic differentiation [7]. To further understand the role of APE1/Ref-1 in the retinoid response, we used E3330, a specific redox signaling inhibitor of APE1/Ref-1 [10–11,13]. To determine whether the redox effects of APE1/Ref-1 are cell line restricted, we examined two

well-studied myeloid leukemia cells lines, HL-60 and PLB, which respond to RA with terminal granulocytic differentiation.

Blocking APE1/Ref-1 redox activity with E3330 results in a dramatically enhanced response to RA while inducing RARs to come off their DNA response element targets. This poses a dilemma to explain mechanistically how this effect is mediated. Several possibilities exist and we will focus on three in this discussion: 1) inhibition of APE1/Ref-1 redox activity inhibits DNA binding of RAR homo- or heterodimers, resulting in an increase in non-genomic effects of activated RARs; 2) inhibition of APE1/Ref-1 redox activity affects the co-activators or the co-repressors involved in RAR gene transcription and/or 3) the activity of NFkB that is induced by RA is blocked by combination treatment with E3330 and blocks the anti-apoptotic effects of NFkB.

The accepted paradigm for RA-induction of its biologic effects is that RA binds receptor RARs which function as transcription factors. Upon binding to the RAREs in a complex which consists of multiple regulatory co-factors, cellular differentiation is achieved through downstream transcriptional targets. This model of RAR transcriptional complexes has been characterized and examined in many different cellular differentiation systems, however identification of downstream transcriptional targets have been elusive. We and others have begun to examine and characterize non-DNA targets for RARs that function to mediate the differentiation response. Recently, we have characterized c-SRC as a direct target for RARs to mediate terminal differentiation of neuroblastoma cells that is RA-dependent [15]. Observations of non-DNA targets for RARs that induce cellular differentiation in a RA-dependent manner fit well with the observations in the present study that RARs coming off DNA targets in a redox-dependent manner can activate or enhance the differentiation response to RA (Figure 6). These data taken together suggest a working model that integrates DNA and non-DNA RAR targets to influence the cellular response to RA. Further studies will be important to characterize the ‘balance’ between DNA and non-DNA targets of RARs to affect differentiation, but the data presented here provides a key observation that redox-mediated detachment of RARs from their DNA RARE targets allows RA-dependent differentiation to proceed in an enhanced manner. Indeed, similar “non-transcriptional” targets have been identified for other NRs, including binding of progesterone receptors to c-SRC and binding of estrogen receptors to MEK [29–30]. Although we are still investigating the interaction of cytoplasmic RAR and c-SRC as a future direction, these studies and others point to a role for extra-nuclear signaling that promotes differentiation [15,31].

Another explanation for the enhanced differentiation following inhibition of APE1/Ref-1 redox activity is that the addition of E3330 to RA in myeloid leukemia cells affects a negative repressor of differentiation which shifts the balance toward activation of differentiation and apoptosis. In order to activate the transcription of the genes necessary for differentiation in response to RA, the balance between co-repressors and co-activators must shift (Figure 6). APE1/Ref-1 may contribute to the RAR transcriptional complex by affecting the binding of nuclear co-repressors (e.g., HDAC) [6]. The activity of histone deacetylases (HDACs) is under redox control, and changes in redox status affect cellular localization. Nuclear export of HDAC allows for co-activator recruitment [28]. Moreover, nuclear receptor co-repressor (NCoR) and silencing mediator of retinoic acid (SMRT) exert transcriptional repression of RARs, AP-1, and NFkB [32–33]. AP-1 and NFkB are well-established targets of APE1/Ref-1 redox activity [9–10], and we show here that APE1/Ref-1 can reduce RARs and increase their DNA binding. Decreased activity of co-repressors and/or nuclear export of HDACs would allow RAR-RXR complexes to bind co-activators and induce expression of genes important for differentiation including BLR1.

A report by Mathieu et al demonstrates a similar increase in apoptosis with the combination of RA plus arsenic trioxide as we observe with the combination of RA plus E3330. RA in combination with arsenic trioxide resulted in an enhancement of RA-induced apoptosis through a blockade of NFκB activity [34]. As E3330 can also inhibit NFκB activity [13], we can attribute some of the effects of the combination of RA plus E3330 to inhibition of NFκB activity. Most likely, there is contribution by several of these pathways to the observed effects we see here with RA in combination with E3330 (Figure 6).

Similar to our findings with neuroblastoma cells, this process requires RA, as E3330 redox inhibition alone is insufficient to trigger differentiation in HL-60 and PLB cells. Similarly, ectopic expression of BLR1 is not enough to drive differentiation and cell cycle arrest, but requires the agonist RA to obtain these effects[26]. RA treatment alone does induce differentiation and expression of BLR1, but not to the extent that is observed with the combination of E3330 and RA.

Literature reports that the retinoic acid receptors (RARs) are under redox control [5]. We confirm this data and demonstrate that APE1/Ref-1 can reduce RARs and enable them to bind to RARE-containing DNA. We are also able to demonstrate that, similar to RAR-α, E3330 could inhibit RXR-α and RAR-γ binding to RAREs (data not shown). E3330 can also inhibit the DNA binding of other transcription factors including AP-1, HIF-1α, and NFκB [10]. Given the extensive pairing of RXR with multiple members of the NR family, combination treatment with E3330 may be an effective strategy with other NRs. Based on the proposed mechanism, we would expect E3330 to exert similar effects on other members of the steroid/thyroid family of NRs. Experiments in HL-60 cells with vitamin D and E3330 support this notion, as the combination enhances monocytic differentiation significantly (data not shown). This approach to potentially activate cellular differentiation pathways and inhibit proliferation pathways may be feasible in other NR family members (e.g. ER, PR) that have direct transcriptional targets (RAREs), as well as cellular protein targets (e.g., NRTKs).

We propose that blocking the redox signaling function of APE1/Ref-1 is a novel approach of directly inhibiting binding of a number of important transcription factors in cancer cells and RAR to RARE-gene targets in particular. E3330 represents a novel class of therapeutic agents that perform this function. Such inhibition of DNA-binding activity would free up specific target gene binding of RARs to allow extranuclear binding and activation to other RAR-specific targets, such as c-SRC, thereby impacting pathways important in terminal differentiation. Studies are underway to understand the cellular signaling networks that are affected following E3330 and RA treatment.

Myeloid leukemia cells may be further sensitized to retinoids by manipulation of the redox status of APE1/Ref-1. This increase in sensitization is accomplished even at 1000-fold (3 logs) lower concentration of RA. Reducing the dose of RA has important clinical implications and could help to eliminate some of the undesirable side effects of this therapy[1–2]. Rising WBC counts coincide with a severe and sometimes fatal coagulopathy, and RA administration can cause differentiation syndrome in 25% of patients [35–36]. Proposed and attempted countermeasures all have drawbacks. Use of corticosteroids can cause headaches, insomnia, mood changes, weight gain and osteoporosis, among other things. Activating the retinoid pathway with ligands of cell surface receptors initiating MAPK signaling [37] tends to potentiate differentiation syndrome. Use of PDGFR (platelet-derived growth factor receptor) increases several differentiation markers [38] but does not increase the G₁-G₀ cell cycle arrest. PDGFR inhibitors also can increase markers and mediators of differentiation syndrome [39].

Ideally, one should be able to avoid the toxicity of differentiation syndrome by being able to accelerate RA-induced promyeloblast differentiation while limiting expansion of cell numbers by inducing an early cell cycle arrest. Here we demonstrate that E3330 appears to do both while potentiating the effects of RA at much lower doses—to achieve the same degree of differentiation previously achieved by RA alone.

Preliminary pharmacokinetic (PK) studies show that 1) E3330 has an approximate half-life of 5.3 hours in mouse plasma, 2) serum levels above 10 μ M are easily achievable, and 3) no toxicities are observed in mice treated with up to 75 mg/kg of E3330 (data not shown). These early data indicate that clinical levels of E3330 can be achieved. Thus, E3330 provides a potential approach to minimizing the risk of differentiation syndrome by limiting leukemia cell expansion and accelerating terminal differentiation. E3330 also is an important investigative tool for studying the signaling pathways involved in cellular differentiation and apoptosis. The clinical implication of the findings reported here are that reagents such as E3330 have potential benefit in enhancing the differentiation response to RA in malignant cells and as such merit further investigation.

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Figure 1. Supershift EMSA assay demonstrating the specificity of binding of RAR α . APE1/Ref-1 stimulates the binding of RAR α (B) to its respective RARE

Panel A: 2 μ g RAR α antibody (C-20: sc-551 Santa Cruz Biotechnology, Inc.) or 2 μ g control antibody (IgG) was pre-incubated with or without 6 μ g nuclear extract from K562 cells that were overexpressing RAR α as the source of RAR α protein in EMSA reaction buffer. EMSA assay was performed as described in Materials and Methods. A supershift band was shown when nuclear extract was pre-incubated with RAR α antibody (Lane 3). Representative EMSA shown; experiment was performed with $n \geq 3$. Panel B: EMSA assay with RARE and nuclear extracts as previously described. Lane 3 contains NE + 0.02mM DTT which is the amount of DTT carried over from reducing APE1/Ref-1 protein. Representative EMSA shown; experiment was performed with $n \geq 3$.

Figure 2. E3330 treatment influences the DNA binding activity of RAR α and the proliferation of HL-60 cells, and E3330 in combination with RA dramatically affects the morphology and proliferation of the cells

A) Effect of E3330 on RAR α binding to RARE; E3330 was incubated in the EMSA reaction mixture containing nuclear extracts from K562 cells as indicated in Materials and Methods. Representative EMSA shown; experiment was performed with $n \geq 3$. **B)** HL-60 cells were counted in triplicate using Trypan Blue after 6 days of E3330 exposure. The mean and standard error from 3 independent experiments are shown. **C)** HL-60 cells were exposed to vehicle control (EtOH), E3330 alone (25 μ M), RA alone (10^{-5} M RA), and E3330 + RA and counted at Day 6. The mean and standard error from one representative experiment are shown. **D)** Morphological changes of HL-60 cells treated with EtOH, 25 μ M E3330, 10^{-5} M RA, and 10^{-5} M RA + 25 μ M E3330 for 6 days and then stained with Wright's stain to assess granulocytic differentiation and apoptosis ($n \geq 5$).

Figure 3. With E3330 present in the media, HL-60 cells proliferate at a slower rate

HL-60 cells were pretreated with E3330 (25 μ M) for six days. Following treatment, HL-60 cells were replated at a cell density of 1×10^5 cell/mL (Day 0), and E3330 was removed (light gray bars) or treatment continued (dark gray bars). Cells were counted in triplicate using Trypan Blue at Day 3 and 6 following replating. The mean and standard error from three independent experiments are shown. Not significant (n.s.), ** $p \leq 0.01$, # $p \leq 0.001$ using Student's t test.

Figure 4. E3330 enhances RA-induced cellular differentiation and increases the percentage of cells undergoing apoptosis in HL-60 and PLB cells compared to RA treatment alone
 HL-60 cells were treated with 10^{-5} M RA in combination with 25 μ M E3330, and PLB cells were treated with 10^{-5} M RA in combination with 45 μ M E3330 for 6 days. **A)** Expression of CD11b, a granulocytic differentiation marker, was measured by FACS analysis using PE conjugated anti-CD11b. Mean relative fluorescence is found in parentheses. IgG-PE was used as a negative control (solid peak). Shown here is one representative experiment. **B)** Quantification of CD11b staining following RA + E3330 treatment. The mean and standard error from 4 independent experiments are shown. **C)** Apoptosis was analyzed by expression of Annexin-V and PI staining. The mean and standard error from four independent experiments are shown. * $p < 0.05$ ** $p < 0.01$ using Student's t test.

Figure 5. E3330 in combination with RA increases expression of BLR1

HL-60 cells exposed to RA (10^{-5} M) in combination with E3330 (25 μ M) and collected at time points following treatment as indicated. Real-time PCR was used to quantitate expression of BLR1. Each time point was done in triplicate with $n \geq 2$. * $p < 0.05$ using Student's t test.

Figure 6. Proposed model demonstrating how inhibition of APE1/Ref-1 can enhance RA-induced differentiation

A) The redox activity of APE1/Ref-1 can be inhibited by E3330, which leads to a decrease in transcription factor binding. **B)** In response to RA, differentiation can be initiated via genomic and non-genomic effects of RARs. Expression of chemokine receptor BLR1 is known to increase in HL-60 cells following RA treatment. **C)** E3330 can inhibit the reduction of RARs, thereby reducing their DNA binding. This reduction in DNA binding potentially results in enhanced signaling through non-receptor tyrosine kinase (NRTK) pathways, leading to an enhancement of differentiation. E3330 can also inhibit NFκB activity which would decrease NFκB's prosurvival effects leading to an enhancement of apoptosis.

Table 1

Dose reduction of RA is possible with the addition of APE1/Ref-1 redox inhibitor E3330, while still achieving similar effects on differentiation and apoptosis.

Treatment	CD11b Relative Fluorescence $\pm$ SE	Percent Apoptosis $\pm$ SE
EtOH	3.5 $\pm$ 0.5	9.2 $\pm$ 2.3
25 mM E3330	9.5 $\pm$ 3.5	14.1 $\pm$ 0.8
10⁻⁵ M RA	235 $\pm$ 51	44.3 $\pm$ 2.5
10 ⁻⁵ M RA + E3330	420 $\pm$ 30*	66.8 $\pm$ 3.6**
10 ⁻⁶ M RA	247 $\pm$ 24	35.9 $\pm$ 1.6
10 ⁻⁶ M RA + E3330	399 $\pm$ 12*	53.9 $\pm$ 1.6**
10 ⁻⁷ M RA	185 $\pm$ 25	30.6 $\pm$ 1.8
10 ⁻⁷ M RA + E3330	281 $\pm$ 30*	40.6 $\pm$ 3.9
10 ⁻⁸ M RA	134 $\pm$ 10	26.8 $\pm$ 4.4
10⁻⁸ M RA + E3330	249 $\pm$ 1*	36.7 $\pm$ 2.4

HL-60 cells treated with RA and E3330 (25 μ M) for 6 days were then analyzed for expression of CD11b, granulocytic differentiation marker, and apoptosis using PI and Annexin-V. The mean and standard error from 3 independent experiments are shown.

*
p < 0.05,

**
p < 0.01, compared to corresponding RA dose using Student's t test.